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REGULAR ARTICLE

Neuroanatomical relationships between FMRFamide-immunoreactive components of the nervus terminalis and the topology of olfactory bulbs in teleost fish

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Abstract The *nervus terminalis* (NT) is the most anterior of the vertebrate cranial nerves. In teleost fish, the NT runs across all olfactory components and shows high morphological variability within this taxon. We compare the anatomical distribution, average number and size of the FMRFamide-immunoreactive (ir) NT cells of fourteen teleost species with different positions of olfactory bulbs (OBs) with respect to the ventral telencephalic area. Based on the topology of the OBs, three different neuroanatomical organizations of the telencephalon can be defined, viz., fish having sessile (Type I), pseudosessile (short stalked; Type II) or stalked (Type III) OBs. Type III topology of OBs appears to be a feature associated with more basal species, whereas Types I and II occur in derived and in basal species. The displacement of the OBs is positively correlated with the peripheral distribution of the FMRFamide-ir NT cells. The number of cells is negatively correlated with the size of the cells. A dependence analysis related to the type of OB topology revealed a positive relationship with the number of cells and with the size of the cells, with Type I and II topologies of OBs showing significantly fewer cells and larger cells than Type III. A dendrogram based on similarities obtained by taking into account all variables under study, i.e., the number and size of the FMRFamide-ir NT cells and the topology of OBs, does not agree with the phylogenetic relationships amongst species, suggesting that divergent or convergent evolutionary phenomena produced the olfactory components studied.

Keywords Nervus terminalis · Nucleus olfactoretinalis · Brain · FMRFamide · Fish

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Introduction

According to the "mosaic evolution hypothesis", natural selection can influence one brain area independently from others, causing changes in certain areas without involving the whole brain (Barton and Harvey 2000; de Winter and Oxnard 2001; Hager et al. 2012; Pinelli et al. 2014). In this manner, the energy costs of maintaining a large brain are minimized (Striedter 2005; Gonzalez-Voyer et al. 2009). A contradictory view is taken in the "concerted evolution hypothesis", which assumes the presence of constraints in the evolution of brain regions attributable to their interdependencies during development (Finlay and Darlington 1995; Finlay et al. 2001; Yopak et al. 2010). This hypothesis implies that the brain is conditioned as a whole in response to specific

selection of certain areas. Most likely, both mosaic evolution and developmental constraints play fundamental roles in driving brain changes (Striedter 2005).

Across species, the brains of fish show great morphological variability, which is, in part, caused by ecological, behavioural and social processes (Ridet and Bauchot 1990; Huber et al. 1997; Kotrschal et al. 1998; Pollen et al. 2007; Salas et al. 2008; Lecchini et al. 2014). This phenomenon is so strong that different natural populations of the same species can differ in the relative size of their brain areas in accordance with the habitat type (Gonda et al. 2011); this intraspecific divergence can happen in a relatively short time (Gonda et al. 2009).

The olfactory bulbs (OBs) are the most variable brain structures, showing positive bivariate allometry in comparison with other brain areas (Gonzalez-Voyer et al. 2009), thus strongly supporting the model of mosaic evolution. Furthermore, different ecotypes of fish, such as wild *Poecilia mexicana* living under different conditions of darkness and exposure to toxic hydrogen sulphide, show habitat-dependent OB-size differences, which are reduced when the ecotypes are reared under the same conditions (Eifert et al. 2014). A high degree of environmentally induced phenotypic plasticity of OBs has also been demonstrated in the nine-spined stickleback (*Pungitius pungitius*), which shows larger OBs only when experimentally exposed to predation (Gonda et al. 2012). Thus, fish OBs appear to be highly interesting structures and excellent models for studying adaptive variability. In this paper, we consider the topology of fish OBs, which might have a sessile or pedunculate position (see Nieuwenhuys 1967) relative to the rest of the telencephalon, together with some neuroanatomical characteristics of the *nervus terminalis* (NT) strictly associated with OBs and to the olfactory nerve (ON).

The NT, also known as "cranial nerve 0", is an enigmatic nerve that is little understood in either evolutionary or physiological terms. It is the most anterior of the vertebrate paired cranial nerves and is composed mainly of small unmyelinated axons including ganglia and slender nerve trunks. It was discovered in a small shark by Fritsch (1878), who referred to it as a "supernumerary nerve". Pinkus (1894, 1895) reported the presence of a similar structure in the African lungfish, *Protopterus annectens*. A little later, in the Australian lungfish, *Neoceratodus forsteri*, Sewertzoff (1902) described a nerve whose course was completely separated from the ON and entered the brain below the telencephalon in the preoptic area. It was named the "*nervus preopticus*". Holmgren and van der Horst (1925) described a second nerve that, unlike the *nervus preopticus*, travelled in association with the ON, in lungfish. At the time, both nerves were considered to belong to the NT system and were designated the anterior root (the branch entering with the ON) and the posterior root (the branch entering at level of the optic nerve) of the NT.

In an attempt to explain the evolution of the NT system, Fiorentino et al. (2002) proposed, on the basis of the supposed ancestral pattern of the NT system comprising two distinct roots (von Bartheld and Meyer 1988), that the cartilaginous fish could have retained only the posterior root, whereas the bony fish could have done the reverse by elaborating the anterior root.

In the current teleosts, the NT consists in a system of cells and fibres that is enclosed in the ON and that extends from the proximity of the olfactory organ to the brain. However, the exact location and morphology of the NT cells are quite variable among species; one can observe single isolated cells or small clusters of cells distributed along the entire extent of the ON pathway (Münz and Claas 1987; Nieuwenhuys et al. 1998). In many species, the NT neurons form a compact cluster (ganglion) that is located between the OBs and the ventral telencephalon. These cells project fibres centrally to the ventral forebrain and peripherally to both the olfactory epithelium (Brookover and Jackson 1911; Rossi and Basile 1968; Oka et al. 1986; Ekström et al. 1988; Kim et al. 1995; Yamamoto et al. 1995; Parhar et al. 1996; Wirsig-Wiechmann and Oka 2002) and the retina (Münz et al. 1981; Stell et al. 1984, 1987; Östholm et al. 1990), suggesting the NT plays a functional role in the physiology of the olfactory and visual systems (Eisthen et al. 2000; Kawai et al. 2009), in addition to modulating behavioural responses (Wirsig and Leonard 1987; Yamamoto et al. 1997; Ogawa et al. 2006; Okuyama et al. 2014). Because of its connection with the olfactory region and the retina, the NT ganglion in teleosts is denominated the "nucleus olfacto-retinalis" (NOR; Münz et al. 1981).

The position of the OBs with respect to the ventral telencephalic area seems to be an important constraint in determining the neuroanatomy of the NT cells, which are generally more peripherally displaced the more pedunculate that the OBs are (Nieuwenhuys et al. 1998; Parhar 2002). The position of the NT cells, as visualized by gonadotropin-releasing hormone (GnRH) immunohistochemistry and the type of OBs are reported to follow a particular evolutionary trend, with basal teleost species having pedunculate OBs and more peripherally displaced NT cells, whereas more derived species tend to have sessile OBs and fewer, more centrally located GnRH-immunoreactive (ir) NT cells (Parhar 2002). In addition to GnRH (Schreibman et al. 1979; Oka and Ichikawa 1990; Yamamoto et al. 1995), the teleost NT cells and fibres also contain FMRFamide-like compounds (Walker and Stell 1986; Ekström et al. 1988; Östholm et al. 1990; Rama Krishna and Subhedar 1992; Vecino and Ekström 1992; Pinelli et al. 2000; Mathieu et al. 2002; D'Aniello et al. 2015). The presence of FMRFamide-ir NT cells in a pre-bulbar position has been reported in species with pedunculate OBs (Bonn and König 1989a; Fujii and Kobayashi 1992; Biju et al. 2003), although in some other species, FMRFamide-ir NT cells have also been observed in postbulbar positions (Rama Krishna and Subhedar 1992). Conversely, in species with sessile OBs, the position of the cells is prevalently at the junction between

the OBs and the rostroventral telencephalon, although fewer FMRFamide-ir cells have also been reported in the ON ventral to the OBs (Ekström et al. 1988; Szabo et al. 1991; Chiba et al. 1996; Pinelli et al. 2000).

In light of this great variability, we describe, in the present study, the neuroanatomical organization of the FMRFamide-ir NT cells system in a set of fourteen different teleost species in order to further explore their relationships with the topology of the OBs. Additionally, we correlate the size and number of FMRFamide-ir NT cells among these species and also with the different positions of the OBs by using a statistical approach, with the aim of determining the way that the topology of the OBs correlates with these variables. Finally, we consider the OBs/FMRFamide-ir NT cells as a whole in order to test whether and to what extent the similarities between these associated structures reflect the phylogenetic relationships among species.

Material and methods

The species used for this study were randomly selected on the basis of their easy availability. We used at least six samples from each of fourteen species. All animals acquired from commercial dealers were adults in a non-reproductive state. They were anaesthetized by immersion in 0.1 % MS-222 (tricaine methanesulphonate ethyl *m*-aminobenzoate; Sigma Chemical, St. Louis, Mo., USA) and decapitated. All experimental procedures were in accord with the Council of the European Communities Directive (86/609/EEC). The brains with olfactory chambers attached were quickly dissected. All samples were fixed in freshly prepared Bouin's fluid fixative for 24 h at room temperature and kept for a week in 75 % alcohol, which was changed daily.

All specimens were then dehydrated in graded alcohols, cleared in xylene and embedded in paraffin (56–58 °C). Serial horizontal or sagittal sections (10 μm thick) were mounted on albumin-coated slides and subjected to immunohistochemical reaction for FMRFamide-like peptide according to the following procedure.

Deparaffinized sections were washed in 0.1 M phosphate-buffered saline (PBS, pH 7.6), then treated with 1 % normal goat serum (Sigma Chemical) in PBS for 20 min to reduce nonspecific staining and subsequently incubated with primary antiserum (rabbit antiserum raised against FMRFamide; Phoenix) at a dilution of 1:10,000 overnight at 4 °C in a dark moist chamber. After two 10-min rinses in PBS, sections were incubated with biotinylated secondary antibody (goat anti-rabbit IgG, 1:150; Pierce) for 1 h at room temperature. After two rinses in PBS (2 × 10 min), purified streptavidin (1:200; Pierce) was applied to the sections for 1 h. The reaction products were visualized by using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, Milan, Italy) with 0.3 % H_2O_2 in TRIS buffer (0.05 M, pH 7.6). The slides were dehydrated and coverslipped in Eukitt mounting media (Sigma Chemical). The immunoreaction appeared dark brown.

Because our goal was not to identify the tetrapeptide FMRFamide in the cells but to stain the NT components in order to avoid any loss of cells during counting, we did not perform specificity tests. However, the antibody used in this study was previously tested for specificity in fish (see Pinelli et al. 2000; Fiorentino et al. 2002). However, we should mention that some FMRFamide antibodies appear to crossreact with proteins containing a similar peptide sequence (Kyle et al. 1995).

Sections were examined by using a Leica DMLB microscope (Nussloch, Germany). All sections in which FMRFamide-ir elements were observed in the olfactory components were captured by using a digital camera (Leica DFC340FX) so that the NT cells could be counted and measured. When the same cell appeared in more than one micrograph, we measured the largest cell section by using the Leica Application Suite. Because the cell shape often seemed not to be round in the sections, we calculated the mean between the long and the short axes (see Jadhao et al. 2001).

For statistical analysis, we defined three relationships of the OBs with the remaining telencephalon: (1) sessile OBs (Type I) in which the olfactory mucosa (OM) was connected to the OB with a long ON and the OBs were in close continuity to the ventral area of the telencephalon; (2) pseudosessile OBs (Type II) in which the OM was connected to the OBs through long ONs, whereas the OBs were connected to the rest of the telencephalon with short peduncles that were evident in brain sections; (3) stalked OBs (Type III) in which an evident peduncle connected the OBs to the rest of the telencephalon. The three positions of the OBs were treated as qualitative variables and were represented in the data set in three columns. For each species, the presence of the Type was indicated with a (1) and the absence of the Type was indicated with a (0).

The estimate of the total number of NT FMRFamide-ir cells was performed on serial sections. We considered only the cells that showed a well-evident nucleus that, given the thickness of the sections, appeared mostly only in one section. When profiles of the same nucleus appeared in more than one section, they were counted only once. For each species, we report the total number (the sum of both sides) and the average size of NT FMRFamide-ir cells measured in sagittal sections for six adult fish of any sex (Table 1).

Because Kolmogorov-Smirnov and Shapiro-Wilk tests showed that the numerical variables were not normally distributed (see Results), we chose a non-parametric statistical approach.

The analysis of correlations between the size and number of cells was performed by using a Spearman correlation, whereas, in a dependence analysis among the size and number

Table 1 Total number (both sides combined) and average size of *nervus terminalis* FMRFamide-immunoreactive cells for six adults of any sex. The disjunctive binary code used to arrange the variable "Type of OBs" in the hierarchical cluster analysis is also shown

Species	Number of cells	Size of cells	Type I	Type II	Type III	Order	Species	Number of cells	Size of cells	Type I	Type II	Type III	Order
Carassius auratus	88	19.64	0	0	1	Cypriniformes	*Corydoras aeneus*	78	16.33	0	1	0	Siluriformes
	70	21.16						71	13.45				
	101	22.83						68	8.34				
	91	17.05						57	9.82				
	75	18.43						63	11.47				
	105	18.67						80	18.71				
Crossocheilus siamensis	86	13.23	0	0	1	Cypriniformes	*Hypostomus punctatus*	49	9.47	0	1	0	Siluriformes
	89	11.39						57	8.99				
	96	13.63						59	10.37				
	88	14.19						54	12.81				
	87	15.33						51	9.09				
	85	10.07						60	13.09				
Gyrinocheilus aymonieri	195	15.03	0	0	1	Cypriniformes	*Pangasius hypophthalmus*	166	10.81	0	0	1	Siluriformes
	217	13.94						184	12.53				
	199	12.56						177	11.89				
	206	16.38						169	13.74				
	189	15.03						181	9.72				
	212	14.73						173	11.29				
Astyanax fasciatus	27	41.34	0	1	0	Characiformes	*Xiphophorus helleri*	73	14.13	1	0	0	Cyprinodontiformes
	32	39.82						69	16.24				
	30	36.12						77	12.51				
	28	38.97						75	16.33				
	27	45.21						72	14.12				
	30	44.03						81	13.54				
Moenkhausia sanctaefilomenae	38	39.41	1	0	0	Characiformes	*Gambusia affinis*	71	12.44	1	0	0	Cyprinodontiformes
	37	33.32						73	15.12				
	41	42.45						75	12.94				
	33	44.23						64	11.88				
	31	36.17						66	15.78				
	32	33.59						68	10.37				
Paracheirodon innesi	7	41.21	0	1	0	Characiformes	*Mesonauta festivus*	17	22.03	1	0	0	Cichliformes
	9	38.82						19	17.32				
	8	41.56						17	18.93				
	6	37.38						16	20.43				
	7	39.45						22	21.09				
	8	37.41						18	17.49				
Pristella maxillaris	18	35.71	1	0	0	Characiformes	*Dicentrarchus labrax*	67	24.71	0	1	0	Perciformes
	15	34.65						78	29.42				
	15	37.97						68	29.42				
	18	33.21						72	26.91				
	16	34.57						81	27.98				
	17	33.94						75	22.87				

of cells through the topology of the OBs, we used a non-parametric Kruskal-Wallis analysis of variance and Mann–Whitney U tests post hoc with the Bonferroni correction.

A hierarchical cluster analysis by using normalized Euclidean distance and average linkage was performed to examine whether the variables under study (number of cells, size of cells and topology of OBs) taken together reflected the phylogenetic relationship across species. The variable "Types of OBs" was arranged by using a disjunctive binary code (see Table 1).

All the statistical analyses were implemented by using IBM SPSS 21 software.

Results

The teleostean species selected for this study showed different positions of the OBs with respect to the ventral telencephalic area, with five species belonging to Type I (sessile OBs), five species belonging to Type II (pseudosessile OBs) and four species belonging to Type III (stalked OBs; see Table 1, which also explains the counting and the sizing of the cells).

Type I topology of OBs was found in *Gambusia affinis*, *Mesonauta festivus*, *Moenkhausia sanctaefilomenae*, *Pristella maxillaris* and *Xiphophorus helleri*.

All species shared a common topology of the FMRFamide-ir NT cells (hereinafter NT cells), which formed a compact group located in the posterior basal OB, just at the border with the rest of the telencephalon (Fig. 1).

The number of NT cells appeared variable among species, with the highest number being counted in *X. helleri* and *G. affinis*, which showed an average of approximately 70 cells (35 on each side), followed by *M. sanctaefilomenae* with approximately 35 (17–18 on each side). The lowest number of cells was recorded in *P. maxillaris* and *M. festivus*.

The reverse trend was registered for the average cell size, in that the largest cells were observed in *M. sanctaefilomenae* and *P. maxillaris,* followed by *M. festivus* and *X. helleri*. The smallest cell size was recorded in *G. affinis*.

Type II topology of OBs was found in *Astyanax fasciatus mexicanus*, *Corydoras aeneus*, *Dicentrarchus labrax*, *Hypostomus punctatus* and *Paracheirodon innesi*.

The NT cells of *A. f. mexicanus* were mainly organized in a single compact cluster located between the ON and the OB (Fig. 2a); only a few cells were observed beyond this cluster along the ON pathway, towards the OM. In *C. aeneus*, the NT cells were distributed in the ON as single cells or small groups of cells near the anterior OBs (Fig. 2b) terminating in

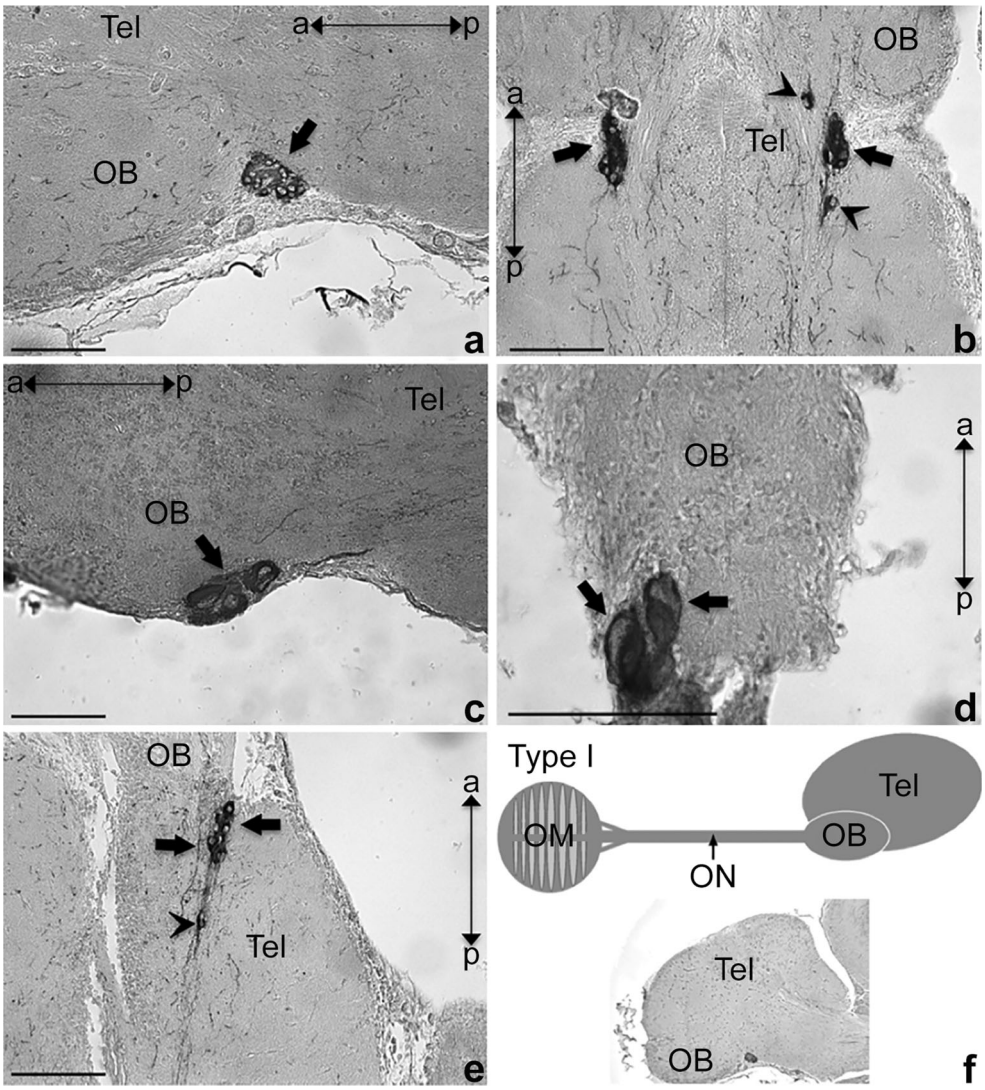

Fig. 1 Brain sections showing FMRFamide-immunoreactive (ir) *nervus terminalis* (NT) cells in species with sessile olfactory bulbs (*OB*), namely Type I. The antero-posterior axis is indicated (*a* anterior, *p* posterior). All species shared a common topology of the FMRFamide-ir NT cells, which formed a compact group (*arrows*) with a few single cells (*arrowheads*) located between the posterior basal OBs and the rest of the telencephalon. **a** Sagittal section of *Gambusia affinis* (*Tel* telencephalon). **b** Horizontal section of *Mesonauta festivus*. **c** Sagittal section of *Moenkhausia sanctaefilomenae*. **d** Horizontal section of *Pristella maxillaris*. **e** Horizontal section of *Xiphophorus helleri*. **f** Representation (*top*) and brain section (*bottom*) from *G. affinis* showing the sessile (Type I) topology of OBs (*OM* olfactory mucosa, *ON* olfactory nerve). *Bars* 100 μm

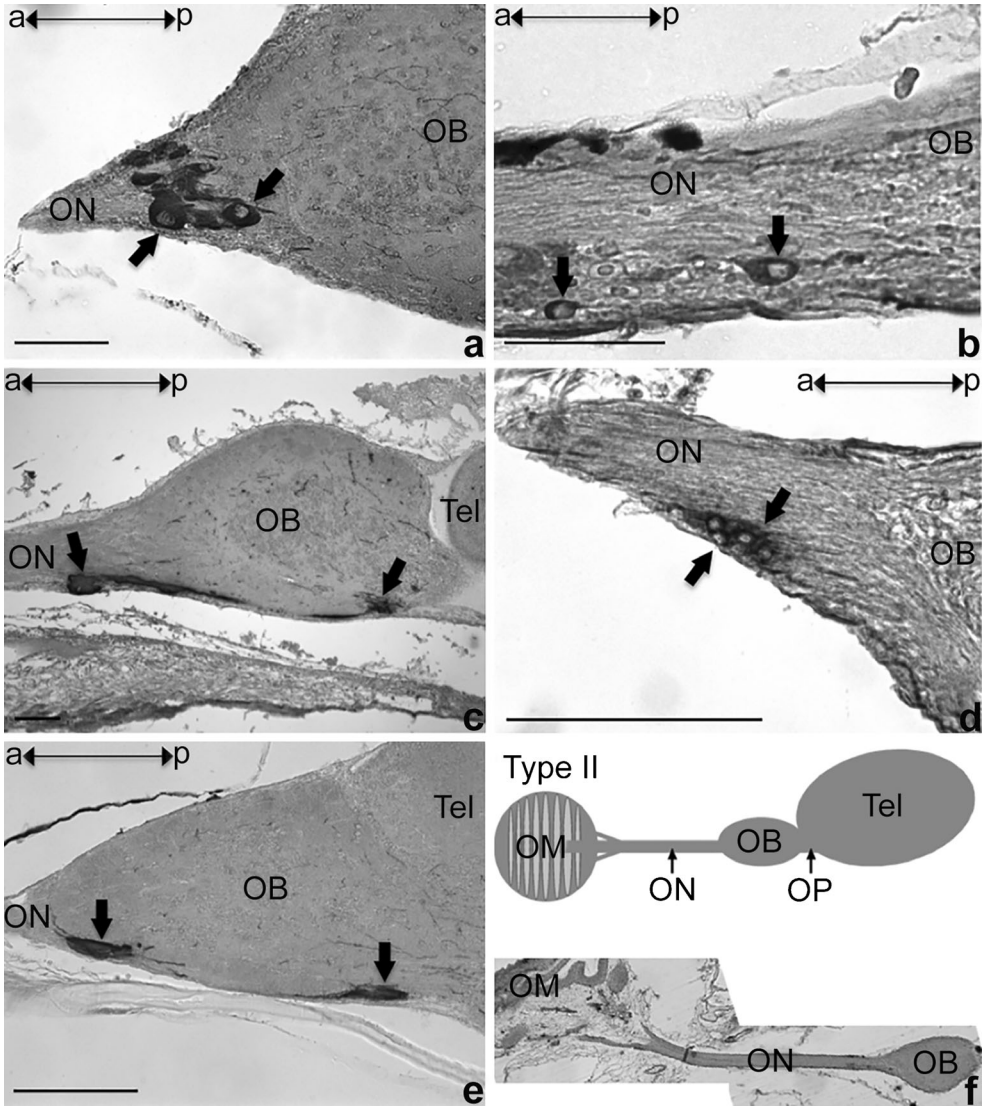

Fig. 2 Brain sections showing FMRFamide-ir NT cells in species with pseudosessile olfactory bulbs (*OB*), namely Type II. The antero-posterior axis is indicated (*a* anterior, *p* posterior). **a** Sagittal section through the OB of *Astyanax fasciatus mexicanus* showing a single compact cluster of cells (*arrows*) located between the olfactory nerve (*ON*) and the OB. **b** Horizontal section through the ON of *Corydoras aeneus* showing NT cells distributed as single cells (*arrows*) along the ON close to the OB. **c** Sagittal section through the OB of *Dicentrarchus labrax*. The NT cells are organized in two clusters, one located anterior to the OB (*left arrow*) and the second one positioned between the OB and the short olfactory peduncle (*right arrow*). **d** Horizontal section through the ON of *Hypostomus punctatus* showing the NT cells organized in a single cluster (*arrows*). **e** Sagittal section through the OB of *Paracheirodon innesi* with the NT cells (*arrows*) organized in two clusters as observed in *D. labrax* (**c**). **f** Representation (*top*) and brain section (*bottom*) from *C. aeneus* showing the pseudosessile (Type II) topology of OBs (*OM* olfactory mucosa, *OP* olfactory peduncle, *Tel* telencephalon). *Bars* 100 μm

proximity to the OM. In *D. labrax*, the NT cells were organized in two clusters, one cluster being located anterior to the OB, at the entrance of the ON and the second cluster being positioned more posteriorly, between the OB and the short olfactory peduncle (OP; Fig. 2c). In *H. punctatus*, the NT cells were organized in a single cluster throughout the short ON (Fig. 2d) and only a few cells were scattered within the ON branches proximal to the OM. Organization similar to that observed in *D. labrax* was found in *P. innesi* (Fig. 2e).

The number of NT cells appeared to be similar, with approximately 70 (35 on each side), in *D. labrax* and *C. aeneus* but fewer were present in *H. punctatus*. In *A. f. mexicanus*, the number of NT cells was approximately half that in *D. labrax* and *C. aeneus*. Few cells were counted in *P. innesi*.

The average size of the NT cells appeared inversely proportional to the number of cells in the species studied but this relationship was not predictive. Indeed, *D. labrax*, which had the most cells, did not have the smallest cells and *P. innesi*, which had the fewest of cells, did not have the largest cells.

Type III topology of OBs was found in *Carassius auratus*, *Crossocheilus siamensis*, *Gyrinocheilus aymonieri* and *Pangasius hypophthalmus*. In *C. auratus*, the NT cells were

positioned in the ON in a loose cluster between the OM and the OB (Fig. 3a). A similar organization was also observed in the other species with Type III topology of OBs (Fig. 3b, *C. siamensis*; Fig. 3c, *G. aymonieri*). In addition, a few scattered single cells or small clusters of NT cells were located posterior to the OB within the OP and in the ventral area of the telencephalon of *P. hypophthalmus* and *G. aymonieri*.

The highest number of cells was recorded in *G. aymonieri*, which had more than 200 cells (more than 100 on each side) and slightly fewer cells were found in *P. hypophthalmus*. *C. auratus* and *C. siamensis* each had fewer than half the number of cells as in *G. aymonieri* but had similar numbers to one another.

The size of the cells appeared largest in *C. auratus*, followed by *C. siamensis* and *G. aymonieri*. The smallest size was recorded in *P. hypophthalmus*. Thus, the inverse relationship between the number and size of NT cells did not apply to fish with Type III topology.

Statistical analysis

Normality tests indicated that data related to both the size and number of FMRFamide-ir cells were not normally distributed (Kolmogorov-Smirnov and Shapiro-Wilk; $P<0.001$ for both). The number of cells showed a negative Spearman correlation ($\rho=-0.584$) with the size of the cells ($P<0.001$). The dependence analysis related to the type of OB topology showed a positive output for the number of cells ($H=47.73$; $P<0.001$; Fig. 4a) and also for the size of the cells ($H=10.24$; $P=0.006$; Fig. 4b). Post hoc tests showed no significant differences between the median of number cells of Types I and II ($U=432$; $P=1.00$), whereas the median of the number cells of Type III appeared significantly higher than that of Type I ($U=11$; $P<0.001$) and Type II ($U=11.5$; $P<0.001$). Similarly, the median cell sizes of Types I and II were not different ($U=422$; $P=1.00$) but the median cell sizes of both Types I and II were higher than the median cell size of Type III (Type I vs. III: $U=179$; $P=0.005$; Type II vs. III: $U=218$; $P=0.041$).

A dendrogram based on similarities obtained by taking into account all variables under study, i.e., the number and size of the FMRFamide-ir NT cells and the topology of OBs, showed some incongruities. Indeed, *G. aymonieri* (Cypriniformes) was grouped with *P. hypophthalmus* (Siluriformes) and neither was grouped with their order. Furthermore, the Characiformes *M. sanctaefilomenae* and *P. maxillaris* appeared closer to the Cyprinodontiformes, whereas the other two Characiformes, namely *A. f. mexicanus* and *P. innesi*, were closer to the Siluriformes (Fig. 5).

Discussion

In the present study, we describe the neuroanatomical organization of the FMRFamide-ir NT cell system in a set of teleost species in order to explore its possible relationship with the topology of the OBs and with respect to the ventral telencephalic area. Several studies have been conducted on fish brain

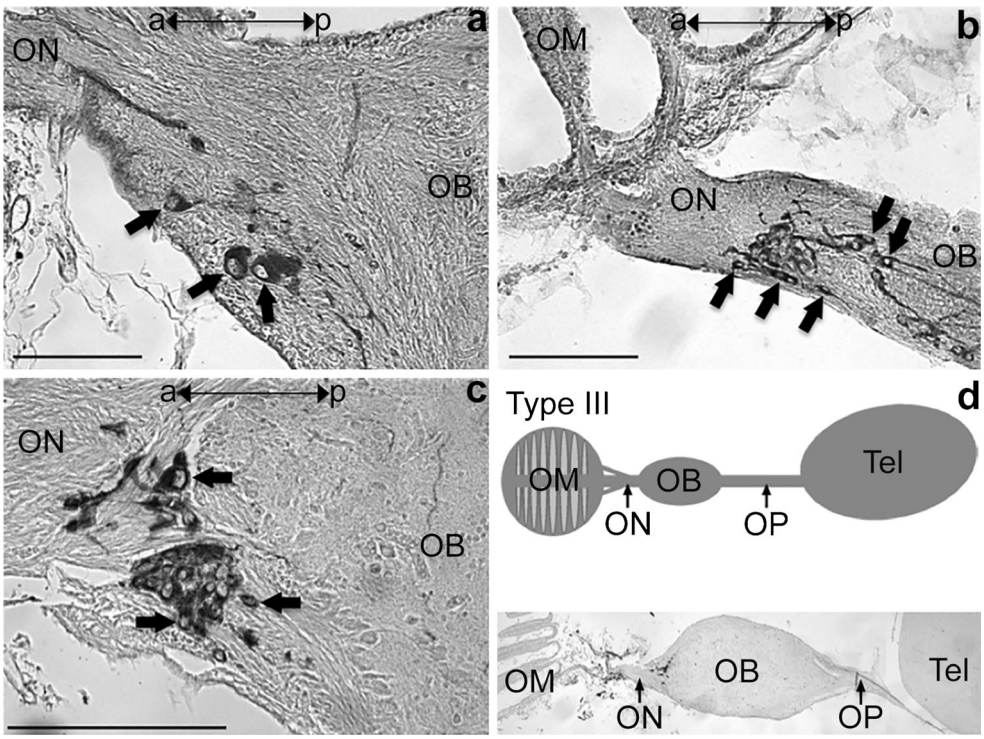

Fig. 3 Brain sections showing FMRFamide-immunoreactive NT cells in species with stalked olfactory bulbs (OBs), namely Type III. The antero-posterior axis is indicated (*a* anterior, *p* posterior). **a** Horizontal section of *Carassius auratus* showing NT cells (*arrows*) arranged in a loose cluster within the olfactory nerve (*ON*). **b** Horizontal section of *Crossocheilus siamensis* with NT cells (*arrows*) disposed in a loose cluster between the olfactory mucosa (*OM*) and the OB. **c** Horizontal section through the ON and OB of *Gyrinocheilus aymonieri* with NT cells scattered or partially clustered (*arrows*) between the ON and the anterior tip of the OB. **d** Representation (*top*) and brain section (*bottom*) from *G. aymonieri* showing the stalked (Type III) topology of OBs (*OP* olfactory peduncle, *Tel* telencephalon). *Bars* 100 μm

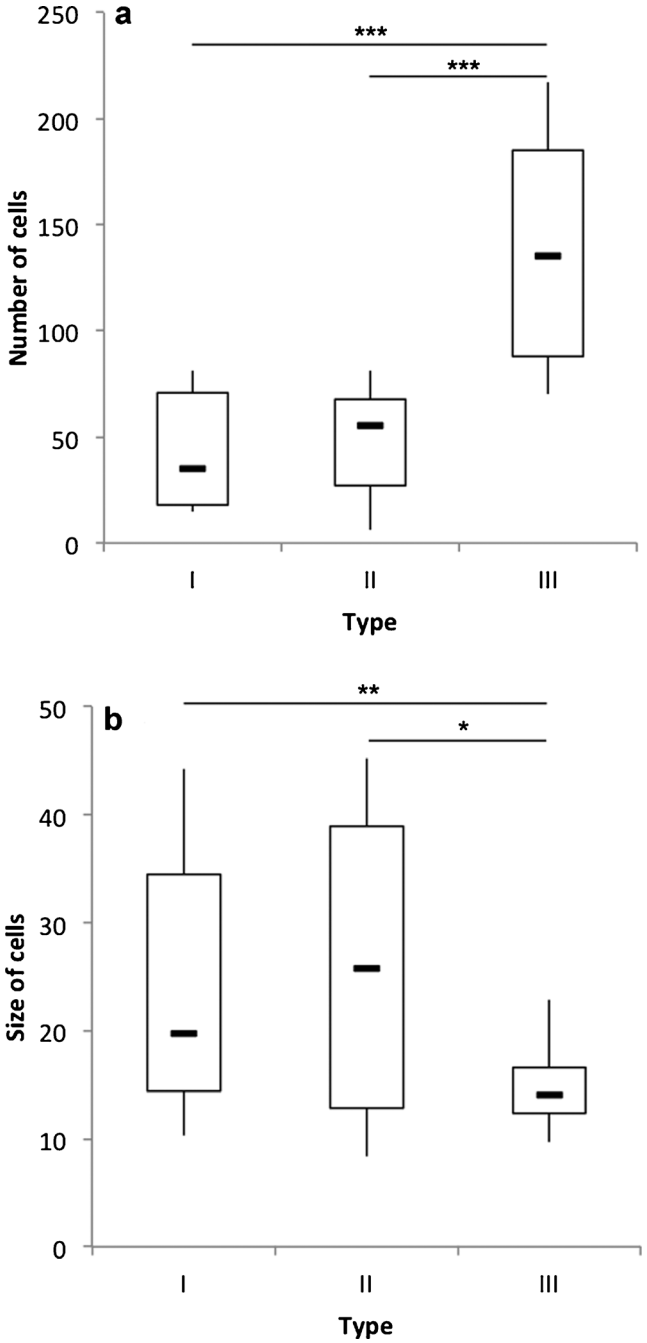

Fig. 4 Dependence analysis between the various olfactory bulb topologies (Types I, II and III) and the number (**a**) and size in micrometres (**b**) of FMRFamide-ir NT cells by using a non-parametric Kruskal-Wallis analysis of variance and Mann–Whitney U test (Bonferroni-corrected). *Short horizontal lines* within the *box* represent medians, *boxes* quartiles and *thin vertical lines* minimum and maximum values. **P* <0.05, ***P* <0.01, ****P* <0.001

to detect FMRFamide-like peptide by immunohistochemistry. In many of these studies, even when the authors have not explicitly referred either to the NT or to the morphology of the telencephalon, one can infer the position of the FMRFamide-ir cells as belonging to the NT and the topology of the OBs from the published drawings (see Table 2). A careful examination of the literature shows that only two species examined in the present study have been evaluated previously, i.e., *Xiphophorus helleri* (Magliulo-Cepriano et al. 1993) and *Carassius auratus* (Stell et al. 1984; Bonn and König 1989a; Fujii and Kobayashi 1992). In *X. helleri*, with Type I topology of OBs, Magliulo-Cepriano et al. (1993) described the presence of FMRFamide-ir NT cells at the junction between the OBs and ventral telencephalon. In *C. auratus*, with Type III topology of OBs, Bonn and König (1989a) and Fujii and Kobayashi (1992) described the presence of FMRFamide-ir NT cells in a pre-bulbar position and only rarely in the OP or in the anteroventral telencephalon. The results of both of these studies are fully in agreement with our data. Among the other studied species with Type III OB topology, Rama Krishna and Subhedar (1992) described FMRFamide-ir NT cells in a postbulbar position in *Clarias batrachus*; this description has also been reported for *Cirrhinus mrigala* (Biju et al. 2003). We also observed FMRFamide-ir NT cells in a postbulbar position in this study in *G. aymonieri*, *P. hypophthalmus* and *C. siamensis*.

To our knowledge, other studies include species with Type I topology of OBs in which the cells are prevalently in a postbulbar position, a result that agrees with our data. However, some of these species also show cells proximal to the OM (*Oncorhynchus nerka*, Östholm et al. 1990) and/or in the ON (*Oncorhynchus nerka*, Östholm et al. 1990; *Apteronotus leptorhynchus*, *Eigenmannia virescens*, *Hypopomus artedi*, Szabo et al. 1991; *Plecoglossus altivelis*, Chiba et al. 1996; *Danio rerio*, Pinelli et al. 2000).

Overall, the pattern of FMRFamide-ir NT cells seems to follow the general trend reported in the literature for GnRH-ir NT cells (Parhar 2002). In particular, fish with stalked OBs tend to have NT cells distributed between the OM and OB, whereas fish with sessile OBs tend to have most of their NT cells located more centrally. This finding is largely expected because the GnRH and FMRFamide-like immunoreactivities co-localize within NT cells (Stell et al. 1984, 1987), although in some cases not completely (Vecino and Ekström 1992; Kyle et al. 1995; Chiba 1997).

Based on ontogenetic studies on zebrafish (Pinelli et al. 2000; Whitlock 2004) showing a peripheral origin of the FMRFamide-ir NT cells from the olfactory placode and a target of the ventral basal telencephalon at the end of development, the NT cell distribution might represent a different degree of cell migration in adults, as proposed previously for GnRH (Parhar 2002). Thus, in fish with stalked OBs, the NT cells do not show extensive migration towards the telencephalon, whereas in fish with sessile OBs, most of the NT cells can be observed between the OBs and the ventral telencephalic area. In species with almost sessile (Type II) OBs, an intermediate condition was observed, with the FMRFamide-ir NT cells largely within the OB, sometimes being organized in one

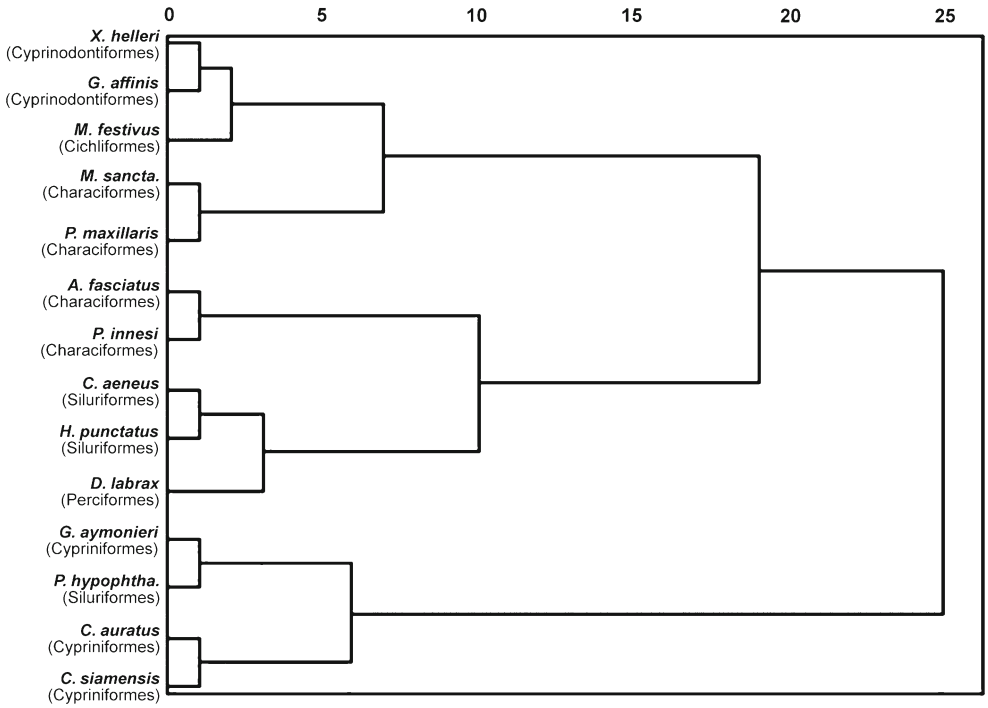

Fig. 5 Hierarchical cluster analysis by using the normalized Euclidean distance and average linkage performed by taking into account the number and size of FMRFamide-ir cells and the topology of the olfactory bulbs. The *numbers* along the *top* represent the ultrametric average distance among observations contained in each cluster of each partition obtained by cutting the hierarchical structure at various levels

large cluster and sometimes being divided into two groups along the ON-OB pathway.

With regard to the relationship between the topology of the OBs and the phylogeny of the various species, it is interesting to note, that among all the species studied (Table 2), the presence of a certain topology of OBs prevails when the fish belong to the same order, as expected. For example, all Gymnotiformes, Salmoniformes, Cyprinodontiformes and Cichliformes studied to date share the same topology of OBs (Type I). However, many instances occur in which the topology of the OBs varies within the same group. For example, amongst the Cypriniformes, which are mostly characterized by the Type III topology of OBs, *Danio rerio* (Pinelli et al. 2000) shows Type I topology. In the Characiformes and Siluriformes, the topology of OBs alternates between Types I and II and Types II and III, respectively. Whereas the Type III topology of OBs appears to be a feature often associated with more basal species, Type I and Type II topologies appear in derived species and in basal species (see Table 2). Thus, our meta-analysis, which confirms the results of Parhar (2002) for the derived species, disagrees with this author for the basal species (Ostariophysi), which might also include species with sessile OBs (i.e., Characiformes and Cypriniformes).

The number and size of FMRFamide-ir NT cells have been little studied. In *Plecoglossus altivelis*, which shows the Type I topology of OBs, Chiba et al. (1996) counted 40–50 FMRFamide-ir NT cells per side of the brain, whereas in brown trout, with the same topology of OBs, Castro et al. (2001) described approximately 50–250 cells. In *C. auratus*, which shows the Type III topology of OBs, Stell et al. (1984) counted a total of 62±4.08 neurons in each olfactory nerve, a number close to that found by Springer (1983) for the ganglion cells on each side but these numbers are based on histological stains and the large size of ganglion cells and probably do not include some of the smaller cells. Stell et al. (1984) reported that about 20 cells on each side are FMRFamide-ir (a total of about 40 cells for both sides combined). This is fewer than the number of FMRFamide-ir cells that we found in the same species (i.e., 88 on average for both sides combined). The most likely explanation for the discrepancy lies in the different techniques used (e.g., fixatives; primary and secondary antisera; thickness of the sections; differences in fluorescence emission or bleaching of the fluorophore), which might have prevented the visualization of cells with a lower content of the antigen. Nevertheless, the two studies together indicate that the FMRFamide-ir population is between about 1/3 and 2/3 of the total number of NT cells, assuming the total number as a minimum of 130 cells (65 on each side) but taking into account that the total number might be higher because smaller cells will have been missed in histological studies. On the other hand, the average soma sizes agree well between all three studies in goldfish. In other teleosts, the size of FMRFamide-ir NT cells appears variable, having been described as approximately 20 μm (*Eigenmannia virescens*, Bonn and König 1989b) or 40 μm (*Xenotoca eisenii*, Bonn and König 1988). For the first time, after the estimation of the number and size of FMRFamide-ir NT cells for each species (see Table 1), we correlated the number and size of FMRFamide-ir NT cells to the topologies of the OBs. Our data show a significant negative correlation between the size

Table 2 Studies related to the neuroanatomical position of FMRFamide-ir NT cells and the topology of olfactory bulbs (OBs). The evolutionary order of teleost was obtained from Betancur-R et al. (2013), from the basal (*top*) to the derived (*bottom*). Abbreviations: *ABC* avidin-biotin complex, *AEC* 3-amino-9-ethyl carbazole, *AP* alkaline phosphatase, *DAB* 3,3′-diaminobenzidine, *IFL* indirect immunofluorescence method, *n.s.* not specified, *OM* olfactory mucosa, *ON* olfactory nerve, *OP* olfactory peduncle, *PAP* peroxidase anti-peroxidase, *PB* phosphate buffer, *PFA* paraformaldehyde, *s.t.* section thickness, *Tel* telencephalon, *Type I* sessile OBs, *Type II* pseudosessile OBs, *Type III* stalked OBs

Basal to derived evolutionary order of teleost	Order	Species	Bulb topology	Cell location	Methods	References
Ostariophysi	Cypriniformes	*Carassius auratus*	Type III	In ON (pre-bulbar), loosely clustered along the rostromedial OB	4 % PFA; cryostat; s.t. 15–40 μm; PAP+DAB/IFL	Stell et al. 1984
				Rostroventral OB	Bouin; Paraplast; s.t. 7-11 μm; PAP+DAB	Bonn and König 1989a
				In ON (pre-bulbar), rarely in OP and ventral Tel	4 % PFA; cryostat; s.t. 15 μm; PAP+DAB	Fujii and Kobayashi 1992
				In ON (pre-bulbar)	Zamboni's fixative/4 % PFA; cryostat; s.t. 25–30 μm; IFL	Kyle et al. 1995
		Carassius auratus	Type III	Loose clusters between OM and OB, rarely in OP and anteroventral TEL	Bouin; paraffin; s.t. 10 μm; streptavidin+DAB	present study
		Cirrhinus mrigala	Type III	ON and glomerular layer of OB as discontinous cluster	Zamboni; cryostat; s.t. 15 μm; streptavidin–peroxidase+AEC	Biju et al. 2003
		Crossocheilus siamensis	Type III	Few scattered cells in ON; loose cluster in the anteromedial OB	Bouin; paraffin; s.t. 10 μm; streptavidin+DAB	Present study
		Danio rerio	Type I	ON, ventral OB and rostroventral Tel	Bouin; paraffin; s.t. 7-15 μm; ABC+DAB	Pinelli et al. 2000
		Gyrinocheilus aymonieri	Type III	Cells between OM and OB, scattered and partially organized in clusters; few cells in OP and anterior basal Tel	Bouin; paraffin; s.t. 10 μm; streptavidin+DAB	Present study
	Gymnotiformes	*Eigenmannia lineata*	Type I	Small group at caudal end of OB	Bouin or Karnovsky; Paraplast; semithin s.t. 1–2 μm; PAP+DAB	Bonn and König 1989b
		Apteronotus leptorhynchus		Within ON, cluster right before OB and one in OB/Tel; also in olfactory epithelium	Bouin; paraffin; s.t. 7 μm; PAP+DAB	Szabo et al. 1991
		Eigenmannia virescens		Within ON, cluster right before OB and one in OB/Tel; also in olfactory epithelium		
		Hypopomus artedi		Within ON, cluster right before OB and one in OB/Tel		
	Characiformes	*Astyanax fasciatus*	Type II	Few cells in ON close to OM; single cluster in ON immediately before OB	Bouin; paraffin; s.t. 10 μm; streptavidin+DAB	Present study
		Moenkhausia sanctaefilomenae	Type I	Compact group at junction between OB and ventral Tel		
		Paracheirodon innesi	Type II	Two clusters: one anterior to OB, just at entrance of the ON and second more posterior, between OB and short OP		
		Pristella maxillaris	Type I	Compact group at junction between OB and ventral Tel		
	Siluriformes	*Clarias batrachus*	Type III		4 % PFA; cryostat s.t. 15-20 μm; PAP+DAB	

Table 2 (continued)

Basal to derived evolutionary order of teleost	Order	Species	Bulb topology	Cell location	Methods	References
				Junction between ON and OB; midventral OB; junction between OB and olfactory tracts		Rama Krishna and Subhedar 1992
		Corydoras aeneus	Type II	Single cells along ON; small group from OM to proximity of OB	Bouin; paraffin; s.t. 10 μm; streptavidin+DAB	Present study
		Hypostomus punctatus	Type II	Few cells proximal to OM; single clusters throughout short ON close to OB		
		Pangasius hypophthalmus	Type III	Cells between OM and OB, scattered and partially organized in clusters; few cells in OP and anterior basal Tel		
Protacanthopterygii	Salmoniformes	*Salmo trutta fario*	Type I	Along ON and rostroventral Tel	4 % PFA; cryostat; s.t. 10-16 μm; PAP+PFA or IFL	Castro et al. 2001
		Oncorhynchus kisutch		Along ON, also near OM; junction between OB and ventral Tel	4 % PFA with 0.25 % picric acid and 0.25 % glutaraldehyde; cryostat; s.t. n.s.; PAP+DAB	Ekström et al. 1988
					4 % PFA with 0.25 % picric acid in Sorensen's PB; cryostat; s.t. n.s. PAP+DAB	Östholm et al. 1989
		Salmo salar		Junction between OB and ventral Tel	4 % PFA with 0.25 % picric acid and 0.25 % glutaraldehyde; cryostat; s.t. n.s.; PAP+DAB	Ekström et al. 1988
					4 % PFA; cryostat; s.t. 10 μm; PAP+DAB	Vecino and Ekström 1992
		Oncorhynchus nerka		Two ganglia within ON: one close to OM and another halfway between OM and OB	4 % PFA+0.25 % picrc acid in Sorensen's PB; cryostat; s.t. 33 μm; PAP+DAB	Östholm et al. 1990
Stomiati	Osmeriformes	*Plecoglossus altivelis*	Type I	Compact mass halfway between OM and OB: few cells scattered in ventromedial OB	Bouin or Karnovsky; paraffin; s.t. 6 to 10 μm; streptavidin-byotin+DAB	Chiba et al. 1996
Percomorphaceae	Anabantiformes	*Colisa lalia*	Type I	Ganglion location on ventromedial forebrain, between OB and Tel	Zamboni; cryostat; s.t. 30 μm; IFL	Wirsig-Wiechmann and Oka 2002
	Cyprinodontiforms	*Poecitia latipina*	Type I	Junction between OB and ventral Tel	Bouin-Hollande+variety of fixatives; paraffin/ cryostat; s.t. 4 μm/10-16 μm; PAP+DAB	Batten et al. 1990
		Xiphophorus maculatus		Junction between OB and ventral Tel	Bouin; polyfin; s.t. 5 μm; PAP/ABC+DAB/AP	Magliulo-Cepriano et al. 1993
		Xiphophorus helleri		Junction between OB and ventral Tel		
		Xenotoca eisenii		In OB or adjacent to it, in rostroventral Tel	Bouin; Paraplast; st. 7-10 μm; PAP+DA	Bonn and König 1988
		Xiphophorus helleri	Type I	Compact group at junction between OB and ventral Tel	Bouin; paraffin; s.t. 10 μm; streptavidin+DAB	Present study
		Gambusia affinis		Compact group at junction between OB and ventral Tel		
	Cichliformes	*Herotilapia multispinosa*	Type I	Junction between OB and ventral Tel	4 % PFA; cryostat; s.t. 10–20 μm; PAP+DAB	Rusoff and Hapner 1990a, 1990b
		Haplochromis burtoni		NOR ventrally at junction between OB and ventral Tel	4 % PFA; cryostat; s.t. 20 μm; IFL	Jadhao et al. 2001

Table 2 (continued)

Basal to derived evolutionary order of teleost	Order	Species	Bulb topology	Cell location	Methods	References
		Mesonauta festivus	Type I	Compact group at junction between OB and ventral Tel	Bouin; paraffin; s.t. 10 μm; streptavidin+DAB	Present study
	Perciformes	*Gasterosteus aculeatus*	Type I	Junction between OB and ventral Tel	4 % PFA with 0.25 % picric acid and 0.25 % glutaraldehyde; cryostat; s. t. n.s.; PAP+DAB	Ekström et al. 1988
		Dicentrarchus labrax	Type II	Two clusters: one anterior to OB, just at entrance of the ON and second more posterior, between OB and short OP	Bouin; paraffin; s.t. 10 μm; streptavidin+DAB	Present study

and number of FMRFamide-ir NT cells. This means that the fewer cells there are, the larger they are. This result might be related to the necessity of producing a sufficient quantity of neuropeptide to satisfy the physiological needs of the species. Of note, the different types of goldfish NT cells have been shown to differ significantly in the extent of axonal collaterals and sizes of the central targets (von Bartheld and Meyer 1986). This supports the view that NT cells can vary in cell size depending on the amount of neuropeptides that they need to supply to their target cells. However, we have not collected physiological data; hence, this conclusion remains speculative at the moment.

The statistical analysis revealed a significant dependence for the number of cells, with Types I and II being equivalent and both Types I and II with significantly fewer cells compared with Type III. In contrast, Type III shows smaller cells with respect to the other two OB topologies. This indicates that displaced OBs are associated with several smaller NT cells, whereas sessile or pseudosessile OBs have fewer larger NT cells.

The dendrogram based on the similarities of the olfactory components under study (i.e., the number and size of the NT cells and the topology of OBs) shows many deviances from the teleost phylogenetic tree (see Betancur-R et al. 2013). Indeed, *G. aymonieri* (Cypriniformes) is grouped with *P. hypophthalmus* (Siluriformes) and not with all other Cypriniformes; *D. labrax* (Perciformes) is located far away from the Ovalentaria (Cyprinodontiformes and Cichliformes), as an outgroup of the Siluriformes, a result that differs from the phylogenetic structure. Additionally, *M. festivus* (Cichliformes) is represented as an outgroup of the Cyprinodontiformes and as being very close to the Characiformes, in an anomalous evolutionary position. This is an interesting outcome showing that, in the olfactory system, adaptive plasticity largely overlaps evolutionary constraints, allowing contingent phenomena of divergence or convergence among species. This conclusion is in accordance with previous observations in cichlid fish demonstrating that the OBs are the most variable brain structures and that they are highly morphologically divergent (Gonzalez-Voyer et al. 2009), so that different natural populations of the same species can differ in the relative size of their brain areas depending on the habitat type or experimental conditions (Gonda et al. 2011, 2012; Eifert et al. 2014). Because phenotypic plasticity has been considered the target of selection (Gonda et al. 2013), the high rate of variability in phenotypic plasticity might be the basis for relatively fast, genetically determined, adaptive variability (see Gonda et al. 2009); this might explain both why phylogenetically unrelated species display a similar organization of their OBs and the associated NT and why the related species can diverge in these structures, as shown in this study.

Several correlations have been found between the brain organization of fish and various ecological and behavioural

patterns, such as diet and feeding habits (Huber et al. 1997; Gonda et al. 2009) and environmental and social factors (Lema et al. 2005; Kihslinger et al. 2006; Pollen et al. 2007; Gonzalez-Voyer et al. 2010; Kotrschal et al. 2012; Lecchini et al. 2014). Some of these factors might also explain the variability that we observed in the position of the OBs and the corresponding neuroanatomy of FMRFamide-ir NT components. Unfortunately, we do not have access to sufficient species, relative to the large number of environmental variables, to investigate this issue. Studies addressing these questions are required.

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