Distribution of Serotonin Nerve Cells in the Rabbit Brain—Immunohistochemistry by the Two-Step ABC Technique Using Biotin-Labeled Rabbit Serotonin-Antibody*

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Summary. The distribution of serotonin neuronal somata in the brain of the rabbit was demonstrated by use of immunoperoxidase technique. The serotonin antibody obtained from a rabbit was applied with the two-step ABC (avidin-biotin-peroxidase complex) method after biotinylation of this antibody.

The location, shape, size and immunoreactivity of these somata were clarified in our permanent preparations without non-specific and background staining. These perikarya were distributed in the raphe nuclei, linear nuclei and interpeduncular nucleus, within and around the medial lemniscus, in the mesencephalic periaqueductal grey lateral to the raphe dorsalis, and in the reticular formation of the brain stem. However, the so-called “masked indolamine cells” were not observed in the hypothalamus or the area postrema, as our experimental animals were not given any pharmacological pretreatment.

These results were essentially similar to those from other mammalian species, especially the rat.

A highly sensitive serotonin antiserum was raised in our laboratory from rabbits using serotonin creatinine sulfate coupled with bovine thyroglobulin (TAKEUCHI et al., 1982a; SANO, 1983). This antiserum was employed for the immunohistochemical demonstration of serotonin neurons in the central nervous system of various vertebrates using a slight modification of the peroxidase antiperoxidase complex (PAP) method by STERNBERGER et al. (1970). We have reported on the distribution of the somata of serotonin neurons in the brains of various vertebrates (chicken: YAMADA et al., 1984; turtle: UEDA et al., 1983; bullfrog: UEDA et al., 1984a; rat and cat: TAKEUCHI et al., 1982a; dog: Kojima et al., 1983; macaque: TAKEUCHI et al., 1982b). This series of phylogenetical studies continued using rabbits. However, non-specific reaction and background immunostaining were marked. These disturbances occurred when the anti-rabbit IgG was used as the second antibody. To avoid these difficulties, the serotonin-antibody was biotinylated and the avidin-biotin-peroxidase complex (ABC) method by Hsu et al.

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(1981a, b, c) was altered for free floating sections and used with the two-step ABC technique. The methodology of biotin-labeling and the application of this technique for immunoelectron- and light-microscopic studies has been described elsewhere (Yamada and Sano, 1985).

With regard to the serotonin neuron system of the rabbit, Feltén and Cummings (1979) detailed the morphological features of raphe nuclei with formaldehyde-induced fluorescence (FIF) histochemistry (Falck et al., 1962) and other techniques; Howe et al. (1983) studied it using the monoclonal antibodies (Consolazione et al., 1981) from rat hybridomyeloma cells for the purpose of avoiding the above drawbacks. In the former study, we could not obtain a comprehensive mapping of the serotonin neuron system because the morphological features of the raphe nuclei were its main focus. The latter study produced results different from ours, e.g., the positive perikarya in the area postrema, which may occur after pharmacological pretreatment. In this study without pharmacological pretreatment, the distribution of serotonin neuronal somata in rabbit brains was revealed in detail with the two-step ABC technique using the biotin-labeled serotonin antibody, along with differences found in the studies of the above researchers.

**MATERIALS AND METHODS**

Fifteen Japanese White rabbits (both sexes, about 2.5–3.5 kg body weight) anesthetized with sodium pentobarbital, were transcardially perfused with PBS (0.1 M phosphate buffer containing 0.9% NaCl) at 4°C, and subsequently with a fixative containing 4% paraformaldehyde, 0.2% picric acid and 0.4% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4, 4°C, 3,000 ml). The brains, taken from the skulls, were immersed in the same fixative without glutaraldehyde for two days. After washing with phosphate buffer containing 20% sucrose, frozen serial sections (20 μm thick) were cut on a cryostat and stored for four days in phosphate buffer containing 0.3% triton X–100. The following treatment was carried out on free floating sections.

The sections were immunohistochemically stained by the two-step ABC technique using the biotin-labeled serotonin-antibody. The details of the immunohistochemical procedure and of the production, purification, biotinylation and specificity of serotonin-antibody have been described elsewhere (Yamada and Sano, 1985). The sections were successively incubated in: 1) biotin-labeled serotonin antibody solution (×15,000), 4°C, about 40 hr; 2) avidin-biotin-peroxidase complex solution (Vector kit form, ×150), 90 min, room temperature; 3) 3, 3′-diaminobenzidine solution containing H2O2 for 15 min.

**Fig. 1.** Diagrams of frontal sections of the rabbit brain stem to illustrate the distribution of somata of serotonin neurons. The black dots indicate serotonin somata.

**Abbreviations**

*A* aqueductus cerebri, *AMB* nucl. ambiguus, *AP* area postrema, *CS* nucl. centralis superior, *DPS* decussatio pedunculorum cerebellarum superiorum, *F* fasciculus longitudinalis medialis, *IO* nuclear complex of inferior olive, *IP* nucl. interpeduncularis, *LC* locus ceruleus, *LI* nucl. linearis intermedius, *LM* lemniscus medialis, *PCM* pedunculus cerebellaris medius, *PO* pontin nuclei, *PY* pyramidal tract, *RD* nucl. raphe dorsalis, *RL* nucl. reticularis lateralis, *RM* nucl. raphe magnus, *RO* nucl. raphe obscurus, *RP* nucl. raphe pallidus, *RPO* nucl. raphe pontis, *RPG* nucl. reticularis gigantocellularis, *RU* nucl. ruber, *SGC* substantia grisea centralis, *SO* superior olive, *III* nucl. and radix of n. oculomotorius, *VIII* nucl. motorius n. trigemini, *VS* nucl. sensorius principalis n. trigemini, *VTS* nucl. tr. spinalis n. trigemini, *VI* nucl. n. abducens, *VII* nucl. and radix of n. facialis, *VIII* nucl. n. vestibulocochlearis, *X* nuclei n. vagus, *XII* nucl. n. hypoglossi
Fig. 1. Legend on the opposite page.
After immunostaining, the sections, mounted on glass slides, were counterstained with cresyl violet and prepared as permanent specimens by the standard procedure.

RESULTS

Serotonin was demonstrated as fine-granular immunoreactive substances filling the perikarya and their numerous processes. The immunopositive, dark brown somata were distributed in the raphe region of the brain stem, and could not be detected in other parts of the brain and spinal cord. A map of the distribution of perikarya is shown in Figure 1.

At the caudalmost area of the brain stem, the medium sized immunoreactive nerve cells (15 × 40 μm in diameter), which showed as spindle or triangular shapes with weak stainability, were found in the nucl. raphe obscurus (Fig. 2). In the nucl. raphe pallidus, many of round or oval serotonin containing somata (20-30 μm in diameter) were distributed (Fig. 3). Another group in the medulla oblongata consisted of triangular, multipolar or sometimes spindle shaped cells (15-35 μm). The somata of this group

Fig. 2. Nucl. raphe obscurus (upper half of photograph) and nucl. raphe pallidus (lower half). ×125

Fig. 2-11. The immunoreactive nerve cells in the brain stem of the Japanese white rabbit. Two-step ABC technique using biotin-labeled rabbit serotonin-antibody. Arrow indicates the midline.
Fig. 3. Nucl. raphe pallidus. ×240

Fig. 4. Cell group lateral to the inferior olive. Arrowheads indicate “paired cells” pattern. ×270
were scattered in a reticular formation, around the nucl. reticularis lateralis, and were present dorsal to the pyramidal tracts and also medial, ventral, and dorsolateral to the inferior olive (Fig. 4). Some of these perikarya were located close to each other, displaying a "paired cells" pattern. Under natural conditions without any pharmacologic pretreatments, the so-called "masked indolamine cells" could not be detected in the area postrema.

At the level between the caudal end of the nucl. n. facialis and the rostral end of the superior olive, variously shaped, weakly reacting nerve cells were scattered in the reticular formation of the pons, especially dorsolaterally adjacent to the pyramidal tracts, ventromedial to the nucl. n. facialis and around the superior olive. In the median portion of the pons, the following two cell groups were distinguished: the nucl. raphe magnus and the nucl. raphe pontis. The lower half of the nucl. raphe magnus consisted of a small number of multipolar somata (20–40 μm) some of which appeared in the "paired cells" pattern, while the upper half was made up of transversely-elongated, spindle-shaped cells (10 x 40 μm) dorsal to the trapezoid body (Fig. 5). The cells in the nucl. raphe magnus were densely immunostained. In the nucl. raphe pontis a few nerve cell bodies were localized. Most of these, which were lying in two rows along the paramedian line, were dorsoventrally-elongated, spindle shaped, and 10 x 30 μm in diameter.

At the level of the isthmus rhombencephali and caudal mesencephalon, groups of serotonin neurons were symmetrically located dorsal to the pyramidal tracts, and within and around the lemniscus medialis on both sides (Fig.6). These were composed of strongly stained cells (20–40 μm) of various shapes, being mostly multipolar. Some of them were located close to each other, showing the "paired cells" arrangement. At the same level, a relatively large number of intensively immunoreactive nerve cells constituted a broad group (Fig. 7). These somata, the majority of which corresponded with the lateral part of the nucl. raphe dorsalis, were scattered within, around, and far lateral to, the fasciculi longitudinales mediales. These laterally spread neurons were in the central grey matter or reticular formation, especially in the subcerulean region and parabrachial area (Fig. 8). Many of them were large in size (40-50 μm), and typically multipolar in form. The "paired cells" arrangement was also observed. Between the right and left fasciculi longitudinales mediales, a large number of round or oval, and weakly stained somata (20–30 μm) were clustered in the median portion of the nucl. raphe dorsalis (Fig. 9). In the nucl. centralis superior, (Fig. 10), nucl. linearis caudalis and intermedius, lay another cell group also consisting of weakly immunoreactive, small sized (about 20 μm in diameter) and round or oval shaped, perikarya. However, they were few in number in the nucl. linearis caudalis and intermedius. Immunopositive somata were also seen in the nucl. interpeduncularis, especially in its caudal portion. They were small and weakly stained (Fig. 11). Though the number was small, other immunoreactive cells were scattered about the tegmentum at the isthmic level and caudal end of the mesencephalon.

DISCUSSION

The existence of serotonergic neurons within the raphe nuclei of the brain stem of the various vertebrates has been confirmed by many investigators using formaldehyde-induced fluorescence (FIF) histochemistry for the demonstration of biogenic amines. However, a detailed histological study on the serotonin neuron system in the rabbit
Fig. 5. Nucl. raphe magnus located around the trapezoid body. × 230

Fig. 6. Cell group situated around the lemniscus medialis. Arrowheads show "paired cells" pattern. × 230
II. YAMADA and Y. SANO: brain has been reported in only two articles, i.e., FELTEN and CUMMINGS (1979) and HOWE et al. (1983).

On the basis of histofluorescence studies on the rat brain, DAHLSTRÖM and FUXE (1964) subdivided the groups of serotonin neurons into nine groups, designated B1 through B9. In the morphological study by FELTEN and CUMMINGS (1979) on the rabbit brain, the nucl. raphe pallidus, obscurus, magnus and pontis correspond respectively to B1, 2, 3 and 5; the nucl. raphe dorsalis corresponds to B6 and 7; and the nucl. centralis superior corresponds to B8 and 9. However, these authors could not find any cells of group B4, and recognized that the nuclei linealis and intermedius did not contain significant numbers of serotonin neurons. They also pointed out that some serotonin somata abutting each other in pairs were observed in the nucl. raphe magnus and pontis of rabbit brains. In our study, these cells were confirmed in the nucl. raphe magnus, but not in the raphe pontis. We also found them in the ventral half of rhombencephalic tegmentum, the lateral portion of, or lateral to, the nucl. raphe dorsalis, and around the lemniscus medialis. As yet, the cells in question have not been ultrastructurally investigated, neither have their electrophysiological functions been precisely understood.

The detailed demonstration by FELTEN and CUMMINGS (1979) was useful as reference for the identification of the raphe nuclei in our study. However, with regard to the serotonergic system, there were the following methodological weakpoints: the rapid fading of yellow fluorescence during microscopic observation; non-permanent preparations; weaker stainability than with immunohistochemistry; the need for pharmacological pretreatment.

On the other hand, HOWE et al. (1983) reported on the distribution of the serotonin neuronal somata of the New Zealand White rabbit in a brief paper. They used the monoclonal antibody (CONSOLAZIONE et al., 1981) of serotonin from rat hybridomyeloma cells, eliminating the technological drawbacks of non-specific or higher background staining when anti-rabbit IgG as the second antibody is used.

Fig. 7. Low magnification of nucl. raphe dorsalis which shows lateral spread. ×65
Serotonin Neurons in Rabbit Brain

HOWE et al. (1983) stated that the serotonin neurons were largely confined to the ventral and medial regions of the brain stem and tended to be clustered in groups resembling the B1–9 groups (including those within the area postrema and nucl. interp. unc.) described for the rat brain. From our research, the occurrence of serotonin neurons in the nucl. raphe pontis (B5 group) was evident but small in number in both the rabbit and rat, whereas HOWE et al. (1983) obtained no evidence of these cells in this region of the rabbit brain. In their report, the portion lateral to the B6

Fig. 8. High magnification of lateral group of nucl. raphe dorsalis.  x 320

Fig. 9. Central portion of nucl. raphe dorsalis.  x 170
and B7 groups, for example, the subcerulean area, contained a larger number of immunoreactive somata than in the rat. In our investigation, this difference was not obvious. They concluded that serotonin neuronal groups in rabbit brains differed considerably from those in rats, particularly with respect to the higher proportion of

Fig. 10. Nucl. centralis superior.  ×150
Fig. 11. Nucl. interpeduncularis.  ×170
serotonin cells located in the lateral regions. They may have compared their observations, not with the immunohistochemical results of STEINBUSCH (1981) or TAKEUCHI et al. (1982a), but with the fluorescent histochemical observations on the rat. The serotonin containing cells were immunohistochemically revealed as being more abundant in the lateral spread of the raphe nuclei and in other areas (particularly the lateral regions) than with fluorescence histochemistry. However, their schematic drawings show that lateral groups of serotonin neurons were much more numerous than in our study, while median groups were quantitatively almost the same as ours. The reasons considered for these discrepancies are: the difference between their New Zealand rabbits and our Japanese rabbits; the difficulty of recognizing positive neurons with fluorescence microscopy (they used the immunofluorescence method); and the influence of pharmacological pretreatment (they used L-tryptophan and/or a monoamine oxidase inhibitor). Some kinds of false, serotonin-positive cells (for example, in the area postrema) were detected after pharmacological pretreatment, but not under physiological conditions. We have termed these cells the “masked indolamine cells” (UEDA et al., 1984b; NISHIDA et al., 1985).

This study has shown that the distribution of serotonin nerve cells in rabbits is essentially similar to other mammals (TAKEUCHI et al., 1982a, b; KOJIMA et al., 1983). Moreover, from the point of view of the mode and the number of lateral spreading cells (i.e., in the ventral half of the medulla oblongata and mesencephalon), the distributional pattern of rabbit serotonin neurons particularly resembled that of rats. This was considered to reflect a characteristic common to rodents.

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