

# Cytoarchitecture of the Telencephalon in the Coral Reef Multiband Butterflyfish (*Chaetodon multicinctus*: Perciformes)

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## Key Words

Neuroanatomy · Forebrain · Teleost · Immunohistochemistry · Behavior · Chaetodontidae

## Abstract

Detailed neuroanatomical studies of model species are necessary to facilitate comparative experiments which test hypotheses relevant to brain evolution and function. Butterflyfishes (Chaetodontidae) boast numerous sympatric species that differ in social behavior, aggression and feeding ecology. However, the ability to test hypotheses relevant to brain function in this family is hindered by the lack of detailed neural descriptions. The cytoarchitecture of the telencephalon in the monogamous and territorial multiband butterflyfish, *Chaetodon multicinctus*, was determined with Nissl-stained serial sections and an immunohistochemical analysis of arginine vasotocin (AVT), serotonin, substance P and tyrosine hydroxylase. The ventral telencephalon was similar to that of other perciform fishes studied, with one major difference. A previously undescribed postcommissural region, the cuneate nucleus, was identified and putatively assigned to the ventral telencephalon. While the function of this nucleus is unknown, preliminary studies indicate that it may be part of a behaviorally relevant subpallial neural circuit that is modulated by AVT. The dorsal telencephalon consisted of 15 sub-

divisions among central, medial, lateral, dorsal and posterior zones. Several regions of the dorsal telencephalon of *C. multicinctus* differed from many other perciform fishes examined thus far. The nucleus taenia was in a more caudal position, and the central and lateral zones were enlarged. Within the lateral zone, an unusual third, ventral subdivision and a large-celled division were present. One hypothesis is that the enlarged ventral subdivision of the lateral zone (potential hippocampus homolog) relates to an enhancement of spatial learning or olfactory memory, which are important for this coral reef fish. This study provides the neuroanatomical basis for future comparative and evolutionary studies of brain organization and neuropeptide distributions, physiological studies of neural processing and insight into the complex social behavior of butterflyfishes.

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## Introduction

Our understanding of the neural regulation of behavior is dependent upon detailed neuroanatomical and connectome studies. When these studies are compared across non-model species (which display differences in behavior or ecological niche), variations in their neuroanatomy can highlight behaviorally relevant circuits. For example,

## Abbreviations used in this paper

5-HT	serotonin	HYP	hypothalamus
AC	anterior commissure	ICL	internal cell layer of the olfactory bulb
AVT	arginine vasotocin	II	optic tract
C	caudal	ir	immunoreactive
CER	cerebellum	LFB	lateral forebrain bundle
D	dorsal	LT	left
Dc	central zone of the dorsal telencephalon	NGS	normal goat serum
Dc1	central zone of the dorsal telencephalon, division 1	NT	nucleus taenia
Dc2	central zone of the dorsal telencephalon, division 2	OB	olfactory bulb
Dd	dorsal zone of the dorsal telencephalon	oc	optic chiasm
Dl	lateral zone of the dorsal telencephalon	OLN	olfactory nerve fiber layer of the olfactory bulb
Dld	dorsal division of the lateral zone of the dorsal telencephalon	POA	preoptic area
Dlp	posterior division of the lateral zone of the dorsal telencephalon	PSP	parvocellular superficial pretectal nucleus
Dlv	ventral division of the lateral zone of the dorsal telencephalon	RT	right
Dlv1	ventral division of the lateral zone of the dorsal telencephalon, subdivision 1	S	unknown sulcus
Dlv2	ventral division of the lateral zone of the dorsal telencephalon, subdivision 2	Se	sulcus externus
Dlv3	ventral division of the lateral zone of the dorsal telencephalon, subdivision 3	Sl	sulcus limitans
Dm	medial zone of the dorsal telencephalon	Slat	sulcus lateralis
Dm1	medial zone of the dorsal telencephalon, division 1	SOF	secondary olfactory layer of the olfactory bulb
Dm2	medial zone of the dorsal telencephalon, division 2	SP	substance P
Dm3	medial zone of the dorsal telencephalon, division 3	Sy	sulcus ypsiloniformis
Dm4	medial zone of the dorsal telencephalon, division 4	T	optic tectum
Dp	posterior zone of the dorsal telencephalon	TC	tela choroidea
Dpx	large-cell division of the lateral zone of the dorsal telencephalon	TEL	telencephalon
Dx	telencephalon	TH	tyrosine hydroxylase
E	entopeduncular nucleus of the ventral telencephalon	V	ventral
ECL	external cell layer of the olfactory bulb	Vc	central nucleus of the ventral telencephalon
GL	glomerular layer of the olfactory bulb	Vd	dorsal nucleus of the ventral telencephalon
		Vi	intermediate nucleus of the ventral telencephalon
		Vl	lateral nucleus of the ventral telencephalon
		Vp	postcommissural nucleus of the ventral telencephalon
		Vs	supracommissural nucleus of the ventral telencephalon
		Vu	cuneate nucleus of the ventral telencephalon
		Vv	ventral nucleus of the ventral telencephalon

the correlation between the size of the dorsolateral telencephalon in two blenniids and their home range provided some functional evidence for the proposed homology between the dorsolateral telencephalon of fishes and the hippocampus of mammals [Costa et al., 2011]. If clear phylogenetic relationships exist, we can compare the neuroanatomical features of different species in order to develop hypotheses about the selection pressures which shape neural circuits of behavior. Perciform fishes are the most speciose and behaviorally diverse order of vertebrates. This makes them an ideal group to identify behaviorally relevant circuits as well as evolutionary processes that might shape brain function. However, one key impediment is that the cytoarchitecture of the perciform fish telencephalon is only described in detail for a few species

(the serranid *Serranus scriba* [Rakic and Lazarevic, 1977]; two centrarchids, *Lepomis cyanellus* [Northcutt and Davis, 1983] and *Lepomis gibbosus* [Fortuyn, 1961]; the osphronemid *Betta splendens* [Marino-Neto and Sabbatini, 1988]; the moronid *Dicentrarchus labrax* [Cerdá-Reverter et al., 2001]; the sparid *Sparus aurata* [Muñoz-Cueto et al., 2001]; the channid *Channa gachua* [Baile and Patle, 2011], and a single cichlid, *Astatotilapia burtoni* [Burmeister et al., 2009]). Detailed descriptions of the telencephalon of behaviorally diverse basal and derived species are important for the development of hypotheses relevant to brain evolution, neural development, comparative function and proximate mechanisms of behavior [Braford, 2009]. For example, Demski and Beaver [2001] surveyed the organization of the telencephalon in multiple

species within the Beryciformes, Perciformes and Tetraodontiformes orders. The results of that study highlighted three brain regions within the dorsal telencephalon (the dorsal part of the lateral zone, the medial zone and the central zone) that were hypertrophied in reef fishes. While this comparative work proposed several interesting and important hypotheses, it did not offer detailed descriptions of the entire telencephalon of these species. Thus, further studies which characterize the entire telencephalon in perciform fishes, particularly from species with well-described social behaviors and those that live in complex environments such as coral reefs, are needed.

The spatially complex environment and high biodiversity on coral reefs are evident in the large number of ecological niches filled by perciform fishes. The evolution of the diverse social behavior and feeding guilds that are apparent on coral reefs may be associated with concurrent changes within the brain. However, to date, there has been no detailed neuroanatomical study in a fish which exclusively inhabits coral reefs, although descriptions of several hypertrophied brain regions are available [Demski and Beaver, 2001]. Butterflyfishes (family Chaetodontidae) are one of the best studied families of coral reef fishes. Closely related sympatric species within this family exhibit diverse social systems, feeding guilds and mating behaviors [Reese, 1975; Ralston, 1981; Tricas, 1985, 1989; Fricke, 1986; Driscoll and Driscoll, 1988; Hourigan, 1989; Tricas and Hiramoto, 1989; Roberts and Ormond, 1992; Cox, 1994; Tricas et al., 2006; Fessler and Westneat, 2007]. Comparative studies between butterflyfishes have yielded insight into the evolutionary selection pressures for mating systems and feeding guilds [e.g. Hourigan, 1989]. However, the organization of the butterflyfish forebrain, a region that would be involved in the regulation of social behavior, remains undescribed. Two previous studies describe several brain regions [Snow and Rylander, 1982; Bauchot et al., 1989], but a more complete study is needed to provide the neuroanatomical support for comparative studies focused on the neural regulation of behavior [e.g. Dewan et al., 2011]. The multiband butterflyfish (*C. multinctus*) is a monogamous corallivore that defends a permanent feeding territory from conspecifics and other food competitors [Tricas, 1989]. The high degree of site attachment as well as the consistency of its social behavior and mate fidelity makes *C. multinctus* an ideal species for foraging ecology, social behavior, acoustic and neuroendocrine studies [Hourigan, 1989; Tricas, 1989; Tricas et al., 2006; Dewan et al., 2008, 2011; Dewan and Tricas, 2011]. The neuroanatomical description of this species will lay the foundation for comparative neu-

rological studies across butterflyfishes. In addition, since *C. multinctus* differs from many other species examined in terms of their mating system, feeding guild and habitat, hypotheses relevant to perciform fish brain function can be proposed.

## Materials and Methods

### Animals

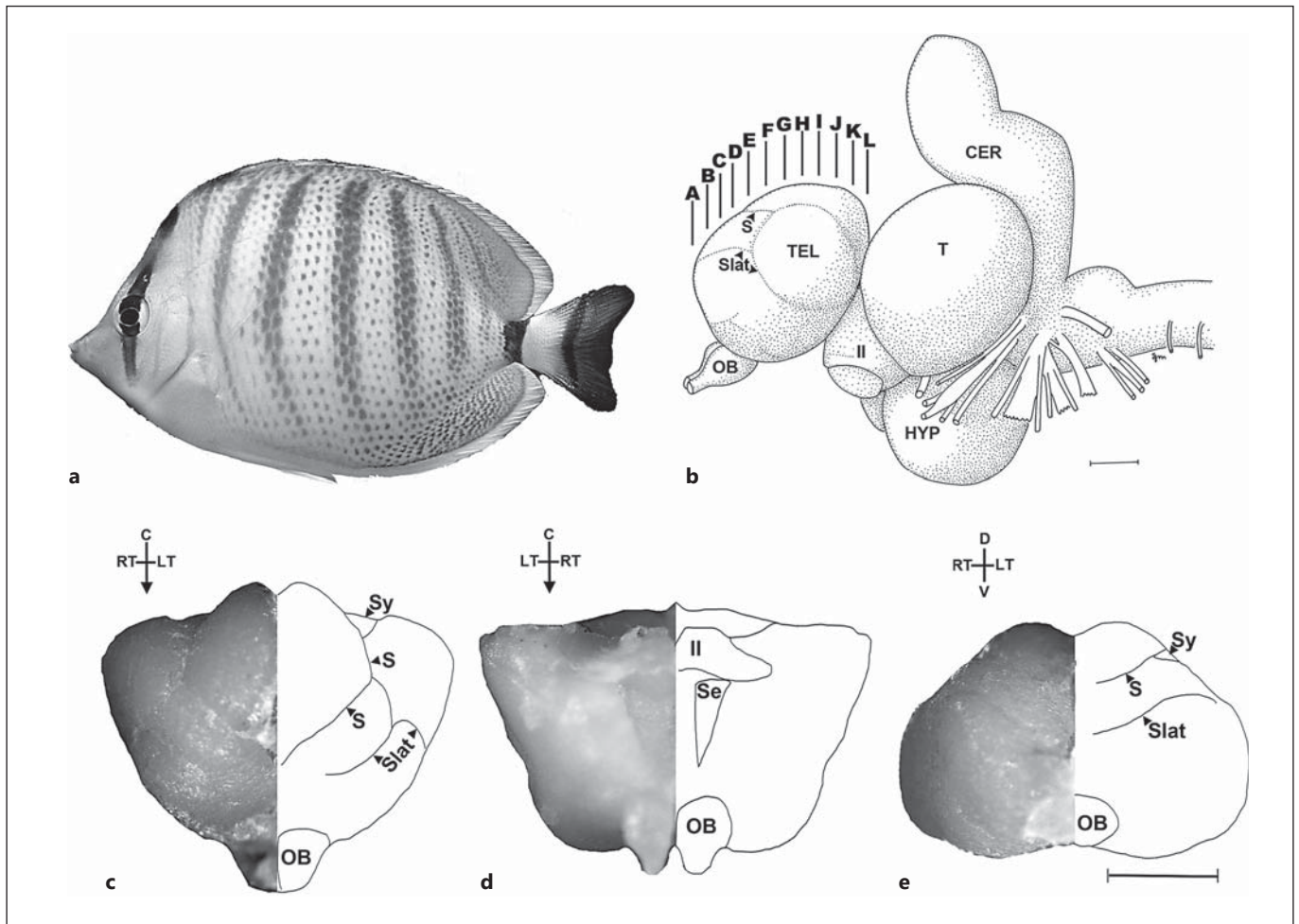
Thirty-two adult male ( $n = 15$ ) and female ( $n = 17$ ) *C. multinctus* were collected with barrier and hand nets from the west and north shore of Oahu (Hawaii, USA). Fish were housed overnight at the Hawai'i Institute of Marine Biology in flow-through saltwater tanks prior to analysis. The original research reported herein was performed under the Institutional Animal Care and Use Committee guidelines for vertebrate care and use established by the University of Hawai'i.

### Histology

Adult male ( $n = 4$ ) and female ( $n = 4$ ) fish were anesthetized with 100 mg/l tricaine methanesulfonate (MS222), measured for standard length, total length and body weight and perfused transcardially with 0.9% heparinized saline followed by alcohol-formalin-acetic acid fixative. Brains were removed and postfixed in the same fixative at 4°C for 3 days. The brains were dehydrated and processed (Leica TP1020 automatic tissue processor) prior to embedding in paraffin (Leica EG1160 paraffin embedding station). Samples were sectioned at 10  $\mu\text{m}$  in the transverse (6 brains) and sagittal planes (2 brains). Serial sections were collected on chrom-alum-coated or SuperFrost Plus slides. Sections were deparaffinized and stained with 0.5% cresyl violet, dehydrated in an ethanol series, cleared in toluene and coverslipped with Cytoseal 60 mounting media.

Telencephalic regions were identified based on their similarities to other published studies (see Introduction). Proposed homologies are based on the density, distribution, size, shape and staining intensity of the perikaryon, the relative abundance of neuroglia and neuroanatomical location. To assist in the identification of these regions in other butterflyfishes and perciform fishes, we have included data on the average soma diameter for each telencephalic region. For these measurements, 20 somata were chosen haphazardly from the entirety of the central section of each region. We calculated cell size by measuring across the short axis of each cell body (perpendicular to the axon, if visible with cresyl violet staining) on high-resolution photographs taken at  $\times 40$  magnification (Sigma Scan Pro 5.0 and Optronics Macrofire camera;  $7.4 \times 7.4 \mu\text{m}$  pixels). We note when cell size changes with rostrocaudal position, but our analysis was restricted to the midpoint of each region. If two clearly different cell types were present within a region, both were analyzed and described. In the text, we have classified the somata by size (small:  $<6.5 \mu\text{m}$ ; medium:  $6.5\text{--}12 \mu\text{m}$ ; large:  $>12 \mu\text{m}$ ). It should be noted that both the acetic acid within the alcohol-formalin-acetic acid fixative and the paraffin embedding process (see above) can cause cell shrinkage. Therefore, cell diameters represent best approximations based on this common protocol.

For the telencephalic atlas, brain sections were photographed in detail at  $\times 4$ . Frequently, several pictures were taken to encompass the entire section. Multiple pictures were made into a com-



**Fig. 1.** The external organization of the brain of *C. multicinctus*. **a** Lateral view of *C. multicinctus*. **b** Lateral view of the brain of *C. multicinctus*. Lines A–L are in the approximate location of the transverse sections represented in figure 2. **c** Dorsal view of the telencephalon. **d** Ventral view of the telencephalon. **e** Rostral view of the telencephalon. All scale bars = 1 mm.

posite using the photomerge function in Adobe Photoshop CS4. All digital photographs were adjusted only for brightness with the exception of figure 1c–e, which were also contrast-enhanced to improve the visibility of the sulci.

#### Immunohistochemistry

Adult male ( $n = 11$ ) and female ( $n = 13$ ) fish were anesthetized by immersion in tricaine methanesulfonate (MS-222), measured for standard length, total length and body weight and perfused transcardially with 0.9% heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, postfixed in the same fixative for 1.5 h at 4°C, rinsed in 0.1 M phosphate buffer and cryoprotected overnight in 30% sucrose in 0.1 M phosphate buffer prior to sectioning. Brains were then embedded with Histoprep, frozen in the cryostat and sectioned at 24 μm in the transverse plane. Alternate brain sections were collected on chrom-alum-coated slides, dried at room temperature

for 2 h and stored at 4°C until reacted. All brains were reacted within 6 days of capture. Six individuals (2–3 males and 3–4 females) were processed for each antibody. Slides were brought to room temperature, surrounded with a hydrophobic barrier, rinsed with 0.05 M PBS and blocked with 0.3% Triton-X 100 in PBS with 2% normal goat serum (NGS) for 1 h (arginine vasotocin, AVT), 5% NGS for 1 h (tyrosine hydroxylase, TH) or 10% NGS for 30 min [substance P (SP) and serotonin (5-HT)]. The primary AVT antibody (online suppl. table 1; www.karger.com/doi/10.1159/000363124) was applied to the mounted sections (final concentration 1:5,000) and incubated overnight (14–16 h) at room temperature in a sealed humidified chamber. The primary TH (1:1,000; Millipore), 5-HT (1:40,000; Immunostar) or SP antibody (1:5,000; Immunostar; online suppl. table 1) was applied to mounted sections and incubated for 16–22 h at 4°C in a sealed humidified chamber. Mounted sections were then rinsed in PBS and incubated with biotinylated goat anti-rabbit



secondary antibody with 2% NGS for 1 h (AVT, 5-HT and SP) or with 5% NGS for 1 h (TH). Sections were rinsed again in PBS, quenched with 0.5–3% hydrogen peroxide in PBS for 30 min, incubated with avidin-biotin-horseradish peroxidase complex for 2 h, rinsed in PBS and reacted with a diaminobenzidine chromogen substrate kit with nickel chloride intensification for 5–6 min (AVT) or 4–5 min (TH, 5-HT and SP). Slides were then soaked in distilled water for 10 min to stop the reaction, counterstained with 0.1% methyl green, dehydrated in an ethanol series, cleared in toluene and coverslipped with Cytoseal 60 mounting media. Alternate sections were stained with 0.5% cresyl violet and processed as above to assist in neuroanatomical identification. The following immunohistochemical controls were also performed in parallel with experimental tissue: the omission of primary antisera, secondary antisera, avidin-biotin-horseradish peroxidase complex solution or diaminobenzidine, all of which resulted in no staining. Additional controls and antibody characterization are described in the online supplementary methods. [www.karger.com/doi/10.1159/000363124](http://www.karger.com/doi/10.1159/000363124).

### Analysis

The presence or absence of immunoreactive (ir) cells or fibers was analyzed by visual scanning ( $\times 40$  magnification, see above) across all telencephalic sections for all individuals. The density of immunoreactive fibers within a region was a qualitative assessment based on the visual scanning of 6 individuals stained with each antibody. Sparse was defined as a couple of fibers (approx. 1–10) across the entire region. Low density was defined as a few fibers (approx. 1–24) in a field of view throughout the region. Moderate density was defined as a higher number of fibers (approx. 25–250) in a field of view throughout the region. High density was defined by dense fibers (approx. 300+) in a field of view throughout the region. This staining was usually visible without magnification. As each region differs in size, low-dense approximations were based on the number of fibers visible in the field of view ( $\times 40$  magnification). If our analysis of fiber densities provided support for the distinction or identification of particular telencephalic regions, it is mentioned in the text; otherwise, immunohistochemical staining is summarized in table 1. The presence of immunoreactive cells within a region is always mentioned in the text.

## Results

The telencephalon of *C. multinctus* (fig. 1b) consists of paired olfactory bulbs (OB), laterally paired hemispheres and at least 5 visible sulci (fig. 1b–e). The immunohistochemical analysis of AVT, 5-HT, SP and TH yielded only TH-ir cells in several ventral telencephalic nuclei. However, further support for the classification of these neuroanatomical regions was also based on strong differences in the density of stained fibers, as described below.

### External Telencephalic Structures

The telencephalon had 5 main sulci, as follows: the sulcus externus (Se), sulcus ypsiloniformis (Sy), sulcus limitans, sulcus lateralis (Slat) and an additional unnamed sulcus. On the ventrolateral surface of the telencephalon, the Se is visible caudal to the OB, where it is narrow. It widens more caudally and ends between the anterior commissure (AC) and preoptic area (fig. 1d, 2h, i, 3f, g, 4l). The lateral portion of the Se is the attachment point of the tela choroidea (asterisk in fig. 2i). This sulcus may serve as a boundary to distinguish the dorsal and ventral telencephalic areas, as the Se is located between the lateral zone of the dorsal telencephalon (Dl) and the ventral telencephalon. On the dorsal surface of the telencephalon and closely associated with the dorsal zone of the dorsal telencephalon (Dd) is the Sy (fig. 1c, e, 2f, g, 4c, d). On the medial surface of each hemisphere is the sulcus limitans. This sulcus is most evident between the supracommissural nucleus of the ventral telencephalon (Vs) and the medial zone of the dorsal telencephalon, division 4 (Dm4), and may also serve as a boundary between the dorsal and ventral telencephalon (fig. 2g). Associated with the border between the dorsal division of the lateral zone of the dorsal telencephalon (Dld) and the ventral division of the lateral zone of the dorsal telencephalon, subdivision 2 (Dlv2), is the Slat (fig. 1b, c, e, 2c–j, 4j). An additional unnamed sulcus was also identified. The unnamed sulcus is associated with the separation between the medial zone of the dorsal telencephalon, division 2 (Dm2), and the medial zone of the dorsal telencephalon, division 3 (Dm3; fig. 1b, c, e, 2d–g, 4c, 5g). The ventricular surface of the telencephalon in *C. multinctus* is very extensive as it includes the medial surface of each hemisphere and continues to the lateral edge, where the tela choroidea attaches. Specifically, the ventricular surface includes the external edge of the Dl, medial zone (Dm) and Dd of the dorsal telencephalon and the Vs, dorsal nucleus of the ventral telencephalon (Vd) and ventral nucleus of the ventral telencephalon (Vv). The meningeal surface is likewise quite diminished, covering a small area along the ventrolateral portion of each hemisphere near the central nucleus of the ventral telencephalon (Vc) and lateral nucleus of the ventral telencephalon (Vl).

### Olfactory Bulbs

The OB are sessile, located immediately rostral to the ventral telencephalon and divided into 5 concentric laminae in the following centripetal order: olfactory nerve fi-

**Table 1.** The size and relative abundance, respectively, of AVT-ir, 5-HT-ir, SP-ir and TH-ir somata and fibers in the telencephalon of *C. multicinctus*

Brain region	Cell diameter (mean ± SD), μm	AVT		5-HT		SP		TH	
		cells	fibers	cells	fibers	cells	fibers	cells	fibers
<i>Dorsal telencephalon</i>									
Dc1	12.48±1.28	–	*	–	***	–	**	–	***
Dc2	16.59±1.90	–	*	–	***	–	**	–	***
Dd	7.67±0.48	–	**	–	***	–	**	–	***
Dld	10.99±0.46 and 6.44±0.35	–	–	–	**	–	**	–	***
Dlp	9.14±0.83	–	–	–	***	–	*	–	***
Dlv1	11.34±0.90	–	–	–	***	–	****	–	****
Dlv2	11.58±0.70 and 7.03±0.43	–	–	–	***	–	***	–	***
Dlv3	10.50±1.01	–	*	–	****	–	****	–	***
Dm1	6.43±0.39	–	*	–	***	–	**	–	***
Dm2	10.86±1.30	–	*	–	***	–	***	–	***
Dm3	10.20±0.58	–	*	–	***	–	**	–	***
Dm4	7.79±0.34	–	*	–	***	–	**	–	***
Dp	9.07±0.48	–	**	–	***	–	***	–	****
Dx	12.88±0.67	–	–	–	***	–	**	–	***
NT	6.70±0.47	–	–	–	**	–	***	–	****
<i>Ventral telencephalon</i>									
E	5.42±0.25	–	*	–	***	–	***	–	**
Vc	9.91±0.75	–	**	–	****	–	***	+	****
Vd	8.21±0.43	–	**	–	***	–	**	+	****
Vi	9.07±1.09	–	**	–	***	–	***	+	****
Vl	7.68±0.85	–	***	–	***	–	****	–	**
Vp	7.22±0.52	–	**	–	***	–	***	+	***
Vs	7.54±0.43	–	**	–	***	–	**	+	***
Vv	6.73±0.77	–	***	–	****	–	****	+	***
Vu	10.92±0.85	–	***	–	***	–	***	–	***

Cresyl violet cell sizes were determined from 20 somata within the center of each region for 6 individuals using Sigma Scan Pro. The presence of immunoreactive cells and fibers was determined from 6 individuals.

\* Fibers are sparse, not in every section; \*\* fibers are present in every section at low densities; \*\*\* fibers are present in every section at moderate densities; \*\*\*\* dense fibers are present in every section; – = no fibers or cells present in any individual; + = cells present in all individuals.

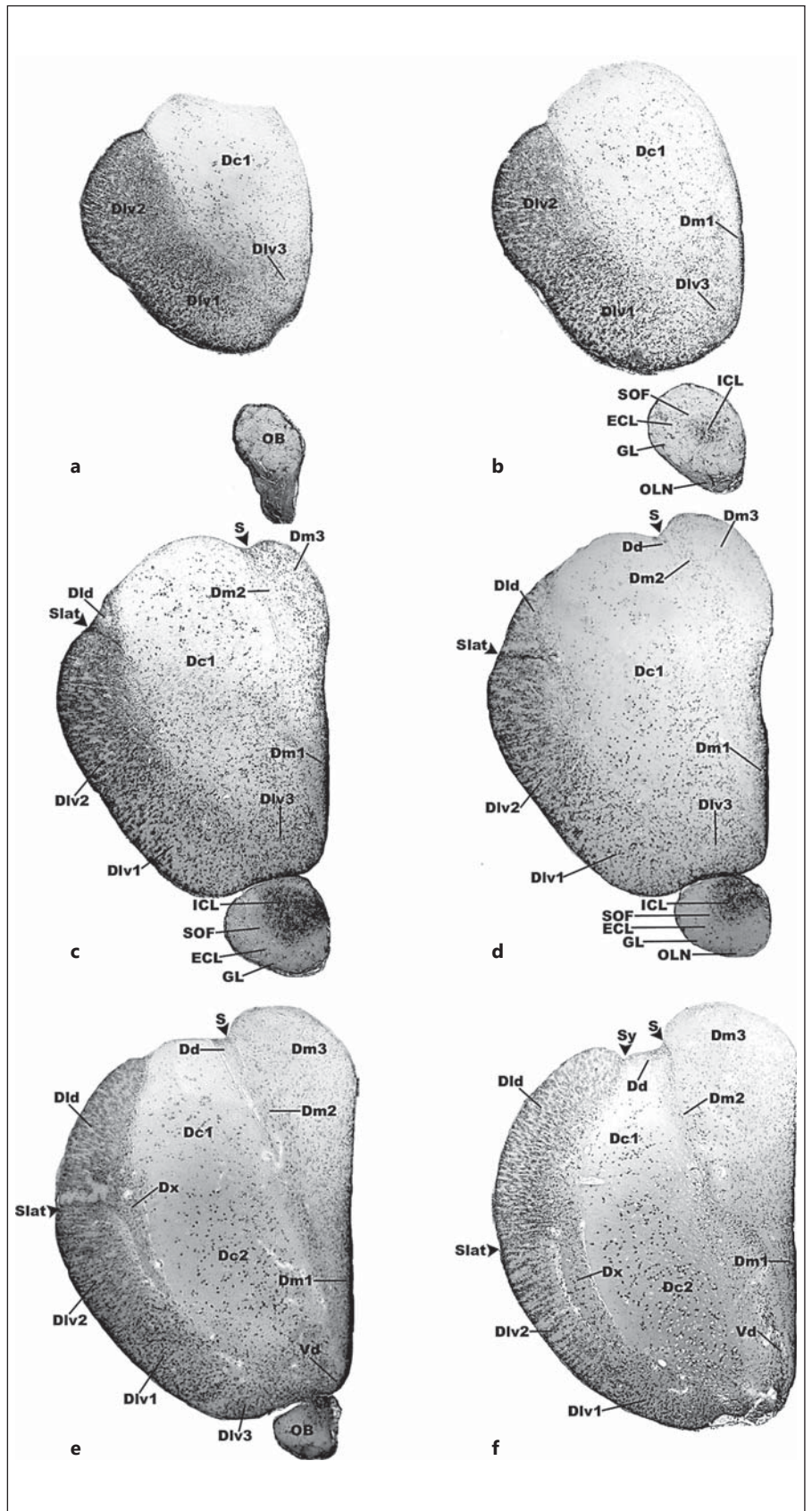
ber layer, glomerular layer, external cellular layer, secondary olfactory fiber layer and internal cellular layer (fig. 1b–e, 2a–e, 3a).

### Ventral Telencephalon

The ventral telencephalon or area ventralis is located caudal to the OB and generally lies ventral and medial to the dorsal telencephalon. The description of nuclei in the ventral telencephalon is based on their position relative to the AC. In total, the ventral telencephalon is divided into 9 nuclei, as follows: 3 precommissural nuclei, the Vd, Vv and Vl; 2 commissural nuclei, the Vs and Vc, and 4 postcommissural nuclei, the intermediate nucleus of the

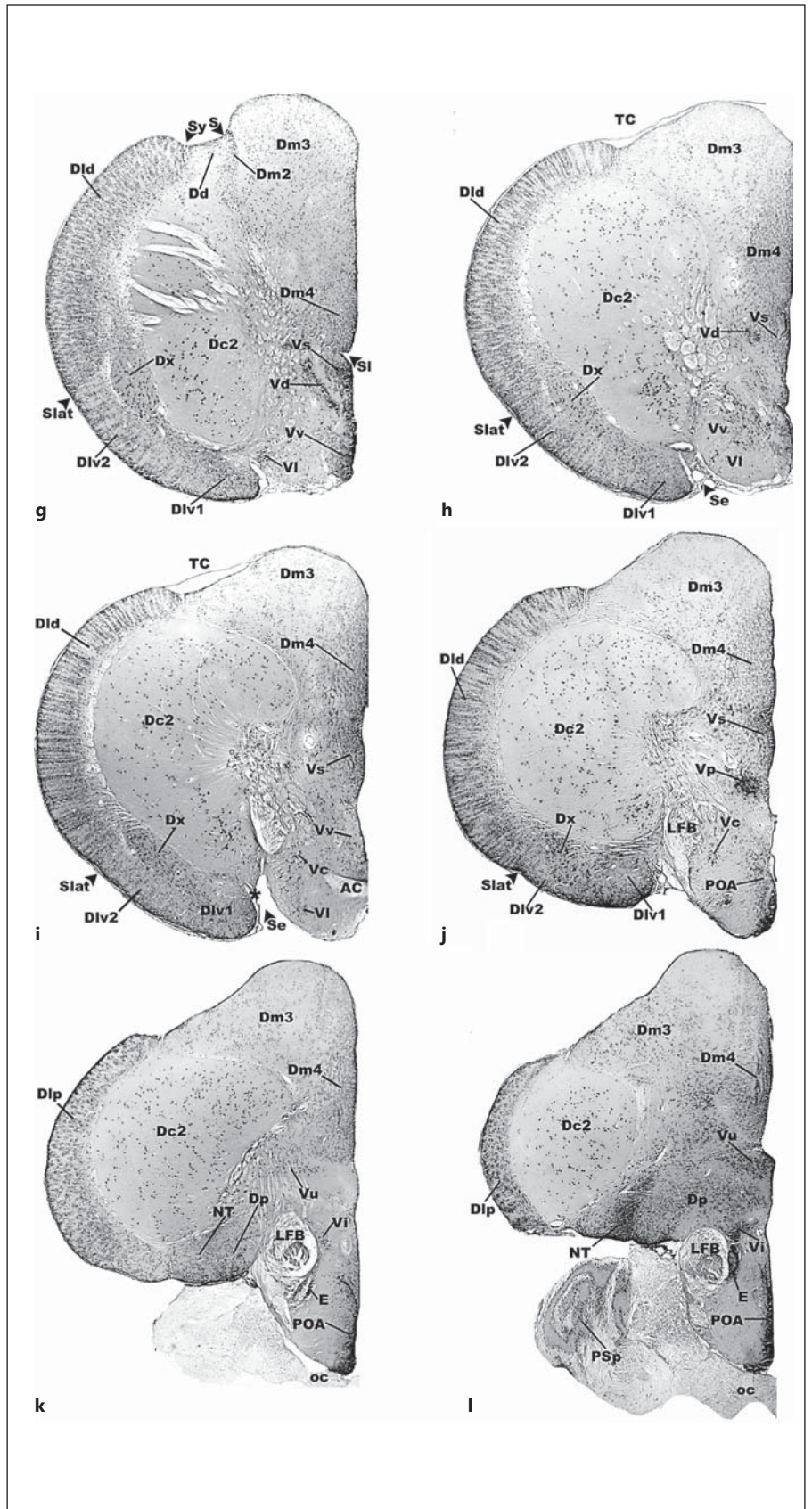
ventral telencephalon (Vi), the entopeduncular nucleus of the ventral telencephalon (E), the postcommissural nucleus of the ventral telencephalon (Vp) and a previously undescribed nucleus herein termed the cuneate nucleus of the ventral telencephalon (Vu).

The most rostral nucleus is the Vd. It begins at the level of the OB along the medial ventricular surface and is gradually displaced dorsally by the Vv (fig. 2e–h, 3b–d). Subsequently, it is displaced laterally by the Vs. It consists of medium-sized, intensely stained, round cells of moderate density. This nucleus has the highest number of TH-ir cells in the telencephalon (table 1; fig. 5a, b).



**Fig. 2. a-f** Transverse sections in a rostral-caudal sequence through the brain of *C. multicinctus* (with **a** representing the rostral-most section). The approximate rostral-caudal location of each section is denoted in figure 1b. Transverse sections were stained with cresyl violet.





**Fig. 2. g–l** Transverse sections in a rostral-caudal sequence through the brain of *C. multicinctus* (with **l** representing the caudal-most section). The approximate rostral-caudal location of each section is denoted in figure 1b. Transverse sections were stained with cresyl violet. The asterisk in panel **i**, denotes the attachment point of the tela choroidea (TC).



The Vv begins caudal and ventral to the Vd along the medial surface and gradually displaces it dorsally and laterally (fig. 2g–i, 3c, e). Caudally, the Vv expands laterally and is organized into laminae parallel to the ventricular margin. This nucleus consists of moderately stained, medium-sized, round cells. The Vv terminates at the level of the AC. The cells of this nucleus are distinct from those of the Vd as they are smaller and less intensely stained. In addition, the Vv contains only a few TH-ir cells near its periphery (table 1; fig. 5c). Immunohistochemical support for the distinction between the Vv and Vd comes from the number of TH-ir cells and the density of AVT-ir fibers in the two cell groups.

Vl remains lateral to the majority of the ventral telencephalic nuclei (fig. 2g–i, 3f, g). It is bounded dorsolaterally by the ventral edge of the central zone of the dorsal telencephalon (Dc) and the medial edge of the ventral division of the lateral zone of the dorsal telencephalon (Dlv). Rostrally, the Vl appears dorsomedial to the Se and continues caudally approximately to the level of the AC. It consists of scattered, medium-sized cells that are moderately stained (table 1).

As the Vd migrates laterally, it is gradually replaced by the Vs. This nucleus forms loose laminae parallel to the ventricular surface (fig. 2g–j, 3c–e, h, i) until it terminates slightly caudal to the AC along the medial ventricular surface. This nucleus consists of moderately stained, medium-sized cells (table 1). This nucleus is distinct from the Vd as its cells are smaller and less intensely stained. Caudally, the Vd and Vs are separated by a cell-free area. A small number of TH-ir cells are present along the periphery of the Vs (table 1; fig. 5b). Immunohistochemical support for the distinction between the Vd and Vs is based on the relative density of TH-ir cells and fibers.

The Vp is caudal to the AC, in a region vacated by the Vv (fig. 2j, 3h–j). This nucleus is located ventral to the Vs and medial to the Dc. It consists of intensely stained, medium-sized cells that are organized into clusters (table 1). This nucleus is distinct from the Vs as the cells are more intensely stained and have a higher density. TH-ir cells are present along the lateral edge of the Vp (table 1). This immunohistochemical analysis did not provide any strong support for the distinction between the Vs and Vp.

Vc is slightly caudal and dorsolateral to Vl (fig. 2i, j, 3g, j). This nucleus consists of scattered, medium-sized cells that stain intensely (table 1). More caudally, the cells of the Vc are interspersed between fiber tracts of the lateral forebrain bundle (LFB). The caudal extent of this nucleus is defined by the appearance of the E. The Vc is distinct

from the Vl as the cells are larger and more intensely stained. TH-ir cells are present near the caudal extent of the Vc, interspersed between fiber tracts of the LFB (table 1; fig. 5d). Caudally, the presence of TH-ir cells provides some immunohistochemical support for the distinction between the Vc and Vl. At rostral levels, this distinction is based solely on somata size and staining intensity.

The Vi begins in the caudal telencephalon, dorsal and medial to the LFB (fig. 2k, l, 3k, l, 4a). It consists of scattered, medium-sized cells that are weakly to moderately stained. Caudally, this nucleus is located ventral to the posterior zone of the dorsal telencephalon (Dp). A small number of TH-ir cells are present along the dorsal edge of the Vi (table 1; fig. 5e).

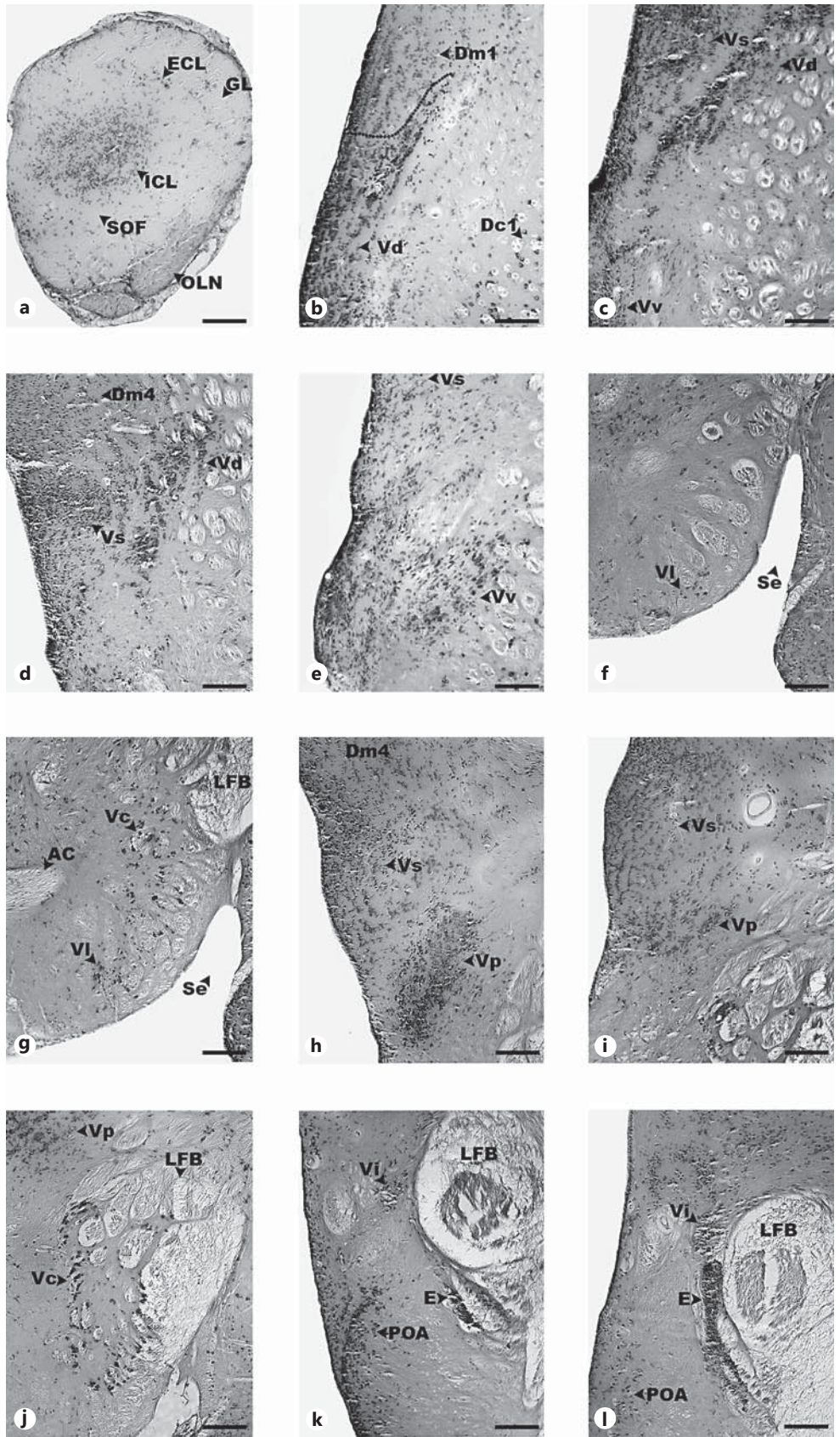
The E is located ventromedial to the LFB (fig. 2k, l, 3k, l, 4a). It consists of small, densely packed, intensely stained somata. This nucleus is located ventral to the Vi, has a moderate density of 5-HT- and SP-ir fibers and a low density of TH-ir fibers (table 1; fig. 5e).

Rostrally, the Vu consists of a diffuse group of lateral cells (fig. 2k). Caudally, this nucleus migrates medially into a cell-free region immediately caudal to the Vp (fig. 2l, 4a). Throughout most of its rostrocaudal extent, this periventricular nucleus is bordered dorsally by the Dm4 and ventrally by the Dp. It consists of medium-sized, moderately stained, scattered somata (table 1). These cells are larger than those in the Dm and Dp and are separated from both regions by a cell-free area across its entire rostrocaudal extent. Our hypothesis is that the Vu is a protuberance of the ventral telencephalon due to the cell-free laminae that isolate it from the dorsal telencephalon and its position immediately caudal to the Vs and Vp. This nucleus has moderate densities of AVT-, 5-HT-, SP- and TH-ir fibers (table 1; fig. 5f).

#### *Dorsal Telencephalon*

In the dorsal telencephalon, we identified 15 regions separated into 5 major zones: the Dm, Dc, Dl, Dd and Dp. There is some debate whether the large cells of the Dc have migrated from a periventricular zone [Braford, 1995; Northcutt, 2006] or a distinct zone [Yamamoto and Ito, 2008; Mueller et al., 2011]. Similar to the later studies, the Dc of *C. multicinctus* occupies a periventricular position (fig. 2a–e), providing some evidence for zonal distinction. Thus, we describe this putative zone within its own section.

The Dm has a large rostrocaudal extent and is divided into 4 divisions. The Dm1 begins on the medial surface of the rostral telencephalon (fig. 2b–f, 3b, 4b). It consists of



3

(For legend see next page.)



small and medium-sized cells scattered laterally and medium-sized cells packed along the ventricular surface (table 1). This division is displaced dorsally by the Vd. At caudal levels, the somata of the Dm1 are organized into parallel laminae. The caudal extent of this division is defined by the lateral expansion of the Dm3.

The Dm2 begins at the dorsomedial surface of the telencephalon (fig. 2c–g, 4c). It consists of a thin dorsoventral strip of scattered, medium-sized somata that are weakly stained (table 1). Rostrally, the Dm2 is bound laterally by the Dc1, and medially by the Dm3. As the Dm3 enlarges, this division migrates until it is bound laterally by the Dd and ventrally by the Dc2. This region is distinct from the Dd and Dm3 as the cells are more densely grouped and more intensely stained and a narrow cell-free lamina exists between these regions (in most sections). More caudally, the cells of this division become smaller and more densely packed. The caudal extent of the Dm2 is marked by the loss of the cell-free zone, and the somata become increasingly difficult to distinguish from those of the Dm3. It is possible that the Dm2 contains the main fiber pathway to the Dd. The immunoreactive fibers from all four neuromodulators (AVT, 5-HT, SP and TH) appeared to course through the Dm2 before terminating in the Dd (table 1; fig. 5g). Further experiments would be needed to confirm this initial observation.

The largest of these divisions, the Dm3, begins medial to the Dm2 along the dorsomedial telencephalic surface (fig. 2c–l, 4c–e). This region consists of medium-sized, weakly stained, scattered cells (table 1). It is distinct from the Dm2 as the cells are more diffuse and are more lightly stained.

The Dm4 is located on the medial surface, dorsal and slightly caudal to the Vd (fig. 2g–l, 3d, h, 4e). This region displaces the Dm3 laterally and has medium-sized, in-

tensely stained cells that are organized in laminae parallel to the medial ventricle (table 1). The immunohistochemical analysis provides no support for the distinction between these 4 divisions of the Dm.

The largest zone in the telencephalon is the Dc, which consists of two large-celled divisions. The most rostral division is the Dc1 (fig. 2a–f, 4b–h). This area begins at the rostral-most extent of the telencephalon, medial to the DL, and occupies a periventricular position. This area consists of large, scattered, moderately stained cells (table 1).

The Dc2 is the largest region in the telencephalon (fig. 2e–l, 4g, i–l). It begins caudal and ventral to the Dc1 and gradually displaces it dorsally. This region consists of large, moderately/intensely stained, scattered cells (table 1). It is distinct from the Dc1 as the cells are larger and more intensely stained. However, the immunohistochemical analysis provided no additional support for the distinction between the Dc1 and Dc2.

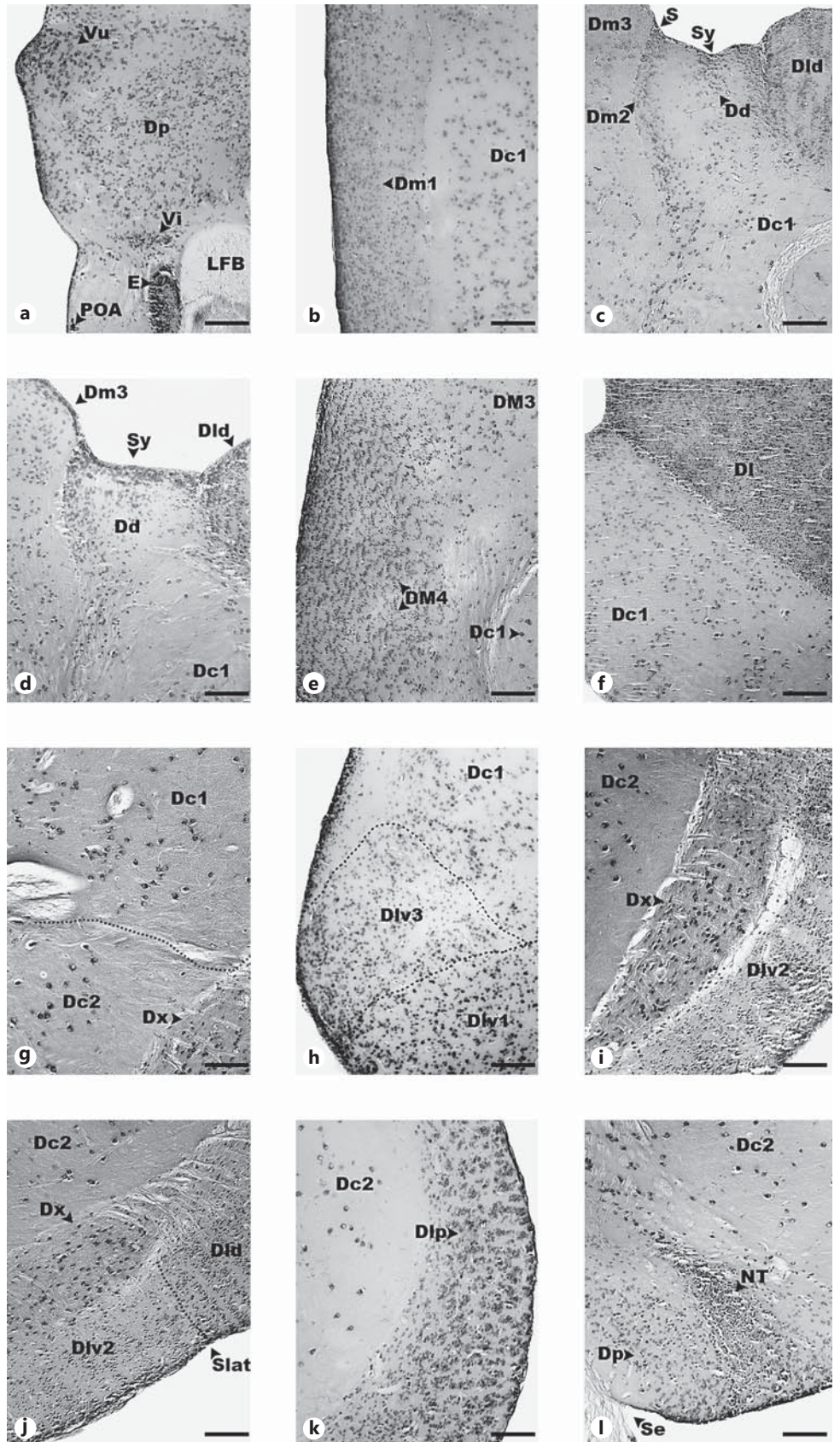
The DL is divided into 3 divisions and 1 large-cell division. The ventral division (Dlv) is further divided into 3 subdivisions. The most rostral subdivision is the Dlv1, which has a large rostrocaudal extent. It consists of medium-sized, moderately stained cells that are organized into loose clusters (table 1; fig. 2a–j, 4h). This area is displaced ventromedially by the Dlv2 and is eventually replaced by the Dp and nucleus taenia (NT).

Slightly caudal and dorsal to the Dlv1 is the Dlv2 (fig. 2a–j, 4i, j). It consists mostly of medium-sized, moderately stained cells that are organized into columnae perpendicular to the ependymal surface (table 1). The Dlv2 is distinct from the Dlv1 as the cells are smaller, organized into columns and stain more weakly. It is eventually replaced by the Dlp. At rostral levels, the Dlv2 has a lower density of 5-HT-ir fibers. The difference in the density of TH-ir fibers provides further support for the distinction between the Dlv1 and Dlv2 (fig. 5h).

At the ventromedial surface of the rostral telencephalon is a noticeable grouping of cells which are similar in size, shape and staining intensity. We propose that this region is a distinct population within the Dlv [ventral division of the lateral zone of the dorsal telencephalon, subdivision 3 (Dlv3); fig. 2a–e, 4h]. The Dlv3 consists of medium-sized, moderately stained cells that are very similar to those in the Dlv1 as well as the Dlv2 and Dld-prior to their organization into columnae (table 1). This subdivision also appears similar to the Dlv3 of Muñoz-Cueto et al. [2001]. This region is distinct from the Vd as the cells are stained more lightly, less dense and separated by a cell-free lamina. This region is also distinct from the Dlv1

**Fig. 3.** Telencephalic regions in the brain of *C. multinctus* (dorsal is up, medial is left). **a** The OB. Section at the level of figure 2d. **b** Rostral dorsal and ventral telencephalon. Section rostral to the level of figure 2f. **c** Ventral telencephalon. Section at the level of figure 2h. **d** Ventral and dorsal telencephalon. Section at the level of figure 2i. **e** Ventral telencephalon. Section at the level of figure 2h. **f** Ventral telencephalon. Section at the level of figure 2h. **g** Ventral telencephalon. Section rostral to the level of figure 2i. **h** Dorsal and ventral telencephalon. Section rostral to the level of figure 2j. **i** Ventral telencephalon. Section at the level of figure 2j. **j** Ventral telencephalon. Section at the level of figure 2j. **k** Ventral telencephalon and preoptic area. Section at the level of figure 2k. **l** Ventral telencephalon and preoptic area. Section caudal to the level of figure 2l. All scale bars = 100  $\mu$ m.





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(For legend see next page.)

as it has smaller and less intensely stained somata. The Dlv3 is gradually displaced laterally by the rostral appearance of the Vd and ventrally by the expansion of the Dc1. The caudal extent of this region is rostral to the appearance of the Vv.

The Dld begins dorsal to the Dlv2 and gradually displaces it ventrally (fig. 2c–j, 4j). It consists primarily of medium-sized, moderately stained cells organized into columnae perpendicular to the ependymal surface (table 1). This region is distinct from the Dlv2 as the cells are smaller and the laminae are wavier. In addition, the Slat delineates the boundary between these two regions. The difference in the density of 5-HT-ir fibers in the Dld compared to the Dlv2 throughout most sections provides some immunohistochemical support for these distinctions (fig. 5i).

The caudal-most region of this zone is the Dlp (fig. 2k, l, 4k). It gradually displaces the Dlv2 ventrally and consists of medium-sized, moderately stained cells that are organized into loose clusters (table 1). This region is distinct from the Dld as the cells are more dense and organized into clusters.

A region that is likely homologous with the gymnotiform large-cell division of the dorsal telencephalon (Dx) of Giassi et al. [2012a–c] was identified in *C. multicaudatus*. While these authors did not assign this region to a particular zone, they postulated that it is most likely associated with the Dl [Giassi et al., 2012a–c; Harvey-Girard et al., 2012]. We agree with this interpretation and thus describe it as a region of large cells in the lateral zone (most likely the ventral division). However, we have maintained the original abbreviation to indicate that the inclusion of this region in the lateral zone is still speculative. The Dx of *C. multicaudatus* is located lateral to both the Dc1 and Dc2 and medial to the Dl (fig. 2e–j, 4g, i, j). Specifically, it

is closely associated with the Dlv2 throughout its rostro-caudal extent. The Dx is easily differentiated from surrounding regions by the large, moderately stained, scattered cells and more intensely stained neuroglia. This region has moderate densities of 5-HT-ir and TH-ir fibers and low densities of SP-ir fibers (table 1; fig. 5j). The lack of AVT-ir fibers in this region provides some support for its association with the Dl (table 1).

Associated with the Sy is the Dd. It is bounded medially by the Dm2 (rostrally) and Dm3 (caudally) and laterally by the Dld and is distinctly separated from these regions by a cell-free lamina (fig. 2d–g, 4c, d). The cells of the Dd are medium-sized, moderately stained and densest on the dorsal, medial and lateral edges (table 1). Within the center of this region, the cells are more diffuse. The density of AVT-ir fibers within the Dd is noticeably higher than surrounding regions and thus provides some support for its distinction (table 1).

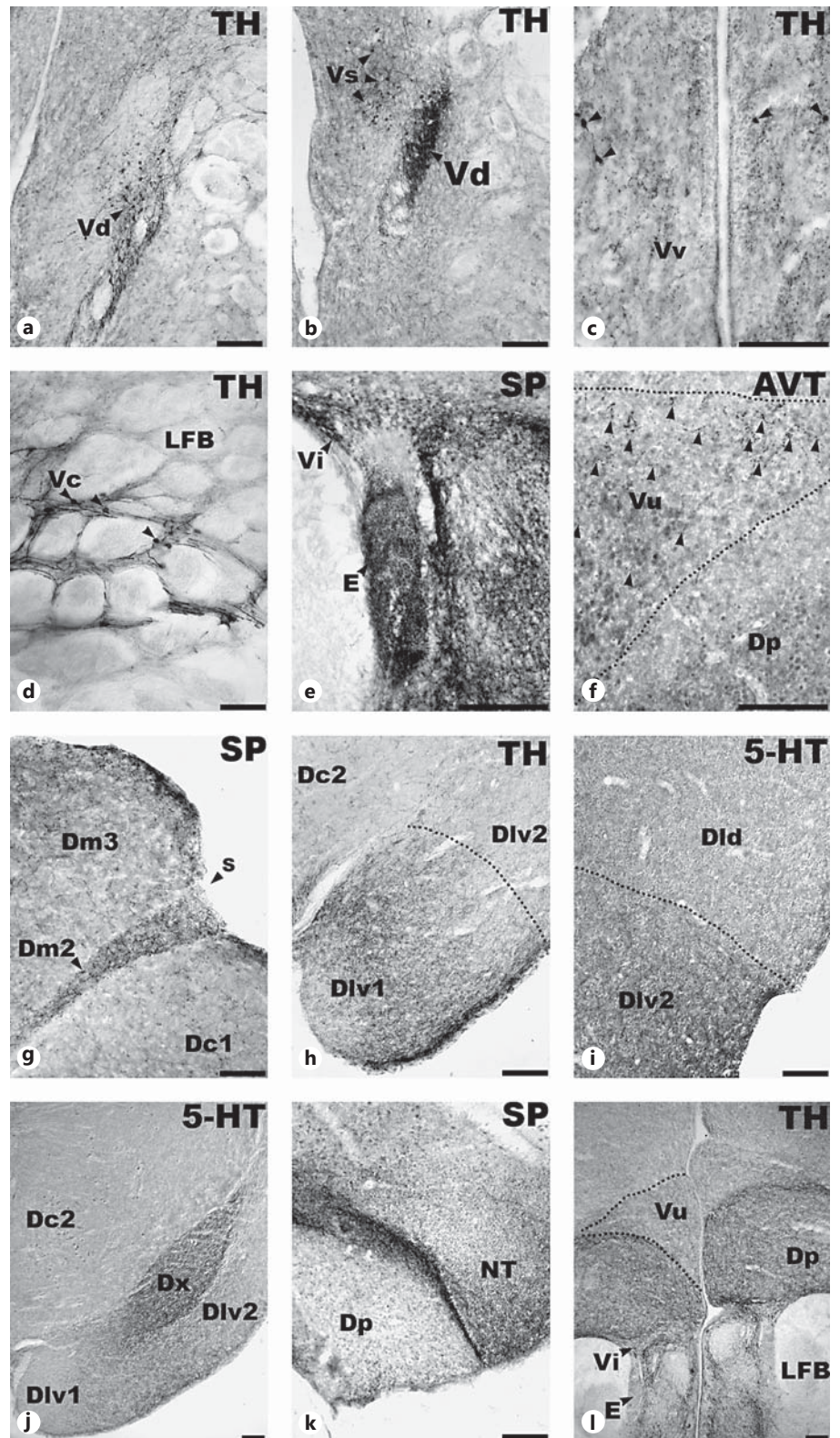
The most caudal zone of the dorsal telencephalon is the Dp. It replaces the Dlv on the ventrolateral surface of the telencephalon (fig. 2k, l, 4l). It consists of medium-sized, moderately stained cells. This zone is distinguished immunohistochemically from the Vu by its high density of TH-ir fibers (fig. 5i).

The NT replaces the ventral aspect of the Dl and is located lateral to the Dp and Se (fig. 2k, l, 4l). Based on this unusual caudal location, it is possible that this region is actually a lateral subdivision of the Dp and the NT is severely reduced. However, based upon the cytoarchitecture and location of this nucleus as well as the uniform cytoarchitecture of the Dp, this possibility seems unlikely. In other perciform fishes (see Introduction), the NT is typically in a more rostral location, ventral to the Dl. In *C. multicaudatus*, this location is occupied by the Dlv1. The possibility that a caudal aspect of the Dlv1 is actually the NT cannot be ruled out but appears unlikely based on the consistency of the cytoarchitecture of the Dlv1 throughout its large rostral-caudal extent. Lastly, the proposed NT of *C. multicaudatus* is the only area in the appropriate region which displays the high density of cells typical of the NT in other species. Thus, the proposed NT of *C. multicaudatus* represents the most likely region of homology; however, it remains possible that this region is severely reduced or indistinguishable with Nissl staining in this species. The cells of this nucleus are medium in size, intensely stained and of high density (table 1). This nucleus forms a triangle, with the apex in a dorsomedial direction. The NT is distinguished from the Dp by a thick SP-ir fiber tract that runs between these two regions (fig. 5k).

**Fig. 4.** Telencephalic regions in the brain of *C. multicaudatus* (dorsal is up, medial is left). **a** Ventral and dorsal telencephalon. Section rostral to the level of figure 2l. **b** Rostral dorsal telencephalon. Section at the level of figure 2c. **c** Dorsal telencephalon. Section rostral to the level of figure 2g. **d** Dorsal telencephalon. Section caudal to the level of figure 2g. **e** Dorsal telencephalon. Section caudal to the level of figure 2k. **f** Dorsal telencephalon. Section rostral to the level of figure 2a. **g** Dorsal telencephalon. Section at the level of figure 2e. **h** Dorsal telencephalon. Section at the level of figure 2d. **i** Dorsal telencephalon. Section rostral to the level of figure 2h. **j** Dorsal telencephalon. Section at the level of figure 2g. **k** Dorsal telencephalon. Section at the level of figure 2k. **l** Dorsal and ventral telencephalon. Section at the level of figure 2l. All scale bars = 100  $\mu$ m.



**Fig. 5.** AVT-ir, TH-ir, SP-ir or 5-HT-ir cells and/or fibers in the telencephalon of *C. multicinctus* (dorsal is up, medial is left, except **c** and **l**, which depict midline). **a** TH-ir cells in the Vd. Section caudal to the level of figure 2f. **b** TH-ir cells in the Vd and fibers in the Vs. Section at the level of figure 2g. **c** Sparse TH-ir cells in the Vv. Section at the level of figure 2h. **d** TH-ir cells in the Vc interspersed between fiber tracts at the rostral edge of the LFB. Section at the level of figure 2j. **e** Dense SP-ir fibers in the E and Vi. Section at the level of figure 2l. **f** Moderate densities of AVT-ir fibers in the Vu while the Dp is devoid of AVT-ir staining. Section at the level of figure 2l. **g** Slightly higher density of SP-ir fibers in the Dm2 compared to the Dm3 and Dc1. Section at the level of figure 2c. **h** Higher density of TH-ir fibers in the Dlv1 contrasted with lower densities in the Dlv2. Section at the level of figure 2h. **i** Lower density of 5-HT-ir fibers in the Dld compared to the Dlv2. Section at the level of figure 2h. **j** Higher densities of 5-HT-ir in the Dx and Dlv2 compared to the Dc2. Section at the level of figure 2i. **k** Higher density of SP-ir fibers in the NT compared with the Dp. In addition, a thick SP-ir fiber tract bisects these nuclei. Section at the level of figure 2k. **l** Dense TH-ir fibers in the Dp contrast with the Vu. Section at the level of figure 2l. All scale bars = 100  $\mu$ m.





**Table 2.** Proposed homologies of the dorsal and ventral telencephalic nuclei in *C. multinctus* with other descriptive studies in teleosts

Butterflyfish <sup>a</sup>	Cichlid <sup>b</sup>	Sea bass <sup>c</sup>	Sea bream <sup>d</sup>	Betta <sup>e</sup>	Sunfish <sup>f</sup>	Cavefish <sup>g</sup>	Catfish <sup>h</sup>	Zebrafish <sup>i</sup>	Goldfish <sup>j</sup>	Snakehead <sup>k</sup>
<i>Dorsal telencephalon</i>										
Dc1	Dc1	Dc1	Dc1	Dc1	Dc1		Dc1-2?			Dc1
Dc2	Dc2?	Dc2	Dc2	Dc2	Dc2	Dc	Dc3	Dc	Dc	Dc2
	Dc3?									
	Dc4-5?				Dc4?	Dc3-4?				
Dd	Ddd?	Dd	Dd?	Dd	Dd	Dd	Dd	Dd	Dd	Dd
Dld	Dld?	Dld	Dld	Dld	Dld	Dld	Dld	Dl	Dld	Dld
Dlm	-	-	-	Dc3?	Dc3?	-	-	-	-	-
Dlp	Ddv	Dlp	Dlp	Dlp	Dlp	Dl	Dlp		Dlv	Dlp
Dlv1	Dlv1?	Dlv1	Dlv1	Dlv	Dlv?	Dlv	Dlv	Dl	Dlvv	Dlv1?
Dlv2	Dlv2?	Dlv2	Dlv2				Dlv		Dlvd	Dlv2
Dlv3	-	-	Dlv3?	-	-	-			-	-
Dm1	Dm1	Dm1	Dm1	Dmv	Dm1	Dm1			Dmr	Dm1
Dm2	Dm2?	Dm2	?		Dm2	Dm2				Dm2
Dm3	Dm3	Dm3	Dm3	Dmd	Dm3	Dm3?	Dm	Dm	Dmc	Dm3
Dm4	Vsm	Dm4	Dm4	d Dmd?	Dm4	Dm4?			-	Dm4
Dp	Dp	Dp	Dp	Dp	Dp	Dp	Dp	Dp	Dp	Dp
NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
<i>Ventral telencephalon</i>										
E	E	E	E	E	E	E	E	Ed/Ev	E	E
Vc	Vc	Vc	Vc	Vc?	Vc	-	Vc	Vc?	Vc	Vc
Vd	Vd	Vd	Vd	Vd	Vd	Vd	Vd	Vd	Vd	Vd
Vi	Vi	Vi	Vi	-	Vi	Vc?	-	-	Vi	Vi
Vl	Vl	Vl	Vl	Vl	Vl	Vl	Vl	Vl	Vl	Vl
Vp	Vdc?	Vp	Vp	Vp?	Vp	-	Vp	Vp	Vp	Vp
Vs	Vsl	Vs	Vs	Vs	Vs	Vs	Vs	Vs	Vs	Vs
Vv	Vv	Vv	Vv	Vv	Vv	Vv	Vv	Vv	Vv	Vv
Vu	-	-	-	-	-	-	Vi?	-	-	-

Nuclei represent proposed well-supported homologies based on location and cytoarchitecture. ? = Putative homologies that are only poorly supported by either cytoarchitecture or location; - = no clear homology; Dc3 = third subdivision of Dc; Dc4 = fourth subdivision of Dc; Dc5 = fifth subdivision of Dc; Ddd = dorsal subdivision of Dd; Ddv = ventral subdivision of Dd; Dlv = dorsal subdivision of Dlv; Dlvv = ventral subdivision of Dlv; Dmr = rostral division of Dm; Dmc = caudal division of Dm; Dmd = dorsal

division of Dm; Dmv = ventral division of Dm; Ed = dorsal division of E; Ev = ventral division of E; Vdc = caudal division of Vd; Vsl = lateral division of Vs; Vsm = medial division of Vs.

<sup>a</sup> Current study. <sup>b</sup> Burmeister et al. [2009]. <sup>c</sup> Cerdá-Reverter et al. [2001]. <sup>d</sup> Muñoz-Cueto et al. [2001]. <sup>e</sup> Marino-Neto and Sabbatini [1988]. <sup>f</sup> Northcutt and Davis [1983]. <sup>g</sup> Riedel [1997]. <sup>h</sup> Bass [1981]. <sup>i</sup> Wullmann et al. [1996]. <sup>j</sup> Northcutt [2006]. <sup>k</sup> Baile and Patle [2011].

## Discussion

The cytoarchitecture and neurochemical organization of the telencephalon of *C. multinctus* is similar to other perciform fishes studied, with several key differences (table 2). Within the ventral telencephalon, a previously undescribed nucleus (Vu) is located between the Dm4 and Dp. It is not yet known whether this nucleus is specific to butterflyfishes or is present in other perciform fishes. Within the dorsal telencephalon, the central and lateral zones are enlarged. We identified two regions associated

with the Dlv (Dlv3 and Dx) that do not have clear homologies to telencephalic regions in other perciform fishes. The Dx does appear to be homologous to the region of the same name in two gymnotiform (non-perciform) fishes [Giassi et al., 2012a-c]. The Dlv3 is most similar to the region of the same name in *S. aurata* [Munoz-Cueto et al., 2001]; however, this homology is unclear based on the unusual rotation of many regions within that species. These regions (Vu, Dlv3 and Dx) are present in at least 5 additional Hawaiian butterflyfish species among 4 genera that exhibit diverse social behavior and ecological niches

within the coral reef environment [Dewan et al., 2011]. While the functional and hodological significance of these regions remains untested, they may have been shaped by evolutionary selection pressures present in the coral reef environment.

Selection pressures associated with the coral reef environment may favor the expansion of particular telencephalic regions. An analysis of the brains of many perciform and non-perciform reef fishes highlighted a striking hypertrophy of the Dld, Dm and Dc [Demski and Beaver, 2001; Demski, 2003]. While these regions are enlarged in *C. multinctus*, only the Dc is significantly larger than the same zone in many non-reef-dwelling perciform fishes (see Introduction, table 2). The function of the enlarged Dc of butterflyfishes is unknown. The dorsal division of the enlarged Dc of reef-dwelling squirrelfishes (non-perciform fishes) is important for visual processing [Demski, 2013]. This region is likely homologous to the caudal portion of the Dc1 or part of the Dc2 in *C. multinctus*. While the visual system of *C. multinctus* is well developed [Losey et al., 2003] and clearly important for social communication [Fricke, 1986], it is unlikely to be the sole reason for the hypertrophied Dc. Neuroanatomical tracing studies indicate that the Dc is interconnected with the OB, tectum, torus semicircularis, cerebellum and hypothalamus as well as many other telencephalic and diencephalic regions [reviewed in Demski, 2013]. These studies underscore the potential importance of this region for behavioral modulation and multimodal stimulus processing. Electrical stimulation of the Dc results in nest-building behaviors in both a perciform and non-perciform fish [Demski, 1983; Satou et al., 1984], but this behavior is not present in butterflyfishes, which are broadcast spawners [Lobel, 1989] and do not build nests. In contrast, *A. burtoni*, a polygynous, nest-building, freshwater cichlid, has an enlarged Dc that consists of 5 subdivisions [Burmeister et al., 2009]. In addition to the enlarged Dc, *A. burtoni* also has specialized cell groups within the Dm and Dl, which may be unique to cichlids [Burmeister et al., 2009]. Thus, the hypertrophy of the Dm, Dld and Dc regions may be advantageous for highly social species with complex visual and multimodal displays such as cichlids and many reef-dwelling fishes (e.g. butterflyfishes). This hypothesis warrants further comparative neuroanatomical studies focused on closely related perciform fishes which occupy diverse ecological niches.

As the Vu has not yet been identified in other fishes, it is possible the expansion of the ventral telencephalon may represent a specialization of butterflyfishes. Butterflyfishes are thought to have undergone a significant spe-

ciation event following their move onto coral reefs approximately 30 million years ago [Fessler and Westneat, 2007; Bellwood et al., 2010]. Although 61% of all corallivores belong to this family [Bellwood et al., 2010], the exploitation of this resource was not likely associated with the diversification of the butterflyfish telencephalon. This is supported by the similarity of the telencephalic organization of butterflyfishes that are thought to have last shared a common ancestor at least 20–25 million years ago [Bellwood et al., 2010; Dewan et al., 2011], while corallivory is proposed to have evolved in this family much more recently, between 3 and 15 million years ago [Bellwood et al., 2010]. However, it is possible that the invasion of coral reefs by butterflyfishes or the rapid cladogenesis that followed may be associated with some aspect of this brain diversification. Unfortunately, the telencephalic organizations of the extant putative relatives of butterflyfishes, the scats (*Scatophagidae*) and angelfishes (*Pomacanthidae*), or more distantly related kyphosids (*Kyphosidae*) and microcanthids (*Microcantidae*), have not yet been analyzed, and evaluation of this brain diversification is therefore not possible at this time. Demski and Beaver [2001] compared the brains of multiple coral reef perciform fishes but did not mention any expansion or cytoarchitectural differences in the ventral telencephalon. Accordingly, there are no leading hypotheses for the function of the Vu, but several possibilities exist. Similar to other ventral telencephalic nuclei, the Vu is innervated by AVT-ir, 5-HT-ir, SP-ir and TH-ir fibers. These data indicate that the Vu is innervated by the preoptic area/ventral hypothalamus, since those regions are the only source of AVT-ir fibers within the butterflyfish brain [Dewan et al., 2008]. However, it is the high density of AVT-ir fiber projections to this region that may provide a clue for its function. Monogamous pair-bonded butterflyfishes (such as *C. multinctus*) have approximately 4 times the density of AVT-ir varicosities within this nucleus compared to polygamous shoaling species [Dewan et al., 2011]. This indicates that the Vu is part of a subpallial neural circuit that can be modulated by AVT, possibly to elicit species-specific social behaviors. Further studies are needed to determine the developmental and evolutionary origin, neural connections and function of Vu.

Within the dorsal telencephalon, the enlarged Dlv of *C. multinctus* was the most unusual region compared to previous studies (see Introduction, table 2). This striking hypertrophy included two regions (Dlv3 and Dx) which are putatively assigned to this division and do not have clear homologies within examined perciform fish brains. The Dlv3 was most similar to the region of the same name

in *S. aurata* [Muñoz-Cueto et al., 2001]. However, this homology is unclear due to the unusual 90° rotation of several areas in this species [Muñoz-Cueto et al., 2001]. The Dx of *C. multinctus* appears to consist of large, migrated cells of the Dlv. This region could be homologous to the third subdivision or lateral division of the Dc in *Ictalurus punctatus* [Bass, 1981], *Nothobranchius furzeri* [D'Angelo, 2013] and *Barbus meridionalis* [Diez et al., 1987]. However, the location and cellular descriptions of these regions do not provide overwhelming evidence for a homology with the Dx in *C. multinctus*. More likely is a homology with the hypothalamic projecting Dx region in two gymnotiform fishes [Giassi et al., 2012a–c]. Harvey-Girard et al. [2012] proposed that the Dl in these fishes differentiated into a Dl proper and an efferent Dx region, a cell group which they hypothesized is embedded within the Dl of other teleosts. If the Dx of gymnotiforms and butterflyfishes are homologous, the selection pressures which may have favored the diversification of this brain region in butterflyfishes are of great interest. However, the dorsal telencephalons of additional species need to be analyzed before hypotheses relevant to these selection pressures can be proposed. If the Dlv3 and Dx regions are associated with the Dlv of butterflyfishes, more general hypotheses relevant to the functional significance of this enlarged region can be proposed.

The function of the enlarged Dlv is unknown but may relate to cognitive/sensory processing in butterflyfishes. It was previously suggested that an enlargement of a dorsal/caudal region of the Dl may represent a specialization in cichlids [Burmeister et al., 2009]. Since both cichlids and butterflyfishes exhibit diverse and complex species-specific behaviors, it is possible that the Dl may play a critical role in the neural regulation of social behavior. Lesion studies in *Carassius auratus* indicate that the Dlv functions in spatial learning [Portavella and Vargas, 2005], and it was hypothesized to be a homolog of the hippocampus of tetrapods as Dlv lesions impair the retention of a spatial task [Broglia et al., 2010]. This division is also larger in females of two blennioid species that must navigate considerably larger home ranges than males of the same species [Costa et al., 2011]. In *Oncorhynchus nerka*, the Dlv is activated upon the olfactory detection of their natal stream [Bandoh et al., 2011]. Thus, the enlargement of this region in butterflyfishes could relate to an enhancement of their spatial/olfactory memory. Recent field studies on pairing behavior of the monogamous and monomorphic *C. multinctus* indicate that olfactory cues are important for mate discrimination in natural settings [Boyle and Tricas, 2014]. The importance of olfactory memory for individu-

al recognition in other butterflyfish species that also exhibit an enlarged Dlv needs to be examined. Spatial memory is known to be critical for territorial butterflyfishes (e.g. *C. multinctus*) that must defend their feeding areas [Reese, 1989]. Spatial memory may also be important for planktivorous butterflyfishes, perhaps to remember feeding stations on the reef, but this has not been tested. However, many of the selection pressures that could enhance spatial memory are unlikely to be unique to butterflyfishes. The fitness of many reef-dwelling species would appear to benefit from a knowledge of site-specific locations of food resources, refuges from predators and resting shelters. Demski and Beaver [2001] compared the brains of multiple coral reef fishes but did not mention any expansion or cytoarchitectural differences within the Dlv. However, it is possible that this expansion is more subtle in other examined coral reef species. Despite this indicative evidence, the significance of the enlarged Dlv of butterflyfishes and its potential role in spatial or olfactory memory needs to be experimentally tested.

The proposed NT of *C. multinctus* is unusual compared to other published studies in terms of its caudal location but not its cytoarchitecture. The NT of most other perciform fishes is located ventral to the caudal portion of the Dlv2 and in many cases rostral to the Dp [e.g. Cerdá-Reverter et al., 2001; Muñoz-Cueto et al., 2001; Burmeister et al., 2009; Baile and Patle, 2011]. In *C. multinctus*, this location is occupied by the Dlv1, a cell region with very uniform cytoarchitecture throughout its rostral-caudal extent. It is possible that the NT in *C. multinctus* is reduced or cannot be distinguished from the caudal portion of Dlv1 based on cell size or staining intensity. However, the NT of *C. multinctus* may be displaced caudally due to the enlarged Dlv. The fact that the cytoarchitecture of the NT in *C. multinctus* is similar to the region of the same name in other perciform fishes provides some support for this hypothesis (table 2). Regardless, further comparative and developmental studies are needed to provide additional support for the proposed homology and to determine whether this caudal location translates to differences in neural connections or function.

The immunohistochemical analysis of the telencephalon of *C. multinctus* yielded only TH-ir cells within the ventral telencephalon. While the telencephalic TH system of *C. multinctus* is overall very similar to most other teleosts, a brief summary of several key differences from other published studies is warranted. The absence of TH-ir cells in the Vl of *C. multinctus* is similar to other studies [Sas et al., 1990; Meek and Joosten, 1993;



Castro et al., 2006]. However, this result differs from *S. senegalensis* and *A. burtoni*, which have at least sparse TH-ir cells in this nucleus [Rodríguez-Gómez et al., 2000; O'Connell et al., 2011]. The Vs of *C. multicolor* has scattered TH-ir cells, similar to at least two other fish species [Meek and Joosten, 1993; Castro et al., 2006; Yamamoto et al., 2010]. However, *S. senegalensis* and *A. burtoni* do not have TH-ir cells in this region [Rodríguez-Gómez et al., 2000; O'Connell et al., 2011]. Lastly, the presence of a few scattered TH-ir cells within the Vp of *C. multicolor* is unusual compared to several other teleosts [Meek and Joosten, 1993; Rodríguez-Gómez et al., 2000; Sas et al., 1990]. However, these cells most likely do not represent a unique cell group but more likely an expansion of a TH-ir group present in other fishes that includes portions of the Vs, Vc and Vi [Sas et al., 1990; Meek and Joosten, 1993; Rodríguez-Gómez, et al. 2000; Rink and Wullimann, 2001; Yamamoto et al., 2010]. Further experiments are needed to determine whether these minor differences in the TH system of *C. multicolor* are of functional significance or result from variations in the neuroanatomical organization of the species or the interpretation of these authors.

In summary, the telencephalon of *C. multicolor* consists of 9 ventral telencephalic nuclei and 15 dorsal telencephalic regions. Most regions have clear homologies to regions within other perciform fishes as they are similar in location, cytoarchitecture and immunohistochemical staining (table 2). The most prominent differences between the telencephalon of *C. multicolor* and other perciform fishes are the previously undescribed cuneate nucleus (Vu) of the ventral telencephalon and the unusual Dlv3 and Dx regions that are associated with an enlarged Dlv. At this time, the function of the Vu is unknown, but from our immunohistochemical analysis, we can surmise that the Vu is innervated at least in part by the preoptic area/ventral hypothalamus. From previous work, we can

postulate that the Vu is part of a subpallial neural circuit that is modulated by AVT in order to elicit species-specific social behaviors [Dewan et al., 2011]. The selection pressures which may have shaped the hypertrophied Dlv region in *C. multicolor* are also unknown but could relate to enhanced spatial memory for navigation of their spatially complex coral reef environment or olfactory memory, which is necessary for the discrimination of mates and other conspecific individuals [Boyle and Tricas, 2014]. This neuroanatomical description, interpreted with extensive studies on butterflyfish natural behavior and ecology, provides an associative basis for future experiments to target the function and evolution of the perciform fish telencephalon. In addition, this neuroanatomical foundation will facilitate future immunohistochemical and physiological studies to address how information on spatial perception, individual recognition, courtship and social behaviors is processed by the butterflyfish telencephalon.

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