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A NOVEL INTRINSIC ELECTRORETINOGRAM RESPONSE IN ISOLATED MOUSE RETINA

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Abstract—Since the discovery of intrinsic photosensitive retinal ganglion cell (ipRGC) was reported in 2002, many features specific to this cell type have been described. However, scare information is available on the retinographic components directly reflecting ipRGC activity. In this study, we identified the electroretinogram (microERG) that reflects the photoresponses by ipRGCs in *ex vivo* preparations of the mouse retina, in which classical photoreceptors (cones and rods) were ablated mechanically and photochemically. MicroERG consisted of three components: a large transient ON response, a small and lazy hump 19 s after the onset of the light, and a large transient OFF response. A complete microERG recording required at least 30 s of light exposure. MicroERG showed the highest spectral photosensitivity at 478 nm. This wavelength corresponds to the peak wavelength in the ipRGCs' photosensitive curve. The psychophysical test using a blue light-emitting diode (LED) light (470 nm) revealed that the absolute threshold illuminance for microERG was greater than 12.26 log photons/s/cm^2 in both ON and OFF responses, whereas microERG was not adapted for dark. The amplitude of microERG increased linearly with irradiance. The sensitivity of temporal frequency was high in microERG (at least 100 Hz), as suggested by the study on melatonin suppression by flickering light in human subjects (Zelter et al., 2014). Melatonin secretion was suppressed by light via ipRGCs and the suprachiasmatic nucleus. These properties of the photoresponse indicate that microERG may reflect the functions of ipRGC as a luminance detector in the mouse retina.

Key words: intrinsically photosensitive retinal ganglion cell, cone, rod, electroretinogram, retina, mouse.

INTRODUCTION

The discovery of the intrinsically photosensitive retinal ganglion cell (ipRGC) led to big paradigm changes in neuroscience. For over 100 years, only cones and rods (classical photoreceptors) were considered capable of transducing light to electrical energy in the retina and transmitting the signals, via the optic nerve, to visual centers in the brain. However, in 2000, Berson, the author and our colleagues discovered novel photosensitive cells in the retinal ganglion cell layer of rodents that could undergo depolarization and generate action potentials intrinsically (Berson et al., 2002; Lucas et al., 2003). These cells contained a special photopigment, melanopsin (Opn4), which activates an intracellular phototransduction cascade, and were named ipRGCs or melanopsin ganglion cells. Thereafter, ipRGCs were shown to project directly into the suprachiasmatic nucleus and olivopretectum, regulating circadian photoentrainment and pupillary responses to the light, respectively (Hattar et al., 2002, 2006).

The spectral sensitivity of ipRGCs is different from that of rods and the two types of cones in the rodent retina. The peak sensitivities of ipRGC, ultraviolet-wavelength (UV) sensitive and middle-wavelength sensitive cones, and rod are 480 nm, 360 nm, 508 nm, and 500 nm, respectively (Lyubarsky et al., 1999; Berson et al., 2002). Moreover, ipRGCs form neural circuits with classical photoreceptors via bipolar and AII amacrine cells (Belenky et al., 2003; Weng et al., 2013). The synaptic inputs from classical photoreceptors expand the dynamic and spectral range of the ipRGC.

Triple knockout mice (TKO mice; *Gnat*$^{-/-}$, *Cnga3*$^{-/-}$, *Opn4*$^{-/-}$) lacking classical photoreceptors and melanopsin are considered to have lost all phototransduction mechanisms, and are therefore expected to completely lack sensitivity to light stimuli (Hughes et al., 2016). In fact, the TKO mice showed no electroretinogram (ERG) responses in a photopic (light) environment (Allen et al., 2010), although a weak ERG response could be recorded in a scotopic (dark) environment (Allen et al., 2010; Semo et al., 2010; Hughes et al., 2016). Moreover, *rd/rd/cl* mice, characterized by degenerated classical photoreceptors, also failed to show any *in vivo* ERG responses in scotopic environment. No studies were performed in these mice in a photopic environment (Allen et al., 2010).

The ERG is an elegant means for evaluating retinal functions in neuroscientific studies as well as ophthalmological testing because it can be recorded

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Abbreviations: ANOVA, analysis of variance; ERG, electroretinogram; M-cone, middle-wave sensitive cone; microERG, ex vivo electroretinogram; UV, ultraviolet-wavelength; UV-cone, ultraviolet sensitive cone.

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simply and noninvasively. The source of each ERG component can be attributed to the electrical activity of different retinal cell types within normal retinae, although ERGs reflect the sum of visual responses by retinal cells.

In this study, we recorded, *ex vivo*, the intrinsic ERG response of a mouse retina in which classical photoreceptors were lesioned mechanically, photochemically, and pharmacologically. The spectral and temporal properties of this response resembled those of ipRGCs. This study reports unusual behavior and unique photosensitive properties of a newly discovered, intrinsic ERG response.

EXPERIMENTAL PROCEDURES

Animal handlings

Adult male C57BL/6J mice were purchased from Kiwa Laboratory Animals Co.,Ltd (Wakayama, Japan). They were maintained on a light–dark cycle of 12-h light and 12-h dark for at least 2 weeks prior to experiments. The procedures in experiments adhered to the guidelines of NIH as well as the ARVO statements for animal experiments, and were approved by the institutional animal experiment board.

Mice were euthanized by intraperitoneal injection of lethal dosage of pentobarbital (80 $mg \cdot kg^{-1}$; Kyoritsu Pharmaceutical Industries Co. Ltd.). The eyes were enucleated and the retinae were isolated for *ex vivo* recordings as described below.

Ex vivo electroretinographic recordings

The protocol for the *ex vivo* ERG (microERG) recording has been previously explained (Berson et al., 2002; Lucas et al., 2003). In brief, retinae were detached from eye cups in Ames' medium with 1.9% sodium bicarbonate and 1.8% dextrose (Ames and Nesbett, 1981; Sigma–Aldrich, MO). The pigment epithelium layer of the retina was peeled off gently with fine brushes to significantly damage outer segments of photoreceptors and physically suppress visual cycles to a large extent. The isolated neural retinae were flat-mounted and placed in a specimen holder for retinographic recordings. The upper side (retinal ganglion cell layer) was superfused with a constant flow of Ames' medium bubbled with 95% O_2 and CO_2.

MicroERGs were recorded using a custom-made silver-wire-in-glass electrode with a tip diameter of about 500 μm. The branching dendrites of the ipRGC extend over a distance of less than 400 μm (Do and Yau, 2010). Therefore, the electrode tip diameter was almost as large as the dendritic field diameter of the ipRGC. The electrode tip touched the upper side of the retina 1–2 mm away from the optic nerve head. To deplete middle-wave sensitive cone (M-cone) and rod photopigments irreversibly, the neural retinae were exposed to strong halogen light (245.7 W/cm^2) for at least 10 min prior to microERG recordings.

MicroERG signals were amplified 1000×, and high-pass and low-pass filtered with a differential amplifier (DP-301, Warner Instruments, Hamden, CT, USA) at 0.1 Hz and 10 kHz, respectively. Hum noise (50 Hz) was removed by an adaptive noise eliminator (Hum Bug; A-M systems, Sequim, WA). The signals were digitized at 1 kHz with a physiological digitizing system (UAS-308S; Unique Medical Co. Ltd, Tokyo, Japan) for further analysis.

Chemicals

To pharmacologically isolate photoresponses attributable to phototransduction in ipRGCs, we added a cocktail containing three glutamatergic blockers to the Ames medium in order to block synaptic transmission from the photoreceptors (Weng et al., 2013). The cocktail included: 50 μM L (+)-2-amino-4-phosphonobutyrate (L-AP4, group III metabotropic glutamate receptor agonist), 40 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX, AMPA/kainate receptor antagonist), and 30 μM D-2-amino-5-phosphonovalerate (D-AP5, NMDA receptor antagonist). MicroERGs were recorded 15–20 min after bath application of this cocktail. These reagents were freshly prepared from aqueous stock solutions. L-AP4 was obtained from Abcam (Cambridge, UK), and DNQX and D-AP5 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). There were no changes in waveform and amplitude in microERGs after 30 min bath application of the cocktail ($n = 4$; Fig. 1). No statistical significance was found in amplitudes of peak to peak ON responses between the two recordings (paired-Student's t-test; mean = 565.63 μV, SD = 170.96 vs mean = 553.53 μV, SD = 163.99, respectively; $t = 0.97$, df = 3, ns).

Visual stimuli

The retinae were stimulated from the photoreceptor side with homogenous full-field narrow-band light. The stimuli were obtained from two types of LEDs, the lambda peaks of which were 360 nm (UV-LED, NS360L-5RLO, Nitride Co. Ltd, Tokushima Japan) and 470 nm (Blue-LED, OSUB5111A–ST, Optosupply Co. Ltd, Hong Kong, China), respectively. Two spectral density curves did not overlap with each other (Fig. 2). Each spectral density curve was narrow enough to mainly stimulate ultraviolet (UV) sensitive cones (UV-cones) and ipRGCs or M-cones (M-cone; Brown 2016). The intensity of light was controlled using natural density filters (Fuji Film Co. Ltd., Tokyo, Japan). The duration of light stimulation was precisely controlled by a physiological stimulator (SEN-3301, Nihon Kohden Co. Ltd., Tokyo, Japan).

To analyze the spectral sensitivity of ipRGCs, light stimuli were delivered by narrow-band filters (430 nm, 460 nm, 472 nm, 509 nm, 523 nm, 569 nm; half-band width 10 nm; Asahi Spectra Co. Ltd., Tokyo, Japan) and a halogen lamp (12V100WHAL-L, Philips Electronic Japan Co. Ltd., Tokyo, Japan). Each photostimulation was manually performed for 5 s. The intensity of light was controlled using neutral density filters as well. All the recordings were performed in an ambient light environment (6.33 $\mu W/cm^2$). Therefore, the retinae were light-adapted throughout the experiments.

Statistical analysis

Statistical analysis was done with repeated two-way ANOVA (analysis of variance). Post-hoc tests were

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