ing frames, could be used to produce antiserums to detect the corresponding human proteins and identify individual gene products. Each of these gene products would then be analyzed as the potential defective protein in HD.

The exact strategy for cloning a disease gene on the basis of its map location will no doubt differ in special details for each disorder. The methods used will probably involve some combination of these new techniques with more standard cloning and somatic cell genetic methods. With HD, new strategies may become evident as each of the techniques used is further refined and additional information is gained concerning the genetics and expression of the defective gene and its precise location on chromosome 4. The rapid pace of technological development in the field of recombinant DNA research also makes it likely that additional useful methods will become available as this work progresses. It should certainly be possible, however, to isolate the HD gene by intensive application of the techniques already at hand.

The characterization of the HD gene and its normal counterpart will certainly provide improved diagnostic capability, and may yield information that will lead to an effective therapy for this disease. Furthermore, the investigation of this specific mutation might yield significant insights into fundamental mechanisms operative in the human nervous system. Perhaps most important, however, is the likelihood that this system will pave the way for application of similar techniques to improve our understanding of many other neurogenetic disorders.

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Chemical Anatomy of the Brain

Tomas Hökfelt, Olle Johansson, Menek Goldstein

The term "chemical anatomy of the brain" encompasses a large field of research. The present article includes a review of some of the histochemical-neuroanatomical methods employed in chemical neuroanatomy and some of the knowledge on systems containing classical transmitters. Among the classical transmitters discussed are the catecholamines, acetylcholine (ACh), and γ-aminobutyric acid (GABA). The more recently discovered neuropeptides and their possible role as cotransmitters or comodulators are also considered.

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Aspects of Methodology

The short history of transmitter histochemistry is to a considerable extent the history of the development of new methods. The first attempts to map transmitter-identified pathways were made by Koelle and Friedenwald (*1*) using ACh esterase (AChE) staining. Thus the enzyme responsible for the breakdown of ACh was taken as a marker for cholinergic pathways. The underlying histochemical reaction was of the metal-precipitation type, and specificity was achieved by using inhibitors of nonspecific esterases.

A different approach was taken for visualizing monoamine neurons. Here the aim was to demonstrate the transmitter itself. Based on the original work of Eränkö (*2*), studies by Hillarp, Falck, and their collaborators resulted in a reliable formaldehyde-induced fluorescence method (Falck-Hillarp technique) for demonstrating norepinephrine, dopamine, and to a lesser extent 5-hydroxytryptamine (5HT) in tissue sections (*3*). Subsequently, many modifications of the original technique were introduced (*4*). These steps improved the quality of sections and the sensitivity of the method, especially for visualizing dopamine systems.

A logical continuation of the formaldehyde fluorescence studies of catecholamine neurons was the introduction of the indirect immunofluorescence method (*5*). The work of Geffen *et al.* (*6*) as well as other studies (*7*) demonstrated that antisera to dopamine-β-hydroxylase (DBH), the enzyme converting dopamine to norepinephrine, could be used to visualize peripheral noradrenergic neurons and gland cells in the adrenal medulla. This approach was rapidly broadened to include visualization of all four enzymes in the synthesis of catecholamines: tyrosine hydroxylase, L-aromat-

ic amino acid decarboxylase (AADC), DBH, and phenylethanolamine-*N*-methyltransferase (PNMT) (*8*). Identifying these enzymes allowed differentiation between various types of catecholamine neurons, and the recent demonstration that some AADC neurons do not contain other catecholamine-synthesizing enzymes (*9*) indicated the existence of neuron populations with a transmitter that was not yet identified. Such differentiation was difficult with the Falck-Hillarp technique, since the excitation and emission spectra of the fluorescence products of the reaction of Formalin with dopamine, norepinephrine, and epinephrine were identical (*10*). Immunohistochemical analysis could also be carried out at the ultrastructural level with horseradish peroxidase (HRP) as a tracer (*11*).

Subsequently, the general applicability of the immunohistochemical technique was extensively exploited. Enzymes—for example the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) (*12*)—and peptides have been mapped (*13*). Of particular interest was the discovery that very small molecules can be analyzed immunohistochemically. Thus Steinbusch *et al.* (*14*) raised antibodies to 5HT and mapped the 5HT neuron systems in detail (*15*). GABA, as well as glutamate neurons, can also be identified with antisera to these two amino acids (*16*).

Immunohistochemistry frequently permits distinct staining of neurons. In fact, under certain circumstances—for example, with the use of thick sections (up to 100 micrometers), immunostained neurons look very similar to Golgi-impregnated cells with extensive dendritic processes clearly visible (*17*).

Immunohistochemistry can be combined with other types of histochemical methods. Of special importance is merging transmitter histochemistry with retrograde tracing of neuronal projections (*18*). Thus, both immunofluorescence histochemistry and HRP immunohistochemistry can be combined with retrograde tracing with HRP or fluorescent retrograde tracers (*19*) (Fig. 1). Other workers have combined immunohistochemistry with such histochemical techniques as Golgi-impregnation procedures (*20*). In this way the transmitters in neuronal loops can be analyzed.

Another technique that may be used to analyze transmitter systems is autoradiography. This method is based on the fact that many neurons have transmitter-specific reuptake systems, the physiological purpose of which is to terminate the action of the transmitter at the synapses (*21*). The technique has been used to trace catecholamine-, 5HT-, and amino acid–containing neurons (*22*).

A major problem with most histochemical techniques, including immunohistochemistry, is specificity (*23*). It is virtually impossible to exclude cross-reactivity with compounds containing the same (or similar) immunogenic amino acid sequences. Sensitivity is of particular concern when analyzing peptides, which are present in the brain in much lower concentrations than classical transmitters such as GABA and the monoamines (*24*). Cell body stores of several peptides can, in fact, often be demonstrated only after treatment of the animals with colchicine, a drug that by interfering with microtubules arrests axonal transport (*25*). Even after this treatment, negative results should be interpreted with caution (see Fig. 2).

A problem not often considered is whether the technique may be "too sensitive." The reasons for considering this question are recent unexpected findings. For example, tyrosine hydroxylase–like immunoreactivity has been observed in the magnocellular neurosecretory hypothalamic neurons that produce the well-known pituitary peptide oxytocin (*26*). So far no evidence has been presented that tyrosine hydroxylase is transported

Summary. The development of sensitive histochemical-neuroanatomical techniques has made it possible to analyze the content of specific compounds in single nerve cells and their processes. In consequence, it has been possible to construct detailed maps of the distribution of various types of neurons on the basis of their transmitter substance. There are now many examples of neurons containing both a classical transmitter and a peptide. In some instances the peptides seem to support the action of the classical transmitters. This interaction may have applications in the prevention and treatment of nervous disease states.

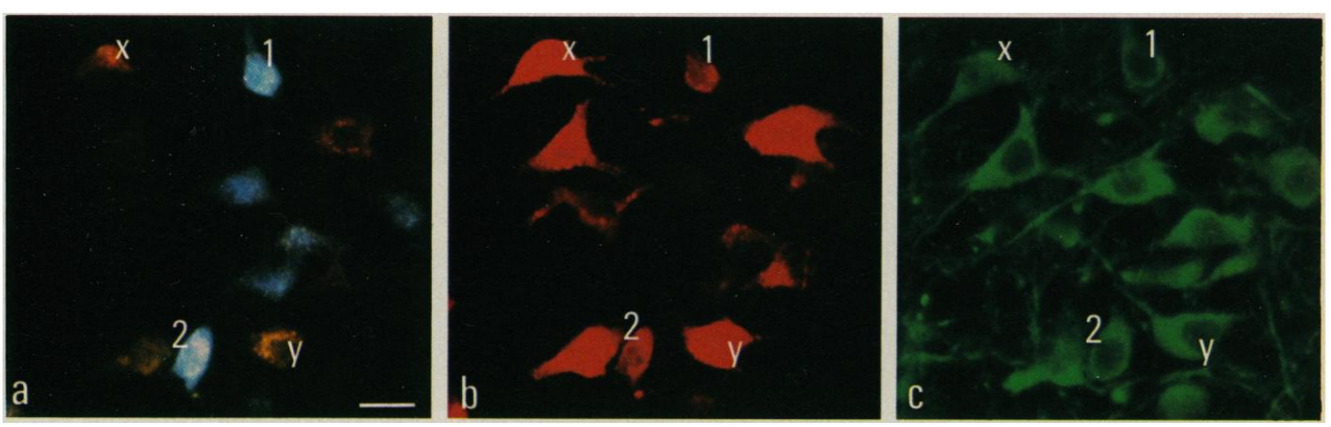

Fig. 1. To determine whether single dopamine neurons in the ventral mesencephalon project to more than one brain region, we used two dyes. Fast blue (FB) and propidium iodide (PI) were used. They exhibit different excitation and emission characteristics and thus show up with different colors in the microscope (*19*). Fast blue was injected into the head of the caudate nucleus and propidium iodide into the caudate tail–amygdaloid complex. Two days later the brain was fixed by Formalin perfusion, and the mesencephalon was sectioned and analyzed in the fluorescence microscope under (a) blue filter and (b) red filter conditions. In the substantia nigra, cells exhibiting both fast blue and propidium iodide colors (indicated by *1* and *2*), as well as only red cells (x and y) were observed. The red propidium iodide shines weakly through blue filters (a). The sections were then processed for indirect immunofluorescence with antiserum to tyrosine hydroxylase as a marker for dopamine neurons and the green dye fluorescein-isothiocyanate as immunolabel. (c) Some neurons containing fast blue and propidium iodide were also immunoreactive to tyrosine hydroxylase (1 and 2), showing that single dopamine neurons can project to both injection sites. Bar indicates 50 μm. [From experiments carried out with L. Skirboll, National Institutes of Health (*19*)]

into the nerve endings where it could be used for transmitter synthesis. Instead, one cannot exclude the possibility that the genomic control of protein synthesis is imperfectly regulated. Perhaps neurons produce small amounts of an enzyme or a peptide that is never of functional significance in the neuron but merely represents a "spillover."

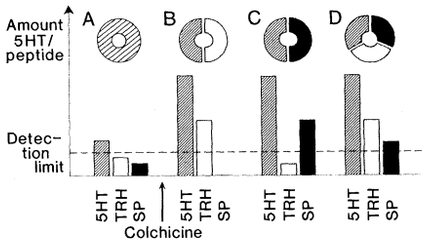

Fig. 2. Schematic illustration of the sensitivity problem in histochemistry, with special reference to analysis of multiple compounds in neuronal cell bodies. (A) 5HT—but not TRH and substance P (SP)—can be seen in cell bodies in untreated rats. After colchicine treatment, some neurons contain (B) 5HT and TRH, (C) some 5HT and substance P, and (D) others 5HT, TRH, and substance P. This may reflect actual absence of one compound [substance P in (B)] or insufficient sensitivity of the technique [THR in (C)].

Classical Transmitters

Some information on systems containing classical transmitters (catecholamines, 5HT, ACh, histamine, and GABA and other amino acids) is summarized below. The most detailed information so far available is for the catecholamines (dopamine, norepinephrine, and epinephrine), thanks to the pioneering studies of Fuxe, Dahlström, and others (*3*). Most studies of catecholamine neurons have been carried out on the rat brain, but tissues from cat (*27*), monkey (*28*), and man (*29, 30*) have also been analyzed. Björklund and Lindvall (*31*) have discussed some quantitative aspects of catecholamine neurons. There are about 50,000 catecholamine neurons in the rat brainstem, of which dopamine neurons constitute 80 percent and norepinephrine neurons 20 percent. About 70 percent of all catecholamine neurons are found in the mesencephalon. The number of epinephrine neurons has so far not been calculated. Quantitatively the catecholamine neurons therefore constitute only a minor fraction of the total number of neurons in the brain. Both norepinephrine and epinephrine neurons give rise to sparse, diffuse fiber networks. However, in some regions dopamine neurons make a sizable contribution to the total innervation of a special area; dopamine fibers constitute 10 to 15 percent of all nerve endings in the rat striatum (*32*) and, regionally in the median eminence, up to one-third of all boutons (*33*). In Fig. 3, a schematic overview is given of the distribution of the dopamine, norepinephrine, and epinephrine cell groups in the rat brain. More detailed information can be found in several original and review articles (*31, 34, 35*).

Dopamine neurons. It was several years before dopamine was recognized as a transmitter in its own right rather than merely a precursor of norepinephrine. Thanks to the work of Carlsson *et al.* (*36*), strong evidence was obtained for high concentrations of dopamine in the basal ganglia, thus suggesting its transmitter role. This was emphasized and confirmed by early formaldehyde fluorescence studies (*37*). Our picture of the central dopamine systems today is complex (Fig. 3). Dopamine cells are found mainly in the mid and rostral parts of the brain—that is, in the mesencephalon, hypothalamus, and olfactory bulb, with scattered cells in some other brain regions. The ventral mesencephalic dopamine cells (Fig. 4) were divided by Dahlström and Fuxe (*3*) into a lateral A9 group in the substantia nigra and a medial, small-sized cell group in the ventral tegmental area termed A10, extending rostrally to the supramammillary area and caudally and dorsally up to the aqueduct, forming a periaqueductal system. It was long assumed that dopamine, in contrast to norepinephrine and 5HT, did not innervate cortical areas. Biochemical work of Glowinski and his collaborators (*38*) strongly suggested occurrence of dopaminergic fiber systems in different cortical areas. This was confirmed by histochemistry, showing in the rat patchy innervation patterns in the cingular, prefrontal, pyriform, and entorhinal cortices (*31*).

The main projection for the A9 nigral dopamine neurons is the nigrostriatal pathway ascending dorsolateral to the medial forebrain bundle through the internal capsule to the neostriatum. Ascending dopamine axons also run within the medial forebrain bundle, projecting to the nucleus accumbens, olfactory tubercle, and septum, as well as to cortical regions. In addition, there is a periventricular fiber system carrying both ascending axons and descending axons. The latter project to the spinal cord (*31*).

Other dopamine cell groups are the A11 cells at the mesencephalic-hypothalamic junction, projecting to the spinal cord (*39*), the A12 cells in the arcuate nucleus, the A13 cells in the zona incerta, and the A14 cells in the periventricular area (A14 group). The most rostral dopamine neurons are periglomerular cells in the olfactory bulb (A16 group) (*40*). Finally, in the retina a small proportion of the amacrine cells are also dopaminergic (A17 group) (*41*). One dopamine system may exist in the lower brainstem. Thus, cells in the medial part of the dorsal vagal motor nucleus contain tyrosine hydroxylase but not DBH (Fig. 5) (*42, 43*). More recently, Jaeger *et al.* (*9*) have reported lack of AADC in these cells and on this basis suggested that they may utilize the amino acid L-dihydroxyphenylalanine (L-dopa) as transmitter.

The histochemical work by Björklund and Lindvall (*44*) has focused on a feature of dopamine neurons that suggests storage and release of dopamine from dendrites in the zona reticulata. Subsequent experimental work supports this hypothesis, indicating that release of dopamine from dendrites may represent an important mechanism for control of neuronal activity in the substantia nigra (*45*). Such dendritic release may also be important in the olfactory bulb (*40*).

Norepinephrine neurons. The pioneering biochemical work of Vogt (*46*) showed an uneven distribution of norepinephrine in the rat brain, suggesting a neurotransmitter role for this amine. Histochemical analysis revealed that the norepinephrine cell groups are confined to the lower brainstem (Fig. 3), with the most rostral ones located in the pons, the A6 group in the locus ceruleus, and the A7 group in the lateral pontine reticular formation. The more caudally located cell groups (A1 to A5) are found mainly in the ventrolateral part of the pons (A5 cell group) and of the medulla oblongata (A1 cell group) and in the nucleus tractus solitarii (A2 cell group) (Fig. 5). Both the A1 and A2 groups extend into the spinal cord (*47*). The projections of these neurons are long and often diffuse; the locus ceruleus innervates both cerebral and cerebellar cortical areas, as well as hypothalamus and spinal cord. The ascending norepinephrine neurons send their axons mainly within the central tegmental tract system through the mesencephalon, whereby the main part of the axons projecting to cortical areas run in the so-called dorsal bundle. These fibers then join the medial forebrain bundle on their way to more rostral parts of the brain. At the level of the lower pons and medulla oblongata the norepinephrine axons run in the medullary catecholamine bundle, which carries both ascending axons from the A1 and A2 groups and descending

axons from the A6 and A5 cell groups to the spinal cord.

Epinephrine neurons. The distribution of epinephrine neurons is much more restricted than that of the other two systems (Fig. 3) (*34, 42, 43, 48, 49*). Two major epinephrine cell group complexes (C1 and C2) have been identified in the medulla oblongata and lower pons. The C1 group represents a rostral continuation of the noradrenergic A1 group and is present in the ventrolateral medulla oblongata (Fig. 6). The dorsal C2 group is located mainly in the dorsal vagal complex with the majority of cell bodies in the solitary tract nucleus, where a parvocellular group can be distinguished in the dorsolateral strip (Fig. 5). Rostrally, a medial cell group can be identified (C3) (*49*). Ross *et al.* (*50*) have observed numerous PNMT-positive cell bodies in the hypothalamus which, however, seem to lack tyrosine hydroxylase, AADC and DBH. Similar neurons have also been observed in the retina (*51*). The nature of these neurons is still unclear.

The main projection group is the C1 group, which gives rise to both ascending and descending pathways. The ascending PNMT axons run in the medullary bundle, in the tegmental tract, and then in the medial forebrain bundle, innervating primarily periventricular regions such as the periaqueductal central gray and various hypothalamic nuclei—for example, the paraventricular and the dorsomedial nuclei. The most rostral fibers are found around the olfactory ventricle and medial to the anterior olfactory nucleus. In the spinal cord, the axons descend in the dorsal part of the lateral funiculus and innervate almost exclusively the lateral sympathetic column.

5-Hydroxytryptamine neurons. Dahlström and Fuxe (*52*) outlined several 5HT cell groups (B1 to B9) in the rat medulla oblongata, pons, and mesencephalon. Extensive 5HT nerve terminal networks were also described in virtually all parts of the brain (*53*). Although attempts to trace 5HT neurons were made by means of autoradiography (*22*) or immunohistochemistry with antisera to tryptophan hydroxylase (*54*) and AADC (*55*), a breakthrough in this field was achieved when Steinbusch *et al.* (*14*) used antibodies to 5HT conjugated with bovine serum albumin to visualize the 5HT systems in an easy and reproducible way (*15*). In the rat, the 5HT neurons have a preferential localization in the midline (raphe) areas, with major cell groups both in the medulla oblongata and at the pontine-mesencephalic level (dorsal raphe nuclei). The former project mainly to the spinal cord and lower brainstem, whereas the dorsal raphe nuclei give rise to extensive projections to the entire forebrain. There may be 5HT cell bodies also in the hypothalamus (*56*).

Acetylcholine neurons. The first attempt to map the central cholinergic neurons were made by Koelle (*57*) and Lewis and Shute (*58*) in the early 1960's. They used the AChE method and outlined several systems with two major ascending pathways belonging to a cholinergic reticular system. With the development of specific antibodies to the ACh synthesizing enzyme, choline acetyltransferase, it has now become possible in a more reliable way to identify central cholinergic neurons and to study their projections both in experimental animals

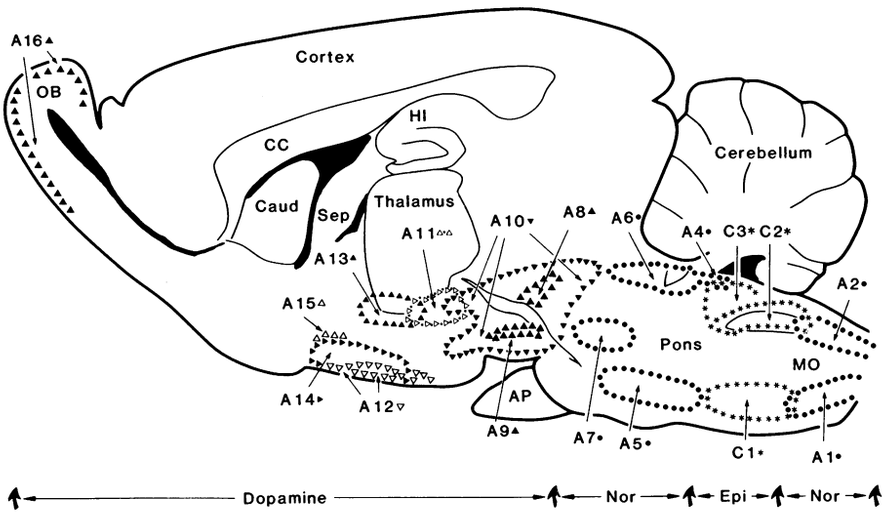

Fig. 3. Schematic drawing of a sagittal section of the rat brain 1.4 millimeters lateral to the midline, drawn from the atlas of Paxinos and Watson (*126*). The approximate distribution of dopamine (triangles), norepinephrine (Nor) (dots), and epinephrine (Epi) (asterisks) cell groups are shown. The nomenclature is according to Dahlström and Fuxe (*52*) and some recent studies (*34, 127*). Each catecholamine principally has its own domain in the brain with dopamine cell bodies (A8 to A16) in the rostral parts of the brain (mesencephalon, hypothalamus, and olfactory bulb) and norepinephrine cells at the pontine (A5 and A6) and lower medullary (A1 and A2) levels, whereas the epinephrine cells lie between the rostral and caudal norepinephrine complexes. One possible exception may be dopamine (or L-dopa) neurons in the dorsal vagal motor nucleus (see text). In this way catecholamine cells can be found almost along the entire brain axis. There is a clear tendency to a dorsal and a ventral catecholamine complex at all levels from the spinal cord to the rostral hypothalamus. Abbreviations: AP, anterior pituitary; Caud, caudate nucleus; CC, crus cerebri; HI, hippocampal complex; MO, medulla oblongata; OB, olfactory bulb; and Sep, septum.

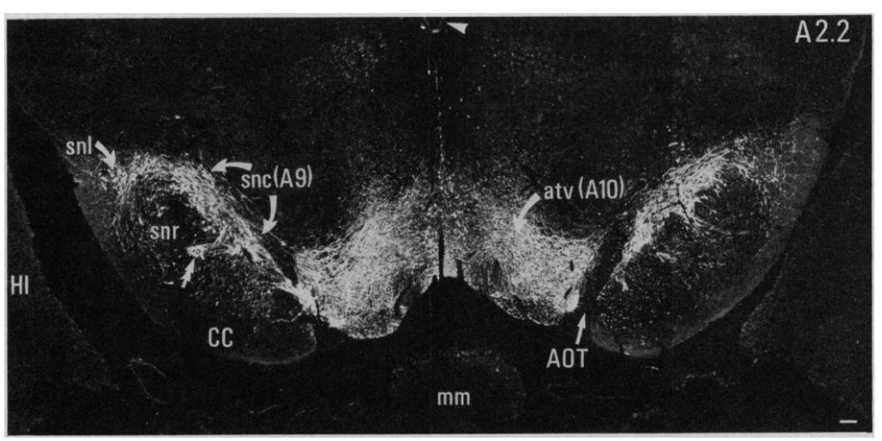

Fig. 4. Immunofluorescence micrograph (montage) of the ventral mesencephalon of the rat after incubation with antiserum to tyrosine hydroxylase. The distribution of dopamine cells is shown with A9 cells, mainly in the zona compacta (snc) and zona reticulata (snr), and with A10 cells in the ventral tegmental area (atv). The arrowhead points to the aqueductus cerebri. The approximate level is 2.2 mm anterior to the frontal zero plane according to the atlas of Palkovits and Jacobowitz (*35*). Abbreviations: AOT, accessory olfactory tract; CC, crus cerebri; HI, hippocampal complex; and mm, mammillary body. Scale bar, 200 μm. This photograph of a section prepared by P. Hökfelt was taken by R. Mårtensson, University of Lund, Lund, Sweden, with a microscope equipped with a newly developed scanning dark-field condenser (*127*).

and human brain (*59*). Large cholinergic cell groups are found in the medial septal nucleus, the limb nucleus of the diagonal band, the nucleus basalis, and the pontine nuclei. Also cortical, striatal, and habenular neurons are immunoreactive to choline acetyltransferase, as are cell bodies in various cranial nerve nuclei. Although the specificity of the AChE staining was often questioned in the past, it is now evident that in several cases there is good agreement between the results obtained with this technique and results obtained with antibodies to choline acetyltransferase (*60*).

Histamine neurons. For many years, biochemical evidence has suggested the existence of histaminergic systems in the brain (*61, 62*). However, a suitable histochemical technique has not been available. More recently, Watanabe *et al.* (*63*) have purified histidine decarboxylase to homogeneity, raised specific antibodies, and observed a restricted hypothalamic histidine decarboxylase–immunoreactive system with immunohistochemistry. Cell bodies are present in the so-called caudal magnocellular hypothalamic nuclei giving rise to sparse but widespread projections to cortical areas, hypothalamus, and the lower brainstem. Antibodies to histamine have also been used for tracing possible histamine neurons (*64*). Whether the systems outlined so far represent the only histamine pathways in the rat brain remains to be seen. The biochemical studies by Schwartz *et al.* (*62*) as well as by others (*61*) suggest a more widespread distribution.

GABA and other amino acid neurons. The amino acid GABA is the major inhibitory transmitter in the central nervous system (*12*). Early attempts to trace GABA neurons in the brain were carried out with autoradiography (*65, 66*). Also possible glycine (*67*) and glutamate (*68*) neurons have been identified with this method. Quantitative evaluations revealed that GABA-accumulating nerve endings often constituted up to 50 percent of the total number of nerve endings (*66*) suggesting a dominant role of this inhibitory transmitter in many brain regions. This impression has been clearly established owing to the pioneering efforts of Roberts and his collaborators, who purified the GABA-synthesizing enzyme glutamic acid decarboxylase, raised specific antibodies (*69*), and initiated immunohistochemical studies (*70*). Knowledge of the GABA systems in the brain is extensive today. Although GABA neurons often represent interneurons—that is, they give rise to local nerve terminal networks—there are also several longer projections described—for example, from the striatum to the substantia nigra or from the posterior hypothalamus to widespread cortical and subcortical areas [(*71*); see also (*16*)].

Neuropeptides

During the last 10 years it has become apparent that large numbers of peptides are present in central neurons. Their possible role as neurotransmitters has been discussed (*72, 73*), and Snyder (*73*) in 1980 anticipated that their number may exceed 200. Subsequent developments have not refuted that view. Krieger (*24*) discussed brain peptides extensively, and her excellent overview need not be repeated in this article.

Intense neuropeptide research over the past 10 years has revealed a number of unexpected findings. For example, the releasing and inhibiting hormones (luteinizing hormone–releasing hormone, thyrotropin-releasing hormone, and somatostatin) isolated and characterized by Guillemin, Schally and their collaborators were not confined to the median eminence, the site for release into hypophyseal portal vessels, and not even to the hypothalamus, but were widespread in the brain and spinal cord. Also unexpected was the finding that the brain contains pituitary hormones as first demonstrated for adrenocorticotropic hormone (*74*). The discovery by Vanderhaeghen *et al.* (*75*) of gastrin- or cholecystokinin-like immunoreactivity represented the first evidence for the presence of a gastrointestinal hormone in the brain, and this concept has been expanded to numerous other peptides.

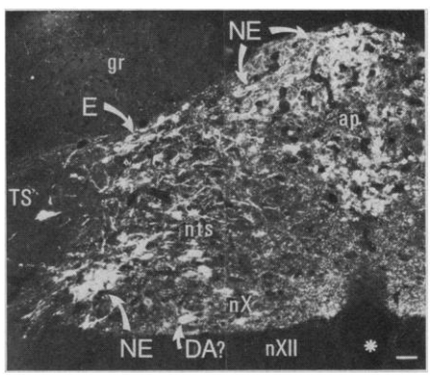

Fig. 5. Immunofluorescence micrograph (montage) of the dorsal vagal complex of the rat after incubation with antiserum to tyrosine hydroxylase. Several catecholamine cell groups are seen. Norepinephrine cells are present in the area postrema (ap) and in the solitary tracts nucleus (nts) and epinephrine cells in the dorsolateral strip of the nts. A few cells, presumed to be dopamine or L-dopa cells, are present in the dorsal vagal motor nucleus (nX). Dense fiber networks are seen in the nts and nX and there is an absence of immunoreactive structures in the hypoglossal nucleus (nXII). DA, dopamine; E, epinephrine; NE, norepinephrine; gr, nucleus gracilis; and TS, solitary tract. Asterisk indicates central canal. Scale bar, 50 μm.

One of the most interesting events in the short history of brain peptides was the demonstration of the opioid peptides methionine enkephalin and leucine enkephalin by Hughes, Kosterlitz, *et al.* (*76*). These two peptides were present in discrete systems in the central nervous system (*77*) and were clearly different from neurons containing β-endorphin (*78*). These peptides have different precursor molecules (*79*). Subsequent to the biochemical work of Goldstein *et al.* (*80*), a third opioid peptide system containing dynorphin-like peptides has been found in the brain (*81*).

Detailed maps of the distribution of various types of peptide neurons have been published, for example, on substance P (*82*), vasoactive intestinal polypeptide (VIP) (*83*), enkephalin (*84*), cholecystokinin (*85*), neurotensin (*86*), somatostatin (*87*), and corticotropin-releasing factor (*88*). Some generalities may be expressed on the basis of these distribution studies. Thus, there are some brain areas which are rich both in peptide-immunoreactive cell bodies and terminals. They include the dorsal horn of the spinal cord, the dorsal vagal complex, various hypothalamic nuclei, the nucleus accumbens, the bed nucleus of the stria terminalis, the amygdaloid complex, especially the central nucleus, and the periaqueductal central gray. Other areas, such as the cerebellum, have low levels of most peptides. The thalamus is also poor in peptides. Cortical areas are particularly rich in some peptides such as VIP, cholecystokinin, and somatostatin. These peptides are present mainly in small interneurons. As a rule, peptides have not been found in larger projection neurons such as the motoneurons of the spinal cord and the cortical pyramidal cells.

Coexistence of Classical Transmitters and Neuropeptides

The multitude of peptides isolated and found in the central nervous system raised the question of their relation to neurons containing classical transmitters. Although the peptide neurons often were present in the same regions as, for example, the catecholamine neurons, the initial impression was that the peptide nerves represented clearly separate systems. For example, whereas several peptides were present in cortical interneu-

rons, no or only occasional catecholamine cell bodies were found in these areas. The immense number of neurons present in the central nervous system would easily allow for all new peptides to be present in separate systems. However, in the peripheral nervous system no such redundancy exists. Studies of the distribution of somatostatin in the periphery suggested that this peptide must be present in a population of sympathetic neurons and that it must be stored with the transmitter norepinephrine (*89*). This finding initiated a systematic search for neurons containing both a classical transmitter and a peptide. As indicated in Table 1, there are now many examples of such situations in the central nervous system [see also (*90, 91*)]. In fact, coexistence of classical transmitters and peptides may represent a rule rather than an exception. As judged from immunohistochemical analyses, however, coexistence does not include entire transmitter-defined neuron populations. For example, only the medullary 5HT neurons contain substance P–like immunoreactivity, whereas the dorsal raphe 5HT neurons seem to lack this peptide.

The functional significance of the occurrence of a classical transmitter and a peptide in the same neuron and their possible release from the same nerve endings is yet unclear, at least with regard to the central nervous system. The complex organization of the brain makes functional analysis of this subject extremely difficult. More favorable conditions do, however, exist in the periphery—for example in the salivary gland and vas deferens, where evidence for two types of interactions have been obtained (*91*). In the salivary gland, the parasympathetic cholinergic neurons contain a VIP-like peptide (*92*) and part of the sympathetic noradrenergic neurons contain a neuropeptide Y–like peptide (*93*). In this model, the peptides seem to support the action of the classical transmitters. Thus, VIP induces vasodilation and enhances the secretory effects of ACh, and neuropeptide Y causes vasoconstriction, as does norepinephrine (*91, 93*). In contrast, in the rat vas deferens, where the noradrenergic neurons also contain a neuropeptide Y–like peptide, the peptide seems to inhibit the release of norepinephrine via a presynaptic action (*94*). Subcellular fractionation studies on the vas deferens indicate a differential storage of norepinephrine and neuropeptide Y, whereby the peptide is preferentially stored in large dense-core vesicles, and norepinephrine is present both in these vesicles and in small synaptic vesicles (*95*). Finally, there is some indication that the release of the two compounds is frequency-dependent, with release of the peptide occurring preferentially at higher frequencies (*91*).

These findings in the periphery may form a basis for speculation on the functional significance of coexistence in the central nervous system. As an example, rat medullary 5HT neurons (*52*) containing substance P (*96, 97*) and thyrotropin-releasing hormones (TRH) (*97*) are discussed. They project to the spinal cord (*97–99*) and probably innervate motoneurons. Pelletier *et al.* (*100*) demonstrated at the ultrastructural level that these nerve endings in the spinal cord contain both 5HT and substance P, possibly in the same large dense-core storage vesicles.

It is known that 5HT activates the stretch reflex (*101*). Barbeau and Bedard (*102*) studied the effect of TRH in chronically spinalized or 5HT neurotoxin-treated rats and observed a marked activation of the stretch reflex after administration of TRH, an effect similar to the one observed after administration of the 5HT precursor 5-hydroxytryptophan (5HTP). The effect of TRH could be blocked by prior treatment with a 5HT antagonist (*102*), suggesting a close relation between the receptors for 5HT and TRH and cooperation of the two compounds at a postsynaptic site, whereby motoneurons involved in the stretch reflex are directly or indirectly influenced.

A further experiment of interest in this context has been carried out by Mitchell and Fleetwood-Walker (*103*). They studied the effect of TRH and substance P on potassium-induced release of tritiated 5HT from spinal cord slices. Neither substance P nor TRH exerted any effects. However, if cold 5HT was added to the bath in a concentration that

Table 1. Coexistence of classical transmitters and peptides in the brain region. Species studied are shown in parentheses.

Classical transmitter	Peptide	Brain region	Reference
Dopamine	Neurotensin	Ventral tegmental area (rat)	(*107*)
	Cholecystokinin	Ventral tegmental area (rat, man)	(*106*)
Norepinephrine	Enkephalin	Locus ceruleus (cat)	(*119*)
	Neuropeptide Y	Medulla oblongata (man, rat)	(*108, 110*)
		Locus ceruleus (rat)	(*108*)
Epinephrine	Neurotensin	Medulla oblongata (rat)	(*107*)
	Neuropeptide Y	Medulla oblongata (rat)	(*108*)
5HT	Substance P	Medulla oblongata (rat, cat)	(*96, 97, 120*)
	TRH	Medulla oblongata (rat)	(*97*)
	Enkephalin	Medulla oblongata, pons (cat)	(*121*)
ACh	VIP	Cortex (rat)	(*122*)
	Enkephalin	Cochlear nerves (guinea pig)	(*123*)
	Substance P	Pons (rat)	(*124*)
GABA	Somatostatin	Thalamus (cat)	(*125*)

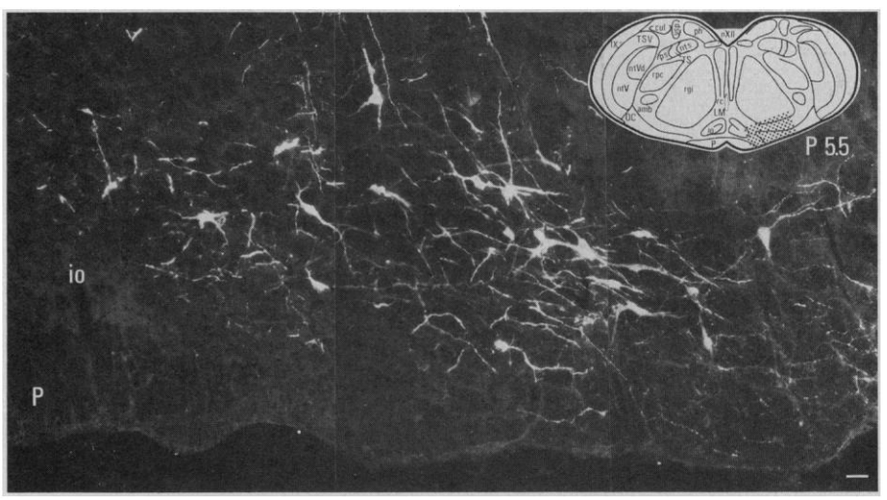

Fig. 6. Immunofluorescence micrograph of the ventral medulla oblongata of the rat after incubation with antiserum to PNMT. Numerous large multipolar cells are seen. The approximate level is 5.5 mm posterior to the frontal zero plane according to the atlas of Palkovits and Jacobowitz (*35*). Dotted area in inset indicates the approximate region shown in the fluorescence micrograph. Abbreviations: io, inferior olive; P, pyramidal tract. For explanation of abbreviations in inset, see (*35*). Scale bar, 50 μm.

blocked the inhibitory, presynaptic 5HT autoreceptor, substance P—but not TRH—counteracted the inhibition of tritium outflow caused by cold 5HT. This indicates that substance P blocks the 5HT autoreceptor. Taken together, these experiments suggest that the two peptides coexisting with 5HT affect 5HT transmission at synapses in the ventral horn in two different ways: TRH by cooperating with 5HT at a postsynaptic site, and substance P by blocking the inhibitory presynaptic 5HT autoreceptor. The net results produced by both peptides, however, tend to enhance 5HT transmission, and both peptides, although by different mechanisms, thus support the action of the main transmitter (Fig. 7).

This hypothesis has recently been tested in a behavioral model. Various parameters of the sexual behavior of male rats were studied after intrathecal application (*104*), at the lumbar level, of 5HT, TRH, and substance P, or combinations of these compounds (*105*). In concentrations up to 50 micrograms, 5HT had no effect, and no effects were seen with substance P. In a dose of 10 μg, TRH caused a small increase in latencies to mount and intromission. However, when 5HT and TRH were given together in concentrations of 50 and 10 μg, respectively, a marked increase (from 20 seconds to 7 minutes) in both mount and intromission latencies was observed. No other parameters, such as the number of mounts or intromissions, were affected. These results could be interpreted along the lines of the hypothesis discussed above. Thus, TRH and 5HT seem to cooperate in affecting these two parameters of sexual behavior at a postsynaptic site. The lack of effect of substance P correlates well with a possible presynaptic action which, in this model, should not be apparent (Fig. 7).

There are, in addition to the 5HT, substance P, and TRH neurons described above, many interesting coexistence situations in the central nervous system (Table 1). For example a cholecystokinin-like peptide is present in a population of the dopamine neurons in the rat ventral mesencephalon (*106*), which may have implications for our understanding of diseases such as schizophrenia. Other dopamine neurons contain a neurotensin-like peptide (*107*). Furthermore, a large proportion of the norepinephrine and epinephrine neurons in the lower brainstem contain a neuropeptide Y–like peptide (*108*), with possible involvement in the regulation of blood pressure and other autonomic functions.

So far, most of the work has been carried out on the rat central nervous system, but preliminary analysis of other species indicate both conservation of the coexistence situation and phylogenetic changes. For example, the coexistence of 5HT, substance P, and TRH described above has been observed also in cat and monkey (*109*). Neuropeptide Y–like immunoreactivity is present also in catecholamine neurons of the human brainstem (*110*). With regard to the mesencephalic dopamine neurons, interesting differences have been encountered. In the rat, marked regional differences exist, with the highest proportion of cholecystokinin-dopamine cells in the pars lateralis of the substantia nigra (100 percent), in the anterior pars compacta (almost 100 percent), and in the A10 area (40 percent), whereas in the posterior zona compacta only few cholecystokinin-dopamine cells are seen (*106*). In the cat, virtually all dopamine neurons in the substantia nigra proper (A9 cell group) contain cholecystokinin, whereas many cells in the midline area (A10) seem to lack the peptide (*111*). Thus, the situation in the cat is to some extent reversed as compared to the rat. In the monkey, cholecystokinin-dopamine neurons have so far been observed only in the midline area (*111*).

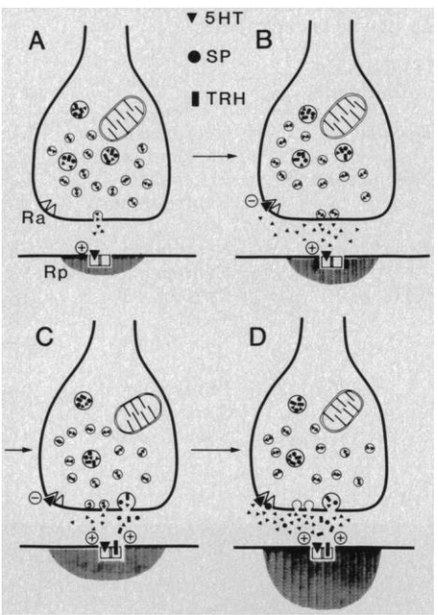

Fig. 7. Schematic representation of a nerve ending in the ventral horn containing 5HT, TRH, and substance P (SP), whereby the small vesicles (diameter about 500 Å) contain only 5HT and the larger vesicles (diameter about 1000 Å) contain, in addition, substance P and TRH. An attempt is made to illustrate a sequence of events occurring hypothetically when presynaptic nervous impulse activity is increased. (A) At low frequency, small numbers of 5HT molecules, released from small synaptic vesicles, cause a small activation ⊕ of the postsynaptic receptor (R_p). (B) Increased release of 5HT results in activation of the presynaptic autoreceptor (R_a) ⊖, which inhibits further 5HT release and prevents a larger postsynaptic response. Further increase in impulse traffic may activate large vesicles and release TRH and substance P, whereby (C) TRH acts together with 5HT on postsynaptic receptors, and (D) substance P blocks the inhibitory 5HT autoreceptor, resulting in a profound postsynaptic activation.

Why should neurons utilize more than one compound in the chemical transmission process? A general hypothesis would be that several messengers could carry more information and that more differentiated messages could be transferred at synapses. The peptide by no means has to be involved in the rapid transmission of nervous impulses at synapses, but may—more or less unrelated to the classical transmitter—participate in regulation of long-term events, such as exerting trophic actions or influencing other intracellular events. However, the findings in the periphery also support the notion that, at least under certain circumstances, the peptides may interact with the classical transmitter. The latter studies indicate, as discussed above, that the peptide may be more important when the neurons are firing at a high rate to induce particularly strong responses. With this in mind, one can view the 5HT, substance P, and TRH system, which as discussed above, project to motoneurons in the ventral horn. Motoneurons are surrounded by thousands of boutons, of which the nerve endings containing 5HT, substance P, and TRH constitute only a small proportion. One may wonder whether such a small system can have any decisive influence on the postsynaptic neuron in competition with the large number of other (excitatory and inhibitory) inputs. One way to "push through" an important message traveling in a small system could be through a concomitant release of multiple messengers—in this case 5HT, substance P, and TRH. This release could lead to a maximal response by strong activation of the postsynaptic receptor through 5HT and TRH and a blockade of the presynaptic inhibitory receptor by substance P (Fig. 7). In this way, a small set of neurons with a message of sufficient importance could perhaps override other influences.

Multiple messengers could also be of importance for regulatory mechanisms at the receptor level. Stimulation or absence of stimulation affects receptor sensitivity and receptor density, leading to "down- and up-regulation," respectively. One way to avoid exaggeration of those phenomena could be through the use of multiple messengers. Thus, if two

compounds released together in low concentrations exert the same effect as one compound released in high concentration, down- and up-regulation of receptors may be less dramatic with less pronounced super- and subsensitivity phenomena.

Agnati and Fuxe and their collaborators (*112*) have proposed that coexisting peptides change the characteristics of the receptors for monoamine transmitters at the postsynaptic level without, at the same time, inducing compensatory responses at the presynaptic level—so-called heterostatic regulation of synaptic functions.

Finally, coexistence phenomena may be of importance for events such as long-term potentiation (*113*) and its possible role in learning processes (*114*) and for various theories dealing with stabilization of synapses (*115*).

Conclusions

The rapid advancement of enzyme and peptide biochemistry combined with the introduction of sensitive histochemical methods has opened up new ways to explore the chemical anatomy of the brain. The results have advanced our understanding of the chemical signaling processes in the nervous system. Indeed, histochemistry represents a valuable link between biochemistry and physiology. Thus, by providing exact knowledge of localization of the messengers in defined neurons, a firm basis can be obtained for physiological and behavioral experiments aiming at improving our understanding of neuronal function under normal and pathological conditions. Of special interest to us has been the finding that neurons may produce and release multiple messengers at their synapses. The physiological significance is still unclear but may influence our view on interneuronal communication and in a larger perspective may be of importance in our efforts to prevent or treat nervous disease states with drugs. For example, substitution therapy as carried out for Parkinson's disease should perhaps aim at including not only a precursor to the classical transmitter dopamine but also one or more still unknown cotransmitters. Furthermore, multiple drug administration may be one way to reduce doses considerably and in this way to avoid side effects.

This field of research is still in an initial phase, and problems such as specificity and sensitivity are still prominent. New techniques are clearly needed. Computer-assisted morphometry will be valuable in quantifying histochemical information (*116*). Hybrid histochemistry in which labeled complementary DNA probes are used to analyze for the occurrence of messenger RNA in single cells has already been used in studies on endocrine cells (*117*) and neurons (*118*). Chemical neuroanatomy can in this way continue to contribute to the challenge of understanding the human brain.

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