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Flicker downregulates the content of crystallin proteins in form-deprived C57BL/6 mouse retina

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ABSTRACT

Image degradation by loss of higher spatial frequencies causes form-deprivation myopia (FDM) in humans and animals, and cyclical illumination (flicker) at certain frequencies may prevent FDM. The molecular mechanisms underlying FDM and its prevention by flicker are poorly known. To understand them better, we have identified proteins that differ in amount in form-deprived (FD) mouse retinas, under steady versus flickering light. Male C57BL/6 mice (age 27–29 days) were randomly divided into three groups: *Experimental* – monocularly form-deprived, and kept under either normal room light ("*FD-Only*") or 20 Hz flickering light ("*FD-Flicker*"), throughout the 12-hour light phase; and *Control* ("*Open-Control*") – kept under normal illumination, without form deprivation. After two weeks of treatment, retinal proteins were extracted and separated by two-dimensional gel electrophoresis (2D-GE); proteins that differ in content in FD-only versus FD-flicker retinas were identified by mass spectroscopy ("MS"), and their identities were verified by western blotting. The contents of three identified proteins differed statistically in FD-only compared to FD-flicker retinas. These proteins were identified by MS as α-A-crystallin, crystallin β A2 and crystallin β A1. Quantitative western blotting showed that the relative amount of α-A-crystallin in FD-only retinas was significantly higher than that in FD-Flicker and control retinas. In conclusion, form deprivation induced significant increases in the amounts of crystallins in mouse retinas. These increases were significantly reduced by exposure to 20 Hz flicker. Since form deprivation is known to induce myopia development, and flicker to prevent it, our data suggest that FD- and flicker-responsive changes in the content of crystallin proteins may be involved causally or protectively in myopia development.

1. Introduction

Myopia (near- or short-sightedness), characterized by excessive axial elongation of the eye and negative refractive error (Curtin and Karlin, 1971), is among the most prevalent of human eye disorders (Sperduto et al., 1983). Over the past few decades, the prevalence of myopia has been increasing rapidly, especially in East Asia (Wu et al., 2001). In one study, 50% of urban children on the Chinese mainland were found to be myopic by age 12, and 70% by age 15 (He et al., 2009); other recent studies have found similarly high prevalence of myopia among urban Chinese children in Hong Kong (Lam et al., 2004), Singapore (Quek et al., 2004), and Taiwan (Lin et al., 2001). A longitudinal study of 345 National Taiwan University medical students showed that the prevalence of myopia progressed significantly, from 92.8% to 95.8%, over a five-year period (Lin et al., 1996). The increasing worldwide prevalence of myopia is attributed mainly to environmental factors, among which prolonged near work is the most frequently cited risk factor (Morgan, 2003; Morgan and Rose, 2005). Although myopia is a major health concern, the mechanisms underlying its development continue to be poorly understood. Moreover, all strategies that have been tried for slowing the progression of myopia, such as myopic defocus (Chung et al., 2002), progressive addition lenses (Gwiazda et al., 2003), and muscarinic receptor antagonist atropine (Chua et al., 2006), have been found to be relatively ineffective. Recent human epidemiological studies suggest that outdoor activities may be protective against myopia progression (Rose et al., 2008; Guggenheim et al., 2012), perhaps as a result of the increased exposure to light, as suggested by the results of laboratory studies in chickens (Ashby et al., 2009) and monkeys (Smith et al., 2012); however, it remains to be seen whether this will lead to a practical myopia-preventing therapy.

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The growth of the eye, like that of other organs, is guided by homeostatic control mechanisms. These mechanisms are at least partly environmental, with visual image quality playing a major role (Wallman and Winawer, 2004). It is widely believed that visually guided ocular growth acts to achieve and maintain emmetropia – the normal state in which the refractive power and the axial length of the eye are matched, so that images of distant objects are focused on the retinal photoreceptors without accommodative effort (Wallman et al., 1981). Failures of homeostatic control are expected to account for many, if not all, cases of refractive abnormalities (Wallman and Winawer, 2004). However, it is difficult in human subjects to identify the control mechanisms that regulate normal ocular growth and to determine which changes in them are responsible for myopia.

Fortunately, considerable insight into mechanisms underlying emmetropia and myopia can be gained from studies in animal models (Howlett and McFadden, 2006; Guggenheim et al., 2002; Tejedor and de la Villa, 2003; Smith and Hung, 2000). Form deprivation, the blurring of vision that is commonly produced by attaching a diffusing goggle over the eye, has been used to induce form-deprivation myopia (FDM) in animal models including chicken (Guggenheim et al., 2002), mouse (Tejedor and de la Villa, 2003), guinea pig (Howlett and McFadden, 2006), tree shrew (Norton, 1999), marmoset (Graham and Judge, 1999) and rhesus monkey (Smith and Hung, 2000). Evidence from these animal studies indicates that the degradation of retinal image quality, especially the attenuation of contrast information at the higher spatial frequencies, may stimulate or permit the development of myopia (Schaeffel, 2006; Hess et al., 2006; Wallman et al., 1978). These increases in myopia-promoting "Go" signals (or reductions in myopia-preventing "Stop" signals) result from disturbances in the temporal and spatial integration of complex visual image components such as spatial and temporal frequency and contrast. Although the mechanisms by which the eye discriminates environmental "Stop" or "Go" signals remain poorly understood, persuasive evidence from experimental myopia supports a leading role for the neural retina (Raviola and Wiesel, 1990; Wallman et al., 1987; Fujikado et al., 1997b), and intrinsic retinal neurons – horizontal (Wu et al., 2007) and especially bipolar and amacrine cells (Rohrer et al., 1995; Fischer et al., 1999; Zhong et al., 2004a) – are likely to play critical roles in the visual control of eye growth and prevention of myopia. The detailed signaling cascade from retina to sclera, which is the ultimate determinant of ocular size and shape (Rada et al., 2006), may be quite complicated and is not well understood. However, animal studies have implicated many candidate transduction cascades and signaling proteins, such as Egr1/zif268 (Fischer et al., 1999; Bitzer and Schaeffel, 2002), glucagon (Feldkaemper and Schaeffel, 2002), sonic hedgehog (Shh) (Qian et al., 2009), transforming growth factor beta (TGF-β) and basic fibroblast growth factor (bFGF) (Rohrer and Stell, 1994), as well as retinal synaptic transmitters and neuromodulators including dopamine (DA) (Stone et al., 1989), retinoic acid (RA) (Seko et al., 1996) and nitric oxide (NO) (Fujikado et al., 1997a).

As shown in the induction of myopia by form deprivation, the loss of higher spatial frequencies – and consequently, reduction of spatial and temporal retinal image contrast – is one of the key risk factors for myopia. If this is true, then one class of myopia-control strategies might employ restoration or supplementation of high spatial- or temporal-frequency information for a few hours every day (Schaeffel, 2006; Hess et al., 2006; Schmid and Wildsoet, 1997). How might this be accomplished? Eye movements are ubiquitous and essential for vision (Steinman and Levinson, 1990), and since eye movements produce image movements across the retina, spatial frequency information is converted to temporal frequency information as patterned images move across the receptive fields of retinal neurons (Rodieck, 1998). Therefore, by mimicking the spatial frequency-dependent effects of moving images on the retina, temporal modulation of image intensity (or temporal contrast, usually periodic and commonly called "flicker") could provide a means of controlling myopia, even with images lacking spatial contrast. Indeed, several studies in animals have found that ~20 Hz flicker suppressed the development of myopia (Schwahn and Schaeffel, 1997; Rohrer et al., 1995; Ayotte et al., 2005). However, the neural circuitry and molecular mechanisms responsible for this effect remain unknown. Since form deprivation induces myopia, and because flicker stimulation is reported to control it, in the current study we used two-dimensional gel electrophoresis (2D-GE) and mass spectroscopy (MS) to screen for proteins whose content in the retina is increased or decreased by these manipulations. The identification of such proteins may help to clarify the pathways involved in the development and prevention of myopia. We chose to use the mouse, which is a promising model for investigating myopia in mammals (Tejedor and de la Villa, 2003; Barathi et al., 2008; Tkatchenko et al., 2010; Faulkner et al., 2007). Although many weeks of form deprivation are required to cause even moderate myopia in mice, changes of gene expression in the mouse retina are detectable by microarray analysis after as little as 30 min of form deprivation (Brand et al., 2007), making this a powerful model for rapid discovery of genes and proteins involved in the control of mammalian eye growth and myopia.

2. Methods

2.1. Animals and generation of animal model

The experiment enrolled 48 wild-type C57BL/6 male mice, ranging from postnatal age 27–29 days (P27-P29). All the mice were obtained from the animal center of Sun Yat-Sen University and kept in a temperature-controlled brooder with free access to food and water. The room temperature was maintained at 25 °C ± 2 °C, and the relative humidity was at 45% ± 5%. The mice were randomly divided into three groups (n = 16 mice in each group): (A) "FD-only" mice with one eye form-deprived, as described below, kept under normal room light (cold light source: fluorescent lamps. 30 Lux at the mouse eye's level. Shiyu Optics, Guangzhou, China); (B) "FD-flicker" mice with one eye form-deprived, but exposed to 20 Hz square-wave flicker (cold light source. 30 Lux at the mouse eye's level. Shiyu Optics, Guangzhou, China) throughout the light phase (Fig. 1); and (C) "Open-control" mice with both eyes not form deprived, kept under normal room light throughout the light phase. The daily lighting cycles for both room light and flickering illumination were 12:12 h light:dark, on at 8:00 am and off at 8:00 pm. Mean illuminance at the level of the animals was determined with a photometer (1332A, TES, Taiwan, China), and was matched to about 30 Lux at the mouse eye's level for all three groups. For convenience, four mice were housed together in one cage; however, given the tendency of mice to cluster in a tight group, which would prevent the eyes from being exposed to either steady or flickering light of consistent illuminance, the individual mice were kept apart by wire-mesh barriers.

For form deprivation, goggles approximately 0.8 cm in diameter and 0.5 cm in depth were extruded from a thin transparent plastic sheet with a hot shaper. A 0.2 cm flange was left around the base of the goggles for periorbital adhesion. A piece of frosted window film was glued to the outer (front) surfaces of the plastic goggles to blur the vision. Flanges of the goggles were fixed to the fur around the eyes with liquid cyanoacrylate super glue (UHU, Germany), and a collar was fitted around the neck to prevent the mouse from removing the goggles. However, since the goggles could still come