The Ultrastructural Localization of Amines, Amino Acids and Peptides in the Brain

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INTRODUCTION

Late in the nineteenth century it was realized that the nervous system consisted of independent cells, each with its own nucleus, membrane, axon and dendrites to communicate with each other (Cajal, 1894). He proposed that the tiny knobs at the site where neurons make contact with other neurons were specialized sites of communication. Sherrington (1906) introduced the term synapse (meaning close together) for these structures. In the peripheral system also synapses were observed at the site where nerves make contact with muscle fibers. Elliot (1904) suggested that adrenalin might be released from sympathetic nerves at these contact sites and thus introduced the idea of chemical neurotransmission, a principle which was only much later established by Loewi (1921). Loewi, who literally did the experiment of his dreams (Loewi 1960), stimulated the vagus nerve of a frog heart attached to a cannula filled with Ringer. When the Ringer's solution of this heart was transferred into a second heart this resulted in slowing down of the heartbeat just as if the vagus nerve had been stimulated, demonstrating that indeed an active chemical substance had been released.

The proposition of Dale (1935) that this principle of chemical neurotransmission would also hold for the central nervous system was met with considerable opposition (Eccles and O'Conner, 1939). Finally, it was not until 1954 that Dale's major former opponent proved this postulate to be true for the spinal cord (Eccles et al., 1954). Consequently it was generally accepted that transfer of information from one neuron to another occurred via release at the site of the synapse of a chemical substance (the neurotransmitter) which is able to induce a change in the postsynaptic membrane potential.

An apparently totally different idea with respect to the transfer of central information was the concept of neurosecretion as proposed by the Scharrers (1937). They suggested that certain substances would be able to release their product into the bloodstream in order to reach distant target structures. Also, this new concept on the functioning of a part of the central nervous system was welcomed "not only with scepticism, but mostly with outright rejection" (Scharrer, 1974). According to the critics, the neurosecretory cells were "nothing more than signs of postmortem changes of degenerative processes". In spite of this strong opposition the Scharrers demonstrated these neurosecretory phenomena throughout the animal kingdom (Scharrer and Scharrer, 1940). In addition to this wealth of comparative data, a major breakthrough was achieved by Bargmann (1949), who applied Gomori's pancreas staining to brain sections and thus revealed a staining of the neurosecretory cells of the hypothalamus with their fiber tracts projecting into the neurohypophysis, and concluded that the stainable material

contained the anti-diuretic, oxytocic and vasopressor principles (Bargmann and Scharrer, 1951). Here Bargmann was probably the first to demonstrate more or less selectively, at the light microscopical level, a substance produced and transported by a neuron.

With the acceptance of these two theories on substances as messengers in nerve cell communication, it is not surprising that hereafter much effort has been spent on the search for the identities of the various substances that are synthesized in the myriads of cell bodies and terminals in the brain (e.g. Hökfelt et al., 1978). And likewise in the visualization of these substances at the light-, and later on, at the electron microscopical level. For example, Barry (1954), who elaborated on Bargmann's work, demonstrated a vast network of Gomori-positive fibers arising from the paraventricular nucleus, reaching many areas of the central nervous system (CNS), pathways which were later on indeed demonstrated to contain vasopressin and oxytocin (Buijs et al., 1978, 1980; Buijs, 1978, 1980; Sofroniew, 1980; Sofroniew and Weindl, 1978). Using sensitive fluorescence staining techniques developed initially by Falck et al. (1962) it was possible to demonstrate neurons with their processes containing amines in the brain (Fuxe, 1965). With the demonstration of an increasing number of substances that were found to be synthesized in and affect the CNS (e.g. Von Euler and Gaddum, 1931), it became necessary to devise criteria which had to be met before one could accept a substance to be called a neurotransmitter (Werman, 1966). Since not all these criteria have been fully met (yet?) for any substance in the brain, one may consider these criteria as a guideline as to how far a certain substance is still away from being a "proven" neurotransmitter. As a result of this inconvenience and the finding of new groups of putative neurotransmitters, a number of other criteria for neuromodulators, neuromediators, neurohumors have been suggested (Barchas et al., 1978; Bloom, 1979; Dismukes, 1979; Siggins, 1979). However, since up until now no true neuromodulator has been demonstrated, the value of all these new names is very questionable and possibly more confusing than helpful (Butcher and Woolf, 1982). As appears from these discussions (Bloom, 1979; Dismukes, 1979) one of the most important criteria is the ability of a neurotransmitter to transmit after release from a neuronal synapse, a presynaptic signal to a postsynaptic neuron by influencing the postsynaptic membrane potential, while a neuromodulator is supposed to influence interneuronal communication through mechanisms mostly different from synaptic transmission (Barchas et al., 1978; Elliott and Barchas, 1980). Consequently, already after Werman (1966) had drawn up his criteria, the ability to prove that a substance is present within a presynaptic terminal would be an important step towards the establishment of a substance as a neurotransmitter within the CNS. Therefore, and because of the need to know where and how these neuroactive substances are localized, ultrastructural localization of putative transmitters became an important goal in brain research.

ULTRASTRUCTURAL LOCALIZATION OF TRANSMITTER SUBSTANCES

In the beginning of the fifties it became possible to demonstrate synaptic terminals at the EM level (Palay, 1956), while the introduction of aldehyde fixation made this technique generally applicable (Sabattini et al., 1963). Hereafter different techniques were developed to demonstrate selectively transmitter substances at the EM level. Until the introduction of immunocytochemistry to demonstrate the transmitters and their synthesizing enzymes directly, most of the techniques employed to demonstrate electron microscopically the classical aminergic or amino acid transmitters depended on the more or less selective uptake mechanisms of these systems (Iversen, 1971; Snyder et al., 1970), or on the use of different fixation methods.

Visualization of amines by fixation

Noradrenaline (NA), for example, can be visualized by means of potassium permanganate fixation of the untreated rat after which small dense-cored vesicles can be seen in areas rich in NA (Richardson, 1966; Hökfelt, 1967). Dopamine (DA) and serotonin, however, can only be visualized as dense-core vesicles as prior to fixation, the brain was treated with these amines by intraventricular injections or via incubation of tissue slices (Ajika and Hökfelt, 1973). Due to the infrequencies by which NA containing dense-core vesicles could be demonstrated in several brain regions, Koda and Bloom (1977) developed a fixation technique with glyoxylic acid paraformaldehyde with subsequent incubation in potassium permanganate. Using this technique they found a close correlation between light microscopically observed fluorescent varicosities and boutons which contained the small dense-core vesicles in the rat dentate gyrus. Unfortunately potassium permanganate fixation is incompatible with the autoradiographic labeling techniques (Bloom, 1973), so that the specificity of this fixation technique for NA alone cannot easily be proven.

Re-uptake of false transmitters

The introduction of false transmitters such as 5-hydroxydopamine (5-OHDA) greatly facilitated the ultrastructural identification of monoamine-containing structures (Tranzer and Thoenen, 1967; Richards and Tranzer, 1970). These substances are so closely related to DA and NA that they are accumulated by the specific uptake processes, but once accumulated they are converted to (toxic) intermediates. Due to the fixation of 5-OHDA by glutaraldehyde and the reduction of OsO_4 , 5-OHDA accumulating in neurons and terminals is demonstrated by forming a dense core in the small 50 nm electron-lucent synaptic vesicles of the monoaminergic nerve terminals (Tennyson et al., 1974; Arluison et al., 1978a). However, especially if 5-OHDA is used in a relatively high concentration, besides DA and NA also serotonin containing structures are readily labeled, as was demonstrated by Richards et al. (1973). Apart from their use as markers, a false transmitter and especially 6-OHDA can be used as an agent degenerating the central catecholaminergic system. The uptake of 6-OHDA will then result in the subsequent degeneration of the accumulating structure (Tranzer and Thoenen, 1968). Thus this method more or less enables the specificity of several other procedures employed to demonstrate the catecholaminergic or serotonergic systems. After the application of 6-OHDA in low concentrations, the catecholaminergic staining must disappear or decrease, while the serotonergic system must remain unimpaired. In addition, the serotonergic false transmitter 5,7-dihydroxytryptamine is an agent which lesions selectively the serotonergic system (Baumgarten and Lachenmayer, 1972). These methods have especially proven their usefulness for the control of specificity after autoradiographic methods to demonstrate either the catecholamines or serotonin in the CNS (e.g. Koda and Bloom, 1977; Aghajanian and McCall, 1980).

Re-uptake of tritiated transmitters

The autoradiographic labeling technique also makes use of the described uptake and release mechanisms of the aminergic and amino acid transmitter system. The selectivity of these uptake mechanisms largely depends on which transmitter is used and in what concentration. Because the high affinity uptake mechanism is a saturable process, the specificity largely depends on what amount of the tracer is administered (Iversen, 1971; Snyder et al., 1970). At

high concentrations the label will also enter neurons and glial cells which do not contain endogenous amines. However, even then, the selectivity of the uptake mechanisms is non-existent. For example, Snyder et al. (1970) demonstrated that DA has a higher affinity of NA terminals than NA itself, while serotonin in concentrations exceeding 10^{-6} M (Snyder et al., 1970) or even 5×10^{-8} M (Ternaux et al., 1977) will also be taken up in DA neurons. Therefore pharmacological or other lesion techniques will be necessary to provide the evidence required for the specificity of the autoradiographic technique. The application of tritiated transmitters occurs by infusion via the cerebral ventricles or via superfusion. In addition, a local microinjection of tritiated transmitters is also used (Arluison et al., 1978b). The in- or superfusion methods are hampered by the lack of penetration (Beaudet and Sotelo, 1981; Descarries et al., 1975, 1977), while the latter method provides only a satisfying labeling at some distance from the injection site (Arluison et al., 1978b).

Autoradiography is thought to be hindered by a loss or a dislocation of the label during the fixation and dehydration process (McGeer et al., 1975). Therefore, usually the tissue is firmly fixed by perfusion with paraformaldehyde glutaraldehyde mixtures with subsequent postfixation using osmium (e.g. Descarries et al., 1980). Therefore, the result is an optimal preservation of the ultrastructure, also of the neurotransmitter-containing structures. Consequently, in spite of the above-mentioned drawbacks concerning specificity, the autoradiographic method is still an important tool in the study of the ultrastructure of the aminergic and amino acid-containing structures (e.g. Aghajanian and Bloom, 1966; Baraban and Aghajanian, 1981; Chan Palay, 1975, 1976; Descarries et al., 1980; Hattori et al., 1973). At the moment, however, this method can only be used to demonstrate the ultrastructural localization of neuroactive substances with high-affinity uptake mechanisms. To demonstrate neuroactive substances directly without the use of high-affinity uptake mechanisms, the method of immunocytochemical localization has been proved to be extremely useful.

Immunocytochemical localization

After the introduction of fluorescent labeled antibodies by Coons et al. (1941), it was not until the beginning of the seventies that such immunofluorescent procedures were used to localize "specifically" transmitter substances within the CNS. Initially, methods had to be developed by which high titre antisera could be raised against small molecular weight substances such as peptides (Skowsky and Fisher, 1973). In addition, the visualization of the tissue bound antisera was improved with the introduction of more sensitive techniques, such as the unlabeled antibody enzyme method using peroxidase antiperoxidase (PAP) complexes (Sternberger, 1974), which is one of the most generally used immunocytochemical methods in brain research nowadays. Although seemingly perfect, this immunocytochemical technique still evinces a number of disadvantages, the most important of which probably is the difficulty experienced in establishing its specificity (this problem is extensively reviewed by Pool et al. (1982)).

To demonstrate compounds immunocytochemically at the electron microscopic level one can choose in principle from 3 techniques: post-embedding staining (e.g. staining of fixed material in ultrathin (plastic) sections), pre-embedding staining (staining of 20–50 μ m thick sections of fixed brain, followed by post-fixation in OsO_4), and non-embedding staining (staining of 100 nm thin cryostat sections, which, however, have not been used up until now to demonstrate transmitter substances).

Post-embedding staining

Post-embedding staining has been used extensively for the localization of peptides, e.g. vasopressin and oxytocin in regions with high concentrations, in the cell body, the median eminence or neural lobe (Silverman et al., 1975; Van Leeuwen and Swaab, 1977; Van Leeuwen, 1978). However, when this technique was used to demonstrate vasopressin at the electron microscopic level in regions with low local concentrations, e.g. in the fibers of the lateral septum, this resulted in no staining or in a non-specific staining (Buijs and Swaab, 1979). For instance, using the normal post-embedding staining procedure as described by Van Leeuwen and Swaab (1977), which permitted vasopressin localization in the neural lobe, an attempt was made to localize vasopressin in ultrathin glutaraldehyde-paraformaldehyde-fixed brain sections with or without osmium postfixation. No reaction was obtained in the lateral septum. Longer incubation times with the first antiserum and during staining resulted in the staining of dense-core vesicles in nearly each synapse and fiber of the lateral septum (Buijs and Swaab, 1979, Fig. 1A,B). Since no staining was observed in controls with vasopressin antiserum preadsorbed with vasopressin-coupled agarose beads, or with normal rabbit serum instead of the first antiserum, it was suggested that the staining was the result of antibodies that were able to bind with vasopressin. When sections from Ho-DI rats, rats which genetically lack vasopressin, were treated with *purified* vasopressin antiserum, staining was again observed on all sizes of dense-core vesicles in the lateral septum (Fig. 1C), indicating that the staining procedure with the longer incubation times was non-specific. Although a satisfying explanation for this non-specificity is still lacking, it might be the result of an overall increase in background staining, (which is always observed in such sections), probably due to sticking of rabbit IgG to the tissue. The false positive reaction occurred not only in OsO₄-postfixed material, but also in glutaraldehyde-paraformaldehyde-fixed material. Moreover, from this result it can be concluded that the presence of unstained dense-core vesicles in the same section is an additional criterion for evaluating the specificity of post-embedding staining.

Pre-embedding staining

Pre-embedding staining has the advantage that it allows a much more intense and precise ultrastructural localization of the transmitter-containing structures together with the preservation of membranes. This disadvantage is, due to e.g. dislocation of the antigen by the incubation procedure and a vigorous staining procedure, a less accurate localization of the transmitter itself (see e.g. Pool et al., 1982). This might explain why in most studies the immunopositive reaction appears to be present around clear vesicles and with endoplasmatic reticulum.

As raising of antisera against e.g. DA and amino acids has failed so far, certainly the ultrastructural localization of these transmitters using antisera cannot be expected at short notice. Consequently, for most of the aminergic or amino acid transmitters only the localization of the synthesizing enzymes is possible and not the transmitter itself. One of the first substances was the enzyme in the glutamate synthesis, glutamate decarboxylase (GAD), which was visualized in axons and synaptic profiles in the rat cerebellum (McLaughlin et al., 1975). For the amines, only data on the immunocytochemical ultrastructural localization of tyrosine hydroxylase and dopamine- β -hydroxylase (DBH), two enzymes in the synthesizing pathway of adrenaline, are available (e.g. Pickel et al., 1976; Olschowska et al., 1981). At present, in spite of the fact that antisera against serotonin have been available for a long time (Steinbusch et al., 1978), no data on the ultrastructural localization of serotonin using these antisera with the pre-embedding staining technique have appeared in literature yet. As the post-embedding staining technique has serious disadvantage (see above and Fig. 1A-C), the

Fig. 1. A: electron micrograph demonstrating dense-core vesicles in a glutaraldehyde-paraformaldehyde- and OsO_4 -postfixed rat brain in the region of the lateral septum contrasted with uranyl acetate and lead citrate. B: the alternating section stained with anti-vasopressin serum using the post-embedding staining procedure demonstrating the positive reacting dense-core vesicles (arrows). m, mitochondria. C: the same positive reaction using a purified anti-vasopressin serum in the lateral septum of a rat genetically lacking vasopressin, demonstrating the false positivity of this reaction (arrows). Bars = 0.25 μm .

data on serotonin localization using post-embedding staining (Pelletier et al., 1981) will have to be interpreted with reservations.

Ultrastructural localization of the transmitter substances themselves was accomplished only by the use of antisera against various peptides at the electronmicroscopical level (Buijs and Swaab, 1979; Chan-Palay and Palay, 1977; Johansson et al., 1980; Pickel et al., 1977, 1979). In general, the reaction product appeared to be present on dense-core vesicles and around clear vesicle-like structures, irrespective of the peptide that has been demonstrated. By way of example, the ultrastructural localization of vasopressin and oxytocin will be discussed in an attempt to find evidence for a different action of these peptides in the CNS as compared to the neural lobe. Vasopressin and oxytocin are found in axons in extrahypothalamic areas in dense-core vesicles of approximately 100 nm, while within the synaptic profiles the immunopositive localization is also found around clear vesicle-like structures (Fig. 2). For vasopressin and oxytocin this immunopositive localization was surprising since from the post-embedding staining localization in the paraventricular and supraoptic nucleus and neural lobe it was known that these peptides were present within 150 nm dense-core granules (Van Leeuwen and

Fig. 2. Vasopressin-positive terminal forming a synapse (arrow) with an unlabeled dendrite (D) in the rat lateral septum, following the pre-embedding staining technique. m, mitochondria; Bar = 0.25 μ m.

Swaab, 1977). This observation of 150 nm granules could be confirmed for these areas by means of pre-embedding staining; thus the localization of these peptides in 100 nm granules outside the hypothalamus seemed not to be due to an experimental artefact. Most probably this means that the system that projects into the brain is totally different from the system that releases the peptides in the neural lobe into the bloodstream. The possibility that the release might also be different in a system innervating neuronal elements is emphasized additionally by the presence of the peptide within synaptic structures.

The immunopositive reaction around the clear vesicle-like structures might also be the result of the presence of these peptides in the cytoplasm, which would result, after fixation and staining, in a positive reaction on membranous structures. On the other hand it might plead in favour of the theory that the smooth endoplasmatic reticulum is a transport vehicle for non-granular, intra-axonal transport of neurosecretory material (Droz et al., 1975; Rambourg and Droz, 1980), and thus gives rise to clear vesicles (Alonso and Assenmacher, 1978).

Fig. 3. Enkephalin-positive terminal forming a synapse (arrow) with an unlabeled dendrite (D) in the rat lateral septum following the pre-embedding staining technique. Leu-enkephalin antiserum obtained from Dr. R.J. Miller, Univ. Chicago. m, mitochondrion; bar = 0.25 μ m.

After the demonstration of peptides as neuroactive substances within the CNS, it was generally assumed that these peptides would be modulators of neurotransmission. The influence of peptides on the turnover of catecholamines in terminal areas (Versteeg et al., 1979); the action of enkephalin on substance P release (Jessell and Iversen, 1977) or on dorsal horn neurons (Zieglgänsberger and Tulloch, 1979) etc.; all these experiments pointed to a mechanism of presynaptic modulation (Jessell and Iversen, 1977). However, surprisingly enough peptidergic synapses have only rarely been found on axonal structures (Hunt et al., 1980) and appear to terminate almost exclusively on dendrites (Figs. 2, 3) (Barber et al., 1979; Buijs and Swaab, 1979; Pickel et al., 1977, 1979; Hunt et al., 1980). Consequently the effects of peptides on the above-mentioned processes will have to be explained via other mechanisms than presynaptic modulation. Other mechanisms have been proposed via which peptides might influence hormonal transmitter release. So the inhibition of enkephalin on AVP and OXT release might be explained via the observed synaptoid contacts of enkephalin-containing axons with pituicytes (Van Leeuwen, 1982), while inhibition of dorsal horn neurons with this peptide is explained by the sensitivity of the dendrites to enkephalin and not via presynaptic inhibition of the excitatory transmitter substance P (Zieglgänsberger, 1982).

NON-SYNAPTIC RELEASE

Apart from specialized synaptic contacts that serve as the channel via which principle the information from one neuron to another is transferred by means of the release of a neurotransmitter, the ultrastructural localization of transmitter substances points to a mechanism of information transfer via non-synaptic release and subsequent diffusion over large distances. Non-synaptic release has already been demonstrated extensively for the release of various hypothalamic hormones, e.g. vasopressin in the median eminence or in the neural lobe, which subsequently acts on the adenohypophysis or the kidney. A comparable non-synaptic release mechanism was also proposed for the putative neurotransmitters DA and serotonin (e.g. Björklund et al., 1973). In addition, a rich serotonin-containing fiber plexus was demonstrated on the ependymal surface of the cerebral ventricles (Richards et al., 1973; Chan-Palay, 1976), from which a non-synaptic release is likely, since a higher uptake of tritiated serotonin could be demonstrated after raphe stimulation (Chan-Palay, 1976), while synapses in this plexus are absent. Already in 1975, independently of these observations, it was proposed on a morphological basis that neurotransmitters might be released from their axons non-synaptically and reach, via extracellular fluid movement, and not via the CSF or bloodstream, their target over a greater distance. The basis for this proposal was laid in the studies of Descarries et al. (1975, 1977) and (Beaudet and Descarries, 1978), who superfused cortical areas in the rat brain with tritiated serotonin or NA, which resulted in labeling of axonal processes and varicosities. Subsequent investigation of serial sections enabled the examination of somewhat less than half of the varicosities in 2 or sometimes 3 sections. Thus 5% of the total number of labeled varicosities was seen to make a typical synaptic contact, whereas, by contrast, 50% of the surrounding non-labeled varicosities exhibited a synaptic contact at random sampling. This led to the suggestion that such non-synaptic release would especially be true for aminergic transmitters (Descarries et al., 1977; Beaudet and Descarries, 1978). A similar figure of varicosities without synaptic contact was proposed by Tennyson et al. (1974), who found a frequency of 2% dopamine-containing boutons in the neostriatum with a synaptic specialization.

However, the inability to find evidence for a synaptic contact is no proof that such contacts

are indeed absent. For example, the results of Arluison et al., (1978a), who found a much higher incidence of synaptic contacts after 5-OHDA labeling in vivo than Tennyson et al. (1974) found in vitro, suggest that the procedure and fixation used may largely determine the outcome of such an observation. The same might be true for the work of Cuello and Iversen (1978), who could not find any evidence for DA-containing dendrites to exhibit synaptic contacts in the substantia nigra, while Wilson et al., (1977) demonstrated the existence of such synapses after a higher dose of 5-OHDA. This argument might also hold for the studies of Descarries et al. (1975, 1977), since Koda and Bloom (1977) demonstrated that, if they were counted randomly, 20% of NA-labeled varicosities in the dentate gyrus exhibited a synaptic specialization. This point was recently supported by an immunoelectronmicroscopical study of Olschowska et al. (1981), who demonstrate DBH immunoreactivity in 10 different brain areas, among which, the dentate gyrus and cortex regions. In this experiment more than 50% of the randomly sampled immunopositive varicosities evinced a synaptic specialization, suggesting that the actual incidence of synapses can be much higher. That such incidence can indeed be 100% is suggested by studies of Groves (1980) and Groves and Wilson (1980), who, in serial sections, found that virtually all (5-OHDA) identified and non-identified varicosities exhibited a synaptic specialization. In addition, the fact that varicosities are labeled does not mean that release from the same varicosities can occur. It is very well possible that the label is taken up at a site distant from a varicosity and subsequently transported retrogradely to a site where it is not released. The possibility of specific uptake of tritiated amines and amino acids with subsequent retrograde transport has been demonstrated by Streit (1980) and Streit et al. (1979).

Vasopressin-containing peptidergic fibers, which are varicose already from the beginning of the fiber near the cell body, although not studied extensively, do not seem to exhibit synaptic specializations at these boutons, but no release from these sites could be demonstrated either, while release after K^+ depolarization could be evoked from regions with synaptic specializations (Buijs, unpublished results). If this also holds for amines, this would be another argument that even if these aminergic fibers do not have synapses at all varicosities, they do not necessarily have release at all these varicosities. In conclusion the evidence for non-synaptic release is too weak at the moment to accept that with such a highly specific and well developed innervation pattern of various transmitter systems in many regions of the CNS, the neuron would use of such a non-specific and random way to communicate with other neurons.

PEPTIDES AS NEUROTRANSMITTERS

If substances have an action on the brain and are found within the brain, an ultrastructural localization study might demonstrate that such a chemical can be found within synaptic terminals. This raises the question whether that compound can indeed transfer a message from one neuron to another via such a synapse. If so, it might simply be considered as a neurotransmitter. But in order to establish (see above) how far such a substance is from being a "true" neurotransmitter, we will follow the criteria of Wermann (1966), as formulated by Barchas et al. (1978). As an example from the new class of putative neurotransmitters — the peptides — it will be determined whether vasopressin and oxytocin, already known for a long time to be neurohormones (Bargman, 1949; Sawyer, 1964), are able to function as neurotransmitters in the CNS.

- (1) The substance must be present in presynaptic elements of neuronal tissue, possibly in an uneven distribution throughout the brain.

The widespread distribution of vasopressin and oxytocin in numerous brain regions (Buijs,

1978; Buijs et al., 1978, 1980; Sofroniew, 1980; Sofroniew and Weindl, 1978; Swaab, 1982; Swanson, 1977) and their localization in synaptic structures (Buijs and Swaab, 1979, Fig. 2) fully meets these important criteria.

- (2) Precursors and synthetic enzymes must be present in the neuron, usually in close proximity to the site of presumed action.

Synthesis of these peptides in the paraventricular and supraoptic nucleus has been demonstrated extensively (Pickering and Jones, 1971; Sachs, 1966; Sloper and King, 1963). No synthesis of peptides in their terminals has been demonstrated up until now (Sachs, 1966); however, extensive transport of these peptides to their site of release, the neural lobe (Pickering and Jones, 1971; Gainer et al., 1977) or the spinal cord (Gainer, personal communication), has been demonstrated.

- (3) Stimulation of afferents should cause release of the substance in physiologically significant amounts.

In vitro studies with slices from regions with many synaptic profiles like, e.g. the rat lateral septum for vasopressin or the solitary tract for oxytocin, demonstrated, respectively, a calcium-dependent K^+ - and veratridine-stimulated vasopressin-oxytocin release. Moreover, such a release could not be demonstrated in regions with vasopressinergic and oxytocinergic fibers lacking synaptic specializations as, for example, in the paraventriculo-supraoptic tract (Buijs, unpublished observations). In addition, in vivo release of vasopressin in the sheep septal region was demonstrated to be negatively correlated with changes in body temperature (Cooper et al., 1979). However, up until now, no experiments have been performed, to our knowledge, to stimulate selectively the peptide-producing cell bodies or their pathways in order to induce release at the terminal regions in the CNS, as has been published for example for NA after locus coeruleus stimulation (Tanaka et al., 1976).

- (4) There should be specific receptors present which interact with the substance; these should be in close proximity to synaptic structures.

Many data are available on the vasopressin and oxytocin sensitivity of a number of brain regions (e.g. Kovacs et al., 1979; Cooper et al., 1979). While the receptor localization for other peptides with many binding sites in terminal fields (see e.g. Palacios and Wamsley, 1982) has been described, the localization of the receptors for vasopressin or oxytocin has not been accomplished yet. Since vasopressin recently became available in a tritiated form, receptor localization might be expected at short notice.

- (5) Interaction of the substance with its receptor should induce changes in postsynaptic membrane permeability leading to excitatory or inhibitory postsynaptic potentials.

Recently Morris et al. (1980) demonstrated that iontophoretically-applied oxytocin inhibited spontaneous and glutamate-induced firing of brainstem neurons in a region that is densely innervated by oxytocin-containing fibers. In addition, Mühlethaler et al. (1981) demonstrated excitation of hippocampal neurons by vasopressin in vitro that could be prevented by the use of a vasopressin antagonist. No electrophysiological effects of peptides after stimulation of their afferents are described, to our knowledge, as it is, for example, for NA (Phillis and Kostopoulos, 1977). However, no direct evidence for any other transmitter has been provided, and yet it has been shown that the neuron one is measuring from electrophysiologically, is indeed directly innervated by these transmitter-containing fibers.

- (6) Specific inactivating mechanisms should exist which stop interactions of the substance with its receptor in a physiologically reasonable time-frame.

Fast re-uptake systems to inactivate peptides have not been described. However, it is not necessarily via such mechanisms that peptides might be inactivated. It has been demonstrated, for example, that synaptic plasma membrane fractions of various regions of the CNS have

aminopeptidase activities capable of degrading oxytocin (Burbach et al., 1980). Apart from such biotransformation, an additional possibility is the removal of peptides via the movement of extracellular fluid into the CSF (cf. Cserr and Ostrach, 1974; Wald et al., 1978).

From these data it will be clear that although the research on the central action of peptides has only recently gained momentum, already many, though not all criteria for establishing these peptides as neurotransmitters can be met fully satisfactorily. In addition, one has to bear in mind that these criteria have been set up with a particular transmitter (acetylcholine) with its action on the neuromuscular junction, in mind. However, even this transmitter cannot meet all the afore-mentioned criteria fully satisfactorily in the CNS. Therefore, instead of inventing new names, one should carefully evaluate the different properties of the several neurotransmitters, of which its ultrastructural localization is just one.

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DISCUSSION

D.C. DAVIES: In view of the fact that 5-OHDA is taken up into NA, DA and 5-HT neurons, just how specific are the high- and low-affinity uptake mechanisms which are important criteria for characterizing a neurotransmitter?

V. CHAN-PALAY: In the case of 5-HT neurons degradation and radiolabeling studies have shown uptake to be extremely specific.

R.M. BUIJS: However, as I already said, uptake of DA is higher in NA terminals than NA itself and 5-HT concentrations of 10^{-6} M will also be taken up in DA terminals according to Snyder et al. (1970).

F. HENN: Effectively, the low affinity system is a safety valve, not functional unless the system is saturated; my guess is that it does not work under in vivo conditions.

A. GROSSMAN: There is considerable evidence that enkephalins act presynaptically to modulate the release of neurotransmitters. Are there any good morphological data to substantiate this, especially for the mediobasal hypothalamus where opiates were implicated in important neuroendocrine control mechanisms?

R.M. BUIJS: I am not aware ultrastructural studies on the localization of enkephalins in the mediobasal hypothalamus. But I want to draw your attention to a paper by Van Leeuwen who demonstrated synaptic-like contacts of enkephalin axons with pituicytes in the neural lobe, suggesting that this might be a regulating mechanism of enkephalins on neuroendocrine processes.

Although in general it is suggested that peptides terminate postsynaptically to modulate transmitter release, it is very rarely that such axo-axonal synaptic contacts have been described for peptides. Generally, peptides are found to terminate on neurons and dendrites, see e.g. Hunt et al. (1980); Buijs and Swaab (1979).

W. ZIEGLGÄNSBERGER: You showed axo-dendritic contacts between enkephalin-containing neurons and dendrites of dorsal horn neurons. This should be taken as evidence for a primarily postsynaptic action for endorphins in this region.

R.M. BUIJS: The slide I showed was from Hunt et al. (1980), demonstrating indeed the larger portion of enkephalin-containing terminals on dendrites.

V. CHAN-PALAY: Challenge to data of Descarries for 95% non-synaptic contact — our studies in cerebellum, brainstem raphe, vestibular nuclei, show at least 60% synaptic terminals.

R.G. HILL: Evidence for axo-axonic synapses in substantia gelatinosa that are not associated with GAD, and hence GABA, is poor in spite of intensive investigation (Barber et al., 1978; Gobel et al., 1980). It is perhaps not surprising, therefore, that Hunt et al. (1980) could not find evidence for functional axo-axonic synapses between met-enkephalin containing neurons and afferent fibers. The pre-synaptic opiate receptors on afferent fiber terminals need to be re-evaluated perhaps as a site of action for exogenous opiates but of limited physiological significance.

L.L. BUTCHER: Because of the potential theoretic importance of "non-synaptic" release, could you comment on the viability of this hypothesis on the basis of the fact that, according to the best data, still only 50% of cortical varicosities make synaptic contact as traditionally evaluated. What about the remaining 50%?

R.M. BUIJS: According to the best data for non-synaptic release, that percentage is 50%. As I pointed out, other data argue for a much higher percentage of synapses. But even if a large percentage of these varicosities remain without synaptic specializations, that does not mean that indeed release occurs from such varicosities. The presence of label in these varicosities can easily be explained by retrograde transport.

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