NEUROPEPTIDES AND BRAIN DEVELOPMENT - A WORKING HYPOTHESIS

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INTRODUCTION

Now that neuroendocrinology has become an established clinical discipline\(^1\), it is hard to imagine that 40 years ago Ernst and Berta Scharrer\(^2\) were at the beginning of their struggle to get the concept of neurosecretion accepted and that recognition of this idea followed only in 1951\(^3\).

The classical Gomori-positive neurosecretory cells \(i.e\). in the hypothalamic supraoptic (SON) and paraventricular nucleus (PVN) that the Scharrers\(^2\) described in mammals were clearly distinguishable from "common" neurons by their size. Recent findings, however, have made any fundamental distinction between endocrine and non-endocrine neurons impossible. Neuroendocrine cells not only exhibit action potentials which are essential for hormone release\(^4\) but they also terminate on other neurons in a synaptoid way (see below). Furthermore, some transmitter producing neurons release their product \(i.e\). dopamine into the capillaries of the portal vessels\(^5,6\). The idea that neuroendocrine cells might terminate on other neurons and modulate thus brain activity has been given important impetus by the development of immunocytochemical techniques. Barry\(^7\), who was the first to describe Gomori-positive extrahypothalamic fibres later pioneered\(^8\) the localization of LHRH fibres throughout the mammalian brain by means of immunofluorescence microscopy. Since then such a vast number of peptide hormone containing nerve fibre systems has been described in the nervous system by means of immunocytochemistry\(^9,10\), that the current list of putative peptide neurotransmitters far outnumbers that of the amines and amino acids which have been proposed as transmitters\(^11\).

The present paper aims to describe some general properties of peptide hormone containing neurons in the adult brain, and to catalogue their presence during early ontogeny in the rat and in man. The hypothesis is put forward that neuropeptides play a role in
brain development, as other factors that are essential for adult brain function, *e.g.* sex- and thyroid hormones and amine- and amino acid transmitters, seem to do.

**NEUROPEPTIDES IN THE ADULT NERVOUS SYSTEM**

The majority of the peptides that are currently known to affect nervous system functions were originally categorized as hormones. However, as these compounds are probably transported to their central sites of action via nerve fibres rather than via the blood stream they do not fall within Starlings definition of hormones. Therefore the more general term "neuropeptides", that was introduced by De Wied *et al.* 12, is used in the present paper.

The centrally active neuropeptides can arbitrarily be divided into three groups on the basis of historical arguments and their peripheral target organs.

1) **Hypothalamic hormones.** In addition to their involvement in kidney function, labour and milk ejection, arginine-vasopressin (AVP) and oxytocin (OXT) have been found to affect memory and transmitter metabolism 13 while lysine-vasopressin influences, in a long term manner membrane properties of neurons14. Also the hypothalamic luteinizing hormone releasing hormone (LHRH), thyrotropin releasing hormone (TRH) and somatostatin (SOM) have been reported to influence nervous system electrical activity 15,16 and to reveal a number of central actions. LHRH was found to facilitate lordosis behavior in the rat17 and to produce the symptoms of premenstrual tension in women18 while TRH was found to increase motor activity and to produce a multiplicity of other central nervous system actions19, and SOM amongst others reduced rapid eye movement sleep20. Although the presence of hypothalamic hormone containing fibres throughout the brain (see below) certainly supports the idea that a number of these central effects can also be of physiological significance, experimental evidence for such central functions is scanty. Only for AVP such an indication is present since its improving effect on passive avoidance, memory and learning can be reversed by injection of anti-vasopressin serum into the lateral ventricle21. In addition, a complete absence of retention of a passive avoidance response was found in the Brattleboro rat which is genetically lacking AVP22. Since, however, homozygous animals showed
only a partial performance deficit in a similar study of Bailey and Weiss23 and no deficit in the study of Celestian et al.24, this point is not yet settled.

II) Pituitary hormones. The central effects of the peptides of the ACTH/LPH family (ACTH, α-MSH, β-LPH, γ-LPH, endorphins and enkephalins) have been studied extensively during the last years10,25. The fact that these peptides remain present in the brain in significant amounts after hypophysectomy26,27,28,29,30,31,32 suggests that these "pituitary hormones" are in addition produced in the brain. This view was recently challenged by Moldow and Yalow33, who favoured the pituitary as the only source since they found pituitary remnants after commercial hypophysectomy and a concentration of ACTH in the brain in direct relation to the distance from the pituitary. Hormones may, in addition, still be produced by the pars tuberalis that might remain functional after hypophysectomy34 while the presence of pituitary tissue in the craniopharyngeal canal35 might be an additional source of hormones. The possibility of retrograde peptide transport via the portal vessels has indeed been shown36. This route cannot, however, explain (1) the high amount of α-MSH-like material in the medulla oblongata, spinal cord and in dorsal root ganglia37, structures that do not have such a direct vascular connection with the pituitary, (2) the presence of enkephalins in cultured spinal cord neurons38 and (3) the occurrence of similar neuropeptides in neurons of lower animals that do not have a pituitary at all: e.g. α-MSH- and ACTH-like material in the pond snail (Lymnaea stagnalis) (P. Schot, unpublished results). Also the localization of α-MSH on the endoplasmatic reticulum of the dorsal root ganglion cell (see below) argues for pituitary hormone production by neurons. This group of pituitary hormones in the nervous system may enlarge in the coming years since prolactin-like material has also been found in nerve terminals in the hypothalamus39.

III) Neuropeptides s.s. The peptides of this group are not primarily known as hypothalamic or pituitary hormones although they have been reported to display endocrine effects. The oldest representative, substance P was found already in 1931 by Von Euler40. It is most probably a sensory transmitter substance in primary afferent synapses in the spinal cord and cranial nerve nuclei in the
brain stem. Angiotensin II is involved in the central regulation of drinking behavior and the stimulation of AVP-release, while bombesin lowers body temperature and induces hypoglycaemia and analgesia. Neurotensin acts centrally by lowering body temperature, vasoactive intestinal peptide (VIP)-containing nerve fibres were found in the brain and in the periphery where it might act as a neurotransmitter, while carnosine is considered to be a putative neurotransmitter in the olfactory pathway. Training induced peptides are scotophobin (the dark avoidance peptide) and ameletin (the noise habituation peptide), while in addition, sleep promoting peptides have been isolated.

Quite a number of the neuropeptides have been found to be common to the nervous system and the gastroenteropancreatic system. In addition, there are several nervous system structures in which a number of different neuropeptides have been demonstrated by means of immunocytochemistry. For example in the dorsal root ganglion neurons somatostatin, substance P and α-MSH-like peptides have all been identified. In addition, the pineal gland appears to contain α-MSH-, AVT-, somatostatin-, angiotensin-,LHRH- and TRH-like material, while Tramu et al. reported that LHRH neurons also contain ACTH-like material. This raises the question whether it are really the same peptides that are present in different systems or only closely related but not identical compounds. Most of the studies are only based on immunocytochemical techniques in which cross reaction with known or unknown related peptides is certainly not excluded.

MODE OF CENTRAL ACTION OF NEOPEPTIDES

The hypothalamic hormones are released by means of the "classical" neuroendocrine cell terminations into the capillaries of the neurohaemal organs, i.e. the neurohypophysis, the median eminence and the organum vasculosum laminae terminalis (OVLT). These endings seem, however, of minor importance for the central actions of neuropeptides, which are most probably effected by synaptoid contacts of the exohypothalamic peptidergic fibres with other neurons. Many of such fibres were found to end with punctate structures around other neurons: e.g. a) vasopressin containing fibres from the
suprachiasmatic nucleus terminating in the lateral septum and the lateral habenular nucleus and b) vasopressin and oxytocin fibres from the paraventricular nucleus terminating in the amygdala\textsuperscript{57,58}. A first attempt has been made by Buijs (in prep.) to visualize these terminations by means of immunoelectron microscopy. Pre-embedding staining revealed very intense staining of these exohypothalamic fibres, but the ultrastructure of the granules was largely lost. The images obtained thus far confirm, however, the idea that the neurohypophysial hormone containing fibres can terminate in a synaptoid way on cell bodies. Other examples of perineuronal neuropeptide containing endings are those of (c) the TRH containing fibres around motoneurons in the spinal cord\textsuperscript{59}, (d) LHRH fibres in the suprachiasmatic nucleus\textsuperscript{60}, (e) somatostatin fibres in the ventromedial nucleus\textsuperscript{61}.

Perineuronal endings were also described for pituitary hormones and neuropeptides \textit{s.s.} containing fibres; (f) \(\alpha\)-MSH-like material containing basket cell fibres were found around the cerebellar Purkinje cells\textsuperscript{37}. Even (g) in spinal cord tissue culture enkephalin containing perineuronal structures have been shown to occur\textsuperscript{38}, while (h) such terminals containing substance P and [Met\textsuperscript{5}]-enkephalin have also been found in the locus coeruleus and the A2 region\textsuperscript{62}. Whether such perineuronal neuropeptide containing endings can also be considered as being predominantly inhibitory as generally proposed for the classical transmitters\textsuperscript{63} has not yet been investigated.

In addition to the perineuronal endings neuropeptide containing terminations are also found on dendrites. In fact, the immunoelectron microscopical work of Buijs on vasopressin and oxytocin containing terminals indicates that such endings are much more common than terminations on cell bodies.

A number of neuropeptides were found to be present in synaptoid structures on granules. [Met\textsuperscript{5}]-enkephalin was localized on 70-95 nm granules in the A2 region\textsuperscript{62}, an intense substance P reaction was observed on 90-120 nm granules in axons in the substantia gelatinosa of the spinal cord\textsuperscript{63a}, and VIP was found in nerve endings in the colon on 120 nm granules\textsuperscript{45}. Immunoelectron microscopical localization of neuropeptides seems, however, not to be confined to granules. In a study of nerve endings in the limbic system (Buijs,
in prep.) only occasionally found AVP staining on granules: staining was generalized throughout the terminals. A similar pattern of staining has been reported for substance P in the spinal cord axons and the locus coeruleus and A2 regions, while \( \alpha \)-MSH-like material has been found on the endoplasmatic reticulum, microtubules and microfilaments in the dorsal root ganglion cells and on microfilaments in the cerebellar basket cell terminations (Van Leeuwen and Buijs, unpublished observ.). Whether such organellar localization is characteristic for neuropeptide transport and indicative of particular intracellular (metabolic?) functions or whether it is due only to the problematic fixation properties of neuropeptides remains to be elucidated.

Neuropeptides have been found to co-exist in endings containing other (putative) neurotransmitters. 5-HT and substance P have both been identified in the raphe nuclei, nucleus reticularis gigantocellularis and nucleus interfascicularis hypoglossi. We observed \( \alpha \)-MSH-like material in the cerebellar basket cell endings that would also contain GABA, while substance P and [Met\(^5\)]-enkephalin have been found in the catecholaminergic neurons of locus coeruleus and A2. Although quite a number of other examples of neurons containing more than one putative neurotransmitter can be given, one can only speculate upon the functional implications of this phenomenon.

THE DUAL FUNCTION

Since hypothalamic hormone producing neurons seem to effect hormone release not only in the hypothalamic neurohaemal organs but also via synaptoid contacts in extrahypothalamic area, the question arises whether the same cell is able to execute both functions. This possibility is reinforced by the bi- or multipolar shape of hypothalamic hormone producing cells, and by the finding of Sherlock et al. who reported that almost all cells of the supraoptic and paraventricular nuclei could be retrogradely stained by HRP from the neurohypophysis. However, electrophysiological studies have so far failed to confirm this possibility (and K. Boer, unpublished observ.). Recently, however, arguments became available which support the suggestion that different cells project towards the neurohypophysis and the extrahypothalamic area. HRP injections placed into the neurohypophysis or the medulla were thought to
label largely different populations of cells$^{69,70,71}$. This controversy might be solved by means of a combination of retrograde transport studies from different regions and immunocytochemical identification of the cells. In the second place, electrophysiology seems a highly appropriate technique which might in addition give information on the nature of exohypothalamic fibres (dendrite vs. axon). However, even this knowledge would not prove the presence or absence of hormone release in exohypothalamic area, since dendrites can at the present also considered to be presynaptic elements that are able to release a wide range of active compounds$^{72}$.

THE PRESENCE OF NEUROPEPTIDES IN DEVELOPMENT

Neuropeptides are present in the brain already early in development, both in the rat and in man. In the fetal rat supraoptic nucleus neurophysins (which are residues of the precursor molecules of vasopressin and oxytocin) can be visualized from the 16th day of gestation, in the median eminence and neurohypophysis from the 17th day and in the paraventricular nucleus from the 19th day of gestation. In the perikarya of the suprachiasmatic nucleus immunoreactive material is demonstrated only from the 7th day postpartum$^{73,74}$. Unpublished radioimmunoassay data from our institute show that between fetal day 17 and 1 day postpartum the brain AVP concentration fluctuates between 4 and 18 ng/g while the much lower oxytocin concentration diminishes from 1.5 to 0.04 ng/g.

LHRH-immunofluorescence positive material was found in the rat OVLT and the external layer of the median eminence of fetuses on day 20.5 of gestation$^{75}$ although the fetal hypothalamic values remained below the detection level of a radioimmunoassay, increasing suddenly on the first day after birth$^{76}$. TRH-like material was found, by means of an in vitro procedure, on the 18-19th day of gestation in fetal rat hypothalami$^{77}$ and radioimmunoassayable somatostatin was present in the day 20 fetal brain of this species$^{77a}$. From day 17 of pregnancy to day 1 postpartum radioimmunoassayable α-MSH concentrations in the brain fluctuate between 1.6 and 0.7 ng/g (own, unpublished results).

In the human fetal hypothalamus the first oxytocin-neurophysin neurons were seen in the 14th week of gestation$^{78}$. Fetal hypothalamic
and cortical tissue at 6 weeks after conception contained radioimmunoassayable LHRH. In some cases at 13-14 weeks even more LHRH activity was found in the thalamus than in the rest of the central nervous system, while after 16 weeks of gestation LHRH was detected in the hypothalamus, thalamus, cerebrum and cerebellum in decreasing concentrations. Sex differences in the hypothalamic LHRH content were found throughout human intrauterine development. Using immunofluorescence, the first LHRH containing hypothalamic neurons were seen as early as the 9th week of gestation.

Somatostatin containing neurons appear in the 14th-16th week of human fetal life. Cross reaction of anti-somatostatin with neurophysins (Van Leeuwen et al., in prep.) might explain the finding of the latter authors that staining with anti-somatostatin was also present in the fetal magnocellular hypothalamic elements.

ß-Endorphin containing neuroblastic cells with short processes were found in the human fetal infundibular region from the 11th week of development.

Fetal endocrine systems might produce hormones that are different from those operating in adult organisms. For example, reports based mainly on bioassays, and radioimmunoassays using antibodies that were not specifically directed to AVT suggest that the fetal pituitary releases the ancestral neurohypophysial hormone vasotocin instead of AVP and OXT. Perks speculated that fetal vasotocin might be involved in the regulation of the amniotic fluid balance. Vasotocin is supposed to be produced, in addition, by some adult brain structures, i.e. in the pineal gland and the subcommissural organ. Using a sensitive and specific radioimmunoassay for vasotocin Dogterom et al. failed, however, to demonstrate vasotocin in sheep and seal fetal pituitaries, in the adult rat, bovine, ovine and mouse pineal gland and in the rat, mouse and rabbit subcommissural organ, while vasotocin could be specifically demonstrated in pituitaries of frogs, chickens and ducklings. Vasopressin and oxytocin were found instead of vasotocin in the various mammalian tissues. Since bioassay indicated the presence of vasotocin in these mammalian structures, they may contain peptides which closely resemble but are not identical to vasotocin. The presence of still unknown neuropeptides in the fetal brain certainly seems possible. Schaller et al. reported e.g. that the phylo-
genetically earliest neurohormone so far found, a growth hormone in Hydra, is also present in the brain of rat embryos. This peptide is different from the other known hypothalamic peptides.

NEUROPEPTIDES AND DEVELOPMENT

Because (1) neuropeptides form an important class of putative neurotransmitters, (2) they are present in the fetal brain from early in development and (3) other transmitters have been found to influence brain development, a role for neuropeptides in brain development can be expected. Research in this field has only relatively recently been started.

In the case of hypothalamic hormones, a first study was performed by Boer et al. (and unpublished results) on the possible involvement of AVP in brain development. Brattleboro rats that are homozygous for diabetes insipidus as a result of a genetic defect in vasopressin synthesis were found to have a retarded body growth, both pre- and postnatally, when compared to heterozygous animals. In addition, postnatal brain development on day 12, 16 and 24, appeared to be retarded as judged from data on dry and wet weight and the DNA, protein and lipid content. These changes persisted in adulthood (180 days). In particular, the cell content of the cerebellum and the medulla oblongata appeared to be seriously affected. Whether this is a direct effect of vasopressin on brain development is currently under investigation. Stunting of physical development has also been reported in human diabetes insipidus, although this phenomenon was thought to be secondary to inadequate caloric intake. Whether developmental disturbances are also found in human hereditary hypothalamic diabetes insipidus is currently under investigation in our institute.

Brain development is also influenced by neuropeptides of the ACTH/LPH family. A subcutaneous injection of ACTH 1-39, 1-24, or 1-16 to neonatal female rats accelerated the time of eye opening, while corticosteroids, ACTH 1-10, 4-10, 11-24, 6-24, 7-16 or α-MSH had no effect. In addition, neonatal injection of ACTH 1-39, 1-24, 1-10 or α-MSH was found to accelerate motor behavior. The effects are most probably mediated not by the adrenal but rather by direct effects on the central nervous system. In agreement with this idea is the finding of Nyakas that ACTH treatment on the 3rd - 5th postnatal day increased passive avoidance on the 22nd -
- 24th day significantly without a concomitant change in the adrenocortical response level. Neonatal injections of α-MSH also influenced the organization of later social behavior as shown by the increased open field body contact of the treated males in adulthood, and improved the performance on learning, memory and attention tasks. In contrast to the eye opening response, this treatment had little or no effect on females. Injection of an ACTH 4-9 analogue into the lateral ventricles of 1-day old rats has different effects on the subsequent behavior of male and female animals. α-MSH and related compounds seem thus to be capable of affecting nervous system organization in a sex dependent way.

Since the organizing effect of α-MSH on the brain, and since endogenous fetal α-MSH has been found to stimulate intrauterine growth, the potential involvement of this substance in fetal brain development was tested in a preliminary study. Anti-α-MSH injected subcutaneously into the rat fetus on day 19 of pregnancy, appeared to inhibit not only body growth but also brain development, as indicated by a decreased brain weight, protein content and lipid content, although total brain DNA was not found to change. This points to an effect of α-MSH on fetal brain cell differentiation rather than on cell multiplication.

No direct information is available concerning the possible influence of neuropeptides on human brain development. However, Precht found a relationship between age-inadequate EEG patterns in newborns and low oestrogen excretion by the mother. The oestrogen excretion is determined by the fetal hypothalamo-pituitary-adrenal system. Since the fetal corticotropic hormones, α-MSH and ACTH, have been shown to influence also brain development in rats (see before), a lack of these hormones might be the common factor in the disturbances of oestrogen secretion and brain development. In the same way might the high IQ's in adrenogenital syndrome theoretically be related to an increased production of the child's adrenotropic hormones.

In conclusion, the relatively few data obtained so far suggest that, in common with the classical neurotransmitters, sex hormones and thyroid hormones, neuropeptides may obey the rule that factors which are of importance for adult brain function are also involved in brain development.
SUMMARY

A rapidly increasing number of centrally active peptides are found in the brain. These neuropeptides can arbitrarily be divided into hypothalamic hormones (e.g. AVP, OXT, LH-RH, SOM), pituitary hormones (e.g. ACTH, α-MSH) and neuropeptides s.s. (e.g. substance P, neurotensin, VIP). They are supposed to execute their central actions via synaptoid contacts with other neurons. The neuropeptides are present early in fetal development, both in man and in the rat. Observations in vasopressin-deficient rats, injections in early development with ACTH, α-MSH and analogues and with antibodies against α-MSH suggest that neuropeptides play an important role in brain development.

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