ABSTRACT

The neural signals controlling the slow pupillary light reflex (PLR) in the turtle are poorly understood. For better understanding of the signals that could be involved, the ciliary nerve was stimulated using constant current pulses to evoke pupil constriction in the enucleated eye of the turtle. Currents were delivered as spike trains using glass tip suction electrodes placed onto the transected end of the nerve and changes in pupil size measured using an infrared video camera. Stimulation consisted of 0.5 s trains composed of 1 ms pulses. Current amplitudes and frequencies were varied to identify the values of these parameters which best evoke constriction. A hyperbolic decay function identified 7 µA as the threshold amplitude which evoked 50% of the maximum constriction (14%) when using trains of 100 Hz. A log-normal distribution function fitted to responses evoked by different frequencies at 100 µA predicted peak response (11%) at 126 Hz. These results suggest that maximal constriction is evoked at stimulation frequencies approaching 126 Hz with threshold current amplitude of 7 µA. Similar signaling in mammals and birds with much faster PLRs suggest that the sluggish source for the turtle's PLR is due to a photoreceptive mechanism in the retina or some other central processing in the brain. In addition, future studies may permit a comparison of these putative optimal parameters with the actual efferent signals recorded from the ciliary nerve in the intact turtle.

INTRODUCTION

The pupillary light reflex (PLR) is the constriction of the pupil in response to illumination of the retina (McIlwain 1996). Although the PLR in mammals has been well-docu-

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MATERIALS AND METHODS

Animals

Animal care and all experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Lafayette College. Four male red-eared slider turtles were used ranging in mass from 0.4 kg to 1.19 kg and carapace length from 14 to 20 cm. Turtles were bought from Kons Scientific Co., Inc. (Germantown, WI). After they were received, they were housed in a 60-gallon tank with a 14:10 light/dark cycle with temperature maintained at 22°C.

Dissection

Turtles were cryoanesthetized at 4°C for one hour prior to euthanasia (Maxwell 1979). Heads were then removed from body, and cranial bones with connective tissues surrounding the eyes carefully dissected to expose the pathways of the cranial nerves going into the back of the eyes. After transecting both the optic nerve and the ciliary nerve distal to the ciliary ganglion, the eye was taken out of the orbit. Eyes were rinsed during dissection and submerged in turtle ringer solution (in mM: 96.5 NaCl, 2.6 KCl, 2.0 MgCl2, 31.5 NaHCO3, 20.0 D-glucose, and 4.0 CaCl2) at pH 7.65 and bubbled with 95/5% CO2/O2. Figures 1 and 2 show, respectively, the stimulation location and dissection within the parasympathetic pathway. Experiments were completed within 24 hours after dissections. Tissues of turtle are resilient to hypoxic conditions (Storey 2007), and reliable electrophysiological measures can be carried out for several hours after euthanasia, as long as 18 and even 36 hours (Granda et al. 1999; Dearworth et al. 2009).

Stimulation and Recording

Eyes of turtles were submerged in turtle ringer solution held in small wells. A suction tip electrode (AM Systems, Inc., Carlsborg, WA) was used to stimulate the ciliary nerve using fire-polished capillary glass tips fit onto the nerve. A photoelectric stimulus isolation unit was used for constant current output, and a Grass stimulator (model S88, Astro-Med, Inc., West Warwick, RI) delivered spike trains to the nerve. A computerized eye tracking system (ViewPoint EyeTracker® Arrington Research, Inc., Scottsdale, AZ) was used to detect and measure the horizontal diameter of the pupil at a sampling resolution of 30 Hz. An infrared light emitting diode (LED) was positioned over the eyes to illuminate the eye, and a camera was positioned at a 45° angle and a distance of approximately 5 cm from the eyes. Based on the range of parameters used in other animal preparations (Jampel 1962; Scheappi and Koella 1964; Pilar and Vaughan 1969a,b; Gamlin et al. 1984), two stimulation paradigms were explored. Nerves were first stimulated using 0.5 s duration, 100 Hz spike trains with a pulse width of 1 ms. Pulse amplitudes ranging from 1 to 400 µA were used. Then nerves were stimulated by trains at 100 µA varying frequencies from 1 to 400 Hz.

Data Analysis

Horizontal pupil diameter was analyzed as a percent change from the pupil size prior to stimulation. Means were calculated for the measure at each time sample and plotted using Microsoft Excel® (Redmond, WA). Sigma plot (SPSS Inc., Chicago, IL) was used to fit mathematical functions to peak mean responses with standard error (SE) in order to predict the values that generated greatest constrictions. Analysis of variances (ANOVA) and t-tests were used for statistical comparison. Fit to the data was done by the method-of-least-squares, and goodness-of-fit by functions was expressed in values of $R^2$. 
RESULTS

The question, what current amplitudes are required to evoke pupil constriction, was first addressed. Figure 3 shows mean pupil responses to 100 Hz trains of increasing current amplitude (1 to 400 µA). Response onset occurred within 66 ms of stimulation and reached greatest maximum within 0.4 s. As current amplitude increased, constriction also increased. When stimulation was terminated, pupils returned to baseline sizes within 0.6 s.

The effects of pulse amplitude on pupil constriction are quantified in Figure 4. Changes of pupil constrictions were statistically significant with different current amplitudes (ANOVA, \( p < 0.05 \)) and appeared to reach a plateau at 100 µA. This is suggested by the response at 400 µA where constriction (–16.36% ± 1.74) was not statistically different from that at 100 µA (t-test, \( p = 0.68 \)). Each constriction evoked by currents less than 100 µA (i.e., 1, 4, 7, 10, and 40 µA), however, was significantly less (t-tests, \( p < 0.05 \)) than that evoked by 400 µA. A two parameter hyperbolic decay function, \( f = ax/(b+x) \), was fitted to the data, where \( a \) is the peak pupil change and \( b \) is the current which produces 50% of the maximum constriction. The best-fit parameters obtained were \( a = -13.82\% \) and \( b = 7.0 \) µA.

The next question addressed was what frequency best drives constriction. Figure 5 shows mean pupil responses for different stimulation frequencies (1 to 400 Hz) with current amplitude fixed at 100 µA. Greatest reduction occurred at 100 Hz (dash-dot-dot trace, Figure 5). As before, pupil sizes were quickly reduced in response to stimulation and returned to their original sizes after stimulation was stopped.

The effects of stimulus frequency on pupil constriction are quantified in Figure 6. Pupil constriction differed as a function of stimulation frequency (ANOVA, \( p < 0.05 \)). Maximum pupil constriction –12.25% ± 2.41 occurred at 100 Hz and was statistically greater than those measured at 1 and 5 Hz (t-test, \( P < 0.05 \)). A log-normal distribution function,
predicted fit parameters were \(a = -10.90\%\), \(x_0 = 125.61\) Hz, and \(b = 2.50\).

**DISCUSSION**

Response dynamics and amplitudes of pupil constrictions were similar to those previously reported for turtle after stimulating the ciliary nerve in the enucleated eye (Dearworth et al. 2009). The current study also found a similar current threshold even though a different function was used for its computation. In Dearworth et al. (2009), a sigmoidal function (Hill equation) identified thresholds as low as 4.2 \(\mu\)A while the current study using a hyperbolic function found a threshold value at 7.0 ± 3.4, SE. When standard error is considered, the overlap indicates computed thresholds which are essentially the same. Our results in turtle also closely resemble those done in pigeon where stimulation was done in the pretectal area of the brain (Gamlin et al. 1984). In that study, 100 Hz trains lasting 1 s and composed of pulses having 0.5 ms width were tested over amplitudes ranging from 1 to 120 \(\mu\)A. That study too used a hyperbolic function fitted to constriction versus current amplitude to describe their data. Extrapolation from their curve (Gamlin et al. 1984, p. 539, Figure 15) computed a threshold at a value equal to 10 \(\mu\)A in close agreement with both the current work and our prior study (Dearworth et al. 2009).

Curve fitting by a log-normal distribution identified maximal constriction evoked by a train frequency centered at 126 Hz with a band-width extending from 10 Hz to 1.5 KHz. This is consistent with observations in other vertebrates. For example, 15 Hz with 5 second train durations have been used to evoke contractions in the pig iris (Scheappi and Koella 1964), 30 through 150 Hz have been used to reliably evoke iris constrictions in pigeons (Pilar and Vaughan 1969a,b), and trains of 100 Hz composed of 1 ms pulse widths produce sustained pupil constrictions in primates (Jampel 1962).

It is not understood then why the PLR in the intact turtle, when compared to bird and mammal, exhibits such slow dynamics relative to the responses obtained by stimulating the relevant efferent pathways in vitro (Dearworth et al. 2009). Since signaling parameters, current thresholds and frequencies, conveyed by efferent pathways to the iris in turtle are similar to that observed in birds and mammals, the sluggishness of the reflex in vivo in turtle must be due to photoreceptive mechanisms or some other central processing done in the brain converging onto efferent pathways to the iris. One possible candidate is melanopsin, a photopigment found in the retina of mammals, and shown to be involved in controlling slow sustained pupillary responses to light (Lucas et al. 2001). Preliminary results from our laboratory have suggested (Lanzone and Dearworth 2007; Boyd and Dearworth 2008) and more recently confirmed (Dearworth et al. 2010) the presence of a 1,004 base pair partial mRNA sequence (GenBank HM197714) in the turtle retina, which aligns with melanopsins in other non-mammalian vertebrates including chicken and frog (Bellingham et al. 2006). Melanopsin, therefore, in turtle could be responsible for its inherently slow PLR (Dearworth et al., 2009). However, then, why is it that the turtle pupil retains the capacity for rapid dynamics in the in vitro preparation? For better understanding, it would be helpful to know how efferent signals involved with generating pupil constriction are modulated at the ciliary ganglion. In future studies, we plan to stimulate the ciliary nerve proximal to and distal to the ciliary ganglion to examine how signals are altered by this circuitry.

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**LITERATURE CITED**


