Ontogeny of melanocortin receptor mRNA expression in rat nervous system and developmental effects of melanocortins in vitro

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ONTOGENY OF MELANOCORTIN RECEPTOR mRNA
EXPRESSION IN RAT NERVOUS SYSTEM
AND DEVELOPMENTAL EFFECTS OF
MELANOCORTINS IN VITRO

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Natural Sciences

presented by

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1998
SUMMARY

Pro-opiomelanocortin (POMC) is the source of two groups of peptides with a wide variety of functions, e.g., the opioid β-endorphin and the melanocortins. The melanocortin family consists of adrenocorticotropic hormone (ACTH) and the melanotropins, of which α-melanocyte stimulating hormone (α-MSH) is best known. Melanocortins are processed from different regions of the POMC-precursor protein, but share a conserved core of four amino acids (His-Phe-Arg-Trp).

The primary function of ACTH is the control of adrenal steroidogenesis while melanotropins were first thought to be implicated mainly in the regulation of pigmentation of skin and fur. In the mid 60ies it became clear, that melanocortins had a wide range of additional effects. They were shown to influence behaviour (grooming/stretching, modulation of sexual and social behavior, learning and memory), and to be strong antipyretics. Just recently they were found to be involved in centrally mediated control of body weight. A number of publications indicated that melanocortins exerted beneficial effects on regeneration of central and peripheral neurons and last but not least, influenced brain development.

During ontogeny POMC, the precursor of melanocortins, becomes detectable very early in rat hypothalamic neurons (gestational day E12.5). The same holds true for binding sites for melanocortins in rat CNS (gestational day 13). They show a distinct stage-and region-specific ontogenetic pattern. Transient peaks of binding site density are often concomitant with phases of neural network formation. These data suggest a role for melanocortins in ontogeny.

Five melanocortin receptors are presently known: the classical α-MSH-receptor (MC1-R) which is typically expressed by melanocytes, the ACTH-receptor (MC2-R) demonstrated in adrenal cortex and on adipocytes, the MC3-R and the MC4-R, both found predominantly in the brain, and finally the MC5-R which is distributed widely in the body and also in low amounts in the brain.
The first part of this thesis was devoted to the question which of the five melanocortin receptors might be present during early ontogeny. The ontogeny of the MC3-R and the MC4-R mRNA, the two main receptors occurring in the CNS, was studied by in situ hybridization in embryos, fetuses and offspring of time-pregnant rats between embryonic day 14 and postnatal day 27. MC4-R mRNA was found to be the predominant species during the entire prenatal period, while the MC3-R was first detected only postnatally. The temporal and spatial pattern of MC4-R mRNA expression in embryonic and fetal central nervous system corresponded closely to the developmental pattern of melanocortin binding sites. The receptor sites in developing cranial nerve ganglia and sympathetic ganglia were also found to be of the MC4-R type. Our data suggest, that the MC4-R might be involved in processes of early ontogeny, while the MC3-R might become important only during postnatal development.

In the second part of this thesis we studied the effects of melanocortins on brain cells in culture. Neurotrophic and regenerative properties of melanocortins have been demonstrated in a number of in vivo and in vitro studies. Since receptor ontogeny was not known, neural tissue was not selected according to receptor density in previous in vitro studies. The tissues studied such as, for instance, cerebral cortex, spinal cord and dorsal root ganglia contained limited amounts of MC3 or MC4-R mRNA. In order to further clarify the influence of melanocortins on brain development, we investigated dispersed cell cultures of fetal rat striatum, a forebrain region exhibiting a marked late fetal to early postnatal peak of MC4-R expression. As receptor expression parallels processes of network formation in many nervous structures, striatum was cocultured with mesencephalic cells of the same stage in order to provide the possibility of developmental interactions.

We studied the in vitro effect of two MSH-type peptides, α-MSH and Nle⁴, D-Phe⁷-α-MSH (NDP), a α-MSH analog with high affinity to all known melanocortin receptors, and of two ACTH-type peptides, ACTH₁⁻³⁹ and ACTH₁⁻²⁴, at concentrations between 3 and 2500nM. In addition, we included the peptide NEI, an alternate splicing product of the melanin concentration hormone (MCH) precursor protein. NEI exhibits similarities to α-MSH at the C-terminus and cross-reactivity with α-MSH antisera.
Terminals of MCH/NEI neurons occur in some brain regions expressing melanocortin receptors but receiving little or no POMC innervation, such as striatum or cortex. The development of the cultures was followed by immunocytochemistry of neuron specific enolase, glial fibrillic acid protein, galactocerebroside, tyrosin hydroxylase, choline acetyltransferase and neurofilaments. Developmental effects were quantitatively assessed by measurement of cell survival rate (MTT assay), growth associated protein (GAP-43), a marker for growth cones (ELISA), neurofilaments as an index of neurite formation (ELISA), and synaptophysin in a synaptosome preparation as an index for nerve terminal differentiation (ELISA). α-MSH and NDP increased neurofilament content in short term cultures (3 days in vitro, with single application of peptides), whereas ACTH1-24 and ACTH1-39 were ineffective. Dose-response curves were bell-shaped, with effective concentrations between 250 and 2500nM for α-MSH and 3.1 and 750nM for NDP (Kd for binding at striatal melanocortin receptor sites: α-MSH 50nM, NDP 0.7nM). α-MSH increased synaptophysin content in synaptosomes harvested from long term cultures (11 days in vitro, with five applications of peptide). GAP-43 protein and survival rate remained unchanged. NEI (250nM) also increased neurofilament content in short-term cultures in analogy to α-MSH. It remains to be elucidated, whether this peptide acts on a melanocortin receptor or exerts its influence through different receptor systems.

In conclusion, we showed that MC-R develop in a complex temporal and spatial pattern with MC4-R being the predominant receptor in early ontogeny and MC3-R developing only postnatally and we provide evidence that melanocortins influence the development of fetal neurons from brain regions with high developmental receptor expression in a ligand-specific way.
2 ZUSAMMENFASSUNG


Heute sind fünf verschiedene Melanocortinrezeptoren bekannt. Der klassische α-MSH Rezeptor (MC1-R) wird hauptsächlich in Melanocyten exprimiert, der ACTH-
Rezeptor (MC2-R) wurde in der Nebennierenrinde und in Adipocyten gefunden. Der MC3 und der MC4-R sind die wichtigsten Melanocortinrezeptoren im Gehirn. Der MC5-R ist in vielen Organen, in kleinen Mengen aber auch im Gehirn nachweisbar.


ronalen Netzwerken verläuft, kultivierten wir die Striatumzellen zusammen mit Mittelhirnzellen des gleichen Entwicklungsstadiums, so dass die Möglichkeit einer Interaktion gegeben war.


3 GENERAL INTRODUCTION

3.1 MELANOCORTINS

Melanocortins are peptide hormones derived from the 31kD polypeptide precursor proopiomelanocortin (POMC). Posttranslational cleavage of POMC leads to three groups of biologically active peptides: the adrenocorticotropins, the melanotropins, and the endorphins. (Mains et al. 1977; Roberts et al. 1977; Eberle, 1988).

![Diagram of POMC processing](Fig. 1)

**Fig. 1** Processing of proopiomelanocortin (POMC) the precursor for the melanocortins and opiates in the mammalian pituitary gland. Processing occurs by proteolytic cleavage at sites of paired basic amino acids. In both the anterior and the intermediate lobes, POMC is processed into ACTH biosynthetic intermediate and into β-lipotropin (β-LPH 1-91). In the anterior lobe, subsequent processing yields two biologically important products, ACTH 1-39 and β-MSH. In the intermediate lobe, ACTH 1-39 is further processed to yield α-MSH and corticotropin-like intermediate peptide (CLIP) (From Strand et al., 1989).
The melanotropins and adrenocorticotropins are grouped together under the term melanocortins; the melanocortin family includes adrenocorticotropic hormone (ACTH\textsubscript{1-39}), its fragment \(\alpha\)-melanocyte-stimulating hormone (\(\alpha\)-MSH), \(\beta\)-melanocyte-stimulating hormone (\(\beta\)-MSH), \(\gamma\)-melanocyte-stimulating hormone (\(\gamma\)-MSH), and fragments and analogues of these peptides.

ACTH, \(\alpha\)-, \(\beta\)-, and \(\gamma\textsubscript{1}\)-, \(\gamma\textsubscript{2}\)- and \(\gamma\textsubscript{3}\)-MSH are processed from three different regions of the POMC precursor, but share a conserved core of four amino acid residues:

\[-\text{His-Phe-Arg-Trp}-\]

### 3.1.1 Distribution and processing of POMC

#### 3.1.1.1 Distribution of POMC in peripheral tissue

a) POMC is expressed in the anterior and intermediate lobe of the pituitary of the hypothalamus.

In the rat, the initial cleavage step occurs between pro-ACTH and \(\beta\)-lipotropin (\(\beta\)-LPH). In the corticotrophs of the anterior lobe, the pro-ACTH-fragment is further processed in ACTH\textsubscript{1-39}, but desacetyl-\(\alpha\)-MSH and \(\beta\)-lipotropin and its fragment \(\beta\)-endorphin are detectable, too.

In the melanotrophs of the intermediate lobe pro-ACTH is further processed into \(\alpha\)-MSH, CLIP (=ACTH\textsubscript{18-39}) and \(\gamma\)-MSH. The POMC-fragment \(\beta\)-endorphin is also secreted. (Catania and Lipton 1993a; Eberle, 1988). These peptides are released into the peripheral circulation.

b) POMC is expressed to a lower extent also in skin, hair bulbs, testes, ovaries, placenta, gastrointestinal tract, kidneys, liver, lung, spleen and heart of several species (Eberle, 1988).
3.1.1.2 Distribution of POMC in the CNS

The occurrence of melanocortins is widespread in the brain in a distribution that is relatively consistent across species (Eberle, 1988). In the adult rat central nervous system, cell bodies with POMC-derived peptide immunoreactivity are mainly localized in the arcuate nucleus and less extensively in the nucleus commissurialis and the nucleus tractus solitarius (Bronstein et al., 1992; Jacobowitz et al., 1978; Eberle, 1988).

Fibres originating in the arcuate nucleus project rostrally to the periventricular regions of the hypothalamus, the preoptic area, olfactory associated areas and the septum. Other fibres project to the thalamus, enter the mesencephalon dorsally and continue into the periaqueductal and periventricular gray, innervate the midline raphe and the reticular formation. Lateral projections reach the lateral preoptic area, the anterior hypothalamic and anterior amygdaloid regions. Caudal projections run in multiple directions, but innervate regions in the posterior hypothalamus, the supramammillary region, the ventral tegmental area of the mesencephalon, and further regions in the brainstem, pons, medulla oblongata and spinal cord (Khachaturian et al., 1985a). Cerebral cortex contains very few, if any, immunoreactive terminals, but POMC-like mRNA has been detected by Northern Blot analysis (Civelli et al., 1982; Bronstein et al., 1982).

Fibres originating from the nucleus tractus solitarius innervate areas of the caudal medullary reticular formations, and possibly also regions of the spinal cord (Khachaturian et al., 1985a).

During ontogeny, an additional site containing cell bodies and processes with immunoreactivity to POMC derived peptides, and thus probably POMC mRNA, is found transiently in cells resembling radial glial cells of a restricted zone of the germinal zone of neonatal rat forebrain (Loughlin et al., 1991).

The highest concentrations of both ACTH and α-MSH in the brain are found in the hypothalamus. Other brain areas containing α-MSH are depicted in Table 1.
<table>
<thead>
<tr>
<th>Brain part</th>
<th>α-MSH (pg/mg wet tissue)</th>
<th>Eskay et al., 1979</th>
<th>Oliver et al., 1978</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forebrain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>1.1 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>1.4 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoptic area</td>
<td>99.8 ± 5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Septum</td>
<td>47.7 ± 4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td>2.8 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>245 ± 20.1</td>
<td>143 ± 22</td>
<td></td>
</tr>
<tr>
<td>Thalamus</td>
<td>11.8 ± 1.0</td>
<td>25 ± 3</td>
<td></td>
</tr>
<tr>
<td>Amygdala</td>
<td>16.7 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.5 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midbrain</td>
<td>16.4 ± 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hindbrain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.2 ± 0.004</td>
<td>2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Pons</td>
<td>5.8 ± 1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medulla</td>
<td>3.6 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinal cord</td>
<td>0.6 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain stem</td>
<td></td>
<td>11 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Cerebrum</td>
<td></td>
<td>6 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Pineal gland</td>
<td></td>
<td>163 ± 44</td>
<td></td>
</tr>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurointermediate lobe</td>
<td></td>
<td>1.1 x 10^6</td>
<td>4.5 x 10^3</td>
</tr>
<tr>
<td>Anterior lobe</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 1* Distribution and quantity of α-MSH in various parts of the rat brain (From Eberle, 1988).

3.1.1.3 Development of the POMC system in the CNS

The first appearance of POMC-derived peptide immunoreactivity in developing rat brain has been observed at embryonic day (E) 12.5 (Khachaturian et al., 1985b) in the ventral diencephalon. From E13 to neonatal age, fibres from the hypothalamic area innervate successively areas containing POMC products in adulthood in diencephalon and mesencephalon and also pons and myelencephalon (Schwatzberg and Nakane, 1982). In the mouse solitary tract nucleus, POMC-derived peptide immunoreactivity appears later compared to the immunoreactivity in the presumptive arcuate nucleus (E10.5 versus E17.5, Elkabe et al., 1989).
The fetal rat pituitary expresses POMC mRNA as early as E13 (Pintar and Lugo, 1987). At E15, the anterior pituitary gland exhibits the first ACTH-immunoreactive cells (Khachaturian et al., 1991), while the intermediate lobe is immunopositive at E17 (Chatelain et al., 1979; Pintar and Lugo, 1987; Khachaturian et al., 1991; Schwartzberg et al., 1982). At E17.5, the pituitary secretes α-MSH (Duff-Davis et al., 1984; Lugo et al., 1996a). α-MSH release by the intermediate lobe of the pituitary is under inhibitory control of dopamine. The neonatal intermediate lobe is sensitive to dopamine, but the feedback loop develops only during the first week postnatally (Duff-Davis et al., 1984).

### 3.1.1.4 Processing of POMC

POMC is formed from the precursor protein in the rough endoplasmic reticulum, translocated to the Golgi system and then packaged into secretory vesicles, whose maturation initiate the cleavage of the precursor molecule (Zimmerman et al., 1980; Smith and Funder, 1988). Several proteolytic enzymes are required for the processing of the peptides. After the removal of the N-terminal signal sequence, cleavage occurs between pairs of basic amino acid residues (lys-lys, arg-lys, arg-arg, lys-arg) by specific endopeptidases. Two of them, PC1 and PC2, have been identified. PC1 cleaves POMC into corticotropin and lipotropin, PC2 cleaves the precursor into α-MSH and β-endorphin (Benjannet et al., 1991). Some of the peptides are then further α-amidated at their carboxyl terminus (α-MSH) and/or α-N-acetylated (α-MSH, and β-endorphin). Amidation is thought to protect the protein from degradation by carboxypeptidases and to be essential for the full biological activity. Acetylation renders β-endorphin inactive in terms of opiate receptor binding and inducing analgesia, while N-acetylation of α-MSH markedly increases potency on melanocytes (Smith and Funder, 1988).

The acetylation status of the peptides depends on the tissue examined: Both, α-MSH (N-mono-or-O-diacetylated) and β-endorphin (N-acetylated) are extensively acetylated in the intermediate lobe of the pituitary gland of most species. In the dorsal caudal medulla, the site of the nucleus tractus solitarius POMC cell group, 80% of α-MSH and 65% of β-endorphin are also acetylated (Emeson and Eipper, 1986; Eberle, 1988). In contrast, the majority of α-MSH and β-endorphin in the adult rat hypothalamus is not acetylated (Emeson and Eipper, 1986; Jégou et al., 1989; Smith and Funder,
However, the proportion of mono- and diacylated forms increases to 60-90% in POMC-nerve terminal areas (Tranchand-Bunel et al., 1990).

3.2 RECEPTORS AND LIGANDS

3.2.1 Receptors and intracellular signalling

The effects of melanocortins are mediated by specific melanocortin receptors (MC-R). Prior to cloning, two MC-R were known from classical physiological and pharmacological studies: The MSH-receptor (now MC1-R) which was specifically found on melanocytes and melanoma cells of a variety of vertebrate organisms (Mountjoy et al., 1992; Chhaljlani and Wikberg, 1992; Xia et al. 1995), and the ACTH-receptor (now MC2-R) which has been demonstrated in adrenocortical cells and adipose tissue (Buckley and Ramachandran, 1981; Mountjoy et al., 1992). Three further receptors were identified by cloning experiments: the MC3-R, MC4-R and MC5-R. The MC3-R (Roselli-Rehfuss et al. 1993; Desarnaud et al. 1994) has been detected by northern hybridization analysis in placenta and brain, primarily in hypothalamus and other limbic structures, and by reverse transcription-polymerase chain reaction also in stomach, duodenum and pancreas (Gantz et al. 1993). The MC4-R (Mountjoy et al. 1994; Alvaro et al. 1996) was found to be expressed only in brain. MC4-R is much more widely distributed than the MC3-R and is found virtually all over the brain. Most recently, the MC5-R has been cloned (Chhajlani et al., 1993; Fathi et al., 1995; Gantz et al., 1994; Griffon et al., 1994; Labbé et al., 1994). This receptor is distributed very widely in the body and is expressed in skin, muscle, thymus, spleen, ovary, testis, adrenal cortex, lung, brain and pars tuberalis of the pituitary. Additionally, receptors of unknown type have been identified in cultured microvascular endothelial cells of murine brain (De Angelis et al., 1995) and in primary rat Schwann cells (Dyer et al., 1993).

The five receptors are 39-61% identical to each other on the amino acid level. The MC1-R and the MC2-R are the most distantly related (39%), whereas the MC3, MC4 and MC5-R seem relatively close (55-61%). This subgroup is related 43-46% to the MC1 and MC2-R.
All five receptors belong to the superfamily of the G-protein coupled receptors (Cone et al., 1996) with 7 transmembrane α-helical structures (Prusis et al., 1995). All of the melanocortin receptors couple to Gs protein and activate adenylyl cyclase. Some reports suggest that the MC3-R may also couple to Gq, resulting in a modest activation of inositol 1,3,4-triphosphate turnover (Konda et al., 1994). For the MC1-R and MC2-R involvement of protein kinase C (Lyson and McCann, 1994; Park et al., 1996) has been discussed. Extracellular Ca$^{2+}$ is required for binding of the ligands to the receptor and for signal transduction at least for the MC1-R (Eberle 1988).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Amino Acids</th>
<th>Sites of expression</th>
<th>Ligand specificity</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1-R</td>
<td>317</td>
<td>Melanocytes, melanoma, low amount in periaqueductal gray matter of the brain</td>
<td>α-MSH&gt;ACTH&gt;β-MSH&gt;γ-MSH</td>
<td>Pigmentation</td>
</tr>
<tr>
<td>MC2-R</td>
<td>297</td>
<td>Adrenal cortex, adipocytes</td>
<td>ACTH</td>
<td>Steroidogenesis</td>
</tr>
<tr>
<td>MC3-R</td>
<td>361</td>
<td>Hypothalamus, limbic system, placenta, gut</td>
<td>α-MSH=β-MSH=γ-MSH=ACTH</td>
<td>Unknown</td>
</tr>
<tr>
<td>MC4-R</td>
<td>333</td>
<td>Hypothalamus, limbic system, cortex, brain stem</td>
<td>α-MSH=ACTH=β-MSH&gt;γ-MSH</td>
<td>Unknown, Body weight control?</td>
</tr>
<tr>
<td>MC5-R</td>
<td>325</td>
<td>Muscle, liver, spleen, lung, brain, adipocytes a.o.</td>
<td>α-MSH&gt;ACTH&gt;β-MSH&gt;γ-MSH</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table 2 Characterization of the presently known melanocortin receptors (MC-R) (modified from Sigriest and Eberle, 1995).

The MC-R displays a unique pharmacological profile for activation by the different melanocortin peptides. The MC2-R exhibits a high specificity for ACTH, while α-MSH is more effective than ACTH on most mammalian MC1-Rs. The MC3, MC4, and MC5-R appear to be less selective, and are more or less equally well activated by α-MSH or ACTH (Cone et al., 1996). The MC3-R binds γ-MSH with higher affinity (Roselli-Rehfuss et al., 1993), while the MC4-R has been reported to show some preference for β-MSH (Schiöth et al., 1996). Desacetylated α-MSH is less potent in activating the MC1-R than α-MSH (Eberle, 1988), though, Schiöth et al. found equal potencies. The MC3, MC4 and MC5-R are equally well activated by the mono- or desacetylated forms (MC4-R) or prefer the desacetylated form (MC3-R, MC5-R (Schiöth et al., 1996).
3.2.1.1 Development of binding sites for melanocortins

Investigations of the development of binding sites for $[^{125}\text{I}]N\text{le}^4,\text{D-Phe}^7-\alpha\text{-MSH}$ ($^{125}\text{I}\text{-NDP}$), an $\alpha\text{-MSH}$ analogue which binds equally well to all known MC-R, have been performed in our laboratory (Lichtensteiger et al., 1996). The study revealed region- and stage-specific, often transient ontogenetic patterns. Binding sites were first detected at E13 in the sympathetic ganglia and transiently, from E13-E15, in the thalamus. The early fetal period was characterized by prominent peaks of receptor density in somatosensory and viscerosensory nuclei (trigeminal sensory nuclei, solitary tract nucleus) paralleled by receptor expression in the cranial ganglia (5th, 7th, 9th, and 10th cranial nerve). During late fetal life, receptor density peaked in the dorsal motor nucleus of vagus and inferior olive. Caudate-putamen, nucleus accumbens, olfactory tubercle and septohippocampal nucleus showed a high perinatal maximum. $^{125}\text{I}\text{-NDP}$ binding sequentially appeared in cerebral cortical areas, starting with late fetal piriform cortex and with highest levels in the entorhinal cortex. Preoptic, septal, hypothalamic and amygdaloid areas known for elevated receptor densities in adulthood, exhibited a slow, peri- and postnatal receptor ontogeny. The sequence of appearance of binding sites in the thalamus, midbrain, pontine flexure, trigeminal sensory nuclei, solitary tract nucleus and cranial nerve ganglia roughly parallels the appearance of POMC immunoreactivity in the developing mouse brain (Elkabes et al., 1989). In contrast, other $^{125}\text{I}\text{-NDP}$ binding sites, for instance in the developing cerebral cortex, cerebellum, hippocampal formation, caudate-putamen, dorsal motor nucleus of vagus and substantia gelatinosa could not be correlated with POMC-data.

The early appearance and distinct pattern of melanocortin binding sites in the developing brain indicated a role of these peptides during ontogeny. Correlation of developmental stages in well studied areas of the brain, such as the sympathetic ganglia, principal sensory nucleus and spinal tract nucleus of the trigeminal nerve, dorsal motor nucleus of vagus, inferior olive or striatum, with binding site ontogeny indicated, that the appearance of $^{125}\text{I}\text{-NDP}$ binding sites coincides mainly with phases of synaptogenesis and the establishment of neural circuitry (Lichtensteiger et al., 1996).
3.2.2 Ligands

3.2.2.1 Structure-activity relationship

There are numerous studies dealing with structure-activity relationships of different fragments or analogues of ACTH1-39. It is of importance to consider the type of test performed when interpreting the results, since different tests yield partly different information on active sites.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Sequence</th>
<th>Receptor activation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH4-10</td>
<td>H-Met¹-Glu-His-Phe-Arg-Trp-Gly¹⁰</td>
<td>MC1/3, MC4, low not MC5</td>
<td>Schioth et al. 1996</td>
</tr>
<tr>
<td>α-MSH</td>
<td>Ac-Ser¹-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly¹⁰-Lys-Pro-Val¹³-NH₂</td>
<td>MC1-5</td>
<td>Schioth et al. 1996</td>
</tr>
<tr>
<td>α-MSH¹¹-¹³</td>
<td>H-Lys¹¹-Pro-Val¹³-NH₂</td>
<td>?</td>
<td>Schioth et al. 1996</td>
</tr>
<tr>
<td>NDP-MSH</td>
<td>Ac-Ser¹-Tyr-Ser-Nle⁴-Glu-His-D-Phe⁷-Arg-Trp-Gly¹⁰-Lys-Pro-Val¹³-NH₂</td>
<td>MC1, 3-5</td>
<td>Schioth et al. 1996</td>
</tr>
<tr>
<td>Org 2766</td>
<td>H-Met-O₂⁴-Glu-His-Phe-d-Lys-Gly⁹</td>
<td>not MC1, MC3-5</td>
<td>Schioth et al. 1996</td>
</tr>
<tr>
<td>BIM 22015</td>
<td>d-Ala⁴-Glu-Tyr-Phe-Arg-Trp-Gly¹⁰</td>
<td>not MC1, MC3-5</td>
<td>Schioth et al. 1997</td>
</tr>
</tbody>
</table>

Table 3 Ligands of melanocortin receptors. The table focuses on ligands discussed in this thesis. The core sequence which is common to all endogeneous melanocortins is highlighted in bold letters.

* γ-MSH structures of different species have been deduced from the respective DNA sequence, but only few γ-MSH peptides have been analyzed by protein sequence determination. Three γ-MSH from synthetic sources, (γ1, γ2 and γ3-MSH) with different chain lengths are known. The structure depicted in the table is derived from rat neurointermediate lobe of the pituitary gland (Eberle, 1988)
Structure-activity studies with linear α-MSH peptides on different melanophores clearly depict two regions within the α-MSH sequence exhibiting melanotropic bioactivity: the central "core sequence" (4-9) and the C-terminal message (10-13), which increases the potency of the core sequence considerably (Eberle, 1988). The structural requirements for inducing steroidogenesis in rat adrenocortical cells include only amino acids (5-10) but amino acids (11-24) are required for receptor binding. The "core sequence" (4-9) has been shown to be important in mediating behavioral effects, too. By contrast, the antiinflammatory/antipyretic action of melanocortins appears to be mediated by the sequence α-MSH11-13. The peptide does not seem to bind to MC3, MC4 and MC5-R as it does not compete with binding of 125I-NDP in binding studies using rat brain homogenates or in vitro autoradiography (Catania and Lipton, 1993b; Tatro and Entwistle, 1994; Lichtensteiger, 1996). Organon 2766, an ACTH4-9 analogue which has been found to be especially potent in behavioral tasks and in central nervous tissue regeneration does not bind to any of the presently known centrally occurring MC-Rs. It is speculated that this ligand may bind to another, yet unknown MC-R (Adan et al., 1994a; Schioth et al., 1996). NDP-MSH, a superpotent α-MSH analogue binds to all known receptors. It differs from α-MSH by inducing Nle⁴ and D-Phe⁷. Recent data from point mutations of the MC1-R suggest, that NDP-MSH has alternate attachment points to the receptor compared to α-MSH (Frändberg et al., 1994).

Only recently, several potent agonists and antagonists, acting specifically on different MC-R have been synthetized. They will permit to study the function of the receptors more detailed (Adan et al., 1994b; Hruby et al., 1995; Cone et al., 1996).

3.2.2.2 NON-POMC-derived ligands

Melanocortins might not be the only ligands binding to the MC-R. One indication to this hypothesis is that the binding constant of α-MSH to the MC4-R in the CNS is surprisingly low. A binding study in transformed cos-cells revealed a Ki value of 640nM for α-MSH for MC4-R compared to 20nM for MC3-R (Schioth et al., 1996). Kd’s of 50 nM and of 5nM were calculated for α-MSH-binding to PN8 rat caudate-putamen (MC4-R), or to arcuate nucleus (MC3-R), respectively (Lichtensteiger et al., 1996). In contrast, EC50 values for cAMP conversion in various cell lines revealed similar values for
MC4-R and MC3-R. They were 1.5-18nM for MC4-R (Alvaro et al., 1996; Mountjoy et al., 1994) and 3.8nM for MC3-R (Roselli-Rehfuss et al., 1990). The hypothesis is further supported by the fact that POMC system and MC-R distribution do not fully overlap. Some areas express MC4-R mRNA with no or very low POMC fiber innervation, such as cerebral cortex, hippocampal formation, cerebellum, caudate-putamen, dorsal motor nucleus of vagus and substantia gelatinosa (Kistler-Heer et al., in press). It is thus not clear where the POMC-derived ligand binding to these receptors may originate from, or if these receptors bind to a ligand that is not POMC-derived.

3.2.2.1 Agouti

It was first shown on melanocytes and hair bulbs, that melanocortins are not the only ligands of the MC1-R. Agouti, a protein which is not derived from POMC but from another precursor, binds to this receptor. The murine agouti gene encodes a 131 amino acid protein which is expressed in the skin of wild type mice between the third and seventh day after birth. It is normally involved in controlling the coat colour of the animals (see also 3.3.1.1.) and has been shown to be an antagonist to the MC1-R and additionally to the MC4-R (Lu et al., 1994). The α-MSH antagonist epitope is localized on the C-terminal domain of the agouti protein (Willard et al., 1995).

3.2.2.2 MCH-positive cell group

In 1978/79 Watson and Akil reported the presence of neurons in the dorsolateral region of the hypothalamus which positively stained with antisera directed to the C-terminus of α-MSH. These neurons projected virtually all over the brain, and in particular reached cortex, dentate gyrus, hippocampus and caudate-putamen (Khachaturian et al., 1985a; Nahon, 1994) and other areas that were not innervated by POMC neurons. They later were identified as melanin concentrating hormone (MCH)-positive cells (Naito et al., 1985). In teleost fish, MCH antagonizes the melanin-dispersing effects of α-MSH, and it was suggested that opposition of MSH effects in the brain might be an ancient role of MCH. This idea is supported by some recent studies showing, for instance, that MCH can antagonize α-MSH in behavioral tests (Gonzalez et al., 1996; McBride et al., 1994; Sanchez et al., 1997). A considerable list of other possible functions of
MCH in the mammalian brain has accumulated: These include a role in water and electrolyte balance, feeding behavior, modulation of cortical arousal, involvement in memory processes and a variety of cognitive functions. However, none of these suggested functions has so far been supported by substantial experimental evidence (Knigge et al., 1996; Nahon, 1994).

The immunoreactivity of the MCH-cells to α-MSH antiserum was attributed to a protein called neuropeptide-glutamic acid-isoleucine, (NEI), which is colocalized with melanin concentrating hormone (MCH). An additional peptide showing cross reactivity with immunosera directed against α-MSH is neuropeptide glycine glutamic acid, (NGE). NEI shows some relationship to α-MSH at the C-terminus (Nahon, 1994).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEI</td>
<td>NH2-Glu-Ile-Gly-Asp-Glu-Asn-Ser-Ala-Lys-Phe-Pro-Ile-COOH</td>
</tr>
<tr>
<td>NGE</td>
<td>NH2-Gly-Ser-Val-Ala-Phe-Pro-Ala-Glu-Asn-Gly-Val-Gln-Asn-Thr-Glu-Ser-Thr-Gln-Glu-COOH</td>
</tr>
</tbody>
</table>

Table 4  Structures of melanin concentrating hormone (MCH), neuropeptide-glutamic acid-isoleucine (NEI) and neuropeptide glycine glutamic acid (NGE). The three peptides are cleaved from different regions of a common precursor protein (Structures provided by A. Eberle).

MCH, NEI and NGE are cleaved from a common precursor. MCH and NEI have been characterized (Bittencourt et al., 1992; Knigge et al., 1996; Parkes and Vale, 1992), whereas NGE is only a putative splicing product. To date, no MCH or NEI receptor has been cloned, and it has been speculated that these peptides might bind to the MC-Rs. MCH does not compete with labeled α-MSH at the MC1-R (Drozdz et al., 1995) or MC3, MC4, and MC5-R (personal communication with E. Hintermann/A. Eberle). No data are available for NEI or NGE.
3.3 FUNCTIONS OF MELANOCORTINS

3.3.1 Peripheral targets

Melanocortins are able to influence various peripheral tissues (Eberle, 1988), however, most knowledge has accumulated concerning the action of melanocortins on melanocytes, the lacrimal gland, the adrenal gland, the cardiovascular system, the immune system and peripheral nerves and muscles. This chapter will concentrate on the action of melanocortins on melanocytes.

3.3.1.1 Melanocytes and Melanoma

α-MSH is active on pigment cells in many different species: it induces rapid skin darkening in lower vertebrates by pigment dispersion in dermal melanophores and stimulates melanin production in humans and mammals. This fact is used to test the activity of melanocortins: in frogskin or melanoma cell bioassays, the darkening of frogskin or melanoma cells is used as parameter for the activity of melanocortins.

Melanocytes are derived from neural crest cells. They migrate as undifferentiated melanoblasts into the dermis and later invade the epidermis and differentiate into melanocytes. They form and maintain dendrites to synthesize and mature melanosomes containing melanin, and secrete them into keratinocytes with which they are associated. The different skin colors in humans are not due to differences in the number or distribution of melanocytes, but are based on differences in the number of melanosomes and the rate at which they are produced, transferred and distributed into keratinocytes, and additionally on differences in the degree of melanization resulting from variations in tyrosinase activity (Eberle, 1988).

The melanin in the fur is produced by melanocytes located above the dermal papilla. These melanosomes contain two kinds of melanin, eumelanin (brown/black pigment) and pheomelanin (yellow/red pigment), which are both synthesized from tyrosine. The rate limiting step in this procedure is the activation of the enzyme tyrosinase. When low levels of tyrosinase are present in the melanocyte, the majority of tyrosine is converted into pheomelanin. At higher levels of tyrosinase, eumelanin is also produced (Eberle, 1988; Hearing and Tsukamoto, 1991). Tyrosinase activity is regulated by
α-MSH. α-MSH induces a 2-5 fold increase in the transcription of the tyrosinase gene and a 5-50 fold increase in the activity of tyrosinase (Halaban et al., 1984; Hoganson et al., 1989; Kwon et al., 1988; Wong et al., 1975). The relative amounts of eumelanin/pheo-melanin are controlled by the genetic loci agouti and extension. Extension encodes the melanocortin receptor which is located within the hair follicle melanocytes, (Robins et al., 1993), agouti the agouti protein (Gschwind, 1966; Gschwind et al., 1972; Lamoureux and Mayer, 1975). Agouti protein is produced in hair follicles, binds to the melanocortin receptor and inhibits temporarily α-MSH induced eumelanin production which results in phaeomelanin expression (Silvers and Russel, 1955).

The role of melanocortins or melanocortin receptors (MC-R) in melanomas is still controversial (Siegrist and Eberle, 1995). According to some reports, melanocortins are able to stimulate proliferation of melanoma cell lines or increase the rate of metastasis, in others, the effect is just the opposite way. MSH induces downregulation of melanocortin receptors in mouse and some human melanoma cell lines, whereas other human melanoma cell lines respond to MSH treatment by an increase in receptor numbers. It is interesting to note that dominant mutations of the agouti gen (viable yellow and lethal yellow), are accompanied by an increased occurrence of neoplasms, obesity, and an increased susceptibility to diabetes (Wolff et al., 1986).

3.3.2 Central actions

Since the pioneering work of De Wied more than three decades ago (De Wied et al., 1964, 1965 and 1966; Mirsky et al., 1953), a large number of behavioral effects of the melanocortins have been described. Additionally, effects on brain development, nerve regeneration, antipyretic, antiinflammatory and centrally mediated cardiovascular effects have been described. Only recently, a new function for melanocortins has appeared. It has been shown that they are involved in body weight control.
3.3.2.1 Blood brain barrier

Melanocortins affecting the central nervous system are generally believed to be of central origin, released by neurons of the arcuate nucleus or the nucleus of the solitary tract. At least partly though, peptides influencing the brain could also be of pituitary origin especially in immature animals. The permeability of the mature blood-brain barrier to peptides is low in general (Banks and Kastin, 1995; Wilson, 1988). However, transfer of melanocortins from blood to cerebrospinal fluid appears to occur (De Rotte et al., 1980), and at least one of the periventricular organs, area postrema, has been implicated in certain actions of α-MSH (Lichtensteiger and Lienhart, 1977). When radioactive α-MSH was injected into the carotid artery, radioactivity was found to be distributed specifically in the brain: it accumulated preferentially in striatum, reticular nucleus (Pelletier et al., 1975), occipital cortex, cerebellum, pons and medulla (Kastin et al., 1976).

3.3.2.2 Behavioral effects

MSH and ACTH have been shown to influence a variety of behavioral paradigms. In general, the focus of these studies has been on attention, learning and memory, sexual and social behavior, grooming and stretching/yawning in rodents.

<table>
<thead>
<tr>
<th>Effect/behaviour</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active avoidance behaviour (shuttle-box, pole-jumping)</td>
<td>facilitated acquisition, delayed extinction</td>
</tr>
<tr>
<td>Passive avoidance behaviour (shuttle-box or platform)</td>
<td>delayed extinction</td>
</tr>
<tr>
<td>Visual discrimination (Y maze)</td>
<td>facilitated acquisition and reversal</td>
</tr>
<tr>
<td>Sexual behaviour</td>
<td>sexual performance, lordosis behaviour</td>
</tr>
<tr>
<td>Social behaviour</td>
<td>increased sociability, reduced territorial aggression, reduced fear</td>
</tr>
<tr>
<td>Grooming behaviour</td>
<td>display of excessive grooming</td>
</tr>
<tr>
<td>Stretching-yawning syndrome</td>
<td>diffuse muscular tremor, stretching, yawning</td>
</tr>
</tbody>
</table>

*Table 5* Effects of melanocortins on behaviour (from Eberle, 1988).
Certain N-terminal portions of the molecule, particularly amino acids 4-9 and 4-10, are believed to be of importance for the behavioral effects. The administration route of melanocortins influences the outcome of the studies. In some cases the peptides were active if applied peripherally (motivation and learning, memory (Sandman et al., 1972), some sexually motivated behavior). For other parameters they were exclusively active if applied centrally (grooming, stretching/yawning (Ferrari et al., 1963; Gispen and Isaacson, 1981)).

3.3.2.3 Antipyretic and antiinflammatory effects

α-MSH is able to decrease fever and inflammation very potently in several species when applied centrally or peripherally. Antipyretic and antiinflammatory activities of α-MSH have been traced down to the C-terminal message sequence Lys-Pro-Val-NH₂ (α-MSH₁₁₋₁₃) (Lipton and Catania, 1997). It is interesting to note that α-MSH₁₁₋₁₃ also exerts marked effects on central DA systems (Lichtensteiger and Monnet, 1979). So far it is not known which of the presently known MC-R mediates these effects: the peptide does not seem to bind to the MC3, MC4 or MC5-R as it does not compete with ¹²⁵I-NDP in binding studies on rat brain homogenates or in vitro autoradiography (Catania and Lipton, 1993b; Tatro and Entwistel, 1994; Lichtensteiger, 1996). It has been speculated (Lipton and Catania, 1997) that α-MSH₁₁₋₁₃ might activate MC1-R, since this receptor has recently been discovered in a murine macrophage cell line, in a human monocyte cell line, in human neutrophils, and to a limited amount also in the brain (Catania and Lipton, 1997).

α-MSH concentration in the circulation is normally regulated within narrow limits, but plasma and local α-MSH concentrations increase in certain inflammatory disorders. α-MSH is upregulated in the septum and in the peripheral circulation during fever. Increased levels of endogenous α-MSH were also measured in several pathological inflammatory conditions, for example infectious disorders (HIV), severe brain injuries, myocardial infarction, rheumatic disorders, etc (Airaghi et al., 1995; Catania et al., 1993c; Catania et al., 1994; Catania and Lipton, 1997).
Although the precise mechanism of action of melanocortins is still unknown, antagonism of cytokines is thought to be responsible for at least a part of their anti-inflammatory and antipyretic activity. Fever or inflammation caused by IL-1, IL-6, TNF-α, IFN-γ, PG E₂ can be inhibited by application of α-MSH. Most remarkably, the peptide appears to act both directly on receptors (?) in inflammatory cells, and indirectly, through receptors within the CNS (Catania and Lipton, 1993a, b; Huh et al., 1997; Lipton and Catania, 1997).

3.3.2.4 Body weight control

Melancortins are thought to be involved in body weight control. Data describing anorexic effects of α-MSH injected intracerebroventricularly (Levine et al., 1986; Poggio et al., 1986; Vergoni et al., 1990) were published already ten years ago. In the last few months, the influence of melanocortins on body weight control has gained new attention. Recently, several reports have confirmed a participation of melanocortin receptors and its ligands in the obesity syndrom (Fan et al., 1997; Huszar et al., 1997; Seeley et al., 1997; Boston et al., 1997) (Fig. 2).

Leptin, a hormone produced by adipocytes, is a key player in novel models of body weight control. Serum concentrations of leptin correlate well with the body mass index in both humans and rodents (Maffei et al., 1995). A loss of body fat leads to a decline in leptin levels, which in turn leads to a state of positive energy-balance and other adaptive changes, such as a decrease in reproductive functions and a reduction of body temperature. Conversely, increased body fat levels cause a rise in leptin levels and a state of negative energy-balance (Maffei et al., 1995). Consequently, mutant mice that lack leptin or functional leptin receptors, such as ob/ob (lack leptin) or db/db mice (lack functional leptin receptor), are hyperphagic, massively obese, hypometabolic, hypothermic, diabetic and infertile (Erickson et al., 1996). The leptin signal is mediated by receptors in the chorioid plexus and the hypothalamus, i.e. in the arcuate nucleus (Lee et al., 1996). Integratory centres of the hypothalamus are known to control energy balance. Lesions of the ventromedial hypothalamic nucleus (VMH), for instance, are associated with an increase in body weight.
Fig. 2 Hypothetical model of body weight control. Leptin, a hormone produced by adipocytes, maintains the stability of body fat mass - a loss of body fat leads to a decrease in leptin, which in turn leads to a state of positive energy-balance. An increase in adiposity results in an increase in the levels of leptin and a negative energy expenditure. The effects of leptin are thought to be mediated by leptin receptors in the choroid plexus and the hypothalamus. The physiological responses to weight loss are probably functionally distinct from the responses to weight gain. Neuropeptide Y (NPY) seems to be an important mediator downstream of leptin in regulating responses to weight loss, while melanocortins play a role in response to weight gain (from Friedman, 1997).

The present hypothetical model for weight regulation states that the physiological responses to weight loss are functionally distinct from the responses to weight gain (Fig. 2). Neuropeptide Y (NPY) is believed to be a downstream signal to decreased leptin levels. NPY is synthesized by neurons of the arcuate nucleus which bear leptin receptors, and secreted from their terminals in the paraventricular nucleus and the VMH.
NPY expression and release from these nuclei are inhibited by leptin (Erickson et al., 1997), and NPY mRNA is increased after food restriction and food deprivation (Brady et al., 1990). Chronic administration of NPY into the hypothalamus of normal animals leads to hyperphagia, insulinemia and an increase in body weight, thus mimicking the phenotype of leptin-deficiency (Zarjevski et al., 1993). Leptin deficient mice consequently have increased NPY levels. Double mutant mice (NPY^/- ob/ob) are less obese than standard ob/ob mice, they are fertile and less affected by diabetes (Erickson et al., 1996). On the contrary, mice deficient only in NPY have normal body weight, indicating additional mechanisms in the hypothalamic response to decreased leptin levels (Erickson et al., 1996).

The downstream signal to increased leptin levels is thought to include melanocortins. Melanocortins are produced in neurons of the arcuate nucleus and transported to different nuclei of the hypothalamus. Intracerebroventricular injections of α-MSH lead to weight loss (Levine et al., 1986; Poggioli et al., 1986; Vergoni et al., 1990), while short-term starvation and food deprivation lead to decreased POMC levels in the arcuate nucleus (Brady et al., 1990). Further evidence for the importance of melanocortins and MC-R in body weight control was provided by the characterization of yellow and viable yellow obese mice (A^y and A^vy) (Fan et al., 1997; Yen et al., 1994), and MC4-R knock-out mice (Huszar et al., 1997). A^y and A^vy mice express the agouti protein ectopically in all tissues examined. The wild type agouti gene locus (a) expresses the agouti protein only in the skin, where it switches the hair follicle melanocytes from black melanin synthesis to yellow, producing a subterminal band of yellow/red pigment in the fur. When the agouti protein is overexpressed in the skin, it inhibits the effects of α-MSH on the MC1-R which leads to a yellow coat color. Mice with the genotype A^vy/a not only have a totally yellow fur, but they also are obese, hyperinsulinemic, hyperglycemic (males) and have a tendency to develop tumors (Yen et al., 1994). This cannot be explained by an antagonistic action of agouti at the MC1-R. It was suggested that agouti might interact with the MC4-R in the hypothalamus, as the peptide was shown to be an antagonist at this type of receptor, too (Lu et al., 1994). Only recently, an agouti-related protein was discovered, whose mRNA is normally expressed in the hypothalamus and which is a selective antagonist at the MC3 and MC4-R (Ollmann et. al., 1997). It was
shown that intracerebroventricular administration of a synthetic melanocortin antagonist at the MC4-R enhances feeding, while administration of an agonist in different models of hyperphagia (fasted mice, ob/ob mice, A^v mice, mice injected with NPY) inhibits feeding (Fan et al., 1997). MC4-R knockout mice develop a phenotype very similar to that of A^vy/a mice (Huszar et al., 1997), thus providing evidence that melanocortin receptors and its agonists/antagonists play an important role in maintaining body weight control.

It was proposed that the weight-reducing effects of leptin are transmitted primarily by the way of POMC neurons. Although it has been shown that intracerebroventricular injections of a non-selective MC4-R antagonist prevent leptin-induced reductions of food intake (Seeley et al., 1997), it is not clear, whether this is entirely the case. The central effects of defective POMC signalling and the absence of leptin on weight gain in double-mutant lethal yellow (A^v/a) leptin deficient (ob/ob) mice were shown to be independent and additive (Boston et al., 1997). Furthermore, deletion of the leptin gene restores leptin sensitivity to A^v/a mice, showing that resistance to leptin in these mice results form desensitization of leptin signalling (Boston et al., 1997).

There are clear differences between various melanocortin peptides in terms of effects on food intake and body weight. α-MSH has been reported to induce anorexic effects when injected intracerebroventricularly to rats (Levine et al., 1986; Poggioli et al., 1986; Vergoni et al., 1990), whereas desacetyl-α-MSH injected into the third ventricle of food-deprived rats had no effect (Tsujii and Bray, 1989). On the other hand intramuscular injection of desacetyl-α-MSH increased food intake and muscle and adipose weight in viable yellow obese mice (A^vy). A^vy mice have significantly higher ratios of desacetyl-α-MSH to α-MSH in pituitary extracts compared to their lean a/a littermates (Bray, 1989). The acetylated form, the main form released by the pituitary gland, and the desacetylated form, mainly present in the hypothalamus, thus appear to have opposite effects on feeding behavior in the CNS and the periphery. However, it should be noted that the α-MSH released from central nerve terminals, can also be acetylated (see above).
3.3.2.5 Interactions with neurotransmitter systems

Most data about interactions of melanocortins with other neurotransmitter systems deal with the release of melanocortins from the pituitary gland. In mammals, the secretion from the pituitary is thought to be tonically inhibited by hypothalamic MSH release-inhibiting factors (MIF), such as dopamine, GABA, enkephalin, serotonin, melanin-concentrating hormone (MCH) and somatostatin, and stimulated by MSH releasing factors (MRF) such as adrenalin/noradrenalin, vasopressin or CRF (Eberle, 1988).

Interactions of the central melanocortin system have been described for the cholinergic, dopaminergic, noradrenergic, serotonergic and GABAergic systems. Atropin blocks the induction of grooming behavior by MSH/ACTH peptides, and administration of ACTH increases acetylcholine synthesis. An alpha-1-adrenoreceptor may be involved (Eberle, 1988; Scimonelli et al., 1992). GABA inhibits α-MSH release from the hypothalamus of the rat (Eberle, 1988; Tiligada and Wilson, 1990). Data on influences of the noradrenergic and serotonergic system are conflicting, increase or decrease of the release of melanocortins have been reported (Eberle, 1988). The glutamnergic system is involved via the NMDA receptor. Superfusion of rat hypothalamic slices with NMDA resulted in increased release of α-MSH (Wayman and Wilson, 1992; Wayman et al., 1994). Melanocortins have also been found to interact with neuropeptides (Eberle, 1988).

3.3.2.5.1 Dopamin

Interactions of melanocortins have been studied most extensively with dopamine (DA). In most species, DA is a potent inhibitor of MSH release from the intermediate lobe of the pituitary (Eberle, 1988). This effect is mediated by D2-receptors (Tonosaki et al., 1995). The development of the tubero-infundibular (arcuate) DA system and the onset of MSH secretion by the intermediate lobe of the pituitary gland (PI) are intimately linked with each other (Davis et al., 1984). When an α-MSH antiserum is injected i.v. to rat pups on two days of the first postnatal week, the characteristic feedback reaction of tubero-infundibular DA neurons to systemic α-MSH fails to develop and is absent in adulthood (Lichtensteiger and Schlumpf, 1986).
Apart from the regulation of intermediate lobe function, melanocortins and DA appear to be linked with each other also in the CNS. Intraperitoneal application of α-MSH, ACTH₁-2₄, ACTH₄-₁₀ caused differential acute responses in DA neurons of arcuate nucleus and substantia nigra (Lichtensteiger and Monnet, 1979). The two DA systems are not only differentially sensitive to the type of melanocortin fragment used, but also to the hormonal status of the animal: the effect on the nigral DA neurons, but not on the arcuate DA neurons, was dependent on the hormonal state of the animals (Lichtensteiger and Lienhart, 1977; Lichtensteiger and Monnet, 1979).

The mode of action of melanocortins on DA systems has not yet been clarified. Cholinergic systems appear to be involved (Lichtensteiger and Lienhart, 1977). Melanocortins also appear to interact with agonist effects on dopamine receptors. ACTH₁-2₄ has been reported to inhibit the binding of dopaminergic ligands to D₂ receptors in rat striatal slices in vitro (Florijn et al., 1991). In adult rat brain slices containing caudate putamen and nucleus accumbens, SCH 23390, a selective D₁ receptor antagonist, was able to block α-MSH induced increases in cyclic AMP levels (Lecanzo et al., 1995).

Central α-MSH release is influenced by DA. D₂ receptor agonists, but not D₁ receptor agonists reduced α-MSH release from rat hypothalamus slices and D₂ receptor antagonists but not D₁ receptor antagonist stimulated increased α-MSH release from the same tissue (Tiligada and Wilson, 1989). By contrast α-MSH content of arcuate nucleus and dorsolateral hypothalamus was not affected by dopaminergic agonists or antagonists (Delbende et al., 1987).

### 3.3.2.6 Development and regeneration of the nervous system

#### 3.3.2.6.1 Development and regeneration of peripheral nerves

Low concentrations of melanocortins have been found to enhance differentiation and regeneration of peripheral neurons in vitro. Peptides devoid of adrenocorticotropic activity (ACTH₄-₁₀, in some cases Organon 2766 and BIM 22015) accelerated in vivo the development of the neuromuscular junction. The peptides are thought to exert two stage-dependent effects during gestation; first they accelerate muscle development, then they modulate nerve development.
The consequences of trophic actions of melanocortins were increased nerve terminal arborization, enhanced electromechanical response of the muscle to stimulation through their nerves, and larger contraction force amplitudes. Neonate rats treated with ACTH peptides were more active than their saline treated litter-mates (Frischer et al., 1985 and 1988; Rose et al., 1988; Strand et al., 1993).

A relatively large number of publications has described effects of melanocortins on peripheral nerve recovery after experimental injury. The model system most often used was experimentally induced sciatic nerve crush in adult rats. The first study on a neurotrophic effect of ACTH1-39 was presented by Strand and Kung (1980) who reported that adrenalectomized rats subjected to sciatic nerve denervation recovered sooner after treatment with peptide, than after treatment with saline. In the following years, a range of other ACTH-derived peptides including α-MSH and Org 2766 were shown to facilitate sensory and motor recovery after sciatic nerve crush, if given in a distinct time-, dose- and application pattern (Bijlsma et al., 1981; Dekker et al., 1987; De Koning and Gispen, 1987; Girlanda et al., 1988; Laquerriere et al., 1994; Van der Zee et al., 1988). The peptides were active only if treatment was started soon enough and lasted long enough. The dose response curve was often bell shaped. Both, acetylated and nonacetylated peptides were active (Gonzalez and Strand, 1981; Van der Zee et al., 1988; De Koning et al., 1986).

Regeneration at the neuromuscular junction after experimental nerve crush in rats was also affected by ACTH/-MSH peptides. ACTH1-39 and other, non-corticotropic melanocortin fragments and analogs, initiated early axonal sprouting and the formation of more and smaller nerve sprouts, increased nerve terminal branching and arborization within the endplate and favored the formation of many small motor units rather than few large units which fatigue more quickly, as seen in the saline treated controls. There were differences in the effects of the various ACTH peptides on nerves and/or muscles: while α-MSH, ACTH4-10, and Organon 2766 mainly influenced nerve regeneration, BIM 22015 possessed mostly myotrophic activities and prevented muscle atrophy following nerve crush (Strand et al., 1991; 1993; 1994a).

Other models where melanocortins were shown to improve pathological disorders of the peripheral nervous system include peripheral neuropathies induced by cisplatin,
streptozotoxin, vincristin, taxol, or neuropathies associated with diabetes mellitus (Gispen et al., 1994; Bär et al., 1994).

3.3.2.6.2 Proliferation in the central nervous system

Early exposure to ACTH peptides has also long-lasting effects on behavior, apparent when these animals are tested as adults (Strand et al., 1989; Beckwith et al., 1977a and b).

Data addressing the question whether melanocortins exert a general trophic action on brain development (and also peripheral organs, such as adrenal gland, muscles and fat deposits) are controversial: s.c. injection of an α-MSH antiserum into E19 rat fetuses reduced brain growth (weight gain) and retarded body growth. By contrast, it did not affect growth if applied in the first postnatal two weeks (Swaab et al., 1978). Administration of peptides yielded controversial results: Fetuses of pregnant rats, treated from E9 to E22 (i.u. or i.p.) with α-MSH, did not differ significantly in fetal weight, crown-rump length, weight of fetal adrenal gland or placenta (Dawson et al., 1993), while des-acetyl-α-MSH, and in a higher dose also α-MSH and NDP, were reported to stimulate neonatal growth after 2 weeks of s.c treatment. All peptides affected muscular and brain tissue, des-acetyl-α-MSH additionally increased fat deposits (Mauri et al., 1995).

In vitro data on positive increased cell proliferation in response to melanocortins have been published for melanocytes (Abdel-Malek et al., 1995) and astrocytes (Zohar and Salomon, 1992). Proliferation of Schwann cells, in contrast, was not influenced (Hol et al., 1996). High doses of melanocortins exert an anti-proliferative effect on melanoma cells (Eberle, 1988). In vitro studies on the proliferation of neural cells are not available.

3.3.2.6.3 Differentiation

Few publications have dealt with effects of melanocortins on fetal nervous tissue (Bär et al., 1990; Darlington et al., 1996; Gispen et al, 1994).

Cell cultures of fetal rat spinal cord and early postnatal rat dorsal root ganglia have been studied most extensively (Hol et al., 1994; Peulve et al., 1994; Van der Neut et al., 1988; Van der Neut et al., 1992). Most often, neurite outgrowth or neurofilament
content and GAP-43 protein were measured. α-MSH, increased GAP-43 and neurofilament content and enhanced neurite outgrowth in spinal cord and dorsal root ganglia explants or dissociated cell cultures of these same structures (Van der Neut et al., 1988; 1990; 1992). Org 2766 was found to increase neurofilament content in E15 dorsal root ganglia prestimulated with βNGF, but was unable to influence neurofilament content in postnatal day (P) 5 dorsal root ganglia (Hol et al., 1994; Van der Neut et al., 1992). Another report mentioned that both Org 2766 and BIM 22015 were active in enhancing neurite outgrowth in E15 neuronal spinal cord and E15 dorsal root ganglia cultures, if these contained only neurons (Strand et al., 1994a). Data for ACTH4-10 and ACTH1-24 are inconsistent: these peptides enhanced neurite outgrowth in intact spinal cord cultures, but elevated GAP-43 content only weakly or not at all when dissociated spinal cord cells were used. The effect of ACTH on neurofilament content or neurite outgrowth was not checked in dissociated cultures (Van der Neut et al., 1988; 1992).

At the level of the brain, melanocortins have been shown to be active in several different neuronal tissues and at different developmental stages: Richter-Landsberg et al. showed that cultures from E16 cerebral cortex responded to α-MSH by an increase in the density of the neural network, the formation of neuronal aggregates and the fasciculation of neurites into neuritic bundles after two weeks in culture (Richter-Landsberg et al., 1987). Daval et al. examined P8 chick cortical hemispheres and observed increased survival and metabolism after 3 days in vitro and treatment of the cultures with ACTH1-24 (Daval et al., 1983). Cortical neurons in general and especially the subgroup of corticospinal neurons of P1 rat brain exhibited increased neurite outgrowth and GAP-43 content after 3 days in vitro and treatment with α-MSH (Joosten et al., 1996). A differential effect of melanocortins was published for rat E14 midbrain serotonergic cell cultures: ACTH4-10, ACHT1-24, ACTH1-39 and Organon 2766, but not α-MSH were able to stimulate (3H)5-HT uptake by serotonergic fetal neurons cultured for 5 days. In contrast, if these cells were co-cultured with E18 hippocampal tissue, one of their target areas, only α-MSH was active (Azmitia and Kloet, 1987). Müller et al. showed that Organon 2766 stimulated the formation of axonal microtubules in tissue obtained from snail ganglia (Müller et al., 1992).
In two studies on cell lines, melanocortins have been shown to induce neurite outgrowth in neuro 2A cells (Adan et al., 1996) and in chromaffin cells (Demeneix and Grant, 1988). With the exception of spinal cord, these studies were all done on regions of CNS and ganglia exhibiting low levels of melanocortin receptor expression during fetal life.

3.3.2.6.4 Regeneration in central nervous system

Numerous experiments have been performed to elucidate the role of melanocortins in central regeneration processes (Darlington et al., 1996; Strand et al., 1993).

Evaluation of effects of melanocortins on lesion-induced plasticity in the CNS is difficult, as it requires the measurement of a behavior or group of behaviors known to be disrupted by the lesion. In contrast, functional recovery in the PNS can be measured for instance by nerve conduction velocity. In addition, the term "recovery" must be interpreted carefully. In principle, a neurotrophic factor could influence the primary site of lesion by protecting neurons from further degeneration, initiating synaptogenesis, proliferation of new neurons, or regeneration of injured neurons. "Recovery" may possibly include compensatory effects as well, if for instance "recovery" is offered by receptor manipulation, collateral sprouting or increased neurotransmitter turnover of the surviving neurons. Melanocortins may act through any of these mechanisms.

In most studies performed, ACTH4-10 or Organon 2766 turned out to be the most potent melanocortins tested. This fact is difficult to interpret, as ACTH4-10 has only a very low affinity at all known melanocortin receptors, and Organon does not bind to them at all (Van Rijzingen et al., 1996). It has been postulated that the two peptides either bind to an as yet unknown melanocortin receptor, or that they modulate directly or indirectly another neurotransmitter system (Darlington et al., 1996; Van Rijzingen et al., 1996).

Beneficial effects of melanocortins have been shown after lesion of the septal area in rats (Isaacson and Poplawsky, 1983), bilateral lesions of the thalamic parafascicular area (Nyakas, et al., 1985), lesions of the septo-hippocampal pathway (Spruijt et al., 1990), lesions of the hippocampus (Hannigan et al., 1985), vestibular lesions (Flohr et al., 1982), lesions affecting projection areas of the dopaminergic system such as nucleus
accumbens (Wolterink et al., 1990) and striatum (Vos et al., 1990), and nigrostriatal 6-OHDA lesions (Antonawich et al., 1993) and after experimental spinal cord injury (Van de Meent et al., 1997). Data on cortical lesions are controversial (Atella et al. 1992; Darlington et al. 1996; McDaniel et al., 1989).

The effect of melanocortins is tissue, lesion and also task dependent; melanocortins more often exert positive effects on recovery models that involve damage to subcortical areas, whereas they seem less effective in models of cortical recovery. Dose, time, onset and duration of peptide administration are of critical importance for the effect of melanocortins (Darlington et al., 1996).

### 3.4 AIM OF STUDY

The early appearance and distinct temporal and spatial ontogeny of melanocortins and α-MSH binding sites in the CNS suggest a developmental role for these neuropeptides. In the present study, we aimed at the identification of the receptor type present in developing CNS on one hand, and at a characterization of effects of melanocortins on developing brain cells on the other hand. In the first part of this work we examined the pre- and postnatal ontogeny of mRNAs encoding for the two main melanocortin receptors in CNS, MC3-R and MC4-R in rat central and peripheral nervous system by in situ hybridization.

In the second part of the thesis, we cultured fetal cells from a brain area chosen for high receptor density according to recent data from this laboratory and investigated the influence of different melanocortins on developmental parameters. None of the earlier in vitro studies dealing with effects of melanocortins on developing nervous system has so far included areas with high melanocortin receptor density.
3.5 METHODOLOGY: GENERAL CONSIDERATIONS

3.5.1 Part 1: Analysis of melancortin receptor ontogeny in rat nervous system by in situ hybridization

In situ hybridization allows the visualization of a specific mRNA in a precise anatomical context within a particular cell or cell group (Watson et al., 1987). A specific mRNA species can be visualized using a labeled complementary nucleic acid (either RNA or DNA) probe as the indicator. The important principle is that two DNA strands or one DNA and one RNA strand hybridize under the proper conditions by forming hydrogen bonds with the potential partner (Watson et al., 1987).

According to previous experiments in our laboratory, the sensitivity of the technique can be increased by the use of two 60mer P<sup>33</sup> labelled oligonucleotides complementary to two different sequences of a given mRNA. Care was taken to choose 60mer oligos with > 50% GC content (increased hybrid stability), without repetetive sequences (danger of internal hybridization), as few as possible base-pair mismatches (decreases hybrid stability), and without sequence identity with other mRNA’s.

Radioactive labelling of oligo probes was performed according to previously established techniques. There are two methods to label oligonucleotides, using either the T4 polynucleotide kinase which adds one labeled reporter to the 5’end of the oligonucleotide, or the terminal transeferase, which adds several labeled reporters at the 3’-end of the oligonucleotide. The terminal transferase reaction yield probes with high specific activity, even if the radionuclids used are of lower specific activity. Usually 35S, but also 33P are used because of their higher sensitivity and better resolution as compared to 32P, though they require longer film exposure times (Emson, 1993). We chose 33P rather than 35S because stronger signals can be obtained after a shorter exposure time compared to 35S because of its higher β-energy and higher specific activity. Additionnally, 33P use has been reported to result in improved background levels compared to 35S (Baskin et al., 1993).

1 Details of materials and methods are given in the appropriate section of part 1.
The strength of the hybrid formed after in situ hybridization of the oligo probe to the target nucleic acid depends on the properties of the oligo probe and on hybridization conditions like temperature, salt and formamide concentration (Emson, 1993). The temperature at which 50% of the duplex molecules in solution dissociate into single strands, \( T_m \), provides a useful index to determine hybridization and washing temperature. Hybridization is usually performed at 15°C below \( T_m \). The calculation of \( T_m \) is based on the following formula (Young, 1990):

\[
T_m = 79.8 + 18.5 \log[Na^+] + 0.584(\% GC) + 0.118(\% GC)^2 - 1.4(\% \text{ mismatched pairs}) - 820/\text{base length} - 0.5(\% \text{ formamide})
\]

The hybridization temperature was calculated with this formula, other hybridization conditions were chosen from protocols in the laboratory (Lauber and Lichtensteiger, 1994).

### 3.5.2 Part 2: Effect of melanocortins on rat fetal striatal/midbrain cocultures

#### 3.5.2.1 Choice of in vitro model system

We decided to study the effects of melanocortins on neuronal development in a co-culture of fetal rat striatum and midbrain. The choice of the regions was based on the following considerations:

- **a)** Striatum belongs to the regions with highest density of \([^{125}I]\)NDP binding sites during ontogeny and strong concomitant MC4-R mRNA (Lichtensteiger et al., 1996, Kistler-Heer et al., in press and this work). Receptor sites become detectable by E16, with a peak at E22.
- **b)** the ontogeny of these regions is well studied, major in- and output regions and developmental events are documented
- **c)** striatum and midbrain are easily dissectible and yield a sufficient number of cells
d) Since MC-R were found to develop in parallel with events of neuritogenesis and synaptogenesis in many areas (Lichtensteiger et al., 1996), we decided to co-culture striatum with midbrain in order to provide an opportunity for interactions. Substantia nigra represents an important target and input region of the striatum. Caudate-putamen cell cultures were also examined in the absence of midbrain cells. We are well aware, that this type of co-culture is still an artificial system, as for instance the mayor part of another input region, the cortex, is missing.

Cultures of striatal cells were prepared from E18 rat fetuses, shortly before the peak of α-MSH binding site density. Striatal and midbrain tissue was dissociated into a single cell suspension, mixed, and seeded into culture vessels. Cell density was adjusted to a fixed value because cell survival and quality of cultures are influenced by cell density (Nakao et al., 1996). The method of dissociated cell cultures was chosen because it allows standardized quantitative determinations of different parameters by enzyme-linked immunoassays.

The culture medium consisted of a defined, serum free medium (DMEM), enriched with several essential vitamins, hormones, trace elements and albumin (Albumax®, Life Technologies). This medium, developed by Honegger and co-workers (Honegger et al. 1979; Riederer et al. 1992; Zurich et al. 1993; Zurich et al. 1994), allows neuronal and glial survival and differentiation for up to 4 weeks in culture.

3.5.2.2 Development of the striatum and its major connections in the rat

The adult striatum comprises mostly (> 95%) medium sized neurons with spiny dendrites which use GABA and neuropeptides as neurotransmitters, and a small number of large cholinergic neurons with long aspiny dendrites.

In the rat neurogenesis of the large, cholinergic interneurons occurs between E13 and E16 (Bayer, 1984). Neurogenesis of the smaller, spiny GABAergic neurons can be divided into two phases. The early born neurons that are localized in the striatal patches (striosomes), are generated between E13-E15. Neurons of the striatal matrix are born during late fetal life (Van der Kooy and Fishell, 1987). Immunostaining for GABA is

Nigrostriatal projections reach the striatum as early as E14 (Moon Edley and Henkenham, 1984), soon after the generation of the first striatal neurons. The first striatoniigral projections, most probably originating from striatal neurons, reach the SN at E16. A second (matrix-neuron) population makes connections with the SN early postnatally (Fishell and Van der Kooy, 1987). Dopamine release and uptake can be measured from E17 (Nomura et al., 1981). In the first postnatal week, both patch and matrix neurons undergo massive cell death (approx. 30%), but cells that have an early projection to the SN are preferentially spared (Fishell and Van der Kooy, 1991).

Another major input to striatum originates from cerebral cortex. In the rat, glutamergic cortical inputs arrive at the end of the first postnatal week in the striatum and mature during the second postnatal week (Iniguez et al., 1990). Glutamergic receptors in striatum are not mature until PN7 (Campochiaro and Coyle, 1978). Projections from thalamus (intralaminar thalamic nuclei) and from raphe are present at birth (Iniguez et al., 1990). Efferent connections from striatum to globus pallidus and entopeduncular nucleus are established already at birth, whereas the pallidal afferents to the striatum are only distinguished from the 14th postnatal day onwards.

The occurrence of NDP binding sites and MC-R mRNA in the striatum thus correlates with the outgrowth of striatoniigral fibres from patch neurons (form E16), the generation of matrix neurons (E18-E21), their innervation by dopaminergic fibres (from E16), and the maturation of the first dopaminergic synapses (from E17). Massive cell death of striatal neurons occurs in the first postnatal week, when the number of NDP binding sites is diminishing in particular in matrix and dorsal patches. Melanocortins acting during this period therefore could influence a number of parameters, beginning with birth of neurons, cell differentiation, fibre outgrowth, and ending with neuronal survival.
3.5.2.3 Choice of developmental parameters for the in vitro study

Endpoints selected included typical markers for cell generation and survival (MTT), neuritogenesis (neurofilament protein), growth cones (growth-associated protein GAP-43), and synaptogenesis (synaptophysin). Neurofilament protein and GAP-43 have been shown to be influenced by melanocortins in other in vitro model systems (Van der Neut et al., 1988; 1992).

3.5.2.3.1 Survival rate

The MTT-assay measures the amount of the purple formazan product generated by mitochondrial dehydrogenases of living cells from 3-(4,5-dimethythiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT). The formazan product is insoluble in the culture medium, but can be dissolved by addition of DMSO (Hefti et al., 1978). The ratio between cell number and optical density (OD) of the formazan is linear (Hansen et al., 1989; Mossmann, 1985). OD is measured with an ELISA-reader. The assay reflects the sum of proliferation or cell death.

3.5.2.3.2 Neurofilament

Neurofilaments belong to the family of intermediate filaments of the cytoskeleton and are uniquely found in neurons. They are distributed in axons, dendrites and cell body, in characteristic longitudinal arrays of single neurofilaments or of neurofilament bundles linked by crossbridges. Mammalian neurofilaments are composed of three different protein subunits with different molecular weights: 73kDa (L, light), 145kDa (M, medium), and 200kDa (H, heavy). L subunits form a core filament around which two filaments, formed by M and H subunits are coiled. The M an H subunits are exposed at the surface and form cross-bridges linking neurofilaments. The main function of intermediate filaments is to provide structural stability to cells. Neurofilaments are responsible for the radial growth and the diameter of an axon (Jacobson et al., 1991; Lodish et al., 1995)

All three forms of neurofilament proteins undergo extensive posttranslational modification: addition of amino acid sequences and phosphorylation at the COOH terminal (by PKC). L and M proteins are found in axons and dendrites. The phosphoryla-
tioned H-subunit is usually restricted to axons and the non-phosphorylated H-subunit is located in somata and dendrites (Riederer et al., 1990). Exceptions are for instance the large dorsal root ganglion neurons which also express phosphorylated neurofilaments in neuronal perikarya, too (Lawson et al., 1984).

Phosphorylation seems to be developmentally regulated. The dephosphorylated form of neurofilament proteins appears earlier in development (Riederer et al., 1990). The temporal pattern of neurofilament expression is tissue specific, but L and M subunits appear earlier in development than H subunits (Sternberger and Sternberger, 1983; Shaw and Weber, 1982; Foster et al., 1987; Riederer, 1990). Phosphorylated H neurofilament is also observed in the cell body compartment of neurons after various types of neuronal injury (Draeger and Hofbauer, 1984; Shaw et al. 1988).

We used an antibody which crossreacts with the phosphorylated epitopes of both the M- and H- neurofilament protein (Wood and Anderton, 1981). It can thus be considered to indicate an advanced stage of maturation.

3.5.2.3.3 Growth associated protein GAP-43

Growth-associated protein (GAP-43) is a neuron-specific membrane-bound phosphoprotein that appears to play a role in the development and functional modulation of synaptic relations. In transgenic mice, overexpression of GAP-43 leads to the spontaneous formation of aberrant connections and new synapses, and to enhanced sprouting after injury, whereas null mutation of the GAP-43 gene disrupts axonal pathfinding and is lethal shortly after birth (Strittmatter et al., 1995). GAP-43 seems to be involved in transducing intra- and extracellular signals to regulate cytoskeletal organization in the nerve ending. It is a substrate of protein kinase C and is able to bind calmodulin in the absence of calcium (Benowitz and Routtenberg, 1997).

GAP-43 is found in cell bodies just after their final cell division and is expressed extensively in elongating axons and growth cones during development and regeneration. GAP-43 content decreases rapidly in most areas of the brain shortly after synapse formation, except for regions associated with high synaptic plasticity in adulthood (limbic and associative regions) (Benowitz and Routtenberg, 1997; Skene, 1989). The amount of GAP-43 in newly formed presynapses is lower than in growth cones, suggesting a
possible correlation between the presence of GAP-43 and the stage of presynaptic terminal development (Burry et al. 1991; 1992). GAP-43 is again upregulated during regenerative processes.

3.5.2.3.4 Synaptophysin

Synaptophysin is a 38kDa integral membrane glycoprotein that copurifies with synaptic vesicles. It is found at terminals of all nerve cells and in a variety of neuroendocrine tissues. It has four transmembrane domains and is present in the membrane of vesicles as a tetrameric homo-oligomer (Trimble and Scheller, 1988). Synaptophysin is, together with other proteins, involved in the synaptic vesicle cycle which consists of vesicle exocytosis with neurotransmitter release, endocytosis of empty vesicles and regeneration of fresh vesicles (Südhof, 1995).

Synaptic transmission depends on exocytotic release of transmitter from two types of vesicles, large dense core vesicles and small synaptic vesicles. Synaptophysin is a typical membrane constituent of small vesicles which are implicated in the release of classical neurotransmitters. Its occurrence in dense core vesicles is low (Mahata et al., 1993).

In mouse brain, the level of synaptophysin dramatically increases from birth to adulthood in a time course that appears to follow the process of synaptogenesis (Knaus et al., 1986). Therefore synaptophysin content has often been correlated with synaptogenesis (Knaus et al. 1986). In hippocampal cultures it was shown that the target cells must have reached a critical age before synaptophysin immunoreactivity is detectable (Fletcher et al., 1994). Data from spinal cord (Bergmann et al., 1991) and cerebellum (Leclerc et al., 1989) suggest that the appearance of the protein precedes the onset of synaptic function. Thus, the presence of synaptophysin in nerve terminals appears to reflect an advanced stage of synaptogenesis but does not necessarily indicate functional synapses.

Synaptophysin immunoreactivity appears first in the perikarya of cells, and later translocates to nerve fibres and presynaptic endings (Erhart-Bornstein et al., 1991; Ovtscharoff et al., 1993). In order to use measurements of synaptophysin as an index of number of synapses, we determined the protein in a synaptosomal fraction.
4 DEVELOPMENTAL PATTERNS OF MELANOCORTIN
MC3 AND MC4 RECEPTOR mRNA

4.1 ABSTRACT

Melanocortins are thought to be involved in neuronal development and regeneration. Pro-opiomelanocortin (POMC), the precursor of α-melanocyte stimulating hormone (α-MSH), γ-MSH, ACTH, and β-endorphin, becomes detectable in rat hypothalamic neurons from gestational day (E) 12.5. We recently described stage- and region-specific ontogenetic patterns of binding sites for the α-MSH analog [125I]Nle⁴, D-Phe⁷-α-MSH ([125I]-NDP), with the first localizations in epithalamus and sympathetic chain at E13. [125I]-NDP binds to all known melanocortin receptors including MC3-R and MC4-R, the predominant melanocortin receptors in nervous system. To identify the receptor type expressed during ontogeny, the developmental pattern of MC3-R and MC4-R mRNA was investigated by in situ hybridization in fetuses and offspring of time-pregnant Long Evans rats between E14 and postnatal day (P) 27. MC4-R mRNA was found to be the predominant species during the entire fetal period. It was localized in all fetal areas exhibiting distinct [125I]-NDP binding, starting with sympathetic ganglia and epithalamus (E14), and including sensory trigeminal nuclei (E16), dorsal motor nucleus of vagus (E16) and cranial nerve ganglia, inferior olive (E18) and cerebellum (E18), striatal regions (E16), and entorhinal cortex (E22). In contrast, MC3-R mRNA was detectable only in the postnatal period, with a fast increase in expression in the ventromedial and arcuate nuclei. The early presence of MC4-R mRNA in central and peripheral nervous system and transient regional peaks of mRNA expression, often concomitant with periods of neural network formation, suggest a role of this receptor type in early ontogeny. The MC3 receptor may be involved in analogous processes during postnatal development.

4.2 INTRODUCTION

The pro-opiomelanocortin (POMC)-derived melanocortins, α-melanocyte stimulating hormone (α-MSH), γ-MSH, adrenocorticotropic hormone (ACTH) and related peptides, are involved in a variety of physiological processes, such as regulation of adrenal steroid synthesis and pigmentation, control of body weight (Fan et al., 1997), reduction of fever (Catania et al., 1993), regulation of heart rate and blood pressure (Li et al., 1996; Van Bergen et al., 1995), and behavior (Klavdieva et al., 1996). One interesting additional feature is their possible role in development and regeneration of the nervous system (Bär et al., 1990; Darlington et al., 1996; Strand et al., 1993). In vitro, melanocortins enhance neural differentiation and neurite outgrowth (Demeneix et al., 1988; Joosten et al., 1996; Van der Neut et al., 1988; Van der Neut et al., 1992); there is also evidence for developmental effects in vivo (Swaab et al., 1976; Li et al., 1986; Strand et al., 1989).

POMC is one of the earliest neuropeptide precursors expressed in the brain, appearing as early as embryonic day E12.5 in rat hypothalamus (Khachaturian et al., 1985). We recently identified melanocortin binding sites in the developing rat central and peripheral nervous system from E13 using \[^{125}\text{I}]-\text{Nle}^4\text{,D-Phe}^7\text{-MSH} ([^{125}\text{I}]-\text{NDP}), a potent \text{α-MSH} analog with similar affinities to all known melanocortin receptors (Lichtensteiger et al., 1996). The study revealed a complex temporal and spatial developmental pattern of melanocortin binding sites. High levels of \[^{125}\text{I}]-\text{NDP} binding were transiently present during fetal and perinatal ontogeny in a number of telencephalic and lower brainstem regions as well as in cranial nerve ganglia and sympathetic ganglia, supporting the idea of an involvement in specific phases of ontogeny.

In order to clarify the possible developmental role of melanocortins, it is important to identify the type of receptor present during early ontogeny. To date, five different melanocortin receptors (MC-R) have been cloned, i.e., MC1-R (Chajlani et al., 1992; Mountjoy et al., 1992), MC2-R (ACTH receptor) (Mountjoy et al., 1992), MC3-R (Roselli-Rehfuss et al., 1993; Desarnaud et al., 1994), MC4-R (Mountjoy et al., 1994; Alvaro et al., 1996), MC5-R (Gantz et al., 1994; Griffon et al., 1994; Labbé et al., 1994). Two of these receptors, the MC3-R and MC4-R, occur primarily in the central nervous system. The MC5-R is also expressed in brain, but in very low amounts (Griffon et al., 1994). We examined the pre- and postnatal ontogeny of MC3-R and
MC4-R mRNA expression in rat central and peripheral nervous system by *in situ* hybridization and found a clear difference in developmental patterns. MC4 is predominant during the entire fetal period and thus, may be of particular importance in developmental processes.

### 4.3 MATERIALS AND METHODS

#### 4.3.1 Animals

Long-Evans rats were bred in our laboratory under controlled conditions (Lights on 02.00 - 16.00 h, 22±1°C) with a standard diet (NAFAG 850, Gossau, Switzerland) and water *ad libitum*. Time-pregnant rats were used for investigations of prenatal and postnatal stages. Nulliparous receptive females were mated with experienced males between 16.00 and 19.00. Pregnant rats were housed in groups of two to four animals and separated two days before parturition. The stage 24 h after the mating period was defined as gestational day E1, the day of birth (E23) as postnatal day P1.

#### 4.3.2 Tissue Preparation

Fetuses were dissected from dams anesthetized by injection of chloral hydrate (400mg/kg s.c.), followed by ether. Postnatal pups were decapitated. The whole body (E14, E16), the head (E18, E22) or the brain (P8, P27) was frozen in isopentane cooled and stored in liquid nitrogen. Serial 10µm sections were cut in a cryostat (Leitz 1720) and mounted to silanized glass slides. Sections were allowed to air dry, then fixed in 10mM phosphate buffered salt solution pH 7.4 (PBS) containing 4% paraformaldehyde for 20 min at room temperature, washed three times in PBS for 5 min, dehydrated with ascending concentrations of ethanol and stored at -80 °C until use.

#### 4.3.3 Oligonucleotides and hybridization

To increase the signal in fetal tissues, we combined two oligonucleotides hybridizing to different sequences of the mRNA species (Lauber and Lichtensteiger, 1994). For both the MC3 and the MC4 receptor, two specific 60 bp long oligonucleotides were used for the *in situ* hybridization. None of the sequences used showed significant simi-
larities to any of the other melanocortin receptors cloned to date, according to computer assisted analysis. For the rat MC3 receptor we used an oligomer complementary to a 5'-region (bp 373-432, 5'-AAG ACC TCT GGC TTG ATG AAA ACC TGC TCG CAG AAC CCA CTG CCA CTC CGG TTG CTG GCA-3') and one complementary to a 3'-region (bp 937 - 996, 5'-TGC GGGGCT ACC CCGTCA GCA GGT GGC AGT GCC GCG ATG CGC TGG ACG TGC AGC CTG GCG-3') of the cDNA (GenEMBL X70667). The sequence of the rat MC4 receptor was not yet published at the time this study was undertaken. Therefore, one oligonucleotide (bp 484 - 543, 5'-GCC TGA AAC CGT GCA AGC TGC CCA GAT ACA ACT TAT GAT GAT CCC AAC CCG CTT AAC TGT-3') was chosen from gene bank data of the human MC4 receptor cDNA (GenEMBL L08603). The the second oligonucleotide (5'-CCG GAG ACA GCA TCC TCT TAT GTG AAG CCT CGC CAT CAG GAA CAT GTG GAC ATA GAGAGA-3') was synthetized according to sequence data kindly provided by R. Cone (Portland, Oregon). The mismatch to the recently published rat MC4 receptor sequence is 11% for the first oligonucleotide and 20% for the second (Alvaro et al., 1996). The four oligonucleotides (PAGE purified) were ordered from Microsynth (Balgach, Switzerland) and labelled at their 3'- end with [α-33P]deoxy-ATP (DuPont-New England Nuclear, Boston, MA) using terminal transferase (Boehringer Mannheim, Switzerland). Labelling efficiency (tail length) was checked by electrophoresis of the oligos on a denaturating 10% urea-polyacrylamide gel. The tailing reactions varied from experiment to experiment and consisted of approximately 10 to 20 nucleotides.

The two labelled oligonucleotides for each receptor were pooled and diluted in hybridization solution to result in approximately 300'000 to 400'000 cpm of each oligonucleotide per slide, depending on the size of the sections. The hybridization solution consisted of 5 x SSC (0.75M NaCl and 75mM sodium citrate dihydrate), 125mg/ml dextran sulfate (Fluka Biochemica, Switzerland), 50% formamide (Fluka Biochemica, Switzerland), 2.2 x Denhardt's solution [0.44mg/ml ficoll (type 400, Pharmacia, Piscataway, NJ), 0.44mg/ml polyvinylpyrrolidone (Merck, Basel, Switzerland), 0.44mg/ml BSA (fraction V, Sigma Chemical Co., St. Louis, MO)], 125M dithiothreitol (Gerbu Gaiberg, Germany), 0.25mg/ml herring sperm DNA (Boehringer Mannheim Germany), 0.25mg/ml polyadenylic acid (Boehringer Mannheim, Germany), and 0.25mg/ml transfer RNA (Boehringer Mannheim, Germany). Fifty to hundred microliters of this solution were added to each slide (containing two sections each) covered with appropriately si-
zed pieces of Fuji Sealon film and incubated overnight in a humified chamber at 43 °C. Nonspecific binding was determined by adding 100-fold excess of unlabelled oligonucleotides to the incubation mixture. After hybridization, slides were washed in two 15 min rinses at 55 °C with 1 x SSC (150mM NaCl and 15mM sodium citrate dihydrate) containing 2mM of dithiothreitol (DTT), then two 15 min rinses at 55 °C with 0.5 x SSC (75mM NaCl and 7.5mM sodium citrate dihydrate) containing 2mM of DTT, one 15 min rinse at RT with 0.5 x SSC with 2mM DTT and finally two quick dippes in distilled water. The sections were dehydrated with a series of ethanol washes in 70%, 96% and 100% ethanol and air dried.

The sections were apposed to an Amersham Hyperfilm β-Max autoradiography film (Amersham, Switzerland) at 4 °C for three months. After three months of exposure, more than 90% of the 33P-labelled isotopes have deposited their energy and further exposure does not greatly enhance the signals. Films were developed manually using developing and fixing solution from Kodak (Kodak, Switzerland). All sections were stained with 1% toluidinblue solution.

4.3.4 Ontogenetic studies

Prenatal stages, E14, E16, E18 and E22, and postnatal stages, P8 and P27, were examined (two independent in situ hybridization series per developmental stage). MC3 and MC4 receptor mRNA localization was studied on neighbouring sections in the same fetus or postnatal brain (sequences of 24 sections, with 4 sections MC4 hot, 4 sections MC3 hot, 4 sections MC4 cold, 4 sections MC3 cold, 4 sections MC4 hot, 4 sections MC3 hot, followed by 2 sections for histology). At E14, MC3-R and MC4-R were studied with separate fetuses. The location of the labelled structures was determined by comparison of the in situ hybridization signal with the adjacent section with non-specific binding and the corresponding stained section. The signals were examined by visual inspection and estimated semiquantitatively. Images of the in situ hybridization, the non-specific binding and the histology, taken with a high resolution video camera and a MCID Type 1 Image Analysis system (Imaging Research, Inc., St. Catharines, Ontario, Canada) were superimposed to determine the exact location and the specificity of the signal. Readings of relative optical density of total and non-specific binding were taken to confirm the specificity of weak signals. Photographs were taken on a Sony co-
lour video printer. Brain areas were chosen to correspond to levels shown in our earlier publication on melanocortin binding sites (Lichtensteiger et al., 1996).

4.4 RESULTS

In preliminary experiments, the structures labelled by one or the other of the MC4-R oligonucleotides were found to be identical. The same was true for the oligos designed for MC3-R (data not shown).

Labelling was prevented in all areas by adding 100-fold excess of unlabelled oligonucleotides (except occasionally in skeletal structures where a higher concentration of cold oligos would be required). Nuclei known to express mainly MC3-R (e.g. arcuate nucleus) or MC4-R (e.g. nucleus accumbens) exhibited the corresponding signals with the oligos used in the present study. Throughout the central and peripheral nervous system, a high degree of overlap of MC3-R or MC4-R mRNA with binding sites for \([^{125}\text{I}]\text{NDP}\) (Lichtensteiger et al., 1996), a high-affinity ligand at all known MSH receptors, was observed. There was a striking difference between the onset of expression of the MC3-R and MC4-R mRNA during development. The mRNA encoding MC4-R could be detected as early as E14 (the earliest stage examined) whereas mRNA for MC3-R became detectable during late prenatal/early postnatal stages.

4.4.1 Ontogeny in sympathetic ganglia

One of the localizations of MC4-R mRNA is in the sympathetic chain. This signal was detected as early as E14, the first stage examined and labelling was higher subsequently (Fig. 3, Table 6). The superior cervical ganglion was labelled at E14, while both, superior cervical and stellate ganglion were strongly labelled at E16. mRNA density was very high as compared to other localizations in the brain throughout development (Tab. 6b). This is analogous to the high density of \([^{125}\text{I}]\text{NDP}\) binding sites in fetal sympathetic ganglia (Lichtensteiger et al., 1996). By E22, MC4-R mRNA had disappeared in the superior cervical ganglion. Throughout development the sympathetic ganglia were devoid of mRNA encoding for MC3-R (Fig. 3, Tab. 6b).
Fig. 3  MC4-R mRNA located by in situ hybridization in sympathetic ganglia and cranial nerve ganglia. A. Histology of (B), sagittal section at gestational day E14, (li) liver, (sc) spinal cord, (sy) sympathetic chain, (t) telencephalon. B. Strong MC4-R mRNA signal in the sympathetic chain (sy). C. Histology of (D), sagittal section at E16, (h) heart, (mo) medulla oblongata, (st) stellate ganglion. D. Presence of MC4-R mRNA in stellate ganglion (st) and trigeminal spinal tract nucleus (s5). E. Section adjacent to (D), in situ hybridization for MC3-R mRNA (negative). F. Non-specific binding in section adjacent to (D), incubated with 100 fold excess of unlabelled MC4-R oligonucleotides. G. Histology of (H), sagittal section at E18, (gf) jugular ganglion, (gs) superior ganglion, (gn) nodose ganglion, (ie) inner ear. H. MC4-R mRNA in nodose (gn), jugular (gf) and superior (gs) ganglion and in trigeminal spinal tract nucleus (s5). Scale bar 500 µm. Nomenclature according to J. Altman and S.A. Bayer, Atlas of Prenatal Rat Brain Development, CRC Press, 1995, and G. Paxinos et al., Atlas of the Developing Rat Brain, Academic Press, 1991.

4.4.2 Diencephalon

4.4.2.1 Thalamus and Epithalamus.

In brain, MC4-R mRNA was first detected at E14 in the area corresponding to the epithalamus just medially to the dorsal origin of the habenulopeduncular tract (Fig 4, Tab. 6a). At E16, a weak signal appeared in the developing anterior and intermediate thalamus. During later fetal and postnatal stages, several thalamic nuclei expressed low amounts of MC4-R mRNA, for instance the paraventricular thalamic nucleus, the lateral posterior thalamic nucleus and the nucleus reuniens at E22 and the posterior thalamic nucleus (triangular part) at P8. All these signals were weak and transient (Fig 5, Table 6a). MC3-R mRNA was not detected in fetal thalamus. At P27, distinct labelling was present in the medial habenular nucleus (Fig 5, Table 6a).
Fig. 4  Early presence of MC4-R mRNA in rat epithalamus. A. Histology of (B), sagittal section through telencephalon (t), diencephalon (d) and midbrain (m). B. Localization of MC4-R mRNA in the epithalamic neuroepithelium (et) by in situ hybridization at gestational day E14. C. Non-specific binding in section adjacent to B, incubated with 100 fold excess of unlabelled MC4-R oligonucleotides. Scale bar 500 µm.
4.4.2.2 Preoptic region and hypothalamus.

Preoptic regions exhibited a late fetal onset of MC4-R mRNA expression with a gradual increase in early postnatal life (Table 6a). The first labelling was detectable at E18 in the medial preoptic area, the lateral preoptic area followed at P8. In the hypothalamus, the paraventricular nucleus exhibited moderate labelling from E18 which stayed high during postnatal life and still was present at P27 (Fig. 5). In the dorsoposterior hypothalamus a transient signal was detected at E18 and had disappeared by E22. The supraoptic nucleus and the dorsal and dorsomedial hypothalamic area expressed MC4-R mRNA only postnatally, at P8 and P27, respectively. By contrast to \[^{125}\text{I}]\text{NDP} binding (Lichtensteiger et al., 1996), which was detectable in mammillary regions at E17 with a strong signal in the ventral premammillary nucleus postnatally, MC4-R mRNA labelling was very low in the mammillary regions. MC4-R mRNA was detectable in very low amounts at P8 in the medial part of the medial mammillary nucleus. The strong signal in the ventral premammillary nucleus seen with \[^{125}\text{I}]\text{NDP} binding can be assigned to MC3-R; the MC3-R mRNA signal was distinct at P27 (Fig. 9, Table 6a).

MC3-R mRNA was detected only after birth. A rather strong signal was seen in the ventromedial hypothalamus, and slightly weaker labelling in the arcuate nucleus from P8 (Fig. 5). These two conspicuous locations are joined by other weak signals in the dorsoposterior and the lateral hypothalamus (Table 6a). Preoptic areas which have been reported to contain MC3-R mRNA in adult rat brain (Roselli-Rehfuss et al., 1993) were devoid of labelling until P27. No mRNA for either MC3 or MC4-R was detected in the developing pituitary, which also exhibited \[^{125}\text{I}]\text{NDP} binding during fetal life (Lichtensteiger et al., 1996).

4.4.3 Mesencephalon

MC4-R mRNA was first detected at E16 in the pretectal area and in the ventral tegmentum. Labelling in the pretectal area was concentrated in the anterior pretectal area by E18 and was visible up to P27 (Fig. 6, Tab. 6b). The number of MC4-R mRNA-containing areas increased by E18. A distinct signal was present in the area of the magnocellular interstitial nucleus of the posterior commissure at E18 and E22. This nucleus is strongly labelled by \[^{125}\text{I}]\text{NDP} at the same developmental stage (Lichtensteiger et al., 1996).
The superior colliculus expressed MC4-R mRNA at E18 (Fig. 7). Ventral tegmentum exhibited an early MC4-R mRNA signal until E22 (Fig. 6), followed later by a MC3-R mRNA signal at P8, which became stronger by P27 (Fig. 6, Table 6b). Other mesencephalic areas containing MC3-R mRNA at P27 are substantia nigra (Fig. 6) and nucleus raphe linearis. The interpeduncular nucleus, which showed high $[^{125}I]$NDP binding at P27, was devoid of MC3-R mRNA or MC4-R mRNA signals at the developmental stages studied (Fig. 6).

### 4.4.4 Somatosensory and viscerosensory brainstem nuclei and cranial ganglia

Prominent expression of MC4-R mRNA was detected from E16 in the principal sensory trigeminal nucleus and the spinal tract nucleus of the trigeminal nerve (Fig. 7, Table 6b). These areas expressed MC4-R mRNA only transiently; the signals had disappeared by E22 and P8, respectively (Table 6b). The subnucleus zonalis at the surface of the posterior section of the trigeminal spinal tract nucleus, which exhibited strong labelling with $[^{125}I]$NDP between P8 and P27 (Lichtensteiger et al., 1996), contained no detectable mRNA for MC3-R or MC4-R at these stages. In the nucleus of the solitary tract, MC4-R mRNA was present from E16 to P27 (Fig. 7, Table 6b).

Some cranial nerve ganglia are strongly labelled by $[^{125}I]$NDP during fetal life (Lichtensteiger et al., 1996). MC4-R mRNA was expressed at E18 in the trigeminal ganglion, geniculate ganglion of the facial nerve, nodose and jugular ganglia of the vagus nerve (Fig. 3), superior ganglion of glossopharyngeal nerve, and vestibular ganglion.
Fig. 6 MC4-R mRNA and MC3-R mRNA in anterior midbrain, E18 - P27. A. Histology of (B), frontal section at E18, (v3) third ventricle, (vl) lateral ventricle. B. MC4-R mRNA at E18. C. MC4-R mRNA at E22. D. MC4-R mRNA at P27. E. MC3-R mRNA at P27. F. Histology of (E), frontal section. Melanocortin receptor mRNA is present in anterior pretectal area (ap; MC4), interstitial magnocellular nucleus of posterior commissure (im; MC4), substantia nigra (sn; MC3), ventral tegmental area (vt; prenatally MC4, postnatally MC3), and hippocampus (hi; MC4). The interpeduncular nucleus (ipd), which shows strong [125I]NDP-binding at P27, is devoid of MC3 and MC4-R mRNA. Scale bar 500μm.
By E22 no MC4-R mRNA was detected in these locations except for the nodose nucleus (Table 6b). No MC3-R mRNA was detected in somatosensory or viscerosensory nuclei and cranial nerve ganglia during the entire period investigated. The follicles of the vibrissae which exhibit intense $^{125}$I]NDP binding from E17 (Lichtensteiger et al., 1996), were devoid of either MC3-R or MC4-R mRNA.

4.4.5 Motor and relay nuclei of lower brainstem, and cerebellum

In the dorsal motor nucleus of vagus, MC4-R mRNA was expressed from E16 throughout ontogeny (Fig. 7, Table 6b). In the hypoglossal motor nucleus, MC4-R mRNA was expressed between E18 and E22. Neither MC3-R nor MC4-R mRNA was detected in the trigeminal motor nucleus, the facial motor nucleus and in the nucleus ambiguus. In the inferior olive MC4-R mRNA was detected first at E18. The principal and medial olivary nuclei were strongly labelled between E22 and P8 (Fig. 7). Thereafter the intensity of the signal decreased rapidly. By contrast, the dorsal cap of Kooy (subnucleus K) exhibited strong MC4-R mRNA expression until P27 (Fig. 7, Table 6b).

The pontine dorsal tegmental nucleus was labelled by E18 and expressed MC4-R mRNA up to P27 (Fig. 7, Tab. 6b). In the dorsal parabrachial nucleus, MC4-R mRNA was first detected at E22 and remained detectable up to P27. MC4-R mRNA in the nucleus of the lateral lemniscus was transiently expressed between E18 and P8. The reticular nuclei of pons and medulla also expressed MC4-R mRNA; the signal was prominent in the dorsal and lateral paragigantocellular reticular nuclei (E18-P27). Very weak, but specific, labelling was also found in some of the raphe nuclei. The raphe linearis showed a specific MC3-R mRNA signal at P27 (Table 6b).

The cerebellum expressed MC4-R mRNA. The mRNA was transiently expressed in the cortex of the developing hemisphere at E18 (Table 6b).
4.4.6 Spinal cord

The spinal cord exhibits $[^{125}\text{I}]$NDP binding sites by E15 (Lichtensteiger et al., 1996). MC4-R mRNA was detected somewhat later, by E16 and E18 (Table 6b). The substantia gelatinosa expressed MC4-R mRNA at P27. No signals for MC3-R mRNA were detected during fetal life (Fig. 7).

4.4.7 Telencephalon

4.4.7.1 Basal ganglia and septal region.

A very faint labelling of the caudate putamen was the first signal seen in this area at E16. By E18, caudate putamen, nucleus accumbens and the olfactory tubercle exhibited strongly positive signals for MC4-R mRNA (Fig. 8, Table 6a). At E22, the mRNA signal showed an essentially homogeneous distribution in caudate-putamen similar to $[^{125}\text{I}]$NDP labelling (Lichtensteiger et al., 1996). Olfactory tubercle and nucleus accumbens remained positive until P27. In the caudate putamen however, MC4-R mRNA expression declined after E22 until P27. mRNA density decreased first in the dorsal patch and finally, positive labelling was observed just in the (rostral part of) fundus striatum.
Fig. 8 Telencephalon, E18 - P27. A. Histology of (B), frontal section at E18. B. MC4-R mRNA at E18. C and E. MC4-R mRNA and MC3-R mRNA, respectively, at E22. E. Histology of (F), frontal section at P8. F. MC4-R mRNA at P8. G and H. MC4-R mRNA at P27. High perinatal signals for MC4-R mRNA are detected in caudate-putamen (cp), nucleus accumbens (ac), olfactory tubercle (ot), and septohippocampal nucleus (sh) with a marked postnatal decrease in caudate-putamen. MC4-R mRNA is further expressed in lateral septal nucleus (ls) and taenia tecta (tt). As shown for E22, the striatal region is devoid of MC3-R mRNA. Scale bar 500μm.

The septohippocampal nucleus was the first region to be labelled at E18 by the oligonucleotides for the MC4-R. The signal was present until P27 (Fig. 8). Dorsal to this structure, the taenia tecta expressed MC4-R mRNA at P27. The lateral septal nucleus expressed MC4-R mRNA from E22 and remained positive until P27, like the septohippocampal nucleus. At P27 the lateral septal nucleus also expressed MC3-R mRNA (Tab. 6a). By E22 a low amount of MC4-R mRNA was expressed in the medial amygdaloid nucleus, but the main increase in mRNA in this area occurred postnatally. At P8 MC4-R mRNA was expressed in medial and basal amygdaloid areas and central amygdaloid nucleus (Fig. 5). At P27, MC4-R mRNA was expressed in the posteromedial amygdaloid nucleus and in the lateral olfactory nucleus. The hippocampal complex exhibited more distinct signals than with [125I]NDP binding (Lichtensteiger et al., 1996). MC4-R mRNA was first detected in the hippocampus at P8. The gyrus dentatus showed a strong signal for MC4-R mRNA at P27, the subiculum and the hippocampus (CA1, CA2 (weakly) and CA3) exhibit a weaker signal. MC4-R mRNA was localized to the granule cell layer in gyrus dentatus and to the pyramidal cell layer in hippocampus. At P27, hippocampus and gyrus dentatus also exhibited a very weak signal for MC3-R mRNA (Fig. 5, 6, 9, Table 6a).
**Fig. 9** Cerebral cortex and hippocampus, E22 - P27. A. Histology of (B), frontal section at E22. B and D. MC4-R mRNA expression in entorhinal cortex (er) at E22 and P8, respectively. C. Histology of (D), frontal section at P8. E. Histology of (F), frontal section at P27. F and G. MC4-R mRNA and MC3-R mRNA expression, respectively, at P27. H. Non-specific binding in section adjacent to (G), incubated with 100 fold excess of unlabelled MC3-R oligonucleotides. At P27, MC4-R mRNA is located in retrosplenial (rs), occipital (oc) and temporal (te) cortex. Hippocampus (CA1-CA3) and dentate gyrus (dg) contain both, MC4-R and MC3-R mRNA at this stage. An MC3 signal is also present in the premammillary region (pm). Scale bar 500μm.

4.4.7.2 Cerebral cortex.

$[^{125}\text{I}]$NDP binding sites appear first in the piriform cortex by E20 and then spread caudally across the entorhinal cortex by E22 (Lichtensteiger et al., 1996). By P8 $[^{125}\text{I}]$NDP binding was seen in the parietal cortex as well, reached the temporal cortex by PN15 and by P27 the retrosplenial, occipital and temporal cortex. A distinct developmental pattern of binding sites could also be observed in the orbital and the cingulate cortex. These developmental patterns in cortex were not as clearly evident in our mRNA data. No MC4-R mRNA was detectable in the fetal piriform cortex, but it appeared by E22 in the entorhinal cortex where it was present until P27 (Fig. 9, Tab. 6a). Weak MC4-R mRNA signals were observed in the cingulate cortex by P8 and in retrosplenial, parietal, temporal and occipital cortex by P27 (Fig. 9, Tab. 6a). No MC3-R mRNA was detected in cerebral cortex during perinatal ontogeny (Fig. 9).
Table 6a. Synopsis of MC3 and MC4 receptor mRNA ontogeny in rat nervous system: Telencephalon and Diencephalon

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<th>Region</th>
<th>E14</th>
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**Table 6b. Synopsis of MC3 and MC4 receptor mRNA ontogeny in rat nervous system: Mesencephalon, lower brainstem, cranial nerve ganglia and sympathetic ganglia**

<table>
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<th>Region</th>
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<td>Superior colliculus</td>
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<tr>
<td>Substantia nigra</td>
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<td>Interpeduncular nucleus</td>
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<td>Lower brainstem, cerebellum, spinal cord</td>
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<tr>
<td>Dorsal tegmental nuc. pons</td>
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<td>Dorsal parabrachial nuc.</td>
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<td>Lateral lemniscal nuc.</td>
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<td>Superior olive</td>
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<td>Principal sensory trigeminal nucleus</td>
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<td>Trigeminal spinal tract nuc</td>
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<td>Solitary tract nucleus</td>
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<td>Dorsal motor nuc. of vagus</td>
<td>+</td>
<td>++</td>
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<td>++</td>
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<td>Hypoglossal nuc.</td>
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<tr>
<td>Dorsal paragigantocell. nuc</td>
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<td>++</td>
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<td>Cerebellum</td>
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<tr>
<td>Spinal cord</td>
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<td>+</td>
<td>(+)</td>
<td>n.s.</td>
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<tr>
<td>Ganglia</td>
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<td>Trigeminal ganglion (V)</td>
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<td>n.s.</td>
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<td>n.s.</td>
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<td>Geniculate ganglion (VII)</td>
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<td>n.s.</td>
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<td>Vestibular ganglion (VIII)</td>
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<td>n.s.</td>
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<td>n.s.</td>
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<tr>
<td>Nodose ganglion (X)</td>
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<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>Superior and jugular ganglia (IX, X)</td>
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<td></td>
<td>n.s.</td>
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<tr>
<td>Sympathetic ganglia</td>
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<td>+++</td>
<td>+++</td>
<td>+</td>
<td>n.s.</td>
<td>n.s.</td>
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</table>

Density of mRNA signals by visual rating (2 observers). (+), +, ++, +++ = very low, low, medium, high. (+) = optical density measurements (MCID) higher than non-specific binding. Empty spaces = no specific signal detectable. n.s. = not studied.

1) Substantia gelatinosa at P27.
4.5 DISCUSSION

The developmental pattern of MC3-R mRNA and MC4-R mRNA in the rat nervous system, as demonstrated by in situ hybridization, closely corresponds to the ontogeny of binding sites for \([^{125}\text{I}]\)NDP (Lichtensteiger et al., 1996), a high affinity ligand for both melanocortin receptor types (Mountjoy et al., 1994; Roselli-Rehfuss et al., 1993; Schioth et al., 1996). The overlap of regions of significant \([^{125}\text{I}]\)NDP binding site density with locations of MC3-R mRNA and MC4-R mRNA is almost complete with respect to both temporal and spatial patterns. The topographical distribution of MC3-R mRNA and MC4-R mRNA observed at 4 weeks of age (P27) is in essential agreement with that found for adult rat brain by in situ hybridization with riboprobes (Roselli-Rehfuss et al., 1993; Mountjoy et al., 1994). This further supports the specificity of the localization obtained with labelled oligonucleotides in the present study. The technique appears to be somewhat limited with respect to sensitivity. Although we combined two labelled oligonucleotides per mRNA species, which enhances the in situ hybridization signal (Lauber and Lichtensteiger, 1994), mRNA could not be demonstrated in certain areas with low \([^{125}\text{I}]\)NDP binding site density.

The overlap of \([^{125}\text{I}]\)NDP binding and melanocortin receptor mRNA suggests that in most areas, the receptors are located postsynaptically on cell bodies or dendrites, since neuronal mRNA generally is restricted to cell bodies, and polyribosomes are preferentially localized beneath the postsynaptic membrane (Steward and Banker, 1992). There are, however, some regions of the developing brain previously found to contain \([^{125}\text{I}]\)NDP binding sites, where mRNA encoding for MC4-R or MC3-R was not detectable. In the case of thalamic areas at E16, piriform cortex at E22, or the postnatal supraoptic nucleus, this might be explained by insufficient sensitivity. By contrast, the interpeduncular nucleus exhibited very strong \([^{125}\text{I}]\)NDP binding at P27 (Lichtensteiger et al., 1996), but was completely devoid of signals for MC3-R mRNA or MC4-R mRNA. Since the habenula contains MC3-R mRNA at P27, it seems possible that the melanocortin binding sites are located presynaptically on terminals of the habenulo-peduncular tract.

The most significant finding of the present study is the difference in the developmental patterns of MC3-R mRNA and MC4-R mRNA. All nervous structures of the fetus exhibiting significant \([^{125}\text{I}]\)NDP binding - brain areas, cranial ganglia, sympathetic
ganglia - contained MC4-R mRNA. By contrast, MC3-R mRNA was detectable only during postnatal development. This does not necessarily mean that MC3-R mRNA is absent at early developmental stages, since we may have been unable to detect low levels of MC3-R mRNA expression. However, there is certainly an important quantitative difference in MC4-R mRNA and MC3-R mRNA expression during fetal life, since the labelling efficiency of the oligonucleotides used for in situ hybridization of MC3-R mRNA was comparable to that of the oligonucleotides for MC4-R mRNA, and both mRNA species were studied in neighbouring sections of the same fetus.

MC3-R mRNA became detectable only during early postnatal life in a restricted number of brain regions, in particular ventromedial and arcuate hypothalamic nuclei and ventral midbrain tegmentum. All of these contain MC3-R mRNA in adulthood, but additional nuclei positive for MC3-R mRNA have been described in the adult brain (Roselli-Rehfuss et al., 1993). Our postnatal MC3-R mRNA data are in good agreement with recent findings of Xia and coworkers (Xia and Wikberg, 1997) who examined the postnatal development of MC3-R in rat diencephalon and mesencephalon. They detected weak MC3-R mRNA positive signals at PN0 (= P1 of the present study) in the hypothalamus and at PN4 (= P5) in the thalamus, and the first prominent signals in ventromedial hypothalamus, arcuate nucleus, and ventral tegmental area at PN7 (= P8, Table 1a). At PN21 (= P22), septal area, amygdaloid complex, anterior hypothalamus, habenula, linear nucleus of raphe and substantia nigra pars compacta contained MC3-R mRNA. We observed additional signals, at P27, in the mammillary and the premamillary region, the dentate gyrus, and the hippocampus. The slight differences between Xia's and our work may be accounted for by methodological differences.

The postnatal rise of MC3 mRNA expression in ventromedial and arcuate nucleus parallels that of [125]NDP binding sites (Lichtensteiger et al., 1996). During that phase, neurogenesis is limited to a small number of cells in parvicellular hypothalamic nuclei (Seress, 1985), whereas neurons of ventromedial and arcuate nucleus are generated between E13-E17 and E12-E17, respectively (Altman and Bayer, 1978). In contrast, nerve fibers and functional synapses develop mainly during late prenatal stages and the first postnatal week (Gennaro et al., 1986; Yamano et al., 1984). In the arcuate nucleus, outgrowing fibres are present from E16 and immature synapses from E17, while mature synapses form mainly between P3 and P6 (Koritsanszky, 1979). This phase seems to be sensitive to manipulations of endogenous α-MSH, which rapidly rises in
plasma during the first postnatal week (Davis et al., 1984). When antiserum against α-MSH was injected i.v. at P5 and P6, the feedback response of arcuate dopamine neurons to α-MSH, tested in adulthood, was abolished, whereas prolactin remained effective (Beckwith et al., 1977b). Antiserum injections on P11 and P12 were ineffective.

MC4-R mRNA appears to represent the dominant mRNA species in fetal central and peripheral nervous system. The two earliest locations detected from E13 by \[^{125}\text{I}]\text{-NDP binding, the sympathetic chain and the posterior thalamic lobe/epithalamic neuroepithelium (Lichtensteiger et al., 1996), express MC4-R mRNA as demonstrated in the E14 fetus. With respect to both \[^{125}\text{I}]\text{-NDP binding and MC4-R mRNA expression, some areas show a steady increase of density to a plateau which is maintained into adulthood (hippocampal formation, amygdala, paraventricular hypothalamic nucleus, dorsal parabrachial nucleus, dorsal motor nucleus of vagus), whereas others exhibit a distinct, fetal or perinatal receptor binding and mRNA peak (entorhinal cortex, caudate-putamen, interstitial magnocellular nucleus of posterior commissure, the two sensory trigeminal nuclei, solitary tract nucleus, inferior olive). Both types of ganglia shown by \[^{125}\text{I}]\text{-NDP binding to possess melanocortin receptors during the fetal period (i.e., certain cranial nerve ganglia and the sympathetic ganglia (Lichtensteiger et al., 1996)), express MC4-R mRNA, again with a marked developmental peak.}.

For several regions with high \[^{125}\text{I}]\text{-NDP binding site density during fetal or perinatal life, we have previously shown that melanocortin receptor sites mostly appear at the end or after neurogenesis and migration; density peaks mainly coincide with phases of synaptogenesis and with the establishment of neural circuitry (Lichtensteiger et al., 1996). The regions include, inter alia, the superior cervical ganglion (Rubin, 1985a and b), caudate-putamen (formation of striatonigral projections) (Fishell and van der Kooy, 1987; Van der Kooy and Fishell, 1987), principal sensory nucleus and spinal tract nucleus of the trigeminus (Al-Ghoul and Miller, 1993 a and b), dorsal motor nucleus of vagus (Kalia, 1992) and inferior olive (Ashwell and Zang, 1992; Merchan-Perez et al., 1993). An interesting parallelism was noted in the time-course of melanocortin receptor peaks in inferior olive and cerebellum. All these regions express MC4-R mRNA. Of the known melanocortin receptors, the MC4 receptor thus appears to be an important candidate for the transmission of developmental signals during early ontogeny.

Neurite outgrowth and GAP-43 content have been found to be increased by α-MSH treatment in three day old cultures of E15 spinal cord (Van der Neut et al., 1988
MC4-R mRNA was detected in rat spinal cord from E16. In three-day co-cultures of rat midbrain and striatal cells taken at E18, the onset of the striatal peak of MC4-R mRNA and $[^{125}\text{I}]-$NDP binding, $\alpha$-MSH enhanced neurofilament production (Kistler-Heer et al., 1996). Effects on neurite and dendrite outgrowth and GAP-43 were also seen in midfetal and early postnatal cerebral cortical cell cultures treated with $\alpha$-MSH (Joosten et al., 1996) or ACTH$_{1-10}$ and ACTH$_{1-24}$ (Richter-Landsberg et al., 1987), but these are difficult to relate to the presence of a defined receptor type. Other data indicate that additional receptors may be involved in the neurotrophic action of melanocortins. Cultures of neonatal dorsal root ganglia also responded to $\alpha$-MSH by increases in GAP-43 and neurofilament (Van der Neut et al., 1992). Specific $[^{125}\text{I}]-$NDP binding was detected in spinal ganglia (Lichtensteiger et al., 1996) but binding site density was very low. It is possible that the mRNA signal for the MC3 or MC4-R was too low to be detected by our method.

In view of the predominant position of the MC4 receptor in fetal nervous system, it is important to know the endogenous ligand present during that stage. One possible source of ligands is the POMC precursor. As discussed for $[^{125}\text{I}]-$NDP binding (Lichtensteiger et al., 1996), the sequential appearance of melanocortin receptors first in posterior thalamus and epithalamus, and later in midbrain, pontine flexure, trigeminal sensory nuclei, solitary tract nucleus and cranial nerve ganglia would agree with the main features of the outgrowth of POMC fibers (Elkabes et al., 1989; Kawai et al., 1986; Schwartzberg and Nakane, 1982). However, some areas which express MC4-R mRNA, have little or no POMC fiber innervation, such as cerebral cortex, hippocampal formation, cerebellum, caudate-putamen, dorsal motor nucleus of vagus and substantia gelatinosa. Moreover, POMC peptides have not been clearly demonstrated in fetal sympathetic ganglia, though POMC mRNA has been located in areas around the dorsal aorta in mouse embryos (De Angelis et al., 1993). A mismatch is also encountered with MC3-R mRNA in the postnatal ventromedial hypothalamic nucleus.

In early postnatal caudate-putamen (P1 - P5), cells of radial glia type containing $\alpha$-MSH and ACTH besides $\beta$-endorphin (Loughlin et al., 1991), could provide the missing MC4 receptor ligands. However, the possibility of additional ligands not derived from POMC cannot be excluded. In the case of the peripheral MC1 receptor, binding has been demonstrated for a completely different protein, agouti (Lu et al., 1994). The affinity of $\alpha$-MSH for the MC4 receptor appears to be low as compared to its affinity for
the peripheral MC1 receptor, with Ki in P8 and adult caudate or accumbens sections 15 - 50 nM (Lichtensteiger et al., 1996; Tatro and Entwistle, 1994) and in transformed cos-cells 640 nM (28). The range of effective α-MSH concentrations in cell systems expressing MC4-R or in cell cultures prepared from fetal spinal cord and striatum at stages with MC4-R expression, varies from 1.5 nM to 100 µM (Van der Neut et al., 1988 and 1992; Roselli-Rehfuss et al., 1993; Kistler-Heer et al., 1996 and submitted). Thus, the nature of the physiological MC4-R ligand in early ontogeny remains uncertain.

In conclusion, the ontogeny of mRNA expression follows a distinct temporal and spatial pattern for MC3 and MC4 receptors. The MC4 receptor predominates during early ontogeny in central and peripheral nervous system and thus may mediate possible developmental actions of endogenous melanocortin receptor ligands.

Acknowledgements

We thank Dr. Roger D. Cone (Portland, Oregon) for the information on a sequence fragment of rat MC4-R.

The study was supported by Swiss National Science Foundation grants no. 31-36643.92 and 31-045862.95.
5 MELANOCORTIN AND MCH PRECURSOR-DERIVED NEI EFFECTS ON RAT FETAL STRIATUM-MIDBRAIN CO-CULTURES

5.1 ABSTRACT

Melanocortins are thought to influence development, but information on effects at the level of the brain is very limited. We cultured dissociated cells from gestational day 18 Long Evans rat striatum, which exhibits a high perinatal peak of melanocortin receptor sites (MC4-receptor) and mRNA together with mesencephalic cells of the same developmental stage for 3 or 11 days in a serum-free medium and measured marker proteins by ELISA. α-Melanocyte-stimulating hormone (α-MSH) and the potent analog Nle\(^4\), D-Phe\(^7\)-α-MSH (NDP) increased neurofilament content in short-term cultures at concentrations of 250 - 2500nM and 3.1 - 750nM, respectively, whereas ACTH\(_{1-24}\) and ACTH\(_{1-39}\) (25 - 2500nM) were ineffective. In long-term cultures, α-MSH (250 - 750nM, 5 applications/11 days in vitro) induced an increase in synaptophysin content of the synaptosomal fraction. Growth-associated protein GAP-43 was not influenced by the peptides. Cell proliferation/survival rate (MTT-test) remained unaffected. These data indicate stimulatory effects of selected pro-opiomelanocortin-derived melanocortins on growth and differentiation of fetal striatum-midbrain co-cultures. An analogous effect was obtained with NEI, an alternate splicing product of the melanin-concentrating-hormone (MCH) precursor with structural similarities to α-MSH at the C-terminus, which increased neurofilament content at 250nM.

\(^3\) Kistler-Heer, V. and Lichtensteiger, W., Melanocortin and MCH precursor-derived NEI effects on striatum-midbrain co-cultures. Peptides, submitted
5.2 INTRODUCTION

In early phases of ontogeny, central nervous system, cranial and sympathetic ganglia exhibit region- and stage-specific, often transient patterns of melanocortin receptor expression, as demonstrated with the α-melanocyte stimulating hormone (α-MSH) analog \([^{125}\text{I}]\text{NDP} ([^{125}\text{I}]\text{Nle}^4,\text{D-Phe}^7-\alpha-\text{MSH}; \text{Lichtensteiger et al. 1996})\). Of the two melanocortin receptor (MC-R) types predominantly expressed in the central nervous system, i.e., MC3-R and MC4-R (Roselli-Rehfuss et al., 1993; Desarnaud et al., 1994; Mountjoy et al., 1993; Alvaro et al., 1996), only mRNA encoding for the MC4-R is detected in fetal rat nervous system, whereas MC3-R mRNA appears during the first postnatal week (Kistler-Heer et al 1996, and in press). The presence of MC3-R mRNA in early postnatal brain has perviously been reported (Xia et al., 1997). The spatio-temporal pattern of MC4-R mRNA expression in fetal nervous system closely corresponds to that of \([^{125}\text{I}]\text{NDP} binding sites. In the majority of neural structures, significant densities of melanocortin receptor sites are found during phases of circuitry formation and synaptogenesis (Lichtensteiger et al., 1996).

These data are suggestive of a role of melanocortin peptides in nervous system development. Melanocortins have been shown to possess neurotrophic and neuroprotective properties \textit{in vivo}, both in peripheral (Bär et al., 1990; Gispen et al., 1994; Strand et al., 1994b; Van de Meent et al., 1997) and central nervous system (Strand et al., 1993; Darlington et al., 1996). Trophic actions have also been demonstrated \textit{in vitro} on a number of nervous structures, including spinal ganglia (Van der Neut et al., 1992) spinal cord (Van der Neut et al., 1988; 1992), raphe neurons (Azmitia and De Kloet, 1987), and cerebral cortical cells (Daval et al., 1983; Richter-Landsberg et al., 1987; Joosten et al., 1996). In addition, melanocortins are able to induce neurite outgrowth in neuro 2A cells (Adan et al., 1996) and in chromaffin cells (Demeneix and Grant, 1988) and stimulate proliferation of cultured astrocytes (Zohar and Salomon, 1992).

However, the brain regions and peripheral ganglia expressing the highest levels of NDP binding sites and MC4-R mRNA expression during ontogeny, have not yet been chosen as models for the analysis of \textit{in vitro} actions of melanocortins. In the present study, we investigated cultures of late fetal rat striatum, a forebrain region exhibiting a marked late fetal to early postnatal peak of \([^{125}\text{I}]\text{-NDP binding and MC4-R mRNA ex-}
pression (Lichtensteiger et al., 1996, Kistler-Heer et al., in press). Striatal cells were co-cultured with mesencephalic cells in order to provide opportunities for striato-nigral and nigro-striatal interactions.

Proopiomelanocortin (POMC)-derived melanocortins (α-MSH, γ-MSH) are considered as potential endogenous ligands at melanocortin receptors. POMC and some of its derivatives are expressed early in brain (Khachaturian et al., 1985b) and in many brain regions, the developmental pattern of melanocortin receptors overlaps to some extent with the development of POMC fiber systems (Lichtensteiger et al., 1996). However, there exist some notable exceptions, such as caudate-putamen and cerebral cortex. These regions are virtually devoid of POMC innervation in adulthood (Khachaturian et al., 1985a), and there is no evidence for the presence of significant amounts of POMC during ontogeny, except for a possible contribution by POMC-containing cells resembling radial glia in caudate-putamen (Loughlin et al., 1991). Therefore, the possibility of endogeneous ligands unrelated to melanocortins should be considered. The agouti protein has been identified as ligand to the peripheral MC1-R as well as to the MC4-R, but it is not normally synthetized in brain (Lu et al., 1994; Huszar et al., 1997). Other possible candidates are NEI and NGE, two alternate splicing products of the melanocyte-concentrating-hormone (MCH) precursor (Parkes et al., 1992; Nahon, 1994). NEI exhibits some similarities to α-MSH at the C-terminus, and NEI shows cross-reactivity to antibodies against α-MSH. Projections of MCH neurons reach, i.a., caudate-putamen and cerebral cortex (Bittencourt et al., 1992; Naito et al., 1986; Nahon, 1994; Knigge et al., 1996).

Here, we are presenting data on trophic effects of melanocortins on striatal-mesencephalic co-cultures and the first evidence for an analogous effect of the MCH fragment NEI.
5.3 MATERIALS AND METHODS

5.3.1 Animals

Long Evans rats were bred in our laboratory under controlled conditions (lights on 02.00-16.00 h, 22±1°C) with a standard diet (NAFAG 850, Gossau, Switzerland) and water ad libitum. Time-pregnant rats were used for tissue collection. Nulliparous receptive females were mated with experienced males between 16.00 and 19.00. Pregnant rats were housed in groups of two to four animals. The stage 24 h after the mating period was defined as gestational day E1.

5.3.2 Tissue culture

Time-pregnant Long Evans dams were anesthetized at gestational day 18 (E18) by an initial s.c. injection of chloral hydrate (400mg/kg) followed by ether. Fetuses were removed, the brain was carefully prepared and placed in a drop of Hank’s balanced salt solution (HBSS, Life Technology, 14170) containing 1% PenStrep stock solution (Life Technology, 10'00U/ml penicillin and 10'000µg/ml streptomycin), for dissection of striatum and midbrain under a binocular microscope. For striatum, a tissue slice was prepared by two transverse cuts behind the olfactory bulbs and posterior to the optic chiasm, respectively. From this slice, cortical, septal and preoptic/hypothalamic areas were removed, leaving a tissue piece consisting of striatum, pallidum, ventral cortex and the anterior amygdala. The midbrain piece was obtained by two cuts, one caudal to the neocortex, one rostral to the cerebellum. The two tissue pieces were cleaned of blood and meninges and pooled.

Tissue was cut into approximately 1mm$^3$ pieces and washed two times with HBSS. Dissociation of cells was carried out by treatment of the tissue pieces with 0.08% trypsin (Fakola, Switzerland, L2133) for 20 Minutes at 37°C. Trypsinisation was stopped by washing two times with culture medium containing 1% albumin (Sigma A8806). The culture medium (CM) found to be most suitable, was a variation of the medium described by Honegger et al. for primary neuronal cultures (Honegger et al., 1979; Riederer et al. 1992; Zurich et al., 1994). It consisted of DMEM (Life Technology, 52100) supplemented by 3.7g/l NaHCO$_3$, 250nM Na$_2$SiO$_3$·5H$_2$O (Fluka), 15nM
Na₂SeO₃ (Sigma S1382), 5nM 3CdSO₄·8H₂O (Fluka, 20920), 10nM CuSO₄·5H₂O (Fluka 61240), 5nM MnCl₂·4H₂O (Fluka 63536), 0.5nM (NH₄)₆Mo₇O₄₀·4H₂O (Fluka 09878), 0.25nM NiSO₄·6H₂O (Fluka 72280), 0.25nM SnCl₂·2H₂O (Fluka 96528), 50nM ZnSO₄·7H₂O (Fluka 96500), 1µM vitamin B12 (Fluka, Switzerland, 95.190), 12µM 1-carnitin (Fluka, 22018), 1mM choline chloride (Sigma, C1879), 1µM thioctic acid (Sigma, T 5625), traces of retinol (Fluka, 95144) and traces of [+]α-tocopherol (Sigma, T1539), 0.9µM insuline (Sigma, I6634), 30nM 3,3',5 triiodo-l-thyronine (Sigma, T6397), 20nM hydrocortisone-21-phosphate (Sigma, H4251), 12.5nM human apo-transferrin (Sigma, T7786), 10ml/l 100x BME vitamin stock (Life technology, 21040), 1g/l Albumax II (Life Technology 11021) and 25mg/l gentamycin sulfate (Fluka 48760). Cells were dispersed by careful, repetitive trituration of the tissue pieces through blue 1ml pipette tips (Treff Lab, Switzerland, 1000µl No.96.1702.6.02). The cells were counted and diluted in culture medium. Depending on the assay, cells were seeded into poly-(l)-lysine (Sigma, P5899, MW>300 000; 5µg/well) coated 96-well flat-bottomed microwell plates (Petra Plastic, Switzerland Typ F-TC-steril, Art. 1.1047), 50ml tissue culture flasks (Falcon, 50ml, 25cm², 3108)) or Labtek 8-well-chamber slides (Nunc, permanox-slide, polystyrene chamber, 177445). Seeding density was 10⁵ cells/100µl culture medium/well for 96-well plates, 2x10⁵ cells/250µl culture medium/well for 8-well labtecs and 8x10⁶ cells/4ml culture medium/flask for tissue culture flasks. Cultures were incubated at 37°C and 10% CO₂.

5.3.3 Treatment of cultures

In short-term cultures (3 days in vitro (DIV)), culturing medium was replaced after the first 24h with new culturing medium containing peptides. In long-term cultures (10 or 11 DIV) the medium was replaced after the first 24h, at the beginning of the 3rd day and at the end of the 4th, 7th, and 9th DIV with fresh, peptide containing medium. α-MSH, ACTH₁-24, ACTH₁-39, NEI and NGE were dissolved in 10mM HCl with 0.1% w/v bovine serum albumine (BSA, Fraction V, Sigma), [Nle⁴, D-Phe⁷]-α-MSH (NDP) in 0.1% w/v BSA to obtain 10⁻⁴M stock solutions. Stock solutions were subsequently diluted with culturing medium to the appropriate concentrations. NEI and NGE were
kindly provided by A. Eberle, Basel, Switzerland. The remaining peptides were purchased from Bachem, Switzerland.

In a preliminary experiment, concentrations of bioactive α-MSH were determined in the culture medium by an *in situ* melanin assay for MSH using mouse B16 melanoma cells in culture (Siegrist and Eberle, 1986) after application of the peptide to the medium. Bioactive α-MSH concentration in the medium at start of the incubation was found to be approximately half of the nominal concentration of α-MSH. The difference may be to absorption effects (nominal concentration: 100nM, bioactive α-MSH concentration: 45nM). The half life of the peptide in the culture was approximately 60h.

### 5.3.4 Quantification of Neurofilament (NF) and GAP-43 protein

After 3 or 10 DIV, cells were fixed for 30 minutes with 4% paraformaldehyde (Fluka 76240) in phosphate buffered saline (PBS, 10mM, pH 7.4). The fixed cultures were rinsed three times with PBS and subsequently preincubated for 15 min at room temperature (RT) with 10% fetal bovine serum (FBS, Life technologies, 10081073) and 0.1% Triton X-100 (Sigma) in PBS. Next, the cultures were incubated for 60 min with 10% FBS in PBS to block unspecific binding sites. Antibodies for the measurement of neurofilaments were diluted in 2% FBS in PBS, antibodies for the GAP-43 assay with 10% FBS in PBS. The first antibody against the neurofilament protein (RT 97, Bohringer) was diluted 1/500, the first antibody against the growth associated protein (GAP-43) (Sigma, G9264) 1/1000, respectively.

Incubation of the fixed cultures was carried out overnight at 4°C. The following morning the cultures were washed three times with PBS and then incubated with the second antibody: anti-mouse IgG (h+1) horseradish peroxidase (HRP)-conjugate from goat (Promega, W4021), diluted 1/2500 for the neurofilament assay, or 1/1000 for the GAP-43 assay, respectively. Antibodies were incubated for 2h at room temperature, then the cultures were washed again three times with PBS and once with distilled water. Peroxidase reactivity was demonstrated with 100μl of a mixture consisting of 1 part 20mM 3,3',5,5'-tetramethyl-benzidine (TMB, Promega W4121) dissolved in acetone:ethanol (1:9) and 19 parts H₂O₂ substrate buffer (200μl 30% H₂O₂ in one liter of
200mM potassium citrate buffer pH 3.95). The reaction was stopped with 100μl of 1M H₂SO₄. The absorbance was read at 450nm on a Anthos Labtec 2001 Elisa-reader (reference wavelength 620nm). Negative controls consisted of a bovine serum albumine (BSA, Fraction V, Sigma) coated lane without cultures that was treated the same way as the sample lanes, and a conjugate control where a culture lane was treated exactly like the sample lines except that the first antibody was omitted. Cell densities used in the assays yielded values in the linear range of the antibodies.

5.3.5 Quantification of synaptophysin in synaptosomes

5.3.5.1 Preparation of synaptosomes

After 11 DIV, cultures in flasks were washed three times with HBSS and then incubated with 0.125% trypsin in HBSS at 37°C for 10 min. Trypsinisation was stopped by adding 10% FCS in HBSS. Cells were detached from the surface, centrifuged at 4°C 200 x g for 10 min and washed once with HBSS. The pellet was homogenized in a glass-teflon homogenizer (Poly Labo, France, 2ml capacity, 8mm diameter, 15-25μm clearance, 10 up and down strokes at 1500U/min, on ice) in 300μl sucrose buffer (0.3M d(+)-sucrose (Fluka, 84100), 5mM HEPES (Merck, 10110) and 0.1mM EDTA (Fluka, 03680), pH 7.4). The homogenizer was washed twice with 100μl sucrose buffer. Homogenate and washing solutions were pooled, transferred into 1.5ml centrifugation tubes (Eppendorf, Germany) and centrifuged for 10 min at 4°C 1000 x g. 450μl of the supernatant was transferred to a thick-wall centrifugation tube (Beckman, 1.5ml, code 357448). The remaining pellet was resuspended twice with 120μl sucrose buffer, centrifuged as above and the supernatants (twice 100μl) were added to the first one. The total supernatant was centrifuged for 30 min at 4°C 17,000 x g. The pellet representing the synaptosomal fraction (Hefti and Lichtensteiger, 1978) was frozen at -80°C until use.

5.3.5.2 Quantification of synaptophysin

Synaptosomes were thawed and homogenized in 100μl PBS pH 7.4 (ice cooled) using a sonicator (Zivy & Cie SA, Switzerland). Protein content was measured in aliquots of the samples according to Bradford, 1976 (Bio-Rad protein assay, Cat. 500-
0006, Bio-Rad, Germany) with BSA (BSA, Fraction V, Sigma) as standard. Microtitre plates (Immunoplate Maxisorp, Nunc 00439454) were coated with 50μl of various dilutions of the samples in PBS pH 7.4 (0.25-0.0025μg protein/μl) for 2h at room temperature. Plates were washed three times with PBS and then incubated with 10% BSA in PBS for 1h at room temperature to block non specific binding. The first antibody (SY 38, Böhringer; 1/100 in 2% BSA in PBS) was incubated overnight at 4°C. The next morning, the plates were washed three times with PBS pH 7.4 and subsequently the second antibody (anti-mouse IgG (h+I) peroxidase (HRP)-conjugate from goat (Promega, W4021), diluted 1/5000 in 2% BSA in PBS) was incubated for 2h at room temperature. Following three washes with PBS and one with distilled water, the peroxidase reaction was carried out as described above. Homogenates were diluted to given protein concentrations in the linear range of optical density (0.025μg/μl to 0.0025μg/μl).

5.3.6 MTT-assay

3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma M5655) solution (5mg/ml sterile PBS pH 7.4,) was diluted with culturing medium (final concentration 0.5mg/ml) and added to the cultures after 3 or 10 DIV. Cells were incubated for 2h with MTT-containing culture medium in the incubator at 37°C and 10% CO₂. The solution was subsequently removed by aspiration and replaced by 100μl dimethylsulfoxide (Fluka 41648), in order to dissolve the purple MTT-formazan product generated by viable cells (Widmer et al. 1993). Absorbance was measured at 540nm in the linear range of the absorption curve(reference filter 620nm) in an Anthos labtec reader.

5.3.7 Immunohistochemistry

In untreated cultures and cultures treated with 500nM of α-MSH, the immunohisto-chemical pattern was studied after 3 and 10 DIV for glial fibrillic acid protein (GFAP, marker for astrocytes), galactocerebrosidase (GALC, for oligodendrocytes), neuron specific enolase (NSE, for neurons), neurofilaments (NF, for cytoskeleton), tyrosin
hydroxylase (TH, for catecholamine neurons) and choline acetyltransferase (CAT, for cholinergic neurons).

For GFAP, GALC, TH, NSE or NF, cultures were washed once with PBS pH 7.4 and fixed with 4% paraformaldehyde in 50mM TRIS buffer pH 7.4 for 20 min at RT. They were then washed three times with TRIS pH 7.4 and subsequently coated for 10 min with 10% goat serum (Life Technologies) and 0.1% Triton X-100 at RT. The first antibody was diluted in 2% goat serum in TRIS pH 7.4 and incubated overnight at 4°C. Dilutions for the anti-GFAP antibody (rabbit anti-cow GFAP, Dako Z 334) was 1/5000, for anti-GAL (mouse anti- GALC, Boehringer, Clone mGalC) 1/20, for anti-TH (mouse anti-TH, Sigma, Clone TH-2) 1/5000, for anti-NSE (rabbit anti-rat NSE, Polysciences, 16625) 1/5000, and for anti-NF (Boehringer, clone RT 97) 1/300. The next morning, cultures were washed three times with TRIS pH 7.4 and then coated for 2h at room temperature with the second antibody: for NF and TH with a 1/1000 dilution of an anti-mouse IgG (h+l) horseradish peroxidase (HRP)-conjugate from goat (Promega, W4021) in 2% goat serum in TRIS pH 7.4, and for NSE and GFAP with an 1/000 dilution of anti-rabbit IgG (Fc) HRP-conjugate from goat (Promega, W4011) in 2% goat serum in TRIS pH 7.4. After three washes with TRIS pH 7.7, the peroxidase reaction was carried out with diaminobenzidine (DAB) as chromogenic substrate. Briefly, cultures were incubated with 200μl of 1% DAB stock solution (Sigma, D5637) in TRIS pH 7.7 for 5 min at RT, then 200μl of 1% (w/v) DAB stock solution and 0.07% (v/v) of H2O2 (32%) in TRIS pH 7.7 were added for 30 min. The reaction was stopped by washing the wells twice with ice cold PBS pH 7.4 and once with distilled water. The upper compartment of the Labtek chamber slides was removed, the cultures were mounted in a mixture of one part glycerin and one part 0.1M sodiumhydrogencarbonate, and examined in an Axiovert 100 light microscope (Zeiss, Germany).

CAT immunohistochemistry was performed according to the instructions given for the first antibody (monoclonal antibody to CAT from rat-mouse-hybrid cells, Boehringer, 770 981). Cultures were fixed for 30 min at room temperature in a solution containing 4% (w/v) paraformaldehyde and 15% (v/v) saturated picric acid solution in 100mM PBS pH 7.2. Following fixation, the cultures were washed several times with PBS pH 7.2 until the yellow color of picric acid had disappeared. Cultures were then
coated for 1h with 10% goat serum in 100mM TRIS pH 7.4 to prevent unspecific binding. The first antibody was diluted 1/100 in a solution consisting of 20% (v/v) goat serum, 2% (w/v) BSA, 0.1% (v/v) Triton X-100 and 0.1% (v/v) sodium azide in TRIS pH 7.4. It was incubated overnight at 4°C and then washed off three times with TRIS pH 7.4. The second antibody (anti-mouse in goat HRP-conjugate, biotinylated not purified, Dianova) was diluted 1/300 in a solution consisting of 1% (w/v) BSA and 20% goat serum (v/v) in TRIS pH 7.4 and incubated for 1h at RT. The cultures were washed three times with TRIS pH 7.4 and subsequently coated for 30 min with ABC complex (Vectastasin Kit, Reactolab, Servion, Switzerland). After three washing steps with TRIS pH 7.7, peroxidase reactivity was demonstrated with DAB as described above.

In controls with omission of the first antibodies, no staining was detected.

5.3.8 Data analysis

Anti-neurofilament ELISA, anti-GAP-43 ELISA and MTT-assay: Each 96-well plate was divided into 4 equal sectors, with two sectors containing 24 control cultures and two sectors containing 24 treated cultures (two treatment groups per plate). In the ELISA series 12 additional wells were used to determine non-specific binding of the antibodies. Edge rows of the plate were not included in the assays. The mean optical density of all control cultures of a given plate was calculated. Then, the optical density of each control as well as treated well was expressed as percentage of control of that plate. One assay was carried out with cells pooled from embryos of one dam. Each experiment was repeated 3-10 times with cells from embryos of different dams.

Statistical analyses were performed using SYSTAT software (Version 5, Illinois, USA). The statistical significance of differences between treated and control cultures was analyzed via a one-way analysis of variance (ANOVA), followed by the Bonferroni test for multiple comparisons.

For the anti-synaptophysin ELISA, two flasks were used for each treatment and for control cultures. Values from treated cultures were expressed as percentage of control cultures. The experiments were repeated 3 to 10 times with embryonic cells from different dams. Results were analyzed with a one sample sign test.
5.4 RESULTS

5.4.1 Immunohistochemical pattern of cultures

After three days of culture of dissociated E18 striatum and midbrain cells, most cells were immunopositive for neuron specific enolase (NSE). They generally exhibited small round cell bodies with short bi- or multipolar processes, but some processes were already considerably longer (10 to 20 times cell body length) (Fig. 10). Approximately half of the cell bodies stained strongly positive for neurofilament (NF), the rest was weakly immunoreactive. NF-positive processes were long and branched. The frequencies of cells immunoreactive for the astrocyte marker glial fibrillar acid protein (GFAP), the oligodendrocyte marker galactocerebroside (GALC), or tyrosine hydroxylase (TH) were all below 1% (Fig. 10). TH-positive cells derived from the dopaminergic midbrain neuron groups showed a small cell body with long, richly branched, multipolar processes. No choline-acetyltransferase (CAT)-positive cells were found at this early stage of culture.

After ten days in vitro, most of the cells still stained positive for NSE. Their processes now were long, thin, extensively branched and partly aggregated into thick fibre bundles. The percentage of NF-positive processes had increased to approximately 75%. The number of TH positive cells had remained constant, with mainly short processes (Fig. 11). CAT positive cells were now identified with big round cell bodies and short thick processes (less than 1%) (Fig. 11). The proportion of GFAP and GALC-immunoreactive glial cells had increased to below 5%. Cultures treated with 500nM α-MSH showed no difference in the proportion of different cell types or morphological patterns compared to control cultures.
Fig. 10 Immunohistochemistry for neuron specific enolase (A), glial fibrillic acid protein (B) and galactocerebrosides (C), markers for neurons, astroglia and oligodendroglia, respectively. Cultures were kept for three days in a chemically defined, serum-free medium. Scale bar (A) 25\mu m, (B) 30\mu m, (C) 50\mu m.
Fig. 11  Immunohistochemistry for tyroxin hydroxylase (A) and choline acetyltransferase (B). Cultures were kept for 10 days in a chemically defined, serum-free medium. Scale bar 25μm.
5.4.2 Survival rate

Cell viability was analyzed with the MTT-assay, based on conversion of MTT to a purple formazan by cellular dehydrogenases (Mosmann, 1983). The ratio between optical density (OD) of the formazan product and cell number was linear for a given culture period (data not shown). The various peptides did not influence survival rate in a systematic way (Table 7). Small differences from controls were noted in short-term cultures for NDP (7% increase at a concentration of 28nM, p<0.01), NEI (103 and 109% at 250 and 500nM, respectively p<0.01, p<0.001, respectively) and for α-MSH in long term cultures (+10% at 2500nM, p<0.001).

<table>
<thead>
<tr>
<th>peptide</th>
<th>DIV</th>
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<tr>
<td></td>
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<tr>
<td>α-MSH</td>
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<td>α-MSH</td>
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<tr>
<td>NEI</td>
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<td>NGE</td>
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<tr>
<th>peptide</th>
<th>DIV</th>
<th>peptide concentration (nM)</th>
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<tr>
<td>NDP</td>
<td>3</td>
<td>104.6 ± 0.9</td>
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Table 7 Cell survival in rat fetal striatum-midbrain co-cultures after 3 or 10 days in vitro (DIV). MTT-test values as percentage of control. Mean values ± SEM. 2-4 experiments with 10 wells each, except α-MSH with 7 to 9 experiments. *p<0.01, **p<0.001 versus control group. In 3 DIV experiments, peptides were added after 1 DIV, in 10 DIV experiments, 5 applications of peptides (see methods).
5.4.3 Neurofilament

The effect of melanocortins on neurofilament (NF) content was measured after three and ten days in vitro. Addition of α-MSH to co-cultures of striatal and mesencephalic cells after the first 24h in vitro induced a significant increase in NF content as determined 48h later, with a bell-shaped concentration-response curve (Fig. 12). The increase in NF ranged between 9.5% at 250 and 500nM α-MSH and 11% at 750nM. The effect disappeared after 10 DIV with repetitive application of the peptide (data not shown). Cultures responded similarly to NDP: In short-term cultures (3DIV) with a single application of the peptide, NF content increased significantly at NDP concentrations between 3nM and 750nM (Fig. 12). The maximum effect was higher than after α-MSH, with 16.8% increase at 250nM (p<0.001). As for α-MSH, this effect disappeared after 10 DIV and multiple treatment with the peptide (data not shown). ACTH1-24 and ACTH1-39 were tested in short-term cultures. ACTH1-24 was active only at the highest dose used (2500nM) whereas ACTH1-39 was ineffective over the whole concentration range (Fig. 12).

Fig. 12 Neurofilament protein in co-cultures of rat fetal striatal and mesencephalic cells after 3 days in vitro. A single dose of α-MSH (A), NDP (B), ACTH1-24 (C) or ACTH1-39 (D) was applied after the first 24h in culture (25-2500nM). Neurofilament protein (NF) was determined with an ELISA. Results of four to eleven individual experiments, each with twelve wells per condition, were combined. The x-axis gives the nanomolar concentration of the peptides applied. The NF levels are expressed as percentage of the corresponding control levels. ANOVA, Bonferroni multiple comparison, *p<0.01, **<0.001 versus control.
A  α-MSH

B  NDP

C  ACTH 1-24

D  ACTH 1-39
The two MCH precursor fragments NEI and NGE were tested in short-term cultures: NEI increased NF content by 22% at 250nM, with a narrow bell-shaped concentration-response curve (Fig. 13). NGE was active only at the highest concentration used, 2500nM (Fig. 13).

NF protein was also measured in the absence of mesencephalic cells. In these cultures, treatment yielded inconsistent values after 3 and 10 DIV (data not shown).

**Fig. 13** Neurofilament protein in co-cultures of rat fetal striatal and mesencephalic cells after 3 days in vitro. A single dose of NEI (A) or NGE (B) was applied after the first 24h in culture and neurofilament protein (NF) was determined after additional 48h in culture with an ELISA. Results of four individual experiments each with 12 wells per condition were combined. The x-axis gives the nanomolar concentration of the peptides applied. The NF levels are expressed as percentage of the corresponding control levels. ANOVA, Bonferroni multiple comparison, *p<0.01, **<0.001 versus control.
5.4.4 GAP-43 formation

GAP-43 protein, determined in short-term cultures, was not influenced by single applications of α-MSH or NDP (Table 8). A small series of cultures was treated with multiple applications of α-MSH and NDP for 10 days, again without effect on GAP-43 (data not shown).

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<th>GAP-43 protein in rat fetal striatal/mesencephalic co-cultures</th>
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<tr>
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<td></td>
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<tr>
<td>α-MSH</td>
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<td>NDP</td>
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Table 8 Growth associated protein GAP-43 in rat fetal striatum-midbrain co-cultures after 3 days in vitro (DIV). ELISA values as percentage of control. Mean values ± SEM. 3-9 experiments with 10 wells each. Application of peptides after 24h in vitro.
5.4.5 Synaptophysin in Synaptosomes of 11 day old cultures

Fig. 14 Synaptophysin protein in a synaptosomal fraction of fetal rat striatal and mesencephalic co-cultures after 10 days in vitro. α-MSH was applied after 1, 3, 4, 7 and 9 days in vitro. Synaptophysin protein was determined with an ELISA. Results of 8 to 9 individual experiments were combined. The x-axis gives the nanomolar concentration α-MSH applied. The synaptophysin levels are expressed as percentage of the corresponding control levels. One sample sign test, *p<0.05, **p<0.005 versus control

Synaptophysin was studied in a synaptosomal preparation of striatum and midbrain co-cultures treated repeatedly with peptides and harvested after 11 DIV. ELISA readings were in the linear range of optical density. Treatment of cultures with α-MSH significantly increased synaptophysin concentrations (Fig. 14). Significant effects were observed at 250nM and 750nM with 9% and 10% increase over control levels. None of the other peptides tested (NDP, ACTH1-24 and ACTH1-39) affected the content of synaptophysin (Table 9).
Synaptophysin in synaptosomes of rat fetal striatum/midbrain co-cultures

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<tr>
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<th>peptide concentration (nM)</th>
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<tr>
<td></td>
<td>25</td>
</tr>
<tr>
<td>α-MSH</td>
<td>106.5 ± 4.8</td>
</tr>
<tr>
<td>NDP</td>
<td>101.3 ± 6.9</td>
</tr>
<tr>
<td>ACTH1-24</td>
<td>97.1 ± 10.1</td>
</tr>
<tr>
<td>ACTH1-39</td>
<td>95.5 ± 3.0</td>
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Table 9 Synaptophysin in synaptosomes of rat fetal striatum/midbrain co-cultures after 11 days in vitro (DIV). ELISA values as percentage of control. Mean values ± SEM. 8 to 9 experiments for α-MSH, 3 - 5 experiments for NDP, ACTH1-24 and ACTH1-39 *p<0.05, **p<0.005 versus control group. 5 peptide applications: DIV 1, 3, 4, 7, and 9 (see methods).

5.5 DISCUSSION

A number of forebrain areas exhibit prominent melanocortin receptor expression during early ontogeny (Lichtensteiger et al., 1996; Kistler-Heer et al., 1996; and in press), but except for early studies on neocortical cells, no data on possible actions of melanocortins in vitro have been published. One part of the present co-cultures system is represented by cells form fetal rat striatum which expresses high densities of melanocortin binding sites (Lichtensteiger et al., 1996) and MC4-R mRNA (Kistler-Heer, 1996; and in press). Striatum was dissected at E18, i.e., at the onset of the steep rise in melanocortin receptor density. At this stage, the medium spiny neurons of the patch compartment (E13-E15) and the small group of large cholinergic interneurons (E13-E16) have been generated, whereas the matrix cells are generated later, between E18-E20 (Bayer, 1984; Van der Kooy and Fishell, 1987; Ebstein et al., 1993). Striatal neu-
rons were co-cultured with mesencephalon in order to provide opportunities for contacts between striatal and mesencephalic cells, since melanotropin receptor expression often parallels circuitry formation and synaptogenesis (Lichtensteiger et al., 1996). Co-culture has also been reported to increase survival of both, mesencephalic DA and striatal cells (Aronica et al., 1996). At E18 DA nigrostriatal fibres have already reached the striatum (Moon Edley and Herkenham, 1984; Schlumpf et al. 1980) and thus are cut during tissue preparation. Formation of the striatonigral projection is starting and continues during the following days (Fishell and van der Kooy, 1987; 1989). It should be noted that the majority of cortical cells forming the corticostriatal projection is missing in the culture system.

α-MSH increased neurofilament (NF) content in short-term striatal-mesencephalic co-cultures with maximal effects at 500/750nM (+11%). Analogous changes in NF have been observed in rat fetal spinal cord explants (Van der Neut et al.,1988) or dissociated spinal neurons (Van der Neut et al., 1992) and in dissociated fetal and early postnatal dorsal root ganglia cells (Hol et al., 1994; Van der Neut et al., 1992).

The concentration-response curve to α-MSH was bell-shaped. Similar biphasic responses are frequently observed in melanocortin-neuron interactions (Van der Neut et al., 1992; Joosten et al., 1996). The α-MSH concentration yielding a significant effect (250nM) was approximately 5-fold higher than the Kd of α-MSH in P8 rat striatum (Lichtensteiger et al., 1996). Data suggest that the effective concentration of α-MSH may depend on culture conditions. Thus α-MSH was effective on NF at 0.1μM in fetal spinal cord explants, and at 100nM-100μM in dissociated cells from dorsal root ganglia and spinal cord, respectively (10nM after prestimulation with βNGF (Van der Neut et al. 1988, 1992; Hol et al., 1994). In addition, the actual amount of bioactive α-MSH in the culture medium was found to be approximately two-fold lower than the nominate peptide concentration.

Analogous effects on NF were observed with NDP, an α-MSH analog with high affinity to all known melanocortin receptors (Schiøth et al., 1996). NDP (Kd = 0.7nM in P8 striatum) was effective at 3nM and elicited a slightly higher maximum increase (16.8%) than α-MSH at lower concentrations (250nM).
In contrast, the two ACTH-type peptides were ineffective (ACTH₁₋₃⁹) or only very slightly active at the highest concentration studied (ACTH₁₋₂⁴). The type of receptors present in the cultures has not been identified. In vivo, fetal striatum and mesencephalon express MC4-R (Kistler-Heer et al., 1996 and in press); MC3-R mRNA appears in mesencephalon at around P7 (Xia et al., 1997; Kistler-Heer et al., in press). Thus, one would expect predominantly MC4-R, possibly combined with a small proportion of MC3-R after prolonged culture periods. Binding studies in expression systems revealed similar Ki values for α-MSH and ACTH₁₋₃⁹ for MC4-R and a slightly higher Ki of ACTH₁₋₃⁹ for MC3-R (Schioth et al., 1996). Autoradiographic analysis of binding to caudate-putamen yielded Ki values for ACTH₁₋₃⁹ similar to α-MSH and for ACTH₁₋₂⁴ slightly lower than for α-MSH (Tatro et al., 1990; Tatro and Entwistle, 1994). EC50 values for cAMP conversion in various cell lines and adult rat striatal slices are similar for α-MSH and ACTH (Alvaro et al., 1996; Florijn et al., 1993; Mountjoy et al., 1994; Roselli-Rehfuss et al., 1990). These data do not provide an explanation for the inefficiency of ACTH-type peptides in our culture system.

The present data suggest that the effect on NF content is a melanotropic rather than an ACTH-type of action. Differential actions of α-MSH versus ACTH-type peptides have also been described for serotonergic neurons in vitro (Azmitia and De Kloet, 1987) and for DA systems in vivo (Lichtensteiger and Monnet, 1979).

In long-term cultures with multiple applications of the peptides, none of them had any effect on NF content. There may exist a time window during which melanocortins are active, or desensitization may have occurred, as shown in non-transfected COS-7 cells with functional melanocortin receptors (Polgar et al., 1996). Also, the effect of melanocortins may have been obscured by additional growth effects in older cultures.

The maximum effects of the peptides were rather small. It cannot be excluded that only a subpopulation of cells was affected. Thus, for instance, in vivo, melanocortin binding sites become preferentially concentrated in striosomes during the second postnatal week (Lichtensteiger et al., 1996). More detailed cytochemical analyses would be required in order to clarify possible differences between subpopulations of cells. It is also conceivable that the melanocortin effect is modulated by additional inputs to the cells, some of which may have been lacking in the culture.
Since NEI, a fragment of the MCH-precursor (Parkes and Vale, 1992) bears some relationship to α-MSH at the C-terminus (-Pro-Ile-NH₂ as compared to -Pro-Val-NH₂ in α-MSH; Nahon, 1994) and since C-terminal fragments of α-MSH are centrally active in vivo (Lichtensteiger and Monnet, 1979; Lipton and Catania, 1997), we tested the effect of NEI on NF. NEI increased NF content in short-term experiments with a very narrow bell-shaped concentration-response curve, exhibiting approximately twice the effect of α-MSH at 250nM NGE, another MCH-precursor fragment with a different peptide sequence, exhibited only a very weak activity at the highest concentration tested.

The effect of NEI is of interest because the endogenous ligand of striatal melanocortin receptors remains uncertain (Lichtensteiger et al., 1996). In perinatal rat forebrain (mainly striatum and cerebral cortex), ACTH peaks at around the time of the MC4-R mRNA peak, while α-MSH remains low until P8 (Monnet-Tschudin et al., 1986). Moreover, there is no evidence for a significant innervation of striatum and cerebral cortex by POMC fibers in adulthood, except for most ventral striatum (Khachaturian et al., 1985a). Radial glia-type cells expressing POMC peptides during the early postnatal period (Loughlin et al., 1991) have been proposed as an alternative source of POMC. On the other hand, MCH immunoreactive fibres project to caudate putamen and cerebral cortex in the adult rat (Bittencourt et al., 1992; Knigge et al., 1996; Naito et al., 1986). Recently, NEI has been shown to elicit grooming in rats after intraventricular injection, similar to MSH/ACTH related peptides (Sanchez et al., 1997). However, it is not known whether significant amounts of NEI are released during perinatal ontogeny. Whether the effect of NEI on NF was mediated by the known melanocortin receptors remains to be clarified by binding studies. An involvement of another receptor type cannot be excluded.

The various peptides did not influence cell proliferation and survival in any systematic way (slightly elevated numbers of living cells (MTT test) in 3 out of 33 experimental groups). Also, there were no distinct changes in the proportion of cells identified by the immunocytochemical markers. In particular, we did not detect a change in the number of astrocytes. The proportion of this cell type has been reported to be increased 1.5-2 fold by melanocortins (Zohar and Salomon, 1992). These observations suggest that the peptide induced increase in NF content did not primarily result from an
increase in cell number but rather, reflects increased growth. This idea is supported by previous observations on other nervous structures (Azmitia and De Kloet 1987; Richter-Landsberg et al., 1987; Van der Neut et al., 1989, 1990, 1992; Joosten et al., 1996), though one study failed to find an effect on neurite outgrowth (Peulve et al., 1994).

In contrast to neurofilament, no changes were seen in the growth-associated protein GAP-43 content. This protein is found in cell bodies just after their final cell division, is expressed in elongating axons and growth cones during development and regeneration, and decreases in most brain areas after synapse formation, except in those that are associated with high synaptic plasticity in adulthood (Skene, 1989; Benowitz and Routtenberg, 1997). In contrast to our study, GAP-43 has been found to be affected in other studies on nerve cell populations after comparable peptide exposure times (Van der Neut et al., 1988; 1992). Van der Neut et al. (1992) reported that the neurotrophic effect of melanocortins on dissociated spinal neurons was higher if the cultures were kept in a relatively poor medium. In addition, GAP-43 was down-regulated as soon as interneuronal contact was established (Van der Neut et al., 1990). Although the medium we used does not contain serum, it is richly supplemented with hormones, vitamins and trace elements and should rather be considered a „rich medium“. Cell density (10^5 cells/96 well-plate well) was high. It is thus possible that the effect of melanocortins on GAP-43 was masked by the culture conditions.

Since the observations on NF indicated an effect of the peptides on growth and differentiation, and since increased densities of melanocortin receptor sites often overlap with phases of synapse formation (Lichtensteiger et al., 1996), we investigated the effect of melanocortins on synaptophysin. Synaptophysin, a component of vesicle membranes (Südhof, 1995), is first located to the perikarya of cells of striatal anlage at E14 but later becomes concentrated in presynaptic terminals restricted to nerve terminals (Ovtscharoff et al., 1993). After prolonged cell culture (11 DIV), a major part of synaptophysin is located in terminals, though some cell bodies remain immunoreactive (unpublished observations; Erhart-Bornstein et al., 1991). In order to assess synaptophysin concentrations reflecting differences in synapse number or quality, we determined the protein in a synaptosomal fraction (Zurich et al., 1994). Long-term cultures exposed to α-MSH exhibited increased levels of synaptophysin in the synaptosomal
fraction, whereas ACTH1-24 and ACTH1-39 were again inactive. This indicates an effect of α-MSH on synapse formation, but the method does not allow to distinguish between changes of the concentration of synaptophysin in secretory vesicles, of the number of vesicles per presynaptic button, or the total number of synapses. The lack of an effect of NDP remains unexplained. It cannot be excluded that following prolonged exposure to the stable MSH analog NDP, receptor densitization prevented a response of the cultured cells to this peptide (Polgar et al., 1996).

In conclusion, our data show that melanocortins exert trophic effects on fetal striatal-mesencephalic co-cultures, as indicated by an increase of neurofilament content in short-term cultures and an increase in synaptosomal synaptophysin in long-term cultures. The effects on neurofilaments were elicited by the two peptides with melanotropic activity, α-MSH and NDP, whereas ACTH1-24 and ACTH1-39 were ineffective. Our study further provides the first evidence for an analogous effect of NEI, an alternate splicing product of the MCH precursor. This peptide co-localizes with MCH in neurons projecting to the forebrain and hence, should be examined further as a possible alternative ligand at some of the melanocortin receptor sites.

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A number of observations point to possible influences of melanocortins on development and regeneration of neural tissue, as outlined in the General Introduction. Five receptors which mediate melanocortin effects are presently known. In this study, we attempted to gain information regarding the receptor type present during different stages of pre- and postnatal ontogeny in the nervous system. We studied the ontogeny of the mRNA of the two most prominent receptor subtypes in the peripheral and central nervous system, the MC3-R and the MC4-R by in situ hybridization. Our most important finding was, that MC4-R mRNA was the predominant species of MC-R during the entire embryonic and fetal period and was found in a wide range of brain structures, cranial nerve ganglia and sympathetic ganglia. In contrast, MC3-R mRNA became detectable only in the early postnatal period and its distribution was much more restricted. We concluded, that the early presence of MC4-R mRNA in central and peripheral nervous system and its transient regional peaks of mRNA expression, often concomitant with periods of neuritogenesis and synaptogenesis, suggest a role of the MC4-R in early ontogeny. The MC3-R, in contrast, might be involved only during postnatal development.

Previous studies showing neurotrophic and regenerative effects of melanocortins on neural tissues in vitro (Azmitia and De Kloet, 1987; Bär et al., 1990; Darlington et al., 1996; Daval et al., 1983; Gispen et al., 1994; Joosten et al., 1996; Richter-Landsberg et al., 1987; Strand et al., 1993; Strand et al., 1994; Van de Meent et al., 1997; Van der Neut et al., 1988, 1992) have been performed with tissues/cells that express low amounts of MC3-R or MC4-R mRNA only. Since the structures studied in vitro were not selected according to melanocortin receptor density, in a second part of this study, we studied the in vitro influence of melanocortins on rat E18 striatum-midbrain cocultures. Rat striatum exhibits perinatally a high transient peak of MC4-R mRNA (Kistler-Heer et al., 1996 and in press) and MSH-binding sites (Lichtensteiger et al., 1996). In vivo MC4-R mRNA and MSH-binding sites peaks are often concomitant with phases of development of neural circuitry. Therefore, we co-cultured striatal cells with mesencephalic cells from the same developmental stage to provide opportunities for
developmental interactions. Nigrostriatal projections reach the striatum in vivo as early as E14 (Moon Edley and Herkenham, 1984), the first striatonigral projections which are most probably of patch origin, reach the substantia nigra at E16. Striatal matrix neurons make connections with the substantia nigra early postnatally (Fishell and Van der Kooy, 1987). In vitro, nigral and striatal cells influence each other reciprocally. Both cell types depict increased cell survival in the presence of the other (Aronica et al., 1996). Rat striatum expresses almost exclusively MC4-R, fetal rat mesencephalon contains MC4-R, additionally, shortly after birth, a limited amount of MC3-R mRNA becomes detectable. The predominant MC-R species in our cell culture system can thus be assumed to be MC4-R. In this culture system α-MSH and NDP, two peptides with melanotrophic activity, increased neurofilament protein in short-term co-cultures. Synaptophysin content in synaptosomes prepared from long-term cultures was also increased by α-MSH. These effects were not due to increased cell survival. The ACTH-type peptides ACTH1-24 and ACTH1-39 were ineffective. NEI, a fragment of the melanin concentrating hormone (MCH) precursor that bears some relationship to α-MSH at the C-terminus and crossreacts with antisera directed against α-MSH, increased neurofilament protein in short-term cultures in a similar fashion like α-MSH. MCH is considered to be a functional antagonist of α-MSH in many aspects, in contrast to NEI which has recently been shown to have antagonistic effects to MCH and to exert effects similar to α-MSH. Thus, both, α-MSH and NEI induce grooming behavior if injected icv, in contrast to MCH that has no effect by itself but antagonizes, grooming behavior induced by α-MSH and NEI (Sanchez et al., 1997). Our data support the notion that NEI might act in a similar fashion like α-MSH.

The fact that NEI is able to exert an effect on neurofilaments similar to α-MSH is especially interesting in view of the fact that the true ligand of the MC-R in the striatum is still under debate. There is no evidence for a significant innervation of striatum by nerve fibres containing POMC, the precursor of melanocortins (Khachaturian et al., 1985a), except for a small number of radial glia-type cells which express POMC peptides during the early postnatal period (Loughlin et al., 1991). Moreover the binding affinity of α-MSH to receptor sites in striatal tissue is relatively low as discussed in the general introduction and part 1.
The questions arises: which is the endogeneous ligand for the MC4-R in the developing rat striatum? Although POMC terminals are lacking in the developing striatum, POMC peptides of peripheral origin cannot completely be excluded. Melanocortins of the pituitary could enter the brain by passing the blood-brain barrier, especially during development, as long as it is immature (Banks and Kastin, 1995). Periventricular organs such as the area postrema have also been shown to be involved in α-MSH actions (Lichtensteiger and Lienhart, 1977), but this would not explain direct effects on striatal melanocortin receptors. Radioactively labelled α-MSH was found to pass the blood brain barrier in adult rats (approx. 0.01%) and accumulate in striatum, occipital cortex, cerebellum and pons and medulla (Pelletier et al., 1975). Rat plasma contains significant amounts of α-MSH from El7 (Davis et al., 1984). However, peripheral sources would yield rather low peptide concentrations in brain. Moreover MC4-R appear in brain long before MSH secretion by the pituitary starts.

It is also possible that another, yet unidentified ligand of Non-POMC-origin binds to these receptors, as shown already for the MC1-R and MC4-R (Lu et al., 1994, in humans also MC2-R, Yang et al. 1997). Agouti, a protein unrelated to the melanocortins and in wild type animals detected only in skin and hairbulbs, is a potent antagonist at MC1-R and MC4-R. Recently, an agouti-related protein whose RNA is normally expressed in the hypothalamus, has been identified in mice (Ollmann et al., 1997). This protein has been shown to be an antagonist at both, the MC3-R and the MC4-R. The new data do not answer the question of the endogeneous agonist at these receptors.

Dynorphin (DynA-(1-13)NH2) has also been reported to bind to melanocortin receptors. However, the affinities found in in transfected Xenopus fibroblast cells are rather low, with Kd values of 87, 43 and 115nM for the human MC1, MC3 and MC4-R, respectively. The peptide acts as an antagonist at the MC-R’s (Quillan and Sadee, 1997). Interactions between the opioid and the melanocortin systems have been known for a long time but they probably are due to functional interactions rather than to interactions at the receptor level.

NEI, an alternate splice product of the MCH precursor protein, is another candidate that could possibly interact with MC-Rs. NEI and MCH, a sister protein derived from the same precursor, are distributed widely in the brain and are detectable in cortex and
striatum, two brain regions containing MC-R but no or very little POMC innervation. NEI shows sequence similarity with α-MSH at the C-terminus and crossreacts with antiserum raised against the C-terminus of α-MSH. The endogenous receptors for MCH, NEI and NGE have so far not been identified. (Nahon et al., 1994). MCH does not bind to any known MC-R and no data concerning binding of NEI or NGE to MC-R have been published (Drodz et al., 1995 and personal communication with E. Hinterman/A. Eberle). It remains to be elucidated, whether NEI acts directly via MC-R or indirectly with the melanocortin system.

With regard to neurotrophic effects of melanocortins in vitro a general problem of this model must be addressed: are we really sure that we are observing a developmental effect rather than a regenerative effect? It is impossible to prepare cell cultures without injury of at least part of these cells. In our system, the early-born patch neurons of the striatum have already established connections with the substantia nigra at the time of tissue collection. Enhanced neurofilament content of short-term cultures after treatment with α-MSH or NDP might thus reflect a neuroprotective and regenerative effect of these compounds, rather than a true developmental effect. The fact that we did not observe an increase in neurofilament content after prolonged culture, could mean either that regeneration was terminated or that there existed a „developmental time window“ for the peptide effect as discussed in Part 2. Regeneration patterns often resemble developmental patterns, such that many proteins reappear that are normally found in neonatal animals, but not or in reduced quantity in the adult animal (e.g. GAP-43) (Bär et al., 1990). In quantitative terms, the nigrostriatal DA neurons constitute a very small fraction in the cell culture system (according to immunohistochemical data) which could hardly influence biochemical indices. In contrast, in cannot be excluded that regenerative processes in GABAergic striatonigral neurons might influence these markers. One difference between development and regeneration is the participation of the immune system in the latter which should be considered in in vivo systems. α-MSH and analoges are known to be neuroimmunomodulators (Lipton and Catania, 1997). The sequence mediating these effects, has been attributed to the C-terminal part of α-MSH, to α-MSH11-13. In contrast, melanotrophic and neurotrophic effects have been attributed to
the core sequence α-MSH4-9. However there is no evidence for an intervention of immune processes in the present in vitro system.

The MC4-R is the predominating receptor type in developing (and adult) striatum. However, it would seem to be premature to associate the observed neurotrophic effects exclusively with this receptor type for a number of reasons:

1. α-MSH exhibits only moderate affinity for the MC4-R in striatum (Lichtensteiger et al., 1996).

2. In spite of the similarity of NEI with α-MSH at the C-terminus, it is not known whether NEI acts on one of the known melanocortin receptors. Moreover, it has so far not been possible to demonstrate competition of the C-terminal peptide α-MSH11-13-NH₂ with [¹²⁵I]-NDP in brain regions rich in MC4-R such as striatum (Lichtensteiger et al., 1996; Tatro and Entwistle, 1994). Other functional effects attributed to the C-terminal sequence, such as effects on immune cells (Lipton et al., 1997) have been thought to be possibly mediated by the MC1-R. However, the latter has been located in small amounts in the periaqueductal gray (Xia et al., 1995), but not in the striatum, or in the septum where α-MSH11-13-NH₂ is thought to elicit reduction of fever (Lipton et al., 1997).

3. NDP, an agonist to all known melanocortin receptors does not mimick centrally mediated cardiovascular effects of γ-MSH, indicating that these effects must be mediated by an additional melanocortin receptor.

4. Org 2766 which does not bind to any known melanocortin receptor (Schiöth et al., 1997) also exerts neurotrophic actions in some model systems (Van Rijzingen et al., 1996). However, this peptide analog was ineffective on fetal spinal cord neurons (Van der Neut et al., 1992) which express MC4-R (this study, Kistler-Heer et al., in press).

In conclusion, we observed neurotrophic effects of α-MSH, NDP and NEI on fetal rat striatal-mesencephalic co-cultures where the MC4-R is the predominant (known) melanocortin receptor. As in other studies on dissociated cells, the effects can be related
either to developmental processes or to regenerative phenomena. Whether the effects of the different peptides are all mediated by the MC4-R or by other, unknown receptors remains to be elucidated.

Neurotrophic factors are proteins that have been observed in vitro and in vivo to promote the survival and differentiation of specific neuronal subpopulations, as well as to stimulate the proliferation of neural precursor cells. A classical neurotrophic factor for the developing striatum is neurotrophin 4/5 (NT4), which promotes survival and differentiation of GABAergic striatal neurons (Ardelt et al., 1994; Widmer and Hefti, 1994). Neurotrophin 3 (NT3) (Nakao et al., 1996; Widmer and Hefti, 1993) and brain derived nerve growth factor (BDNF) mimick NT4/5 effects, but less pronounced (Mizuno et al., 1994; Widmer and Hefti, 1994). Nerve growth factor increases survival and differentiation of striatal cholinergic neurons (Hartikka and Hefti, 1988). In contrast, α-MSH, NDP and NEI did not influence survival of striatal-mesencephalic co-cultures, but influenced markers for cell differentiation. They probably should not be considered as true neurotrophic factors, but rather might represent co-factors modulating developmental processes in the central nervous system.
7 OUTLOOK

In experimental model systems melanocortins showed promising effects on a wide range of neuro- and immunopathological conditions. They elicited beneficial effects on neuropathies caused by diabetes mellitus, vincristine, taxol and cisplatin and improved recovery in models of peripheral and central nervous system injury (Bär et al., 1990; Gispen et al., 1994; Strand et al., 1993). Potent antiinflammatory actions of melanocortins have been shown for peripheral inflammations of both the acute and chronic type (Lipton and Catania, 1997). The peptides also influenced inflammatory processes in brain induced by tumor necrosis factor-α (TNF-α) (Lipton and Catania, 1997; Rajora et al., 1997) or brain stem ischemia (Huh et al., 1997). Melanocortins have been applied in a wide dose range to a number of different species (including humans) and were found to have few or no significant side effects (Eberle, 1988, Lipton and Catania, 1997; Van der Hoop et al., 1990). The mechanisms underlying the above mentioned effects have not yet been elucidated. In particular, it is not yet known which types of melanocortin receptors are involved in the various effects. Further research is necessary to match receptor and ligand data and to develop ligands crossing the blood brain barrier in order to provide new insight in this fascinating field of research.
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ABSTRACTS


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