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GABAergic amacrine cells and visual function are reduced in PAC1 transgenic mice

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ABSTRACT

Pituitary adenylate cyclase activating polypeptide (PACAP) and its high affinity receptor PAC1 are expressed in mammalian retina and involved in processing light information. However, their roles during retinogenesis remain largely elusive. Previously, we have generated transgenic mice overexpressing the human PAC1 receptor, and shown that PACAP signaling is essential for normal development of the central nervous system. In this study, we show for the first time that PACAP signaling plays an important role in the development of retina, particularly in the genesis of GABAergic amacrine cells. Overexpression of the PAC1 receptor leads to an early exit from retinal proliferation, reduced production of GABAergic neurons, and a marked decline in visual function. These data demonstrate that an appropriate level of PACAP signaling is required for normal retinogenesis and visual function. This finding may have implications in GABAergic neuron-related neurological conditions.

1. Introduction

Vision of mammals is achieved through the retina, a highly organized laminar structure consisting of seven cell types in three cellular layers. The outer nuclear layer (ONL) is composed of rod and cone photoreceptors. The inner nuclear layer (INL) contains Müller glia, horizontal, bipolar and amacrine neurons. The innermost ganglion cell layer (GCL) is occupied by ganglion neurons and displaced amacrine cells. Amacrine cells in the INL are the major inhibitory neurons. They synapse on bipolar, ganglion and other amacrine cells, and relay integrated outputs to ganglion neurons. At least 26 amacrine subtypes are identified (MacNeil and Masland, 1998) and most of them are either glycinergic (~35%) or GABAergic (~40%) (Vaney, 1990; Davanger et al., 1991; Crooks and Kolb, 1992).

Accumulating evidence shows that appropriate GABAergic signaling is critical for vision. For example, the *rd* mutants develop deteriorated vision due to a mutation in a photoreceptor-specific phosphodiesterase, meanwhile the GABA content is strikingly increased in the *rd* retina (Murashima et al., 1990). Drugs elevating GABA signaling also affect vision. Vigabatrin is an effective antiepileptic drug that binds GABA-transaminase irreversibly (Hammond and Wilder, 1985). Its clinical use causes a variety of visual dysfunctions (Hilton et al., 2002; Hosking and Hilton, 2002). On the other hand, reduced GABA-immunoreactivity is found in the *spastic* mice with impaired vision (Yazulla et al., 1997). Stiff-man syndrome is a rare neurological disorder (Solimena and De Camilli, 1991). It associates with severe bilateral visual deterioration (Steffen et al., 1999) and reduced GABA signaling in the majority of the patients due to the presence of an autoantibody against a GABA-synthesizing enzyme (Solimena et al., 1988, 1990; Grimaldi et al., 1993).

In retina, GABA is produced largely by GABAergic amacrine cells. Amacrine cells and other retinal cell types are born in a sequential order during retinogenesis (Young, 1985; Cepko et al., 1996), a process involving numerous transcription factors. Pax6 is a master gene required for maintaining retinal progenitors and producing all retinal cell types except amacrine cells (Marquardt et al., 2001; Marquardt and Gruss, 2002), while Math3 and NeuroD are needed for the genesis of amacrine cells (Morrow et al., 1999; Inoue et al., 2002). Bhlhb5 is involved in the specification of amacrine cells and cone bipolar cells (Feng et al., 2006), and Islet-1 is essential for the differentiation of bipolar and cholinergic amacrine cells (Elshatory et al., 2007). Ptf1a affects differentiation of GABAergic, glycinergic and horizontal cells (Fujitani et al., 2006; Nakhai et al., 2007), whereas Barhl2 selectively regulates glycinergic

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; GCL, ganglion cell layer; GLYT1, glycine transporter 1; INBL, inner neuroblast layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONBL, outer neuroblast layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PACAP, pituitary adenylate cyclase activating polypeptide; Tg, transgenic; WT, wild type.

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amacrine cell specification (Mo et al., 2004). Pax6 also modulates amacrine cell genesis, and in the *Pax6*$^{flox\Delta}$ retina, 31.1% amacrine cells are GABAergic, but only 0.4% amacrine cells are glycinergic (Marquardt et al., 2001). However, little is known about roles of neuropeptide signaling during retinogenesis.

The neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) and its receptor PAC1 are expressed in rat INL and GCL (Seki et al., 1997, 2000) and involved in light transmission. PAC1 deficiency alters light-induced resetting of rhythmic behavior (Hannibal et al., 2001), while the role of PACAP signaling during retinogenesis remains mysterious. We have generated transgenic (Tg) mice bicistronically expressing the human *PAC1* and a *lacZ* reporter from a 130 kb transgene. The transgene faithfully reproduces the expression of the endogenous *PAC1* gene, and Tg mice manifest hydrocephalus-like brain abnormalities (Lang et al., 2006). In the present study, we show for the first time that the PAC1 receptor is expressed throughout retinogenesis, that PACAP signaling modulates the production of GABAergic amacrine cells and that elevated PACAP signaling in mice leads to a reduced INL with decreased GABAergic amacrine cells and substantially impaired visual function.

2. Methods

2.1. Animals

PAC1-overexpressing mice were generated in a mixed genetic background of CBA/Ca: C57Bl/6JCrl and genotyped as described before (Lang et al., 2006). Three lines of Tg mice were backcrossed to C57Bl/6JCrl (CharlesRiver UK) for 3 generations. Adult mice used in this study were 3–5 month-old littermates with no grossly abnormal appearance. All experimental procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and were approved by the Ethical Review Committee, University of Aberdeen, and the Home Office (London, United Kingdom). All analyses were carried out on 3 lines of mice, and the results were pooled together, as they all showed reduced retina.

2.2. Tissue preparation and histological analysis

Adult and newborn mice were killed humanely with a lethal dose of sodium pentobarbitone. The eyeballs and embryos were fixed with 4% paraformaldehyde in PBS for 2 h at 4 °C. After cryoprotection with 20% sucrose in PBS overnight, 12 μm vertical sections of retinas and horizontal sections of embryos were prepared with a cryostat (CM1850; Leica Microsystems) and mounted on Polysine slides (VWR). Eight sets of serial sections were produced for each retina, and were stained with cresyl violet, or antibodies (see below). Some sections were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and counterstained with Neutral Red as described (Lang et al., 2006).

2.3. Morphometrics, cell scoring and statistics

Images were taken at 10×, 20× or 40× objective lens as specified, under an Axiovert 40CFL microscope (Zeiss, Germany) at a linear retinal region with equivalent retinal eccentricity near the optic nerve head as previously reported (Elshatory et al., 2007). Confocal images for cell counting (where specified) were captured with a confocal scanning laser microscope (LSM 510 META, Carl Zeiss, Gottingen, Germany). All quantifications were carried out on images with the aid of AxioVision Rel. 4.5 software (Zeiss, Germany). The thickness of retina or individual layers was measured on cresyl violet or H&E stained sections. Immunofluorescent images stained with cell subtype-specific markers were superimposed with nuclear staining of Hoechst 33342 for counting. Immunopositive cells were counted from different layers of retinal images. The number of cells on individual layers was determined by Hoechst-stained nuclei. Area sizes (in μm^2) of individual layers were measured by AxioVision Rel. 4.5 software. Cell density was obtained by dividing the total cell number with the area size. Optic nerves were imaged under a stereomicroscope after brain dissection. The diameter of optic nerve was measured at a 1.5 mm distance off the chiasm with AxioVision Rel. 4.5 software.

For each mouse, 2–4 images were captured and the mean values were used for statistics. Data were presented as mean and s.e.m., and analyzed by one-way ANOVA for statistical significance. Sample sizes were specified in the Results section. $p < 0.05$ was considered to be significant.

2.4. BrdU labeling and TUNEL assay

A pulse of 5-bromo-2′-deoxyuridine (Sigma–Aldrich) was injected into timemated pregnant females (i.p., 50 mg/kg body weight) 1 h prior to embryo harvesting. For BrdU immunostaining, sections were treated with 0.4% pepsin (Sigma–Aldrich) in PBS for 30 min at 37 °C, denatured with 2 N HCl for 30 min at 37 °C, and neutralized with 0.1 M sodium borate (pH 8.5) for 10 min at room temperature. BrdU incorporation (BD Biosciences) and TUNEL (Roche Diagnostics) assays were executed according to the manufacturers' instructions.

2.5. Immunohistochemistry

Sections were immunohistochemically processed as described (Lang et al., 2006) with primary antibodies including: goat anti-Brn3b (1:200; Santa Cruz), rabbit anti-PACAP (1:2000, Bachem), rat anti-Ki67 (1:100, Dako), rabbit anti-PKCα (1:1000; Sigma–Aldrich), mouse anti-Vimentin (1:100; DHSB, University of Iowa), mouse anti-calbindin D-28K (1:1000, Sigma–Aldrich), mouse anti-syntaxin (1:2000, Sigma–Aldrich), goat anti-GLYT1 (1:4000, Chemicon International), rabbit anti-GABA (1:1000, Sigma–Aldrich), mouse anti-acetylated tubulin (1:1500; Sigma–Aldrich), mouse anti-BrdU (1:200; BD Biosciences), rabbit anti-phospho-histone H3 (1:100, Cell Signaling), rabbit anti-$p57^{Kip2}$ (1:100, Abcam), rabbit anti-human PAC1 (1:50, Abcam), rabbit anti-cleaved caspase 3 (1:100, Cell Signaling). The secondary antibodies were FITC-conjugated donkey anti-rabbit IgG (1:400; Invitrogen), FITC-conjugated donkey anti-mouse IgG (1:400; Invitrogen), FITC-conjugated donkey anti-goat IgG (1:400; Invitrogen), Texas red-conjugated donkey anti-rabbit IgG (1:1000; Invitrogen), Texas red-conjugated donkey anti-rat IgG (1:1000; Invitrogen), Texas red-conjugated donkey anti-mouse IgM (1:1000; Invitrogen), Texas red-conjugated donkey anti-mouse IgG (1:1000; Invitrogen).

2.6. Optokinetic response test

The optokinetic response test was performed as described elsewhere (Thaung et al., 2002; Hart et al., 2005). Briefly, the visual tracking drum (with a diameter of 30.5 cm and height of 54.5 cm) was motorized. A removable card with black and white vertical stripes was fitted around the inner wall of the drum. Each mouse was allowed to acclimatize for 1 min on an elevated platform (8.0 cm in diameter, 20.0 cm in height) in the centre of the visual tracking drum. The drum was then rotated clockwise for 1 min at 2 rev./min. After 1 min interval, the drum was rotated counter clockwise for another minute. Wild type (WT, $n = 16$) and Tg ($n = 11$) mice at 5–6 months were sequentially tested with cards of a stripe width subtending 2° (5.3 mm wide stripes) and 4° (10.6 mm stripes). Only mice showing no clear head tracking were then tested with grating at 8° (21.1 mm stripes). The test was recorded on a video camera and head movements were scored three times on a large TV screen by independent observers. A mouse was scored as "responsive" if it made at least one head tracking movement during the 2-min test, and as 'non-responsive' if it failed to make any head tracking movement. The total numbers of tracking in the 2-min test made by individual mice at each grating were also scored, and two groups of mice were compared statistically by one-way ANOVA.

3. Results

3.1. PAC1 overexpression leads to a reduced retinal thickness

Previously we have described Tg mice overexpressing the human PAC1 and β-galactosidase bicistronically with a 130 kb P1-artificial chromosome that faithfully reproduced the endogenous expression pattern (Lang et al., 2006). PAC1 is known to be expressed in retina (Seki et al., 1997). To explore the effect of PAC1 overexpression on Tg retina, we examined sections of retina histologically, and observed a reduction in the Tg retina (Fig. 1A–D). We subsequently quantified retina thickness from 10 WT and 12 Tg adults, and found that the reduction in the Tg retina was significant statistically (Fig. 1E). The lamination effect of the Tg retina was investigated by comparing WT and Tg retinal layers morphometrically with AxioVision Rel. 4.5 software. No significant difference was detected in the ONL (64.2 ± 1.9 *vs.* 63.9 ± 1.4 μm, $p = 0.75$). Consistently, the outer and inner segments (OS + IS, 48.1 ± 3.5 *vs.* 42.5 ± 2.4 μm, $p = 0.10$, Fig. 1F), the projections of the photoreceptors, were not grossly altered. However, the INL was reduced in Tg mice by ~15% in thickness (43.3 ± 1.9 *vs.* 36.6 ± 1.2 μm, $p < 0.01$, Fig. 1F).

To examine whether Tg and WT retina differ in cell density, we quantified cells in individual cell layers from 40× retinal images of