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Área de Psicobiología

Identificación y caracterización funcional y anatómica de las áreas del palio del telencéfalo de los peces teleósteos implicadas en procesos emocionales

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Identification and functional and anatomical characterization of the teleost telencephalic pallium areas involved in emotional processes

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Área de Psicobiología**Identificación y caracterización funcional y anatómica de las áreas del palio del telencéfalo de los peces teleósteos implicadas en procesos emocionales**

Trabajo presentado por Manuel Reiriz Rojas para optar al grado de Doctor por la Universidad de Sevilla

Los Directores,

Dr. Cosme Salas García **Dr. Emilio Durán García**

1. INTRODUCTION

1.1. The evolution of the forebrain of vertebrates.

Traditional theories about vertebrate forebrain evolution have described the evolution of this neural structure as a linear series of increasing complexity. These theories placed the fishes in the first step of the 'evolutionary leader', as the most primitive and less 'evolved' vertebrate group, followed by the amphibians, reptilians and birds, to end with the superior cerebral and cognitive stage of mammals, and specially humans, who posses new and more complex neural mechanisms and circuits which have been acquired along the successive evolutionary stages (Ariëns, et al 1936; MacLean, 1990; Noback, 1983; Papez, 1929). According with these traditional theories, the telencephalon of fishes consisted on a structure dominated by olfactory inputs, with simple circuits sustaining elemental ´instinctive´ or ´reflexive´ forms of behavior and with limited capabilities for learning and plasticity processes. Consequently, fishes should exhibit rigid and fixed behaviors rather than the flexible ones that characterize mammals and that are generally associated with the expansion of the six-layered neocortex (Ariëns et al., 1936; MacLean, 1990; Noback, 1983; Papez, 1929). However, these old theories seem to be inadequate and anthropocentric in the light of the recent data coming from the different fields of Comparative Neurosciences and Evolutionary and Developmental Biology. Thus, increasing neurobiological and psychobiological evidence challenges these traditional notions on brain and cognition evolution, showing that fishes, as land vertebrates, also show complex learning and

memory capabilities based on comparable neural mechanisms and brain systems.

The telencephalon of vertebrates is considered one of the most variable structures throughout the phylogeny (Figure 1.1). In fact, the telencephalon of vertebrates is more variable in shape, size or organization than other brain regions like the cerebellum, the mesencephalon or the spinal cord (Butler & Hodos, 2005; Nieuwenhuys, Donkelaar, & Nicholson, 1998; Northcutt, 1995) Nevertheless, recent neuroanatomical, embryological, and genetic studies show that the telencephalons of all vertebrates are comparable and that they retain a huge number of primitive features.

These studies have shown that one of the primitive features that have been preserved throughout the vertebrate forebrain evolution is the division of the telencephalon in a pallium, located in a dorsal position in the telencephalic hemispheres, and a ventrally located sub-pallium. Although in some cases the exact delimitation between the pallial and the sub-pallial boundaries is under discussion, both regions can be easily distinguished in each vertebrate group. Morphogenetic studies have been proved to be particularly relevant to determine the boundaries between the pallium and the sub-pallium in vertebrates. For example, the technique of immediate early genes expression has notably contributed to the description of the boundaries between these two domains in amphibians, reptiles, birds and mammals (Bachy, Berthon, & Rétaux, 2002; Brox, Puelles, Ferreiro, & Medina, 2003, 2004; Bulfone et al., 1999; Garda, Puelles, Rubenstein, & Medina, 2002; Smith, 1968).

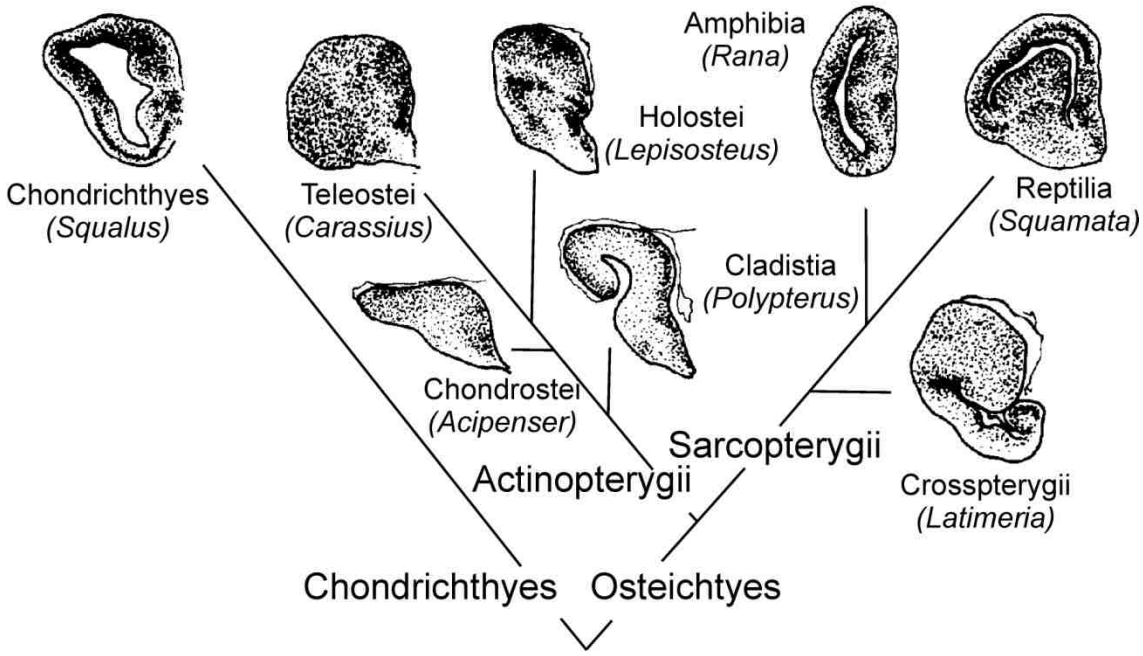


Fig 1.1. Cladogram representing transversal sections of the telencephalon of the main vertebrate radiations. Note the morphological and cytoarchitectonical variation existing among the different groups. Modified from *Northcutt, 1995; Northcutt and Braford, 1980*.

Multiple data indicate that the telencephalon of teleost and tetrapods share a basic pattern of organization with equivalent pallial and sub-pallial divisions, and even more, comparable pallial subdivisions, some of which considered homologous to the hippocampus and the amygdale of the land vertebrates (Braford, 2009; Broglio et al., 2005; Broglio, Rodríguez, Gómez, Arias, & Salas, 2010; Butler & Hodos, 2005; Nieuwenhuys et al., 1998; Nieuwenhuys, 2011; Northcutt, 2008; Salas, Broglio, & Rodríguez, 2003; Wullimann & Mueller, 2004; Yamamoto et al., 2007).

1.2. The telencephalon of the teleost fishes

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 their brain exhibit a remarkable range of variation. However, the telencephalon of actinopterygian fishes presents a unique morphological feature that makes it equal among them but quite different compared to the telencephalon of the remaining vertebrate groups. In this sense, the telencephalon of actinopterygian fishes consist of two main solid hemispheres separates by a single ventricular cavity (Braford, 1995; Nieuwenhuys, 1963; Northcutt & Kaas, 1995; Northcutt, 2006, 2008; Northcutt & Kicliter, 1980; Striedter & Northcutt, 2006), instead of the telencephalon with paired intra-hemispheric ventricular cavities that characterize non-actinopterygian vertebrates. This variation is caused by the different processes underlying the forebrain development in actinopterygians and non-actinopterygian vertebrates during the embryogenesis. Thus, whereas the forebrain of actinopterygian fishes undergoes a process of eversion or outward folding of the walls of the ispheres, the forebrain of the non-actinopterygian vertebrates develops by a process of evagination or inward bending (Figure 1.2) (Braford, 1995; Nieuwenhuys, 1963; Northcutt, 1995; Northcutt, 2006, 2008; Northcutt & Kicliter, 1980; Striedter & Northcutt, 2006).

These developmental peculiarities have traditionally hindered the aim of identifying homologies among vertebrates. However and despite of the differences between the telencephalon of actinopterygians and non-actinpterygian vertebrates, the process of differentiation of the pallial regions

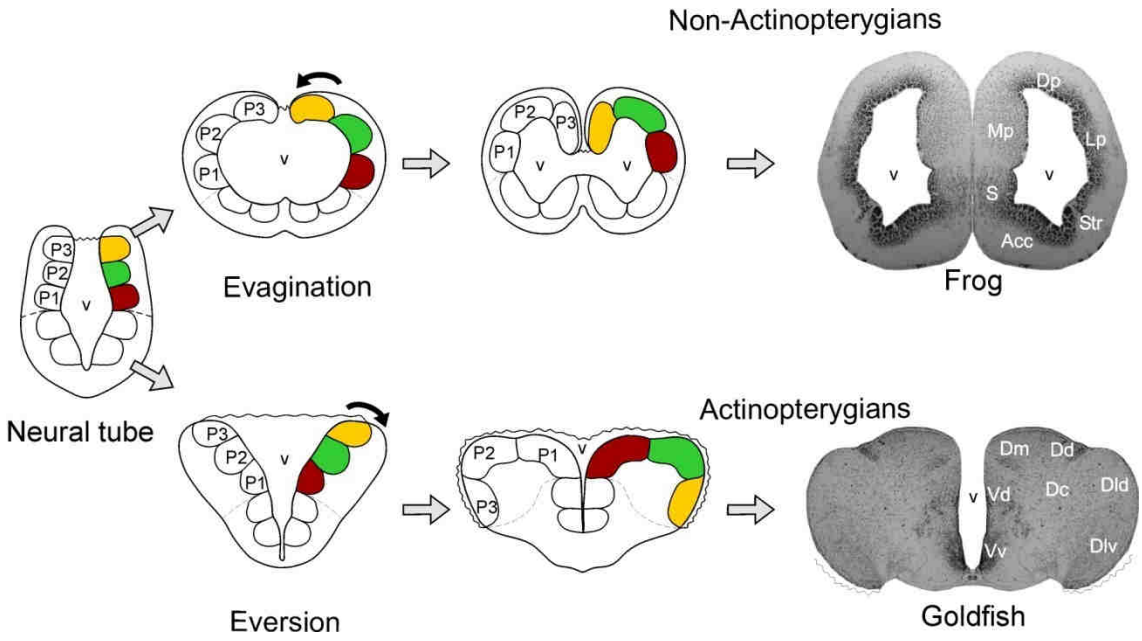
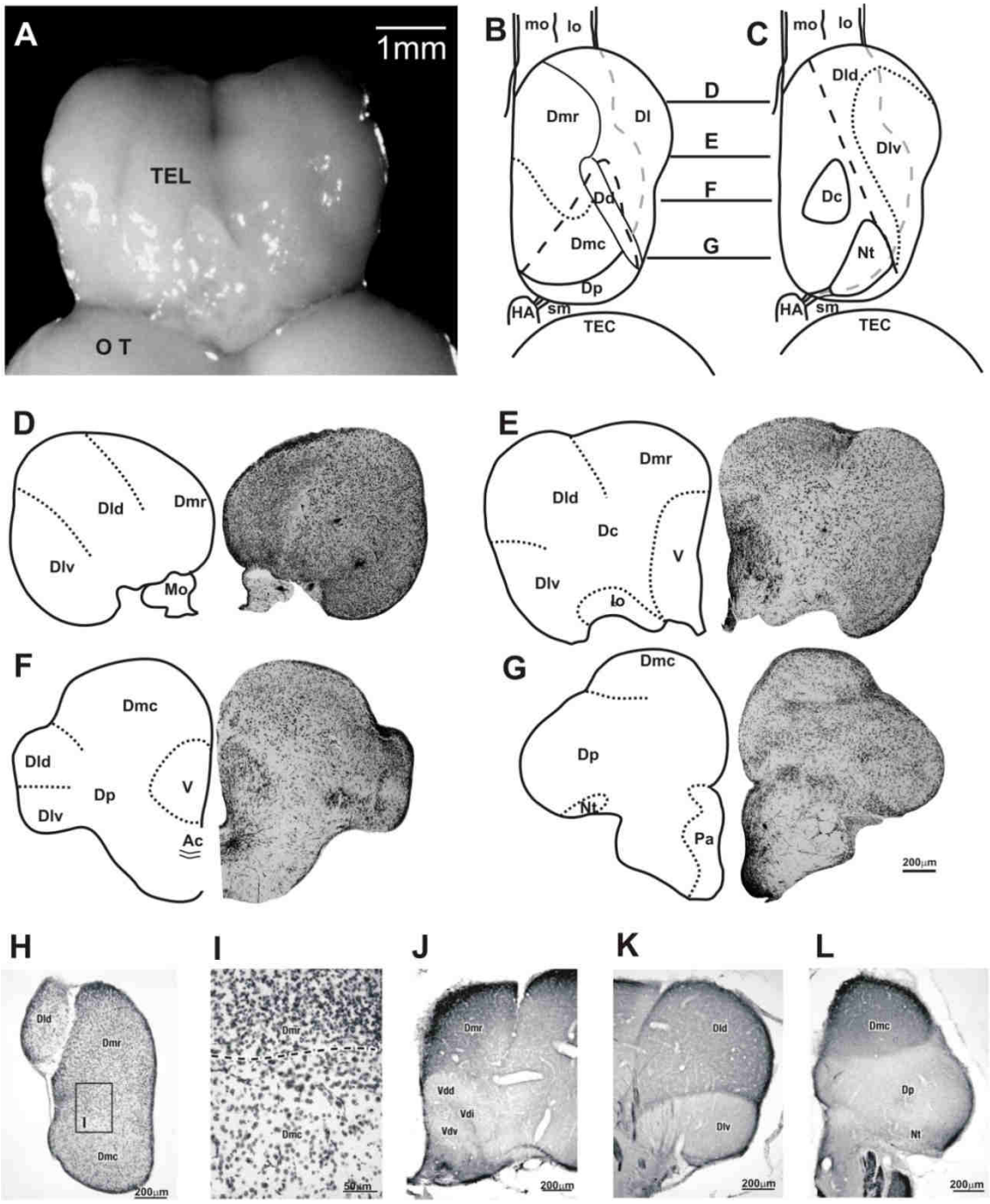


Fig 1.2. Comparison of the embryonary development of the telencephalon in non-actinopterygian and actinopterygian vertebrates. An eversion process occurs during the embryonary development of the telencephalon in actinopterygians. Due to this eversion, the telencephalon of actinopterygian fishes consist of two main solid hemispheres separated by a single ventricular cavity. The result of the evagination process in non-actinopterygian vertebrates are two ventricular cavities, each of them localized within one hemisphere. Abbreviations: Acc, nucleus accumbens; Dc, central division of the area dorsalis; Dlv, ventral subdivision of the lateral division; Dld, dorsal subdivision of the area doralis; Dm, medial division of the area dorsalis; Dp, posterior division of the area dorsalis; Lp, lateral pallium; Mp, medial pallium; P1, P2, and P3, Three main pallial subdivision; S, septal nuclei; Str, striatum; V, ventricle; Vd, dorsal nucleus of area ventralis; Vv, ventral nucleus of area ventralis. *Modified from Nieuwenhuys et al, 1998.*

occurring during the development of the vertebrate telencephalon seems to be re conservative that it was initially thought, in such a way that it is seems to be plausible that a basic organizational pattern of the telencephalon have remained constant along the vertebrate phylogeny (Wullimann & Mueller, 2004; Wullimann & Rink, 2002). It is thought that the major implication of the eversion process relative to evagination is the reversal of the topography of the telencephalic pallium. Even so, it should be to take into account that in both, actinopterygian and non- actinopterygian vertebrates, the topology of the

telencephalic pallium remains preserved. For example, it has been shown that some immediate early genes existing in both amniotes and teleost, have equal expression telencephalic domains, delimiting therefore, comparable regions in topological terms (Akimenko et al., 1994; Ganz et al., 2012, 2015; Hauptmann & Gerster, 2000; Püschel et al., 1992) Particularly, these studies have indicated that the dorsal and the ventral subdivision of the telencephalon of teleost fishes correspond to the pallium and the subpallium of land vertebrates, respectively. More precisely, the pallium of teleost fish has been traditionally subdivided in medial, dorsal and lateral regions (Butler & Hodos, 2005; Karten, 1997; Medina & Reiner, 2000; Nieuwenhuys et al., 1998; Northcutt, 1981, 1995; Northcutt & Kaas, 1995) which, in turn, have been considered homologous to the three main pallial divisions of the tetrapod telencephalon (Braford, 1995; Butler & Hodos, 2005; Northcutt, 1995; Wullimann & Rink, 2002) .

Fig 1.3. Cytoarchitectonics of the goldfish (*Crassius auratus)* telencephalic pallium. **A.** Photography of a dorsal view of the goldfish telencephalon. **B-C.** Reconstruction of the dorsal surface of the telencephalic pallium. In **B**, all the pallial division can be identified from a dorsal view, but only the rostral (Dmr) and caudal (Dmc) subdivision of the medial division can be seen in their entirely. The boundary between these two Dm region is marked by the dot line. From the surface, only the caudal pole of Dp can be seen. This structure lies beneath Dm and its boundary is marked by a dashed line. The dorsal division of the dorsal pallium in goldfish (Dd) separates Dm from Dl along the rostrocaudal axis. The dashed gray line marks the attachments of the tela choroidea. The full extent of the other pallial divisions can be visualized in a reconstruction that does not include Dm, Dd or Dp (**C**). In this reconstruction, the boundary of the large-celled subdivision of caudal Dm (Dc) can be indentified medially and a dashed oblique line marks the dorsomedial border of Dl. The lateral pallial division in goldfish can be subdivided in a ventral (Dlv) and dorsal (Dld) subdivisions. The dotted line marks the boundary between these two structures. As in the reconstruction before, the attachment of the tela choroidea is indicated by the dashed gray line. **D-G.** Photomicrographs of cresyl violet-stained transverse sections that correspond with the lines drawings in figures B and C. **H-I.** Photomicrography of horizontal sections where differences in cell density and size are shown between the rostral and caudal subdivisions of Dm. **J-L.** Photomicrography of coronal sections showing the calretinin distribution in Dmr (**J**), Dl (**K**) and Dmc (**L**). 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; Io, lateral olfactory tract; mo, medial olfactory tract; Nt, nucleus taeniae; Pa, preoptic area; sm, stria medullaris; OT, optic tectum; TEL; telencephalon; V, area ventralis; ac, anterior commisure. *Modified from Northcutt, 2006.*



1.3. Cytoarchitectonics of the goldfish telencephalic pallium

The telencephalic pallium or area dorsalis telencephali in goldfish can be divided into five regions: medial (Dm), dorsal (Dd), central (Dc), lateral (Dl) and

posterior (Dp) (Braford, 2009; Mueller & Wullimann, 2009). Figure 1.3 illustrates the pallial subdivisions in goldfish.

The medial area of the dorsal pallium (Dm) in goldfish is the closest area to the subpallium and is continued with the Vs at the commissural level (Braford, 1995; Braford, 2009). According to Northcutt (2006), Dm is cytologically characterized by a superficial layer of packed granule-like cells overlying a core of larger cells whose size gradually increases with depth. Nevertheless, the area where deeper cells are located in Dm has traditionally been designated as a separate, central pallial division (Dc). Dm in goldfish can be divided in two main regions: rostral (Dmr) and caudal (Dmc). Although both regions have a moderate concentration of calretinin in its neuropil, a superficial layer of cells that are much larger in Dmc than the superficial granulate -like cells of the rostral Dm easily distinguishes these two regions.

The pallial division Dl can be easily separated from Dm by the sulcus ypsiloniformis and citoarchitectonically by a marked increase in the cell size that 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). Thus, Dl is found along the entire dorso-ventral axis and in media-lateral axis Dl comes to occupy about 2/3 of the telencephalon from the most lateral border until the ypsiloniformis sulcus. Therefore Dl is, as in other teleosts, one of the largest areas of the pallium of the goldfish and it is easy to distinguish due to it is close proximity to ypsiloniformis sulcus.

Unlike most pallial divisions, Dl has the largest cells in a medial segment, not in the core (Northcutt, 2006) and can be divided in two main regions, dorsal (Dld) and ventral (Dlv). Unlike the ventral subdivision, the dorsal subdivision is characterized by a moderate concentration of calretinin (Northcutt, 2006), as well as by large cells that decrease in size with depth. It is easy to distinguish the border between Dld and Dlv in a caudal position due to a shallow sulcus between the two regions although this border is hard to distinguish in the rostral pole. In addition, the ventral subdivision of Dl can be divided in a dorsal and ventral part. A large region of scattered cells forms the dorsal part of the ventral subdivision of Dl, whereas the ventral part is formed by a smaller region of densely packed granule-like cells.

In transverse sections, the two parts of the ventral subdivision of Dl, as well as the dorsal subdivision of Dl are easily distinguished at the level of the anterior commissure. Dld and 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 any subdivision of Dl can be fully appreciate, and the ventral caudal pole is dominated by a superficial ring of medium sized cells surrounding a core of large and densely packed neurons that characterized Dp. The border between Dlv and Dp could be the harder border to distinguish in the goldfish pallium, but the transition from Dlv to Dp is much more evident in the horizontal plane. In this plane, the caudal pole of Dlv is marked by different sulcus, and there is actually a narrow, cell-free zone between Dlv and Dp (Northcutt, 2006).

1.4. Recent hypothesis on the pallial homologies between teleost and land vertebrates

The telencephalic pallium of teleost shows a notable structural variety. In fact, different subdivisions of its five main divisions have been described in different teleost species (Burmeister, Munshi, & Fernald, 2009; Giassi, Harvey-Girard, Valsamis, & Maler, 2012; Nieuwenhuys & Meek, 1990; Northcutt, 2006), which makes difficult the identification of homologies among these areas and the pallial nuclei in other vertebrate species. In addition, the different interpretations of the eversion process (See Nieuwenhuys, 2011) and the neurochemical and connectional data of the implied regions (Braford, 1995; Folgueira, Anadón, & Yáñez, 2004a; Kaslin & Panula, 2001; Mueller, Dong, Berberoglu, & Guo, 2011; Nieuwenhuys & Meek, 1990; Northcutt, 2006, 2008; Wullimann & Mueller, 2004; Yamamoto et al., 2007) have lead to the proposal of different models of homologies, most of them proposing conflicting or partially conflicting ideas (Figure 1.4).

The “partial pallial eversion model” (Mueller, Dong, Berberoglu, & Guo, 2011; Mueller & Wullimann, 2009; Wullimann & Mueller, 2004) based on connectional and gene expression data, proposes a partial eversion followed by a cellular migration. The “eversion-rearrangement theory” by Northcutt and Bradford suggests that differential expansion of the ventricular surface of some pallial zones and the differential proliferation and migration of neuroblasts from the different ventricular zones might result in displacement or shifting of the different pallial subdivisions (Braford, 1995, 2009; Northcutt & Kicliter, 1980). In the “new eversion model”, the eversion was suggested to occur in a

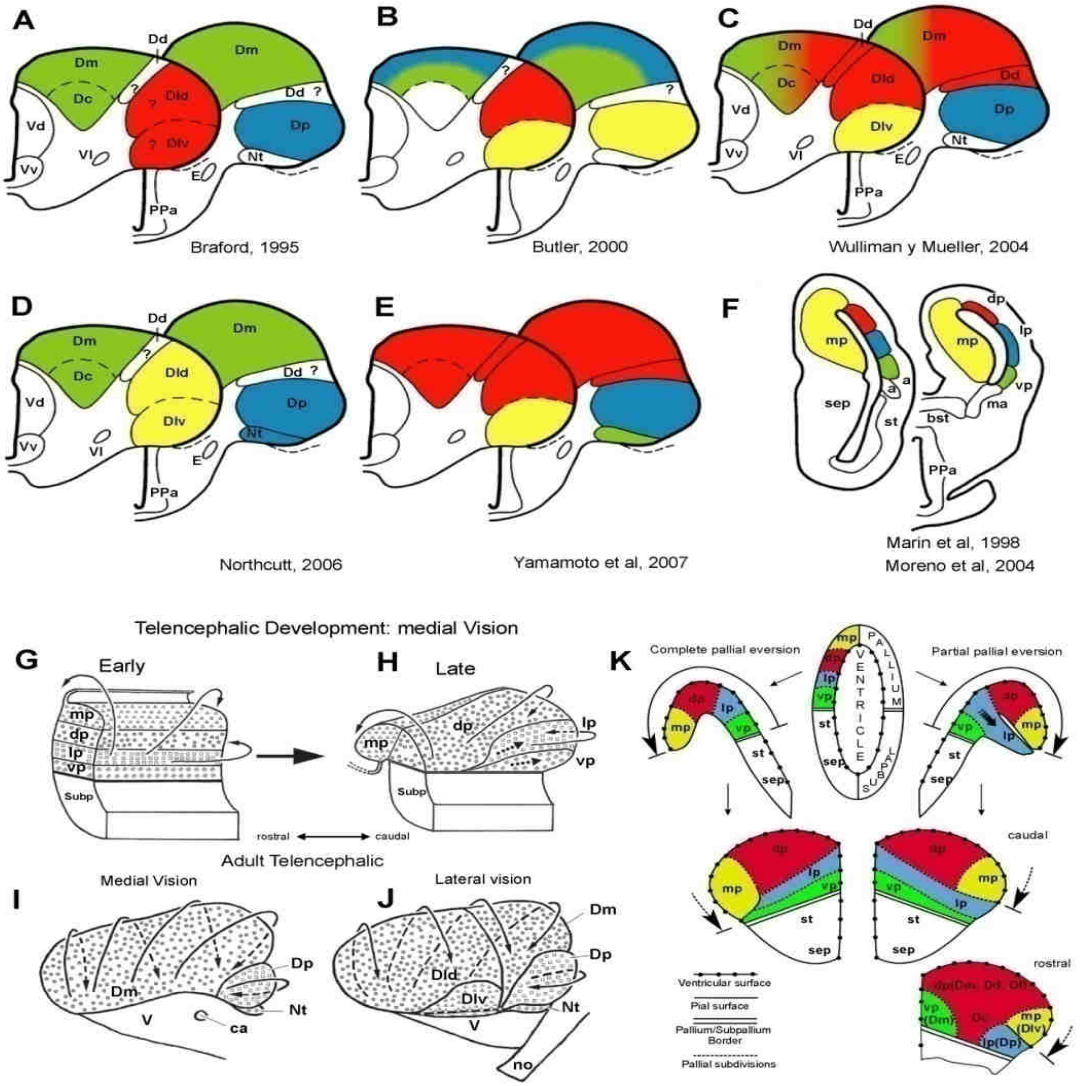


Fig 1.4. Recent hypothesis about pallial homologies in teleosts. Each hypothesis is illustrated using a rostral and a caudal section of the right telencephalic hemisphere of goldfish (**A-E**) using a color code which represents the possible homologous regions with a representative amphibian (*Rana*, **F**). Question mark indicate uncertainty regarding the homology. (**G-J**) Schematic representations showing the eversion hypothesis proposed by yamamoto et al (2007). (**G**) Early development stage when the pallial organization is still similar to other vertebrates. (**H**) Late stage of telencephalic organization if the pallial development followed the direction of the arrows. (**I-J**) Medial (**I**) and lateral (**J**) view of adult goldfish telencephalon. (**K**) Schematic comparison of the complete pallial eversion versus the incomplete eversion model proposed by Wulliman and Muller, 2004. Abbreviations: aa, anterior amygdaloid area; ac anterior commissure; Bst, bed nucleus of the stria medullaris; 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; Dm, medial subdivision of area dorsalis; dp, dorsal pallium; Dp, posterior division of area dorsalis; E, emtopeduncular nucleus; lp, lateral pallium; ma medial amygdala; mp, medial pallium; Nt, nucleus teniae; on, optic nerve; PPa, anterior parvocellular preoptic nucleus; Subpsubpallial 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 Muller, (2004).*

caudolateral direction leading to a shift of the arrangement of the different pallial subdivisions(Yamamoto et al., 2007). Yamamoto and colleagues propose a complex eversion that implies cell reorganization in the pallial areas, meaning that ventral pallial and lateral pallial homologous are not present in the rostral but only in the caudal pallium. Finally, (Butler, 2000) and (Nieuwenhuys, 2011) claim that the teleost telencephalon develops by a simple eversion process.

Although differences exist among these hypotheses in the interpretation on the pallial development, there is a general consensus that at least some pallial structures in the telencephalon of actinopterygians are homologous to specific regions in the cortex of tetrapods. Thus, Dlv, the ventral part of the lateral pallium subdivision (Dl) is considered homologous to the medial or hippocampal pallium of amphibians and other tetrapods (Northcutt, 2006; Portavella, Vargas, Torres, & Salas, 2002; Rodríguez et al., 2002; Yamamoto et al., 2007). What it is still under discussion is whether the entire Dl, i.e., Dld plus Dlv, or by contrast Dlv exclusively, are comparable to the hippocampal pallium. In this regard, Northcutt suggests that the hippocampal pallium in actinopterygians also involves the dorsal part of the lateral pallium subdivision (Dld). However, Butler, Wullimann and Mueller (2000), and Yamamoto and colleagues (2007) claim that Dld is homologous to the isocortex and therefore, that only Dlv might be considered homologous to the hippocampal pallium of actinopterygians. In addition, some authors (Butler, 2000; Nieuwenhuys, 2011) have proposed that the posterior region of pallium (Dp), a region commonly considered homologous to the piriform cortex on the basis of it massive olfactory inputs (Mueller and wulliman, 2009; Northcutt, 2006; Yamamoto et al.,

2007), is actually a specialized part of the medial pallium or hippocampus.

Although with the exception of Yamamoto and co-workers, there is also a general consensus that at least part, if not all, of Dm of teleosts is homologous to the pallial amygdala of amphibians and other tetrapods. This hypothesis is based primarily on topological, genetic, and connectional data and assumes that Dm or at least part of it, is derived from the ventral pallium and therefore, that constitutes a limbic area lying between the subpallium and the olfactory pallium (Braford, 1995; Northcutt & Kicliter, 1980; Puelles & Rubenstein, 1993; Wullimann & Rink, 2002). However, Yamamoto and co-workers (Ito & Yamamoto, 2009; Yamamoto et al., 2007; Yamamoto & Ito, 2008. See figure 1.4) propose that Dm, Dd and the dorsal part of Dl should be homologous to the dorsal pallium of amphibians and the isocortex of 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 of tetrapods (Yamamoto et al., 2007). Nevertheless, other authors suggest that the preglomerular complex has multiple embryonic origins and remain skeptical about that homology (Northcutt, 2008). Finally, Nieuwenhuys (2009) proposed that Dm is homologous to the lateral pallium based on topology.

1.5. Anatomical and functional organization of Dm of teleost fishes

As we previous mentioned, there is a wide consensus between the comparative neuroanatomists that at least part of Dm of teleost fish is

homologous to the amygdala of tetrapods, although some discrepant views has been suggested. For example, based in the pattern of connectivity, Yamamoto and colleagues (2007) have proposed that Dm, together with the dorsal part of Dl, must be considered homologous to the isocortex of tetrapods.

The amniote amygdala is an structure located in the anterior region of the temporal lobe that is critical for emotional learning and memory (LeDoux, 2000; Maren, 2001; McGaugh, 2004). From an anatomical point of view, the amygdala is formed by set of nuclei that include the basolateral complex, subdivided into the lateral, basolateral and basomedial nucleus; the extended amygdala, which include the central and medial amygdala and the bed nucleus of the stria terminalis; and the intercalate cell masses (Duvarci & Pare, 2014; Janak & Tye, 2015; Sah, Faber, Armentia, & Power, 2003). Functionally, the amygdaloid complex in amniotes can be subdivided in olfactory, frontotemporal, and autonomic systems (Swanson & Petrovich, 1998). The olfactory system can be divided in two different regions. Firstly, an accessory olfactory (or vomeronasal) component dominated by the medial amygdala (MeA). This region is a key for the perception of pheromonal stimuli involve in recognition (Newman, 1999) and detection the odors of predators (Canteras, 2008). The second region of the olfactory system is the main olfactory component, dominated in mammals by the posterior basolateral amygdala, the basomedial amygdala, and the posterior amygdala (Swanson & Petrovich, 1998). The origins of these two regions are different. Thus, the first subdivision has a subpallial origin whereas the second subdivision has a ventropallial origin (Brox

et al., 2004; Bupesh, Legaz, Abellán, & Medina, 2011; García-López et al., 2008; Waclaw, Ehrman, Pierani, & Campbell, 2010).

The second amygdala complex component suggested by Swanson and Petrovich (1998) is the frontotemporal amygdaloid system. This subdivision has a ventropallial origin (Brox et al., 2004; Waclaw Ehrman, Pierani, & Campbell, 2010). This system is composed by the basolateral amygdala (BLA) and the lateral amygdala (LA). The third component of the amygdaloid complex is the autonomic amygdala. This component is formed by the central extended amygdala (CEXA), composed of central amygdala (CeA) and bed nucleus of the stria teminalis (BNST) which has a subpallial in origin.

Numerous studies demonstrated that the amygdala of amniotes shares its basic developmental, hodological and neurobiological features (Moreno & Gonzalez 2007). In addition, homologous territories of all the main amygdaloid subdivisions have been recognized in amniotes characterized by the expression of common patterns of developmental genes (Medina., et al 2002; Moreno & González 2006). In addition the organization of the anamniote amygdaloid complex is also conserved in its main essential features, supporting the idea that the basic plan of organization is shared in mammals, birds, reptiles, and also in amphibians (Moreno & Gonzalez, 2007).

1.5.1. Hypothesis regarding the homology of Dm

As result of the eversion process, the medial region of the area dorsalis telencephali (Dm), topographically correspond to the lateroventral pallium of non-everted brains has it is located at the boundary between the dorsal and the

ventral telencephalon (Maximino, Lima, Oliveira, Batista, & Herculano, 2013; O'Connell & Hofmann, 2011; von Trotha, Vernier, & Bally-Cuif, 2014). Accordingly, several studies support the idea that Dm correspond to the amniote pallial amygdala based on the conexion pattern, gene expression, developmental and comparative evidences, neurochemical distribution, and behavior (Braford, 2009; Desjardins & Fernald, 2010; Mueller & Wullimann, 2009; Nieuwenhuys, 2009; Northcutt, 2006, 2008; Portavella, Torres, & Salas, 2004; Wullimann & Mueller, 2004).

Although a complete vomeronasal system is not present in bony fishes (Døving & Trotier, 1998), they are able to detect social cues, food odors, sex pheromones and skin extract from conspecifics (Ubeda-Bañon et al., 2011). In this sense, it has been described that the ventral part of the precommisural Dm of the trout receives direct inputs from the olfactory bulb (Folgueira et al., 2004a). This cells projecting to this subregion of Dm from the medial portions of the olfactory bulb are calretinin-positive (Gayoso, Castro, Anadón, & Manso, 2011). In crucian carps (*Carassius carassius*) this subregion of Dm has neurons selective and sensitive for skin extract (Hamdani & Døving, 2003; Lastein, Hamdani, & Doving, 2008). These hodological and functional data suggest that the ventral subdivision of the precommisural Dm is part of a putative fish vomeronasal system (Maximino et al., 2013). In fish (and amphibians) the differences between the frontotemporal and the olfactory amygdaloid system has not been yet described and it seems to be more adequate to consider the pallial amygdala as a single multisensorial region which receives olfactory information as well as polymodal inputs from brainstem and thalamus (Davies,

Martínez-García, Lanuza, & Novejarque, 2002; Martínez-García, Novejarque, & Lanuza, 2007; Medina, Bupesh, & Abellán, 2011; Moreno & González, 2006, 2007).

In addition the characterization of the distribution of markers in the telencephalon of teleost fish, as for example the cannabinoid receptor gene Cb1, a marker of basolateral amygdala in mammals (Mailleux & Vanderhaeghen, 1992; Matsuda, Bonner, & Lolait, 1993) support also the homology between the amniote pallial amygdala and Dm (Harvey-Girard, Giassi, Ellis, & Maler, 2013; Lam, Rastegar, & Strähle, 2006; Ruhl, Moesbauer, Oellers, & Emde, 2015). On the other hand, different markers distribution support the idea that Vs and Vp of Zebrafish larvae could be homologous to the centromedial amygdala and bed nucleus of the stria terminalis (Mueller, Wullimann, & Guo, 2008). The pattern of distribution of other markers molecules supports also the hypothesis of homology of Dm with the pallial amygdala. For example, while CRF expression is lacking in Dm (as occur in frontotemporal amygdala), urotensin I expression is moderate in the rostral but no in the caudal area of the zebrafish Dm (Alderman & Bernier, 2007). Moreover, in goldfish vasotocin inmunoreactive fibers terminate in Dm just rostral to the anterior commissure (Thompson & Walton, 2009). In addition, in the catfish, low levels of cocaine and amphetamine regulated transcript peptide (CART), a peptide enriched in the medial amygdala of rodents (Olmos et al., 2004) are found in Dm (Subhedar, Barsagade, Singru, Thim, & Clausen, 2011). These peptides are expressed or secreted at neurons of the vomeronasal amygdala of

mammals, but are also present in the BLA (Koylu, Couceyro, Lambert, & Kuhar, 1998; Sofroniew, 1980).

1.5.2. Hodological data

Hodological data regarding Dm in teleost fish has been obtained in trout (Folgueira et al., 2004a) and goldfish (Northcutt, 2006; Yamamoto et al., 2007). In mammals, the amygdala receives primary sensory information. The lateral nucleus of the amygdala receives visual, auditory, somatosensory, olfactory and gustatory inputs from the cortex and thalamus (LeDoux, 2007; Swanson & Petrovich, 1998). Similarly, it has been demonstrated in different teleost fishes that Dm received olfactory, gustatory, auditory, visual and mechanosensory information (Folgueira, Anadón, & Yáñez, 2004b; Northcutt, 2006; Yamamoto et al., 2007). Moreover, as occur in mammals, the putative homologue region of the hippocampus in fish, Dl, projects to Dm (Northcutt, 2006). The projections from the anterior part of Dm extend to pre- and postcommissural portions of Dm (Maximino, Silva, Gouveia, & Herculano, 2011). This area could be thus considered the interface between the putative basolateral and central amygdala and expresses transcription factors that are markers of the intercalated cell masses (Shah, Medina-Martinez, Chu, Samaco, & Jamrich, 2006; Waclaw et al., 2010; Zerucha & Prince, 2001). Finally, the pattern of afferences of Dm suggests that there are no differences between accessory and olfactory amygdaloid system in teleosts (Maximino et al., 2013; Swanson & Petrovich, 1998).

1.5.3. Behavioral data

Dm receive stimulus from different sensory modalities, including nociceptive information. Thus, galvanic stimulation of the tail at noxious intensities produces potentials in Dm in salmons (Nordgreen, Horsberg, Ranheim, & Chen, 2007). Some studies suggest the existence of a spino-parabrachial-amygdalar pathway in teleost involved in aspects of nociception (Bernard et al., 1996). Moreover, a C-fos (a marker of stimulus induced neural activity) inmunoreactivity is increased in Dm in Chinese mudskippers (*Periophtalmus cantonensis*) when these animals are agitated by stirring the water (Wai, Lorque, Webb, & Yew, 2006), and increased of C-fos mRNA in Dm has been described in zebrafish after the animal exposure to a light/dark box, a procedure that is considered anxiogenic for this species (Lau, Mathur, Gould, & Guo, 2011).

Lesion studies suggest that Dm is essential for the acquisition of fear conditioning. Thus, lesions in caudal Dm produce deficits in the habituation of the startle response in *Rutilus rutilus* (Laming & McKee, 1981) and goldfish (Rooney & Laming, 1986). Nevertheless, an opposite results was observed in *Betta splendens* (Marino-Neto & Sabbatini, 1983). Lesions in Dm but not in Dl impair the acquisition of active conditioned avoidance response in goldfish (Portavella, Salas, Vargas, & Papini, 2003; Portavella, Torres, Salas, & Papini, 2004). These results are similar than those obtained in mammals after amygdalar lesions (Aggleton, Kentridge, & Sembi, 1992; Davis, 1992) and suggest that Dm could have an important role in emotional learning and

memory as it occurs with the amygdala of mammals (Kim & Jung, 2006; Maren, 2001; Medina, Repa, Mauk, & LeDoux, 2002; Parkes & Westbrook, 2010).

Taste aversion learning in goldfish is also dependent of Dm. In an experiment from our laboratory (Martín, Gómez, Salas, Puerto, & Rodríguez, 2011) goldfish learn 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 (associated with the discomfort) and other by saline, both after 10 minutes delay. Animal with lesions in Dm were no able to learn the task. In contrast, when the damage was in Dl (the region homologous to the hippocampus) there was not effect in the acquisition of the taste aversion procedure. Thus, the Dm lesion in goldfish produces a similar effect on the taste aversion procedure than that produced by the lesion of the amygdala in mammals (Bermúdez-Rattoni et al., 2004 Bernstein et al., 1999; Lamprecht and Dudai, 2000; Yamamoto et al., 1994). In this sense, neuroanatomical data show that gustatory and visceral inputs converge in the Dm, supporting the taste aversion learning (Folgueira, Anadón, & Yáñez, 2003; Folgueira et al., 2004a; Northcutt, 2006; Yoshimoto & Yamamoto, 2010) and suggesting that this region, like the amygdala of mammals, could be a site for the taste malaise integration in teleosts.

Altogether, these functional data support the hypothesis that Dm in teleost fish, as the amygdala in mammals, is essential for emotional learning and memory system, and provide further support concerning the hypothesis of homology between these both structures (Davis & Whalen, 2001; Fanselow &

Ledoux, 1999; Kim & 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).

Thus, multiple anatomical, hodological, genetic and functional data support the idea that Dm is homologous to the amygdala in tetrapods, and only Yamamoto and colleagues (2007) disagree with this hypothesis. Nevertheless, it is not clear if the entire Dm can be considered homologous to the amygdala or only a specific subregion of it should be considered as such. In this sense, according to Northcutt (2006) Dm is a homogeneous region of the area dorsalis telencephali that could be subdivided in two main subregions: Dmr and Dmc. Both subregions have similar calretinin concentration and projections. Both, Dmr and Dmc received projections from the suprachiasmatic nucleus, all preglomerular nuclei and the posterior thalamic nucleus. On the other hand, both structures project to the anterior tuber, hypothalamus and nucleus diffusus. These similarities support the idea that Dm, as a unitary entity (Dmr + Dmc), is homologous to the amygdala.

In contrast, Butler (2000), and Wulliman and Muller (2004) suggest that not all the Dm region would be homologous to the ventral pallium of the amniotes but only a part of it. Supporting this proposal, a recent study in our lab (Rodriguez-Expósito, 2014) suggest that only the medial and precommisural region of the medial pallium in goldfish is critically involved in the acquisition of fear classical conditioning and plays a role similar to that of the amygdala in mammals (Blanchard, 1972; Cousens and Otto, 1998; Iwata et al., 1986;

LeDoux, 1993; Maren et al., 1996; Sananes & Davis, 1992). Thus, this study suggests the possibility that not the entire Dm, but only a restricted subregion of it, would be considered homologous to the amniote pallial amygdala. In this sense, based on calretinin-like immunoreactivity, Castro et al (2003) have identified at least four zones in Dm of trouts along the rostrocaudal axis. The zebrafish telencephalon is also heterogeneous in the dorso-ventral axis and shows different markers which clearly distinguish a dorsal and ventral region (castro et al.,2006).

1.6. General objectives

The main objective of the present work was to further investigate the functional identity of the dorsomedial subdivision of the area dorsalis telencephali (Dm) of goldfish. Particularly, we analyzed the possible involvement of this region in the nociceptive somatosensory processing and in the generation of affective and emotional states associated with noxious stimulation and pain. Special emphasis was done in the identification of differentiated functional subregions within the Dm area. The implication of the functional results obtained in this work will be discussed in the light of the different hypotheses of homology about the telencephalic pallial areas of teleost fish in order to clarify the bases of the evolution of the telencephalic pallium in vertebrates.

Four experiments were included in the present doctoral dissertation manuscript. In Experiment 1, we used voltage-sensitive dye imaging to map the areas of goldfish telencephalic pallium involved in the processing of nociceptive information. In Experiment 2, we used intracerebral electrical microstimulation to map systematically the surface of the telencephalic pallium in order to identify the areas involved in the generation of motor and visceral components of escape, fearful-like, emotional responses. In Experiment 3, we used a conditioned place aversion procedure (CPA) to behaviorally study whether the activation by means of intracerebral electrical stimulation of the pallial areas identified in the Experiment 2 produced unpleasant, emotionally negative, or fearful states in goldfish. Finally, in Experiment 4, we tested, by means of selective pallial lesions in goldfish trained in a conditioned place aversion

procedure, if the pallial areas identified in the Experiments 1, 2 and 3 are critical for the generation of the affective and fear components of the nociceptive stimulation involved in fear conditioning.

2. EXPERIMENT 1. Voltage-sensitive dye imaging (VSD) of pallial nociceptive activity

2.1. Introduction

Based on topological, developmental, histochemical, hodological and functional evidence, the pallial Dm area of teleost fish has been proposed as homologous to the pallial amygdala of tetrapods (Broglio et al., 2005; Northcutt & Kicliter, 1980; Northcutt, 2006). Like the amygdala in land vertebrates, the Dm area of teleost fish is involved in the generation of motivated and emotional related behaviors (Broglio et al., 2005; Desjardins & Fernald, 2010; Lau et al., 2011; Martín et al., 2011; Portavella, Torres, & Salas, 2004). Previous studies have reported that Dm receives gustatory, auditory and somatosensory diencephalic inputs (Butler & Hodos, 2005; Northcutt, 2006; Wullimann & Rink, 2002) and that it presents also gustatory, auditory and somatosensory related activity (Echteler, 1985; Kanwal, Finger, & Caprio, 1988; Prechtl et al., 1998). Prechtl and collaborators (1998) have described electrophysiological responses evoked by four different sensory modalities in Dm, which led them to suggest the existence of segregated sensory areas in the fish pallium. Unfortunately, their electrophysiological approach did not allow them to determine how stimuli of different modalities were represented in the pallium and therefore, to precisely describe the location and the topological relationships of the different sensory areas. More specifically, Ocaña (2009) have reported that somatosensory stimulation activates two different regions in Dm. A significant nociceptive input has also been identified from among all the sensory pathways

that convey to Dm (Wullimann & Rink, 2002) what suggest the existence of a spino-parabrachial-amygdala pathway involved in the autonomic and affective aspects of nociception in teleosts (Bernard, Bester, & Besson, 1996). Thus, increased *c-fos* expression was observed in the Dm area of teleost fish exposed to noxious, unpleasant or anxiogenic situations (Lau et al., 2011; Maximino, Marques de Brito, Dias, Gouveia, & Morato, 2010; Ramsay et al., 2009; WAI et al., 2006). In addition, electrophysiological activity in the medulla, cerebellum, tectum and telencephalon can be recorded in response to mechanosensory noxious stimulation (Dunlop & Laming, 2005) and transdermal electrical stimulation at noxious intensities produce evoked potentials in the Dm (Nordgreen et al., 2007).

In the present experiment, we used in vivo voltage-sensitive optical imaging recording to identify the areas of the telencephalic pallium of goldfish that are activated by noxious transdermal electrical stimulation, and to locate precisely their borders and their correspondence with the cytoarchitectonic and histochemical landmarks.

2.2. Methods

2.2.1. Animal preparation for in vivo voltage-sensitive dye imaging

Goldfish (*Carassius auratus*) 10-11 cm in length, measured from the mouth to the beginning of the caudal fin, were obtained from the vivarium of the University of Seville. 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. 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, high sensitivity to low voltage levels, good signal-to-noise ratio, and small decline of fluorescence over time (Cha et al, 2009; Onimaru & Homma, 2003; Prechtl, Cohen, Pesaran, Mitra, & Kleinfeld, 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 $CaCl_2$ 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. After staining, the telencephalon was washed twice with Ringer solution to eliminate the unbound dye and to keep it moist.

For the recording session, goldfish were immobilized with an intraperitoneal injection of Flaxedil (5 µg/g animal body weight; Gallamine

triethiodide, Sigma-Aldrich) to eliminate artifacts caused by body movements. All animal procedures were performed in accordance with Directive 2010/63/UE of the European Community Council and Spanish legislation (R.D. 53/2013).

2.2.2. Stimuli

The noxious stimulus consisted of an electric shock (150 µs, 5 mA) provided by a stainless steel bipolar electrode (diameter = 100 µm) subdermally implanted on the left side of the rostral extreme of the dorsal fin. The electric shock was provided by a current constant current isolation unit (SiU-90, Cygnus Technology Inc) connected to a digital stimulator (PG-4000, Cygnus Technology Inc) that controlled the duration and timing of the pulse.

2.2.3. Optical imaging and analysis

Optical images were acquired using a commercially available system (MiCAM01; Scimedia, Brain Vision; Tominaga, Tominaga, Yamada, Matsumoto, & Ichikawa, 2000). 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 150W tungsten halogen lamp (MHF-G150LR; Moritex). The light beam was passed through a 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 (PLAN APO; Leica Microsystems) and a 1x projection lens, so an area of 4.6x 3.3 mm was covered by the image sensor.

The imaging field was centered on the dorsal surface of the right 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 200Hz (5 frames/ms). To avoid the contaminating effect caused by the shutter opening on the recorded responses, imaging acquisition was begun 400 ms after the illumination shutter was opened. During each trial, the VSD signals were collected for 1,700 ms. A period of 300 ms was included before stimulation was initiated. To improve the signal-to-noise ratio, 16 successive trials were averaged. The intertrial interval was 30 s.

Data analysis was performed using BV-analyzer software (Brain Vision). The acquired images were detrended to compensate for dye bleaching, two-dimensionally averaged (spatial filter, 5 x 5 pixels) and low-pass filtered using smoothing algorithms. To evaluate the changes in optical signal intensity, we used the percentage fractional fluorescence change (% $\Delta F/F$), 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 8 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 several parameters: maximum activity, latency, time to peak and duration. The maximum or peak activity ($\Delta F/F_{max}$) was quantified by averaging the fractional fluorescence change values of a 3 x 3 pixel square positioned over the more activated region. Latency was defined as the time interval from stimulus onset up to 25% of $\Delta F/F_{max}$, and the time to peak was defined as the interval from stimulus onset to $\Delta F/F_{max}$. Duration was defined as the time interval in which the activity was over 25% of $\Delta F/F_{max}$.

2.2.4. Cytochrome oxidase histochemistry and Nissl staining

Sections of goldfish telencephalon were stained for either Cytochrome Oxidase (CO) or Nissl, both of them techniques contributing to reveal borders of pallial areas. To this purpose, animals were initially anesthetized (ethyl 3-aminobenzoate methanesulphonate, MS-222, Sigma) and perfused transcardially with cold PBS 0.1M (pH 7.4) followed by cold fixative solution (2% paraformaldehyde and 0.5% glutaraldehyde in PBS 0.1 M, pH 7.4). After 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). Brains

were sectioned in horizontal and sagittal planes with a cryostat (Leica CM 1850). For each brain, two series of adjacent sections (30 μm) were collected on clean slides. One series was processed for CO histochemistry using previously described procedures (Broglio et al., 2010; Uceda et al, 2015). First, 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 0.1M (pH 7.4). The slides were rinsed three times with PBS, dehydrated, and coverslipped with DPX (Sigma). The second series was Nissl-stained (cresyl violet acetate 0.5%), dehydrated, and coverslipped with DPX.

2.3. Results

2.3.1. Identification of different subdivisions within the medial region of area dorsalis telencephali

The first result in this experiment was the identification, based on macroanatomical observations, of different subdivisions within the medial region of area dorsalis telencephali (Dm). Several shallow sulci in the dorsal surface of the goldfish telencephalon served as external macroscopic landmarks to demarcate different pallial subdivisions within Dm. One of these macroanatomical landmarks is the sulcus ypsiloniformis that turns rostrally to the mid-sagittal line and separates Dl from Dm. More caudally along the rostro-caudal exe, the sulcus ypsiloniformis continues bordering laterally the medial region of the area dorsalis telencephali (Dm) and separates this region from

Dld, the dorsal subdivision of the lateral region of the area dorsalis telencephali (Figure 2.1 A). A more detailed analysis of the Dm pallial surface shows two more sulci that we have named *rostral sulcus* and *caudal sulcus* of Dm, respectively. These sulci divide Dm in three well-differentiated and prominent lobes that we named DmI, DmII and DmIII and that occupy, respectively, the rostral, intermediate, and caudal regions of Dm (see Figure 2.1 A and B, C). A well-differentiated bulge, rostrally delimited by the rostral sulcus, was also observed in the anteromedial region of DmII (asterisk in Figure 2.1 A). Finally, a shallow sulcus, clearly visible in a sagittal section (Figure 2.1 B), that we have named *sulcus posterior,* and that is located in the most caudal portion of the telencephalon, separates DmIII from Dp, the posterior region of the area dorsalis.

The borders and subdivisions revealed by the sulci and lobes above described can also be identified by means of cytoarchitectonic and histochemical techniques. For example, the sulcus ypsiloniformis, the external landmark separating Dld from DmII and DmIII, is revealed by a marked increase in cell size in the medial border of Dld as well as by the existence of a cell-free zone between Dld and DmII observed in horizontal Nissl stained sections (Figures 2.1 C and I). This border is also revealed by cytochrome oxidase (COX) histochemistry as it shows a marked transition from a highly stained (COX-dark region) Dld zone to a poorly stained (COX-lighter regions) DmII and DmIII zones (Figure 2.1 J). In Nissl stained horizontal sections, Dld can also be distinguished from DmIII as they are separated by a strip of densely packed medium sized cells that constitutes Dd, the dorsal region of area dorsalis

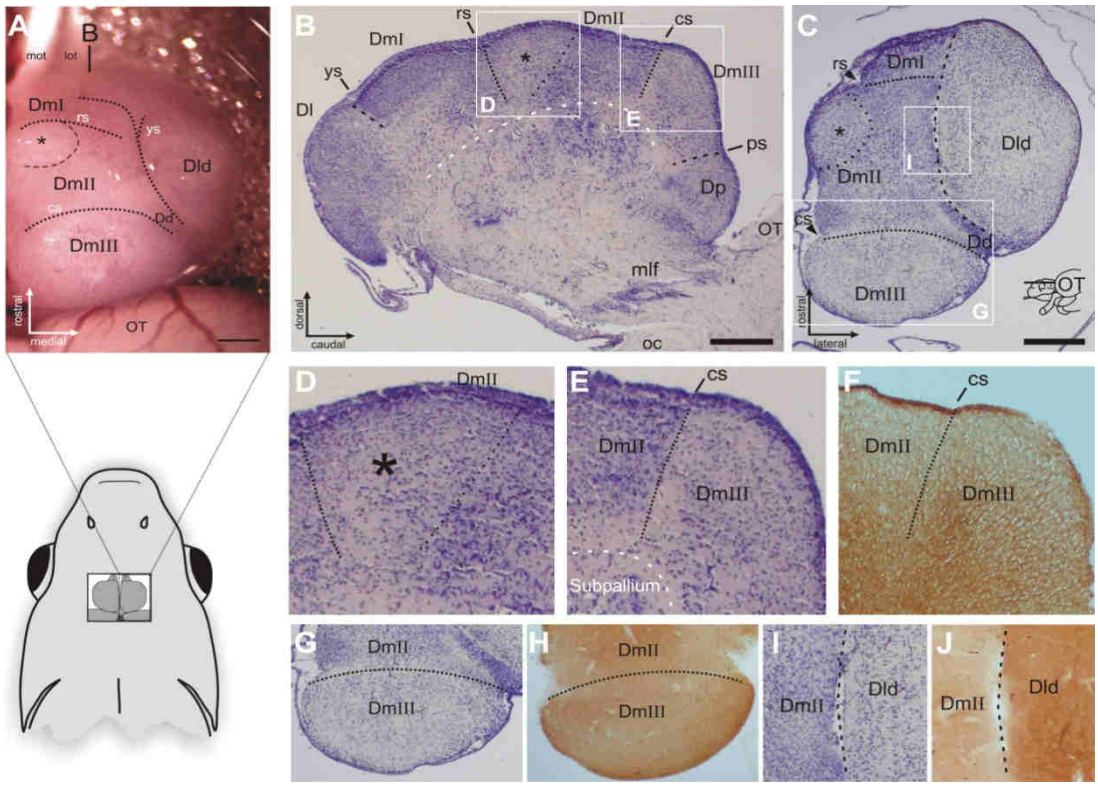


Fig 2.1. Subdivisions of the goldfish telencephalic pallium. A. Dorsal photograph of goldfish pallial surface showing the subdivisions within the medial region of area dorsalis telencephali (Dm) based on the existence of different sulci (dotted lines) **B**. Photomicrograph of a Nissl stained sagittal section of goldfish telencephalon. Note that the different sulci of Dm can also be observed in this section **C**. Photomicrograph of a Nissl stained horizontal section of goldfish telencephalon. Note in B and C the citoarchitectural differences observed between the three different subdivisions of the Dm as well as between the Dm subdivisions and Dld. **D-E.** Magnifications of the zones demarked by squared in B. **F.** Cytochrome oxidase (COX) staining of the same pallial region showed in E. **G.** Magnification of the caudal region of the pallium signaled by a square in C. Note that the frontier between DmII and DmIII is easily discernible. **H.** COX staining of the pallial region showed in G. **I.** Magnification of the frontier between DmII and Dld (signaled by a square in C). **J.** COX staining of the pallial region showed in I. Note that Dld presents a high level of cytochrome oxidase staining in comparison with the Dm. Asterisks denotes the anteromedial bulge identified in DmII. Abbreviations: cs, caudal sulcus; Dd, dorsal division of the area dorsalis; Dl, lateral division of the area dorsalis; Dld, dorsal subdivision of lateral division of area dorsalis; DmI, subdivision I of the medial division of the area dorsalis; DmII, subdivision II of the medial division of the area dorsalis; DmIII, subdivision III of the medial division of the area dorsalis; Dp, posterior division of the area dorsalis, lot, lateral olfactory tract; mot, medial olfactory tract, OT, optic tectum; oc, optic chiasma; ps, posterior sulcus; rs, rostral sulcus; ys, Ypsiloniformis sulcus.

(Figures 2.1 C). The borders between Dm subdivisions can also be identified in Nissl and COX sections. Thus, a smaller and more densely packed population of superficial cells can be observed in DmIII compared to DmII in Nissl sagittal

sections (Figure 2.1 E). Additionally, a free-cells strip separating both areas can easily be observed in horizontal and sagittal sections processed with Nissl and COX histochemistry (see Figures 2.1 E, F, G and H). Finally, the prominent bulge described in the most anteromedial region of DmII is cytologically characterized by the presence of extremely little unpacked cells (asterisk in Figures 2.1 D).

2.3.2. In vivo VSD imaging

We used in vivo VSD imaging, which allows the simultaneous recording of activity in different neuron populations with high spatio-temporal resolution (Chemla & Chavane, 2010; Grinvald & Hildesheim, 2004), to functionally localize the pallial regions responsive to a noxious stimulus (Figure 2.2).

After staining the pallial surface of the goldfish telencephalon with a VSD (Di-2-ANEPQ, Molecular Probes) we recorded, in six different experiments, the pattern of activity evoked by an electric shock (150 µs, 5 mA) delivered at the base of the dorsal fin (Figure 2.2). Noxious stimulation evoked a complex stereotyped spatiotemporal pattern of activity in the telencephalic pallium of animals (Figures 2.3 A and 2.4).

The optical signal appeared in the most medial region of DmIII with a latency of 56±3.8 ms, and spread laterally across a large part of it in the few subsequent milliseconds. This pallial region, the most medial part of DmIII, has been proposed as the primary somatosensory pallial region of goldfish and is characterized, as the S1 of mammals and sauropsids, by receiving ascending diencephalic somatosensory inputs in a topographic manner and consequently,

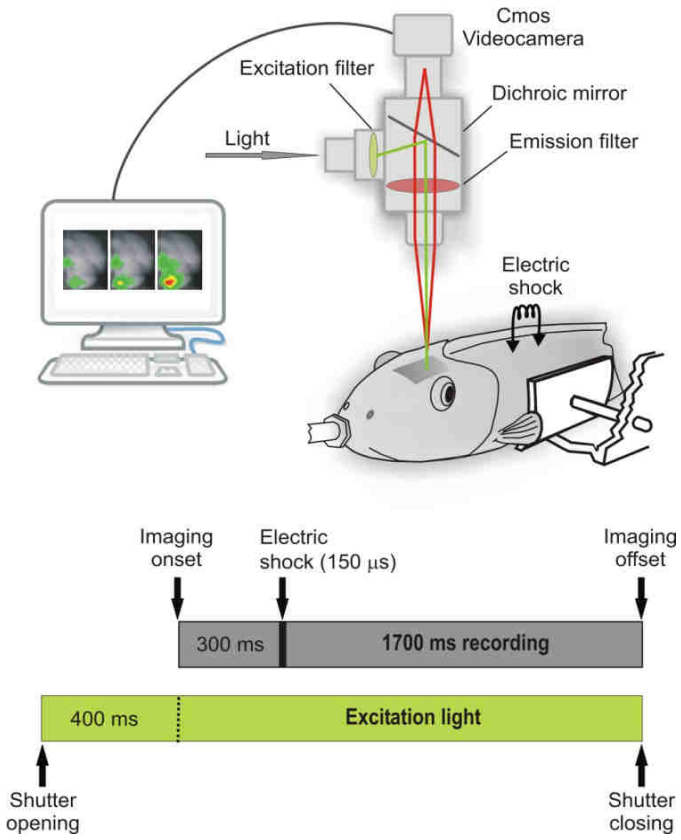


Fig 2.2. Voltage sensitive dye (VSD) imaging setup and stimulation sequence. On the top Schematic representation of the experimental setup showing the animal preparation and the epifluorescence microscope. On the bottom Schematic representation showing the aperture and closing temporal sequence of the shutter in which the excitation light (530nm, green bar) was present, the recording period (grey bar), and the stimulation.

by presenting a somatotopic representation of the body surface (Ocaña et al., 2016). Approximately 85 ms after the noxious stimulation, a second depolarization appeared in the bulge localized in the most anteromedial region of DmII (asterisks in Figures 2.1). The analysis of the spatiotemporal pattern of the optical signal in the two depolarized regions revealed biphasic responses characterized by a rapid depolarization, a peak of activity, a slow hyperpolarization, and finally, an optical signal similar to that observed during the pre-stimulus period (see curves in Figure 2.3 B). However, a more detailed analysis showed interesting differences in the characteristics of the activity

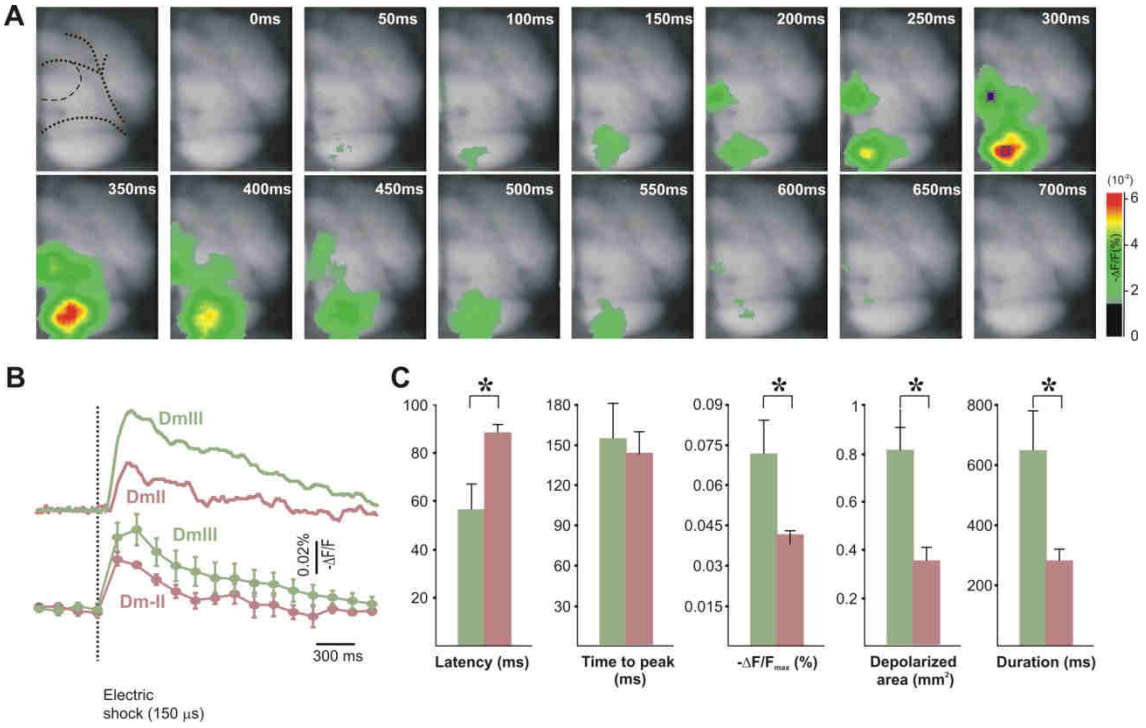


Fig 2.3. Voltage sensitive dye (VSD) imaging of pallial sensory activity evoked by noxious stimulation. A. Representative example of the spatiotemporal pattern of pallial activation following noxious stimulation. The percentage fractional fluorescence change for each pixel is color-coded as indicated in the scale bar. The time elapsed from the stimulus onset is indicated in each frame. **B**. Curves showing the time course of the VSD signal measured at the region with the maximal activation (3 x 3 pixels square; black asterisk) for the experiment represented in A (top), and averaged across animals (bottom; n = 6). The somatosensory stimulation depolarized two regions with different activity pattern (DmII and DmIII; see text). The pink and green curves show the time course of the VSD response recorded in DmII and DmIII for the example showed in A, and averaged across animals, respectively. The dotted line marks the stimulus. **C.** Histograms showing the mean (n=6) for the main parameters of the responses evoked in DmII (green) and DmIII (pink).

evoked in DmIII and DmII (Figure 2.3 C). Thus, whereas latency was significantly shorter in DmIII than in DmII ($t_{(10)}$=-3.912, $p < 0.05$), the amplitude and the duration of the response, and the extension of the depolarized area were higher in DmII than in DmIII ($t_{(10)}$ = -2.404, $p < 0.05$; $t_{(10)}$ = 2,725, $p < 0.05$; $t_{(10)}$ = 6.185, $p < 0.05$ for response amplitude, response duration and depolarized area, respectively). However, no significantly differences were observed in the time to reach the peak of activity between DmII and DmIII ($t_{(10)}$ = 0.650, $p > 0.05$).

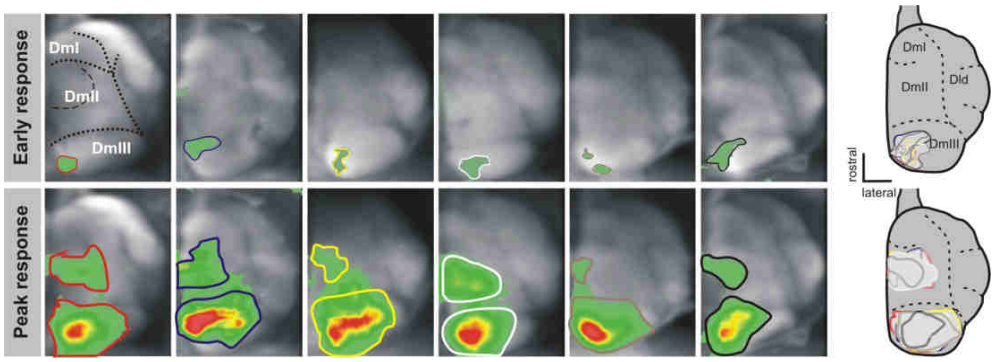


Fig 2.4. Consistency of the evoked response across animals. Optical images of the early (25% of the peak value) and peak responses evoked by noxious stimulation in 6 representative animals. The schematic representations of the telencephalon surface show the superimposed contour plots of the early and peak responses (n = 6). The outlines of the activated areas were aligned using the telencephalic borders and the caudal and ypsiloniform sulci as landmarks. Although the shape of the contour plots for differed across animals, the relative position of the responsive areas was highly consistent. Scale bar = 500 μm**.**

These differences regarding the amplitude, duration and latency between DmIII and DmII depolarization lead us to consider that DmIII plays a primary role in the processing of the sensory aspect of noxious or pain stimulation and on the contrary, that DmII might be associated with the processing of fearful or unpleasant attributes of the stimulus, as occurs to the amygdala and limbic structures of the mammalian brain (Apkarian., 2005; Lannetti, Domenico & Mouraux, 2010; Vierck, 2007). In fact, it has been proved that the most ventromedial region of Dm in teleosts (the bulge in DmII) classically called Dmv (Nieuwenhuys, Bauchot, & Arnoult, 1969), is critically involved in behaviors with significant emotional value (Broglio et al., 2005; Desjardins & Fernald, 2010; Lau et al., 2011; Martín et al., 2011; Portavella, Torres, & Salas, 2004), so that this region has been proposed as the homologue of the mammalian pallial amygdala (Braford, 1995; Wullimann & Mueller, 2004; Wullimann & Rink, 2002).

To test whether the activity in DmII is certainly involved in the unpleasant attributes of a stimulus and taking into account that in fishes, as in mammals,

motor responses to painful stimuli include fast-starts reactions motivationally induced (Chandroo et al., 2004), we stimulated a group of four animals with electric shocks of different intensities (1, 3 and 5 mA, Figure 2.5) and analyzed the different motor behavioral responses and the areas activated in the dorsal telencephalic pallium by these stimuli. Initially, we recorded the characteristics (latency, amplitude, and velocity) of the motor escape responses produced by stimuli of different intensities and then, we paralyzed the animals with Flaxedil (Sigma-Aldrich) and recorded the pallial activity evoked by the same stimuli. We observed that low intensity stimulus did not evoke any motor response but evoked a slight depolarizing response restricted to DmIII (Figure 2.5). However, stimuli of moderate (3 mA) and high (5 mA) intensity evoked fast-start escape responses of low and high amplitude respectively (Figures 2.5 B-D).

In both cases, a complex pattern of pallial activity similar to that described above was obtained (Figure 2.5 A). This activity included the initial depolarization of the DmIII, responsive to the sensory aspects of the painful event, followed by a depolarization of the DmII anteriomedial bulge, probably associated with the unpleasant and cognitive aspect of painful stimulation. The obtained responses spread on the same pallial regions that they did in the previous experience but the spatio-temporal pattern of the optical signal varied according to the shock intensity (Figure 2.6 A). The quantification of the main parameters of the evoked pallial responses across animals (Figure 2.6 B) showed that the activity in DmII and DmIII is stimulus-strength dependent as an increase of the stimulus intensity caused a significant augmentation in the amplitude of the response as well as in the extension of the depolarized area in

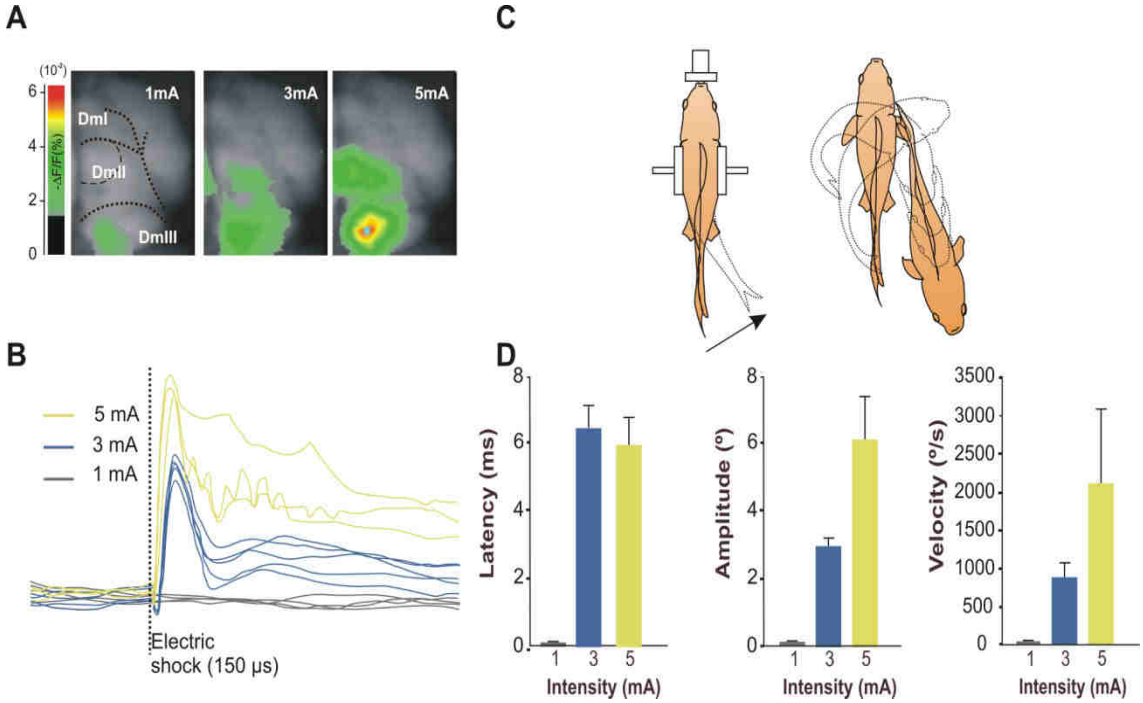


Fig 2.5. The spatiotemporal dynamics of the evoked response depends on stimulus strength. A. The frames show the peak responses recorded following stimulation with shock of different intensities. **B**. Curves representing the amplitude of the tail movement (scape response) evoked by the noxious stimulation. Note that low intensity stimulation did not evoke a scape response. **C.** Examples of the motor response evoked when the animal is fixed in the experimental chamber (left) and the scape response evoked in a freely moving animal. **D.** Histograms showing the mean effects of stimulus strength variation on the parameters of the tail movements evoked by noxious stimulation (n = 4).

DmIII ($F_{(2,11)} = 11.233$, $p < 0.05$ and $F_{(2,11)} = 18.452$, $p < 0.05$, for amplitude and depolarized region, respectively) and in DmII ($F_{(2,11)} = 13.249$, $p < 0.05$ and $F_{(2,11)} = 15.118$, $p < 0.05$, for amplitude and depolarized region, respectively).

Taken together, our results suggest that the electric shock that activated the bulge located in the anteromedial region of DmII is a painful stimulus that elicits a fast-escape response in all the animals tested.

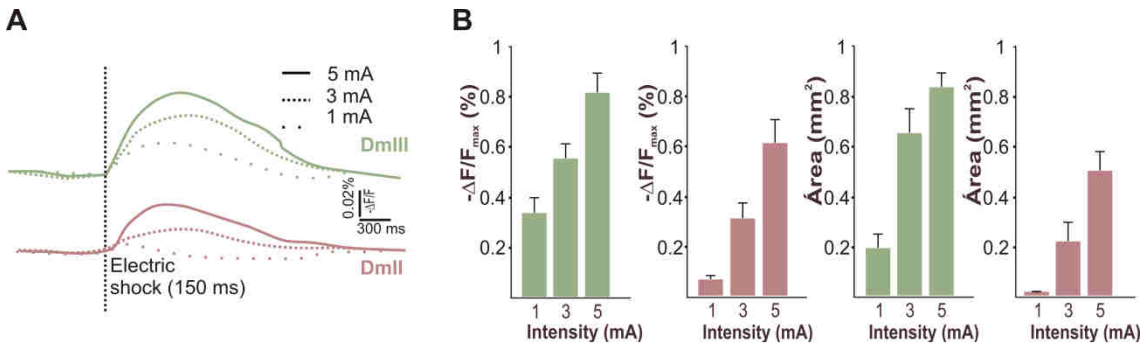


Fig 2.6. Parameters of the recordered optical signal in DmII and DmIII. A. The curves depict the time course of the optical signal evoked by the different stimuli at the epicenters in DmII and DmIII **B.** Histograms showing the mean effects of stimulus strength variation on the parameters of the pallial evoked responses (n = 4).

2.4. Discussion

The results in this experiment indicate that, in functional terms, the goldfish Dm region can not be considered a single pallial division. Otherwise, in vivo voltage-sensitive dye imaging reveals that it could be functionally subdivided in, at least, three different areas, DmI, DmII and DmIII, whose locations and borders clearly coincide with conspicuous cytoarchitectonic and histochemical landmarks. Furthermore, the functional profiles of activity evoked in each one of these three subdivisions by a transcutaneous nociceptive somatosensory stimulus were markedly different. Thus, no evoked activity was recorded in DmI, being this area largely unresponsive to nociceptive stimulation, but does it was in DmIII and DmII, although their functional patterns of activation differed clearly between them. Specifically, whereas DmIII seemed to process the sensory aspects of the noxious stimulation, DmII seemed to codify the fearful or unpleasant attributes of that stimulus.

Present results, and those obtained previously in our laboratory (Ocaña, 2009) suggest that DmIII might be considered as the primary somatosensory area of the goldfish pallium and therefore, comparable to the primary somatosensory area in the dorsal pallium or isocortex of other vertebrates. DmIII seems to be unimodal, that is, it contains neurons responding exclusively to primarily somatosensory information independently of the fact that this information is derived from non-painful or nociceptive mechanosensory stimulation (Ocaña, 2009). In addition, their responses present the shorter latencies of all the pallial areas. Moreover, this area contains and ordered somatotopic map of the body surface (Ocaña, 2009). These functional characteristics strikingly resemble to those of the primary somatosensory area of the dorsal pallium or isocortex of land vertebrates. Some hodological studies carried out in teleost fish have shown that certain specific areas of Dm receive somatosensory and octavolateral inputs from the lateral nuclei of the preglomerular complex (Ito & Yamamoto, 2009; Murakami, Ito, & Morita, 1986; Murakami, Fukuoka, & Ito, 1986; Northcutt, 2006; Striedter, 1991; Yamamoto & Ito, 2005). This ascending pathway is strikingly similar to the ascending somatosensorial one [dorsal column nuclei - inferior colliculus (torus semicircularis) - dorsal thalamus - somatosensory area of the dorsal pallium] described for amniotes (Bruce, 2007; Cordery & Molnár, 1999; Desfilis, Font, & García-Verdugo, 1998; Desfilis, Font, Belekhova, & Kenigfest, 2002; Funke, 1989a, 1989b; Hall, Foster, Ebner, & Hall, 1977; Hoogland, 1982; Korzeniewska & Güntürkün, 1990; Künzle & Schnyder, 1983; Pritz & Stritzel,

1987; Schneider & Necker, 1989; Siemen & Künzle, 1994; Wild, 1989; Wild, 1987, 1997).

The present results also show that the functional pattern of activity of DmII was significantly different to that of DmIII. The activity in DmII seemed to be evoked exclusively by noxious mechanosensory or painful electrical stimulation but not by mild non-painful somatosensory stimulation. In addition, the activity in DmII showed longer latencies and higher intensity levels and durations than in DmIII (Figures 2.3 C; Ocaña, 2009). Moreover, the amplitude of the responses in DmII increased when the noxious or painful properties of the stimulus increased (see Figure 2.5 and 2.6). In addition, DmII, unlike DmIII, seemed not to present a somatotopically ordered representation of the body surface, nor to receive and process unimodal sensory inputs, but multimodal ones. In this sense, besides responding to aversive somatosensory stimulation, DmII also activates by auditory and gustatory stimuli during fear classically conditioning (Ocaña, 2009; Uceda, 2015). Finally, the activation of DmII is related to the generation of defensive-escape behaviors and emotional-like responses (Figure 2.5). All these functional characteristics closely parallel those described for the pallial amygdala of the land vertebrates (Broglio et al., 2005, 2011). In fact, based on developmental, molecular, connectional, and functional evidence, it has been proposed that at least a part of the Dm is homologous to the pallial amygdala of amniotes (Braford, 1995; Broglio et al., 2005; Butler, 2000; Marino-Neto & Sabbatini, 1983; Nieuwenhuys & Meek, 1990; Northcutt, 1995; Portavella, Salas, Vargas, & Papini, 2003; Salas et al., 2006). As a whole, the present functional results suggests that, like the mammalian

amygdala, the area DmII of the teleost fish pallium is involved in processing nociceptive somatosensory information and in the generation of emotional responses to noxious stimulation. In teleost fish, nociceptive ascending pathways from the medullar dorsal root ganglions, the trigeminal nucleus and the vagal nuclei, ascend through the spino-thalamic tract and are relayed to the parabraquial nucleus, the nucleus of the tractus solitarius, the hypothalamus, and the ventral thalamic area to finally reach Dm (Chandroo et al., 2004; Wulliman and Rink, 2002; Yamamoto et al., 2007). In addition, previous studies have recorded somatosensory evoked potentials in specific brain regions to putative noxious stimuli in fish (Dunlop & Lamin, 2005; Ludvigsen et al., 2014; Nordgreen et al., 2007). Similarly, in mammals, ascending somatosensorial pathways convey nociceptive information to the primary somatosensory area of the dorsal pallium or isocortex (S1) which contributes to the processing of the sensory and perceptual dimensions of the painful stimulation, for example, discriminating the location or the intensity of the stimulus (Gebhart et al., 2009; Lannetti, Domenico & Mouraux, 2010; Vierck, 2007). In contrast, the nociceptive information conveyed by these ascending pathways to the amygdala and other pallial areas like the insular cortex (Vierck, 2007) are involved in the neural processing of the affective and emotional dimensions of pain and in the orchestration of the autonomic and motor responses associated with the pain experience (Bushnell et al., 1999; Duquette, Roy, Leporé, Peretz, & Rainville, 2007; Sah, Faber, Armentia, & Power, 2003; Shih et al., 2008).

To more precisely determine the morphofunctional demarcation of the pallial surface in goldfish and particularly to clarify the involvement of DmII and

DmIII in the emotional processing, an exhaustive mapping of the telecephalic pallium of goldfish was performed using electrophysiological stimulation.

3. EXPERIMENT 2. Mapping of the areas of the goldfish telencephalic pallium involved in triggering escape and defensive, emotional-like motor and visceral responses

3.1. Introduction

In mammals, the electrical stimulation of the amygdala produces complex patterns of behavioral and autonomic responses that highly resemble fear states (Davis & Whalen, 2001). For example, the stimulation of particular sites in the amygdala produces bradycardia as well as motor responses and patterns of behavior like freezing or escape. In addition, the activation of a single site in the amygdala evoked, in turn, the activation of a variety of target areas to which it projects that are, by themselves, critical for the generation of each one of the specific components of fear expression, as well as the experience of fear (LeDoux, 2000). It has been reported that focal electrical stimulation of Dm in free-swimming teleost fish elicits arousal, and defensive and escape responses (Savage, 1971). In fishes, startle and escape responses elicited by threatening or noxious stimulation are accompanied by visceral changes in heart rate (mainly, bradycardia) and in ventilatory rate. In fact, bradycardia has been used as a physiological indicator of fear states (Dunlop et al. 2005).

To confirm the findings obtained in the previous optical imaging recording experiment (Experiment 1), in the present experiment we used electrical microstimulation mapping to systematically explore the dorsal surface of the telencephalic pallium of goldfish in order to identify the pallial areas whose

stimulation produce escape, emotional-like responses and associated visceral responses, and to correlate these functionally identified areas with macroanatomical, cytoarchitectural and histochemical landmarks. In particular, in this experiment we tested whether the intracerebral electrical stimulation of the areas DmII and DmIII produces escape or fearful-like responses.

3.2. Methods

The experimental procedures were performed in accordance with Directive 2010/63/UE of the European Community Council and Spanish legislation (R.D. 53/2013).

3.2.1. Surgery

Goldfish (*Carassius auratus*) 11-13 cm in length (n = 7), measured from the mouth to the beginning of the caudal fin, were obtained from the vivarium of the University of Seville. 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 (see figure 3.1). 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. 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

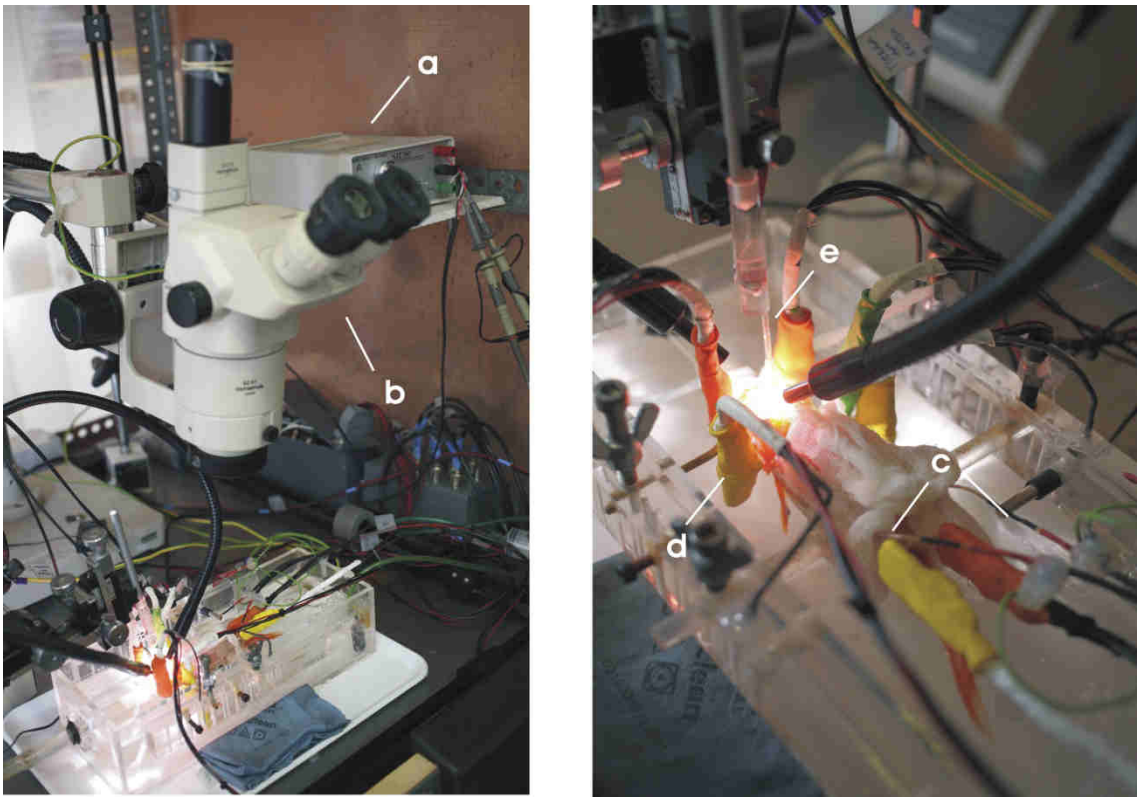


Fig 3.1. Dorsal view of the experimental preparation. On the left a general view of the preparation is shown and on the right a detail of the animal in the experimental chamber can be observed. a, constant-current stimulus isolation unit; b, binocular microscope; c Heart rate electrodes signal; d, Hall-effect sensors; e, electrode.

was removed to expose the telencephalon. Experiments started at least 2 hrs after surgery when the animal had completely recovered from anesthesia. The reappearance of spontaneous breathing, eye and fins movements were considered signs of recovery.

3.2.2. Movement Recording System by Hall-effect sensors

Movements of the caudal fin were registered using a movement recording system based on Hall-effect sensors (Rodríguez, Salas, Vargas, & Torres, 2001; Salas, Torres, & Rodríguez, 1999). Magnets weighing 25 mg

(dimensions 2 x 2 x 1 mm, width x height x depth) were affixed to the caudal fin of the animal by means of surgical suture and cyanocrilate glue. These magnets produce a roughly uniform magnetic field that could be detected by the high-sensitivity Hall-effect sensors (Honeywell). The Hall-effect sensors produce an output voltage directly proportional to the intensity of the magnetic field to which they are exposed, and thus, inversely proportional to the distance between the magnet and the sensor. The movements of the fish caudal fin produced displacements of the magnets relative to the Hall-effect sensors and the alterations produced in the magnetic fields can be detected by the recording system and transformed into a precise measure of the position of the fish appendages. The analog signal produced by the Hall-effect sensors was acquired by a CED Power1401 intelligent laboratory interface (CED, Cambridge) running Spike 2 software and analyzed off-line.

3.2.3. Electrocardiographic recording

To monitor heart rate, bipolar silver cup electrodes were placed in the skin under the pectoral fins on both sides of the ventral thoracic cavity. The electrocardiographic (ECG) signal was amplified (50,000x), band-pass (0.1-50 Hz) filtered by a differential amplifier (NL905, Digitimer), and acquired by a CED Power1401 intelligent laboratory interface running Spike 2 software (CED, Cambridge).

3.2.4. Electrical microstimulation mapping

Glass-insulated stainless steel wire (25 µm in diameter) microelectrodes with impedances ranging from 300 to 500 kΩ were used for electrical

microstimulation of the pallium. The electrical stimulation consisted of a train of pulses delivered by a constant-current stimulus isolation unit (pulse train: 50 Hz; train duration: 2 s; pulse width: 2 ms; Neurodata, SIU 90). Current intensity varied or was maintained fixed according to the nature of the study. In the particular case of the study on the variation of stimulus parameters on motor escape responses, wide ranges were used for frequency (10–100 Hz), train duration (0.5–3 s), and intensity (5-50 μA).

Electrode penetrations were made perpendicular to the pallial surface and stimulation delivered at a depth of 50 μm, measured from the pallial surface. In the experiments carried out to map the responses along the midline of the pallium stimulation was delivered at a depth of 50, 250 and 450 μm. For each stimulation site, the initial current intensity was 5 μA; if no response occurred, it was increased until a motor o bradycardia response was elicited (up to 100 μA). Threshold current was defined as the lowest current reliably evoking a motor or bradycardia response. At least 5 min were left between two electrical microstimulation deliveries. Different penetrations were made with the same microelectrode, which was advanced with a microdrive. The position of the electrode on the pallial surface was always monitored under visual control. The coordinates of each stimulation site in the anteroposterior and mediolateral axes and its position respective to the pallial sulci and other macroanatomical landmarks in the telencephalic surface was registered. In addition, electrolytic lesions (3-5 μA for 10 seconds) were made in sites of interest for reconstruction of the electrode positions. Non-visible electrode tracts were reconstructed on

the basis of their relative positions to the electrolytic lesions and the identified electrode tracts.

3.2.5. Heatmaps of escape and bradycardia responses

In the present experiment two types of microstimulation mapping sessions were carried out. In threshold mapping sessions, we aimed to identify the pallial areas from which escape and bradycardia responses could be evoked at minimal current intensity. To generate the pallial threshold maps for escaping and bradycardia responses, the location of the stimulation sites were reconstructed over an image of the dorsal surface of the telencephalic hemisphere or a sagittal section of the goldfish telencephalon. In the case of the threshold heat maps of escape responses, the minor effective current intensity to evoke an escape response was noted for each one of these points. Heat maps were then generated using the stimulation sites coordinates and threshold intensity data in Sigmaplot 11.0. The range for the color scale was set between 0 to 50 μA. In the case of threshold heat maps of bradycardia responses, the percentage of bradycardia was calculated in each one of the stimulated site as [(X-Y)/X] x 100, where Y was the number of heart beats during the 5 or 10 sec that followed the electrical microstimulation of the pallium, and X was the number of beats in a 5 or 10 sec baseline period immediately preceding the stimulus. A bradycardia response was considered when the percentage of bradycardia was higher than 15%. The minor effective current intensity to evoke bradycardia responses was noted for each of the stimulated sites. Then heat maps were generated using the stimulation sites coordinates and threshold intensity values.

In the second type of experimental sessions, we aimed to establish how the amplitude of escape and bradycardia responses varied depending of the stimulated site. For this purpose, the intensity current remained constant for all stimulated sites in the pallial surface and the variation in the amplitude of motor and bradycardia responses were registered. Thus, fixed current intensity heat maps where created using the coordinates of each stimulated site and the amplitude of bradycardia responses (percentage of bradycardia), the amplitude of tail movement or the number of tail beats.

3.2.6. Statistical data analysis

Data were analyzed with one-way analyses of variance, adjusted for multiple comparisons using a Bonferroni correction. Group data is reported as mean ± SEM. In all tests, $P < 0.05$ was considered to indicate statistical significance. Statistical computations were performed with SPSS software.

3.3. Results

In a first experiment we tested whether escape and bradycardia responses could be elicited by electrical microstimulation in different regions of the goldfish pallium. For this purpose, the whole pallial dorsal surface was stimulated at a depth of 50 µm and the threshold current necessary to evoke escape or bradycardia responses were noted. Examples of the responses obtained after the microstimulation in a single site is shown in Figure 3.2. The areas in which stimulation evoked escape movements and bradycardia

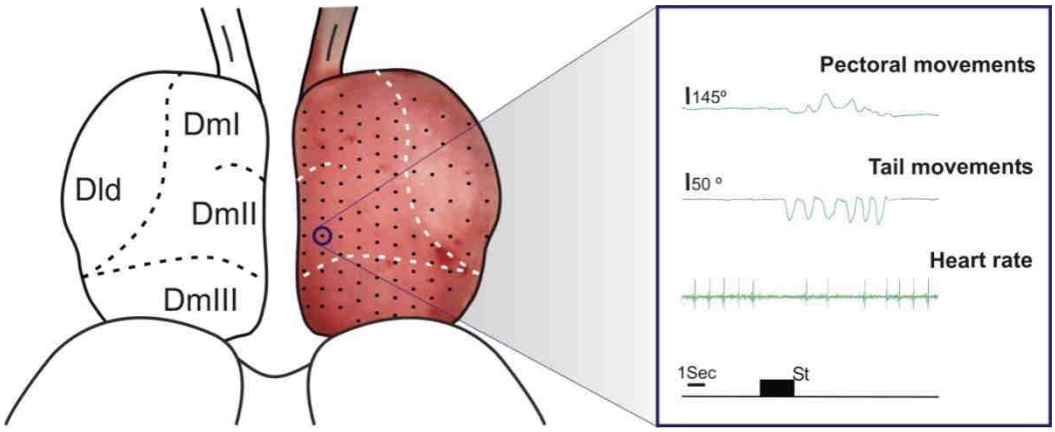


Fig 3.2. Representative example of the tail and pectoral movements and heart rate response evoked after the stimulation of DmII. On the left, a schematic representation of a dorsal surface of goldfish telncephalon with a real dorsal view of a experimental animal, showing the stimulation sites. On the right, examples of the tail, pectoral and heart rate responses after the stimulation of one representative DmII site marked in the schematic representation. The black rectangle represent the stimulus duration. Abbreviations: Dl, lateral division of the area dorsalis; Dml, subdivision I of the medial division of the area dorsalis; DmII, subdivision II of the medial division of the area dorsalis; DmIII, subdivision III of the medial division of the area dorsalis.

responses are indicated in Figure 3.3 with the lowest threshold in red (5–10 µA).

The results showed that the lowest threshold electrical microstimulation eliciting escape-like responses, characterized by tail beats and bradycardia, were obtained in DmII. Interestingly, it has been observed that free-moving goldfish with electrodes implanted in this pallial region exhibited avoidance responses characterized by rapid and abrupt displacements from their initial position (see experiment 3). Although stimulation in some sites of Dml and DmIII also evoked escape-like responses, this type of coordinated movements were consistently evoked at low thresholds when stimulation was delivered in sites located in DmII (Figure 3.3).

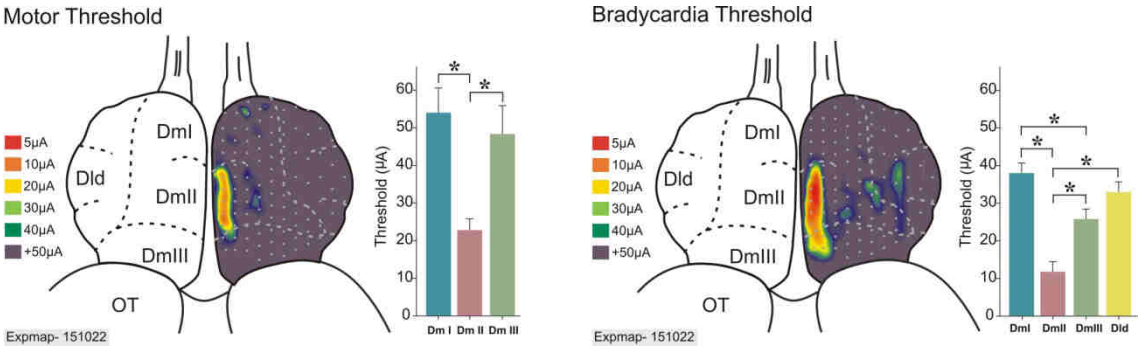


Fig 3.3. Threshold maps in surface for escape-like and bradycardia responses. Schematic view of the dorsal surface of the goldfish telencephalon showing the stimulation sites and the representative color-code contour plots of the thresholds for motor (left) and bradycardia (right) responses in an experimental animal. Bar graphs show the thresholds to evoke motor (left) and bradycardia (right) responses on the surface of each one of the pallial areas analyzed (blue: DmI, pink: DmII, green: DmIII and yellow: Dld). The five stimulation sites with the lower current values in each zone were used to represent the motor and bradycardia thresholds. Note that no motor response was found in Dld. On the contrary, note that the lower thresholds to evoke motor and bradycardia responses were found in DmII. Asterisk denote statistical signification. Abbreviations: Dld, dorsal subdivision of the lateral division of the area dorsalis; DmI, subdivision I of the medial division of the area dorsalis; DmII, subdivision II of the medial division of the area dorsalis; DmIII, subdivision III of the medial division of the area dorsalis; OT, optic tectum.

To determine whether the threshold for generating escape-like responses varied between the three different subregions of Dm (DmI, DmII and DmIII), we analyzed the current intensities necessary to evoke this particular response in these areas. A one-way ANOVA indicated that there were significant differences in the current intensity needed to evoke escape movements in the different subdivisions of Dm ($F_{(2,12)} = 12.72$; $P < 0.05$). Post-hoc analyses revealed that threshold values in DmII were significantly lower than those observed in DmI and DmIII (both Ps < 0.05) and that no differences were observed between the current intensity needed to evoke the escape responses in DmI and DmIII ($P > 0.05$) (Figure. 3.3).

The threshold heat map generated for bradycardia responses is shown in Figure 3.3. In this case, our data also suggests that DmII has a critical role in

generating this particular component of the goldfish nocifensive or fear response, as the lowest thresholds were observed in this pallial region. In addition, a relative low threshold region for generating bradycardia responses was also localized in the dorsal subdivision of the lateral division of the goldfish pallium (Dld). However, the electrical microstimulation of this pallial region did not evoke escape-like motor responses (see Figure 3.3), which suggest that the bradycardia responses observed in Dld could not be related with a genuine fear response. The comparison of the intensity current needed to evoke bradycardia responses from the different subdivisions of Dm (DmI, DmII and DmIII) and Dld showed significant differences ($F_{(3,16)}$ = 22.802; P = 0). Post-hoc analyses revealed that the DmII region showed significant lower threshold values than those observed in DmI, DmIII and Dld (all Ps < 0.05). Finally, there were no significant differences between the current intensities needed to evoke bradycardia responses in Dld and those needed in DmI or DmIII (both Ps > 0.05) (Figure 3.3.).

In a next series of microstimulation experiments we aimed to study how the magnitude of the escape and the bradycardia responses varied along the different subdivisions of the pallium when a fixed microstimulation current intensity value was used. Thus, we used two different intensity values in each animal and studied how the site of stimulation influenced or determined the amplitude of the evoked response. Figure 3.4 shows the heat amplitude response maps obtained in a particular experiment after the stimulation of the whole dorsal pallial surface at a depth of 50 µm.

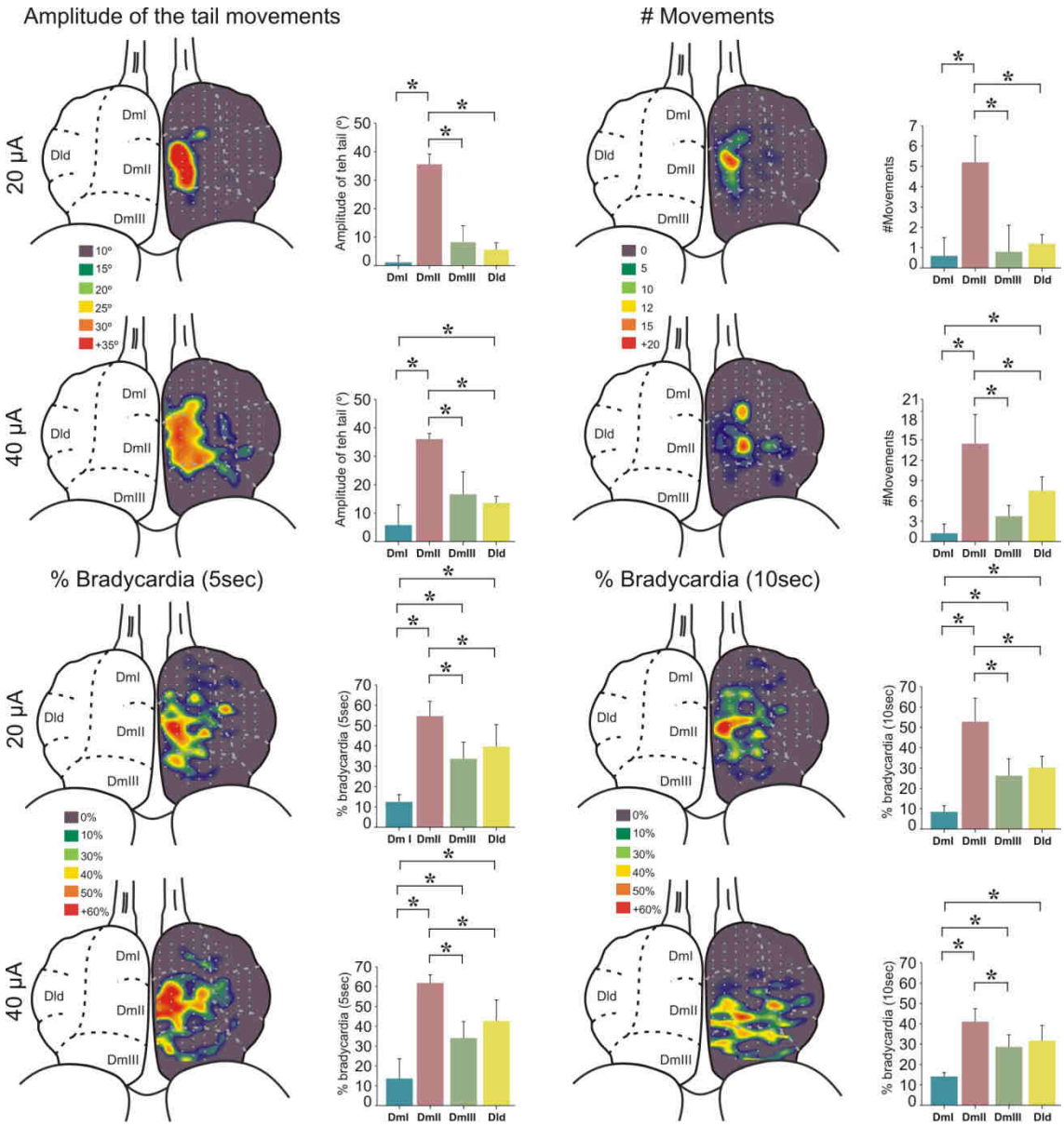


Figure 3.4. Maps in surface of escape-like and bradycardia responses evoked at fixed current intensities. Schematics of the dorsal surface of the goldfish telencephalon showing color-code contour plots representing the amplitude and the number of the tail movements, and the bradycardia percentage in the 5 and 10 seconds following stimulation when two different intensity values (20µA or 40µA) were used. Note that the maximal responses are evoked when DmII is stimulated. Bar graphs show the amplitude of the responses after the stimulation on the surface of each one of the pallial areas stimulated (blue: DmI, pink: DmII, green: DmIII and yellow: Dld). The five responses with higher amplitude in each zone were used to represent the amplitude value Asterisk denote statistical signification. Abbreviations: Dld, dorsal subdivision of the lateral division of the area dorsalis; DmI, subdivision I of the medial division of the area dorsalis; DmII, subdivision II of the medial division of the area dorsalis; DmII, subdivision III of the medial division of the area dorsalis.

To determine the low intensity value to be used in each one of this experiment, we determined the current intensity threshold needed to evoke an escape-like response by microsimulation in DmII (including its motor and bradycardia components) and then 15% of this value was added to be sure that suprathreshold values were used. The high intensity value to be used was defined as the double of low intensity values. The areas from which higher amplitude responses were obtained are shown in Figure 3.4, with the high amplitude responses in red. One-way ANOVA analyses were conducted to compare the amplitude of the responses evoked in the different pallial subdivisions with low and high intensity currents. The results obtained with low and high intensity current were very similar and statistical significant differences were observed in the amplitude of tail movements ($F_{(3,16)} = 83.553$, $P < 0.05$ and $F_{(3,16)} = 22.891$, $P = 0$ for low and high intensity currents respectively), number of tail movements ($F_{(3,16)} = 21.621$, $P < 0.05$ and $F_{(3,16)} = 24.664$, $P < 0.05$; for low and high intensity currents respectively), percentage of bradycardia in an interval of 5 seconds ($F_{(3,16)} = 24.589$, $P < 0.05$ and $F_{(3,16)} = 26.635$, $P < 0.05$) and percentage of bradycardia in an interval of 10 seconds ($F_{(3,16)} = 27.431$, $P < 0.95$ and $F_{(3,16)} = 18.379$, $P < 0.05$). As expected, post-hoc analysis showed that the DmII region, in particular its more medial subregion, can be considered as a core region for generating escape and fear-like responses, as the responses evoked by either low or high intensity electrical microstimulation of this pallial area were always of a significantly higher amplitude than those evoked from DmI, DmII or Dld (All Ps < 0.05; see graphs in Figure 3.4). Only an exception was found to this general rule; the percentage of bradycardia (measured in an

interval of 10 seconds) evoked by high intensity stimulation in DmII and Dld was not significant different (P > 0.05). By contrast, rostral to the DmII-DmI border (rostral sulcus of Dm), the amplitude of the evoked responses decreased abruptly, and even high current intensity stimulation (40 µA) was unable to evoke noticeable escape-like or bradycardia responses. Taking together, the results above described indicate that fear-like responses, with motor (escape-like) and visceral (bradycardia) components, can be elicited by electrical microstimulation of Dm, in particular the present results suggests that DmII can be considered a core region for generating this type of fear-like responses.

Finally, we aimed to determine whether the current intensity needed to evoke an escape-like response or whether the amplitude of the responses varied along the dorsoventral axis of the different subdivisions of the Dm region. Thus, electrode penetrations perpendicular to the pallial surface were made in a parasagittal section (from rostral to caudal part) separated 100 µm from the midline (see Figures 3.5 and 3.6). We stimulated at three different depths (50 µm, 250 µm and 450 µm); thus avoiding the subpallial region, whose more dorsal border is located approximately 500 µm below the pallial surface. The depth distribution of low threshold activation is represented in Figure 3.5, and the amplitude of the evoked movements in Figure 3.6.

One-way ANOVA analyses indicated that there were not statistical significant differences along the dorsoventral axis of the three different subdivisions of Dm for the thresholds needed to evoke motor ($F_{(2,20)} = 0.932$, $P > 0.05$; $F_{(2,69)} = 0.998$, $P > 0.05$; $F_{(2,21)} = 0.102$, $P > 0.05$; for DmI, DmII and DmIII, respectively) and bradycardic responses ($F_{(2,20)} = 0.932$, $P > 0.05$; $F_{(2,69)} =$

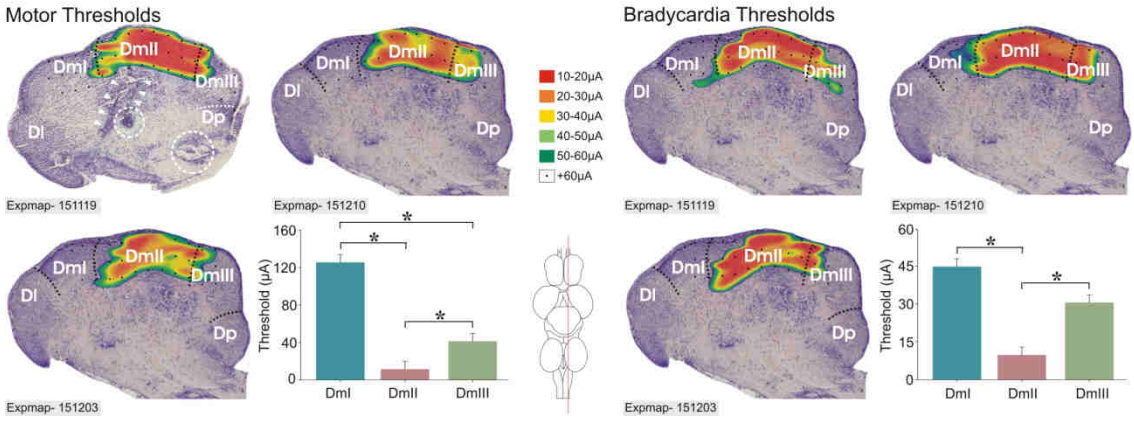


Figure 3.5. Threshold maps in depth for escape-like and bradycardia responses. Photomicrographs of a representative sagittal section of the goldfish telencephalon showing the stimulation sites and the color-code contour plots representing the threshold needed to evoke motor (left) and bradycardia (right) responses in three different animals. The inset representing a goldfish brain with a red line along its rostro-caudal axis illustrates the telencephalic section level in the medio-lateral axis. The picture of the map at the top left belongs to a real experimental goldfish and shows two different lesions (discontinuous white circles) and two microelectrodes traces (white triangles). The five stimulation sites with the lower current values in each one of the three zones of Dm were used to represent the motor and bradycardia thresholds in the bar graph. Note that the lower thresholds to evoke motor and bradycardia responses were found in DmII. Asterisk denote statistical signification. Abbreviations: Dl, lateral division of the area dorsalis; DmI, subdivision I of the medial division of the area dorsalis; DmII, subdivision II of the medial division of the area dorsalis; DmII, subdivision III of the medial division of the area dorsalis; Dp, posterior division of area dorsalis OT, optic tectum.

0.998, $P > 0.05$; $F_{(2,21)} = 0.102$, $P > 0.05$; for DmI, DmII and DmIII, respectively). In addition, the observation of the heat threshold maps reconstructed under sagittal sections of the goldfish telencephalon showed that the core of the functional identified subregion of Dm corresponds with a cytoarchitectonically well differentiated area delimited by macroscopic landmarks. In particular, the rostral sulcus that separates DmI from DmII (see again figure 2.1) can be considered as a milestone to identify the pallial region which requires the lowest current intensities to evoke a fear response. Rostral to the rostral sulcus, the thresholds increased abruptly, and even with current intensities above 100 µA no escape-like responses were elicited.

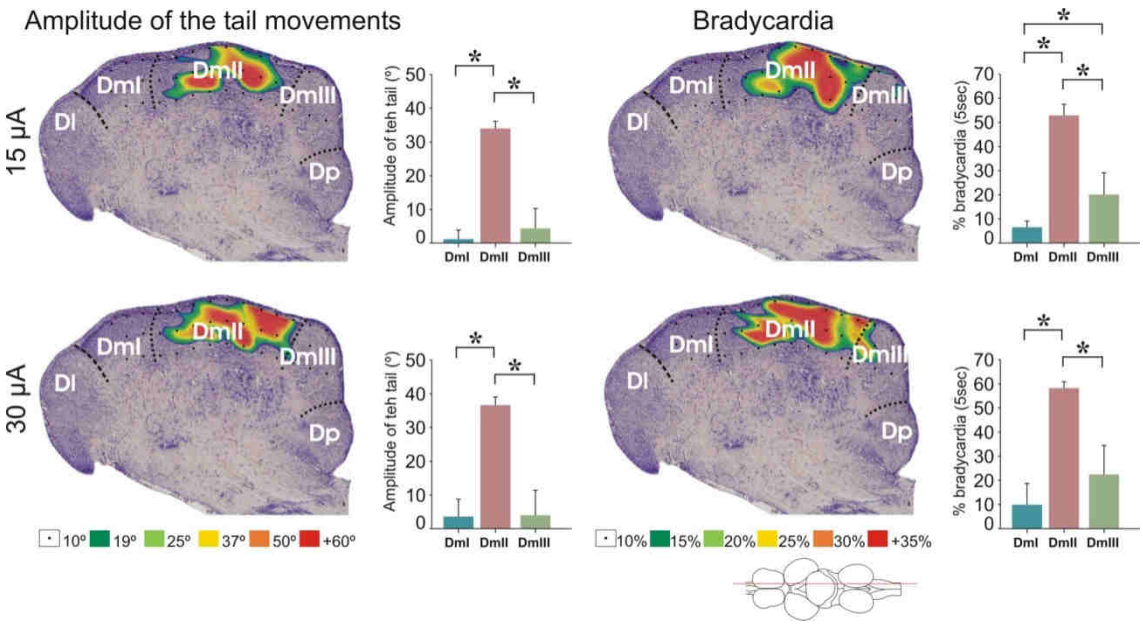


Figure 3.6. Maps in depth of the amplitude of the tail and bradycardia responses at fixed intensities. Photomicrographs of a representative sagittal section of the goldfish telencephalon showing the stimulation sites and the color-code contour plots representing the amplitude of the tail movements and percentage of bradycardia in the 5 seconds following stimulation when two different intensity values (15µA or 30µA) were used. The inset representing a goldfish brain with a red line along its rostro-caudal exe illustrates the telencephalic section level in the medio-lateral exe. Bar graphs show the amplitude of the responses after the stimulation in depth of each one of the pallial areas stimulated (blue: DmI, pink: DmII and green: DmIII). The five responses with the higher amplitude in each zone were used to represent the amplitude value. Asterisk denote statistical signification Abbreviations: Dl, lateral division of the area dorsalis; DmI, subdivision I of the medial division of the area dorsalis; DmII, subdivision II of the medial division of the area dorsalis; DmII, subdivision III of the medial division of the area dorsalis; Dp, posterior division of area dorsalis.

The amplitude heat maps (Figure 3.6) showed that the stimulation sites in which high amplitude escape-like and bradycardia responses were evoked were always located in DmII, independently of whether low or high intensity current values were used. In fact, a one-way ANOVA comparisons for the amplitude of the evoked responses in the three main divisions of Dm showed statistical significant differences for the amplitude of tail movements ($F_{(2,12)}$ = 105.082, $P < 0.05$ and $F_{(2,12)}$ = 62.122, $P < 0.05$; for low and high intensity,

respectively) and for the amplitude of the bradycardia responses ($F_{(2,12)}$ = 78.297, $P < 0.05$ and $F_{(2,12)}$ = 41.462, $P < 0.05$; for low and high current intensity values respectively). Again, DmII was the core region and post-hoc analyses revealed that the responses of higher amplitude were always evoked from this pallial region (all Ps < 0.05).

In a next step, we study whether the amplitude of the evoked response (evoked by low and high intensity currents) were related to the depth of the stimulated site. Thus, a one-way ANOVA per region was conducted in order to compare the amplitude of the evoked responses. When low intensity current was used, there were not statistical significant differences along the dorsoventral axis of the three different subdivisions of Dm in the amplitude of the escape-like ($F_{(2,8)}$ = 1.455, $P > 0.05$; $F_{(2,15)}$ = 1.391, $P > 0.05$; $F_{(2,6)}$ = 0.942, $P > 0.05$; for DmI, DmII and DmIII, respectively) and the bradycardia responses ($F_{(2,20)}$ = 0.932, $P > 0.05$; $F_{(2,69)}$ = 0.998, $P > 0.05$; $F_{(2,21)}$ = 0.102, $P > 0.05$; for DmI, DmII and DmIII, respectively). Similarly, there were not statistical significant differences along the dorsoventral axis of the three different subdivisions of Dm in the amplitude of the motor ($F_{(2,6)}$ = 0.942, $P > 0.05$; $F_{(2,8)}$ = 4.09, $P > 0.05$; $F_{(2,14)}$ = 0.734 $P > 0.05$; for DmI, DmII and DmIII, respectively) and bradycardia responses ($F_{(28)}$ = 1.410, $P > 0.05$; $F_{(2,15)}$ = 0.6, $P > 0.05$; $F_{(2,6)}$ = 1.110 $P > 0.05$; for DmI, DmII and DmIII, respectively) evoked with high current intensity stimulation.

Once identified the pallial region in which escape-like responses were evoked by means of electrical microstimulation, we focused on studying the effects of stimulus parameters on motor escape-like response features. Thus,

whether variations in current strength, pulse rate and duration of the electrical stimulus affected the amplitude and latency of tail movements, the duration of the response and the number of tail beats were studied. The quantitative effects of the systematic variation of the stimulation parameters on the evoked escape-like responses were determined at a single site in the core of DmII pallial region. Figure 3.7 illustrate a representative example of the quantitative effects of stimulation parameters on the escape response evoked from a single site. Changes in the parameters pulse rate and train duration influenced the evoked responses in a similar way. Thus, for a stimulus train of 2 seconds, and an intensity current 25% higher than the current intensity threshold, an increase in the pulse rate from 15 to 50 Hz produced a noticeable increase in the number of tail beats and the duration of the response. When pulse rate exceeded 50 Hz, a negative effect was found as the number of the tail beats and the duration of the response decreased. With regard to the amplitude of the movement, the increase in the pulse rate from 15 to 50 Hz led to a notable increase in this parameter. However, when pulse rate exceeded 50 Hz the amplitude of the evoke response reached a plateau level. On the other hand, the increase in the pulse rate from 15 to 50 Hz, resulted in a notable decrease in the latency of the evoked response, whereas increasing the pulse rate above of 50 Hz had no a clear effect on the latency, suggesting that the maximal effect was reached at this frequency value. As indicate for the pulse rate variations, when the duration of the train of stimulus was varied (with constant pulse rate and current intensity) an increment in the number of tail beats, amplitude of tail movements and duration of the movements was observed. Once the train duration exceeds

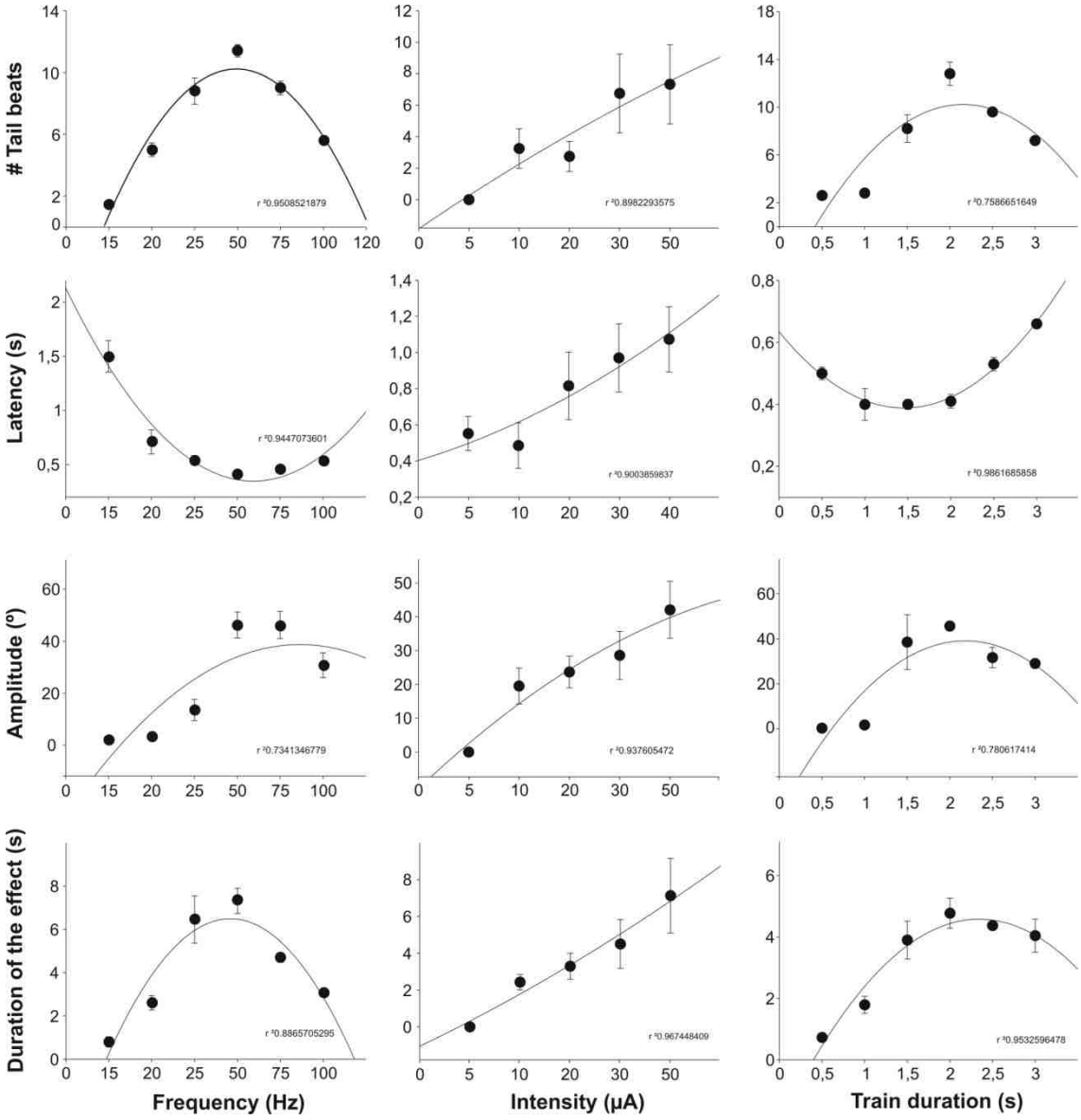


Fig 3.7. Effects of the variation of the stimulation parameters on the tail movement characteristics in DmII. The represented data are based on the movements evoked by the stimulation in a single stimulation site of the DmII telencephalic region for a single animal. For each graph, each point represents the average of 5 measurements. The column on the left represents the effects of pulse rate (frequency) variation on number of movements, latency, amplitude, and movement duration. Current intensity and stimulus duration were fixed at the threshold level plus 20% and 2 seconds, respectively. The column on the middle represents the effects of current strength (intensity) variation on number of movements, latency, amplitude, and movement duration. Frequency and stimulus duration were fixed at 50Hz and 2 seconds, respectively. The column on the right represents the effects of the variation of the stimulus duration on the number of movements, latency, amplitude, and movement duration. Frequency and current intensity were fixed at 50 Hz and the threshold level plus 20%, respectively. Changes in the tail movement characteristics were represented by line regressions.

2 seconds a detrimental effect was found and a decrement in the parameters above described was observed. In the case of the latency, we found that a train of 1.5 or 2 seconds was the most effective stimuli as increments in the duration above these values resulted in an increment in the latency of the response. Finally, the effect of the current intensity in the parameters here studied was different that those described for the pulse rate and train duration variations as the increase in current intensity from 5 to 50 μA led to a clear increment in the number of tail beats, latency, amplitude and duration of the evoked response.

3.4. Discussion

In the present experiment we systematically mapped the dorsal surface of the telencephalic hemispheres of goldfish by means of focal electrical microstimulation to identify the pallial areas evoking escape-like emotional responses. The results in this experiment show that DmII is a pivotal neural centre for the generation of defensive emotional responses. It has been previously reported that the electrical stimulation of the telencephalon of goldfish produces arousal reactions and negative reinforcement, and that the lowest threshold sites are located postero-medially the telencephalic hemispheres (Boyd & Gardner, 1962; Quick & Laming, 1988; Savage, 1971).

The present results show that intracerebral electrical microstimulation of DmII triggered behavioral (motor) responses as immobility or escape and fearful responses, accompanied by physiological (visceral) responses as heart rate and opercular beat rate changes that closely resemble naturally-induced fear integrated responses. The lowest thresholds of intracerebral microstimulation

needed to elicit fear-like motor responses, including escape, and visceral responses consisting in bradycardia and ventilatory rate changes were consistently found in the DmII area. Very low intensity electrical microstimulation in DmII elicited arousal-like responses characterized by a period of immobility accompanied of bradycardia and opercular beating cessation (unpublished observations; data not showed in the present work). However, as the current intensity increased, coordinated motor responses of the dorsal, pectoral and caudal fins of increasing amplitude, resembling escape responses, were evoked (see figures 3.3, 3.4, 3.5 and 3.6). These escape-like responses were accompanied of visceral responses as bradycardia, heart rate acceleration, and significant decreases or increases in the opercular beat rate. Changes in heart and ventilatory rate have been considered a robust and reliable indicator of pain experience in teleost fish (Reilly, Quinn, Cossins, & Sneddon, 2008; Sneddon, 2003b, 2009). It has been previously described that intradermal administration of noxious chemical agents, as well as painful electrical stimulation, produces heart and gill ventilation rate alterations in fish (Reilly et al., 2008; Sneddon, 2003b). The changes in heart and ventilatory rate observed in the present experiment in response to painful stimulation are similar to the physiological responses reported in mammals and humans experiencing nociceptive events (Kato, Kowalski, & Stohler, 2001). Furthermore, it has been described that the administration of morphine and other opioids produce analgesia and suppress or attenuate the pain-elicited behaviors in mammals (Ebersberger, Anton, Tölle, & Zieglgänsberger, 1995; Kavaliers & Innes, 1992) and in teleost fish (Ehrensing, Michell, & Kastin, 1982; Sneddon, 2003a),

demonstrating that these behavioral and physiological changes are associated to pain (Sneddon, 2003a; Sneddon, 2009).

The present results show that, in functional terms, the Dm area of the goldfish telencephalon is a heterogeneous pallial region in which only DmII presents a functional profile that resembles that of the pallial amygdala of the land vertebrates. In contrast, DmI proved to be largely unresponsive to the electrical stimulation. No escape and emotional-like responses were produced in this area with current threshold bellow 100 uA. In fact, the rostral sulcus separating DmII from DmI seemed to constitute a clear functional border between these two regions as the intensity threshold rose abruptly in the stimulation sites located rostrally to this neuroanatomical landmark. This result is in agreement with those of the Experiment 1 showing that no evoked neural activity was recorded in DmI in response to transdermal painful electrical stimulation. Our results in this experiment also contribute to the functional dissociation between DmII and DmIII areas. Results in Experiment 1 showed significant evoked noxious somatosensory related neural activity in both, DmII and DmIII areas. However, the present results show that the electrical microstimulation in DmII, but not in DmIII, triggers low threshold emotional-like responses. This finding strongly supports our interpretation that DmIII is a pallial area that can be considered functionally equivalent to the mammalian primary somatosensory cortex, as it is involved in the processing of the sensory and perceptive dimensions of the noxious stimulation, whereas DmII is functionally similar to the amygdala, as it is involved in the processing of affective and emotional dimensions of the nociceptive experience, as well as in the

organization and generation of the behavioral responses to the noxious stimulus.

Finally, it should be mentioned that the electrical stimulation in some sites of the Dld area produced vegetative responses as bradycardia at relatively low thresholds (Figure 3.3). However, these heart rate decelerations were not accompanied of fear-related behaviors or escape-like responses. It has been reported that Dld receives mainly visual inputs (Murakami et al., 1983) and that can be considered the main visual area of the telencephalic pallium in teleost fish' possibly comparable to mammalian primary visual cortex (Ocaña, 2009). Thus, these vegetative manifestations could be considered as an arousing or attentional reaction forming part of a sensory orientation response generated by the stimulation of the primary visual sensory pathway (Quick & Laming 1988).

As a whole, the results of the Experiment 2 agree with, confirm and extend those obtained in Experiment 1 in which the neural activity evoked by noxious somatosensory stimulation was recorded by voltage-sensitive dye optical imaging. Arguably, pain is a complex cognitive and neural phenomenon that includes, besides the mere processing of the nociceptive sensorial information and the expression of the behavioral, neurovegetative, and endocrine components of the emotion, higher cognitive, affective and emotional components (Baliki & Apkarian 2015; Broom et al., 2001; Lannetti & Mouraux 2010; Meyer et al., 2007; Segner, 2012). Unfortunately, the results of Experiment 2 do not allow us to rule out the possibility that the behavioral and physiological responses elicited by the intracerebral electrical stimulation in DmII were merely reflexive motor reactions, lacking the high emotional

components that characterize the pain experience. To assess the existence of these high order cognitive components of pain, appropriate behavioral procedures are required in order to reveal some degree of behavioral flexibility, the capability to learn associations between the context and the noxious stimulus, the memory formation of fear conditioned associations, and the anticipation and avoidance of the consequences of the pain experience. Thus, in the subsequent experiment (Experiment 3) we aimed to assess whether intracerebral electrical stimulation in different pallial subdivisions, especially in DmII that produce painful-like related responses, produces also a negative valence; that is, whether the intracerebral electrical stimulation results in an aversive experience.

4. EXPERIMENT 3. Conditioned place aversion (CPA) induced by intracerebral electrical microstimulation of Dm pallial area.

4.1. Introduction

The results of the Experiment 1 revealing distinct patterns of activation of DmII and DmIII areas of the goldfish telencephalic pallium produced by nociceptive somatosensory stimulation suggest that whereas DmIII is involved in the processing of the sensory and nociceptive components of the noxious stimulus, DmII is involved in the processing of the affective or emotional components of the painful experience. In addition, the results of the Experiment 2 showed that intracerebral electrical stimulation in DmII, but not in DmI, DmIII or Dld, produced an integrated behavioral response that included motor (escape-like responses) and visceral (bradycardia responses) components, and that closely resembles the defensive natural fear and escape behavior naturally occurring in response to painful stimulation.

Pain is a complex phenomenon consisting of both a sensory, nociceptive dimension and an affective-emotional component (Broom, 2001; Lannetti, Domenico & Mouraux, 2010; Segner 2012). While the sensory component of pain is relatively accessible and easy to approach, its affective component has proved to be more elusive to the experimental scrutiny, and it can only be revealed by suitable behavioral experimental paradigms as the conditioned place aversion (CPA) procedure. CPA, is one of the most used behavioral procedures for the study of the motivational effects of neural manipulations and pharmacological treatments in experimental animals, and it constitutes a valuable model to analyze the motivational aspects of pain and aversive

stimulation (for a revision see Tzschentke, 2007). In the CPA procedure, the primary motivational property of a natural or an artificial aversive stimulus serves as the unconditioned stimulus. When it is explicitly paired with one of two previously neutral places or set of environmental stimuli, the animal develops a preference for the non-paired place, avoiding the paired environment. Noxious painful stimulation produces CPA in mammals (Suzuki, 2001; Suzuki, Ise, Maeda, & Misawa, 1999). In teleost fish, CPA procedure has been used to assess the aversive, unpleasant or potentially fearful quality of electrical shock stimulation (Ehrensing et al., 1982; Millsopp et al., 2008; Savage, 1971) and the negative reinforcing effects of the exposition to noxious chemical substances or drugs (Mathur, 2011; Ninkovic, 2006; Serra et al., 1999).

The objective in Experiment 3 was to test whether the intracerebral electrical microstimulation in different pallial subdivisions of goldfish telencephalon produces an aversive or unpleasant effect that could be revealed trough conditioned place aversion behavior. With this aim, four groups of free moving animals with intracerebral electrodes chronically implanted in the main pallial subdivisions (DmI, DmII, DmIII and Dld), and a control group receiving transdermal noxious electrical stimulation, were compared in a successive reversal CPA task.

4.2. Methods

4.2.1. Subjects and microelectrode implantation

The experiment was carried out on thirty eight goldfish (*Carassius auratus*) of 11-13 cm body length obtained from the vivarium of the University of

Seville. Prior to the experiment, thirty one of these animals were randomly assigned to one of the four implantation groups: DmI (n = 8), DmII (n = 8), DmIII (n = 7), and Dld (n = 8). An additional control group of non-implanted animals (n = 7) receiving transdermal electrical shocks was used as a reference to validate the effectiveness of the CPA procedure in goldfish. The animals were maintained in 200 l tanks with aerated and filtered water at 19±1 ºC and on a 14/10-light/dark cycle for at least two weeks before the experiments. All fish were identified individually based on phenotypic features and all animal procedures were performed in accordance with Directive 2010/63/UE of the European Community Council and Spanish legislation (R.D.53/2013).

For implantation, each animal was immobilized in a metacrilate surgical chamber. A plastic tube inserted into the mouth of the animal provided a constant flow of aerated water through the gills. This water contained an anesthetic agent (tricaine methanosulfonate MS222, Sigma, USA) in a concentration of 1:20,000 that was maintained stable during the whole microelectrode implantation surgical session. Once the animal was anesthetized, a small piece of the skull was removed. The underlying fat tissue was gently removed to expose the telencephalic lobes. A monopolar glass-insulated stainless steel wire microelectrode (25 µm in diameter) was implanted 100 µm in deep in any of the four target area designed for this experiment. Sulci and lobes on the pallial telencephalic surface served as macroanatomical landmarks to determine the implantation sites. Implantation was visually controlled using a binocular microscope. Once the electrode was implanted, it was fixed to the skull with cyanoacrylate glue and the opening in the cranium

sealed and covered with dental cement. After the implantation, the animal was located in a new surgery chamber with clean aerated water for reanimation and returned to its home tanks for, at least, four days before the beginning of behavioral training.

4.2.2. Apparatus

The apparatus consisted of an 83 x 31 x 26 cm (length x width x height) PVC tank filled with aerated and filtered water at 19±1 ºC to a depth of 20 cm. The apparatus was divided into two zones identical in size, that differed in the color of their walls and floor -one zone was colored in white and the other in orange (Figure 4.1). A plastic container disposed close to the experimental tank served as resting area during inter-phase intervals. Two 60 W LED lights placed on the ceiling of the experimental environment provided diffuse illumination to the apparatus. A ceiling-mounted video camera was used to monitor the behavior and to record the trajectory paths of the fish.

4.2.3. Conditioned place aversion (CPA) procedure

One day prior to the experiment, the animals were handled individually in order to habituate them to manipulation. Animals in all groups were trained in three consecutive phases. Initially, the fish were habituated individually to the apparatus by allowing them to explore freely the two zones in the experimental tank during 1 h. The initial preference of animals for the two zones was determined at the end of this period. After habituation, an acquisition phase began in which animals underwent four 12-min stimulation trials and four 12-min non-stimulation trials presented in an alternating sequence. In the stimulation

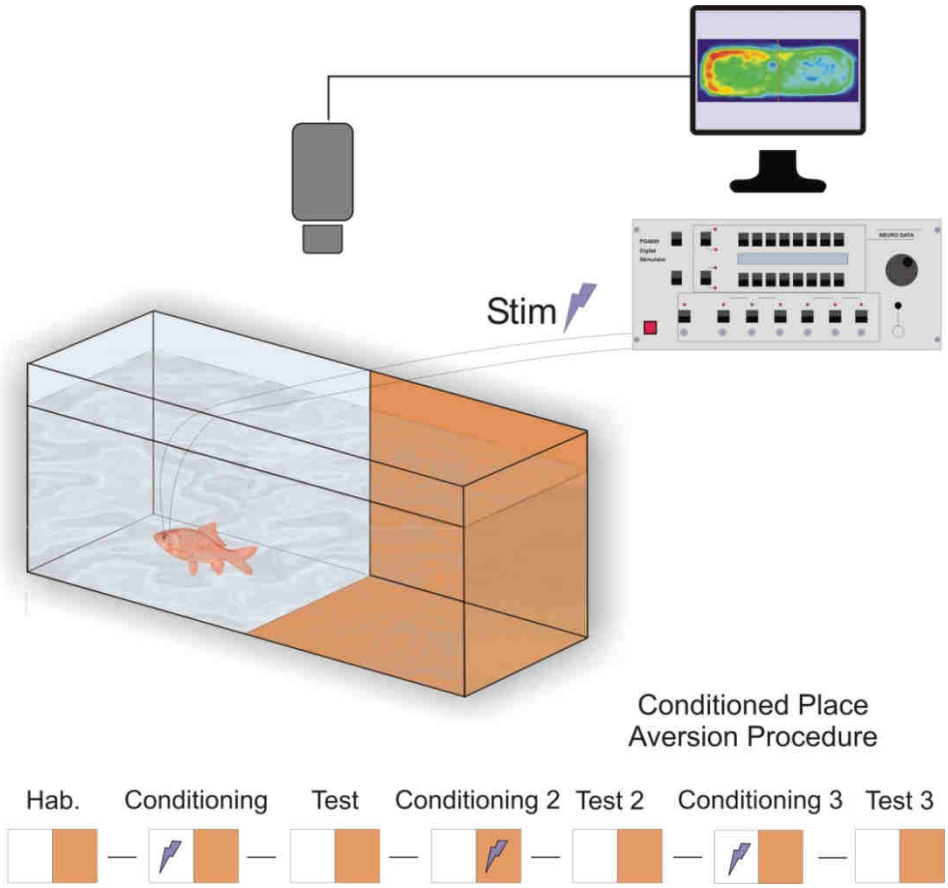


Fig 4.1. Conditioned place aversion (CPA) procedure. Goldfish with implanted microelectrodes in DmI, DmII, DmIII, and Dld were used in this experiment. On the top, a schematic illustration of the experimental setting used in this experiment is shown. On the bottom, the schematic representation illustrates the CPA procedure with successive reversals used in this experiment. Animals in all the groups underwent a habituation, and a conditioning and test phases followed by two successive reversals of this CPA procedure. The cerebral microstimulation used in the first conditioning was delivered in the zone preferred by the animals during the habituation (initially preferred zone). Note that, for each one of the two successive conditionings of the successive reversals, the unconditioned stimulus was delivered in the zone preferred by the animals in the test trial of the previous place conditioning.

trials, animals, confined in their initially preferred zone by means of a plastic barrier separating the two zones in the tank, received five stimuli with an inter-stimulus interval (ISI) of 2 min. The stimulus used in these trials consisted of a train of cathodal pulses (train duration, 2 s; pulse rate, 50 Hz; pulse width, 2 ms) delivered through a stimulator (Neuro Data PG 4000 Digital stimulator)

connected to a constant-current stimulus isolation unit. The appropriate current intensity was individually adjusted for each animal by applying progressive increments of 5 μA until they exhibit an evident motor response (X± 70 μA; mean ± SEM). For each stimulus, current strength was calculated by monitoring the voltage across a 1 kΩ resistance connected in series with the stimulating electrode. In the non-stimulation trials, animals were confined in the non-initially preferred zone and did not receive any stimulus. After this acquisition phase, animals were transferred to the resting area for 10 min. Once this period was elapsed, the plastic barrier in the experimental tank was removed and the animal returned to it to swim freely during 35 minutes in a test phase. This phase allowed us to estimate whether the stimulation during acquisition had changed the initial preference exhibited by the animal during habituation. After the test trial, two successive reversals of the previous conditioning phase (Conditioning 1) were performed. Therefore, two additional conditioning phases (Conditioning 2, and Conditioning 3) in which the relation between preferred zone and the potentially aversive stimulus was reversed relative to the previous conditioning phase were carried out for all groups. Thus, for each one of these successive reversals, the stimulation trials were performed in the zone preferred by the animals in the test trial of the previous place conditioning, whereas the non-stimulation trials were performed in the non-preferred zone. Two test trials (Test 2 and Test 3) with the same characteristics than that performed after Conditioning 1 (Test 1) were carried out after Conditioning 2 and Conditioning 3, respectively (Figure 4.1). To avoid a positional bias, in both the habituation and

the test trials carried out in this experiment, the start point coincided with a central point in the contacting area of the two zones in the middle of the tank.

Goldfish in the control group did not received intracerebral microstimulation. For these animals, the CPA procedure used was exactly the same except that the stimulus consisted of an external electric shock delivered through two four-bars grids arranged internally along the longitudinal walls of the apparatus. The position and distance among the bars were calculated to ensure a homogeneous effect of the stimulation at any point of the apparatus (Figure 4.2). The electric shock consisted of a single DC pulse of 2 s in duration with an inter-stimulus interval (ISI) of 1 min delivered by an electric generator (ac 50 Hz, adjustable voltage) connected to the bars grids. The intensity of the electric shock was adjusted for each animal to the lowest value as to elicit a robust escape response (6.18 ± 0.22 V; mean ± SEM).

4.2.4. Data analysis

Two different measures, percentage of total time spent in each compartment and change in percentage preference were analyzed in this experiment. The percentage of total time spent on white and orange zones in the habituation and test phase was calculated for animals in all the groups. The total duration of these recordings (60 min for habituation and 35 min for test 1, test 2, and test 3) were used. For each one of the three conditioning sessions that animals underwent in this experiment, change in percentage preference was expressed as the percent of time in the previously preferred zone minus the percent of time in the same zone during its respective test phase and weight to the percentage value in the previously preferred zone. Specifically, this

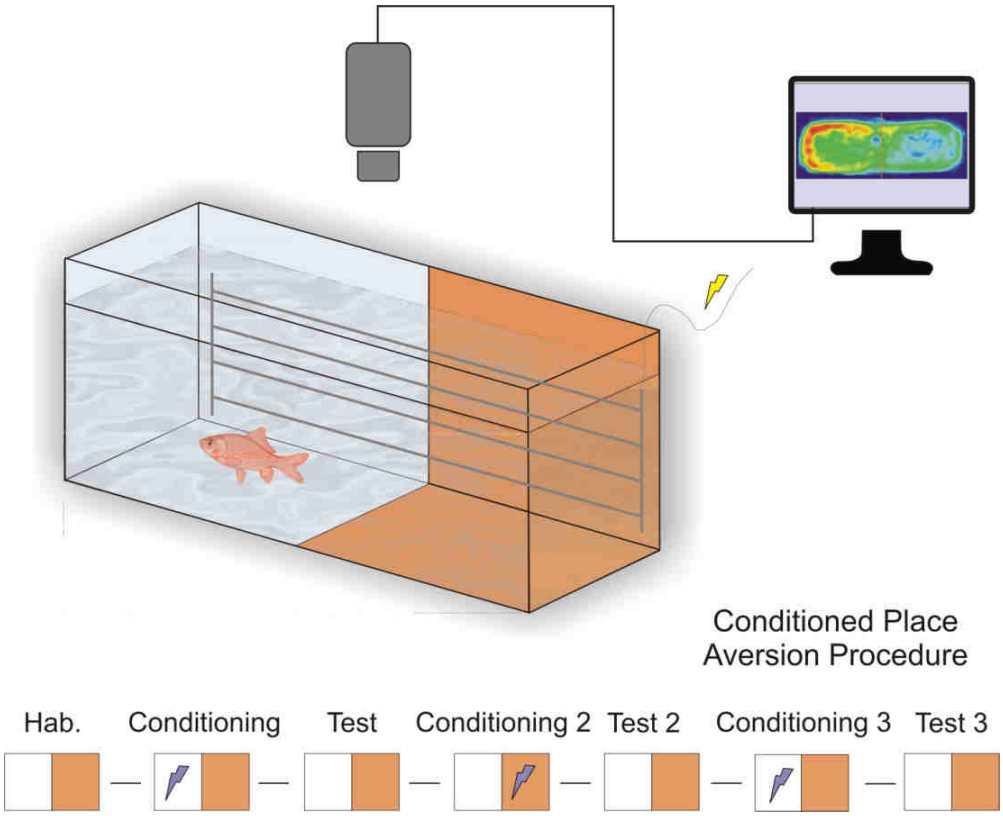


Fig 4.2. Conditioned place aversion (CPA) procedure. Non-implanted goldfish were used in this experiment. On the top, a schematic illustration of the experimental setting used in this experiment is shown. On the bottom, a schematic representation illustrates the CPA procedure used in this experiment. The unconditional stimulus used in the first conditioning was delivered in the zone preferred by the animals during the habituation (initially preferred zone). Note that in the two additional conditioning phases (Conditioning 2, and Conditioning 3) of the reversals, the relation between the preferred zone and the unconditional stimulus is reversed relative to the previous conditioning phase.

measure was calculated as (X-Y) / X, where, for each one of the three conditionings, X was the percent of time in the preferred zone in Habituation, Test 1 or Test 2, and Y was the percent of time in the preferred zone in Test 1, Test 2, and Test 3, respectively. A one-way repeated measures ANOVA was used to analyze the differences in percentage of total time in the initial preferred zone through the successive reversals. As the percentages of total time spent in each one of the two zones are complementary measures, only the analysis in

one of these two zones was performed. Repeated-measures ANOVA was also used to analyze the differences in change in percentage preference. Post hoc tests with Bonferroni correction were used when ANOVA indicated significant overall differences between means. Student's t-tests were used when necessary. All group data were 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. Video-tracking software such as ANY-maze (Stoelting Co, USA) was used to record the trials and to quantify the behavioral variables.

4.2.5. Histology

Once completed the behavioral tests, animals were deeply reanesthetized (1:5000 solution of tricaine methanesulfonate MS222, Sigma, USA) and a small electrolytic lesion was made using a cathodal pulse to facilitate the histological identification of the electrode tip location (5-10 µA/10s), followed by intracardially perfusion with a phosphate buffer and MAW as fixative solution. Brains were removed from the skull and were cut in coronal or sagittal slices of 20 µm and were stained with cresyl violet to locate the stimulation sites.

4.3. Results

4.3.1. Histological analysis

The location of the electrode tip was carried out by analyzing the electrolytic brands in sagittal sections (Figure 4.3). For electrode to meet the

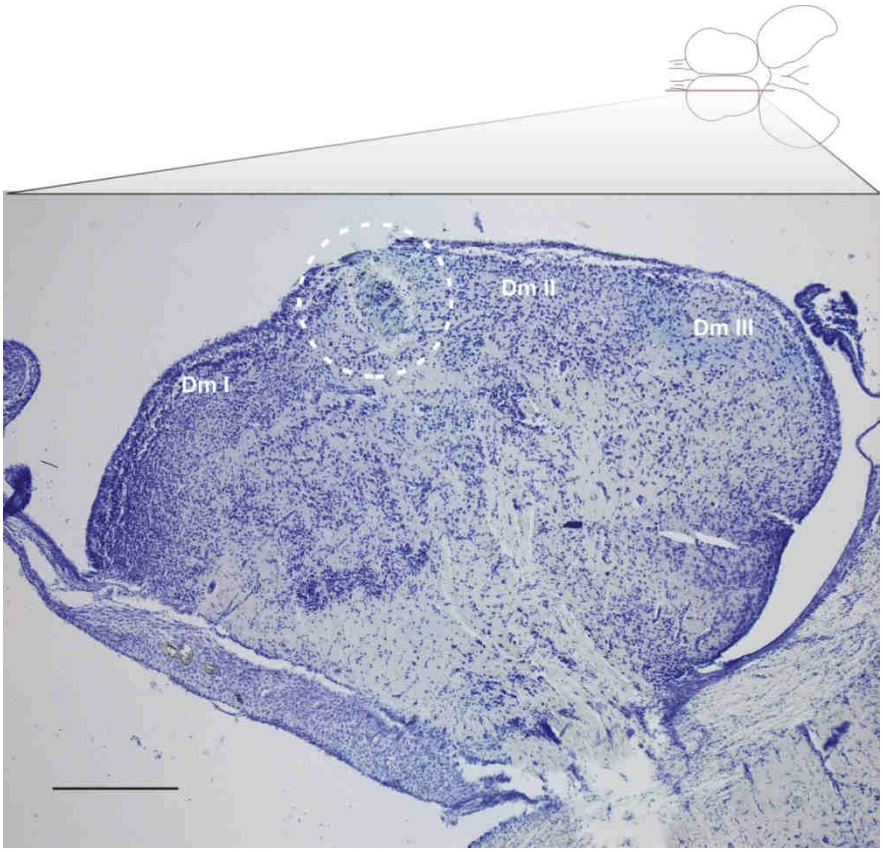


Fig 4.3. Microelectrode location. Photomicrography of a sagittal section of the goldfish telencephalon showing an example of electrolytic lesion produced by a microelectrode implanted in DmII when a cathodal pulse (0.1mA/10s) is used. The dotted-lined circle indicates the electrolytic lesion site and therefore, the electrode tip location. The schematic dorsal view of the goldfish forebrain on the top signals the level of the photographed sagittal section in the latero-medial exe of the telencephalon (red line). Abbreviations: *DmI,* subdivision I of the medial division of the area dorsalis; *DmII,* subdivision II of the medial division of the area dorsalis; *DmII,* subdivision III of the medial division of the area dorsalis. Scale bar denoting 500µm

criteria for inclusion in this study, the electrode tip had to be located in the intended target area. Beside the electrolytic brands, the exactly position of the electrodes was reconstructed using the penetration marks made in the nervous tissue by the electrode and the different notes taken during the surgery regarding the electrode position concerning the major landmarks of the telencephalon surface as the ypsiliformis, rostral or caudal sulci.

In order to representate the exact position of the different electrodes used in this experiment, the anatomical variation in the telencephalic surface of the different animals were normalized and the stimulation sites of each animal were reconstructed in a dorsal view of the goldfish telencephalon (Figure 4.4).

4.3.2. Validation of CPA in goldfish

Frequently, animals in the control group exhibited an initial preference for a particular zone during habituation (Figure 4.5) as revealed by the comparison between the percentage of time they spent in each one of the two compartments of the experimental apparatus (Student's t-test, $t_{(6)} = -3.995$, $p < 0.05$). This result was taken in to account to proceed with the CPA procedure and their successive reversals.

The results of the successive place conditioning reversals in the control group revealed that, when tested in each one of the test trials, animals consistently inverted their preference relative to the previous place conditioning phase. The one-way repeated measures ANOVA revealed a significant effect for test ($F_{3,218} = 59.520$, $p < 0.05$). Posthoc multiple comparisons adjusted by Bonferroni showed that the preference for a particular zone reverted systematically through the successive reversals conditionings coinciding with the alternate electric shock-place pairing schedule (all ps < 0.05). In addition, it has to be noted that the different reversed conditionings carried out through the three successive reversals elicited marked place preference changes as revealed by the profound difference in the percentage of time that the fish spent in each one of the two zones of the tank in three successive tests. Thus, the

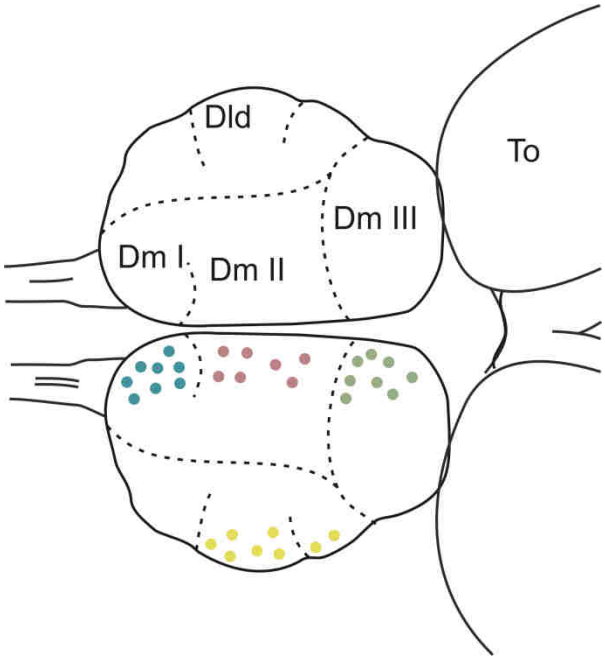


Fig 4.4. Distribution of the implantation sites of the microelectrodes. Schematic dorsal view of the telencephalon of goldfish showing a reconstruction of the implantation sites of the microelectrodes used in this experiment. Each circle represent a microelectrode site, and color of circles denotes the region in which the microelectrode was implanted (blue: DmI, pink: DmII, green: DmIII and yellow: Dld). Abbreviations: *Dld,* dorsal subdivision of the lateral division of the area dorsalis; *DmI,* subdivision I of the medial division of the area dorsalis; *DmII,* subdivision II of the medial division of the area dorsalis; *DmII,* subdivision III of the medial division of the area dorsalis; *OT,* optic tectum.

Student's t-tests showed that, in every one of the three tests trials, fish spent significantly more time in one zone than in the other (all ps < 0.05). These results indicate that the change in place preference exhibited by the control animals through the successive reversals is determined by the last place-shock association experienced by the animal. As a whole, the results of successive reversals in the control group reveal that the CPA procedure used in this experiment is a reliable tool to assessing the aversive nature of an experience or stimulus in goldfish.

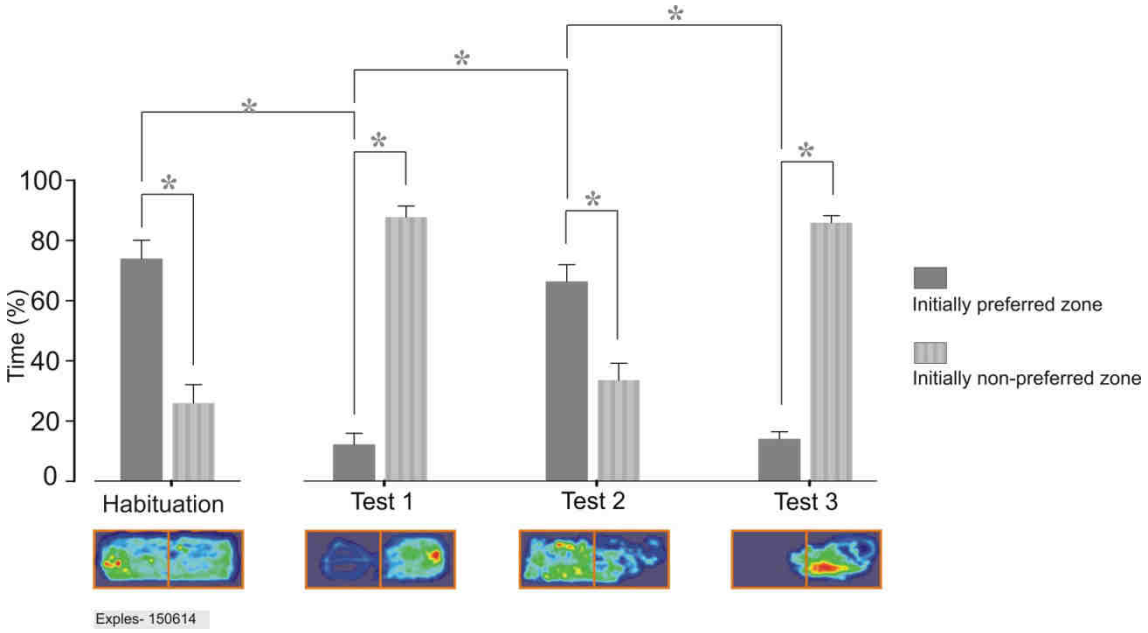


Fig 4.5. Reliability of the Conditioned place aversion (CPA) procedure. The graph shows the percentage of time spent by the animals of the control group in the preferred and non-preferred zone in the habituation and in the test phases of the successive reversal experiment. Note the alternation in the preference for a particular zone exhibited by the animals through the successive tests. A decrement in the percentage of time in the initially preferred zone is observed in Test 1 compare to habituation. An increase is observed in Test 2 compared to Test 1 and, anew, a decrease is observed in Test 3 compared to Test 2. Drawings in the bottom show examples of the occupancy of one individual animal in the habituation and in the test phases of this experiment. Note also here the alternation in the preference for a particular zone through the successive tests.

4.3.3. Involvement of dorsal telencephalic areas in the CPA during successive reversals

The results of this experiment in which animals with chronically implanted electrodes were trained in the CPA procedure revealed that the intracerebral electrical microstimulation of DmII systematically induced a change of the initial place preference after the first place conditioning, and that the preference for a particular place was changing after the conditioning phases of the subsequent successive reversals. On the contrary, the microstimulation of DmI, DmIII and Dld did not produce place preference aversion as the animals in these groups

maintained their original preference through the whole experiment (Figure 4.6). Successive reversals in the CPA demonstrate that there was a particular contingency relationship between the electrical stimulation of DmII and the place of the environment in which this stimulation was delivered what explains the change of preference through the successive reversal. In addition, since this change of preference supposed the avoidance of the zone in which the animal was previously stimulated, it might be considered that the intracerebral electric microstimulation of the DmII region of goldfish, but not those of DmI, DmIII, or Dld, have an aversive nature or negative 'valence'.

The results in this experiment showed that animals in all groups exhibited an initial preference for a particular zone during habituation (Figure 4.6) as revealed by the comparison between the percentage of time that animals in each group spent in each one of the two environments during this phase (Student's t-test, all ts < -2.569, all ps < 0.05). This result is important and is a necessary condition to proceed with the CP procedure and the successive reversals.

The analysis of change in the percentage of preference through the three successive reversals showed that the electrical microstimulation delivered on DmII, but not those delivered on DmI, DmIII, and Dld during the respective training phases caused a change on the preference that the animals had exhibited in the previous experimental phase (Figure 4.7). Accordingly, a 3 X 4 (test x group) repeated measures ANOVA revealed a significant effect for group ($F_{3,26} = 22.222$, $p < 0.05$), but not for test ($F_{2,52} = 0.466$, $p > 0.05$), nor for test by

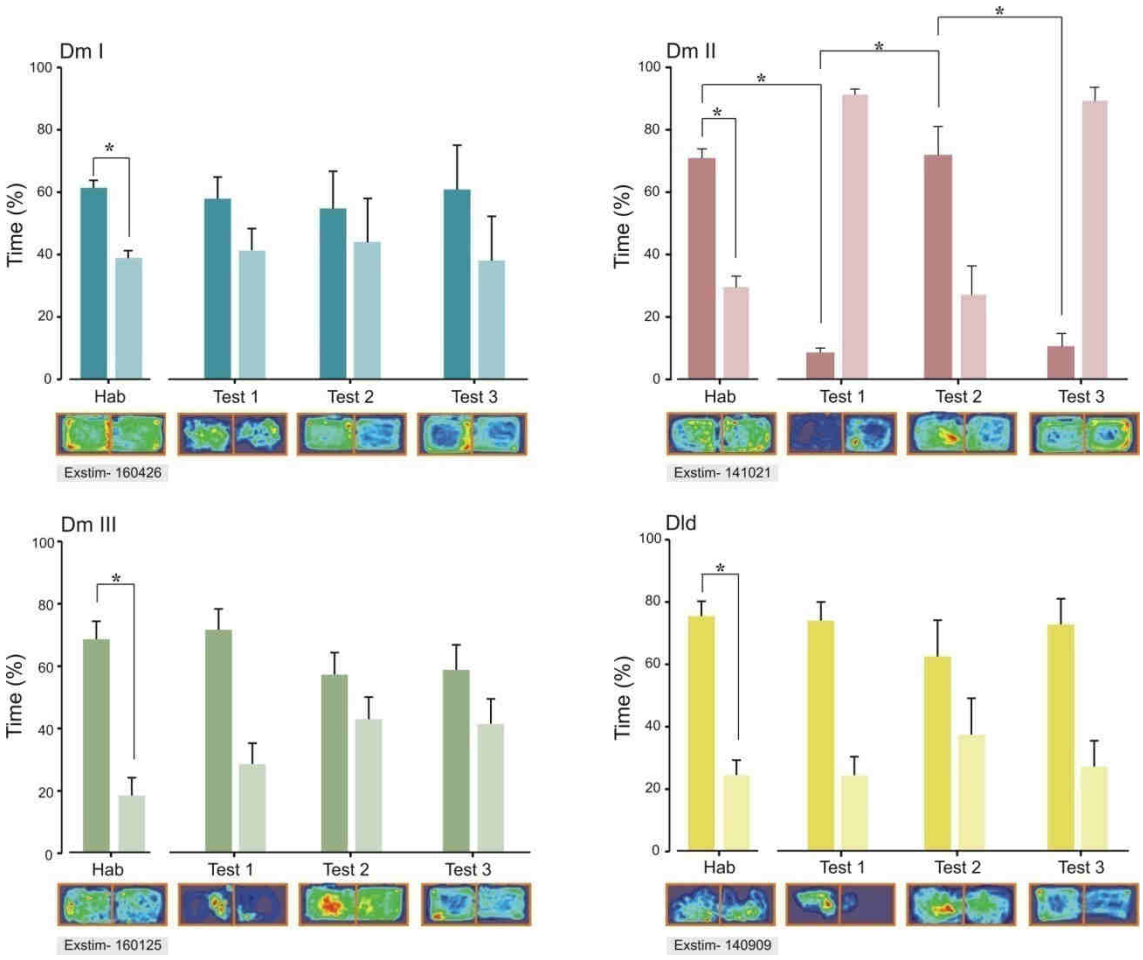


Fig 4.6. **Microstimulation of the DmII region evokes an effect of alternation of the preferred zone through the successive reversals.** Graphs show the percentage of time spent by animals in the DmI, DmII, DmIII, and Dld groups in the two compartments of the tank in the habituation and the three tests. Color of histograms denotes the region in which the microelectrode was implanted (blue: DmI, pink: DmII, green: DmIII and yellow: Dld). Note that only animals in the DmII group chose significantly one of the two compartments of the tank in each one of the three successive tests, and that this preference was alternated through the successive reversals. Drawings in the bottom show examples of the occupancy of one individual animal in the habituation and in the test phases of this experiment. Note also here that only animals in the DmII group exhibited an alternated pattern in the preference for a particular zone through the successive tests.

group interaction ($F_{6,52}$ = 0.355, p > 0.05).The analysis of the percentage of time spent by the animals of the DmII group in the initially preferred zone confirmed this change in the preference through the successive reversals. A one-way

repeated measures ANOVA was carried out for each one of the groups in this experiment. The ANOVA for the DmII group showed a significant effect for the test factor ($F_{3,21}$=85.116, $p < 0.05$). Interestingly, posthoc multiple comparisons adjusted by Bonferroni showed an alternation effect on the preferred zone through the successive reversals (Figure 4.6). Thus, animals in this group spent significantly less time in the previously preferred zone in Test1 than in Habituation ($p < 0.05$), significantly more time in Test 2 than in Test 1 ($p < 0.05$) and, again, significantly less time in Test 3 than in Test 2 ($p < 0.05$). In addition, the percentages of time that animals spent in the preferred zone in Habituation and T2, and in T1and T3 were statistically equal (both $p > 0.05$ for Hab-T2 periods and T1-T3 periods, respectively). In addition, no significant effect of the test factor was revealed by the ANOVA for any of the remaining groups ($F_{3,18}$ = 0.128, $p > 0.05$ for DmI group; $F_{3,18}$ = 1.207, $p > 0.05$ for DmII group, and $F_{3,18}$ = 0.439, $p > 0.05$ for Dld group). The absence of a significant effect of the “test” factor for animals of DmI, DmIII, and Dld groups confirms that no alternation in the preferred zone was produced, suggesting that the cerebral microstimulation in any of these pallial regions had not an aversive value.

4.4. Discussion

The present results show that electrical microstimulation of the DmII area induced place-associated aversive effects. Goldfish receiving electrical stimulation in DmII, but not in DmI, DmIII or Dld, changed consistently their place preference through the successive reversals and avoided the zone in which they have been intracerebrally stimulated. The activation of DmII by

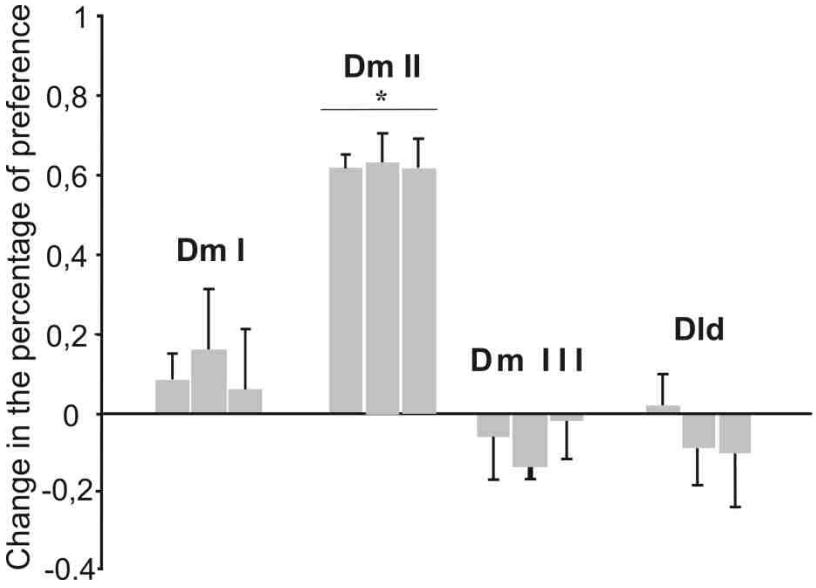


Fig 4.7. Intracerebral microstimulation of the Dm II region increased the change of preference after conditioning. Graph showing the change in the percentage of preference (the percent of time in the previously preferred zone minus the percent of time in the same zone during its respective test phase and weight to the percentage value in the previously preferred zone) of animals in DmI, DmII, DmIII, and Dld groups. For each group, each histogram represents the change in the percentage of preference in each successive reversal. Note that only animals in the DmII changed significantly their place preference in the three tests of this experiment.

intracerebral stimulation when the animals were in a zone of the tank in which they spent previously the most of the time produced a change in the affective valence associated with that area (Balleine & Dickinson, 2000; DeCoteau, Kesner, & Williams, 1997; Ragozzino & Kesner, 1999) that led consequently to the exhibition of place aversion and avoidance behaviors.

Through the two successive reversals, animals in the DmII-implanted group consistently and readily avoided the zone of the tank in which they have been previously received intracerebral stimulation. Furthermore, intracerebral stimulation in the present experiment produced changes in the percentages of place preference as high as those produced by the painful and fear-inducing

somatosensory stimulation caused by a transdermal noxious electric shock (Figs 4.5 and 4.6). Thus, the intracerebral stimulation of the DmII area in this experiment constituted an effective substitute for a painful external somatosensory aversive reinforcer and has a similar motivational value. Presumably, the electrical stimulation of DmII acted like a strong primary negative reinforcer, producing an internal state of discomfort, pain or fear, which, in turn, was associated with the exteroceptive cues, like the context, place or other allocentric cues, in which the aversive stimulation occurred. Thus, the electrical stimulation of DmII may have activated neural circuits involved in the negative-incentive attribution processing (Balleine & Dickinson, 2000; DeCoteau et al., 1997). These results agree with and confirm the results obtained in the Experiment 1 suggesting that the neural activity observed in the area DmII after the administration of a transdermal noxious somatosensory stimulus is related with the involvement of this area in the processing of the affective and emotional components of the painful experience and the results of the Experiment 2 showing that the activation of the neurons of DmII by electrical microstimulation produced pain-related escape-like responses and hearth rate changes that were associated to an aversive unpleasant state. It seems, therefore, that DmII, but not DmI, DmIII or Dld is part of a brain circuit involved in negative incentive attribution processing. In mammals conditioned place preference can be produced by intracerebral stimulation of the amygdala (Tzschentke, 2007) or other structures related with the pathways for pain perception, like the insular cortex (García, Zafra, & Puerto, 2015) or the parabrachial complex (Simon, Garcia, & Puerto, 2011; Simon, Molina, & Puerto,

2009; Simon, Zafra, Molina, & Puerto, 2008). The possible homologies and comparative and evolutionary consequences of these findings will be discussed in the General Discussion section.

Regarding the DmIII region, the present findings strongly support a role for this area in the somatosensory processing of the nociceptive stimuli, but not in the processing of the affective dimension of pain in goldfish. The activation of DmIII after a brief transdermal noxious stimulus observed in Experiment 1 might be indicative of the involvement of this area in the processing of the sensory qualities of the nociceptive stimulation, like the stimulus location in a somatotopic representation, or the stimulus strength. In fact, no pain-related escape-like responses were evoked by intracerebral electrical stimulation of DmIII in Experiment 2. Moreover, no aversive or unpleasant state was obtained by cerebrally stimulating this area in Experiment 3, as revealed by the absence of conditioned place aversion. These results reveal the functional similarity between DmIII and the primary somatosensory areas of the neocortex of land vertebrates. In the same line of reasoning, the results in this experiment allow us to disregard the aversive nature of the physiological responses (e.g. bradycardia) evoked by intracerebral stimulation in Dld in Experiment 2, and reinforce the idea that these visceral responses can be considered arousal- and sensory orientation-related responses. As discussed below, according with this conclusion, Dld has been considered a putative teleostean homologue of the primary visual cortex of tetrapods (Demski, 2003; Saidel, Marquez-Houston & Butler, 2001).

In Experiment 4, the last experiment of this serial work, we analyzed the effects of the lesions to DmI, DmII, DmIII and Dld on conditioned place aversion. This complementary experimental approach based on the lesions of different pallial regions of goldfish will provide a further confirmation of the findings obtained in the Experiments 1, 2 and 3.

5. EXPERIMENT 4. Effects of selective pallial lesions on a conditioned place aversion procedure

5.1. Introduction

The results of Experiment 3 showed that the neural activation of DmII by intracerebral electrical microstimulation produced strong aversive place conditioning, indicating that the activation of this area induces an aversive or unpleasant state and that this pallial area is a critical neural centre involved in the processing of the affective or emotional dimension of the nociceptive stimulation. In contrast, the intracerebral stimulation of DmI, DmIII or Dld did not produce CPA. Thus, it should be expected that, using a reverse experimental approach, only the lesions of DmII area, but not those of DmI, DmII or Dld, should impair the conditioned place aversion produced by a noxious external transdermal somatosensory stimulus. In this sense, it has been reported that, in mammals, conditioned place aversion is impaired by lesion or inactivation of the amygdala (Darvas, Fadok, & Palmiter, 2011; Gracy, 2001; Watanabe, Nakagawa, et al., 2002; Watanabe, Yamamoto, et al., 2002; Xu et al., 2012; Zanoveli, Ferreira-Netto, & Brandão, 2007).

In the present experiment, we trained animals with lesions in DmI, DmII, DmIII and Dld, and sham-operated goldfish in a CPA procedure in which a noxious transdermal electrical shock was delivered when the animal was in its preferred zone of the experimental apparatus.

5.2. Methods

5.2.1. Subjects

Thirty nine goldfish (*Carassius auratus*), 11–13 cm in length obtained from the vivarium of the University of Seville, were maintained for 2 months prior to the experiment in 200 l glass aquaria with aerated and filtered water at 19 ± 1 ◦C on a 14/10 h light/dark cycle. The animals were fed twice a day with dry food for pond fish (Tetra pond, Ulrich Baemsch, GmbH, Melle, Germany). Prior to the experiment, each animal was randomly assigned to one of the following surgical groups: sham operation (Sh; n = 8), rostral dorsomedial pallium ablation (DmI; n = 8), medial dorsomedial pallium ablation (DmII; n = 7), caudal dorsomedial ablation (DmIII; n = 8) or dorsolateral dorsal ablation (Dld; n = 8). All animal procedures were performed in accordance with Directive 86/609/CEE of the European Community Council and Spanish legislation (Royal Decree 53/2013).

5.2.2. Surgery

The telencephalic pallium ablations were performed by aspiration according to the methods described previously (Durán et al, 2010). Briefly, the animals were anesthetized by immersion in a solution 1:20,000 of tricaine methanesulfonate and then immobilized in a surgical chamber. An adjustable tube was introduced into the mouth of the animal to ensure a constant flow of aerated water through the gills. The concentration of aesthetic in the water was kept at 1:20,000 during the surgical procedure. Surgery was performed under visual control by means of a binocular microscope. The cephalic skin and a

section of the dorsal skull were removed carefully, and the fatty tissue inside was aspirated to expose the brain. The sulcus lateralis, sulcus limitans telencephali, sulcus ypsiliformis, rostral and caudal sulci of Dm, and the anterior commissure were used as anatomical references to determine the location and extension of the to-be aspirated nervous tissue. The lesions were performed by means of a glass micropipette connected to a manual vacuum system. After ablation, the piece of skull was replaced in its original position, fixed with cyanocrilate glue and covered with dental cement. Sham operations were performed in the same way as in the surgical groups, except that the brain tissue was not injured. After recovery of the anesthesia the animals were returned to their home aquarium and allowed to recover for 4 days before behavioral training.

5.2.3. Conditioned place aversion (CPA) procedure

The apparatus used in this experiment was the same used with the animals in the control non-implanted group of the previous experiment. Briefly, the apparatus consisted of an 83 x 31 x 26 cm (length x width x height) PVC tank filled with aerated and filtered water at 19±1ºC to a depth of 20 cm. This tank was divided into two zones identical in size, while different in the color of their walls and floor. Two four-bars grids arranged internally along the longitudinal walls of the apparatus and connected to an electric generator (ac 50 Hz, adjustable voltage) were used to provide the electrical stimulus (Figure 4.2). A plastic container disposed close to the experimental tank served as resting area during the inter-phase intervals. Two 60W LED lights placed on the ceiling of the experimental environment provided diffuse illumination to the

apparatus. A ceiling-mounted video camera was used to monitor the behavior, and to record the trajectory paths of the fish.

The day prior to the experiment, the animals were handled individually in order to habituate them to manipulation. Animals in all groups were trained in three consecutive phases. In an initial phase, the fishes were habituated individually to the maze by allowing them to explore freely the white and orange environments in the experimental tank during 1 h. The initial preference for the two zones for each animal was determined at the end of this period. Once habituated, animals underwent a conditioning phase consisting of four 6-min stimulation trials and four 6-min non-stimulation trials presented in an alternating sequence. All the conditioning trials were performed with the animals being individually confined in one of the two colored zones by means of a plastic barrier that divided the tank. In the stimulation trials, animals were confined in the initially preferred zone and received five mild electric shocks (a single DC pulse) of 2 s in duration, and an inter-stimulus interval (ISI) of 1 min. The intensity of the electric shock was adjusted for each animal to the lowest value as to elicit an evident escape response (6.25 ± 0.31 V; mean ± SEM). In the non-stimulation trials, animals were confined in the non-preferred zone and did not receive any electric shock. After this conditioning phase, animals were transferred to the resting area for 10 min. Once this period was elapsed, the plastic barrier in the experimental tank was removed, the animals were returned to it, and allowed to swim freely during 35 minutes in a test phase. This phase was designed to assess whether the electric shock stimulation during conditioning changed the initial preference exhibited by the animal in

habituation. For both, habituation and test trial carried out in this experiment, the start point coincided with a central point in the contacting area of the two zones in the middle of the tank.

5.2.4. Data analysis

Three different measures, percentage of total time, change in percentage preference, and mean travelled distance were analyzed in this experiment. The percentage of total time spent on white and orange zones in the habituation and test phase was calculated. The total duration of these recordings (60 min for habituation and 35 min for test 1) were used. Change in percentage preference, expressed as the percent of time in the initially preferred zone during habituation minus the percent of time in the same zone during Test and weight to the habituation value of each animal, was also determined. Mean travelled distance for animals was calculated to assess the possible effect of the different pallial lesions on the locomotion and navigational abilities of the animals. Student´s t-test was used to analyze the differences in percentage of total time. Repeated-measures ANOVA was used to analyze the change in percentage preference and mean travelled distance. Post hoc tests with Bonferroni correction were used when ANOVA indicated significant overall differences between means. Additional Student's t-tests were used when necessary. All group data were 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. Video-tracking software such as ANY-maze (Stoelting Co, USA) was used to record the trials and to quantify the behavioral variables.

5.2.5. Histology

At the end of the experiment, the brains of the animals in all the experimental groups were hitologically analyzed to determine the real extent of the different lesions. Once deeply anesthetized with a solution 1:5000 of tricaine methanesulfonate (MS-222, Sigma), animals were perfused transcardially with 50 ml of phosphate saline solution (PBS 0.1 M; pH 7.4), followed by 125 ml of MAW (methanol, acetone and water in 2:2:1 proportion) as fixative solution. The brains were removed from the skulls, post fixated with the same fixative solution for 10 days, embedded in paraffin wax and cut in transverse 20 µm sections with a rotator microtome. The sections were deparaffinized in xilene, hydrated and Nissl stained (cresyl violet acetate 0.5%). The damaged area was assessed for each animal by reconstructing the location and the extent of the lesion on the plates of a goldfish brain atlas elaborated in our laboratory.

5.3. Results

5.3.1. Histological analysis

The histological analysis showed that the lesions were homogeneous in size and location (Figure 5.1). The Dld lesions damaged the dorsal part of the area dorsalis pars lateralis (Dld), and, in some cases, also the dorsal boundaries of the ventral part of the same area (Dlv). The analysis of the lesions in Dm showed that all the three regions were damaged almost completely in their rostrocaudal extends. Thus, DmI lesions affected the rostral lobe of Dm and extended from the rostral pole of the telencephalon until the

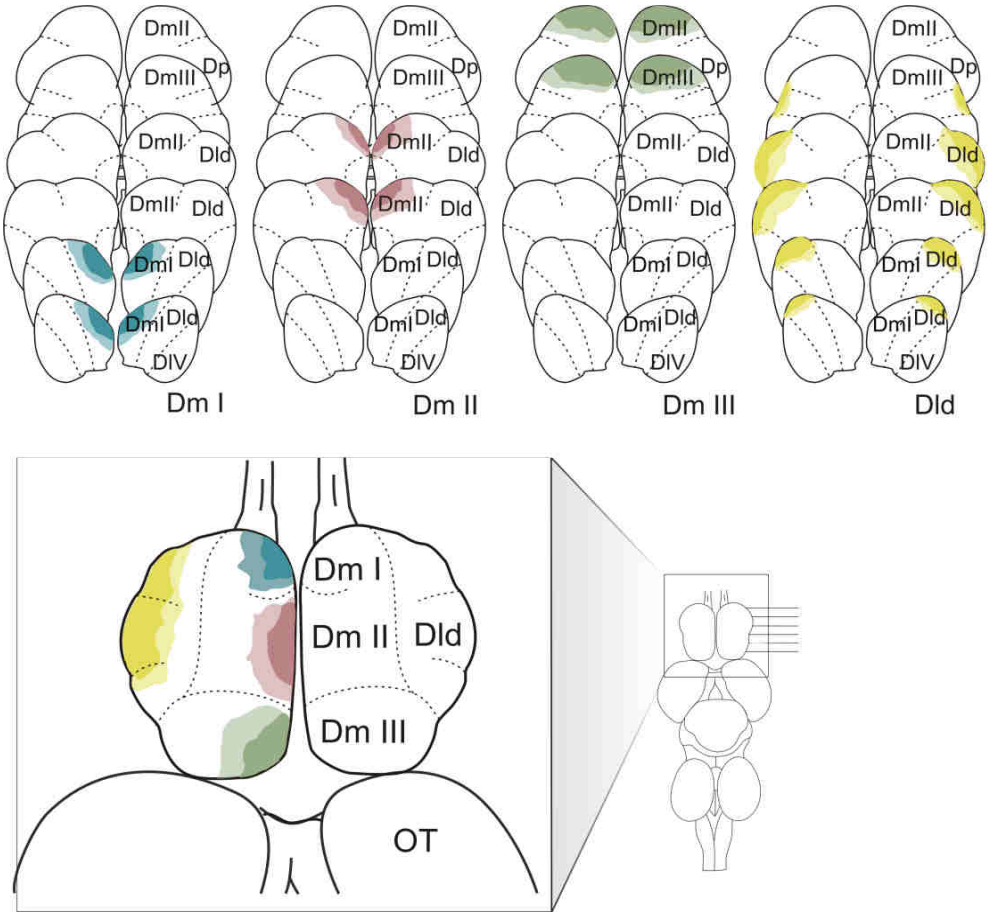


Fig 5.1. Pallial lesions. Top: Transversal sections showing the maximal (pale colored) and the minimal (intense colored) extent of the four pallial lesions performed in this experiment (DmI, DmII, Dm III and Dld). Bottom: Dorsal view of the goldfish brain showing the six transversal section levels in the telencephalon (right). A magnified view of the dorsal surface of the goldfish telencephalon showing the maximal (pale colored) and the minimal (intense colored) extent of the four pallial lesions performed in this experiment (left). Abbreviations: *Dld,* dorsal subdivision of the lateral division of the area dorsalis; *DmI,* subdivision I of the medial division of the area dorsalis; *DmII,* subdivision II of the medial division of the area dorsalis; *DmII,* subdivision III of the medial division of the area dorsalis; *OT,* optic tectum.

rostral sulcus. No animals with DmI lesions presented apparent damage in DmII region. DmII lesions affected the intermediate lobe of Dm, but not the rostral and the caudal ones. This lesion scarcely affected the area dorsalis pars centralis (Dc) and the subpallial area ventralis pars dorsalis (Vd). The DmIII lesions affected the region between the caudal sulcus and the caudal border of

Dm and did not damaged DmII. The histological analysis did not reveal any cerebral damage in the sham animals.

5.3.2. Involvement of the dorsal telencephalic areas in the CPA procedure

A previous important result in this experiment was that the animals in all groups, independently of the pallial lesion they had, exhibited an initial preference for a particular zone during habituation (Figure 5.2) (Student's t-test $t_{(7)} = -3.517$, $p < 0.05$; $t_{(6)} = -3.304$, $p < 0.05$; $t_{(7)} = -2.951$, $p < 0.05$; $t_{(7)} = -3.329$, $p < 0.05$; $t_{(7)} = -3.218$, $p < 0.05$ for DmI, DmII, DmIII, Dld and Sham groups, respectively).

The results of the test phase carried out after the conditioning phase showed that the animals in the Sh, DmI, DmIII and Dld groups, but not those in the DmII group, changed their place preference as revealed by the fact that they avoid consistently the compartment in which they have received the electric shock during the conditioning phase (the initially preferred compartment) (Figure 5.2). In contrast, only a mild preference decrease for the initially preferred compartment (which was paired with the electric shock) was observed in the animals in the DmII group.

Accordingly, t-tests revealed that whereas animals in the DmI, DmIII, Dld and Sham spent significantly less time in the compartment where the received the electric shock (Student's t-test $t_{(7)} = 11.526$, $p < 0.05$; $t_{(7)} = 8.186$, $p < 0.05$; $t_{(7)} = 8.525$, $p < 0.05$; $t_{(7)} = 6.256$, $p < 0.05$ for DmI, DmIII, Dld and Sham, respectively) animals in the DmII group spent the same time in both environments ($t = 1.856_6$, $p > 0.05$). Furthermore, the percentagen of time that

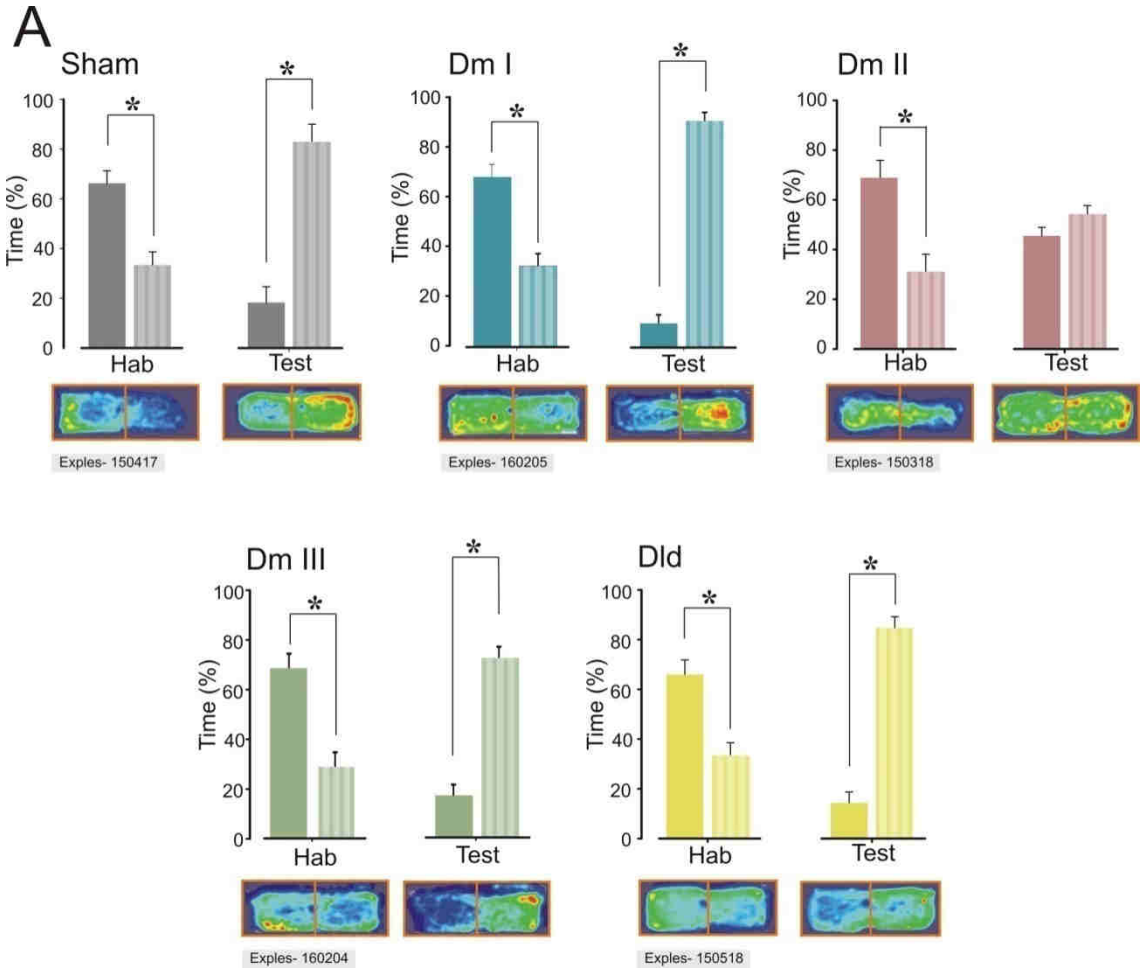


Fig 5.2. Effects of the pallial lesions on a CPA task. Graphs showing the percentage of time spent by goldfish of each experimental group in the initially preferred (solid bars) and the initially non-preferred (hatched bars) compartments during the habituation and the test phase. Note that animals in all groups except those in the DmII group spent significantly less time in the initially preferred zone than in the initially non-preferred zone in the test phase after conditioning. Note also that, except for the DmII group, this temporal distribution was opposite to that exhibited during the habituation. Drawings in the bottom show examples of the occupancy of one animal per group in the habituation and in the test phase of this experiment. Note also here, except for the DmII group, the opposite temporal distribution of animals in their preference compared to that in the habituation.

animals in the Sh, DmI, DmIII and Dld groups spent during the test in the compartment not associated with the electric shock was similar among them (all ps > 0.05) and differed significantly from that of the animals in the DmII group (all ps < 0.05). The analysis of the change in percentage preference (Figure

5.3) revealed that all groups, except DmII showed a strong change in the percentage preference (Sh = 0.75 ± 0.08, DmI = 0.84 ± 0.08, DmII = 0.33 ± 0.09, DmIII = 0.75 ± 0.08 and Dld = 0.07 ± 0.8; mean ± SEM). In addition, the overall analysis of this index with a one-factor ANOVA showed an noticeable between group difference ($F_{4,34}$ = 6.09, $p < 0.05$). Particularly, paired comparisons showed that the change in the percentage of preference of animals in DmII was significantly lower than those of the animals in the remaining groups (Figure 5.3 All ps < 0.05). The analysis of the mean travelled distance during habituation and test phases revealed that none of the pallial lesions performed in this experiment caused a impairment in the locomotion or motor activity of the animals. Thus, a 2 X 5 (phase x group) repeated measures ANOVA revealed no significant effect for phase ($F_{4,34}$ = 0,11, $p > 0.005$), group ($F_{4,34}$ = 0,83, $p > 0.005$), or the phase by group interaction ($F_{4,34}$ = 0,72, $p > 0.005$). Thus, the results in this lesion study revealed that only animals with lesion in the area DmII but not those with lesions in DmI, DmIII and Dld regions, or those with the Sh operation, were impaired in their ability to avoid the zone of the tank paired previously with the electric shock.

Finally, it has to be noted that not one of the different types of lesions performed in this experiment produced an observable deficit in the capability of the animals to perceive and respond to the electric shock since the animals in all groups normally react to the shock with fast escape responses, and other natural nocifensive behavioral patterns and no difference between groups was Observed for distance travelled just after stimulation ($F_{4,34}$ = 0.73, $p > 0.05$).

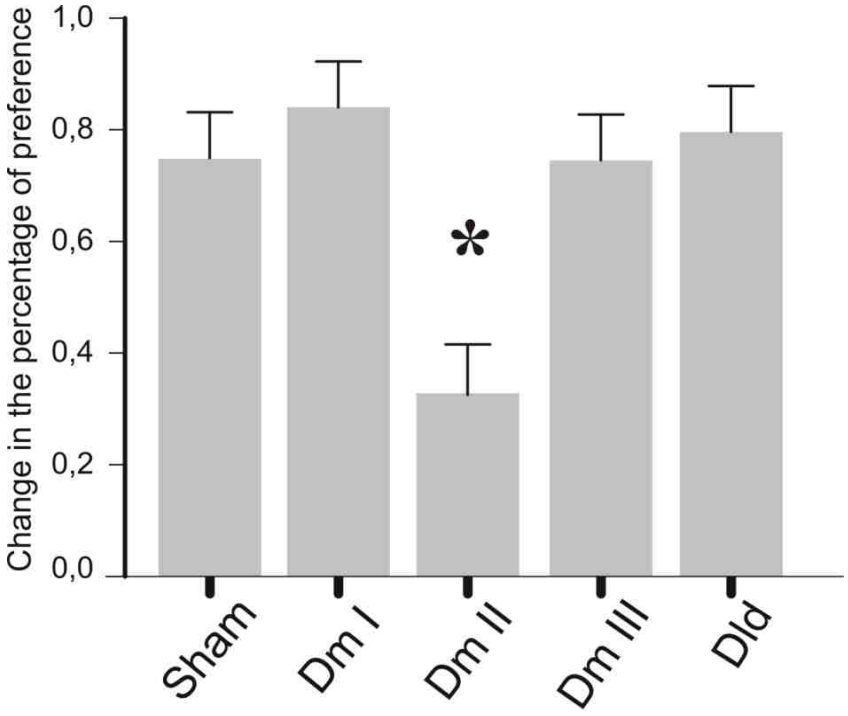


Fig 5.3. Dm II lesions impairs the change of preference after conditioning. Graph showing the change in the percentage of preference (percent of time in the initially preferred zone during habituation minus the percent of time in the same zone during test and weight to the habituation value) for animals in all the groups. Note that animals in all groups except those in the DmII one changed their place preference, after that the initially preferred zone were associated to the electric shock during the conditioning phase.

5.4. Discussion

The results of Experiment 4 showed that DmII plays a critical role in aversive place conditioning in which a noxious transdermal electrical shock was associated with the preferred compartment of the tank. Lesions of DmII, but not those of DmI, DmIII or Dld severely impaired the acquisition of electric shock-induced CPA in goldfish, but interestingly they did not affected the spontaneous nociceptive or nocifensive behavior in response to the electric shocks (i.e., erection of the dorsal fin, immobility near the walls of the experimental apparatus, escape responses, and c-start like responses.

The present results suggest that the DmII area is selectively involved in processing of the aversiveness of nociceptive stimulation and in the formation of place aversion memories and fear conditioning. Similar results have been obtained in mammals after lesioning the amygdala (Darvas et al., 2011; Gracy, 2001; Watanabe, Nakagawa, et al., 2002; Watanabe, Yamamoto, et al., 2002; Xu et al., 2012; Zanoveli et al., 2007) or limbic structures (Johansen, Fields, & Manning, 2001). Furthermore, lesions of the amygdala (Coover, Ursin, & Levine, 1973; Werka, Skår, & Ursin, 1978) or the insular cortex (Grossman, Grossman, & Walsh, 1975; Kaada, Rasmussen, & Kveim, 1962; Pare & Dumas, 1965) produce deficits in avoidance learning in rats. Amygdala lesions impairs avoidance learning in formalin-induced and footshock-induced CPA in rats, but does not significantly affect their reflex nociceptive behaviors (Gao, Ren, Zhang, & Zhao, 2004; Tanimoto, Nakagawa, Yamauchi, Minami, & Satoh, 2003), and the formalin-induced CPA produces a c-Fos activation in the amygdala associated with the expression of the emotional component of pain (Lei, Zhang, & Zhao, 2004).

In agreement with the present results, previous experiments have shown that the telencephalon of teleost fish is involved in avoidance learning (Overmier & Curnow, 1969; Overmier & Papini, 1986). More specifically, it has been demonstrated that Dm constitutes a critical pallial center in the neural system supporting fear conditioning in teleost fish (Portavella, Salas, Vargas, & Papini, 2003). Indeed, DmII lesion produces deficits in the acquisition of place aversion conditioning that strikingly resemble those produced by the damage to the amygdala in mammals (Watanabe, Yamamoto, et al., 2002; Xu et al., 2012).

Furthermore, the Dm region of teleost fish seems to be essential for the normal acquisition of several tasks where an aversive component is present, as avoidance conditioning (Lau et al., 2011; Portavella, Salas, Vargas, & Papini, 2003; Portavella, Torres, Salas, & Papini 2004), taste aversion learning (Martín et al., 2011), or conditioned place aversion in the present experiment.

In conclusion, the present results indicate that the dorsomedial telencephalic pallium of goldfish is a fundamental component of the circuitry necessary for processing the emotional dimension of nociceptive stimulation and therefore, for the acquisition of CPA. The consequences of these findings for the hypothesis of homology of the different areas of the telencephalic pallium of teleost fish will be discussed in the following section.

6. GENERAL DISCUSSION

The telencephalon of vertebrates is characterized by a remarkable degree of morphological diversity. Particularly, the telencephalon of actinopterygian fishes presents a very distinct divergent morphology compared to that of the rest of vertebrates. This divergence is caused by the particular developmental process that undergoes the telencephalon of actinopterygian fishes during embryogenesis, i.e., the eversion, instead of the evagination occurring in non-actinopterygian vertebrates, of the telencephalic alar walls (Nieuwenhuys, 2011; Northcutt & Kicliter, 1980; Northcutt, 1995; Striedter & Northcutt, 2006). Typically, this conspicuous developmental difference has hampered the comparison of the different areas of the telencephalon between actinopterygians and tetrapods. Several contending hypothesis of homology have been proposed for the different pallial subdivisions of actinopterygians. In particular, the dorsomedial subdivision of the area dorsalis telencephali (Dm) has been proposed by a broad consensus to be homologous to the pallial amygdala (ventral pallium) of tetrapods based on topological, connectional, developmental and functional data (Braford, 2009; Northcutt, 2006, 2008; Portavella et al., 2002; Portavella, Torres & Salas, 2004; Wullimann & Mueller, 2004). However, Nieuwenhuys (2009) and Butler (2000), based on topological considerations, propose an alternative hypotheses in which Dm is considered homologous to the ventral (pallial amygdala) and the lateral (piriform) pallia conjoined. Furthermore, Yamamoto et al (2007), based on connectional data, proposes that Dm plus Dd and Dld are homologous to the dorsal pallium of tetrapods.

Based on neurochemical, connectivity, and molecular evidence, topological considerations, the patterns of expression of early genes, and neurobehavioral data, Dm of teleost fish has been considered homologous to the pallial amygdala (or ventral pallium) of land vertebrates (Braford, 1995; Braford, 2009; Desjardins & Fernald, 2010; Mueller & Wullimann, 2009; Northcutt, 2006, 2008; Portavella, Torres, & Salas, 2004; Wullimann & Mueller, 2004). Since eversion implies the reversal of the topography, but not the topology, of the pallial areas of actynopterygians relative to that of the non-actynopterygians (Nieuwenhuys, 1962a, 1962b, 1964; Nieuwenhuys, 1969; Northcutt, 2006), it would be expected that Dm, the actynopterygian homologue of the ventral pallium (VP) of amniotes, lies in the most proximal (medial) position of the teleostean telencephalic pallium, lateral and adjacent to the non-everted subpallium (Fig. 1.2 and 1.3).

Interestingly, the connectivity pattern of the teleostean Dm resembles strikingly that of the land vertebrate amygdala. Dm receives somatosensory, auditory and gustatory inputs from the hypothalamus, the thalamus and the preglomerular complex (Butler & Hodos, 2005; Northcutt, 2006; Yamamoto & Ito, 2005), and Dmv, the most ventral part of the precommisural Dm, receives a direct input from the olfactory bulb via the medial olfactory tract (Folgueira et al., 2004a; Nieuwenhuys, 2009), as well as intrapallial projections from Dc, Dd, and Dld, and from the subpallial nuclei Vd, Vv, Vl, Vs and Vi. This evidence strongly suggests that the pattern of connectivity of the teleostean Dm is reminiscent of the hodological pattern of the amygdala of tetrapods (Moreno & González, 2007; Northcutt, 2008; Swanson & Petrovich, 1998).

More precisely, it has been proposed that, as in amniotes, teleost fish posses a pallial and a subpallial amygdala. More precisely, it has been proposed that, as in amniotes, teleost fish posses a pallial and a subpallial amygdala. Based on gene expression studies, Dm has been identified as the homologous of the pallial amygdala of tetrapods Ganz et al (2015), and the supracommissural (Vs) and the postcommisural (Vp) nuclei of the area ventralis telencephali of teleost fish as the homologous of the dorsal and ventral part of the central amygdala and the bed nucleus of the stria terminalis of tetrapods. Thus, these authors proposed that Dm (pallial amygdala) and Vs and Vp (subpallial amygdala) constitute the amygdaloid complex in teleost fish. Dm might be considered homologous to the frontotemporal amygdaloid system, while the Vs and Vp would be homologous to the postcommissural amygdala (Swanson & Petrovich, 1998).

Functional data also support the hypothesis of the homology of Dm with the amygdala of mammals. Like the amygdala of mammals (McGaugh, 2004; Phelps & LeDoux, 2005; Maren, 2001), the Dm area of teleost fish is involved in behavioral processes with significant emotional and affective components requiring emotional learning and memory (Marino-Neto & Sabbatini, 1983; Martín et al., 2011; Portavella, Torres, & Salas, 2004; Portavella, Torres, Salas & Papini, 2004). For example, an impairment has been described after Dm lesion in the acquisition and retention of a conditioned avoidance responses in goldfish (Lau et al., 2011; Portavella, Salas, Vargas & Papini, 2003, Portavella, Torres, Salas & Papini, 2004), which is similar to that found in amygdala lesioned mammals (Aggleton et al., 1992; Davis, 1992). In addition, like the

amygdale lesion y mammals, Dm lesions impair taste aversion learning (Martín et al., 2011). Interestingly, neuroanatomical data have shown that gustatory and general visceral inputs converge in Dm (Folgueira et al., 2003, 2004b; Northcutt, 2006; Yoshimoto & Yamamoto, 2010), suggesting that this pallial region, like the amygdale of mammals, could be the neural site for the taste-malaise integration necessary for the formation of taste aversion memory in teleosts. Present results indicate that DmII is a pivotal centre for conditioned place aversion in teleost fish. In mammals conditioned place aversion critically depends on the amygdala (Azar, Jones, & Schulteis, 2003; Darvas et al., 2011; Fidler, Bakner, & Cunningham, 2004; Gracy, 2001; Risinger & Oakes, 1994; Stinus, Cador, Zorrilla, & Koob, 2005; Tzschentke, 1998; Watanabe, Yamamoto, et al., 2002; Xu et al., 2012).

However, it should be taken into account that Nieuwenhuys (2009) and Butler (2000) propose an alternative hypothesis regarding the identity of Dm. In this sense, these authors suggest that Dm is formed not only by derivatives of the teleostean homologue of the ventral pallium of land vertebrates (i.e. pallial amygdala), but also by those ones of the lateral pallium (i.e. olfactory cortex). Supporting this view, recent studies on the conservation of gene expression patterns during embryogenesis indicate that the lack of both, a positive *tbr1* area and a negative *emx* gene area in Dm suggests that teleost fish do not have a proper ventral pallial subdivision as in tetrapods (Ganz et al., 2015). Instead, Dm might rather be either, a pallial structure homologous exclusively to the lateral pallium of land vertebrates or a combined complex of regions homologous to the ventral and the lateral pallium, conjoined (Ganz et al., 2015).

Recent studies support the last view in which at least part of Dm of teleost can be considered homologue of the pallial amygdala of tetrapods (Ganz et al., 2015). For example, the expression of the cb1 cannabinoid receptor, typically observed in the basolateral amygdala of mammals (Mailleux & Vanderhaeghen, 1992; Matsuda et al., 1993), can also be found in the Dm area of teleost fish (Aoki et al., 2013; Harvey-Girard et al., 2013; Lam et al., 2006). In addition, the $GABA_a$-benzodizepine receptors, a noteworthy indicator of the limbic character of the tetrapod amygdala, are also present in Dm (Wolkers et al., 2015). All these data are in agreement with the hypothesis of Nieuwenhuys (2009) and Butler (2000) regarding a combined identity of Dm. Consequently, the possibility that some specific subregions of Dm could be comparable to the olfactory pallium of non-actinopterygian vertebrates should be investigated in future studies.

As a whole, our results support the hypothesis of Wulliman and Mueller (2004) proposing that only part of Dm (in our case, DmII) is homologous to the amygdala. The results in this work provide multiple and convergent functional evidence suggesting that only DmII should be considered homologous to the basolateral amygdala of mammals. The basolateral amygdala of mammals is considered the main regulatory neural centre mediating fear responses and modulating different downstream components of the fear related behavior, as well as an essential component of the neural circuitry responsible for emotional learning and memory (LeDoux, 2000; Maren, 2001; McGaugh, 2004). The general organization and the connectivity pattern of the amygdala seems to be highly conserved across vertebrates (Medina et al., 2011; Price, 2003) and its

involvement in the processing of the emotional component of pain and fear seems also to be generalized across vertebrates (Whalen & Phelps, 2009), to monkeys (Davies et al., 2002; Kalin, 2004; LeDoux, 2000; Mason, Capitanio, Machado, Mendoza, & Amaral, 2006). The basolateral amygdala receives inputs of several sensory systems and the activation of the neurons within this nucleus has been shown to be sufficient to associate the incoming sensory information with unconditioned fear responses and to produce behavioral responses to nociceptive and unpleasant stimuli (Johansen et al., 2010).

The results in this study revealing striking functional similarities regarding DmIII of goldfish and the primary somatosensory cortex of mammals leads us to propose a hypothesis of homology between these two structures (see also Yamamoto 2007). The somatosensory pallium of teleost fish resembles the avian somatosensory pallium in two important characteristics: a) unlike the mammalian neocortex, it is not laminated; b) its thalamo-pallial circuitry closely parallels that of amniotes (Butler & Hodos, 2005). The dorsal thalamus of teleost fish project to the pallium, and also the preglomerular nuclear complex, located in a more caudal position in the diencephalon, serves as the major relay center of sensory projections to the pallium (Northcutt, 2006; Yamamoto & Ito, 2005). Particularly, the lateral nucleus of the preglomerular complex constitutes the main efferent region sending somatosensory projections to the Dm area (Ito and Yamamoto 2009; Northcutt et al 2006; Yamamoto and Ito 2005). Both, the central posterior nucleus in the dorsal thalamus and the preglomerular nuclear complex receive reciprocal projections from the pallium (Northcutt, 2006; Yamamoto & Ito, 2005). In addition, the teleost fish pallium has intrinsic,

GABAergic pallial neurons, and reciprocal pallial glutamatergic to the Project to the diencephalon (Butler, 2012). Furthermore, electrophysiological recordings from the neurons in the dorsal thalamus of goldfish present similar functional profiles that are similar to that of the mammalian and avian dorsal thalamic neurons (Llinas & Steriade, 2006).

Pain perception in fish is a highly controversial issue. Some authors, based on the supposed absence of the necessary neural structures and circuits (i.e., a neocortex and talamocortical neural circuits) required to support it (Leonard, 1985; Rose, 2002), state that fish are incapable of pain perception. Present study strongly contradicts this view and provides compelling evidence revealing that the neural mechanisms required for processing both, the sensory-perceptual and the emotional dimensions of the painful experience are present in the telencephalic pallium of teleost fish and also that they are likely homologues to the somatosensory areas of the neocortex and the pallial amygdala of tetrapods. Nociceptors responding to intense mechanical pressure, heat, and noxious chemicals that present identical anatomical and physiological features to those described in mammals, have been described in teleost fish (Ashley, Sneddon, & McCrohan, 2006, 2007; Sneddon, 2002; 2003a, 2004). These nociceptors, as occurs in land vertebrates, convey nociceptive information through the spinothalamic and the trigeminal tract (Finger, 2000; Luiten, 1975; Sneddon, 2004), and their stimulation produces evoked potentials in the midbrain and forebrain of teleosts (Dunlop & Laming, 2005; Nordgreen et al., 2007). The pain-related processing has also been demonstrated in the telencephalon of teleost fish (Dunlop & Laming, 2005; Ludvigsen et al., 2014;

Nordgreen et al., 2007; Reilly et al., 2008). For example, Dunlop and Laming (2005) recorded electrophysiological signals in different areas in the brain, including the telencephalon, produced by mechanosensory and noxious stimulation. The electrical stimulation of the skin at noxious intensities evokes a potential in the dorsal part of the dorsal telencephalon in the contralateral hemisphere (Nordgreen et al., 2007). In addition, gene expression increases in response to noxious stimulation has been described in the midbrain, hindbrain and forebrain of teleost fish (Reilly et al., 2008). Changes in gene expression occurred mainly in the forebrain, which indicates the importance of this brain structure to the pain processing in teleost fish (Reilly et al., 2008; Sneddon, 2009). Other evidence supporting nociceptive processing regards the presence opiates and opioid receptors in the fish brain (Alvarez et al., 2006; Buatti & Pasternak, 1981; Li, Keith, & Evans, 1996; Porteros, García-Isidoro, Barrallo, González-Sarmiento, & Rodríguez, 1999) as well as endogenous encephalins (Schulman, Finger, Brecha, & Karten, 1981; Vecino, Ekström, & Sharma, 1991). In mammals, these endogenous substances and their receptors are present in the neural structures involved in the processing of pain information, as the spinal cord, the raphe nucleus, the reticular formation, the periaqueductal gray, the thalamus and the telencephalon (Simantov, Kuhar, Uhl, & Snyder, 1977). In teleost fish, these substances and their receptors are distributed in a similar pattern (Gonzalez-Nunez & Rodríguez, 2009; Snow, Renshaw, & Hamlin, 1996; Vecino et al., 1992). Furthermore, the administration of morphine and other opiate substances diminished or abolish avoidance learning in fish (Ehrensing et al., 1982; Sneddon, 2003b). Interestingly, the injection of midazolan, (that

enhances the effect of the neurotransmitter GABA on the $GABA_A$ receptors) in the rostral part of Dm (probably, the DmII area) blocks the inhibition of the nocifensive response to formaldehyde promoted by restrain stress, but did not alter the nocifensive response (Wolkers et al., 2015). Stress-induced antinociception in fish is mediated through the release of endogenous opioid and cannabinoid substances (Alves, Barbosa, & Hoffmann, 2013; Wolkers et al., 2013, 2015). These results suggest that rostral Dm might be involved in a descending modulatory nociceptive pathway similar to that described in mammals (Butler & Finn, 2009), and that, like the amygdala of mammals (Fox & Sorenson, 1994; Harris & Westbrook, 1996; Helmstetter, 1992, 1993; Leite-Panissi, Coimbra, & Oliveira, 2003; Lico, Hoffmann, & Covian, 1974; Mena, Mathur, & Nayar, 1995; Werka, 1994, 1997; Werka & Marek, 1990), the rostral Dm might be critical for the induction of endogenous analgesia. These results revealing the role of Dm in the modulation of stress-induced antinociception in fish are in agreement with the results in this study regarding the critical importance of Dm in the teleostean nociceptive information processing, and suggest that this pallial subdivision might have a similar function to that of the mammalian amygdala. Like the amygdala in mammals (Butler & Finn, 2009; Fox & Sorenson, 1994; Leite-Panissi et al., 2003; Lico et al., 1974; Mena et al., 1995; Werka, 1994, 1997), DmII of teleost fish seems to plays a pivotal role in mediating conditioned and unconditioned fear, pain processing, and stress-induced analgesia.

As it is always associated with a “feeling” or negative attribution, pain can be considered more than a mere sensory experience. In this line, it has been

reported that fish not only display “reflex” protective motor behaviors and vegetative reactions as a response to a noxious stimuli but, as revealed by behavioral evidence, they can also learn to avoid and anticipate it (Davis & Klinger, 1994; Ehrensing et al., 1982). As a whole, the present results suggest that goldfish reactions to noxious stimulation are not simply “reflexes” executed by lower brain centers but, on the contrary, they require further neural processing at the level of higher brain centers. The present findings allowed us to identify these higher brain centers in goldfish as well as the areas of the telencephalic pallium that constitute the emotional neural system involved in the processing of the affective components of pain and fear conditioning in teleost fish. These neural systems seem to be highly conserved in vertebrates.

7. CONCLUSIONS

1. The dorsomedial area of the area dorsalis telencephali (Dm) of goldfish can be subdivided macroscopically, citoachitecturally, histochemically, and functionally in at least three main subregions: DmI, DmII, and DmII.

2. DmIII can be identified as the primary somatosensory area of the goldfish telencephalic pallium. This region, apparently involved in the processing of the sensory attributes of the nociceptive stimulation, present a somatotopically ordered representation of the body surface in which the sensory attributes of the nociceptive stimulus, like location or intensity, are represented.

3. DmIII seems not to be directly involved in the processing of the affective and emotional components of pain, nor in the processes of attribution of negative incentive values of the noxious stimulation. Thus, the activation of this area by intracerebral electrical microstimulation did not produce neither fear or escape responses in restrained animals nor was sufficient as to produce conditioned place aversion in free-swimming goldfish. In addition, Dm III lesions did not produce any observable deficit in conditioned place aversion produced by a transdermal electric shock.

4. DmII does seem to be involved in the processing of the emotional components of the painful experience. Indeed, intracerebral electrical stimulation of this area produced escape-like responses and visceral fear-like

reactions in restrained animals and conditioned place aversion. Furthermore, selective lesions of DmII severely impaired conditioned place aversion in goldfish.

5. These functional findings support the hypothesis of homology between DmIII and the somatosensory areas of the neocortex of mammals and between DmII and the pallial amygdala, respectively.

6. DmI is largely unresponsive to the noxious stimulation and does not seem to be directly involved in the nociceptive processing.

7. As a whole, the results in this study suggest the presence of complex, high order telencephalic mechanisms in teleost fish necessary to process both, the sensory/nociceptive and the affective/emotional dimensions of the painful stimulus.

8. In addition, the presence of these high order telencephalic mechanisms conjoined to ascending nociceptive pathways suggest that teleost fish are capable of feel pain, and that these neural circuits are probably equivalent and possibly homologous to those supporting pain experience in mammals.

9. Finally, the present findings suggest that mechanisms involved in pain perception, including the higher telencephalic and pallial centers, are highly conserved in vertebrates.

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