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The M4 muscarinic acetylcholine receptor play a key role in the control of murine hair follicle cycling and pigmentation

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Abstract

Cholinergic receptors of the muscarinic class (M1-M5) are expressed in epidermal keratinocytes and melanocytes as well as in the hair follicle. Knockout (KO) mice of all five receptors have been created and resulted in different phenotypes. KO mice with a deletion of the M4 muscarinic acetylcholine receptor (M4R) present a striking hair phenotype, which we have analyzed here in greater detail by quantitative histomorphometry. Earlier studies revealed a retarded hair follicle morphogenesis in M4R KO mice, compared to age-matched wild type controls. On day 17, when mice enter the first hair growth cycle, the KO mice still showed a slightly retarded catagen phase. Subsequently, hair follicles of the KO mice stayed in a highly significantly prolonged telogen phase, while wild type mice had already far progressed in the hair cycle by entry into anagen. Most strikingly, the M4R KO mice did not engage in follicular melanogenesis and failed to produce pigmented hair shafts. The current pilot study suggests that the M4R plays a fundamental role in the control of the murine hair follicle cycling and is an essential signaling element in the control of hair follicle pigmentation.

Keywords

M4 muscarinic acetylcholine receptor; hair cycle; hair follicle; pigmentation; keratinocytes; melanocytes

Introduction

The class of muscarinic acetylcholine receptors (mAChRs) consists of five subtypes, M1-M5. Within the epidermis, all five subtypes of mAChRs, including M4 mAChR (M4R), are expressed in both keratinocytes and melanocytes (Ndoye et al., 1998; Buchli et al., 2001). Even-numbered mAChRs, M2 and M4, selectively couple pertussis toxin-sensitive G_i-proteins that consequently inhibit adenylate cyclase (AC), weakly stimulate protein kinase C, open inwardly rectifying K⁺ channels, and inhibit Ca²⁺ flux (reviewed by Grando et al., 2006).

Keratinocytes of the stratum spinosum show the highest expression of M4R within the epidermis (Ndoye et al., 1998). Recently an important role of M4R in keratinocyte migration was realized by comparing wound healing in M4R wild type and knockout (KO) mice: M4R KO mice showed significantly decreased epithelialization rate concomitant with a reduced

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migration distance of keratinocytes (Chernyavsky et al., 2003,2004). The M4R KO mice exhibited changes in the integrin expression, which shifted from migratory integrins, such as α_5 , α_V , β_5 , in wild type towards the sedentary integrins α_2 and α_3 (Chernyavsky et al., 2004).

In epidermal melanocytes, muscarinic stimulation increases the intracellular free Ca^{2+} concentration. Regulation of this ion plays a fundamental role in the control of melanocyte dendricity (Meyer zum Gottesberge, 1995). Moreover, the uptake of the essential amino acid L-phenylalanine, which serves as substrate for melanin biosynthesis, involves the Ca^{2+} -dependent Phe- Na^+ /ATPase antiporter (Schallreuter and Wood, 1999). Activation of M2R and M4R is thought to inhibit melanogenesis via the inhibition of AC and reduced cAMP synthesis. This represents a negative feedback regulation to the $\alpha\text{MSH/MC1R}$ and catecholamine/ β_2 adrenergic receptor ($\beta_2\text{AR}$) response in melanocytes (Gillbro et al., 2004;Kurzen and Schallreuter, 2004).

The mAChRs are also expressed in the inner root sheath and the central cell layer of human hair follicles (Kurzen et al., 2004). In an earlier study, distinct hair follicle development phenotypes had been noted in 1 day old M3R KO and M4R KO mice: while the first displayed advanced hair follicle morphogenesis, the latter appeared significantly retarded compared to their wild type littermates (Chernyavsky et al., 2004).

Exploiting murine hair follicle cycling as a model for exploring the role of mAChR in a complex neuroectodermal-mesodermal interaction system *in vivo* (Paus and Cotsarelis, 1999;Slominski and Paus, 1993;Schmidt-Ullrich and Paus, 2005;Peters et al., 2006), we have now examined the hair phenotype of selected mAChR KO mice more closely, and have concentrated here on M4R. In this pilot study, we show that the M4R-coupled pathway plays a fundamental role in murine hair follicle cycling and pigmentation.

Materials and Methods

The M4R KO mice were kindly provided by Dr. J. Wess (Molecular Signaling Section, LBC-NIDDK, NIH, Bethesda, MD, USA). Skin samples of M4R KO mice and their wild type littermates were harvested from the back at days 17, 34 and 42 post partum (dpp). This allows one to reliably assess differences in the entry in to hair follicle cycling (dpp 17 = first catagen), and in subsequent hair cycling activity (dpp 34 = first anagen, dpp 42 = second telogen) (Paus et al., 1999;Müller-Röver et al., 2001). For cryosectioning, skin samples were embedded as described elsewhere (Paus et al., 1999), and 6 μm sections were prepared. At each time point, histomorphometric analysis was performed on 25 to 50 hair follicles in Giemsa-stained skin sections. Hair cycle stages were determined and grouped as described elsewhere (Paus et al., 1999;Müller-Röver et al., 2001). In addition, melanin was visualized histochemically, using Masson-Fontana stain (silver nitrate deposition). Mice skin of early developmental stages (dpp1 and dpp8) were included to demonstrate that melanogenesis is absent even in the developing hair follicle. Photographs were taken with a digital camera attached to an Olympus BH-2 microscope.

Results

Quantitative histomorphometry of postnatal hair follicles reveals defected cycling in the pelage hair follicles of M4R KO mice. On dpp17, i.e. when murine hair follicles enter the first catagen phase (thereby initiating hair follicle cycling), we observed a slight delay in catagen development in M4R KO mice. Instead, a dramatic difference in KO hair follicle cycling was observed on dpp34, compared to wild type mice (Fig 1). At this time point, wild type skin showed only fully pigmented, growing anagen IV-VI hair follicles. In marked contrast, the hair follicles of the KO mice were still in telogen, indicating a substantially retarded progression

through the first hair follicle cycle, i.e. from the first catagen at around dpp17 via the first telogen (dpp 21-25) through the first anagen phase (ca. dpp 28-40) (Paus et al., 1999; Müller-Röver et al., 2001). On dpp42, the majority of back skin hair follicles in both KO and wild type mice was in telogen (Fig 1).

One of the most striking differences noted between the wild type and KO mice was a failure to produce pigmented hair shafts by M4R $-/-$ mice, which was noticeable already on dpp1. The differences in pigmentation became striking on dpp8 and dpp34 (Fig 2). All melanin synthesis in truncal mouse skin is restricted to pelage hair follicles, and follicular melanogenesis is strictly coupled to the anagen phase of hair follicle cycling (anagen III-VI) (Slominski and Paus, 1993; Tobin and Paus, 2001). Since the examined KO mice did not enter the first genuine anagen phase, it was expected that no melanin could be detected by Masson-Fontana stain (Fig 2). This is in sharp contrast to the wild type mice that demonstrated the expected melanin granules in the precortical hair matrix of their anagen IV-VI hair follicles, i.e., where the hair follicle pigmentary unit resides (Tobin and Paus, 2001; Slominski et al., 2005).

Discussion

In the current pilot study, we provide evidence that M4R signaling is important for the control of murine pelage hair follicle cycling, and is indispensable for hair follicle pigmentation. Even though the current findings remain to be confirmed by analyzing larger groups of M4R KO and wild type mice and by the analysis of additional time points, they already enroll mACHR into the ever-growing list of key modulators in hair follicle biology (Stenn and Paus, 2001; Alonso and Fuchs, 2006) and designate M4R-mediated signaling as a particularly intriguing target for the therapeutic manipulation of hair growth and pigmentation.

In human skin, cholinergic signaling has been shown to be important for the control of keratinocyte differentiation, adherence and migration (Grando et al., 2006). Since the functional inactivation of the M4R-coupled pathway appears to block murine hair follicles in the resting phase of the hair cycle (telogen) and prevents cyclic regeneration of the hair follicle by entry into anagen, it is reasonable to conclude that M4R-mediated signaling also is critical for murine hair follicle keratinocyte migration and differentiation (which are key elements of hair follicle cycling) (Paus and Cotsarelis, 1999; Stenn and Paus, 2001). Previous work has revealed that, while M2R KO mice show striking central nervous system defects, M4Rs are dispensable for central muscarinic responses (Gomez et al., 2001), suggesting that M4R plays a more important role in peripheral muscarinic responses. The results of the current study, which document striking peripheral defects in M4R KO mice, support this concept.

Crawling locomotion of keratinocytes is activated by stimulation of M4R, and delayed wound healing was observed in M4R KO mice (Chernyavsky et al., 2004). M4R activation apparently counteracts the stimulatory effects of β 2AR on AC, leading to a reduced cAMP production. On the other hand, adrenergic stimulation and AC activation inhibit keratinocyte migration accompanied by reduced expression of migratory integrins (Chernyavsky et al., 2004). The transformation of telogen into anagen hair follicles morphologically recapitulates key aspects of hair follicle development and leads to the regeneration of a large keratin fiber-producing biofactory, the anagen hair bulb, which requires highly coordinated keratinocyte migration (Scott, 2002). Thus it is reasonable to speculate that the observed arrest of M4R KO hair follicles in telogen is, at least in part, due to severe abnormalities in M4R-dependent hair follicle keratinocyte locomotion (e.g., sedentary integrins may serve as signals that inhibit anagen development). In this context it is noteworthy that a retarded hair follicle morphogenesis has been observed in 1 day old M4R KO mice (Chernyavsky et al., 2004). Hair follicle morphogenesis also requires perfectly coordinated hair follicle keratinocyte differentiation,

adherence and migration. In subsequent studies it will be tempting to compare e.g. the migratory characteristics of cultured outer root sheath keratinocytes from M4R KO and wild type mice in various stages of hair follicle development and cycling.

Human epidermal melanocytes express M4R (Buchli et al., 2001). The current study suggests that functional M4R are also expressed by murine hair follicle melanocytes *in vivo*, and are absolutely required here for follicular melanogenesis. Lack of M4R in the hair follicle melanocytes of KO mice was not compensated by the M2R-coupled or other related pathways, suggesting a pivotal role of M4R in the physiologic control of murine follicular melanogenesis (it is as yet unclear whether murine hair follicle melanocytes express M2R). A negative feedback mechanism of M4R and M2R on the α MSH/MC1R and catecholamine/ β 2AR response has been proposed in human melanocytes (Kurzen and Schallreuter, 2004).

As shown more than a decade ago by Evinger et al. (1994), cholinergic agonists acting through both nicotinic and muscarinic receptors induce gene expression of phenylethanolamine N-methyltransferase (PNMT), an enzyme converting norepinephrine into epinephrine. In this context, M4R was shown to be the most important one, at least in bovine adrenal chromaffin cells (Evinger et al., 1994). This important link connects cholinergic and adrenergic signals and may be involved in the regulation of hair follicle pigmentation.

Despite their different signaling mechanisms, M4R and M3R act upon a common effector step, which involves Rho proteins (Chernyavsky et al., 2004). Interestingly, Rho signaling plays an important role in melanocyte dendrite formation, as shown for epidermal melanocytes (Scott, 2002). Whether these signals are also involved in follicular melanocyte dendricity and/or melanogenesis now needs to be dissected in follow-up studies.

In this study we only looked at postnatal skin. A more comprehensive and systematic study should include both fetal stages of hair follicle morphogenesis and later hair follicle cycles so as to assess e.g. how long the dependence of murine pelage hair follicles on M4R for anagen development persists, and whether later cycles become M4R-independent. Early during embryogenesis, undifferentiated melanoblasts migrate from the neural crest to the skin where they later differentiate to form dendritic melanocytes and immigrate into hair follicles (Peters et al., 2002; Tobin and Paus, 2001; Slominski et al., 2005). Thus, it is important to clarify whether the observed defect in hair follicle pigmentation arises from a defect in the (M4R-dependent) control of follicular melanin synthesis, or whether M4R KO mice show a deficiency in the migration and/or differentiation of their neural crest-derived precursor cells into the hair follicle so that functional hair follicle pigmentary units never get assembled, resulting in absent hair color.

In conclusion, M4R-mediated signaling is critical for normal hair follicle cycling and pigmentation in mice, at least during the first genuine hair cycle. One critical next step is to explore to which extent this also holds true for human hair follicle biology.

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Figure 1. Histomorphometric analyses of hair follicles of wild type and M4 mAChR KO mice
 About 25 to 50 hair follicles per group were included in the analyses and the percentage of hair follicles per stage of hair cycle (dpp17, 34, 42) was summarized in the graphs. At day 17 (dpp17), when the first genuine hair cycle started, a slight retardation of catagen was visible in the M4R KO mice (green lines) compared to wild type (yellow lines). The retardation becomes more prominent on dpp34 when wild type mice progresses into anagen IV-VI while M4 mAChR KO animal stay in telogen. On dpp42, only telogen hair follicles can be found in both groups. Representative images of hair follicles in the skin of M4R wild type and KO mice supplement the graphic presentation. Magnification x100.

Figure 2. Masson-Fontana-stain of skin samples of wild type and M4 mAChR KO mice
Brown-black color indicates the presence of melanin granules (arrows). Wild type mice formed normally pigmented hair shafts on dpp1, dpp8 and dpp34. The hair follicles of M4R KO mice did not contain melanin at this time points. Magnification x100. Insets provide a close view of the hair follicle pigment localization on dpp8 and dpp34 and non-pigmented hair bulbs, respectively. DP: dermal papillae; WT: wild type; M4R^{-/-}: M4 mAChR KO.