Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, pp. 3086–3089, July 1977
Neurobiology

Serotonergic neurons in the peripheral nervous system: Identification in gut by immunohistochemical localization of tryptophan hydroxylase

(myenteric plexus/serotonin/monoamines/tissue culture/neurotransmission)

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Communicated by Cyrus Levinthal, April 29, 1977

ABSTRACT **A specific antibody to tryptophan hydroxylase [L-tryptophan, tetrahydropteridine:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4] has been used to localize the enzyme immunohistochemically in neurons of the mammalian gut. The enzyme was found in perikarya of intestinal neurons of mice, rats, and guinea pigs. Neurons containing the enzyme survived for up to 3 weeks in organotypic tissue culture and were intrinsic to the gut. These neurons are probably serotonergic and are the first such neurons to be found in the peripheral nervous system.**

Serotonin (5-HT) has gained general acceptance as a neurotransmitter in the central nervous system. Most of the cell bodies of the central 5-HT neurons lie in the nuclei of the median raphe (1–3). Although no similar acceptance of a role of 5-HT as a neurotransmitter in the peripheral nervous system as yet exists, recent evidence has indicated that 5-HT might be the transmitter of some peripheral neurons (4–7). These neurons are in the enteric nervous system of the mammalian gut (4–10).

A population of the neurons of the myenteric plexus resembles central serotonergic neurons in several respects, including a selective high-affinity uptake mechanism for 5-HT (11–13). The characteristics of this mechanism (ion dependence, structure–activity relationship, and sensitivity to antagonists) are similar in both types of neurons (11–13). Moreover, both brain and myenteric plexus contain a specific high-affinity 5-HT binding protein (14–16).

In the brain, tryptophan hydroxylase [L-tryptophan, tetrahydropteridine:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4], the enzyme catalyzing the first step in the biosynthesis of 5-HT from tryptophan, is largely restricted to serotonergic neurons and is therefore used as a marker for these neurons (17). The demonstration of tryptophan hydroxylase in neurons of the enteric nervous system would strongly support the view that these enteric neurons are serotonergic. The present study utilized an immunohistochemical method that previously was used to demonstrate tryptophan hydroxylase in central serotonergic neurons (18). The method was applied to intestinal tissues of mice, rats, and guinea pigs. Tryptophan hydroxylase was found in intestinal neurons.

MATERIALS AND METHODS

Adult rats were anesthetized with pentobarbital and perfused with paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.2, for 15 min) as described (18). The intestine was removed and postfixed for 6 hr in 0.21% picric acid/6% formaldehyde in 0.1 M phosphate buffer (pH 7.4), washed overnight in 0.1 M phosphate buffer (pH 7.4), embedded in paraffin, and sectioned at 5 μm. Longitudinal muscle with attached myenteric plexus dissected from the guinea pig ileum (11) was stretched onto cardboard with the myenteric plexus exposed and fixed for 6 hr in picric acid/formalin. This tissue was processed as a whole mount. Cultures of 18-day fetal mouse intestine were grown from explants consisting of either hemisections of small intestine or dissected muscularis externa containing the myenteric plexus (8–10, 19). After 3 weeks' growth, cultures were fixed in picric acid/formalin and processed as whole mounts.

The sections of rat intestine, the whole mounts of longitudinal muscle with attached myenteric plexus from guinea pigs, and the cultures from mice were incubated with rabbit antiserum to tryptophan hydroxylase (purified from raphe nuclei of rat brain), goat antiserum to rabbit IgG, and peroxidase–antiperoxidase complex (18, 20). Peroxidase activity was then localized with 3,3′-diaminobenzidine and hydrogen peroxide (18, 20). In this bridge technique, the unlabeled rabbit antibody to tryptophan hydroxylase combines with the enzyme in the tissue, the goat antiserum bridges the rabbit antibody to the peroxidase–antiperoxidase complex, and the peroxidase activity localizes the tryptophan hydroxylase. Controls consisted of tissues exposed to antiserum obtained from rabbits prior to their immunization with tryptophan hydroxylase or to antiserum from which specific antibody had been removed by absorption with the antigen, tryptophan hydroxylase. As an additional control, cultures were exposed to antiserum to tyrosine hydroxylase, a marker for catecholaminergic neurons (21). The extrinsic adrenergic innervation degenerates after 3 weeks in culture (9) and therefore this enzyme should not be present. All controls were negative and no tyrosine hydroxylase was found in the cultures.

RESULTS

In sections of rat intestine, ganglion cells were found to contain peroxidase reaction product marking the presence within them of tryptophan hydroxylase. Reactive ganglion cells were found isolated, or in small groups, in both myenteric and submucosal plexuses (Fig. 1). The reactive cells appeared to be small neurons. Staining was confined to perikaryal cytoplasm. Nuclei were unstained and, although long processes containing reaction product appeared to leave the perikarya, staining could not be followed into processes unequivocally identified as axons. In whole mounts of guinea pig longitudinal muscle with attached myenteric plexus, reaction product marked small neurons within the myenteric plexus (Fig. 2). In these, staining was seen in the cytoplasm of their perikarya and neuritic processes. The cells resembled Golgi type II neurons in configuration. Smooth muscle was nonreactive.

Cultures of fetal mouse intestine also contained cells that

Abbreviation: 5-HT, serotonin.

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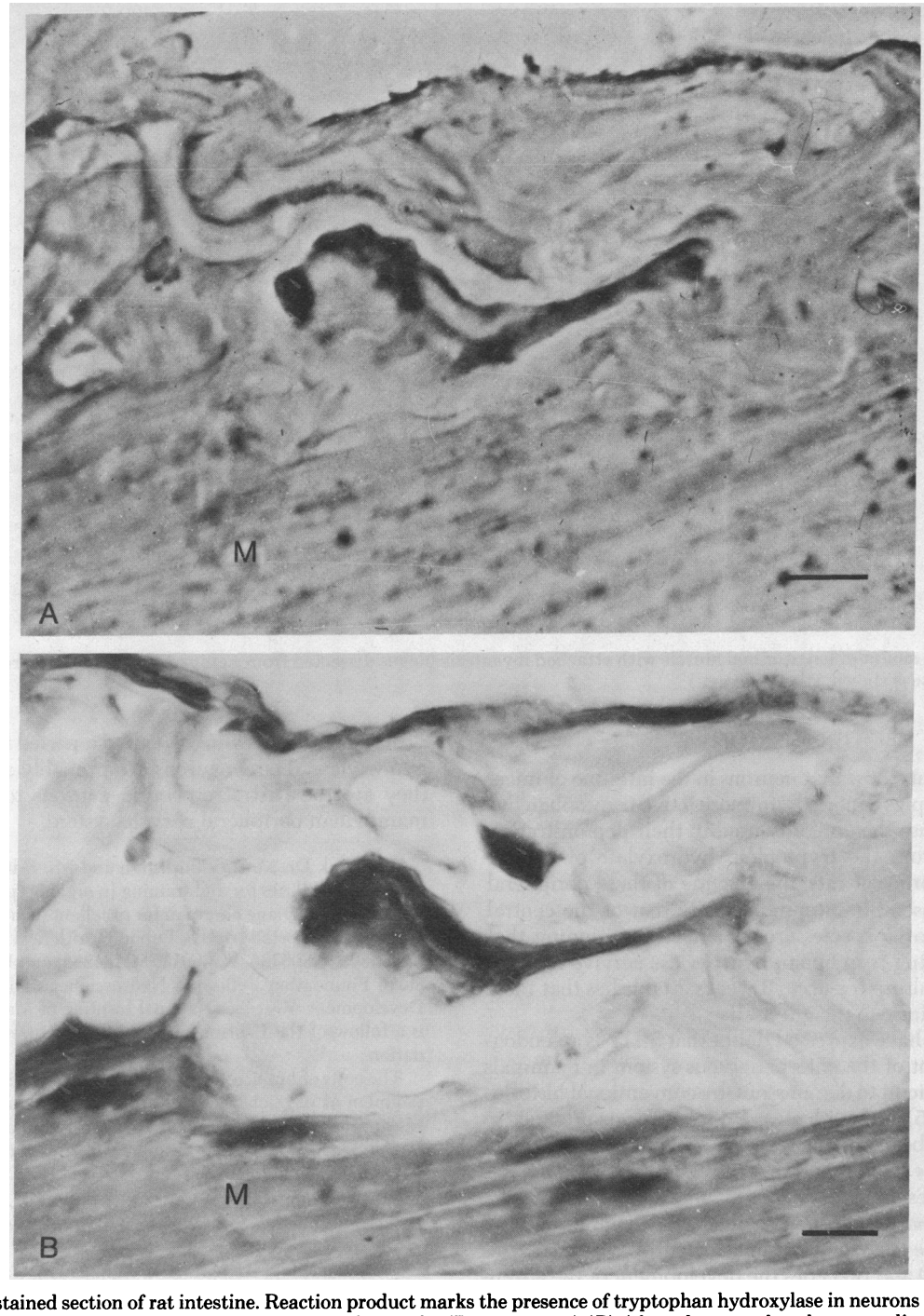

FIG. 1. (*A*) Unstained section of rat intestine. Reaction product marks the presence of tryptophan hydroxylase in neurons of the submucosal plexus. Nuclei are nonreactive. M = circular layer of smooth muscle. (Bar = 30 μm.) (*B*) After photography, the cover slip was removed and the section shown in *A* was stained with hematoxylin and eosin. M = circular layer of smooth muscle. (Bar = 30 μm.)

stained for tryptophan hydroxylase (Fig. 3). These cells usually occurred in groups and tended to be confined to one region of the cultures. Staining of the cultures with Bodian's silver stain indicated that this region contained a dense neural plexus. Reaction was particularly intense in the perinuclear region of the reactive cells, giving them a circular appearance. However, this appearance was misleading because peripheral portions of the perikaryal cytoplasm were unstained. The reaction product appeared granular at higher magnification. The cells sent neurites, which also contained reaction product, into the underlying plexus. This reaction product was particularly intense in varicose swellings that occurred along the processes and at terminal expansions that resembled growth cones.

The potential presence in the cultures of cells other than neurons that might contain tryptophan hydroxylase was examined. Cultures were stained to demonstrate mast cells with pinacyanol erythrosinate (22) or toludine blue and argentaffin cells with Gomori's methenamine silver (23). One mast cell was found in each of 2 of 28 cultures and three argentaffin cells were found in 1 of 20 cultures. It should be noted that the mucosa was either dissected away or it degenerated and so was not present in these cultures (21). Therefore, those cells in the cultures that contained tryptophan hydroxylase were not mast cells or argentaffin cells.

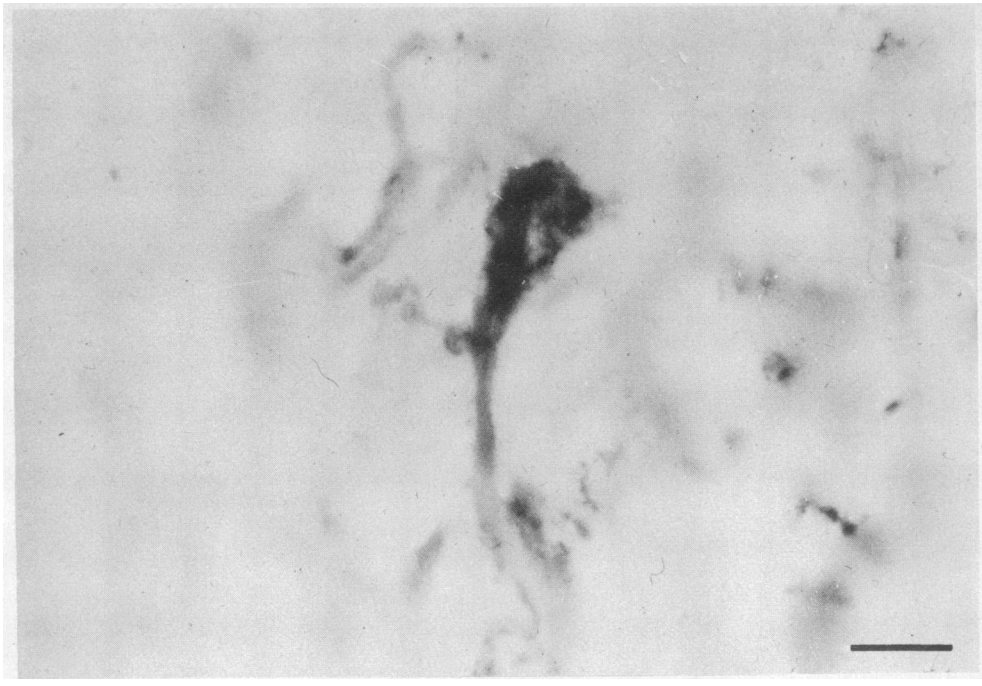

FIG. 2. Whole mount of longitudinal muscle with attached myenteric plexus dissected from a guinea pig. A single neuron reactive for tryptophan hydroxylase is shown. (Bar = 30 μm.)

DISCUSSION

These experiments show that neurons in the intestine of mice, rats, and guinea pigs contain immunoreactive tryptophan hydroxylase. Because the antibody used in their demonstration was obtained from the tryptophan hydroxylase of central serotonergic neurons of rats, the enzyme of these peripheral neurons must resemble immunologically that of the central neurons and also be species crossreactive. The finding that neurons containing tryptophan hydroxylase survive in organotypic tissue culture for up to 3 weeks establishes that these neurons are intrinsic to the gut itself.

Some authors have expressed doubt that 5-HT is an endogenous constituent of the enteric nervous system in mammals because it is difficult to demonstrate by conventional histofluorescence (24, 25), although clear evidence for its presence in cyclostomes has been obtained (26). However, the mammalian intestine has now been found to contain neurons with the following properties.

1. These neurons have a specific uptake mechanism for 5-HT (11, 13). Because this uptake is unaffected by chemical sympathectomy (12) and precedes the development of the adrenergic innervation of the gut in ontogeny (27), the neurons responsible for 5-HT uptake are not adrenergic. Furthermore, because neuronal uptake of 5-HT persists in intestine grown for 3 weeks in organotypic tissue culture (3), the neurons that take up 5-HT must, like those with tryptophan hydroxylase, be intrinsic to the gut itself.

2. Intestinal neurons not only take up 5-HT but also synthesize it from L-tryptophan (3). Histochemical evidence suggests that intrinsic neurons are responsible for this synthesis (3). This suggestion is supported by the observation that intestine cultured for 3 weeks continues to convert L-[^{3}H]tryptophan to 5-[^{3}H]HT. When these cultures are processed histochemically by the Falck-Hillarp technique (28) they are found to contain neurons that show fluorescence with the spectral characteristics of 5-HT (9).

3. Myenteric neurons, like central serotonergic neurons, have a 5-HT binding protein (5) and, as reported herein, tryptophan hydroxylase. These neurons are probably serotonergic. If so, they are the first serotonergic neurons to be found in the mammalian peripheral nervous system.

We thank Dr. Murray Bornstein and Ms. Edith Peterson for providing the facilities for and training in organotypic tissue culture. We also thank Ms. Diane Sherman for excellent technical assistance. This work was supported by U.S. Public Health Service Grants NS07436, GM00895, NS06735, NS06911, MH24285, and HL18974. T.H.J. is a Sloan Foundation Fellow in Neuroscience and V.M.P. is a Career Development Awardee (National Institute of Mental Health). C.F.D. is a fellow of the Pharmaceutical Manufacturers Association Foundation.

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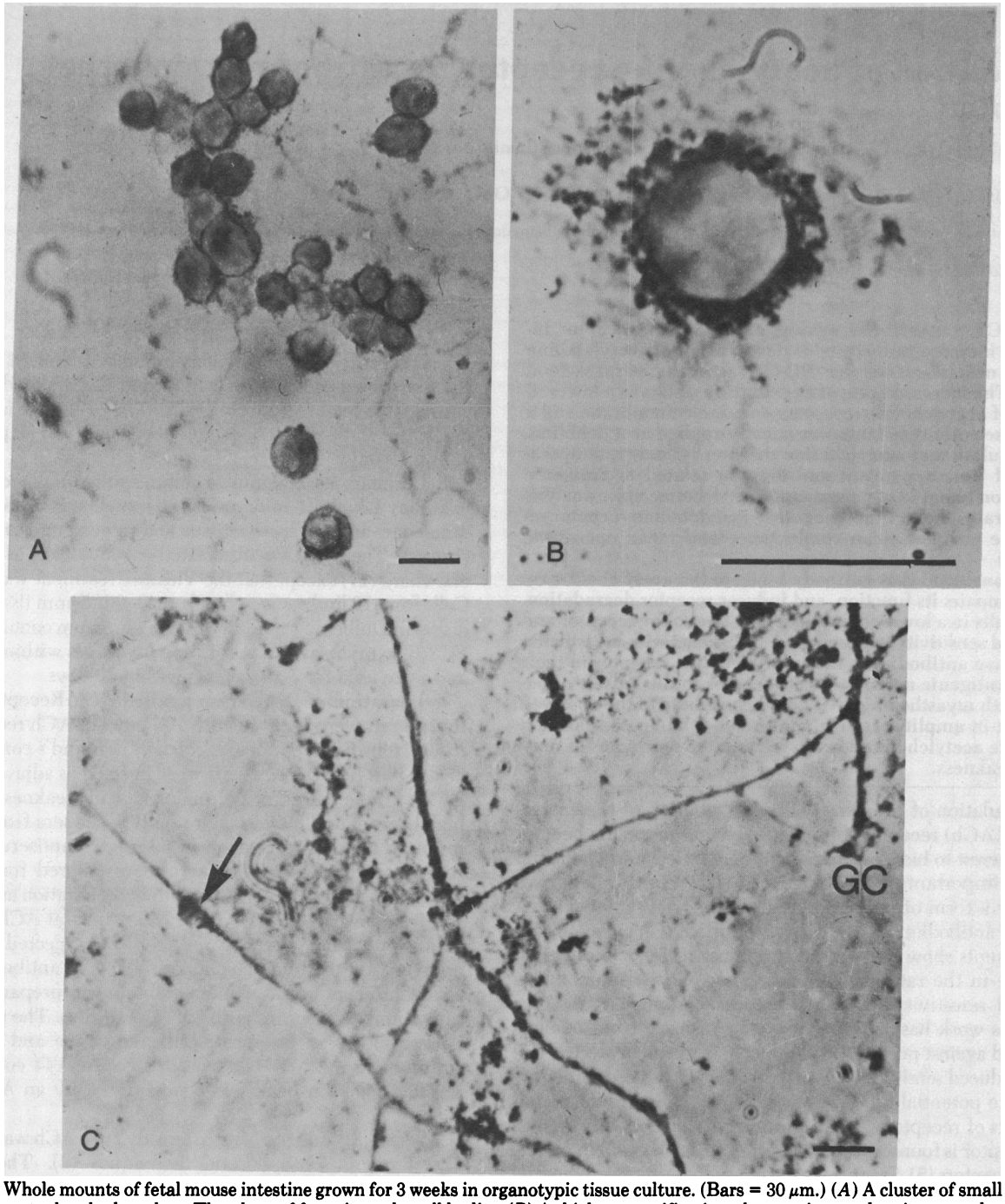

FIG. 3. Whole mounts of fetal mouse intestine grown for 3 weeks in organotypic tissue culture. (Bars = 30 μm.) (*A*) A cluster of small neurons containing tryptophan hydroxylase. The plane of focus is on the cell bodies. (*B*) At higher magnification, the reaction product is seen to be granular and concentrated in the perinuclear region. A slender neuritic process passes out of the plane of focus to the right. (*C*) Reaction product is seen in neuritic processes, particularly at varicose expansions (arrow). A growth cone (GC) also contains reaction product.

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