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Distribution of serotonin immunoreactive neurons in the visual system of the beetle *Harmonia axyridis* *

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异色瓢虫视觉系统中 5-HT 阳性神经元的分布 *

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摘 要 本文运用树脂石蜡（Colophony-Paraffin，CP；专利号：ZL98125709.7）组织包埋切片技术，结合免疫组织化学链酶菌抗生物素蛋白-过氧化物酶（Streptavidin-Peroxidase，SP）双染法，对异色瓢虫视觉系统中 5-羟色胺（5-HT）能神经元的分布进行了初步研究。结果显示，异色瓢虫视觉系统的结构及 5-HT 免疫反应系统相对比较特殊。5-HT 阳性神经元胞体数目较少，染色显著，并聚集成群。根据胞体定位、细胞形态及轴突走向，可大体分为 5 群，其中包括 1 群呈弱反应的光感细胞。5-HT 阳性膨大纤维支配所有的视神经纤维网，并呈柱状或分层排列模式。结果表明 5-HT 作为经典的神经递质在昆虫的视觉信息处理过程中可能发挥重要的调节作用，且主要以远距离的广域神经调节模式为主，并在特定区域和 GABA 有伴随现象。此外，昆虫视觉系统中 5-HT 的含量还可能与其明暗适应的生理调节方式具有相关性［动物学报 51（5）：912－918，2005］。

关键词 异色瓢虫 5-羟色胺（5-HT） 视觉系统 树脂石蜡（CP） 免疫组织化学

Key words *Harmonia axyridis*，Serotonin（5-HT），Visual system，Colophony-Paraffin（CP），Immunohistochemistry

Serotonin（5-hydroxytryptamine，5-HT），the biogenic monoamine neurotransmitter，usually functions as a neurotransmitter，and like other biogenic amines，it is often associated with neuromodulation（Homberg and Hildebrand，1989b）. In the last few decades，5-HT has been extensively studied using biochemical，immunohistochemical，physiological，and pharmacological approaches（Homberg，1994）. Many insect species have been reported to express intense 5-HT-immunoreactivity within the visual system（Bishop and O'Shea，1983；Nässel and Klemm，1983；Schürmann and Klemm，1984；Wegerhoff，1999；Zhang et al.，2003）. The findings have led previous authors to suggest that 5-HT plays a critical role in circadian rhythms（Homberg，1994），through modulation of activities of other interneurons in the optic lobes and perhaps photoreceptors which may be involved in arousal or diurnal neuronal activity（Nässel，1988）.

Except for notable studies on *Tenebrio molitor*（Wegerhoff and Breidbach，1989，1991；Wegerhoff，1999），relatively little information is available on the neuroanatomy and the distribution of classical neurotransmitters in the central nervous system of Coleoptera，the largest order of Insecta. Our own studies of 5-HT-IR neurons in the visual systems of ants and butterflies（Zhang et al.，2003；Niu et al.，2004）have revealed that the 5-HT content in ants is much higher than that in butterflies under the same experimental conditions，perhaps reflecting different life styles and living environments. In particular，these differences raise the possibility that 5-HT plays a role in the light-dark adaptation of insects. However，so far，the possible relationship between

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5-HT and insect light-dark adaptation has only rarely been discussed. To further explore such a relationship, we have chosen to study 5-HT neurons in the visual system of the beetle *H. axyridis*, which shares a similar light variation environment with the ant.

H. axyridis, a multicolored natural enemy of aphids, has an extensive distribution in Asia (Wang and Shen, 2002). For a long time, it has been used as an important genetic and ecologic model for the study of biological and ecological characteristics, such as variation in elytra color polymorphism and gene mosaicism (Wang and Shen, 2002). The beetle is also a good model for anatomical and immunohistochemical studies because of its concentrated nervous system and well-defined optic neuropils. In this paper, we describe the distribution, number, and projections of 5-HT-IR neurons in the visual system of the beetle *H. axyridis*. Our aim is to provide a basis for understanding insect vision neuroanatomy as well as neurophysiology.

1 Material and methods

1.1 Experimental animals

Forty adult Asian ladybird beetles *H. axyridis* were collected from the wild in Huinan Country, Jilin Province, China.

1.2 Preparation of the specimen

The heads together with pronotums were quickly isolated from the bodies and fixed in 4% paraformaldehyde at 4℃ for 3 hours. After thoroughly rinsing in 0.1 mol PBS, the samples were dehydrated through ethanol in ascending concentrations, cleared in tertiary-Butanol at 35℃ for 24 hours, and embedded in CP embedding reagent at 53℃ for 4 hours (Bao et al., 1999). The pronotums were removed during the embedding process. The specimens were then sectioned with a rotatory microtome. Serial sections (6 μm) were cut in the frontal, horizontal, or sagittal planes, and mounted on Poly-L-Lysine-coated glass slides.

1.3 Immunohistochemistry for serotonin

The sections were deparaffinized in xylene, rehydrated through graded ethanol, and rinsed in 0.1 mol PBS and then incubated with endogenous peroxidase blocking solution (Maixin-Bio, China) for 10 min at room temperature and rinsed with 0.1 mol PBS. After blocking with normal rabbit serum (Maixin-Bio, China) for 10 min, the sections were incubated sequentially with rabbit 5-HT antiserum (Sigma, Pre-divided by Boster, China; diluted at 1∶2 000 in 0.1 mol PBS) overnight at 4℃. On the next day, after washing with PBS, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (Maixin-Bio, China) for 10 min, washed with PBS, and incubated with HRP-streptavidin peroxidase (Maixin-Bio, China) for 10 min. Subsequently, the sections were covered with a chromogenic agent solution of 3, 3′-diaminobenzidine and H_2O_2 (DAB, Maixin-Bio, China) for 3 – 5 min, rinsed with distilled water, and counter-stained with Ehrlich-hematoxylin. Finally, they were dehydrated through alcohol, cleared in xylene and mounted with neutral balsam. Two different experimental controls were carried out: (1) no-primary antiserum control, which involved running the whole immunohistochemical procedure excluding the primary antiserum; (2) no-counter stained control, the procedure of which was performed without counter-staining with Ehrlich-hematoxylin. The results were observed and photographed under an Olympus microscope.

2 Results

The head of *H. axyridis* is of the prognathous type. For consistency, however, we still consult the orientation in hypognathous insects to describe the localization of 5-HT immunoreactivity in the brain of *H. axyridis*.

2.1 Compound eyes

The compound eyes of *H. axyridis* are apposition eyes. Under the optical microscope, in each ommatidium, 6 major cells could be observed clearly (Plate Ⅰ: A). The most prominent feature of individual ommatidia was the extensive distribution of pigment granules. Besides being abundant in pigment cells (Pc), the spherical pigment granules are also present in high quantities in the cytoplasm of retina cells (Rc) (Plate Ⅰ: B, C, D), extending even into postretinal fibres (Prf) following the axons (Plate Ⅰ: E). The cornea (Co) of each ommatidium was shaped like a shallow cup (Plate Ⅰ: C), and the crystalline cone (Cc), secreted from the four crystalline cone cells (Plate Ⅰ: F), was arranged in the pattern of an equilateral triangle in longitudinal section (Plate Ⅰ: B, C). In addition, at the lateral distal part of cytoplasm in each photoreceptor, there was a specific columnar structure with strong refractivity, which was transparent in no-primary antiserum control sections (Plate Ⅰ: B) but was slight labelled by the anti-5-HT antibody (Plate Ⅰ: A, C, D).

2.2 Neuropils

In the current study, we found that all neuropil regions of the optic lobe, the lamina, medulla, and lobula complex contained 5-HT-IR fibres arrayed in different patterns such as columns and layers. The following schematic drawing is offered to give a brief overview over the visual system organization and the 5-HT immunoreactivity (Fig.1).

2.2.1 Lamina (La) The lamina could be subdi-

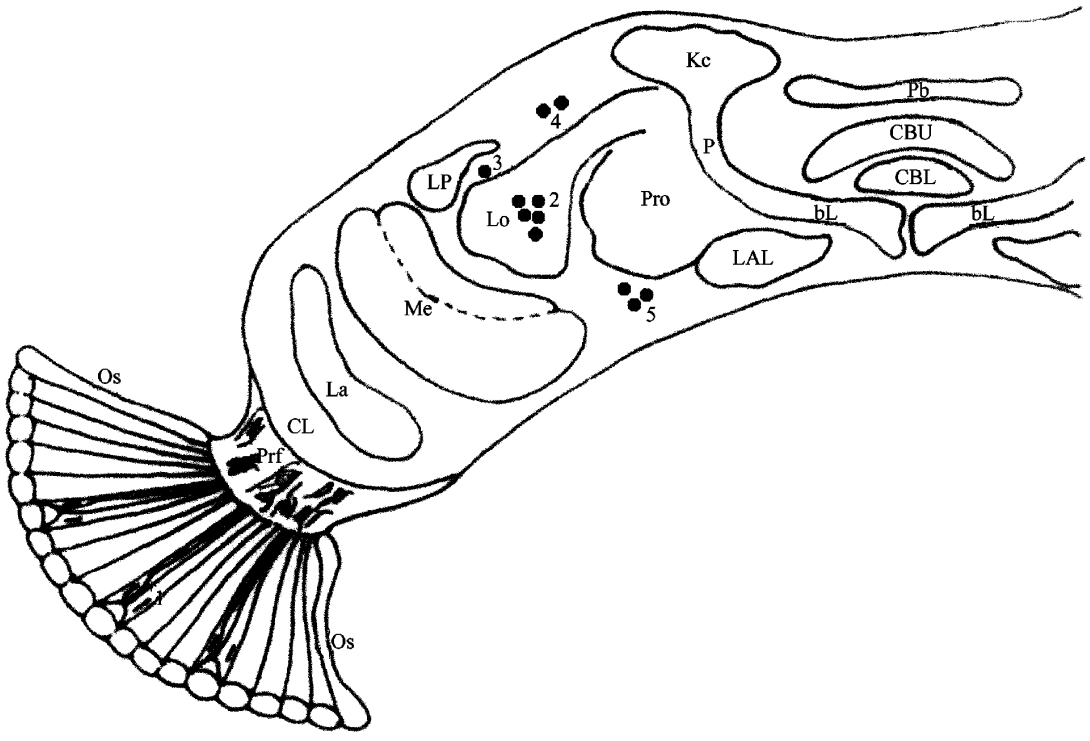

Fig.1 Basic organization of visual system and the localization of 5-HT-IR neurons in the visual system of *H. axyridis* in frontal view

Os: Orbital skeleton. Prf: Postretinal fibres. CL: Cell body layer. La: Lamina. Me: Medulla. Lo: Lobula. LP: Lobula plate. Pro: Protocerebrum. LAL: Lateral accessory lobe. Kc: Kenyon cells. P: Pedunculus. Bl: β-lobe. Pb: Protocerebral bridge. CBU: Upper central body. CBL: Lower central body. Numbers refer to cell body clusters.

vided into a cell body layer that contained the somata of monopolar cells and a fibre layer (Plate Ⅰ: G, H). A columnar organization perpendicular to the layers was a general feature of lamina (Plate Ⅰ: G, H). The fibre layer contained many 5-HT-positive fibres with varicosities throughout the entire neuropil (Plate Ⅰ: H), while the cell body layer was totally devoid of any immunostaining (Plate Ⅰ: E, G, H). In the frontal sections, a large 5-HT-positive neurite projecting from the lateral protocerebrum to the proximal edge of the medulla through the inferior part of the lobula could be observed clearly. It further divided into two main branches, which sent a few arborizations into the medulla and lobula, and then ran along the dorsal surface and the ventral brim of the medulla respectively and finally formed numerous varicose arborizations in the lamina.

In this beetle, adjacent to the dorsal-lateral edge of the lamina, we also observed a structure, comprising abundant pigment granules (Plate Ⅰ: H). It might be an extraocular photoreceptor (EP) organ (Yasuyama and Meinertzhagen, 1999), which was red in unstained section but became dense brown after the immunostaining.

2.2.2 Medulla (Me) The medulla, the largest and most complex neuropil in the optic lobe of *H. axyridis*, was connected by the outer chiasma (Och) outwards to lamina and by the inner chiasma (Ich) inwards to the lobula complex (Plate Ⅰ: G). Both chiasms were lightly labelled by 5-HT-antiserum (Plate Ⅰ: G). The neuropil of the medulla could be divided into eight layers, based upon variations in 5-HT immunoreactivity, which were interconnected to each other with numerous thin positive fibres (Plate Ⅰ: I). 5-HT-positive varicose terminals mainly invaded the first, third, fifth and seventh layers, whereas the other four layers only showed slight brown background (Plate Ⅰ: I). The width of each layer in the medulla was approximately similar (ca. 11－15 μm) except for the most proximal one (ca. 28 μm) (Plate Ⅰ: I). In addition, the accessory medulla (AMe) (Homberg, 1994), located at the dorsal-posterior edge of the medulla, was innervated by 5-HT-positive fibres (Plate Ⅰ: J).

2.2.3 Lobula complex (LC) In *H. axyridis*, the lobula complex was subdivided into the lobula (Lo) and the lobula plate (LP) located dorsal to the medulla and lobula (Plate Ⅰ: G). Only a few thin 5-HT varicose fibres were found innervating the proximal part of lobula, and no obvious pattern was detected (Plate Ⅰ: G). The lobula plate also showed weak 5-HT immunoreactivity (Plate Ⅰ: G). Moreover, there were several large irregularly organized fibres from the lobula complex projecting to the medi-

an protocerebrum centripetally (Plate Ⅰ: G).

2.3 The distribution of immunoreactive somata

The 5-HT-IR neurons in *H. axyridis*, dark brown after immunostaining, usually clustered into groups. According to the location of the perikarya in visual system and the course of the primary neurites, they could be mainly distinguished into 5 groups (group 1－5) (Fig.1). Most of the 5-HT-IR neurons distributed in the central visual system (ca. 40 per optic lobe) were tangential neurons.

Group 1 comprised photoreceptors that presented weak 5-HT immunoreactivity (Plate Ⅰ: A, C, D). In each visual column, most of the major retinular cells showed weak immunoreactivity with brown pigment granules (Plate Ⅰ: C, D). Their immunoreactive axons projected into the optic lobe together with unlabelled postretinal fibres (Plate Ⅰ: E).

Group 2 contained about 20 immunoreactive somata (8－11 μm in diameter), situated between the medulla and the protocerebrum over the entire anterior surface of the lobula (Plate Ⅰ: K). The majority of neurites of these cells sent branches into the medulla and a small number projected into the protocerebrum. In both cases, they formed extensive varicose arborizations.

Group 3 contained only 1 reactive cell body (ca. 6 μm in diameter), located dorsal-anterior to the lobula (Plate Ⅰ: L). Its primary neurite was directed towards the medulla through the dorsal margin of the lobula, with small branches invading the lobula.

Group 4 consisted of 5 relatively large, strongly immunoreactive somata (10－12 μm in diameter), which lay between the lobula complex and the Kenyon cells and sent processes mainly toward the protocerebrum (Plate Ⅰ: M).

Group 5 comprised at least 15 positive neurons (8－11 μm in diameter), which was located in a ventral area between the medulla, lobula and lateral protocerebrum (Plate Ⅰ: N). The primary neurites of these somata mainly entered the medulla through its inferior edge, and a large number of these immunoreactive processes innervated the protocerebrum as well, thus possibly integrating the optic lobes and the other brain regions.

2.4 Fibre tracts

In *H. axyridis*, a number of fibre tracts originated from the optic ganglia and projected to the median protocerebrum. In the horizontal section, the most prominent intertubercle tract (Reischig and Stengl, 2002) (Plate Ⅰ: O), the thicker fibre tract between the lobula and anterior optic tubercle, was observed clearly. It appeared that a few thin fibres of these tracts showed 5-HT immunoreactivity.

2.5 Controls

All sections in the no-primary antiserum control showed negative labelling (Plate Ⅰ: B). While, sections that were not counter-stained only showed low-level background immunostaining (Plate Ⅰ: I).

3 Discussion

Compared with other insects, the ommatidia of *H. axyridis* have several specific morphological features. For each visual column, in the distal region, there were six retina cells accompanied with a unique refractive columnar structure. Other particular characters included a shallow cup-like cornea, an equilateral triangle crystalline cone, and a well-developed pigment system. In *H. axyridis*, we could clearly discern the crystalline cone, including the four crystalline cone cells with elliptic nuclei, which probably suggest that not all beetles belong to acone eyes. Most of the photoreceptors in this beetle showed weak 5-HT immunoreactivity. This result was also supported by the fact that a few postretinal fibres showed weak 5-HT immunostaining. There were no descending fibres projecting to compound eyes. Thus, these positive fibres would be ascending ones originating from the positive photoreceptors. In summary, in addition to histamine (HA), the main neurotransmitter of photoreceptors (Homberg, 1994), 5-HT may be also involved in the complexity of light modulation in this insect. However, the exact localization of these positive photoreceptors in the retinulae cannot be identified exactly under the optical microscopy.

The lamina, the first relay station of the insect visual information processing, is where the axons of retina cells make synaptic connections with monopolar cells. In *H. axyridis*, the lamina showed dense, fibrous immunostaining with 5-HT varicose fibres throughout the entire neuropil. This is very similar to that of ant *Camponotus japonicus* (Zhang et al., 2003), but obviously differed from other species, such as the praying mantis *Tenodera sinensis* (Leitinger et al., 1999), bee *Apis mellifera* (Schürmann and Klemm, 1984) and butterfly *Mimathyma schrenckii* (Niu et al., 2004), whose laminas have been reported to display weak or only a single layer of 5-HT immunoreactivity. Thus, 5-HT may function as a significant modulator at the first step of visual information processing in the lamina of the beetle and ant. In the present study, the medulla of *H. axyridis* could be divided into eight layers according to variations in 5-HT immunoreactivity. Previously, six layers were distinguished in the medulla of *H. axyridis*, based upon GABA immunostaining; two of those layers could be further subdivided into two sublayers (Tian et al., 2003). Therefore, the 5-HT immunoreactivity stratification

in the medulla of *H. axyridis* roughly matches that of GABA.

The overall pattern of 5-HT immunoreactivity in the visual system of *H. axyridis* was similar to that in other species, but there were significant differences in number and morphology. In *H. axyridis*, there were about 40 5-HT-IR neurons in each optic lobe, and most of them were probably tangential neurons with similar morphology. In contrast, 110 – 140, 300 – 350, and at least 25 5-HT-IR neurons per hemisphere, not only tangential ones, were found respectively in the optic lobes of the cockroach *Periplaneta americana*, sphinx moth *Manduca sexta* and mantis *Tenodera sinensis* (Bishop and O'Shea, 1983; Homberg and Hildebrand, 1989a; Leitinger et al., 1999). The variations in number and morphology of 5-HT positive neurons between species are likely to reflect the volume of the brain, living status, and visual modulation style of insects.

In the visual system of *H. axyridis*, apparently, we could observe several phenomena that were rendered as follows. The number of 5-HT positive neurons is small, and the localization of somata only restricted to certain area between the medulla and the protocerebrum with extensively distributed varicose fibres. Besides, the 5-HT-IR fibres of the lamina and the lobula were resolved in the superior protocerebrum. In addition, a few thin fibres of the fibre tracts showed 5-HT immunoreactivity. Collectively, the above phenomena supported that 5-HT mainly modulate distant and extensive neural interactions. Comparisons of 5-HT and GABA immunoreactivity in the visual system of *H. axyridis* indicate that 5-HT and GABA positive fibres share some common projection areas in each optic neuropil, such as the proximal part of the lamina and layer 1, 3, 5 and 7 of the medulla. Colocalization of these two substances in the same areas suggests a possible interaction between 5-HTergic and GABAergic pathways involved in visual information processing.

Our previous studies showed that butterflies exhibit weak 5-HT immunoreactivity in the optic lobe and that the immunoreactive fibres follow no obvious pattern (Niu et al., 2004). In contrast, all the three optic ganglia of the ant exhibited stratification and varicose-like immunoreactive fibres (Zhang et al., 2003). In our present investigation, 5-HT immunoreactivity in the optic lobe of the beetle *H. axyridis*, just like the ant, was relatively stronger than that in butterflies. It is known that the beetle and ant share environments with similar light variation. The head of the beetle can flexibly move into the pronotum when the beetle faces danger. In addition, in the daily life, the beetle takes activities such as flying and foraging in the light, but it usually chooses shadow to take a rest. Similarly, during the foraging activity, the ant always frequently shuttles from nest to ground. Thus, the behaviour of these two insects causes fast changes in the intensity of light falling on the eyes. In contrast, the butterfly undergoes activities in an environment with no sharp changes in light intensity. Therefore, the correlation between 5-HT content in the optic lobes and relative changes in light intensity experienced by different insects suggests that 5-HT may be greatly involved in light-dark adaptation in insects.

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Explanation of Plate

Plate Ⅰ　The 5-HT immunoreactivity in the visual system of *Harmonia axyridis*

A. Cross section through the ommatidia showing the visual column (Vc) constitution and the 5-HT immunoreactivity in the retinulae (↖). ↘ pointing to the positive columnar structure.

B. Frontal section of no-primary antiserum control showing the coloration of each Vc. ↘ pointing to the negative columnar structure.

C. Frontal section showing group 1 (↖) of 5-HT-IR photoreceptors. ↘ pointing to the positive columnar structure.

D. Frontal section also showing the immunoreactive photoreceptors (↖) and the columnar structure (↘).

E. Frontal section showing a few of postretinal fibres (Prf) with slight 5-HT immunoreactivity (↙).

F. Frontal section showing crystalline cone and the four crystalline cone cells with elliptic nuclei (↗).

G. Frontal section through the optic lobe showing the overall 5-HT immunoreactivity in the lamina (La), medulla (Me), lobula (Lo), and lobula plate (LP).

H. Frontal section showing the extraretinal photoreceptor (EP) organ. → pointing to the 5-HT-IR varicose terminals in the La.

I. Frontal section through the optic lobe showing the 5-HT immunostaining stratification (①－⑧).

J. Horizontal section showing the accessory medulla (AMe).

K. Frontal section showing the group 2 (◣) of 5-HT-IR neurons.

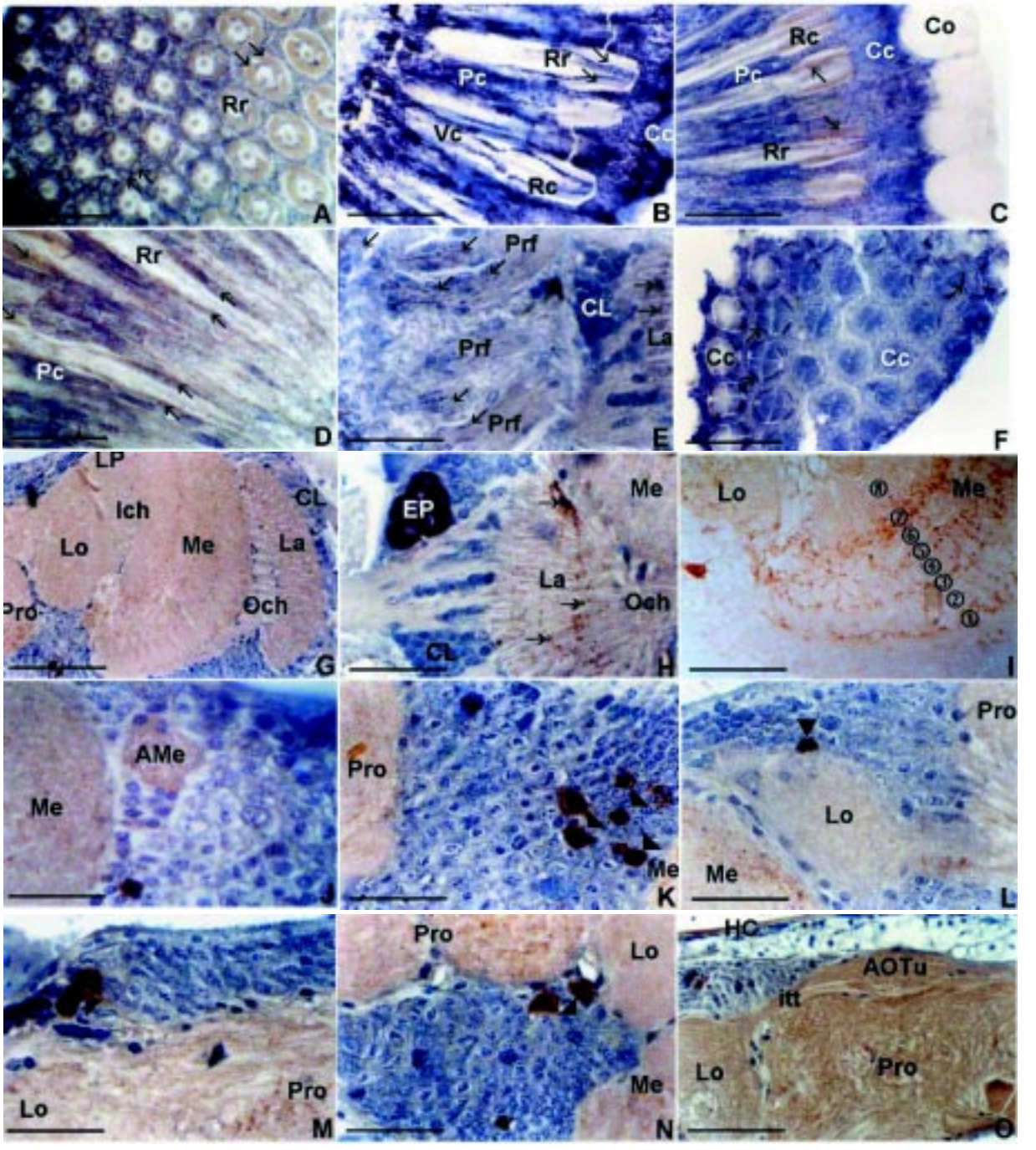
L. Frontal section showing the group 3 (▼) of 5-HT-IR neurons.

M. Frontal section showing the group 4 (◤) of 5-HT-IR neurons.

N. Frontal section showing the group 5 (◢) of 5-HT-IR neurons.

O. Horizontal section showing the intertubercle tract (itt).

Rr: Retinal rod. Pc: Pigment cell. Cc: Crystalline cone. Rc: Retina cell. Co: Cornea. CL: Cell body layer. Och: Outer chiasma. AOTu: Anterior optic tubercle. HC: Head capsule. Scale bar: A, D, I, J = 30 μm; B, C, E, F, H, K, L, M, N = 40 μm; G, O = 100 μm.



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