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Proopiomelanocortin and melanocortin receptors in the adult rat retinotectal system and their regulation after optic nerve transection

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Abstract

The aim of this study was to characterise the expression of the melanocortin system in the normal and injured rat visual system. Using real-time polymerase chain reaction and immunohistochemistry, we detected melanocortin MC_3, MC_4 and MC_5 receptors and proopiomelanocortin in adult retina and superior colliculus. Melanocortin MC_4 receptor mRNA was the most abundant receptor. Melanocortin MC_3, MC_4 and MC_5 receptors were localised to the ganglion cell and inner nuclear layers and the melanocortin MC_3 and MC_4 receptors were localised to retinal ganglion cells. Transection of the optic nerve leads to ganglion cell death and both melanocortin receptor and proopiomelanocortin expression decreased in superior colliculus after transection whereas the expression was unchanged or even increased in the retina. α-Melanocyte-stimulating hormone elicited neurite outgrowth from embryonic retinal explants. Together, these data implicate a role for the melanocortin system in the adult rat retina and that melanocortins can stimulate neurite growth from retinal neurons.

Keywords: Axotomy; Injury; mRNA expression; POMC (proopiomelanocortin); Melanocortin receptor; Retina

1. Introduction

The melanocortins are a family of bioactive peptides derived from proopiomelanocortin (POMC) (Eipper and Mains, 1980). The melanocortins are formed by post-translational modifications of POMC in a tissue-specific manner. Adreno-corticotropic hormone, α-melanocyte-stimulating hormone (α-MSH), β-MSH, or γ-MSH are formed by proteolytic cleavages of POMC by two main proteolytic enzymes, proconvertases 1 and 2 (Benjannet et al., 1991). Other biologically active peptides derived from POMC are β-lipotropin, γ-lipotropin and β-endorphin (Eberle, 1988). The melanocortins are synthesised in various regions in the central nervous system and in peripheral tissues and participate in multiple physiological functions (Adan and Gispen, 2000). The biological effects of melanocortins are mediated by the activation of melanocortin receptors. This activation is antagonised and modulated by two endogenous physiological antagonists, agouti-signaling protein and agouti-related protein, that bind the melanocortin receptors (Dinulescu and Cone, 2000). In all, five melanocortin receptors have been cloned and characterised (melanocortin MC_{1-5} receptors) (Abdel-Malek, 2001; Chhajlani and Wikberg, 1992; Gantz et al., 1993; Mountjoy et al., 1992) that mediate the physiological functions, including stimulatory effects on pigmentation, adrenal function, energy homeostasis, feeding, sebaceous gland lipid production, steroidogenesis in immune and sexual function (Butler and Cone, 2002; Wikberg et al., 2000). Melanocortin receptors represent a distinct family of G-protein-coupled receptors and all five receptors are functionally coupled to adenylate cyclase and mediate their effects by activating cAMP-dependent signaling pathways. Melanocortin receptors differ from each other in their tissue distribution and in their binding affinity for the melanocortins and their antagonists (Abdel-Malek, 2001). The main sites of melanocortin receptor expression include: melanocytes, immuno- and inflammatory cells for melanocortin MC_1 receptor; adrenal cortex and adipocytes for melanocortin MC_2 receptor; central nervous system, stomach, duodenum and pancreas for melanocortin MC_3 receptor; central nervous system for melanocortin MC_4 receptor and exocrine gland; sebaceous glands and adipocytes for melanocortin MC_5 receptor (Fathi et al., 1995; Wikberg et al., 2000; Wong et al., 1997; Xia et al., 1995). Notably for this study is that POMC and melanocortin

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receptors have been found in the developing chicken eye indicating presence of the melanocortin system in the eye and retinal cells (Teshigawara et al., 2001).

Previous work has shown that melanocortins may have activities on developing and injured neurons that resemble those of neurotrophic factors (Gispen et al., 1994; Hol et al., 1995; Muller et al., 1994; Plantinga et al., 1995; Van de Meent et al., 1997; Van der Neut et al., 1992). The classical neurotrophic factors with the neurotrophin gene family members as prototypes are secreted proteins that exert their trophic functions via receptor tyrosine kinases (Barde, 1988; Lewin and Barde, 1996). Recent results show that retinal ganglion cells respond with increased neurite growth and survival to the stimulation of classical neurotrophic factors if they are co-stimulated with agents that increase intracellular cAMP (Meyer-Franke et al., 1995, 1998). The described neurotrophic activity of melanocortins and the fact that their main signalling pathway involves modulation of intracellular cAMP concentration prompted us to study the expression of the melanocortin system in the retina. The expression has not previously been characterised in the retina and this work shows that melanocortin receptor and POMC mRNA are expressed in the rat retina and in particular in retinal ganglion cells. In addition to the normal expression, we have studied retina and superior colliculus, which is one of the main central retinal ganglion cell targets, after optic nerve transection. This axotomy leads to subsequent retinal ganglion cell death (Villegas-Perez et al., 1993). The induced death occurs via apoptosis (Berkelaar et al., 1994) and can be postponed but not halted by classical neurotrophic factors (Aguayo et al., 1996; Di Polo et al., 1998; Peinado-Ramón et al., 1996). Responsiveness of retinal ganglion cells to neurotrophic factors is decreased after axotomy but is restored by cAMP elevation (Meyer-Franke et al., 1998). The survival of axotomised retinal ganglion cells can be improved if they are simultaneously stimulated by several trophic factors together with cAMP elevation. Thus, this suggest that the death of retinal ganglion cells after axotomy is not caused solely by the loss of retrograde trophic stimuli but also by a loss of complementing intracellular signaling and trophic responsiveness (Shen et al., 1999). In addition to the characterization of the retinal localization of melanocortin receptors, the aim of this work was to study whether the melanocortin receptors contribute to an injury-induced receptor system. We have also used retinal explant cultures to study if retina responds to α-MSH stimulation in a similar way as has been shown for other peripheral and central neurons.

2. Materials and methods

2.1. Animals and surgical procedures

We used adult female DA rats (225–250 g; B&K Universal, Sollentuna, Sweden). For all experimental manipulations, animals were anesthetised with an intraperitoneal injection mixture of xylazine (Rompun; Bayer) and ketamine (Ketalar; Parke-Davis), 10–15 and 30–100 mg/kg body weight, respectively. Rats were housed in standard cages, fed ad libitum and maintained in temperature-controlled rooms with a 12:12-h light/dark cycle with light intensity ranging from 8 to 24 lx. Experiments were carried out in accordance with European Community guidelines and the Association for Research in Vision and Ophthalmology statement for use of animals in ophthalmic and vision research. The local ethics committee (Uppsala djurförsöksetiska nämnd) for experimental animals scrutinised the procedures. Retinal ganglion cells were retrogradely filled with True Blue (Molecular Probes) from the superior colliculi, using previously described techniques (Peinado-Ramón et al., 1996; Vidal-Sanz et al., 1988; Villegas-Perez et al., 1993). Animals were sacrificed 7 days after tracer application by an intraperitoneal injection of 3–4 ml pentobarbital (100 mg/ml, Apoteket, Sweden). For intraorbital optic nerve transection (Vidal-Sanz et al., 1987; Villegas-Perez et al., 1993), an incision was made in the skin covering the superior orbital rim, the superior orbital contents were dissected and superior and external muscles were sectioned. Following rotation of the eye, the dura mater sheath was longitudinally opened and the optic nerve was completely transected as close as possible to the eye with care taken to avoid damage to the retinal blood supply. Animals were sacrificed 4 or 14 days after the transection.

2.2. RNA preparation and real-time quantitative reverse transcriptase PCR (RT-PCR)

Total RNA was purified from retina and superior colliculus using Trizol (Invitrogen). The RNA condition was checked using the Agilent RNA 6000 Assay (Agilent Technologies, USA), which analyses RNA degradation using capillary electrophoresis. After treatment with RNAse free DNAse (Promega), 2 μg of total RNA was used for cDNA synthesis with TaqMan reverse transcriptase using random hexamer primers (PE Applied Biosystems, Foster City, CA). Analysis of gene expression with polymerase chain reaction (PCR) was performed using the SYBR® Green I assay in combination with ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). Primers for melanocortin MC_{2-5} receptors (Table 1) were designed with Primer Express software (Applied Biosystems). The rat melanocortin MC_1 receptor is not available and was therefore not included in the analysis. PCR reactions were carried out on duplicate cDNA samples with activation of AmpliTaq Gold DNA Polymerase for 10 min at 95 °C and 50 cycles were run using two-step PCR (95 °C–15 s, 60 °C–60 s). Each sample was assigned a C_T (threshold cycle) value corresponding to the PCR cycle at which fluorescent emission, detected real time, reached a threshold above baseline. Initial target mRNA levels were calculated and relative differences between operated and

Table 1
Primers used for the real-time RT-PCR analysis

Target cDNA	Primers (5′–3′) forward/reverse	Amplicon size (bp)
MC_2 receptor	TCTTCCAGGTCAATGGCATGT	73
	TGGGCTCCGAAAGGCATATA	
MC_3 receptor	CTTCCTCCACCTGGTCCTCA	66
	TGCGCCGTGTAGCAGATG	
MC_4 receptor	AACCTGCACTCACCCATGTACTT	70
	TCGAAACGCTCACCAGCA	
MC_5 receptor	CTGGCCCGGAACCATGT	66
	GGCCCTTTGCCTCACAGA	
POMC	TGCAGACTCGACCTCTCGG	70
	TTTCAGTCAAGGGCTGTTCATCT	
β-Actin	CTTCAACACCCCAGCCATG	69
	GTGGTACGACCAGAGGCATACA	

unoperated animals were determined. PCR products were gel-separated to confirm a band of the expected size. As controls, the relative mRNA levels of housekeeping genes β-actin, β2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase, and hypoxanthine phosphoribosyl transferase were measured in each group of normal and injured tissues. None of these genes showed any differences in mRNA levels between groups and thus were not affected by optic nerve transection (results not shown). β-Actin was chosen as a reference gene in each PCR run. All collected data was normalised against β-actin levels and statistically analysed using Analysis of variance (ANOVA). *P*-values less than 0.05 were considered significant.

2.3. Immunohistochemistry

Adult rats were deeply anesthetised and perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Four eyes were post-fixed, cryoprotected and 12 μm sections were collected using a cryostat and mounted on Superfrost Plus slides (Menzel–Gläser). Sections were pre-incubated in PBT (PBS with 0.3% Triton X-100) and incubated overnight with anti-α-MSH (1:50; Euro Diagnostica), anti-MC_3 receptor (1:50; Alpha Diagnostic), anti-MC_4 receptor (1:50; Alpha Diagnostic), or anti-melanocortin MC_5 receptor (1:50; Alpha Diagnostic) in PBT with 5% of appropriate serum. For detection of primary antibody, a biotinylated secondary antibody (1:50; Vector Laboratories, Burlingame, CA) and a Vectastain ABC™ Elite kit (Vector Laboratories) was used with diaminobenzidine substrate. The length of the ABC reaction was individually adjusted for the antibodies to give a clear immunostaining pattern. Patterns representative for the four animals are shown. For fluorescent detection, Alexa Fluor 594-conjugated antibody (1:50; Molecular Probes) was used. Negative controls were performed with secondary antibodies only. Slides were mounted with glycerol, viewed and documented in a Zeiss Axioplan microscope. A region 1 mm lateral to the nerve exit is depicted in the figure.

2.4. Bioassay

Chicken embryos were staged according to Hamburger and Hamilton (1992) and 0.4 mm retinal punches of central embryonic day 6 chick retina (stage 30) were collected and placed in collagen gels (100 μl). Neurobasal medium (100 μl; Invitrogen; supplemented with 0.05 M HEPES and 1% penicillin/streptomycin) was added to the gels together with 4–4000 ng/ml [Des-Acetyl]α-MSH (Des-Ac-MSH) or [Nle^4, D-Phe^7]α-MSH (NDP-MSH) (Neosystem Groupe, Strasbourg, France). After 2 days in culture, the mean maximum outgrowth was measured as previously described (Carri and Ebendal, 1987; Hallböök et al., 1996; Karlsson et al., 2002) using a Zeiss Axioplan microscope.

3. Results

3.1. Melanocortin MC_3, MC_4, MC_5 receptor and POMC mRNA expression in retina and superior colliculus

Among the melanocortin receptors, the melanocortin MC_3 and MC_4 receptors are mainly expressed in the nervous system whereas melanocortin MC_1 and MC_2 receptor are expressed in melanocytes and in the adrenal cortex. In order to study the melanocortin receptor mRNA expression in the rat retina and superior colliculus, we used quantitative real-time PCR with primers for melanocortin

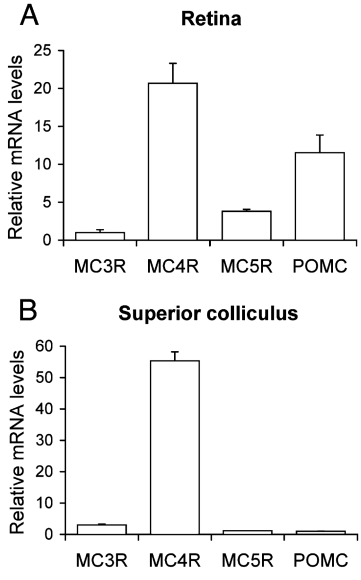

Fig. 1. Melanocortin receptor and POMC mRNA levels in the normal adult rat retina and superior colliculus. Melanocortin receptors and POMC mRNA levels were analysed in adult rat retina and superior colliculus using real-time quantitative RT-PCR. (A) Relative melanocortin MC_{3-5} receptors and POMC mRNA levels in rat retina. Melanocortin MC_3 receptor mRNA level was assigned a relative index of 1. (B) Relative melanocortin MC_{3-5} receptors and POMC mRNA levels in rat superior colliculus. POMC mRNA level was assigned a relative index of 1. Bars represent means ± S.E.M. ($n=4$).

MC_{2-5} receptors (Table 1). The melanocortin MC_1 receptor sequence is not available from rat and was not included in the analyses. Analysing the relative levels of the melanocortin receptors mRNA in normal retina using RT-PCR showed that melanocortin MC_{3-5} receptor mRNA could be detected in retina whereas the melanocortin MC_2 receptor mRNA level was not above background. The melanocortin MC_4 receptor mRNA levels were highest, followed by melanocortin MC_3 and MC_5 receptor mRNA (Fig. 1A). Although relatively low compared to those of melanocortin MC_4 receptor, the melanocortin MC_3 receptor mRNA levels were clearly above baseline RT-PCR analysis levels. The mRNA levels encoding the precursor peptide POMC were high in the normal retina and comparable to those of the receptors (Fig. 1A). It should be noted that comparison of the relative level of different amplified fragments only gives an indication of their relative level of expression.

In rat, all retinal ganglion cells project to the superior colliculus, which is one of the main targets for visual central innervation. Similarly to the retina, melanocortin MC_4 receptor mRNA levels were high in superior colliculus. Melanocortin MC_3 and MC_5 receptor levels as well as POMC mRNA levels were low but above baseline RT-PCR analysis levels in superior colliculus (Fig. 1B).

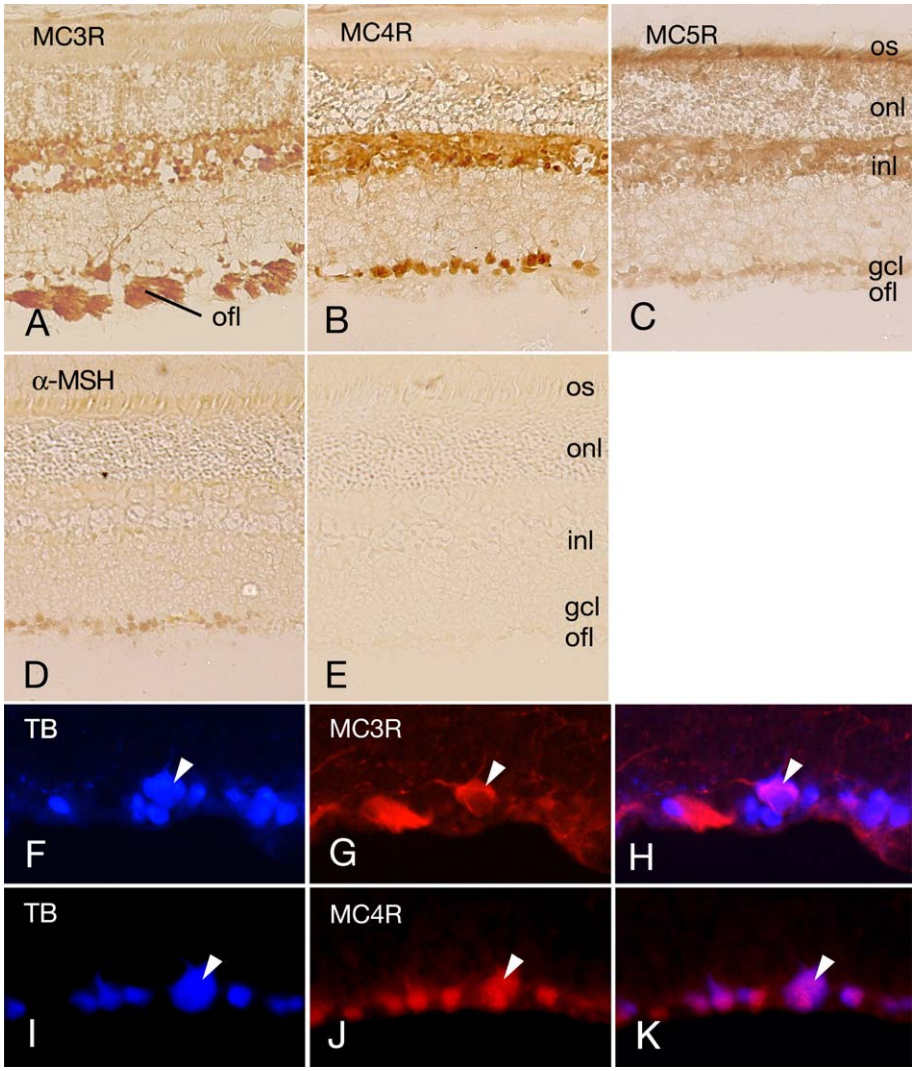

Fig. 2. Expression of melanocortin MC_{3-5} receptors and α-MSH and in the adult rat retina. Micrographs showing immunohistochemistry for (A) melanocortin MC_3, (B) MC_4, (C) MC_5 receptors and (D) α-MSH. (E) Negative control without primary antibodies. The immunoreactivity in A–E is visualised using diaminobenzidine staining. (B) Note melanocortin MC_3 receptor immunoreactivity in large retinal ganglion cells as well as in the ofl. (F–K) Micrographs showing fluorescent immunohistochemistry of retrogradely labelled retinal ganglion cells using the tracer TrueBlue (TB). (F) True Blue-labelled retinal ganglion cells in the ganglion cell layer (blue), (G) melanocortin MC_3 receptor immunoreactivity in the ganglion cell layer (red) and (H) merge of images shown in panels F and G. (I) True Blue labelled retinal ganglion cells in the ganglion cell layer (blue), (J) melanocortin MC_4 receptor immunoreactivity in the ganglion cell layer (red) and (K) merge between images shown in panel I and J. Arrow heads indicate the same cell in F–H and in I–K. gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer; ofl, optic fibre layer; os, outer photoreceptor segments. Scale bar in E is 100 μm, also valid for A–D. Bar in K is 20 μm, also valid for F–J.

3.2. Localization of melanocortin receptor and α-MSH immunoreactivity in the rat retina

Using specific peptide melanocortin receptor antibodies, distinct patterns of immmunoreactivity could be seen for melanocortin MC_{3-5} receptors in rat retina (Fig. 2A–C). Melanocortin MC_3 receptor immunoreactivity was found on cell soma in the inner nuclear layer and ganglion cell layer. Strong staining was seen on a portion of the cells in the ganglion cell layer and on separate processes protruding from these cells that spanned into the inner plexiform layer. Strong staining was also seen in the optical fibre layer containing retinal ganglion cell axons. The optical fibre layer is not a continuous layer and the retinal ganglion cell axons are organised in bundles that give a broken appearance, shown in Fig. 2A,G,H. Weaker staining could be seen in the plexiform layers that separate the nuclear layers. Mainly large cells in the ganglion cell layer were stained for melanocortin MC_3 receptor and when staining retinas with retinal ganglion cells that had been retrogradely labelled from the superior colliculus with True Blue, it was shown that those cells were retinal ganglion cells (Fig. 2F–H). This also confirmed that melanocortin MC_3 receptor immunoreactivity could be seen on retinal ganglion cell dendritic trees within the inner plexiform layer. Weaker melanocortin MC_3 receptor immunoreactivity was also found on smaller retinal ganglion cells.

MC_4 receptor immunoreactivity was found on cell soma in the inner nuclear layer and ganglion cell layer (Fig. 2B), likely on both amacrine and bipolar cells. Melanocortin MC_4 receptor immunoreactivity was found both on large and small True Blue-labelled retinal ganglion cells with immunoreactivity mainly localised to the cell somas (Fig. 2J,K). A marked difference from melanocortin MC_3 receptor was that melanocortin MC_4 receptor immunoreactivity was not seen in the optical fibre layer. Melanocortin MC_4 receptor immunoreactivity could also be seen on cells in the ganglion cell layer that were not labelled for True Blue. Those cells are likely displaced amacrine cells (Fig. 2J,K). There were clearly more True Blue-labelled cells in the ganglion cell layer that were immunoreactive for melanocortin MC_4 receptor than melanocortin MC_3 receptor (Fig. 2A,B).

MC_5 receptor immunoreactivity was found in the inner nuclear layer, ganglion cell layer and in the outer segments of photoreceptors (Fig. 2C). The staining for melanocortin MC_5 receptor was much weaker and less distinct than those for the melanocortin MC_3 and MC_4 receptor. In order to clearly illustrate the MC_5 receptor immunoreactivity in Fig. 2A,C, the incubation time of the peroxidase with substrate for the micrograph shown in Fig. 2C was approximately three times longer for MC_5 receptor than for the MC_4 receptor.

Distinct α-MSH immunoreactivity, although weak, was detected mainly in the ganglion cell layer of the adult rat retina using an antibody that distinguishes all forms of α-MSH. The staining was clearly above background (Fig. 2D,E). Controls for unspecific staining were performed for the different antibodies and they did not produce any unspecific background (Fig. 2E).

3.3. MC receptor and POMC mRNA levels in retina and superior colliculus after optic nerve transection

The mRNA levels of melanocortin MC_3, MC_4, MC_5 receptors and POMC in retina and superior colliculus were

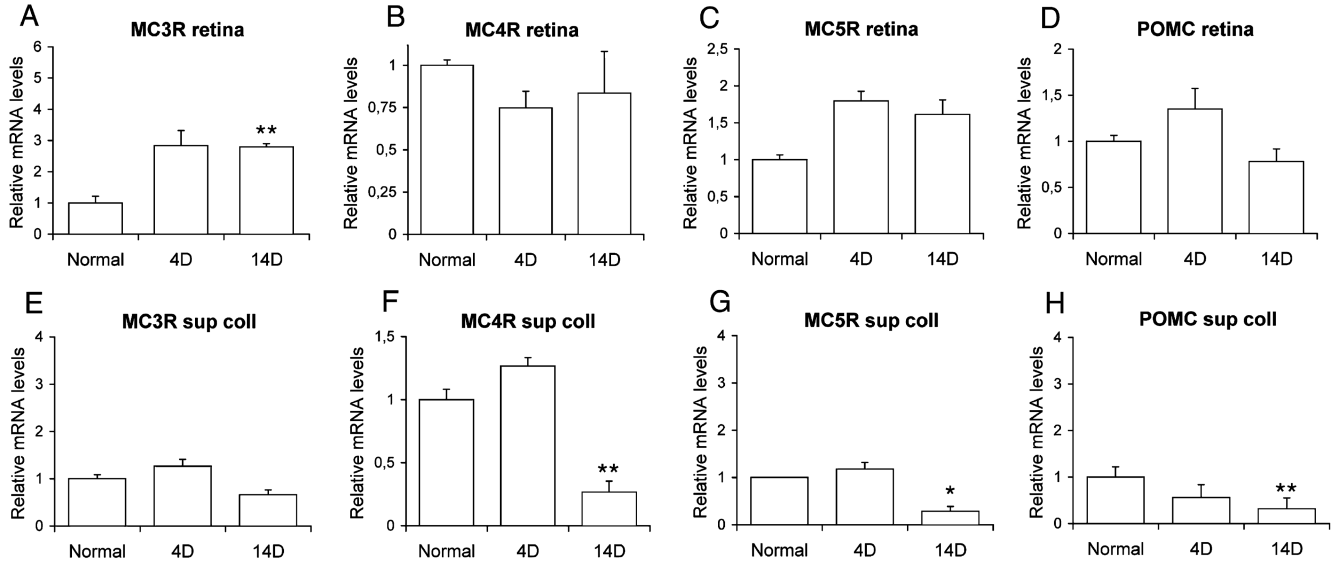

Fig. 3. Melanocortin receptor and POMC mRNA levels in retina and superior colliculus after optic nerve transection. Melanocortin receptors and POMC mRNA levels were analysed in adult rat retina (A–D) and superior colliculus (E–H) 4 and 14 days after optic nerve transection using real-time quantitative RT-PCR. (A, E) Relative melanocortin MC_3, (B, F) MC_4, (C, G) MC_5 receptors and (D, H) POMC mRNA levels. mRNA levels in normal tissue were assigned a relative index of 1. Note that the scale on the *Y*-axis is adapted so that the bar sizes better reflect the relative levels among the melanocortin receptors. Values are means ± S.E.M. ($n = 6$). *Significant difference compared to normal mRNA level (ANOVA, $P < 0.05$), **Significant difference (ANOVA, $P < 0.01$).

analysed using RT-PCR analysis 4 and 14 days after optic nerve transection. The mRNA levels were related to those of the normal retina and superior colliculus and are shown as relative to the levels in the normal tissues. The relative levels of melanocortin MC_3 receptor mRNA increased almost threefold in the retina 4 days after transection from initially relatively low levels in the normal retina (Fig. 1A), and 14 days after injury this increase was significant (Fig. 3B). The high levels of melanocortin MC_4 receptor as well as melanocortin MC_5 receptor mRNA in normal retina (Fig. 1A) were not significantly changed in the retina after optic nerve transection. Similarly, the levels of POMC mRNA did not change significantly after the nerve transection (Fig. 3D).

MC_4 receptor mRNA is clearly the receptor with highest expression in the superior colliculus (Fig. 1B). Fourteen days after injury, melanocortin MC_4 receptor mRNA levels had dropped significantly to about one fourth of those in the normal superior colliculus (Fig. 3F). A drop in mRNA levels could be seen for all of the four analysed genes. Both the levels of melanocortin MC_5 receptor and POMC dropped significantly although from initially very low levels in the normal superior colliculus (Figs. 1B and 3G,H). β-actin or the other control genes did not show any differences in mRNA levels between groups and thus were not affected by optic nerve transection.

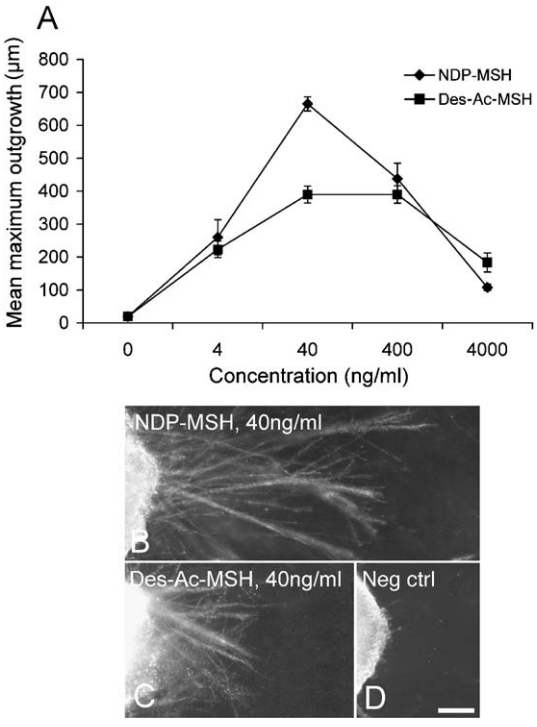

Fig. 4. Neurite outgrowth from embryonic avian retinal explants in culture after stimulation with α-MSH. Pellets from avian embryonic day 6 retina were placed in collagen gels and stimulated by deacetylated α-MSH (Des-Ac-MSH) or NDP-MSH at concentrations varying from 4 ng/ml to 4 μg/ml. Neurite outgrowth was measured after 2 days in culture. (A) Diagram showing dose-dependent neurite outgrowth elicited by [Nle^4, D-Phe^7]α-MSH (NDP-MSH, diamonds) or Des-Ac-MSH (squares). Dark field micrographs showing neurite outgrowth stimulated by (B) NDP-MSH at 40 ng/ml, (C) Des-Ac-MSH at 40 ng/ml or (D) vehicle. Scale bar in D is 100 μm, also valid for B and C.

3.4. Effects of α-MSH on neurite outgrowth from embryonic retinal explants

Previous studies have shown that embryonic and neonatal neurons can be stimulated with melanocortins and respond with neurite outgrowth. To study the effect of melanocortins on retinal cells, we used a bioassay to measure neurite outgrowth. The assay is based on the capacity of avian embryonic retinal explants to grow neuritic processes when stimulated by trophic agents (Carri et al., 1998; Carri and Ebendal, 1986; Hallböök et al., 1996). De-acetylated α-MSH (Des-Ac-MSH) was chosen because it has been shown to be able to promote outgrowth from dorsal root ganglia whereas acetylated forms of α-MSH did not (Haynes and Semenenko, 1989). The synthetic analogue NDP-MSH has an increased stability and increased potency in activating adenylate cyclase in relation to α-MSH (Sawyer et al., 1980). Both Des-Ac-MSH and NDP-MSH stimulated a robust dose-dependent neurite outgrowth from embryonic chick retinal explants as compared to control (Fig. 4A,D). Maximum neurite outgrowth with Des-Ac-MSH was achieved at 40 and 400 ng/ml and at 4000 ng/ml there was an obvious decrease in neurite outgrowth. Maximum neurite outgrowth with NDP-MSH was achieved at 40 ng/ml, and as with Des-Ac-MSH, there was a marked decrease in neurite outgrowth at higher concentrations (Fig. 4A).

4. Discussion

In this work we show that the melanocortin system is present in the adult rat retina. Melanocortin MC_3, MC_4 and MC_5 receptors as well as the POMC mRNA are expressed in retina although at different levels. Melanocortin MC_4 receptor mRNA is clearly the most abundant melanocortin receptor mRNA in the retina. Melanocortin MC_4 receptor mRNA was also abundant in the superior colliculus, which is one of the main regions in the rat brain that receive visual information via the optic nerve. Injury to the optic nerve, such as optic nerve transection, leads to a subsequent retinal ganglion cell death. We studied the regulation of melanocortin receptor and POMC mRNA expression after optic nerve transection. In retina, melanocortin receptor and POMC mRNA expression was unchanged or increased after optic nerve transection whereas the expression decreased in the superior colliculus. This is in agreement with that the regulation of the melanocortin system in retina and superior colliculus is in part activity-dependent. The melanocortins have been shown to exert neurotrophic activities on several different neuronal populations and in agreement with this

we show that embryonic retina respond to α-MSH stimulation with neurite outgrowth.

The three melanocortin receptors in this study could be detected in retina using immunohistochemistry and the antibodies gave distinct patterns of the receptor localisation in the adult retina. We could also detect α-MSH in retina using the same method. The receptor antibodies labelled cells in the inner nuclear layer and ganglion cell layer, and using retrograde filling of retinal ganglion cells we could establish that melanocortin MC_3 and MC_4 receptor are expressed in retinal ganglion cells. The melanocortin MC_5 receptor antibody did not clearly label any identified retinal ganglion cell although labelling was found in the ganglion cell layer. Cells in the entire inner nuclear layer were labelled for the receptors (Fig. 3A–C), suggesting that the retinal inter neurons and horizontal, bipolar and amacrine cells express the receptors. We show that the melanocortin MC_3 and MC_4 receptor expression in the ganglion cell layer is neuronal (Fig. 3F–K) but we cannot exclude that retinal glia cells and the Müller glia cells express the receptors. The primary site of nervous system melanocortin receptor expression is in neurons, but melanocortin receptor expression has also been detected in astrocytes (Wong et al., 1997). The localisation of melanocortin MC_5 receptor immunoreactivity distinguishes itself from those of the melanocortin MC_3 and MC_4 receptors in that it is weaker and that it is found in photoreceptor segments. This suggests that photoreceptors express melanocortin MC_5 receptor. Alternatively, it may be accumulation of melanocortin MC_5 immunoreactivity in outer Müller glia end-feet structures. The diffuse melanocortin MC_5 receptor immunoreactivity over both the outer and inner parts (ganglion cell layer) of the retina could also be the result of receptor localised to Müller glia cells. Melanocortin MC_3 and MC_4 receptor immunoreactivity were co-labelled with True Blue showing that retinal ganglion cell express these receptors. The patterns were different in that melanocortin MC_3 receptor immunoreactivity was on larger retinal ganglion cells and that the melanocortin MC_4 receptor could be found on a substantial portion of True Blue-unlabelled cells in the ganglion cell layer. This suggests that displaced amacrine cells in the ganglion cell layer express melanocortin MC_4 receptor in addition to the identified retinal ganglion cells. In all, these results show that the studied melanocortin receptors are expressed by several of the retinal neuronal types including a large proportion of the retinal ganglion cells. Further neuronal identification will require co-labelling with additional retinal inter-neuron markers. Fig. 2 shows the pattern of immunoreactivity and it should be noted that the intensity of the immunohistochemistry staining of the MC_5 receptor was individually adjusted to give a clear pattern and thus Fig. 2 cannot be directly compared to the mRNA levels for the melanocortin receptors and does not represent the actual levels of receptor protein. A difference between the melanocortin MC_3 and MC_4 receptors is the conspicuous melanocortin MC_3 receptor immunoreactivity in the optic fibre layer as well as on retinal ganglion cell dendrites spanning several layers in the IPL. This suggests that melanocortin MC_3 receptor is present on the complete retinal ganglion cell including axons and dendrites as opposed to the melanocortin MC_4 receptor that is mainly localised to the soma of retinal ganglion cells. If this reflects a difference in function between the receptors remains to be studied. The α-MSH immunoreactivity seen over cells in the ganglion cell layer may very well reflect the site of synthesis; however, we think it is equally likely that the pattern represents α-MSH peptides that are bound to melanocortin receptors on retinal ganglion cells. α-MSH bind to all the melanocortin receptors (Adan and Gispen, 2000) and the antibody used in this study does not distinguish between de- and acetylated forms of α-MSH.

An overwhelming part of the research on the melanocortin MC_3 and MC_4 receptor system is focused on their role in energy homeostasis. POMC, melanocortin MC_3 and MC_4 receptor knock-out mice are obese and this confirms the role for melanocortin MC_3 and MC_4 receptors as mediators of neuronal modulation in the hypothalamic neuropeptidergic system of energy homeostasis (Butler and Cone, 2002). The most likely role for the melanocortins in the retina and visual system is as neuronal modulators. One of the most severe effects in the superior colliculus after optic nerve transection is the deprivation of all visual input to the superior colliculus neurons. Our results show that mainly melanocortin MC_4 receptor mRNA is expressed in the superior colliculus but melanocortin MC_3 and MC_5 receptor as well as the POMC mRNA can be detected. The higher levels of melanocortin MC_4 receptor mRNA is in agreement with previous autoradiographic data showing that a selective ligand for melanocortin MC_4 receptor gave stronger labelling in the superior colliculus region compared to a melanocortin MC_3 receptor selective ligand (Lindblom et al., 1998). The levels of all of the mRNAs drop after the retinal ganglion cell axotomy. The decrease in melanocortin MC_4 receptor mRNA levels is clear (Fig. 3F). Melanocortin MC_3, MC_5 receptor and POMC mRNA levels also drop but from already very low levels (Fig. 3E,G,H). It is likely that the drop is due to the decreased visual input suggesting that the expression is in part activity-dependent. This effect may be indirect since increased activity does not seem to change POMC mRNA expression in other regions of the brain (Plata-Salaman et al., 2000).

Optic nerve transection cause retinal ganglion cell death and after 14 days more than 90% of the retinal ganglion cell are lost. The loss of retinal ganglion cell after optic nerve transection and the fact that retinal ganglion cells express both melanocortin MC_3 and MC_4 receptor mRNA would lead to the presumption that mRNA levels for these receptors would decrease in retina after transection. However, our results show that this is not the case for the melanocortin MC_3 or MC_4 receptor mRNA levels. The level of the more abundant melanocortin MC_4 receptor mRNA is relatively unchanged (Fig. 3B) and unexpectedly the levels of mela-

nocortin MC_3 receptor mRNA increase significantly after optic nerve transection. This may be explained by an up-regulation of the expression in the remaining cells including the remaining retinal ganglion cells or it may be due to increased expression in other cells in the retina. The increase of melanocortin MC_3 receptor mRNA levels after the injury could be the result of a neuromodulary compensatory response.

Using the embryonic chicken retinal explants, we tested if stimulation with α-MSH could elicit trophic responses from retinal neurons. Chicken retina was used for practical reasons. The available rat retina is very small. Neurite outgrowth has been used as an indicator for trophic activity (Carri and Ebendal, 1986; Hallböök et al., 1996) even though this does not automatically mean that survival is supported when outgrowth is stimulated. For embryonic neurons, outgrowth is a strong indication of survival (Ebendal, 1989; Ebendal et al., 1995). Stimulating the embryonic day 6 retinal explants with α-MSH induced a robust outgrowth from the explants (Fig. 4). The de-acetylated form of α-MSH (Des-Ac-MSH) as well as the synthetic analogue NDP-MSH have been shown to be effective in stimulating trophic responses from avian neurons (Haynes and Semenenko, 1989) and was used in our assay. Both Des-Ac-MSH and NDP-MSH bind and activates melanocortin MC_3 and MC_4 receptors (Roselli-Rehfuss et al., 1993) and our results show that both NDP-MSH and Des-Ac-MSH could stimulate neurite outgrowth in the bioassay. NDP-MSH is the most potent agonist for melanocortin MC_{3-5} receptors measured as elevated intracellular cAMP production (Adan and Gispen, 1997; Hol et al., 1995). The expression of melanocortin receptors in the embryonic avian or rat retina is not yet studied. Our results suggest that embryonic neurons express any of the melanocortin receptors that can be activated by α-MSH. This is in agreement with results obtained from 1-day-old avian retina where some of the melanocortin receptors are expressed (Teshigawara et al., 2001); however, the embryonic expression of these molecules in chick and rat retina has to be confirmed. The dose–response curves of Des-Ac-MSH and NDP-MSH both had inverted U-shaped forms. This may be due to a desensitization of receptors at high ligand concentrations. Similar effects have been seen when stimulating other embryonic neurons with the same ligands (Hol et al., 1995). Inhibition or lack of stimulation at high concentration is often seen when adding higher concentration of protein neurotrophic factors to embryonic neurons (Hallböök et al., 1988). Both cyclic AMP and Ca^{2+} are involved in regulating neurite outgrowth (Kilmer and Carlsen, 1984; Lankford and Letourneau, 1989). For example, forskolin, which activates adenylate cyclase directly, could stimulate neurite outgrowth from neuroblastoma cells (Adan et al., 1996) and intracellular Ca^{2+} may control neurite outgrowth by regulating the stability of actin filaments (Lankford and Letourneau, 1989). It is likely that the neurite outgrowth we see is mediated by ligand binding to melanocortin receptors and activation of signal transduction pathways in which cAMP and Ca^{2+} play vital roles.

In conclusion, we present data that show that the melanocortin system is present in the adult retina in particularly in retinal ganglion cells. We show also that embryonic retinal neurons can respond to α-MSH stimulation with neurite outgrowth and that the melanocortin receptor expression is retained in retina but not the superior colliculus after optic nerve transection. This indicates that the melanocortin system is not generally upregulated after optic nerve transection. Our conclusion is that this modulatory system does not take a major part in a hypothesized inducible endogenous repair system that could be present as a compensatory system for injuries in the visual system. Such system is present in the peripheral nervous system and successfully contributes to increased peripheral neuronal survival after injuries (Trupp et al., 1995). The lack of compensatory upregulation of this kind of modulatory system in the visual system may contribute to why neurons do not survive and regenerate in the central nervous system. However, the levels of melanocortin MC_3 receptor mRNA is significantly upregulated in retina after transection and may therefore contribute to an injury-induced receptor system locally in retina.

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