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Antibodies as Molecular Probes in Neurobiology

Identification of Chemically Defined Neurons and Synapses in Tissues and Tissue Cultures

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Abstract

Immunocytochemical localization of 5-hydroxytryptamine (5-HT) in the nervous system and aggregate tissue cultures was performed employing an antibody to 6-OH-1,2,3,4-tetrahydro-β-carboline. A number of immunochemical and biochemical tests with the antigen and the antibody and some procedural changes in the methodology applied for immunolocalization revealed the anti-5-HT–like affinity of the antibody, if applied in paraformaldehyde-fixed tissues.

Studies in the hypothalamus, striatum, brainstem, spinal cord, and pineal gland show the complexities of the serotoninergic system. Ultrastructural immunocytochemistry with the preembedding technique reveals that 5-HT synapses are of the asymmetric type. The presynaptic element contains clear, round, small vesicles, with some large dense-core vesicles. The contacts are made with the somata and primary, secondary dendrites or with spines of non–5-HT neurons. Presynaptic dendrites are found in the n. raphe dorsalis, contacting non–5-HT dendrites.

Double immunocytochemical methods demonstrated contacts of 5-HT fibers on enkephalin containing neurons of the spinal trigeminal nucleus and on somatostatin containing neurons of the medullary reticular formation. In vitro studies of cultured mesencephalic neurons were performed with the method of aggregating cultures. Such development of a miniature organized nerve tissue was followed up to 35 d in culture. Organization of the neuropil and synaptogenesis was studied using standard electron microscopy. The differentiation of neurons and astrocytes was studied using antibodies to 5-HT and GFAP. Serotonin immunoreactivity could be observed in neuronal bodies and processes at light microscope level as early as the fourth day of culture. The length of neuronal projections increased to form extensive networks. The GFAP immunolabeling could be observed in astrocytes, forming a perinuclear ring, after 5 d of culture. Later, immunoreactivity spread to glial processes acquiring a differentiated fenotype.

Index Entries: Serotonin (5-hydroxytryptamine) in the CNS; antinociceptive system and serotonin; fixative selection in immunocytochemistry; 6-OH-1,2,3,4-tetrahydro-β-carboline as an immunogen; tissue culture and neurotransmitter expression; presynaptic dendrites; immunocytochemical methodology; ultrastructural immunocytochemistry; schizophrenia and serotonin; dendrodendritic synapses.

Introduction

Discovery of the subcellular location of 5-HT and other neurotransmitters in the rat brain by De Robertis and colleagues in the 1960s (Pellegrino de Iraldi and De Robertis, 1963; Pellegrino de Iraldi et al., 1963; Zieher and De Robertis, 1963; Jaim Etcheverry and Zieher, 1968); the location of 5-OH-tryptophan decarboxylase in these synaptic endings (Rodríguez de Lores Arnaiz and De Robertis, 1964); the studies of the chemical nature of 5-HT receptors (Fiszer and De Robertis, 1969); and the contributions of the Swedish school (Falck et al., 1962; Dahlström and Fuxe, 1964; Fuxe and Ungerstedt, 1968; Fuxe and Johnsson, 1974; Furness et al., 1977) paved the way for systematic investigation of the monoaminergic systems.

The development of antisera to tryptophan hydroxylase (Joh et al., 1975), 5-HT (Steinbush, Verhofstad and Juosten, 1978; Steinbusch, 1981; Steinbusch, Verhofstad and Joosten, 1983; Consolazione et al., 1981; Consolazione and Cuello, 1982), and other neurotransmitters and related enzymes gave birth to a number of important contributions and a growing new field (i.e., chemical neuroanatomy). This made possible the detailed description of chemically defined neuronal populations and pathways and their synaptology and connectivity, at the level of the light (LM) and electron microscope (EM).

Using other neuronal and glial antigen markers increased knowledge of the different types and subtypes of cells and the relation between neurons and glia, as well as gliovascular relationships. In addition, the application of antibodies to the study of the nervous system during neurogenesis has helped to elucidate the intimate mechanisms leading to final assembly of the brain. Developmental phenotypic expression of the neuroblast genome and the action of epigenetic influences could thus be followed from the earliest stages to its final maturation.

Antibodies were also applied to tissue cultures in an effort to clarify interactions of neurons and extracellular molecules, glial cells, and other neurons. Systematic reviews and extensive references may be found in the works of Sternberger et al. (1970), Sternberger (1986), Pickel (1982), Emson (1983), Priestley and Cuello (1982,1983), Cuello (1983), Björklund and Hökfelt (1984), and Priestley (1987). Recent methodological advances may be found in several publications (Cuello et al., 1984; Heimer and Záborszky, 1989; Bullock and Petrusz, 1989).

Also, 5-OH-tryptamine has been extensively studied in the brainstem and spinal cord of different species. The main physiological and pharmacological actions of 5-HT are related to hypothalamic endocrine regulation, thermoregulation, regulation of blood pressure, appetite, antinociception, extrapyramidal and spinal cord control of motility, arousal, and sleep (Scheibel et al., 1975; Cooper et al., 1978; Fuller, 1986; Mylecharane et al., 1989). Serotonin precursor antagonists and monoamine oxidase (MAO) inhibitors have been extensively used in different pathological conditions, including depression and alcoholism.

In this work, a limited review of some relevant data from the literature produced in our and other laboratories will be presented in an effort to offer an updated perspective of the potential of immunocytochemical methodology applied to nerve tissue and tissue cultures and some of their methodological limitations.

Methodological Considerations

Requirements for Immunocytochemical Studies in the Nervous System

The problems of antibody specificity, method specificity, controls, false-positives, false-negatives, and other artifacts have been extensively discussed in a classical publication by Vandesande (1979); in articles by Cuello (1983) and Sternberger (1986), and in our publications (Brusco et al., 1982; Pecci Saavedra et al., 1983; Peressini et al., 1984; Tandler and Pecci Saavedra 1984; Tandler et al., 1986).

Irrespective of the selected immunocytochemical staining method employed (fluorescent dyes, peroxidase–antiperoxidase, or avidin-biotin-peroxidase complex), it is important to know the properties of the antibody (Ab) in some detail. The commmercial source or the origin of the Ab is important, but in each case, special tests need to be applied to assure proper localization and specificity. We shall give an example of an antiserum to 5-HT produced in our laboratory by a method originally devised for radioimmunoassay (RIA) determinations (Ranadive and Sehon, 1967; Peskar and Spector, 1973; Grota and Brown, 1974; Geffard et al., 1982).

The nature of the antibody obtained in rabbits with an antigenic complex 5-HT–bovine serum albumin (BSA) was systematically studied (Brusco et al., 1983; Pecci Saavedra et al., 1982,1983a,b; Peressini et al., 1984; Tandler and Pecci Saavedra, 1984; Tandler et al., 1986). It was shown that 5-HT treated with paraformaldehyde in order to form a complex with BSA suffered an extramolecular and an intramolecular Mannich reaction (Blicke, 1942; Ho and Walker, 1971) resulting in a cyclization of the lateral chain of 5-HT (Pecci Saavedra et al., 1983; Tandler and Pecci Saavedra, 1984; Tandler et al., 1986). Thus, instead of 5-HT, the antigen complex contained 6-OH-1,2,3,4-tetrahydro-β-carboline (Fig. 1).

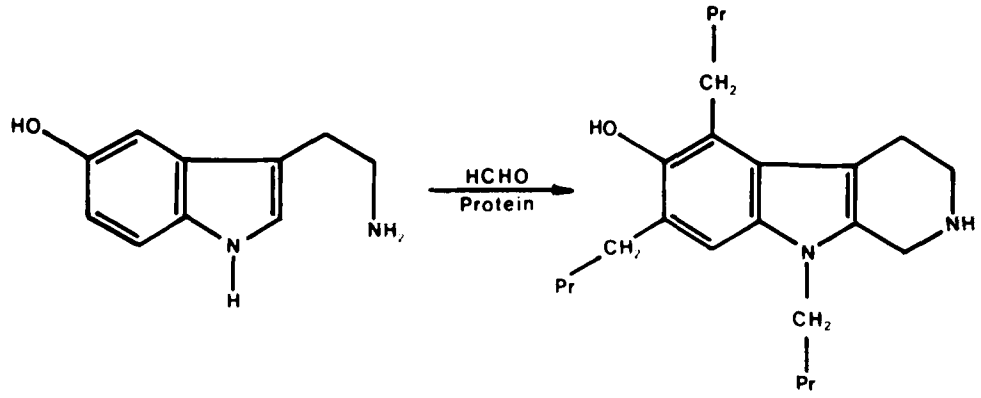

Fig. 1. The reaction between 5-HT, formaldehyde, and BSA gives rise to a conjugate in which the 5-HT molecule suffers a cyclization of its lateral chain and the 6-hydroxy-1,2,3,4-tetrahydro-β-carboline ring system attaches to active groups of the protein at positions 9, 5, and/or 7. The secondary amine (NH) group of the newly formed ring remains free, not being involved in coupling to the protein.

The antigen complex used as the immunogen shows a maximum of emission (Em.)- and excitation (Exc.)-induced fluorescence with the following values: Em.; 510–525 nm and Exc., 315–385–415 nm. These values are similar to those obtained treating tryptamine with formaldehyde vapors (Björklund et al., 1971; Jonsson, 1971), which are known to produce also the cyclization of the lateral chain (Peressini et al., 1984). The spectrophotometric studies showed that the 5-HT–BSA complex contains about 40 residues of 5-HT (or of its derivative) per BSA molecule (Tandler and Pecci Saavedra, 1984). From the above considerations, it is evident that the antigen used by us, as well as by most authors in this field, is not 5-HT but a cyclicized derivative (i.e., 6-OH-1,2,3,4-tetrahydro-β-carboline). Milstein et al. (1983) suggested a similar change for their monoclonal Ab (MAb) to 5-HT.

The potency of 6-OH-1,2,3,4-tetrahydro-β-carboline is 100-fold greater than that of 5-HT, as measured by inhibition tests performed with a modified Larsson (1981) test (Peressini et al., 1984). Schipper and Tilders (1983) arrived at similar conclusions employing a different test. For all of these reasons, we advise that new antibodies be tested for all possible causes of molecular changes in the immunogen and tissues in which they are to be detected (i.e., changes resulting from the coupling agent or the fixative).

Selection of Fixative Solution

As mentioned in the preceding paragraph, the antigen used by us and other authors is not 5-HT but a cyclic derivative (i.e., 6-OH-1,2,3,4-tetrahydro-β-carboline). Thus, the antibody obtained is directed to the latter molecule and not to 5-HT, as is still referred in the literature and listed in commercial catalogs. Despite this circumstance, the antibody is suitable to label 5-HT containing neurons and synapses. We have explored the apparent discrepancy since crossreactions in vitro between 5-HT and 6-OH-1,2,3,4-tetrahydro-β-carboline do not appear to exist. We found that the ability of our Ab to label 5-HT neurons and synapses is dependent on the fixative used for the tissue preparation: If 4% paraformaldehyde is used alone or with low concentrations of glutaraldehyde (i.e., 0.1%) the Ab behaves "anti-5-HT–like." If instead glutaraldehyde alone or *p*-benzoquinone is used as the fixative, the labeling is very faint or nil (Peressini et al., 1984; Tandler et al., 1986). It is known that glutaraldehyde and *p*-benzoquinone do not produce cyclization of the lateral chain of 5-HT (Pearse, 1980).

Technical Protocols

Animal Preparation and Tissue Sectioning

Pharmacological pretreatment is employed in order to enhance the intraneuronal amount of serotonin and thereby the intensity of the staining. Prior to fixation, the rats receive ip injections of nialamide (200 mg/kg/, a monoamine oxidase inhibitor, and L-tryptophan (100 mg/kg), a serotonin precursor. Adult animals are anesthetized with ip sodium thiopenthal, and then fixed by cold perfusion with 4% paraformaldehyde + 0.25% glutaraldehyde solution in a 0.1*M* phosphate buffer, pH 7.4. The perfusion is performed by inserting a needle into the left ventricle. The fixative is preceded by a quick wash of the circulatory system with 30 mL of a saline solution (0.9% w/v NaCl) containing 0.5 mL of 0.4*M* $NaNO_2$ and 2500 IU of heparin. Brains are dissected and immersed for 6 h in the same fixative solution. After rinsing for 18 h in a phosphate buffer to which 5% w/v sucrose is added, they are sectioned (40-µm thick) with a vibratome at 4°C and maintained in this solution until the immunocytochemical procedures are started.

Immunocytochemical Peroxidase–Antiperoxidase Procedure

Sections are collected in small test tubes and treated for 30 min with Triton X-100 0.2% v/v in phosphate-buffered saline (PBS). After three washes with PBS + 0.025% v/v Triton X-100 (PBSX), 5 min each, sections are incubated in 3% v/v normal sheep serum in PBS for 30 min. A further incubation is performed in anti-5-HT protein serum 1:10,000, diluted in 1% v/v normal sheep serum in PBSX for 24 h. Sections are then washed extensively (five washes) with PBSX prior to incubation in sheep antirabbit immunoglobulin-G (IgG) diluted 1:50 in PBSX for 30 min. After three washes with PBSX, 5 min each, sections are incubated in rabbit peroxidase–antiperoxidase complex (Sternberger et al., 1970; Sternberger, 1986) diluted 1:100 in PBSX for 30 min. Then, sections are washed twice in PBSX and once in TS (0.05*M* Tris buffer + NaCl 0.9% wv) for 5 min. All incubations are carried out in a rotary shaker at 4 °C.

Development is performed in a solution of 0.05% w/v diaminobenzidine and 0.1% hydrogen peroxide in TS for 3–8 min at room temperature. In order to enhance the immunocytochemical reaction the development is carried out in a solution of 0.035% w/v diaminobenzidine, 2.5% w/v nickel ammonium sulfate, and 0.1% v/v hydrogen peroxide in 0.1*M* acetate buffer, pH 6.0 (Hancock, 1984). When the sections are to be observed exclusively with LM, the inhibition of endogenous peroxidase (0.5% hydrogen peroxide in absolute methanol, 30 min) is performed prior to starting the immunocytochemical procedure. For double labeling studies at LM level, the first immunocytochemical reaction is silver gold substituted according to Görcs et al. (1986).

EM Procedure

Sections are processed for EM as follows:

1. Postfixation in 2% OsO_4 in 0.1*M* phosphate buffer, pH 7.4, at 4°C for 1 h;
2. Staining with uranyl acetate;
3. Embedding in epon; and
4. Flat mounting between two plastic coverslips.

The preferred region for ultramicrotomy is selected by LM. Thin sections are mounted on regular grids and stained with lead citrate (Reynolds, 1963) before EM observation.

Cell-Culture Techniques

Wistar rat embryos, 14–15 d of gestation, are removed by caesarean operation from timemated rats. The area limited by the vertex of the mesencephalic flexure and the caudal portion of the metencephalon is dissected in cold (Ca^{2+}, Mg^{2+}, free) Hanks' solution. The neural tissue is mechanically dissociated and the cell suspension is allowed to decant. The supernatant suspension is seeded in Erlenmeyer flasks containing Dulbecco modified Eagle's medium (DMEM) and kept in a bath at 37°C, rotating at 80 rpm

(Moscona, 1961; Morris and Moscona, 1971; Halgren and Varon, 1972; Marks and Seeds, 1978).

EM Procedure for Cultures

The aggregates are fixed with 3% v/v glutaraldehyde in 0.1*M* phosphate buffer pH 7.4 during 60 min at 4°C. They are then postfixed with 2% w/v osmium tetroxide, stained with uranyl acetate, and embedded in Epon 812. The ultrathin sections are stained with lead citrate.

Ultrastructural Immunocytochemistry

Structural and ultrastructural studies applying the 5-HT–like antibody were performed in monkeys (Pecci Saavedra et al., 1981; Pasik et al., 1982; Pecci Saavedra et al., 1983; Pasik et al., 1983), rats (Pecci Saavedra et al., 1983; Brusco et al., 1983; Pecci Saavedra et al., 1986), chicken (Flores et al., 1988), and molusks (Flores et al., 1983; Flores et al., 1986a,1986b,1988,1991). Applications of polyclonal and monoclonal Ab to the localization of 5-HT to LM studies were reported by Steinbusch et al. (1978); Lidov et al. (1980); Steinbusch (1981); Consolazione et al. (1981); Consolazione and Cuello (1982); Morrison et al. (1982); Morrison et al. (1984). The first ultrastructural studies were presented by us at the meeting of the American Association of Anatomists (Pecci Saavedra et al., 1981) and to a symposium organized by Caputto and Ajmone Marsan in Córdoba, Argentina (Pecci Saavedra et al., 1983) (Figs. 2–4).

Studies in the hypothalamus of *Macaca nemestrina* showed the presence of 5-HT neurons in the area of n. paraventricularis and n. supraopticus, confirming the expectations suggested by previous results with the Falk-Hillarp technique (Falck et al., 1962) and radioautography (Beaudet and Descarries, 1979; Descarries et al., 1979). The findings of Palkovits et al. (1974) are in accord with local production of 5-HT in the hypothalamus. Indeed, they demonstrated that surgical isolation of the hypothalamic areas is unable to lower the serotonin content of the hypothalamus (Brownstein et al., 1976).

Ultrastructural analysis of serotoninergic afferents to motoneurons (Fig. 2C), descending from the raphe nuclei, revealed that the contacts are axosomatic and axodendritic and that the synapses belong to the asymmetric type, suggesting an excitatory function. Similar types of synapses were found in the striatum (Pasik et al., 1983). Studies performed in the globus pallidus showed axosomatic and axodendritic contacts of the asymmetric type. Postjunctional dense bodies were a constant in the pallidal junctions (Figs. 3A–C; 4A–C).

Observations of synaptic endings in the pineal gland, pioneered by Pellegrino de Iraldi (Pellegrino and De Robertis, 1963; Pellegrino de Iraldi et al., 1963; Pellegrino de Iraldi and Guedet, 1969), showed that afferent fibers form synaptic endings in extracellular vascular spaces. Distribution of the neurotransmitter revealed dense deposits in synaptic vesicles, axoplasm, and attached to membranes, a distribution similar to that encountered in the CNS. In experiments in which both superior cervical ganglia were extirpated, results showed the absence of 5-HT–containing processes (*see* Fig. 1 in Heredia Chons et al., 1989). Observation of the pineal gland—a peripheral organ—demonstrated that no apparent differences are found between the central and peripheral synaptic endings when immunocytochemical procedures are applied.

Studies performed in the raphe dorsalis of the rat with EM showed that the labeled dendrites appear in synaptic contact with unlabeled dendrites (Pecci Saavedra et al., 1982; *see also* Fig. 5 in Pecci Saavedra et al., 1986). A presynaptic role of dendrites had been proposed in different territories of the CNS, including the neuronal circuitry of the n. dorsalis raphe. Wang and Aghajanian (1978,1982) proposed dendrodendritic interactions between neurons of the raphe nuclei as the physiological substratum for the synchronization of inhibitory interactions, as detected by electrophysiology. They suggested that the neurons of the raphe nuclei have a pacemaker or homeostatic function. It has been demonstrated that the stimulation of dorsal raphe 5-HT neu-

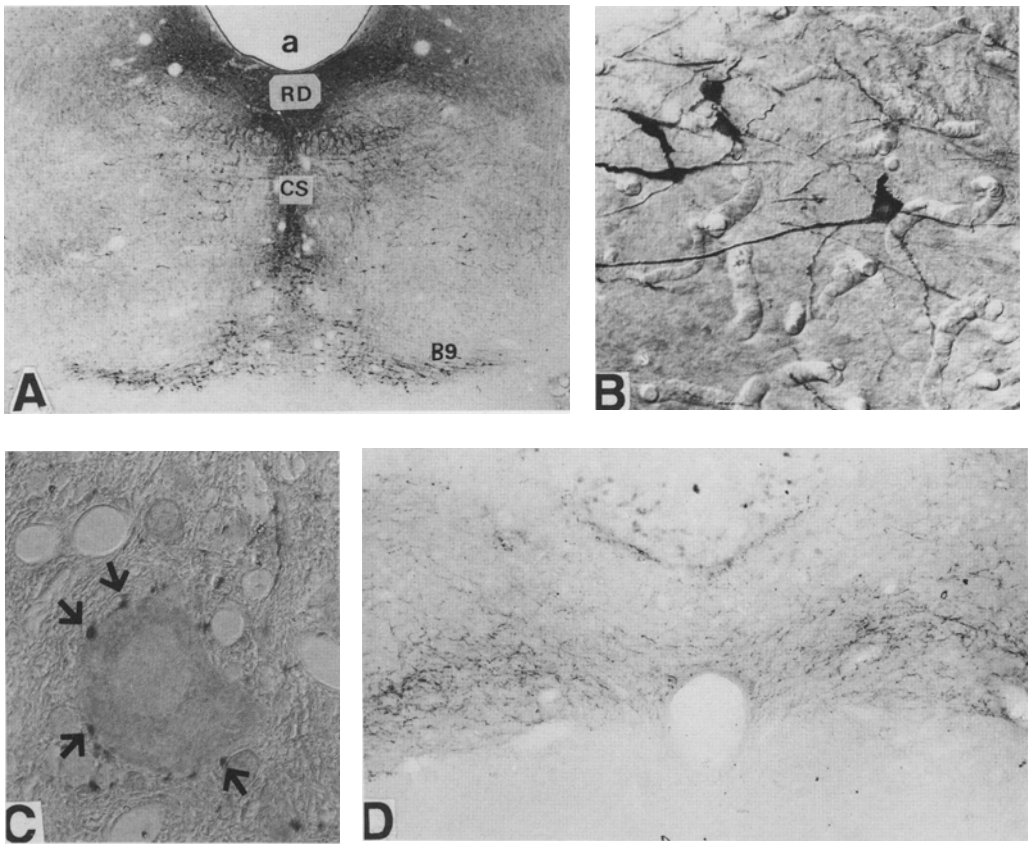

Fig. 2. A: Section of the rat mesencephalon showing the distribution of labeled neurons in the n. raphe dorsalis (RD) and the n. centralis superior (CS) with its lateral extensions (B9 groups). Differential interference contrast, DIC; a=aqueduct. B: Serotonergic neurons of the B9 group (DIC) C: Serotonergic synaptic contacts (arrows) on α-motoneuron of the rat spinal cord. One-micron paraffin section (DIC). D: Transverse section of the rat medulla showing high concentrations of beaded 5-HT axons in the nucleus of the tractus solitarius.

rons produce a synchronous neuronal discharge in the area (Wang and Aghajanian, 1982). Felten et al. (1974) and Felten and Harrigan (1980) proposed, from Golgi-stained sections, that dendrodendritic contacts were present in the nuclei. Our findings of presynaptic dendrites connecting labeled dendrites with unlabeled ones are in accord with the suggestions of the above-mentioned authors.

Kelly et al. (1989) have published an interesting comment on the autoregulation of raphe neurons in which new references to the subject may be found, including a publication of Chazal and Ralston (1987) confirming our original observations of presynaptic dendrites (Pecci Saavedra et al., 1982; Pecci Saavedra et al., 1986). The inhibitory effects of 5-HT on dorsal raphe neurons were thus clearly explained.

Double Immunocytochemical Staining Methods

Using two primary Ab developed in different species combined with two different cromogens (i.e., DAB and α-chloronaphthol) demonstrates the presence of two antigens in the same section observed with LM (Vandesande, 1983,1988). This procedure allows characterization of chemistry of neuronal networks, in particular, the identifi-

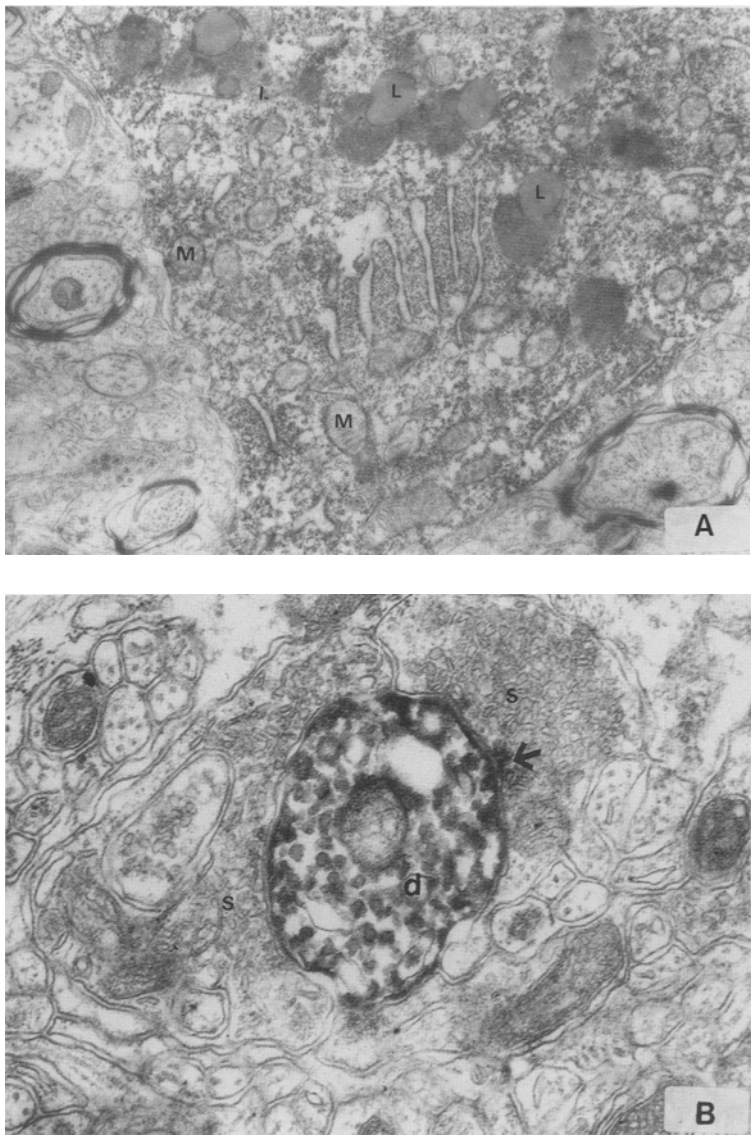

Fig. 3. Electron micrographs of thin sections from the rat mesencephalon in the region of the n. raphe dorsalis labeled with our antibody anti-5-HT. A: Immunocytochemically labeled somata of a raphe neuron. m = mitochondria; l = lipofuchsin granules; and G = Golgi apparatus. B: Immunostained dendrite branching (d) receiving multiple synaptic contacts (s). These synaptic terminals contain abundant pleomorphic vesicles, and one has an evident presynaptic density (arrow).

cation of the neurotransmitters in pre- and postsynaptic elements. Silver was introduced to intensify the staining. The precipitate becomes black, thus being easily differentiated from a single brown DAB precipitate (Gallyas et al., 1982; Gallyas and Merchenthaler, 1988; Merchenthaler et al., 1989).

Silver-gold substitution was introduced in double immunocytochemical EM studies of the CNS (Görcs et al., 1986). The use of this technique allowed us to study the connectivities of 5-HT fibers and neuropeptide-containing neurons in the medullary reticular formation (López Costa et al., 1991) and in the spinal trigeminal

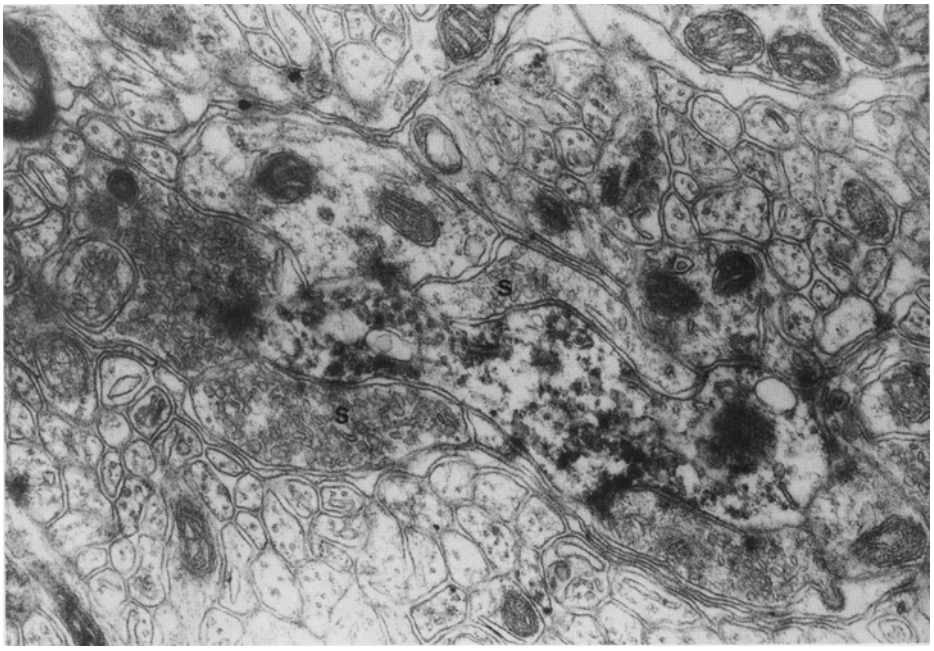

Fig. 4. Electron micrograph showing a serotonergic dendrite receiving multiple synaptic contacts. Pre- and postsynaptic densities are clearly evident in these contacts.

nucleus, pars caudalis. The substantia gelatinosa of the spinal cord and the lamina II (LII) of the spinal trigeminal nucleus (sVn) are two of the main sites in the transmission of nociceptive information. Primary afferents contact projection neurons whose axons arrive at the thalamus. Local interneurons in the LII participate in complex circuits involved in controlling the transmission of nociceptive information. Some interneurons contain peptides. The existence of enkephalin (ENK), somatostatin (SOM), neurotensin (NT), cholecystokinin (CCK), and substance P (SP) has been clearly demonstrated in the dorsal horn of the spinal cord (Hunt, 1983). In addition, descending fibers coming from the n. raphe magnus (NRM), n. raphe obscurus (NRO), n. raphe pallidus (NRP), and the adjoining reticular formation (RF) (n. Ret. Gigantocellularis pars α, pars ventralis, and n. ret. paragigantocellularis) (Gebhart, 1982; Willis, 1982) take part in antinociception (PAG and NRM) and in the nociceptive (RF) control of the incoming sensory information.

Serotonin is one of the neurotransmitters involved in the descending antinociceptive control of pain transmission. Electrical stimulation of NRM induces analgesia and an increment of 5-HT release in the spinal cord. Iontophoretic application of 5-HT in dorsal horn neurons inhibits pain (Randic and Yu, 1976; Headly et al., 1978). Other evidence also demonstrates an antinociceptive role for 5-HT (Messing and Lytle, 1977, Yaksh and Tyce, 1979).

Evidence suggests that serotonin exerts a postsynaptic control of nociceptive transmission, although the complexity of the interrelations is large (Basbaum and Fields, 1984; Dubner et al., 1984). Among the possibilities, 5-HT fibers could contact either projecting neurons or local interneurons. In our work, we demonstrate that 5-HT fibers contact the somata and dendrites of ENK-containing neurons of lamina I (LI) and II. Probably, serotonin regulates the liberation of ENK in the spinal cord, and this is the manner by which 5-HT induces analgesia. This was previously suggested using radioautography and immuno-

cytochemistry (Glazer and Basbaum, 1984). However, this is the first time that the fact is clearly demonstrated with a double immunocytochemical method. Serotonin fibers also contact somatostatin cells in the reticular formation (López Costa et al., 1991), although the richness of ENK contacts on the somatostatin population is more abundant than the former. The reticular formation is known as a site with complex functions, including nociception. However, the functional role of 5-HT and peptides in the reticular formation requires further study.

Neural Cell Cultures from Recognized Areas of the Brain

The cell culture technique offers the advantage of yielding an amount of material sufficient for immunocytochemical, biochemical, and pharmacological studies. The technique of reaggregating cultures (Moscona, 1961; Morris and Moscona, 1971; Halgren and Varon, 1972; Marks and Seeds, 1978) was adapted for obtaining spherical pieces of newly formed tissues reproducing the tridimensional network of a nervous system (Peressini et al., 1989).

The population of the newly formed miniature nerve system could be enriched by seeding in the cell tissue flasks selected areas of the brain containing high concentrations of the preferred neurons (i.e., 5-HT neurons). The cell suspension was seeded and cultured in rotatory flasks maintained at 37°C for periods of up to 35 d. Yamamoto et al. (1981) and Privat (1982) also cultured dissociated cells from the mesencephalon in monolayer cultures. This technique has the advantage of providing a tridimensional space for neurite growth, thus reproducing with greater validity the conditions of the development of the nervous system, permitting a new neuropil to develop in vitro.

Once the cultures are allowed to grow for periods of 3–35 d, LM, EM, and immunocytochemical techniques for various antibodies can be applied (Fig. 5). The aggregates are spherical, reaching sizes of about 0.1 mm in 10 d. Since most aggregates are formed with cells obtained from the mesencephalon of rat embryos in their second week, both neuroblasts and differentiated neurons participate in the aggregate development. At the early stages, most cells are loosely packed, with some rosette formations reminiscent of the neural tube with some mitosis found at its apical surface. At first, the cells appear undifferentiated, with sparse neuropil. The tissue is loose, and the first neurites and axons cones can be visualized.

Following this initial week, the neurons acquire the typical differentiated stage, and some astrocytes and oligodendrocytes are also discerned. At the end of the third week, synapses, some fully mature, are recognized. Regarding the cells forming the rosette neural tube-like structures, they appear as mature ependymal cells. The 5-HT cells stained with our antibody are bipolar, multipolar, oval-shaped, or round. Dendrites, in numbers from 2 to 5, emerge from the soma (Fig. 5B), ramifying in all directions with bifurcations and trifurcations. The appearance of the labeled neurons is very similar to that seen in adult (i.e., the dorsal raphe).

It is known that 5-HT neurons differentiate by expressing their neurotransmitter, immediately after becoming postmitotic (Goto and Sano, 1984; Lauder and Bloom, 1975a,b; Lauder et al., 1982a,b). This being the case, the presence of some 5-HT–labeled neurons in the fourth d of culture implies either that 4 d are sufficient for the neuroblasts to reach maturity or that some neurons are already present in the suspension used for seeding the cultures. We demonstrated that the latter is the case since in both the suspensions and the small pieces of the mesencephalon dissected to obtain the cells, the 5-HT immunocytochemistry revealed positively stained elements. This fact, of course, does not deny the possibility that true differentiation takes place in our cultures because typical neuroblasts as well as mitosis are observed in the tissue pieces used for obtaining the cell suspensions and in the aggregates of early age.

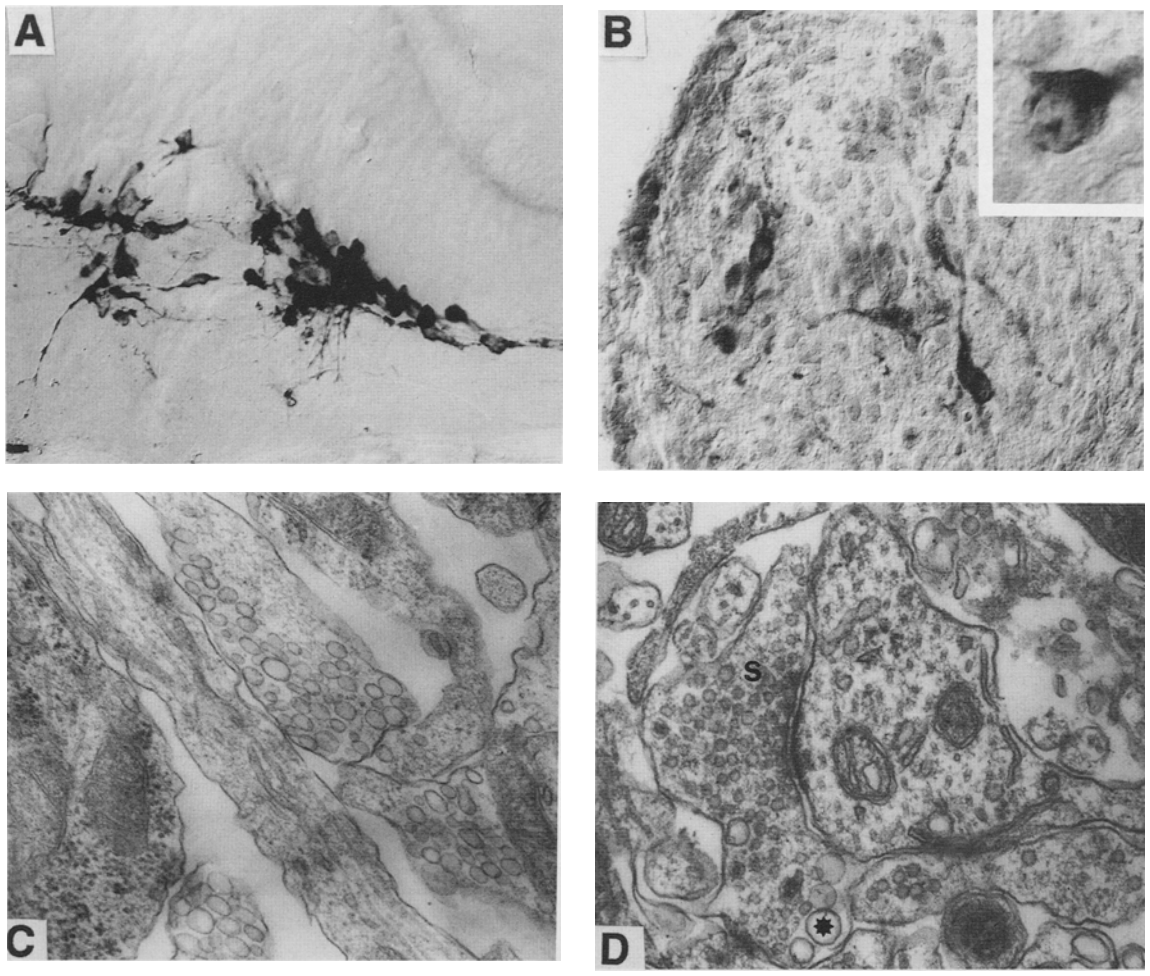

Fig. 5. A: Transverse section through the rhombencephalon of an E14 rat embryo. This figure illustrates the stage of differentiation reached by 5-HT neurons at the moment of dissociation prior to starting tissue culture. Note the intense immunostaining of somata and the scarcity of 5-HT positive processes (DIC). B: Thirty-day-old aggregate immunocytochemically stained with an antibody to 5-HT. Serotonergic neurons are mainly bipolar. Their somata and dendrites appear intensely immunostained (DIC). Inset, higher magnification of a serotonergic neuron at the same age of culture (DIC). C: Electron micrograph of an aggregate after 10 d in culture. A high concentration of growth cones containing clear large- and medium-sized vesicles is present. D: Electron micrograph of a 35-d-old aggregate showing an axodendritic asymmetrical synapse. Note the presence of large clear vesicles (typical of growth cones) coexisting with synaptic vesicles.

The 5-HT neurons can appear randomly distributed in the aggregate or forming small clusters by grouping in selected areas of the sections. It was not possible yet to define if in the latter cases, the clustering depends on some kind of "sorting out" (Seeds, 1983). Considering that membrane recognition of specific molecules is needed to form this type of cell aggregation (Levitt et al., 1976; Wallace and Lauder, 1983), it is possible that in some instances (i.e., those in which 5-HT neurons form such clusters) molecular membrane maturation is in a more advanced stage than in those cases in which 5-HT cells were found randomly distributed. Further study, possibly with

morphometric analysis and markers of specific neural cell adhesion molecules (CAMs), is necessary to clarify this point.

Regarding the networks of 5-HT beaded fibers, which are abundant in the later stages of the mature aggregate, it is of interest to point out that a net difference is found between the periphery of the sections and the center. In fact, the density of the plexus is much higher in the periphery than in the center. The difference could not be ascribed to a problem of antibody penetration since the protocol was performed in the sections but not in the whole aggregate. In summary, the miniature nervous system formed in vitro not only contained all the features found in the differentiating embryonic tissues (Lauder and Bloom, 1975a,b; Lauder et al., 1982a,b) but also exhibited some signs of regional organization (i.e., clusters and peripheral concentrations of fiber plexi). In addition, a study was performed (López Costa et al., 1988) on the distribution of astrocytic cells, the result of which could be easily correlated to the astrocytic distribution in vivo during CNS development.

Viability of Aggregate Cultures

Maturation of the aggregate cultures was followed by LM and EM. In general, preservation of the newly organized tissue was good, as explained in some detail in the preceding paragraphs. Definite proof of the normal differentiation, neurite growth, and synapse formation was given by EM observations. Figure 4 presents examples of the process of *synaptogenesis* clearly visualized between 6 and 35 d of culture. At first, neurite growth is characterized by the development of neural processes filled with microtubules, microfilaments, and some mitochondria, The distal tips of growing neurites feature pear-like enlargements containing oval and round vesicles of several sizes, with a medium diameter of 160 nm (Fig. 5C). At intermediate stages, the growth cones, described above, start losing the large vesicles up to a point in which typical synaptic vesicles appear (Fig. 5D). Finally, the pre- and postsynaptic membranes complete their differentiation with the development of a definitive synaptic contact. Synaptic vesicles increase in number, forming clusters next to the "active points" (Fig. 5D). During differentiation of the neuropil, as just described, the extracellular space starts to become loose and acquires a tight and compact organization between d 5 and 35 of the cultures. Likewise, glial cells are easily identified (López Costa et al., 1988), either as astrocytes or oligodendrocytes, at about 20 d.

New Perspectives

Methodological Considerations

The new roads of present-day chemical neuroanatomy have an important potential area of research in combination with different Ab applying double and triple immunostaining of different neurotransmitters (i.e., 5-HT and peptides). They also show potential in combination with immunocytochemical methods with retrograde tracing techniques, anterograde axonal tracing methods, radioautography at LM and EM levels, traditional Golgi technique, electrophysiological recordings of identified neurons, *in situ* hybridization for RNA, microdissection, and quantitative receptor radioautography. The processing and analysis of neuroanatomical images with systems equipped with bit tablets, video-cameras, digiters, and software for processing, analysis, and quantification of the data allow indepth studies with sophisticated computerized morphometric methods (Marko et al., 1988; Heimer and Záborszky, 1989; Bullock and Petrusz, 1989). The laser beam microscope (LBM; i.e., the confocal microscope) is becoming another valuable tool to improve the knowledge and comprehension of spatial organization of neurons, neuropil, and glial cells.

Considerations Relating Serotonin to Pathological Conditions

The role of serotonin and serotonin receptors (Hamon et al., 1988) in the pathogenesis of schizophrenia has been considered by several authors (Garelis et al., 1975; De Lisi et al.,1981; Jackman et al., 1983; Gelders et al., 1986; Lerer et al., 1988). Recently, Doty (1989) related the disease to abnormalities in interhemispheric processes, possibly caused by deficits produced by some unknown viral infection (Mohammed et al., 1990; Doty and Davies, 1991). The pshycotropic drug 3,4-methylenedioxy-metamphetamine (MDMA, "ecstasy") can induce chronic cerebral dysfunction in nonhuman primates. McBean et al. (1990) found specific neurotoxicity to 5-HT cells when MDMA was injected. In relation to this, it is well known that amphetamine can produce an acute schizophrenic syndrome.

The finding of a serotonin depletion following nasal viral infection (Mohammed et al., 1990) in adult rats infected during infancy reinforces the retrovirus/transposon hypothesis of schizophrenia (Crow, 1987). These developments, again relating serotonin to neuropathological conditions, open important avenues of research for continuation of the efforts toward a major understanding of the serotoninergic system and schizophrenia using basic neuroanatomical, neurochemical, and neuropharmacological methods.

Other psyhchiatric conditions (i.e., depression, Alzheimer disease, bulimia, and anorexia) also have been related to changes in the metabolism of serotonin. Indirect pharmacological evidence for the participation of serotonin in several of these conditions is available (Crow et al., 1984; Arai et al., 1984; Meltzer and Lowry, 1987; Murphy et al., 1980; Jimerson et al., 1990; Yates et al., 1990; McBride et al., 1990). Obviously, further research in humans and in nonhuman primates is required to elucidate the physiopathological mechanisms underlying these disorders.

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