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Melanocortins and the brain: from effects via receptors to drug targets

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Abstract

The lack of specific receptors (and antagonists) has hampered the research on the neural mechanism of action of adrenocorticotropic hormone (ACTH)- and melanocyte-stimulating hormone (MSH)-like peptides. Yet the original observations in the 1970s already pointed to cAMP as a possible mediator of ACTH/MSH effects in neurons. The cloning of melanocortin receptors since 1992, the identification of at least two subtypes (melanocortin MC_3 and MC_4 receptors) that are present in neural tissue and the development of selective and potent agonists as well as antagonists have markedly furthered the position of melanocortins as important neuropeptides. In this paper we discuss the role of especially the receptor subtype melanocortin MC_4 in various behaviors including grooming behavior and feeding behavior and consider new insights in the interaction between the opioid and the melanocortin system at the level of the spinal cord (i.e. pain perception). Finally, based on new data obtained in molecular pharmacological studies on brain melanocortin receptors, we suggest a general concept for selective receptor–ligand interaction: ligand residues outside the peptide core-sequence may direct the conformation of the residues in the ligand core-sequence that interact directly with the receptor-binding pocket and thereby determine selectivity.

Keywords: ACTH (adrenocorticotropic hormone); MSH (melanocyte-stimulating hormone); Melanocortin receptor; Brain melanocortin system; Peptide–receptor interaction; Avoidance; Grooming; Food intake; Pain

1. Introduction

De Wied's (1969) hypothesis about the role neuropeptides play in the brain in shaping behaviour has been and remains of enormous significance. It has inspired the world's neuroscientists to both explore further the insights it was based on and challenge the underlying facts and assumptions. The developments reviewed here illustrate the fact that the availability, and use, of ever more refined techniques will continue to reveal the vast ramifications of the neuropeptide concept.

2. The revival of neuropeptides

The neuropeptide concept formulated by De Wied suggested the existence of neuropeptide receptors at a time when only amine receptors (receptors for dopamine, serotonin, noradrenalin, histamine, etc.) had been identified as important receptors for neurotransmission in the brain. Now, almost half of the drugs prescribed act directly or indirectly (e.g. reuptake inhibitors) on G protein-coupled receptors (Drews, 2000). Although approximately 250 human genes (excluding odorant receptors) for G protein-coupled receptors have been identified (in the public domain) most drugs still act via amine receptors (Fig. 1a). It is estimated that the human genome contains between 500 and 1000 G protein-coupled receptors in total and a significant number of these are neuropeptide receptors. Homologous recombination in mice has demonstrated that not only does ablation of genes encoding for amine receptors results in clear phenotypic changes, but ablation of genes encoding for neuropeptide receptors and for neuropeptide precursor genes also result in clear phenotypic changes. For instance, melanin-concentrating hormone gene knockouts have a low bodyweight, melanocortin MC_4 receptor knockouts are obese, vasopressin knockouts have diabetes insipidus, corticotropin releasing hormone (CRF_2)-receptor knockouts have increased anxiety and orexin knockouts display narcolepsy (Bohus and De Wied, 1998; Chemelli et al., 1999; Huszar et al., 1997; Kishimoto et al., 2000; Shimada et al., 1998). Thus, from a biological point of

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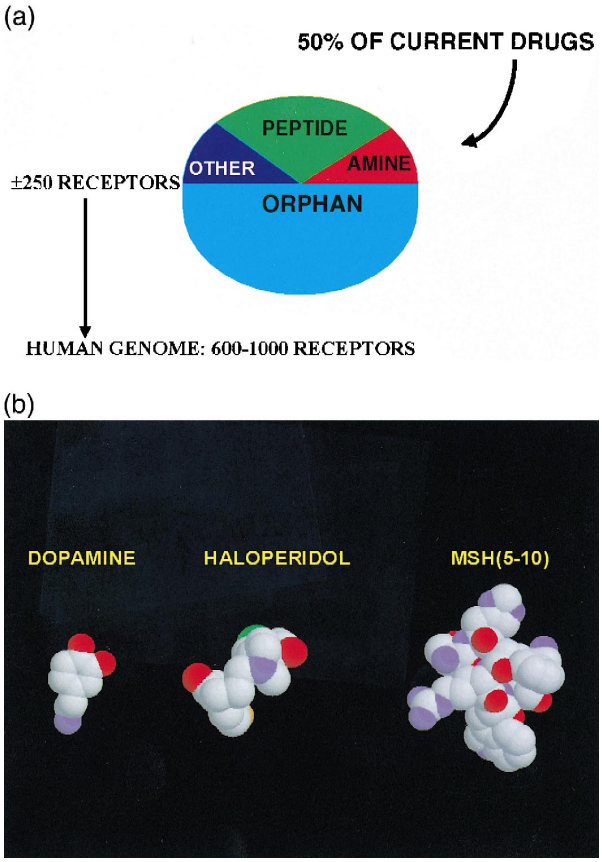

Fig. 1. (a) Classification of G protein coupled receptors (GPCRs) based on ligand. Segments represent approximate shares of the total number (250) of GPCRs identified. The endogenous ligand has not been identified (orphan) for most receptors. The estimated number of GPCRs in the human genome is also indicated. (b) The structures of dopamine, haloperidol and MSH-(5–10) are depicted. Note that the complexity and occupation of space are greater for MSH-(5–10) than for the amines.

view, neuropeptide receptors seem as important as amine receptors, and in the near future, will become increasingly important drug targets.

From a chemical point of view, the occupation of chemical space and the complexity of endogenous ligands are much less for amine receptors than for neuropeptide receptors (Fig. 1b). This, combined with the importance of catecholamine signaling in the (para)sympathetic nervous system, is the main reason why ligands that have been discovered mimick or block the action of amines on their receptors, whereas the equivalent was only achieved recently for neuropeptides. Thus, the fact that drugs like haloperidol, reserpine and propranolol have been available for decades helped with early promotion of amine receptors as drug targets. The only exception to this, demonstrating that neuropeptide receptors were also important drug targets, are the opioid receptors. Clearly, this was because of the availability of morphine as non-peptide ligand. The lack of, in particular, neuropeptide antagonists was the main reason why the importance of neuropeptides was not generally recognized by pharmacologists when De Wied formulated his neuropeptide concept. Current examples of drugs for (neuro)peptide receptors are losartan (angiotensin receptor), octreotide (somatostatin receptor) and desmopressin (vasopressin receptor). Non-peptide ligands have now been developed for cholecystokinin receptors, neurokinin receptors, angiotensin receptors and vasopressin/oxytocin receptors, providing tools to investigate the role of these receptors and to evaluate these receptors as drug targets (Gether et al., 1993; Perlman et al., 1997). In this brief review we use the brain melanocortin system to illustrate the initial rise in importance of neuropeptides, the technical problems encountered in the early days of neuropeptide research and the renewal of interest in this field.

3. Effects of melanocyte stimulating hormone (MSH) and adrenocorticotopic hormone (ACTH) on the brain

In a time before the era of receptor binding assays, of immunohistochemistry and molecular neurobiology, De Wied (1969) based his concept on the observations that pituitary peptide fragments affected animal behaviour via an extra-endocrine target effect that presumably involved a peptide–brain interaction. The concept therefore predicted brain action/production and processing of peptides. As one of David de Wied's first students given the task to unravelling the neurochemical actions of neuropeptides in the brain, one of us (WHG) focused on peptides derived from ACTH and MSH. The original studies were inspired by the ideas of the sixties that learning and memory processes involved RNA and protein metabolism in the brain. Hence, peptides taken to exert effects on learning and memory were thought to alter these neurochemical mechanisms. Later, the studies were guided by the principle that a given cell, when the target for peptide action, be it endocrine target cell or neuron, would respond to ACTH and MSH by recognition and activation of the hypothetical receptor, a subsequent increase in cAMP, the only second messenger known at the time, followed by cell/tissue specific responses at the DNA/RNA/protein level. One should realize that, in the early 1970s, there were no receptors characterized for ACTH and MSH anywhere in the body, the pro-opiomelanocortin (POMC) system was not known and POMC pathways in the brain had not been described.

In retrospect, the early search for the neurochemical action of MSH and ACTH was unsuccessful regarding its primary aims, the precise mechanisms of neuron activation by these peptides. The rationale of the studies was good, the methodology was often inadequate and interpretation of the results was hampered by the lack of factual knowledge about neuron function. However, many important issues arose which yielded new and fascinating information about peptide action in the brain or about signal

transduction in neurons. For instance, the role that melanocortins play in novelty-induced grooming behaviour and/or the neurosubstrate of grooming could never have been described in such detail without this information (Spruijt et al., 1992). Peptide-sensitive, non-cAMP dependent phosphorylation by protein kinase C of a specific presynaptic protein B-50 involved in cell shape changes, vesicle recycling, synaptic plasticity and phospholipid metabolism (Oestreicher et al., 1997) could never have been found if the search for the neurochemical action of MSH/ACTH had not been launched at the time when no receptors were available.

4. Peptide–cell interaction in the pre-receptor era: cAMP discovered as second messenger mediating effects of MSH and ACTH

Most of our work was based on a presumed multiple mechanism of action of MSH/ACTH-like peptides which would be co-determined by the developmental state of the nervous system at the time of peptide action. The peptide was considered to act as neurohormone, neuromodulator or neurotransmitter (Fig. 2). The neurohormonal mechanism was thought to reflect the trophic influence exerted by peptides on their endocrine targets and to become of significance during brain development and in neural repair (see Strand, this volume). The neurotransmitter and neuromodulator function were the brain correlates of direct hormonal action in the periphery and should explain the behavioural effects of these peptides in adult animals.

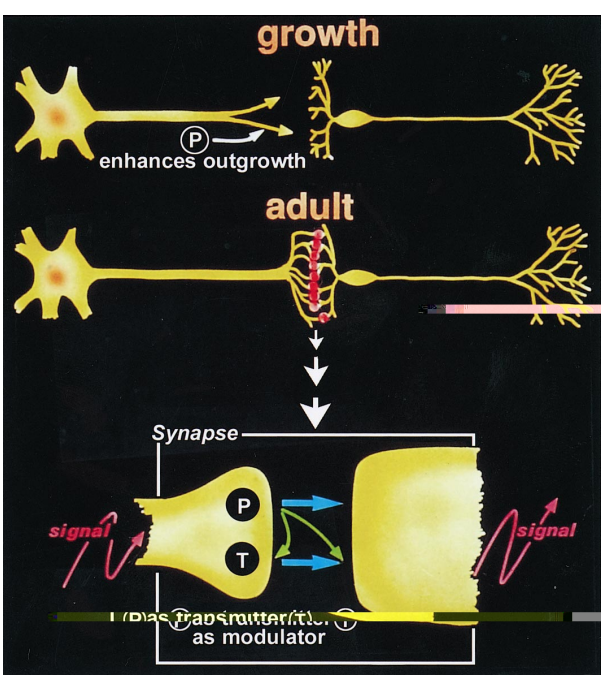

Fig. 2. Multiple actions of neuropeptides. In the top panel, the role of the peptide as paracrine factor or neurohormone is depicted, emphasizing the trophic influence of the peptide in the developing nervous system. The lower panel illustrates the role of the peptide as transmitter carried out by a colocalized neurotransmitter.

The second messenger in the peripheral target cells of ACTH and MSH was presumed to be cAMP and thus a number of studies in the brain focused on the effects of ACTH/MSH on the enzyme, adenylate cyclase. In broken cell preparations of rat brain subcortical areas, ACTH had a biphasic effect on the activity of adenylate cyclase: concentrations below 25 μM stimulated, whereas concentrations of 0.1 mM and higher inhibited activity. Slices of rat neostriatum incubated with ACTH had a dose-dependent increase in cAMP accumulation, and intracerebroventricular (i.c.v.)-injected ACTH significantly increased cAMP in rat septum 60 min following the injection and did not do so in a variety of other brain areas analysed at various times after peptide application (Wiegant et al., 1979a). As the inhibitory effect on cAMP production was marked and therefore easy to study, this aspect of the peptide–brain interaction attracted much attention. The fact that high concentrations were required was considered to result from the poor experimental conditions for in vitro work as compared to those for work with the intact adrenal or brain.

Interestingly, a later, much improved, experimental approach confirmed and extended some of the early findings on melanocortins and brain cAMP. ACTH and MSH stimulated adenylate cyclase in brain tissue slices with efficacies in the nM range. Again, neostriatum and the septal region were found to be especially sensitive to the peptides in this respect. However, as Wiegant et al. (1979a) had found, the maximal stimulatory effects were rather modest compared to that what was documented for the peripheral targets of these peptides. Nonetheless, it was suggested that the binding of endogenous ACTH or MSH to a putative ACTH/MSH receptor in certain brain regions would lead to the activation of a signal transduction pathway that used cAMP as second messenger (Florijn et al., 1993b). These suggestions were made plausibe by results of studies on the role of cAMP in the neurotrophic mechanism of action of melanocortins in fetal spinal cord and dorsal root ganglia in culture (Hol et al., 1993, 1994). Meanwhile, in 1992 a melanocortin receptor family was cloned, which consisted of at least five members. Three of these members could be identified in neuronal tissue, all being G-coupled seven transmembrane spanning domain receptors, positively coupled to adenylate cyclase.

5. POMC

Although direct effects of melanocortins on the brain, i.e. independent of the adrenal gland, had been described since the 1950s (Ferrari, 1958; Miller and Ogawa, 1962; Mirsky et al., 1953) it had remained unclear whether melanocortins had a physiological role in the brain. The

demonstration of melanocortin peptide immunoreactivity in brain in the late 1970s was the first indication that there was a brain melanocortin system (Holmes and Weber, 1986; Jacobowitz and O'Donohue, 1978; Watson et al., 1978). Cloning of the POMC gene in the early 1980s and the finding of its expression in hypothalamus and brainstem (Drouin and Goodman, 1980; Gee et al., 1983; Nakanishi et al., 1979; Nakanishi et al., 1981; Schwartzberg and Nakane, 1983), further pointed to the existence of a brain melanocortin system. It was only in the late 1980s that binding sites for melanocortins could be demonstrated in the brain (Tatro, 1990; Tatro and Reichlin, 1987). However, at this stage research on the functional role of melanocortin receptors was hampered by the lack of melanocortin receptor antagonists.

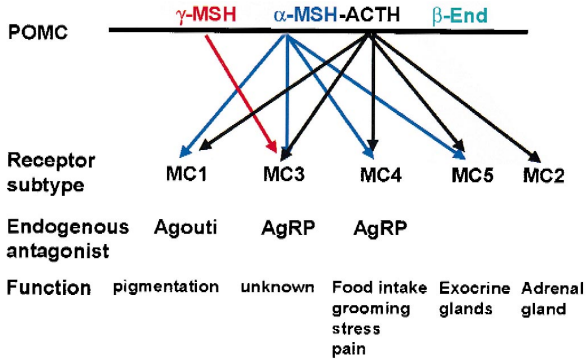

Fig. 3. The melanocortin system. Pro-opiomelanocortin (POMC) and its products MSH, ACTH and endorphin are shown. Arrows indicate for which melanocortin MC receptor subtype the POMC products are agonists. Agouti and agouti-related protein (AgRP) act as endogenous melanocortin receptor antagonists.

6. Cloning of melanocortin receptors

The lack of melanocortin antagonists and of the demonstration of specific binding sites in the brain had long raised doubts about the existence of brain melanocortin receptors. The cloning of melanocortin receptors and the demonstration that some of the members of the melanocortin receptor family were expressed in the brain were thus milestones in this field of research.

Cloning of the melanocyte MSH (MC_1) receptor and adrenal ACTH (melanocortin MC_2) receptor was soon followed by the cloning of three other melanocortin receptors: melanocortin MC_3, MC_4 and MC_5 (Barrett et al., 1994; Chhajlani et al., 1993; Chhajlani and Wikberg, 1992; Gantz et al., 1993a, b; Low et al., 1994; Mountjoy et al., 1994; Roselli-Rehfuss et al., 1993). All melanocortin receptors belong to the G protein-coupled receptor superfamily. These studies confirmed that melanocortin receptors mainly couple to the cAMP signal transduction pathway. All melanocortin receptors are activated by ACTH, and all melanocortin receptors except the melanocortin MC_2 receptor are activated by α-MSH. The melanocortin MC_2 receptor is only activated by ACTH, and not by the other melanocortins (Fig. 3). The shortest peptide showing activity at micromolar concentrations on all melanocortin receptors is ACTH-(4–10). The N- and the C-terminal three amino acids of α-MSH increase the affinity of the melanocortin peptides, with the C-terminal amino acids being the most important in this respect (Adan et al., 1994a).

The cloning of melanocortin receptors also opened the way to identification of melanocortin receptor antagonists, which were essential tools to determine which of the effects of melanocortins are mediated via the known melanocortin receptors. Screening of a large collection of ACTH-(4–9) and ACTH-(4–10) led to the identification of four ACTH-(4–10) analogs which were characterized as antagonists for melanocortin receptors (Adan et al., 1994b). Two of these analogs antagonized the melanocortin MC_3 receptor, and three antagonized the melanocortin MC_4 receptor. Surprisingly, one of the melanocortin MC_4 receptor antagonists, [Pro^8,Gly^9,Pro^{10}]ACTH-(4–10), was identical to a peptide that had been named Semax by a Russian group studying effects of ACTH peptides on learning and attention (Ashmarin et al., 1995). It may thus be that effects of Semax are mediated via antagonism of melanocortin MC_4 receptors.

Another approach was based on pigmentation assays in frogs and lizards. This led to the identification of other agonists and antagonists for the melanocortin receptor family. For example, [D-Trp^7, D-Phe^{10}]-α-MSH-(6–11)-amide which was discovered to be an antagonist in a lizard skin pigmentation bioassay (Castrucci et al., 1994), also antagonized the melanocortin MC_3 receptor (Tatro and Entwistle, 1993a), and displaced [^{125}I][Nle^4,D-Phe^7]-α-MSH bound to specific brain regions (Tatro and Entwistle, 1994). Especially modifications of phenylalanine modify the activity of melanocortin peptides. Increasing hydrophobicity apparently changes the peptide into an antagonist, since substitution with naphthalene or para-iodo-phenylalanine yields potent antagonists (Hruby et al., 1995). On the other hand, parachloro- and parafluoro-phenylalanines at this position in the peptide yield potent agonists (Hruby et al., 1995), indicating that this position in the melanocortin peptide is essential for receptor (in)activation. Recently, More selective antagonists for the melanocortin MC_4 receptor been described (Schioth et al., 1998).

7. Avoidance or grooming

One of the first effects reported for melanocortins on the brain was the modulation of learning and memory (De Wied, 1966). Extensive structure activity studies with modified ACTH analogs and an active avoidance learning task (the pole jump test) led to the development of

[$MetO_2^4$,D-Lys^8,Phe^9]-ACTH-(4–9) (ORG2766), an ACTH-(4–9) analog lacking activity on steroidogenesis and pigmentation (Van Nispen and Greven, 1986). However, ORG2766 has neither activity at nor affinity for the cloned melanocortin receptors, suggesting that its effects on learning and memory are mediated via other receptors.

ACTH-(4–10) stimulates the facilitation of retention of active avoidance behaviour whereas it is inactive to induce excessive grooming behaviour (De Wied, 1966; Wiegant et al., 1978). Surprisingly, substitution of Phe at position 7 in ACTH-(4–10) for its D-enantiomere yielded a peptide that could induce excessive grooming behaviour, but had an effect opposite to that of ACTH-(4–10) on extinction of active avoidance behaviour (Beckwith et al., 1989; Fekete and De Wied, 1982; Kobobun et al., 1983; Wiegant et al., 1978). Since ORG2766 is not a melanocortin receptor ligand, and ACTH-(4–10) is a poor activator of melanocortin receptors, the effects of melanocortins on avoidance behaviour are probably not mediated via the cloned melanocortin receptors.

The effects of ACTH fragments and ACTH peptides on avoidance behaviour could be differentiated pharmacologically from effects of α-MSH on nerve regeneration (Bijlsma et al., 1981; Van Der Zee et al., 1991) as well as from stimulatory effects of intracerebroventricularly administered melanocortins on plasma corticosterone levels (Wiegant et al., 1979b). This was early evidence for the existence of multiple melanocortin receptor subtypes in the nervous system. ACTH-(1–10) and ACTH-(4–10) did not elicit excessive grooming behaviour (Gispen and Isaacson, 1986) and poorly activated the melanocortin MC_4 receptor in vitro (Adan et al., 1994a, b). [D-Phe^7]ACTH-(4–10) (Gispen and Isaacson, 1986; Kobobun et al., 1983) and ACTH-(4–13) induced excessive grooming behaviour, but with a response less than that response to α-MSH. The order of potency of [Nle^4,D-Phe^7]-α-MSH (NDP-MSH), α-MSH and ACTH-(4–13) for eliciting excessive grooming behaviour correlated with the order for activation of the melanocortin MC_4 receptor in vitro (Adan et al., 1994a, b). [D-Phe^7]ACTH-(4–10) was active, whereas ACTH-(4–10) had no or little activity on both the grooming response and the melanocortin MC_4 receptor in vitro.

The fact that melanocortin MC_4 receptor antagonists block α-MSH-induced excessive grooming behaviour (Adan et al., 1994b) is further evidence suggesting the melanocortin MC_4 receptor mediates melanocortin-induced grooming. SHU9119 (Ac-Nle^4-c[Asp^5, D-2-Nal^7, Lys^{10}]α-MSH-(4-10)-NH_2) a potent competitive mela-

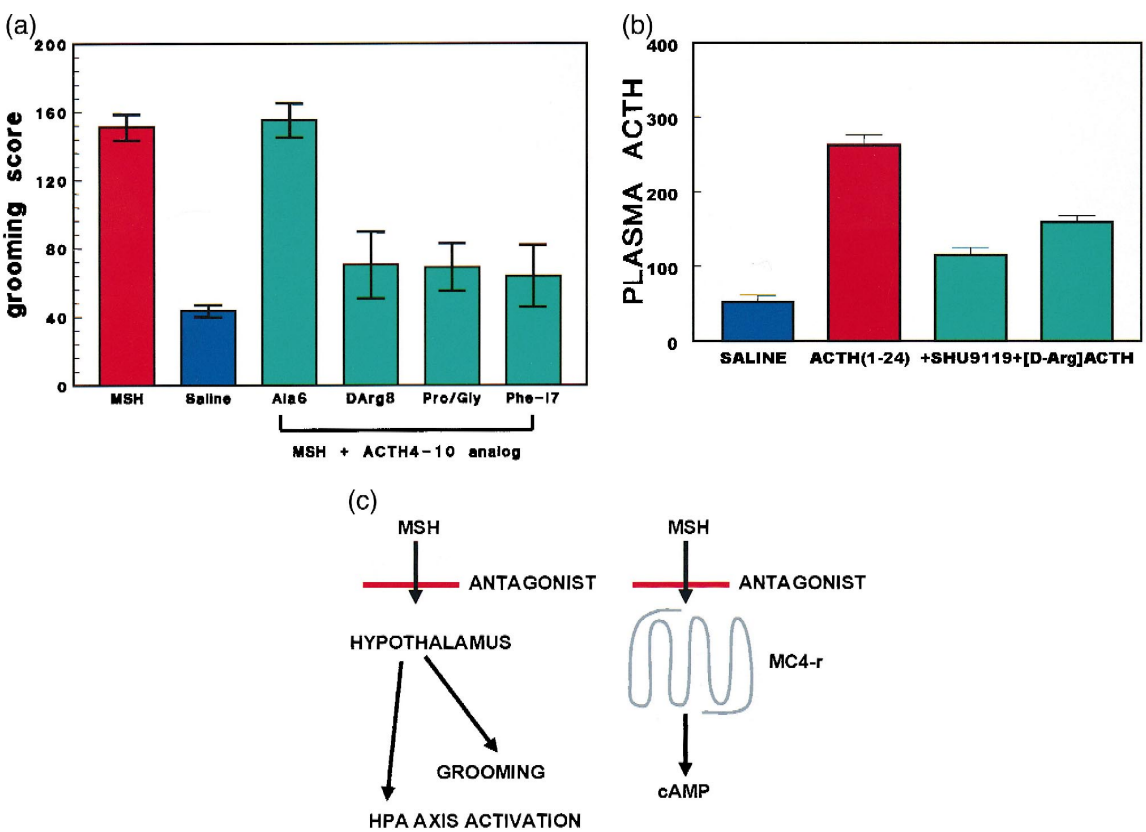

Fig. 4. (a) MSH stimulates grooming behaviour and melanocortin MC_4 receptor antagonists (Pro/Gly = [Pro$^{8,\ 10}$, Gly^9]ACTH-(4–10); D-Arg = [D-Arg^8]ACTH-(4–10); Phe-I7 = [paraiodo-Phe^7]ACTH-(4–10)) block MSH-induced grooming whereas Ala6 (= [Ala^6]ACTH-(4–10) which does not antagonize MC_4) does not. (b) ACTH-(1–24) injected into the brain stimulates plasma ACTH levels. Melanocortin MC_4 receptor antagonists (SHU9119 and [D-Arg^8]ACTH-(4–10)) block this stimulation. (c) Diagram displaying the putative mechanism of melanocortin receptor mediated effects on grooming and on stimulation of hypothalamo-pituitary-adrenal (HPA) axis activity.

nocortin receptor antagonist of human melanocortin MC_3 and MC_4 receptors (pA_2 value 8.3 and 9.3, respectively) but not of human melanocortin MC_1 and MC_5 receptors (Hruby et al., 1995) also inhibited melanocortin-induced grooming behaviour at the low dose of 150 ng, whereas the other melanocortin MC_4 receptor antagonists, with lower pA_2 values, were effective at the dose of 15 μg. Furthermore, the melanocortin MC_4 receptor is the only melanocortin receptor for which mRNA is expressed in all areas (Mountjoy et al., 1994) implicated in α-MSH-induced grooming behaviour (i.e. periaquaductal gray, substantia nigra and paraventricular nucleus) (Bressers et al., 1995; Spruijt et al., 1986; Spruijt and Gispen, 1984; Spruijt et al., 1992). Together, these findings make it probable that the melanocortin MC_4 receptor mediates the effects of melanocortins on excessive grooming behaviour in rats. We demonstrated recently that the increased grooming activity observed when a rat is exposed to a novel environment is also blocked by SHU9119 (Adan et al., 1999). This suggests that activation of the endogenous melanocortin system (and its consequence: novelty-induced grooming) is one of the responses that follow mild emotional stress. Therefore, we tested the validity of this suggestion more directly. We demonstrated that activation of the melanocortin system (and in particular the melanocortin MC_4 receptor) results in activation of the hypothalamo-pituitary-adrenal axis (Fig. 4) (Von Frijtag et al., 1998).

8. Expression of melanocortin receptors in brain

In situ hybridization, using melanocortin MC_3 (Roselli-Rehfuss et al., 1993) and MC_4 (Mountjoy et al., 1994) receptor probes, has demonstrated mRNA expression in multiple nuclei of the brain. There is a good overlap of these expression sites with [^{125}I]NDP-MSH binding sites that had been described before (Low et al., 1994; Tatro and Entwistle, 1993b; Tatro and Entwistle, 1994), suggesting that the sites of melanocortin MC_3 and MC_4 receptor mRNA expression lead to functional receptor protein. The expression of the melanocortin MC_4 receptor in the paraventricular nucleus may link the activity of the central and of the periphal melanocortin system since the paraventricular nucleus regulates the activity of the hypothalamo-pituitary-adrenal axis via its parvocellular vasopressinergic and corticotropic neurons. This site of melanocortin receptor expression may form the substrate for the influence of melanocortins on the hypothalamo-pituitary-adrenal axis (Von Frijtag et al., 1998).

Most of the regions that express dopamine receptors also express the melanocortin MC_4 receptor. Furthermore, the melanocortin MC_3 and MC_4 receptors are expressed in the ventral tegmental area and the melanocortin MC_4 receptor is also expressed in the substantia nigra. Expression of melanocortin receptors in nuclei containing serotonergic and dopaminergic cell bodies, as well as in projection areas of these aminergic neurons, suggests an interaction between these aminergic systems and the melanocortin system. Indeed, an interaction between melanocortins and the dopaminergic system has been described (Florijn et al., 1993a,b).

Melanocortin MC_5 receptor mRNA has been reported to be expressed in the brain, based on results of the reverse transcriptase-polymerase chain reaction, RNase protection and Northern blotting (Barrett et al., 1994; Gantz et al., 1994; Griffon et al., 1994; Labbe et al., 1994). Thus far, no in situ hybridization has been performed in the brain with melanocortin MC_5 receptor probes, nor are there antibodies available against this receptor subtype. Therefore, the site(s) of melanocortin MC_5 receptor expression in the brain is (are) still unknown. RNase protection assays suggest that the melanocortin MC_5 receptor is expressed in cerebellum (Fathi et al., 1995; Griffon et al., 1994); however, this could not be reproduced by others (Alvaro et al., 1996). The melanocortin MC_3 and MC_4 receptors are not expressed in cerebellum. There is no specific binding of [^{125}I]NDP-MSH to the cerebellum (Tatro and Entwistle, 1993b), suggesting that there are no functional melanocortin receptor binding sites in the cerebellum. Since virtually all NDP-MSH binding sites in brain overlap with sites of melanocortin MC_3 and MC_4 receptor mRNA expression, the question remains as to whether melanocortin MC_5 receptors are functionally expressed in the brain.

9. Interaction with opiates

The melanocortin and opioid system have opposite activities in many tests, and have therefore been considered as functional antagonists. For instance, naloxone inhibits the full expression of excessive grooming behaviour observed after intracerebral melanocortin injections (Aloyo et al., 1983; Gispen and Wiegant, 1976). Conversely, melanocortins have been reported to antagonize opiate self-administration as well as opiate analgesia (Contreras and Takemori, 1984; Szekely et al., 1979). Recently, it was demonstrated that morphine treatment influences the expression of the melanocortin MC_4 receptor in the periaquaductal grey region and in the striatum (Alvaro et al., 1996). The anatomical link between the melanocortin system and the opioid system in the periaquaductal grey area may form the substrate for the effects of α-MSH on the development of opioid dependence.

The functional antagonism between the opioid and the melanocortin system (Gispen et al., 1976) that had been described earlier, before the cloning of opioid and melanocortin receptors, now is based on more solid evidence. First, β-endorphin and α-MSH originate from the same precursor molecule, POMC. Full processing of the POMC precursor will result in storage of β-endorphin and

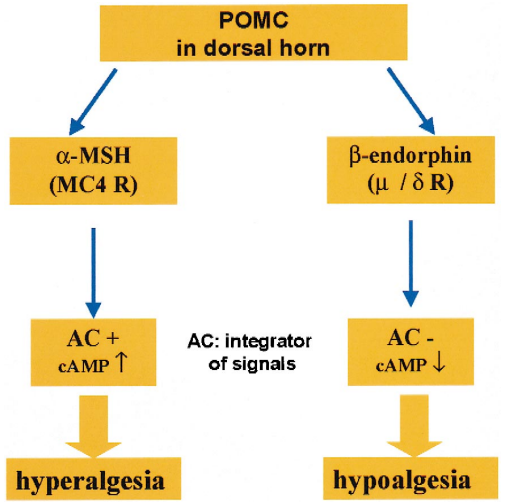

Fig. 5. The spinal cord melanocortin system and pain. Endorphins and melanocortins released from POMC neurons descending from the brainstem project on melanocortin MC_4 receptor expressing neurons in the dorsal horn of the spinal cord to modulate transmission of nociceptive stimuli. These latter neurons integrate these oppositely acting signals (MSH and endorphins) at the level of adenylate cyclase (AC) to modulate transmission of nociceptive stimuli.

α-MSH in the same synaptic vesicles. Thus, the release of α-MSH will always be accompanied by the release of β-endorphin at the same anatomical site. Interestingly, opioid receptors are negatively coupled to adenylate cyclase whereas melanocortin receptors are positively coupled to adenylate cyclase. Opioid and melanocortin receptors are colocalized in, for instance, locus coeruleus cells and, in these cells, they regulate adenylate cyclase activity in opposite ways (Rene et al., 1998). Thus, the activity of the melanocortin and that of the opioid system are integrated at the single cell level by co-expression of its receptors in the same cell. Recently, our group (Vrinten, Adan and Gispen) found that the melanocortin receptor antagonist, SHU9119, suppresses allodynia in a neuropathic pain model in the rat, whereas administration of a melanocortin receptor agonist aggravates pain. Thus, the activity of the melanocortin system is also involved in the modulation of sensitivity to painful stimuli (Fig. 5).

10. Melanocortins and obesity

Up to 1994, the role of the brain melanocortin system in the regulation of food intake and bodyweight had not been a major issue in the literature on melanocortin effects.

The first evidence that the brain melanocortin system was involved in the regulation of body weight came from Lu et al., demonstrating that Agouti was not only an antagonist for the melanocortin MC_1 (MSH) receptor, but also for the melanocortin MC_4 receptor (Lu et al., 1994). Prior to this report, it was known from genetic studies in the mouse that the agouti locus was epistatic to the extension locus. It had been suggested that the extension locus encoded the receptor, and that the agouti locus encoded for the ligand for this receptor. The extension locus encodes for the melanocortin MC_1 receptor and loss of function mutations of the extension locus result in lack of dark (eumelanin) pigmentation. Therefore, it was expected that Agouti was a melanocortin MC_1 receptor antagonist. In some mutations of the agouti locus, like viable and lethal yellow (A^y), there is ectopic overexpression of Agouti. These mice are not only yellow, but also obese. It was hypothesized that the ectopic expression of agouti in the brain would result in antagonism of a brain melanocortin receptor. Lu et al. (1994) demonstrated that agouti antagonized not only the melanocortin MC_1 receptor, but also the melanocortin MC_4 receptor. This provided an explanation for the fact that the A^y mouse was not only yellow (because of blockade of the MSH receptor in skin) but also obese because of blockade of the brain melanocortin MC_4 receptor. Further evidence was provided by the demonstration that mice homozygous or heterozygous for deletion of the melanocortin MC_4 receptor become obese (Huszar et al., 1997). Moreover, the identification of the agouti-related protein (AgRP) as endogenous Melanocortin receptor antagonist expressed in the arcuate nucleus, its upregulation when food intake is restricted, and the fact that ectopic overexpression of AgRP in transgenic mice results in obesity (Ollmann et al., 1997) further emphasized the importance of the melanocortin system in the regulation of body weight (Fig. 6). Another line of evidence showed that the melanocortin MC_3/MC_4 receptor antagonist, SHU9119, stimulated food intake (Fan et al., 1997). In view of these findings, the lack of an antagonist prior to the cloning of melanocortin receptors was probably the

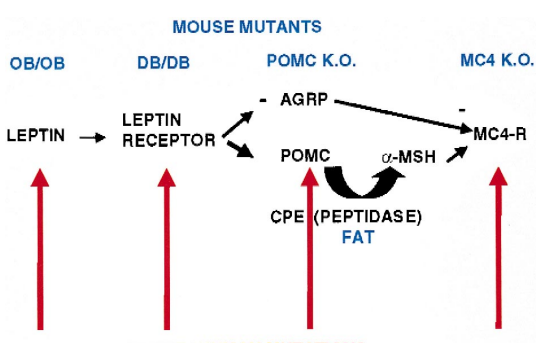

Fig. 6. Four mouse mutants resulting in obesity are obese (ob/ob), diabetic (db/db), and the knock-outs POMC −/− and melanocortin MC_4 receptor −/−. The products of these genes are in a linear neuroendocrine signalling cascade. Loss of function of one of these genes results in obesity, not only in mice, but also in humans (indicated by red arrows). Gain of function mutations of AgRP, also results in obesity, probably due to chronic blockade of the melanocortin MC_4 receptor. The fat mouse may also have a mutation in the same cascade, since, in these mice, carboxypeptidase E is mutated. This enzyme is necessary for the proper processing of POMC into MSH.

main reason why melanocortin receptors were not recognized as receptors modifying food intake. It was the genetic studies that identified the importance of the melanocortin system in the regulation of food intake and body weight.

Neuropeptide (receptor) gene knockouts and the design of selective ligands thus have been, and remain, essential for elucidating the role of neuropeptide receptors.

11. Molecular pharmacological studies of brain melanocortin receptors

Studies, performed by Oosterom in our laboratory, focused on the molecular interaction between neuropeptide ligand and receptor revealed the existence of residues in receptor and neuropeptide ligand that determine ligand selectivity. For instance, when studying the molecular interaction between α- and γ-MSH on the melanocortin MC_3 and MC_4 receptors, for which we constructed chimeric melanocortin MC_3/MC_4 receptors and chimeric α/γ MSH peptides, we identified a single amino acid (Tyr) in the receptor (Tyr^{268} in melanocortin MC_4 receptor) and a single amino acid in γ-MSH (Asp in γ-MSH), which are essential for selective ligand–receptor interaction (Oosterom et al., 1999) (Fig. 7). Asp in [Nle^4]Lys-γ_2-MSH, is located at position 10, where [Nle^4]α-MSH has a Gly. [Nle^4, Gly^{10}]Lys-γ_2-MSH, where Asp replaces Gly, is a high-affinity ligand for the melanocortin MC_4 receptor, which demonstrated that Asp is essential for melanocortin MC_3 receptor-selective interaction.

Selective receptor–ligand interaction could occur through a mechanism of exclusion, or a model of specific recognition. Thus, the Tyr^{268} of the melanocortin MC_4 receptor may hinder [Nle^4]Lys–γ_2-MSH interaction, whereas the absence of this Tyr residue in the melanocortin MC_3 receptor may allow [Nle^4]Lys–γ_2-MSH to interact with high affinity for this receptor. Alternatively, the Asp residue in [Nle^4]Lys–γ_2-MSH may interact specifically with a residue in the melanocortin MC_3 receptor.

His^{264} of the melanocortin MC_4 receptor is the equivalent of the His^{260} in the melanocortin MC_1 receptor and may, as was suggested for the melanocortin MC_1 receptor (Frandberg et al., 1994; Lung et al., 1996), also be important for interaction with γ-MSH. His^{264} is essential for melanocortin peptide activation of the melanocortin MC_4 receptor as well (Adan et al., 1997). His^{264} of the melanocortin MC_4 receptor is located only four residues deeper into transmembrane 6 (TM6) than Tyr^{268}. Therefore, Tyr^{268} may mask His^{264} and thereby exclude an interaction with [Nle^4]Lys–γ_2-MSH.

The presence of Glu^5 and Asp^{10}, as in [Nle^4, Asp^{10}]α-MSH, leads to a marked decrease in melanocortin MC_3 and MC_4 receptor affinity, probably because of repulsion of these two acidic residues. This is consistent with the high affinity of cyclic lactam-(cyclization between residues 5 and 10) and disulfide-bridged (cyclization between residues 4 and 10) melanocortin derivatives (Al-Obeidi et al., 1989; Haskell-Luevano et al., 1997b, c; Hruby et al., 1995; Sawyer et al., 1982). Thus, these data suggest that, in the active conformation, the residues in positions 4/5 and 10 are in close proximity. The presence of Asp^{10} in [Nle^4]Lys-γ_2-MSH probably decreases the chance that the core sequence will be in the optimal conformation for binding to the melanocortin MC_4 receptor.

To investigate whether presentation of a constraint core sequence determined melanocortin MC_4 receptor selectivity, the affinity of cyclic melanocortin peptides with a structurally constrained core sequence was tested. [Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH-(4-10) (MT-II) and [D-Tyr^4]MT-II, displayed a marked increase in affinity compared to NDP-α-MSH for the melanocortin MC_4 receptor, but not for the melanocortin MC_3 and MC_4 (Tyr^{268}Ile) receptors (Oosterom et al., 1999). Thus, mutation of Tyr^{268} in melanocortin MC_4 receptor towards the Ile residue present on the corresponding position in the melanocortin MC_3 receptor increased [Nle^4]Lys-γ_2-MSH affinity and decreased affinity for the high-affinity melanocortin MC_4 receptor ligands, MT-II and [D-Tyr^4]MT-II. Thus, a single amino acid residue determines melanocortin MC_3/MC_4 receptor selectivity for different ligands and melanocortin MC_4 receptor binding increases when the core sequence is presented in a constrained conformation.

It was shown that cyclic peptides have a higher affinity than α-MSH at all melanocortin-receptors (Schioth et al., 1997). Strikingly, all cyclic peptides displayed higher or similar affinity for the melanocortin MC_4 receptor, than for the melanocortin MC_3 receptor (Schioth et al., 1996; 1997), while linear peptides always seem to show a lower affinity on the melanocortin MC_4 receptor than on the melanocortin MC_3 receptor. In more detail, peptides with a disulfide bridge and without the C-terminus appear to be favourable for the melanocortin MC_4 receptor. This effect is further enhanced by replacement of L-Phe by D-Phe. Moreover, peptides with a lactam bridge, SHU9119 and MT-II, have a higher affinity at the melanocortin MC_4 receptor as compared to the melanocortin MC_3 receptor (Fan et al., 1997). Therefore, for design of new melanocortin receptor selective ligands, cyclization may be appropriate for the melanocortin MC_1 and MC_4 receptors. In contrast, modification in linear MSH peptides may be more valuable for melanocortin MC_3 and MC_5 receptor selectivity, as was suggested by Haskell-Luevano et al. (1997b). A similar model was proposed for the opioid receptors in which linear dynorphin A analogues were generally more selective for the μ-opioid receptor while cyclic constrained-dynorphin A peptides had slight selectivity for κ- vs. δ-opioid receptors or were non-selective (Arttamangkul et al., 1997). Together, these data suggest that the message sequence residues do not interact with conserved neuropeptide receptor residues in the same manner. Indeed, it has been shown that the His–Phe–Arg–Trp

(a)

1 2 3 4 5 6 7 8 9 10 11 12 13

α-MSH: Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

γ_2-MSH: Ac-Lys-Tyr-Val-Nle-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2

	MC3R	MC4R
α-MSH	+	+
γ_2-MSH	+	-
[Gly^5]α-MSH	+	+
[Asp^{10}]α-MSH	-	-
[Gly^5,Asp^{10}]α-MSH	+	-
[Gly^{10}]γ_2-MSH	+	+

(b)

	MC3	loop	1st half	Y^{268}I	MC4	2nd half
α-MSH	+	+	+	+	+	+
γ_2-MSH	+	+	+	+	-	-

(c)

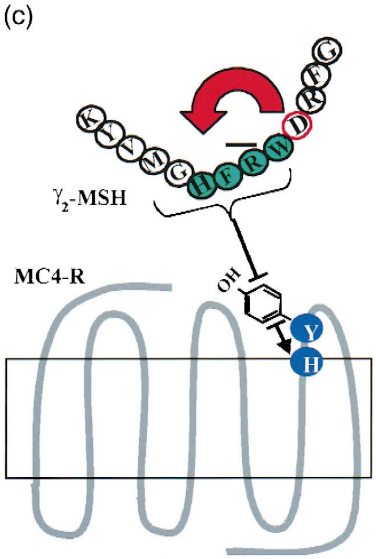

Fig. 7. (a) The sequence of α- and γ-MSH is given. The table indicates whether or not the ligand shown potently activates the melanocortin MC_3 or MC_4 receptor. (b) The activity of α- and γ-MSH on wild type, mutated and chimeric melanocortin MC_3 (red) or melanocortin MC_4 (grey) receptors is shown. (c) Model describing why γ-MSH is not able to activate the melanocortin MC_4 receptor. The Asp residue influences the conformation of the core sequence (in green: His–Phe–Arg–Trp), thereby preventing this core from interacting with the binding pocket of the melanocortin MC_4 receptor. Replacement of Asp by Gly in γ-MSH allows the ligand to interact with the melanocortin MC_4 receptor.

pharmacophore interacts differently with all melanocortin receptors (Haskell-Luevano et al., 1997a).

12. Neuropeptide pharmacophores

We offer a general concept for selective receptor–ligand interaction that may apply to all peptide receptors: ligand residues outside the peptide core sequence may direct the conformation of the residues in the ligand core sequence that interact directly with the receptor binding pocket, and thereby determine selectivity. There are several examples that emphasize this critical role of residues positioned outside the core of true contact residues. Opioid peptides require the Tyr–Gly–Gly–Phe core for opioid action but opioid receptor specificity is dependent on the C-terminal extension of this core. More specifically, it was proposed for deltorphin C that N-terminal residues may be critical for determining the selective interaction of δ-opioid receptors (vs. the μ-opioid receptor) with the Asp residue in the ligand (Bryant et al., 1997). In addition, cyclic lactam peptide analogues of dynorphin A with D-Asp incorpora-

tion in different positions were suggested to exert conformational effects on nearby amino acids which help to discriminate between the κ-, μ- and δ-opioid receptors (Lung et al., 1996). Furthermore, alanine scan and molecular dynamic simulation of neuropeptide Y revealed that it may bind with distinct active conformations to the neuropeptide Y_1 vs. Y_2 receptor and thereby directs selectivity (Beck-Sickinger et al., 1994).

These data are important for the design of selective ligands and of drugs for neuropeptide receptors. The picture emerges that it is usually only three or four residues in the neuropeptide ligand which are essential for potency and selectivity. The other amino acids in the neuropeptide serve to stabilise the proper positioning of these real interacting residues. The impact of this is that small molecular weight compounds can be made as peptidomimetics. Thus, to design drugs in neuropeptide research it is essential to identify the interacting amino acids and to stabilise the proper conformation of these residues in chemical space. Therefore, a promising field in medicinal chemistry is the synthesis of new chemical entities in which amino acid side-chains (and derivatives thereof) are positioned on non-peptide backbone scaffolds and tested for activity on neuropeptide receptors. This may provide the tools required to elucidate neuropeptide receptor function and may form the basis for the next step: the design of orally active, non-peptide drugs acting on neuropeptide receptors.

13. Conclusions

The melanocortins are a family of peptides that are relative latecomers to the class of neuropeptides. Once their receptors were cloned, it became clear that the presence of several receptor subtypes, in distinct brain areas overlapping the previously described projection regions of the brain POMC system, could explain the multitude of known effects of ACTH and MSH on brain function. The understanding of these latter effects, based on early work, have helped modern molecular neuropharmacology to open new vistas. Development of specific new ligands for melanocortin receptor subtypes must be paralleled by insight into brain mechanisms to allow the design of drugs for relevant and clearly defined indications. The early behavioural studies by David de Wied are pivotal in this respect as they predicted the possibility of multiple targets for melanocortin action in brain function.

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