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A ROLE FOR THE OUTER RETINA IN DEVELOPMENT OF THE INTRINSIC PUPILLARY LIGHT REFLEX IN MICE

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Abstract—Mice do not require the brain in order to maintain constricted pupils. However, little is known about this intrinsic pupillary light reflex (iPLR) beyond a requirement for melanopsin in the iris and an intact retinal ciliary marginal zone (CMZ). Here, we study the mouse iPLR *in vitro* and examine a potential role for outer retina (rods and cones) in this response. In wild-type mice the iPLR was absent at postnatal day 17 (P17), developing progressively from P21–P49. However, the iPLR only achieved ~30% of the wild-type constriction in adult mice with severe outer retinal degeneration (*rd* and *rdcl*). Paradoxically, the iPLR increased significantly in retinal degenerate mice >1.5 years of age. This was accompanied by an increase in baseline pupil tone in the dark to levels indistinguishable from those in adult wild types. This rejuvenated iPLR response was slowed by atropine application, suggesting the involvement of cholinergic neurotransmission. We could find no evidence of an increase in melanopsin expression by quantitative PCR in the iris and ciliary body of aged retinal degenerates and a detailed anatomical analysis revealed a significant decline in melanopsin-positive intrinsically photosensitive retinal ganglion cells (ipRGCs) in *rdcl* mice >1.5 years. Adult mice lacking rod function (*Gnat1*$^{-/-}$) also had a weak iPLR, while mice lacking functional cones (*Cpfl5*) maintained a robust response. We also identify an important role for pigmentation in the development of the mouse iPLR, with only a weak and transient response present in albino animals. Our results show that the iPLR in mice develops unexpectedly late and are consistent with a role for rods and pigmentation in the development of this response in mice. The enhancement of the iPLR in aged degenerate mice was extremely surprising but may have relevance to behavioral observations in mice and patients with retinitis pigmentosa.

Key words: melanopsin, development, *rd* mice, pigmentation, pupillary light reflex, retinitis pigmentosa.

INTRODUCTION

Pupil constriction is maintained during daylight hours by the pupillary light reflex (PLR), a neural pathway traditionally thought to involve input from different components of the retina, relay via midbrain nuclei and output to muscles of the iris via the ciliary ganglion (Alexandridis, 1985; Lucas et al., 2003; Guler et al., 2008; Lall et al., 2010; Chen et al., 2011). However, it has been known for some time now that the irises of fish and amphibians (Seliger, 1962; Barr and Alpern, 1963), birds (Tu et al., 2004) and some mammals, including rats (Bito and Turansky, 1975; Lau et al., 1992) can constrict in response to light independently of the brain.

Recently, it has been demonstrated in both anaesthetized and conscious preparations that mice also retain an intrinsic pupillary light reflex (iPLR) following axotomy (Xue et al., 2011; Semo et al., 2014). In both studies, the iPLR was sufficient to maintain pupil constriction over a range of physiologically relevant light intensities and was absent in adult mice lacking the melanopsin gene (*Opn4*$^{-/-}$). In addition to a dependence upon melanopsin and phospholipase C β4 (Xue et al., 2011), the iPLR in mice requires cholinergic neurotransmission (Semo et al., 2014; Schmidt et al., 2014a) and is also inhibited by selective damage to the ciliary marginal zone (CMZ) of the retina (Semo et al., 2014).

In the mouse eye, melanopsin is expressed in the iris (Xue et al., 2011), ciliary body (Semo et al., 2014), retinal pigment epithelium (Peirson et al., 2004) and retina. In the retina, melanopsin is expressed by intrinsically photosensitive retinal ganglion cells (ipRGCs), a heterogeneous population of neurons sending axons to a variety of subcortical brain structures, including the midbrain olivary pretectal nucleus (OPN), which mediates the conventional PLR (Hattar et al., 2006; Baver et al., 2008; Chen et al., 2011). In addition to this, ipRGCs also send axonal collaterals up into the inner plexiform layer of the retina, which may mediate a novel form of retrograde visual

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Abbreviations: ANOVA, analysis of variance; Brn3a, *Pou4f1* transcription factor; Brn3b, *Pou4f2* transcription factor; CMZ, ciliary marginal zone; *Cnga3*$^{-/-}$, cone-specific cyclic nucleotide-gated channel knockout mouse; *Cpfl5*, cone photoreceptor function loss 5 mouse; *Gnat1*$^{-/-}$, rod alpha transducin knockout mouse; iPLR, intrinsic pupillary light reflex; ipRGC, intrinsically photosensitive retinal ganglion cell; LCMD, laser capture microdissection; OPN, olivary pretectal nucleus; *Opn1mw*^{R}, red cone knock-in mouse; *Opn4*$^{-/-}$, melanopsin knockout mouse; P, postnatal; PCR, quantitative real time polymerase chain reaction; PLR, pupillary light reflex; RGC, retinal ganglion cell; TBP, TATA-binding protein.

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signaling (Zhang et al., 2012; Joo et al., 2013). In rodents, ipRGCs are more common in the superior and temporal retina (Hannibal et al., 2002; Hattar et al., 2002; Vugler et al., 2008; Galindo-Romero et al., 2013; Hughes et al., 2013; Nadal-Nicolas et al., 2014; Valente-Soriano et al., 2014), with a discrete, melanopsin-rich plexus in the extreme retinal periphery (CMZ) of rats and mice (Vugler et al., 2008; Semo et al., 2014). In the mouse CMZ, we have shown that Brn3b-negative melanopsin neurons send projections directly into the ciliary body (Semo et al., 2014), a finding which complements recent reports of a direct retinal projection from ipRGCs into the mouse iris (Schmidt et al., 2014a).

In addition to being intrinsically light responsive, ipRGCs also receive synaptic input from rods and cones (Dacey et al., 2005; Schmidt et al., 2008; Weng et al., 2013). The genetic elimination of ipRGCs has shown them to be required for non-image forming vision in mice (Guler et al., 2008) and the current thinking is that ipRGCs integrate rod and cone signals with their own melanopsin-driven light responses to control important aspects of non-image forming and image-forming vision (Lucas et al., 2003; Panda et al., 2003; Brown et al., 2010, 2012; Ecker et al., 2010; Estevez et al., 2012; Allen et al., 2014; Schmidt et al., 2014b).

To date, nothing is known about the development of iPLR in mice beyond an apparent requirement for melanopsin from birth. As such, we were keen to examine the time course of iPLR development in wild-type mice and to use mice lacking functional rods and cones to explore if melanopsin alone is sufficient for iPLR development. This appeared to be a sensible question to ask given the known routing of rod/cone signals through ipRGCs and emerging evidence of a direct retinal contribution to the iPLR. As our established intraocular axotomy procedure was not feasible on retinal degenerate mice (Semo et al., 2014), we chose to validate and use a new *in vitro* approach here.

Our new method proved to be a good way of studying the iPLR in mice, giving comparable results to previous *in vivo* experiments. In retinal degenerate mice the *in vitro* pupillometry was correlated with molecular analysis of melanopsin expression in iris/ciliary body and a detailed anatomical assessment of ipRGC survival. This revealed a paradoxical increase in the strength of the iPLR response in aged retinal degenerates that occurred in parallel with a significant decline in the number of melanopsin-positive ipRGCs.

EXPERIMENTAL PROCEDURES

Animals

All procedures were conducted according to the Home Office (UK) regulations, under the Animals (Scientific Procedures) Act of 1986 and associated guidelines, with local ethics committee approval. All animals were housed under a 12-h light, 12-h dark cycle (lights on at 07:00, lights off at 19:00), with food and water available *ad libitum*. The following strains/genotypes of mice were used in our experiments: wildtype C57BL/6 (Harlan, UK); wildtype C3H/He; mice lacking either rods (*rd/rd*) or rods and cones (*rd/rd cl*), which are both on the C3H/He background; melanopsin knockout ($Opn4^{-/-}$) mice and triple knockout ($Opn4^{-/-}$, $Gnat1^{-/-}$, $Cnga3^{-/-}$) mice, which are both on a C57BL/6-129 mixed strain background (Hattar et al., 2003; Lucas et al., 2003); red cone knock-in ($Opn1mw^{R}$) mice which are on a C57BL/6 background (Lall et al., 2010) and were obtained from the colony maintained at the University of Manchester, UK; Cone photoreceptor function loss 5 (*Cpfl5*) mice (Pang et al., 2012), which are on a mixed C57BL/6 background; rod α-transducin knockout ($Gnat1^{-/-}$) mice which are on a mixed 129/Sv-BALB/c background (Calvert et al., 2000) and albino mice of either the BALB/c or MF1 strain (both from Harlan, UK). The mice used in our experiments were of mixed sex and ranged in age from postnatal day 17 (P17) to 30 months. Unless otherwise stated, all mice came from colonies maintained at UCL-Institute of Ophthalmology, UK.

In vitro pupillometry to isolate the iPLR

The methodology used here to isolate iPLR in mice is similar to that used for recording iPLRs from the isolated anterior chamber (Semo et al., 2014). However, instead of dissecting away the posterior segment, here we used a relatively simple whole-eye preparation to study pupillary constriction in the intact, isolated mouse eye. Following the initial experiments described in the sections 'Irradiance response under light and dark-adapted conditions' and 'Influence of stimulus duration on the dark-adapted iPLR', all subsequent experiments were carried out as described below in this section, with both eyes from a single mouse studied in darkness following a period of overnight dark adaptation. Occasionally, the PLR video acquisition software crashed and data from individual eyes were lost (hence the disparity between eye and animal numbers below).

On the morning of experimentation (between 08:00 and 11:00), mice were killed by cervical dislocation under red light. Eyes were removed with scissors and placed carefully (corneal surface upwards) onto on a custom-made Perspex indentation and covered with 4 drops of Neurobasal® culture medium (Invitrogen, 12348-017), which had been preheated to 37 °C. Eyes were illuminated with an infra-red light source and then stimulated with broad-spectrum white light originating from a xenon-arc lamp (Lambda DG-4, Linton Instrumentation). The stimulating light was heat filtered (preventing the passage of wavelengths > 600 nm) and then guided through a fiber optic cable, which terminated 1.5 cm away from the cornea, delivering 63 mW/cm^2 to the eye. The iPLR was recorded under infrared illumination, with 30 s of baseline recording in darkness followed by 60 s of light stimulation and a further 60 s of post-stimulation recording.

As described previously (Semo et al., 2010, 2014), pupil area was measured off-line at 1-s intervals by an observer using bespoke MATLAB software, with all iPLR measurements expressed as normalized pupil area (relative to the baseline pupil area). The baseline pupil area was also estimated in mm^2 following the calibration of video images using a scale bar placed at the level of