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Transgenic mice with ocular overexpression of an adrenomedullin receptor reflect human acute angle-closure glaucoma

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ABSTRACT

Glaucoma, frequently associated with high IOP (intra-ocular pressure), is a leading cause of blindness, characterized by a loss of retinal ganglion cells and the corresponding optic nerve fibres. In the present study, acutely and transiently elevated IOP, characteristic of acute angle-closure glaucoma in humans, was observed in CLR (calcitonin receptor-like receptor) transgenic mice between 1 and 3 months of age. Expression of CLR under the control of a smooth muscle α-actin promoter in these mice augmented signalling of the smooth-muscle-relaxing peptide adrenomedullin in the pupillary sphincter muscle and resulted in pupillary palsy. Elevated IOP was prevented in CLR transgenic mice when mated with hemizygote adrenomedullin-deficient mice with up to 50% lower plasma and organ adrenomedullin concentrations. This indicates that endogenous adrenomedullin of iris ciliary body origin causes pupillary palsy and angle closure in CLR transgenic mice overexpressing adrenomedullin receptors in the pupillary sphincter muscle. In human eyes, immunoreactive adrenomedullin has also been detected in the ciliary body. Furthermore, the CLR and RAMP2 (receptor-activity-modifying protein 2), constituting adrenomedullin receptor heterodimers, were identified in the human pupillary sphincter muscle. Thus, in humans, defective regulation of adrenomedullin action in the pupillary sphincter muscle, provoked in the present study in CLR transgenic mice, may cause acute and chronic atony and, thereby, contribute to the development of angle-closure glaucoma. The CLR transgenic mice used in the present study provide a model for acute angle-closure glaucoma.

Key words: adrenomedullin, angle-closure glaucoma, calcitonin receptor-like receptor (CLR), intraocular pressure, pupillary palsy, receptor-activity-modifying protein (RAMP), smooth muscle α-actin.

Abbreviations: ACG, angle-closure glaucoma; AM, adrenomedullin; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CREB, cAMP-response-element-binding protein; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IOP, intra-ocular pressure; PACAP, pituitary adenylate-cyclase-activating peptide; RAMP, receptor-activity-modifying protein; SMαA, smooth muscle α-actin.

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INTRODUCTION

Progressive visual field loss due to degeneration of retinal ganglion cells and atrophy of optic nerve fibres are characteristic signs of glaucoma. Anatomically, glaucoma is subdivided into open- and closed-angle forms. Primary glaucoma occurs in approx. 70 million people worldwide, half of them with ACG (angle-closure glaucoma) [1,2]. The prevalence of blindness in ACG is twice that of other forms of glaucoma [3]. ACG has acute, intermittent/subacute and chronic forms [4], and results from increased IOP (intra-ocular pressure) caused by the iris obliterating the trabecular meshwork [3,5]. This leads to reduced and blocked aqueous outflow and anterior peripheral synechiae between the iris and cornea. Several causative genes have been identified in primary open-angle glaucoma and in malformations of the anterior eye segment [5–10], but the pathogenesis of ACG is largely unknown. ACG is characterized by reduced anterior chamber depth, corneal size and curvature and lens thickness. In structurally predisposed eyes, drug-enhanced dilation of the pupil in dim light can provoke ACG [11,12]. Apparently, acute and chronic atony of the pupillary sphincter muscle contributes to the pathophysiolgy of acute ACG, but the underlying mechanisms remain unclear. Thus animal models reproducing the human disease are a prerequisite for an understanding of ACG pathophysiology and the development of new treatment strategies.

Whereas the DBA/2J mouse with pigmentary glaucoma [8,13] has been widely used as a rodent model of chronic ACG, there is no hereditary animal model available for acute and intermittent/subacute ACG. Iris pigment dispersion and iris stromal atrophy, provoking ACG in DBA/2J mice, are caused by a single recessive mutation in the *Gpnmb* gene and by two mutations in the *Tyrp1* allele respectively, that reveal two defective melanosomal proteins [7]. DBA/2J mice have elevated IOP between 6 and 16 months of age. The earliest onset and the most severe form of chronic ACG has been observed in animals that carry homozygous mutations in both the *Gpnmb* gene and the *Tyrp1* allele [8].

AM (adrenomedullin) is a 52-amino-acid smooth-muscle-relaxing polypeptide identified in many tissues including the eye. The peptide belongs to the calcitonin family of peptides that includes CGRP (calcitonin gene-related peptide), intermedin (AM2) and amylin [14]. AM and the neuropeptide CGRP are potent vasodilators, and CGRP shares smooth-muscle-relaxing activity with AM.

Interestingly, patients with primary open-angle glaucoma have elevated levels of AM in the aqueous humour, and those with proliferative vitreoretinopathy exhibit increased AM in the vitreous fluid [15,16]. In cats and rats, AM is localized in the iris ciliary body [17,18]. Furthermore, AM-induced cAMP production has been observed in the iris sphincter muscle from different mammalian species including humans [17,18]. In the cat, AM dose-dependently inhibited carbachol-induced contraction of the isolated sphincter muscle, and the resting tension of the isolated bovine iris sphincter was transiently decreased by AM [18,19]. This indicates that the pupillary sphincter is a target of ocular AM. AM is, furthermore, produced and secreted by retinal pigment epithelial cells, where the peptide presumably is an autocrine/paracrine growth-stimulating factor up-regulated by hypoxia and inflammatory cytokines [16]. In inflammatory conditions in the eye, AM may also originate from fibroblasts, macrophages, glial cells and vascular endothelial cells.

Molecularly defined receptors for AM and CGRP, linked to cAMP production, are CLR (calcitonin receptor-like receptor)/RAMP2 (receptor-activity-modifying protein 2) and CLR/RAMP1 heterodimers respectively [20]. Upon interaction with RAMP3, the CLR is a mixed type AM/CGRP receptor. Thus the CLR, unlike other members of the family B1 of G-protein-coupled receptors with seven transmembrane domains, requires associated RAMP to determine ligand selectivity. Intermedin interacts with all three CLR/RAMP heterodimers (CLR/RAMP1, CLR/RAMP2 and CLR/RAMP3), but, unlike AM and CGRP, at high nanomolar concentrations only [21].

In the present study, we have generated transgenic mice that express rat CLR in smooth-muscle-containing tissues ($CLR^{SM\alpha A}$ mice; where SMαA is smooth muscle α-actin). $CLR^{SM\alpha A}$ mice overexpress CLR/RAMP2 AM receptors in the pupillary sphincter muscle, resulting in enhanced AM-induced sphincter muscle relaxation. Importantly, certain $CLR^{SM\alpha A}$ mice had acutely and transiently elevated IOP between 1 and 3 months of age before chronically elevated IOP became evident. Transient or chronically elevated IOP was not observed in wild-type littermates and in $CLR^{SM\alpha A}$ mice with only one intact AM allele and reduced plasma and organ AM concentrations. This indicates that pupillary palsy observed in $CLR^{SM\alpha A}$ mice is mediated by endogenous AM of iris/ciliary body origin. Taken together, $CLR^{SM\alpha A}$ mice represent a hereditary model for acute ACG.

MATERIALS AND METHODS

Generation of $CLR^{SM\alpha A}$ and $CLR^{SM\alpha A} \times AM^{-/+}$ mice

All protocols for experiments with animals were approved by the Kantonales Verterinaeramt Zurich. The transgene designed for expression of the rat CLR in mouse smooth-muscle-containing tissues consisted of an SMαA promoter fragment [22] linked to a DNA sequence encoding the signal sequence of the CD33 protein [23], a V5 epitope tag (GKPIPNPLLGDST) and rat CLR lacking the signal sequence (Figure 1A). Four independent $CLR^{SM\alpha A}$ founders were obtained by pronuclear

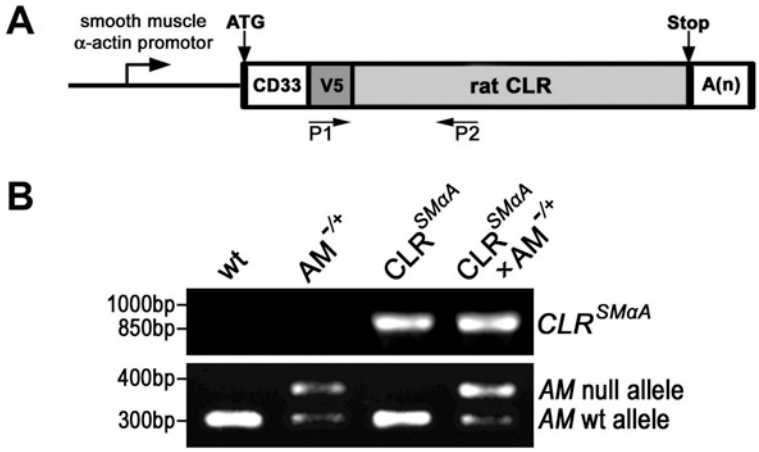

Figure 1 Characterization of $CLR^{SM\alpha A}$ and $CLR^{SM\alpha A} \times AM^{-/+}$ mice

(A) The transgene consisted of an SMαA promoter fragment, a DNA fragment encoding the signal sequence of the CD33 protein, a V5 epitope tag, the rat CLR and the polyadenylation signal of the bovine growth hormone gene [A(n)]. P1 and P2 indicate the positions of the oligonucleotide primers used for the identification of CLR^{SMaA} mice by PCR amplification of transgene-specific sequences from DNA isolated from tail biopsies. (B) Agarose gel electrophoresis of PCR-amplified DNA from mouse tail biopsies showing a predicted 880 bp transgene-derived product in $CLR^{SM\alpha A}$ mice, which was absent in control (wt) littermates. A 300 bp PCR product amplified from DNA of wild-type and $AM^{-/+}$ mice represented intact AM alleles. In $AM^{-/+}$ mice, an additional 380 bp product indicated the AM-null allele.

injection of the transgene into B6D2F1 × B6D2F1 oocytes [24]. The founders and their offspring were genotyped by PCR analysis of genomic DNA extracted from tail biopsies, using transgene-specific forward (5′-GGCCCTGGCCATGGAAGAAGG-3′) and reverse (5′-TGGGACCATGGATGATGTAGAGG-3′) primers. PCR products representing the transgene had a predicted size of 880 bp (Figure 1B). $CLR^{SM\alpha A} \times AM^{-/+}$ mice were obtained by mating hemizygote $CLR^{SM\alpha A}$ mice with hemizygote AM-knockout animals [19]. Offspring with the $AM^{+/+}$ or $AM^{-/+}$ genotype were identified by PCR analysis of tail biopsy DNA with AM gene-specific forward (5′-GGCTCCTTAAGTTGCGCA-3′) and reverse (5′-ACGTAGAAGAACTTATTAAACCGCA-3′) primers. All primers were purchased from Microsynth. PCR products of the intact and defective AM alleles were DNA fragments of 300 and 380 bp respectively.

Genomic sequencing

Sequence analysis in both directions of PCR-amplified DNA fragments that spanned the sites with potential mutations in the *Gpnmb* and *Tyrp1* genes was carried out by Microsynth. The primers used for amplification and sequencing of the *Gpnmb* gene fragment and two distinct *Tyrp1* gene domains had the sequences 5′-CTGAACACGAAGACGTTAGC-3′ (*Gpnmb* forward), 5′-CCGAGTAAGGAGAAGAACAG-3′ (*Gpnmb* reverse), 5′-AACTCTGTTTTTGCCTTTCTG-3′ (*Tyrp1a* forward), 5′-AAGGTGACTCCTGACCTATG-3′ (*Tyrp1a* reverse), 5′-CAGGTTGTCTCAATTCACAG-3′ (*Tyrp1b* forward) and 5′-CATGAACCCACGTGATCAG-3′ (*Tyrp1b* reverse).

Reverse transcription and semi-quantitative PCR

Microdissected iris/ciliary body tissue samples from wild-type and $CLR^{SM\alpha A}$ mice were frozen in liquid nitrogen immediately after preparation. mRNA was isolated using the RNeasy kit (Qiagen), and cDNA was generated with M-MLV reverse transcriptase (Promega). Semi-quantitative PCR was carried out with gene-specific primers (Microsynth) for AM (forward, 5′-CCCTACAAGCCAGCAATC-3′; reverse, 5′-CGTCCTTGTCTTTGTCTGTT-3′), the CLR (forward, 5′-GGCATCCGGATAGTAATAG-3′; reverse, 5′-CAATGCCAAGTAGTGGTACC-3′), the V5-CLR (forward, 5′-CTCGGTCTCGATTCTACG-3′; reverse, 5′-CAATGCCAAGTAGTGGTACC-3′), RAMP2 (forward, 5′-CCCTCCGCTGTTGCTGCTG-3′; reverse, 5′-AGGAACGGGATGAGGCAGATG-3′) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; forward, 5′-GGGTGGAGCCAAACGGGTC-3′; reverse, 5′-GGAGTTGCTGTTGAAGTCGCA-3′). PCR products were analysed by agarose gel electrophoresis.

Immunohistochemistry and *in situ* hybridization

Immunofluorescent staining of sections of paraffin-embedded mouse eyes was carried out with antibodies to SMαA (Sigma) and the V5 epitope tag (Bethyl), and with Alexa Fluor®546-labelled secondary antibodies to mouse IgG and Alexa Fluor®488-labelled antibodies to rabbit IgG (Molecular Probes). Human samples were retrieved from the archives of the Institute of Clinical Pathology, University of Zurich, Zurich, Switzerland.

The use of archival eye tissue for immunohistochemical analysis has been approved by the Ethical Committee of the University Hospital Zurich, Zurich, Switzerland. On these sections, AM, CLR and RAMP2 were visualized with antibodies from Santa Cruz Biotechnology, and the Vector elite AP-ABC kit (Vector Laboratories) [25].

In situ hybridization was carried out on adjacent sections with the protocols provided with the digoxigenin RNA labelling and detection reagents from Roche Applied Science. RAMP2- and CLR-specific antisense and sense (control) probes were generated from cDNA encoding the corresponding human proteins.

Autoradiography

Binding of ^{125}I-AM and -CGRP to 20 μm frozen sections of mouse pupillary sphincter was carried out as described previously [26]. Briefly, sections were incubated at 4 °C for 24 h with 7 fmol ^{125}I-rAM (rat AM) or -hCGRP (human CGRP) (74 TBq/mmol) in 250 μl of binding buffer [50 mmol/l Tris/HCl (pH 7.5) containing 3 % (w/v) BSA (Sigma)]. Non-specific binding was estimated with sections incubated in parallel with 1 μmol/l non-labelled rAM or hCGRP. The sections were then rinsed with binding buffer, air-dried and apposed to X-ray film (Hyperfilm^{TM}; GE Healthcare BioSciences). Subsequently, sections were scraped from the slides to measure total and non-specific binding in a γ-counter.

CREB (cAMP-response-element-binding protein) phosphorylation in iris sphincter smooth muscle cells

Microdissected pupillary sphincter cells were obtained by treatment with collagenase IA and elastase (Sigma), followed by trituration and filtration through a 70 μm cell strainer (Becton Dickinson). The cells were cultured in Ham's F12/DMEM (Dulbecco's modified Eagle's medium) (1:1, v/v) supplemented with 10 % (v/v) fetal bovine serum (GibcoBRL) and antibiotics. Cells were cultured on glass slides and pre-incubated at 37 °C for 6 h in Ham's F12/DMEM (1:1, v/v) supplemented with 0.1 % BSA (Sigma). Cells were then stimulated at 37 °C for 15 min with AM (Bachem) in the absence or presence of the AM antagonist AM-(20–50) (Bachem) or CGRP (Bachem) at the indicated concentrations. Subsequently, the cells were fixed with 2 % formaldehyde and immunostained with rabbit antibodies to phospho-CREB (Cell Signaling) and with biotinylated goat anti-(rabbit IgG) antibodies (Vector) and Cy3-labelled streptavidin (Sigma). Smooth muscle cells were identified with antibodies to SMαA [27].

Measurement of IOP

Acute or transient rises of IOP were measured indirectly by indentation tonometry, as described in detail by Gross et al. [28], in mice that were anaesthetized by intraperitoneal injection of 40 μl of PBS containing 0.5 mg of ketanarkon and 0.1 mg of xylazin (Streuli-Pharma) per 10 g of body weight. The same technique was used for IOP measurements in at least ten CLR$^{SM\alpha A}$, CLR$^{SM\alpha A}$ × AM$^{-/+}$ and AM$^{-/+}$ mice and wild-type littermates at 1 and 3 months of age.

Pupillary constriction

Pupillary constriction was stimulated by short xenon light flashes in dark-adapted CLR transgenic mice and control littermates [29]. Topical application of carbachol (carbamylcholine chloride), AM and AM-(20–50) was carried out in anaesthetized animals.

Statistical analysis

Results are shown as means ± S.E.M. Graphs and statistical analysis were done with Prism 4.01 (GraphPad Software).

RESULTS

Overexpression of AM receptors in the pupillary sphincter muscle of CLR$^{SM\alpha A}$ mice

Four transgenic mouse lines have been generated that express a V5 epitope-tagged CLR (V5–CLR) under the control of an SMαA promoter (CLR$^{SM\alpha A}$ mice) in corresponding tissues, including the pupillary sphincter muscle (Figure 2A). Immunofluorescent staining with specific antibodies localized the V5–CLR in SmαA-expressing cells. mRNA encoding endogenous AM, and CLR and RAMP2, which constitute a heterodimeric AM receptor, were identified in iris/ciliary body extracts of CLR$^{SM\alpha A}$ mice and of wild-type littermates (Figure 2B). Receptor autoradiography on pupillary sphincter muscle sections indicated increased specific binding of ^{125}I-AM in CLR$^{SM\alpha A}$ mice compared with control littermates (Figure 2C). Specific binding of ^{125}I-CGRP, on the other hand, was undetectable (results not shown). Moreover, AM, unlike CGRP, increased the percentage of cells accumulating nuclear phospho-CREB through activation of adenylate cyclase/PKA (protein kinase A) signalling (Figure 2D). With 0.1 μmol/l AM, the number of phospho-CREB-accumulating cells in CLR$^{SM\alpha A}$ mice was twice that of control animals. The AM antagonist AM-(20–50) suppressed AM-induced nuclear phospho-CREB accumulation in both CLR$^{SM\alpha A}$ mice and control littermates. Taken together, CLR$^{SM\alpha A}$ mice overexpress an AM receptor linked to cAMP production in the pupillary sphincter muscle.

Acute transient increase in IOP observed in CLR$^{SM\alpha A}$ mice is suppressed in CLR$^{SM\alpha A}$ × AM$^{-/+}$ animals

Certain CLR$^{SM\alpha A}$ mice, unlike wild-type littermates, had abrupt transient rises in IOP up to a mean level

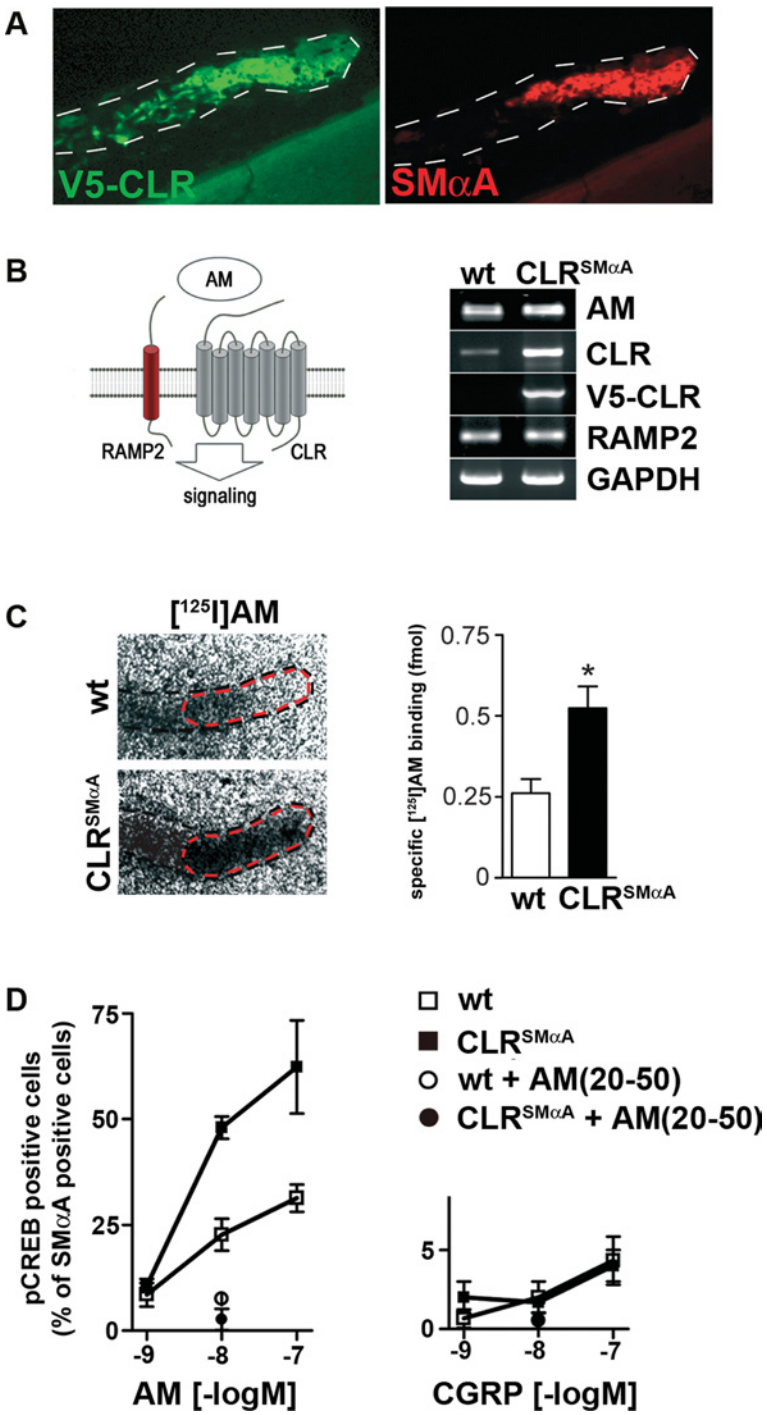

Figure 2 AM receptors are overexpressed in the pupillary sphincter muscle of CLR$^{SM\alpha A}$ mice

(A) CLR$^{SM\alpha A}$ mice express V5–CLR in SMαA-expressing cells of the pupillary sphincter muscle. (B) CLR associated with RAMP2 is a G-protein-coupled AM receptor [20]. Reverse transcriptase-PCR showing expression of AM and the CLR/RAMP2 AM receptors in iris/ciliary body tissue of control (wt) and CLR$^{SM\alpha A}$ mice. V5–CLR was expressed in CLR$^{SM\alpha A}$ mice alone. GAPDH was used as a reference. (C) Specific binding of ^{125}I-AM to the pupillary sphincter muscle was higher in CLR$^{SM\alpha A}$ mice compared with wild-type mice. Results are from three mice per genotype, averaged from three sections per eye and are equal to six sections per mouse. $^{*}P < 0.05$ compared with wild-type. (D) Nuclear phospho-CREB (pCREB) immunostaining indicated an increased number of AM-responsive cultured pupillary sphincter smooth muscle cells in CLR$^{SM\alpha A}$ mice compared with wild-type littermates. The AM antagonist AM-(20–50) suppressed CREB phosphorylation, but CGRP was ineffective. Results are from at least three mice per genotype.

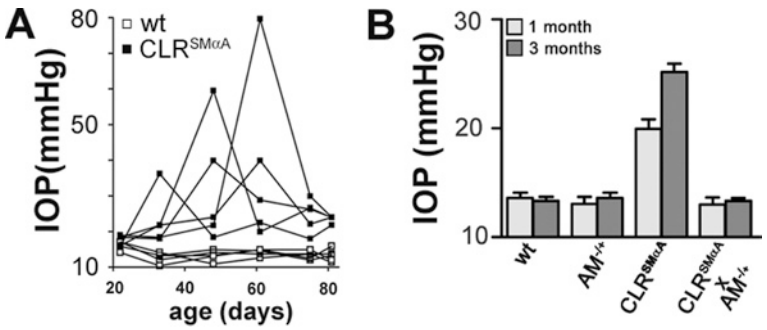

Figure 3 IOP in CLR$^{SM\alpha A}$ and CLR$^{SM\alpha A}$ × AM$^{-/+}$ mice

(A) Transient acute increases in IOP in individual CLR$^{SM\alpha A}$ mice and baseline values in control littermates. (B) In control (wt), AM$^{-/+}$ and CLR$^{SM\alpha A}$ × AM$^{-/+}$ mice, unlike in CLR$^{SM\alpha A}$ animals, IOP was in the normal range up to 3 months of age. Results are from at least ten mice per time point.

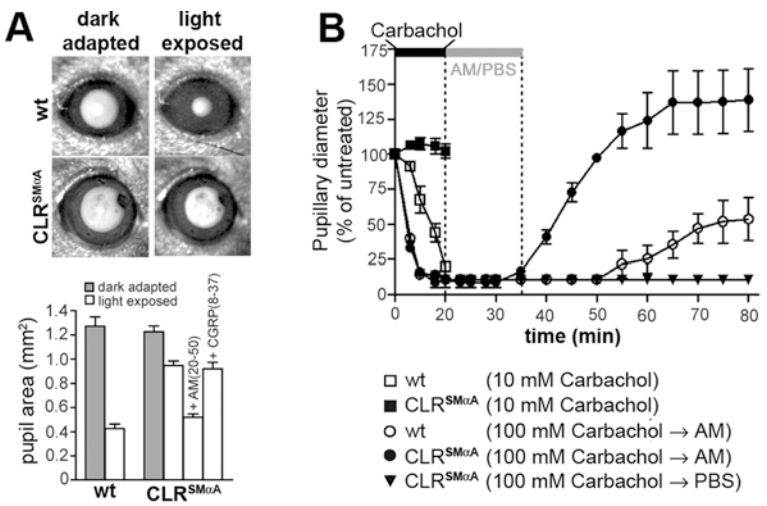

Figure 4 Pupillary constriction in response to a light flash or carbachol is impaired in CLR$^{SM\alpha A}$ mice

(A) Pupillary constriction in dark-adapted eyes in response to a brief xenon light flash was impaired in 6-week-old CLR$^{SM\alpha A}$ mice, but was restored by topical application of 100 μmol/l AM-(20–50). The CGRP antagonist CGRP-(8–37) was ineffective. (B) Carbachol-induced pupillary constriction required higher doses in CLR$^{SM\alpha A}$ mice (100 mmol/l) than in control littermates (10 mmol/l). Topical administration of 1 μmol/l AM relaxed carbachol-pre-contracted pupils of CLR$^{SM\alpha A}$ mice more rapidly and caused a larger diameter than in control mice. Results are from at least three mice per time point.

of 48.2 ± 7.3 mmHg between 30 and 70 days of age (Figure 3A). In these mice, the IOP values measured before and after the acute rise were slightly higher than those observed in control littermates. Interestingly, mating of hemizygote CLR$^{SM\alpha A}$ mice with hemizygote AM-knockout (AM$^{-/+}$) mice, resulting in up to 50 % lower plasma and organ AM concentrations [30], revealed CLR$^{SM\alpha A}$ × AM$^{-/+}$ offspring with IOP indistinguishable from wild-type and AM$^{-/+}$ littermates (Figure 3B). In contrast, CLR$^{SM\alpha A}$ littermates with intact AM genes had increased mean IOP at 1 and 3 months of age. These findings, together with the observed expression of AM in the iris/ciliary body, suggest that overexpression of the CLR in the pupillary sphincter muscle of CLR$^{SM\alpha A}$ mice enhanced its responsiveness to endogenous AM, thereby causing functional defects in the anterior chamber of the eye that result in initially acutely and transiently, and later chronically, elevated IOP.

Functional defects in the pupillary sphincter muscle of CLR$^{SM\alpha A}$ mice

Dark-adapted CLR$^{SM\alpha A}$ mice exhibited impaired pupillary constriction in response to light exposure (Figure 4A). However, topical administration of the AM antagonist AM-(20–50) normalized the pupillary response to light stimulation in CLR$^{SM\alpha A}$ mice. The CGRP antagonist CGRP-(8–37) on the other hand was ineffective. This suggested an AM-mediated functional defect of the sphincter muscle. A general defect was excluded by the topical application of carbachol (Figure 4B). Interestingly, CLR$^{SM\alpha A}$ mice required a higher dose of

Table 1 ***Gpnmb*** **and** ***Tyrp1*** **genotypes of V5–CLR transgenic mice and control littermates**

mu, mutation; wt, wild-type.

Mice	Gpnmb			Tyrp1a			Tyrp1b		
	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-
V5-CLR	0	1	3	2	2	0	2	2	0
wt	0	2	3	4	1	0	4	1	0
sequence									
mu		GCTGA C			CTATG G			GCATC G	
wt		GCTGA		CTGTG			GCGTC		

+/+ wt
+/- mutant hemizygous
-/- mutant homozygous

carbachol than wild-type littermates. Moreover, AM reversed the carbachol-induced pupillary constriction in wild-type and CLR$^{SM\alpha A}$ mice, and the dilatory response to AM occurred more rapidly and to a larger diameter in CLR$^{SM\alpha A}$ mice than in wild-type animals. Taken together, AM-dependent relaxation of the pupillary sphincter muscle was enhanced in CLR$^{SM\alpha A}$ mice, and AM counteracted light- and carbachol-induced constriction of the pupils in both wild-type and CLR$^{SM\alpha A}$ animals.

Gpnmb and *Tyrp1* mutations in CLR$^{SM\alpha A}$ mice

The V5–CLR transgenic mice used in the present study were generated in B6D2F1 animals with a mixed genetic background of C57BL/6J and DBA/2J mice and were therefore potential carriers of homozygous mutations in the *Gpnmb* and *Tyrp1* genes, which have been reported to cause pigmentary glaucoma in DBA/2J mice. Randomly selected CLR$^{SM\alpha A}$ mice and control littermates were therefore genotyped by sequence analysis of PCR-amplified DNA fragments that spanned the sites with potential mutations. Three out of four CLR$^{SM\alpha A}$ mice and three out of five wild-type animals carried homozygous *Gpnmb* mutations, but the *Tyrp1* allele was either wild-type or hemizygous for the reported mutations *Tyrp1a* and *Tyrp1b* (Table 1). Thus predisposition to pigmentary glaucoma was considered low and equal in CLR$^{SM\alpha A}$ mice and control littermates.

AM is expressed in the ciliary body of human eyes, and the CLR/RAMP2 AM receptor is localized in the pupillary sphincter muscle

Immunoreactive AM was localized in the ciliary body of human eyes obtained at autopsy (Figure 5A). Moreover, CLR and RAMP2, which constitute the heterodimeric AM receptor, were identified in SmαA-expressing cells of the pupillary sphincter muscle (Figure 5B). *In situ* hybridization with CLR- and RAMP2-specific RNA

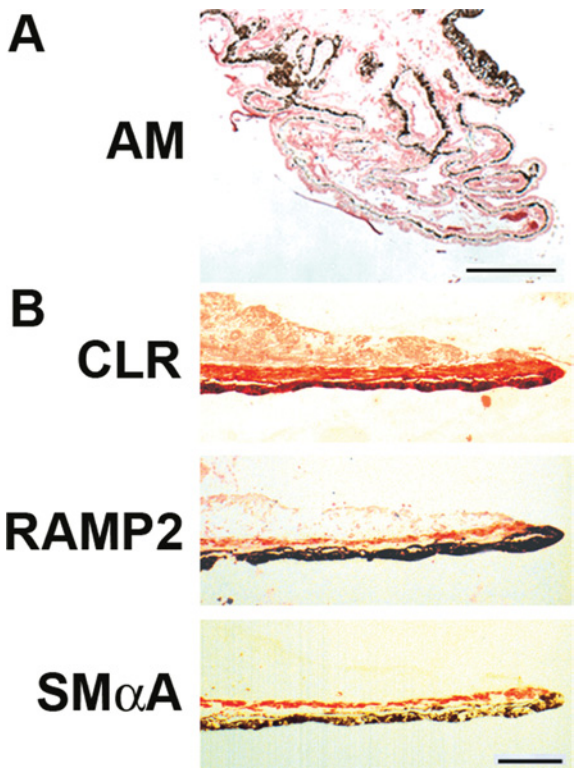

Figure 5 Expression of AM in the human ciliary body and the CLR/RAMP2 AM receptor in human pupillary sphincter muscle

(A) AM immunohistochemical staining (red) in the ciliary body. (B) Staining (red) of adjacent sections through the human pupillary sphincter showing co-localization of CLR and RAMP2 in SMαA-expressing cells. Representative paraffin sections of eyes obtained at autopsy from human subjects with no history of eye disease were used. Scale bars, 250 μm.

probes revealed a corresponding localization (results not shown).

DISCUSSION

AM, initially identified as a potent vasodilatory and smooth-muscle-relaxing polypeptide, is produced in several tissues, including the eye. In human eyes, AM has been identified in the aqueous humour that is produced by the iris ciliary body [16]. Moreover, AM has been observed in the ciliary body of cats and rats [17,18]. Studies in several mammalian species, including humans, have identified the pupillary sphincter as a target of ocular AM, and AM receptors linked to cAMP production have been identified [17,18].

To assess the biological relevance of the AM receptor in the pupillary sphincter muscle, we have generated transgenic mice that overexpress the CLR in this organ. These mice developed normally up to 2 weeks of age, but, later, between 1 and 3 months, certain $CLR^{SM\alpha A}$ mice had transient increases in IOP characteristic of acute glaucoma. This was not observed in control littermates.

Functional analysis of the eyes of $CLR^{SM\alpha A}$ mice *in vivo* showed impaired pupillary constriction in response to flash light in dark-adapted animals. The defect was abolished with the topical application of the AM antagonist AM-(20–50). Thus acute stimulation of pupillary constriction in $CLR^{SM\alpha A}$ mice confirmed the predicted enhanced sensitivity to endogenous ocular AM that prevented normal contraction. A general functional defect of the iris sphincter muscle in $CLR^{SM\alpha A}$ mice was excluded by the observed normal constriction in response to topical administration of carbachol. In the cat, AM antagonized carbachol-induced pupillary constriction [18]. These findings have been confirmed and extended in the present study in mice. In $CLR^{SM\alpha A}$ mice with carbachol-pre-constricted eyes, pupillary relaxation in response to AM occurred more rapidly and to a larger diameter than in equally treated wild-type littermates. The findings again demonstrate increased AM sensitivity of the iris sphincter muscle in CLR-overexpressing mice.

In humans, prolonged pupillary sphincter relaxation leads to ACG [11,12,31]. In the present study, overexpression of an AM receptor in the pupillary sphincter muscle of $CLR^{SM\alpha A}$ mice revealed enhanced relaxation of the iris in response to endogenous ocular AM. Thus, in line with observations in patients with acute ACG, chronic relaxation of the iris sphincter muscle by endogenous AM appeared to transiently obstruct the aqueous outflow in $CLR^{SM\alpha A}$ mice as early as between 1 and 3 months of age. Suppression of the effects of endogenous ocular AM was therefore attempted. Repetitive topical application of the AM antagonist AM-(20–50) was ineffective in $CLR^{SM\alpha A}$ mice, presumably because of its low potency. We therefore took advantage of $AM^{-/+}$ mice that have up to 50 % lower levels of AM in tissues and the circulation [30]. Mating $AM^{-/+}$ mice with $CLR^{SM\alpha A}$ animals produced $CLR^{SM\alpha A} \times AM^{-/+}$ mice with IOP indistinguishable from wild-type animals up to 3 months of age. $AM^{-/-}$ mice are not available as they die *in utero* as a result of defective cardiovascular morphogenesis [30]. The normal IOP in $CLR^{SM\alpha A} \times AM^{-/+}$ mice is in accordance with the proposed mechanism for the development of acute glaucoma in $CLR^{SM\alpha A}$ mice. The 50 % lower expression of AM in $CLR^{SM\alpha A} \times AM^{-/+}$ mice compared with $CLR^{SM\alpha A}$ animals rendered the enhanced AM sensitivity of the CLR-overexpressing sphincter muscle ineffective and rescued the mice from increased IOP. Taken together, our results indicate, but do not prove, that the increased sensitivity of the sphincter muscle to endogenous AM causes chronic relaxation of the sphincter muscle and, as a consequence, obstruction of the aqueous outflow system.

Interestingly, CGRP was reported to regulate IOP in the eye of cats and rabbits [32,33]. CGRP was recognized, together with PACAP (pituitary adenylate-cyclase-activating peptide), in sensory C-fibres in the eyes of rabbits. Together with PACAP, CGRP is released from the iris and the ciliary body by capsaicin [34]. Intravitreal injections of CGRP in cats and rabbits facilitated trabecular outflow, and intracameral administration caused in addition a breakdown of the blood aqueous barrier. As a result, a sustained decrease in IOP was observed. The present study demonstrates that the pupillary sphincter muscle is not a principal target of CGRP. Receptor autoradiography revealed ^{125}I-AM binding to sphincter muscle sections that was 2-fold higher in $CLR^{SM\alpha A}$ mice than in control animals, but ^{125}I-CGRP binding was undetectable. Moreover, nanomolar concentrations of AM, unlike CGRP, stimulated cAMP production in sphincter smooth muscle cells in primary culture, as reflected by nuclear phospho-CREB accumulation. However, CGRP at micromolar concentrations probably cross-reacts with AM receptors in the pupillary sphincter muscle. This may have occurred in rabbits that had transiently elevated IOP upon intravitreal injections of microgram amounts of CGRP, amounting to estimated micromolar concentrations in the aqueous humour [35]. In accordance with our present findings with AM in mice, high concentrations of CGRP may have caused transient pupillary palsy and angle closure and, as a consequence, impaired aqueous outflow. However, physiological concentrations of approx. 10^{-10} mol/l CGRP in the aqueous humour of mice only increased to 10^{-8} mol/l in response to laser irradiation of the iris [36]. Taken together, CGRP predominantly facilitates trabecular outflow with a reduction in IOP, and AM, relaxing the pupillary sphincter muscle, dramatically increases IOP.

Genotyping of randomly selected $CLR^{SM\alpha A}$ mice by DNA sequencing of PCR-amplified fragments of the *Gpnmb* gene and the *Tyrp1* allele revealed low and equal risk of DBA/2J mice-like pigmentary glaucoma

in $CLR^{SM\alpha A}$ animals and control littermates [7]. These findings and the early onset of acute transient increases in IOP in $CLR^{SM\alpha A}$ mice, which were not observed in control littermates, indicate that the phenotype described in the present study is not related to the pigmentary glaucoma reported for DBA/2J mice [7,13]. Thus we conclude that sustained increased AM sensitivity and, as a result, chronic relaxation of the pupillary sphincter muscle in $CLR^{SM\alpha A}$ mice induces acute transient increases in IOP. Rescue of this phenotype in $CLR^{SM\alpha A} \times AM^{-/+}$ mice with a reduction in AM tissue content supports the notion that the overexpressed AM receptor is causative for acutely elevated IOP.

In view of our findings in mice, we have investigated the expression of AM and its receptor in human eyes obtained at autopsy. *In situ* hybridization and immunohistochemical analysis have revealed the expression of AM in the ciliary body. Moreover, CLR and RAMP2 have been recognized in SMαA-containing cells of the human iris sphincter muscle. Thus balanced expression of AM and its receptor in these structures of the human anterior eye appear relevant for the regulation of IOP.

In conclusion, ocular AM reduces pupillary sphincter muscle tone. As a result, overexpression of the CLR/RAMP2 AM receptor in the iris sphincter muscle of mice leads to chronically enhanced AM activity in the eye and provoked acute transient increases in IOP observed in acute ACG in humans. Thus enhanced signalling of AM in the pupillary sphincter muscle appears to contribute to the pathogenesis of acute glaucoma. Aberrant ocular functions of AM and CGRP have not been associated to date with the pathogenesis of human acute glaucoma. Nevertheless, ocular AM and its receptor in the iris sphincter may present novel targets for the treatment of ACG, and $CLR^{SM\alpha A}$ mice may provide a model for drug screening.

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