Immunohistochemical Observation of Serotonin Neurons in Newborn Mouse and Rat Brainstem Cultures*

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Summary. Serotonin neurons in newborn mouse and rat brainstem cultures were studied by peroxidase-antiperoxidase immunohistochemistry using a serotonin antibody. The intensity of the immunohistochemical reaction in the neuronal somata decreased gradually with time and was hardly detectable after one month of culturing. On the other hand, the immunoreactivity of the processes noted at the early stages later on became more intense and covered the full extent of the fibers. From the early stages of the cultures, the axon of each serotonin neuron formed not only a network by branching, but also a true anastomosis with the axonal networks of the other serotonin neurons.

Since the introduction of immunohistochemistry for detecting serotonin in the central nervous system (CNS), as initiated by STEINBUSCH and his associates in 1978, several important morphological studies on the distribution of serotonin neurons and their processes in the mammalian CNS have been carried out (STEINBUSCH et al., 1978; STEINBUSCH, 1981; KOHLER et al., 1981). We also succeeded in obtaining stable and detailed results using the modified peroxidase-antiperoxidase (PAP) technique and antibodies against serotonin which were prepared in our laboratory (TAKEUCHI et al., 1982a, b, c). Research on the serotonin neuron system in the CNS of various vertebrates strongly suggests that axons of serotonin neurons not only branch out but also make a vast anastomotic plexus of varicose fibers (SANO et al., 1982). In the present study, our modified PAP-technique for the demonstration of serotonin was successfully applied to the raphe neurons of the mouse and rat maintained in vitro, and possibility of anastomosis between axonal networks of serotonin neurons is discussed.

MATERIALS AND METHODS

Tissue blocks, including neurons of the nucleus raphe dorsalis, were removed from the brainstem of newborn mice and rats, explanted to collagen coated coverslips and maintained using Maximow’s double coverslip assembly. The nutrient medium was composed of equal parts of Gey’s balanced salt solution, bovine serum ultrafiltrate, horse serum, extract of 9-day-old chick embryo and glucose in amounts of 600 mg % of final concentration. This medium was replaced every three days.

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Fig. 1. Serotonin immunoreactive somata showing various shapes. Ten-day-old cultures of mouse (a) and rat (b and c) brainstem. × 350
The immunohistochemical procedure was as follows. After a rinsing in 0.1 M phosphate-buffered saline (PBS, pH 7.4), the cultures were immersed in a fixative containing 0.2% picric acid and 4% paraformaldehyde in 0.1 M PB for 3-6 hrs, then incubated in PBS containing 0.3% Triton X-100 for 1 hr. The PAP immunohistochemical staining was accomplished by sequential application of 1) normal goat serum (diluted 1:200 with PBS containing 0.3% Triton X-100) for 2 hrs, 2) rabbit anti-serotonin serum (1:16,000) for 12 hrs, 3) goat anti-rabbit IgG (Miles-Yeda Ltd. 1:200) for 3 hrs, 4) rabbit peroxidase-antiperoxidase complex (Daco, Co. 1:200) for 90 min and 5) 3,3-diaminobenzidine (Sigma Chemical Co.) containing H2O2 for 10 min.

The primary antiserum was obtained by immunizing male rabbits with serotonin coupled with bovine thyroglobulin (for details of the preparation of the antiserum and the specificity of the immunohistochemical staining, see TAKEUCHI et al., 1982b).

In the controls, immunoreactive staining was not achieved when the primary antiserum was absorbed with serotonin creatinine sulfate (at least 10 μM in final dilution).

RESULTS

The cultures of tissue blocks including the dorsal raphe nucleus of the mouse and rat displayed axonic outgrowth with glial proliferation. Compared to the rat cultures, those of the mouse exhibited a more abundant axonic proliferation. The following description, therefore, will be mainly based upon observations of the mouse brain.

Immunoreactive nerve fibers grew in radial fashion from the explant, where their somata were located. The distal branching parts of the outgrowing nerve fibers showed an intensive immunoreactivity, while the perikarya and proximal portions of the

Fig. 2. A dense network composed of fine serotonergic varicose fibers. Note the two large swellings at the distal branches of a thick serotonergic fiber. Ten-day-old culture of mouse brainstem. × 350
Fig. 3 and 4. Legends on the opposite page.
axons of serotonin neurons were occasionally negative in reactivity. Glial and fibro-blast cells revealed no staining for serotonin.

The tissue block of the brainstem (including the nucleus raphe dorsalis) cultured for 10 days produced an increase in cellular elements, both neuronal and non-neuronal. At this stage of culturing, neuronal somata were intensely stained for serotonin and displayed various shapes—stellate, ovoid and round—similar to those seen in the adult brain (Fig. 1). Their processes with varicosities (ca. 1-8 μm) could be demonstrated through the entire length, even to the most distal portions where large Herring’s body-like swellings of varicosities of the serotonin fibers were often observed (Fig. 2).

Fig. 5. Network of thick meandering immunoreactive fibers with intense staining at the peripheral portion of the culture. Three-month-old culture of mouse brainstem. × 350

Fig. 6. Arborization of the immunoreactive varicose fibers. Three-month-old culture of mouse brainstem. × 350

Fig. 3. Anastomoses of serotonergic axons originating from different neurons. Ten-day-old culture of mouse brainstem. × 200

Fig. 4. A vast and tortuous extension of immunoreactive fibers from the cluster of explanted tissue (asterisk) where few serotonin immunoreactive somata were found. One-month-old culture of mouse brainstem. × 200
The orientation of the fibers was fundamentally radial, but even at this early stage, a complex plexus by anastomoses of immunoreactive fibers had already formed in some cultures, particularly in the mouse tissues (Fig. 3). In these specimens, the axonal ramification origination from different serotonin neurons was a true network formed by a protoplasmic continuity. At times, however, immunoreactive fibers were interposed with smaller unstained cells. Serotonin immunoreactivity was more intense in the distal than in the proximal portions of the fibers. The same tendency was also seen at the later stages.

After one month of culturing the mouse tissues, a remarkable extension of immunoreactive fibers was achieved, whereas few immunoreactive neuronal somata could be detected in the explant (Fig. 4). Around this stage, it was almost impossible to follow immunoreactive fibers up to their origin, particularly near the cluster of the explant.

Serotonin immunoreactive fibers differed in caliber, and the majority seemed, light-microscopically, to form a circular structure of various sizes, as seen in the CNS of the adult animals. Compared to cultures at the early stages, the networks composed of serotonin-containing axons in the one-month-old cultures were markedly extensive and complicated. Intervaricose segments were very thin, but the continuity of the fibers was confirmed using a higher magnification.

In the three-month-old culture in vitro, extensive networks of serotonin immunoreactive fibers were still maintained. In comparison with findings in the culture at one month, serotonin immunoreactivity was remarkably intensified in the fibers. On the other hand, some immunoreactive neuronal somata of various intensities of staining were observed in the explant. Additionally, the immunoreactive fibers were somewhat thicker and varicosities were larger than in the earlier stages (Fig. 5). At this later stage, immunoreactive fibers were seen to branch off at the varicosities (Fig. 6).

DISCUSSION

For an immunohistochemical study on serotonin neurons in the CNS, tissue culture techniques are most advantageous as the entire neuron, including the somata and processes, can be observed in one single specimen (Sano et al., 1967). To determine whether the axons of serotonin neurons form a true anastomotic plexus, our modified PAP method for demonstration of serotonin was able to be applied successfully in vitro, because serotonin-containing processes extended into the thin layer. Thus, observation of the mutual protoplasmic continuity among the fibers in a complicated immunoreactive plexus was made feasible.

We had already proposed that axons of serotonin neurons not only branch, but form a true network with repeated anastomoses as well (Sano et al., 1982). Furthermore, serotonin neurons should be classified as a third type of neuron as they belong to neither the Deiters type nor the Golgi type. Judging from the morphological features of the serotonin neuron together with its function, it seems probable that serotonin neurons form the "rete nervosa diffusa" proposed by Golgi (Sano et al., 1982).

Previous investigators described a "permanent anastomosis" of collateralized plexuses derived from cultured spinal ganglion cells, using Hél’s hematoxylin staining or the silver impregnation method (Esaki, 1929; Levi, 1934). Hild (1954) also pointed out the existence of direct cytoplasmic continuity through observation of cultured neurosecretory cells of the supraoptic nucleus, but he did not refer to the neuron theory or Golgi’s reticular theory. We found in our cultures the formation of a network originat-
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ing from the immature serotonin neurons. In contrast to the aforementioned earlier reports of cultured nerve cells, there is no need to discuss whether the observed plexus consisted of only neuronal elements, because of the specificity of the present immunohistochemical staining for serotonin. Nevertheless, the possibility is not excluded that serotonin neurons may tend to adhere in a cultured environment, in which case a glial sheath is not formed. The results of our study support the possibility of the syncytial continuity of plural serotonin neurons, in vivo.

At the early stage of culturing, the somata of serotonin neurons were already evident, yet a previous report of sympathetic chains taken from rats and mice did not reveal any fluorescent somata of catecholamine neurons at this stage of the culture (Sano et al., 1967). Prochiantz et al. (1979) also reported that, by glyoxylic acid fluorescence histochemistry, catecholamine fluorescence in embryonic mouse neurons grown in culture could not be detected until the third week of culture. These discrepancies may be due to the sensitivity of the methods and/or different materials (catecholamine and indoleamine neurons).

At the later stages of culture, the serotonin immunoreactivity became visible over the entire length of the neuronal axons, while the immunoreactivity of neuronal somata decreased. A similar result was reported by Yamamoto et al. (1981) in serotonin neurons in dissociated cultures of embryonic rat brain.

At one month in culture, an extensive network of the serotonin fibers displayed a strong immunoreaction, particularly in the distal portions, while immunoreactivity in the neuronal somata could hardly be detected. Such a change found in vitro is postulated to occur in the postnatal development of serotonin neurons, in vivo. Using the formaldehyde-induced fluorescence (FIF) method, Levitt and Moore (1978) reported that the fluorescent intensity of serotonin neurons became increasingly difficult to demonstrate, and at only the tenth postnatal day, the neurons fluoresced almost as weakly as they did in normal adult preparations. Despite the difference in the method used, these data are useful for the interpretation of the change in the intensity of immunoreactivity in the culture.

Yamamoto et al. (1981) found that over the first month in culture, the total number of neurons decreased, whereas the percentage serotonin neurons increased tenfold, and they pointed out the preferential survival of serotonin neurons under conditions of dissociated culture. In our experiment, serotonin immunoreactive somata at the later stage were hardly detectable in the explant. Taking the presence of the extensive networks of immunoreactive fibers into consideration, it is reasonable to assume that serotonin neurons do occur in the explant. The failure to detect the somata of the serotonin neurons at the one-month stage may possibly be due to technical differences, blocks and dissociated cultures. Nevertheless, the block culture seems to more adequately represent physiological states than does the dissociated culture.

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