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A system to measure the pupil response to steady lights in freely behaving mice

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HIGHLIGHTS

- We present a system to measure the pupillary response to steady lights of freely behaving mice.
- Our method uses a low-cost, portable device that automatically acquires close-up images of mouse eyes.
- Our method takes advantage of the intrinsic nature of mice to inspect objects of interest.
- Our method does not require manual restraint, anesthesia or training of mice.
- Our method can be used in optomotor or operant behavior testing chambers.

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ABSTRACT

Background: Transgenic mice are widely used for the study of basic visual function and retinal disease, including in psychophysical tests. Mice have a robust pupillary light reflex that controls the amount of light that enters the eye, and the attenuating effects of the pupil must be considered during such tests. Measurement of the size of pupils at various luminance levels requires that mice remain stable over prolonged periods of time; however, sedation of mice with anesthesia and/or manual restraint can influence the size of their pupils.
New Method: We present a system to measure the pupillary light response to steady lights of freely behaving mice using a custom-built, portable device that automatically acquires close-up images of their eyes. The device takes advantage of the intrinsic nature of mice to inspect objects of interest and can be used to measure pupillary responses in optomotor or operant behavior testing chambers.
Results: The size of the pupils in freely behaving mice decreased gradually with luminance from a maximal area in the dark of 3.8 mm^2 down to a minimum 0.14 mm^2 at 80 scotopic cd/m^2. The data was well fit with a Hill equation with Lo equal to 0.21 cd/m^2 and coefficient h = 0.48.
Comparison with existing methods: These values agree with prior measurements of the pupillary response of unrestrained mice that use more laborious and time consuming approaches.
Conclusions: Our new method facilitates practical, straightforward and accurate measurements of pupillary responses made under the same experimental conditions as those used during psychophysical testing.

1. Introduction

Transgenic mice are widely used for the study of basic visual function and retinal disease (Nishina and Naggert, 2003; Pinto and Enroth-Cugell, 2000). These studies compare structure-function changes at the cellular and retinal levels by combining traditional approaches such as immunohistochemistry, electrophysiology and biochemistry (Huberman and Niell, 2011; Pinto and Enroth-Cugell, 2000). More recently, these traditional approaches have been complemented with psychophysical tests that assess the limits of vision in mouse (Busse et al., 2011; Histed et al., 2012; Naarendorp et al., 2010; Prusky et al., 2004) and can track visual changes in models of

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retinal degeneration (McGill et al., 2012; Umino et al., 2006), retinal transplantation (Pearson et al., 2012) and gene therapy treatment (Boye et al., 2013; Pang et al., 2011). A primary objective of these visual psychophysical assays is to determine the minimal amount of light or contrast that is necessary to elicit a visual response in animals (also known as visual threshold) (Stebbins, 1970). However, mice have a robust pupillary light reflex that controls the amount of light that enters the eye (Pennesi et al., 1998): as luminance increases the iris muscles constrict and close the pupil. Hence, the attenuating effects of the pupil must be considered, particularly when it is important to know the precise level of retinal illumination (Lyubarsky et al., 2004).

Characterizing the response of the pupil to light is a straightforward procedure with human subjects, but can be a challenging undertaking in mice. Measurement of the size of mice pupils at various luminance levels requires that mice remain stable over prolonged periods of time. This is generally accomplished by gently restraining the mice by hand while the measurements are performed (Guler et al., 2008; Hattar et al., 2003; Lucas et al., 2003).

Intrinsically photosensitive melanopsin-containing retinal ganglion cells (ipRGCs) mediate the pupillary light reflex (Berson et al., 2002; Hattar et al., 2002; Provencio et al., 2000); ipRGCs also receive input regarding luminance from rod and cone photoreceptors (Altimus et al., 2010; Dacey et al., 2005; Guler et al., 2008; Lall et al., 2010; Viney et al., 2007; Weng et al., 2013). However, the size of pupils in restrained mice can be influenced by an autonomic response triggered when restrained (Bitsios et al., 1996) irrespective of background illumination. An option that reduces animal anxiety for assaying the pupillary light reflex when restrained is to immobilize (Pennesi et al., 1998) or sedate the mice during the procedure (Kuburas et al., 2014; Thompson et al., 2011). Small amounts of ketamine/xylazine anesthesia do not interfere with the pupillary light response to *brief* light flashes (Thompson et al., 2011) but the effect of ketamine/xylazine anesthesia on the size of the pupil in *steady* lights has not been determined. More importantly, it is unknown whether the pupil size determined with either of these procedures is the same as the size of the pupil of freely behaving mice exposed to similar luminance environments.

In view of these uncertainties, in an earlier study of the optomotor response, we measured the pupillary light response of unrestrained mice standing on a pedestal inside the enclosure formed by the four video LCD monitors that provided the visual stimulation (Umino et al., 2012). However, a practical difficulty we faced with this setup is that mice do not always orient themselves in the direction of the camera, making the acquisition of high quality images of the pupil a problematic, time demanding task. To address this problem we built a portable device that automatically acquires eye images of unrestrained mice as they explore an object of interest. Here we describe this automated system and demonstrate its application for the measurement of steady-state pupil responses in mice. To determine the accuracy of this method we compare pupillary responses measured with the new system to the responses obtained previously in unrestrained mice standing on a pedestal (Umino et al., 2012) and those obtained in gently restrained mice under a Ganzfeld illuminator. Finally, we present an empirical formula that describes the relationship between size of the pupil and luminance in freely behaving C57BL/6J mice within the OptoMotry© enclosure.

2. Methods

Adult (3–4 months of age), female C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were maintained at SUNY Upstate Medical University (Syracuse, NY). Mice were fed ad libitum a standard diet and maintained on a 14-h light/10 h-dark cycle (lights on at 6 am). All measurements were performed in the early evening hours (6pm to 10 pm), when mice increase their levels of activity. Mice were dark-adapted for 2 hours prior to the measurements of their pupil areas and handled in dim red lights to minimize the effects of light adaptation. The procedures in this study were approved by the SUNY Upstate Medical University Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (2011, National Academies Press, Washington, DC) and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.1. Measurement of the pupillary light response to steady lights in freely behaving mice

We measured the pupillary light response to steady lights of freely behaving mice using a custom-built, low-cost portable device that automatically acquires eye images of unrestrained mice (Fig. 1A). The device was designed to fit inside illuminated enclosures such as those used for optomotor (Fig. 1B) or operant behavior testing and consisted of a transparent plexiglas box (11 cm × 11 cm × 10.5 cm) with a small circular aperture (0.6 cm in diameter) drilled on a side wall at 1.25 cm from the elevated floor and 0.3 cm from the side panel (Fig. 1C). During experiments a plexiglas divider was introduced to reduce the 'behaving surface' by approximately half of the chamber floor dimension in order to minimize the area that the animal was allowed to explore. The floor was elevated 1.25 cm from the bottom of the chamber and was perforated by uniformly spaced holes 0.6 cm in diameter. Four aeration holes were drilled into the lid of the chamber.

Mice placed inside the enclosure spend significant amounts of time exploring the aperture on the side panel (Fig. 1A) which provides the opportunity to acquire close-up images of their pupils. High resolution, infrared images of the pupil were captured using a CCD Webcam (Logitech HD Pro Webcam C920, Newark, CA,) after replacing the IR blocking filter in the camera's light path with an IR high pass filter (Edmund Optics, Barrington, NJ). A cosmetic blue LED located inside the camera and the autofocus feature were inactivated. Infrared illumination was provided with an array of IR LEDs (940 nm emission wavelength) aligned with the camera's view (Fig. 1A, C). The webcam was attached to the lid of the chamber and focused at the level of the aperture and oriented to provide a frontal view of the pupil as the mice explored the aperture. Size calibrations were performed with a graticule positioned at the level of the aperture. Calibrations were repeated with the graticule positioned 5 mm proximal and 5 mm distal relative to the aperture in order to cover all possible locations where the eye may be positioned as the mouse inspects the aperture. Images remained on focus and the same calibration factor (39 pixels/mm) applied to all three locations indicating that the quality and size of the image does not vary significantly within the range of possible eye locations. Video acquisition was triggered with motion detection software (Webcam Zone Trigger, Montreal, QC) every time the nose of the mouse crossed the plane of the aperture. Each video clip was a minimum 5 s in duration but automatically extended to longer recording times as long as activity continued to be detected at the aperture. Video resolution was 1920 × 1080 pixels per frame acquired at 15 Hz. Off-line analysis of pupil areas is described below.

2.2. Illumination

For the purpose of this study, the portable device was placed inside an enclosure formed by four video LCD monitors that provided white (broad spectral) light stimulation, an environment routinely used to measure the optomotor response of mice