Retinal crosstalk in the mammalian visual system
Xiaolan Tang¹, Radouil Tzekov²,³, and Christopher L. Passaglia¹,²

¹Department of Chemical & Biomedical Engineering, University of South Florida, Tampa, FL 33620
²Department of Ophthalmology, University of South Florida, Tampa, FL 33612
³The Roskamp Institute, Sarasota, FL 34243

Running Head: Retinal crosstalk in mammals

Correspondence: Dr. Christopher L. Passaglia, Department of Chemical and Biomedical Engineering, University of South Florida, 4202 E Fowler Ave, Tampa, FL 33620. phone: 813-974-7140, email: passaglia@usf.edu

Keywords: centrifugal fibers, retino-retinal projection, retinopetal fibers, electroretinogram
ABSTRACT (250 words max; actual count: 250)

The existence and functional relevance of efferent optic nerve fibers in mammals has long been debated. While anatomical evidence for cortico-retinal and retino-retinal projections is substantial, physiological evidence is lacking as efferent fibers are few in number and severed in studies of excised retinal tissue. Here we show that interocular connections contribute to retinal bioelectrical activity in adult mammals. Full-field flash electroretinograms (ERGs) were recorded from one or both eyes of Brown-Norway rats under dark-adapted (n=16) and light-adapted (n=11) conditions. Flashes were confined to each eye by an opaque tube that blocked stray light. Monocular flashes evoked a small (5-15μV) signal in the non-illuminated eye, which was named “crossed ERG” (xERG). The xERG began under dark-adapted conditions with a positive (xP1) wave that peaked at 70-90ms and ended with slower negative (xN1) and positive (xP2) waves from 200-400ms. xN1 was absent under light-adapted conditions. Injection of tetrodotoxin in either eye (n=15) eliminated the xERG. Intraocular pressure elevation of the illuminated eye (n=6) had the same effect. The treatments also altered the ERG b-wave in both eyes, and the alterations correlated with xERG disappearance. Optic nerve stimulation (n=3) elicited a biphasic compound action potential in the non-stimulated nerve with 10-13ms latency, implying the xERG comes from slow-conducting (W-type) fibers. Monocular dye application (n=7) confirmed the presence of retino-retinal ganglion cells in adult rats. We conclude that mammalian eyes communicate directly with each other via a handful of optic nerve fibers. The crosstalk alters retinal activity in rats, and perhaps other animals.
INTRODUCTION

The standard view of the visual system stipulates that the axons of retinal ganglion cells exit the eyes to form the optic nerves, with nasal fibers projecting along the contralateral optic tract and temporal fibers along the ipsilateral optic tract to the lateral geniculate nucleus and other brain areas. This view applies primarily to the adult visual system. A diversity of connections exists during embryogenesis and early development, which gets pruned to form the mature pathway (Innocenti 1981; McLoon and Lund 1982; Nakamura and O'Leary 1989). For example, it has been reported that a cluster of retinal ganglion cells (ca. 5-200) sends axons to the opposite eye in frogs (Bohn and Stelzner, 1979, 1981; Toth and Straznicky 1989; Tennant et al. 1993), rats (Bunt and Lund 1981; Bunt et al. 1983; Sefton and Lam 1984; Gastinger et al. 2006; Avellaneda-Chevrier et al. 2015; Nadal-Nicolas et al. 2015), and rabbits (Muller and Hollander 1988). The number of retino-retinal axons peaks at birth and dwindles into adulthood so most studies have not ascribed any particular function to the misrouted cells. Those that remain are presumed to either be in the process of degeneration (Bohn and Stelzner 1979) or to have branches that synapse with proper targets elsewhere in the visual system (Tennant et al. 1993; Nadal-Nicolas et al. 2015).

A role of retino-retinal connections in vision cannot, however, be excluded solely based on low axon counts in adulthood. Beginning with Cajal’s work over a century ago, anatomical studies have shown that the optic nerves of adult mammals, including human (Honrubia and Elliott 1968), contain sparse populations of efferent fibers that originate from multiple locations, including the brainstem and hypothalamus (Terubayashi et al. 1983; Labandeira-Garcia et al. 1990; Gastinger et al. 2006; Reperant et al. 2006), in addition to the contralateral eye. Some may even come from auditory, olfactory, and somatosensory centers (Spinelli and Weingarten, 1966; Nikitopoulou-Maratou et al. 1980; Stell et al. 1984; Walker and Stell 1986; Francis et al. 2013). The efferent fibers branch widely in the eye, covering over a quarter of the retinal surface (Gastinger et al. 2006). Their branches terminate in the inner and
outer plexiform layers, inner nuclear layer, and ganglion cell layer depending on fiber type (Honrubia and Elliott 1970; Usai et al. 1991; Schutte 1995; Gastinger et al. 2001). Hence, there may be few retinal efferents in adult mammals, but activation of just one could have broad influence on visual information processing.

Despite the anatomical evidence for efferent optic nerve fibers, centrifugal control of retinal function in mammals has long been a controversial subject (Brindley and Hamasaki 1966; Schnyder and Kunzle 1984). Fueling the controversy is a lack of convincing physiological evidence for efferent effects on retinal activity. Early research on the electroretinogram (ERG) noted that illumination of one eye evoked a small ERG-like signal in the other eye of cats (Auerbach and Feinsod 1973) and that the amplitude of the ERG b-wave was reduced in humans if both eyes are illuminated (Motokawa et al. 1956; Hellner 1964). However, interocular effects were subsequently attributed to methodological artifacts, including light scatter into the non-illuminated eye, consensual pupillary or blink reflexes, and volume conduction of neural signals from the illuminated eye to the contralateral eye electrode (Horsten et al. 1961; Nagaya et al. 1962; Johnson and Massof 1982; Peachey et al. 1983; Seiple and Siegel 1983; Chou and Porciatti 2012). Little-to-no research has since been reported on retino-retinal interactions in mammals. There has been some support for cortico-retinal interactions. Electrical stimulation of the optic tract in rabbits and dorsal raphe nucleus in rats was shown to induce antidromic spikes that invade the retina (Dodt 1956) and to alter the ERG b-wave and oscillatory potentials (OPs) (Lorincz et al. 2008). ERG changes were also observed after severing or pharmacologically blocking the optic nerve of rabbits and cats, though not always of the same form [decrease in a-wave, increase in b-wave, and no effect on b-wave but increase in OPs] (Jacobson and Suzuki 1962; van Hasselt 1969; Auerbach and Feinsod 1973; Molotchnikoff et al. 1989) and not by everyone (Brindley and Hamasaki 1962). Efferent optic nerve fibers were implicated as well by the centrifugal effect of cortical cryoinactivation (Molotchnikoff and
Tremblay 1983, 1986) on retinal ganglion cell spike trains and slow-wave and REM sleep (Galambos et al., 1994, 2001) on the amplitude of light-evoked retinal and optic nerve signals in rats.

We investigated interocular communication by recording the bioelectrical activity of both eyes while visually stimulating one eye of adult rats. We describe a new light-evoked signal that can be recorded in the eye that is not illuminated. We present multiple lines of evidence that this signal is neural in origin and comes from the illuminated eye. We show that the crosstalk is likely mediated by a small group of ganglion cells that send axons to the opposite eye and that the spike activity of these cells alters light responses in the recipient retina.

MATERIALS AND METHODS

Animal preparation

Brown-Norway rats (27 males, 6-8 months, 300-400 g, Harlan Laboratories Inc.) were housed under a 12hr-12hr light-dark cycle and fed a standard daily diet. On the day of experimentation the animal was anesthetized with an intraperitoneal injection of ketamine hydrochloride (75 mg/kg) and xylazine (7.5 mg/kg), supplemented as needed, and given an intramuscular injection of dexamethasone (3 mg/kg). Cannulas were surgically inserted into the trachea for mechanical ventilation, the femoral vein for intravenous drug delivery, and in some experiments the femoral artery for blood pressure measurement. The animal was then mounted in a stereotaxic apparatus in a light-tight booth. ECGs were recorded with needle electrodes, and body temperature was maintained with a rectal thermometer and thermal blanket. Corneas were fitted with clear contact lenses, pupils were dilated with a drop of 1% cyclopentolate hydrochloride, and eye movements were paralyzed with an intravenous injection of gallamine triethiodide (40 mg/kg). Anesthesia and paralysis was sustained for the remainder of the experiment via an intravenous infusion of ketamine (30 mg/kg/hr), xylazine (1.5 mg/kg/hr), gallamine (40 mg/kg/hr),
dextrose (600 mg/kg/hr), and 0.9% physiological saline. Data were collected while vital signs remained at acceptable levels (heart rate >225 beats/min, rectal temperature 37-38 °C, blood pressure >80 mmHg).

Animals were euthanized with Euthasol (50 mg/kg) given to effect at the end of experiments, which could last up to 12 hrs. All procedures were approved by the Institutional Animal Care and Use Committee of the University of South Florida in accordance with NIH guidelines.

Electrophysiological recordings

Figure 1 illustrates the experimental setup. ERGs were recorded from both eyes with a custom ring-shaped gold electrode placed on the corneal limbus. Platinum needle electrodes (Natus Neurology Inc., Warwick, RI) inserted in the temples and tail served as references and ground, respectively. Recorded signals were differentially amplified (2000x) and filtered (0.1-1000 Hz) by a multi-channel bioamplifier (Xcell-3x4, FHC Inc., Bowdoin, ME) and digitized at 1000 Hz. Light stimuli were produced by a green LED (Vishay TLCTG5800, Newark Electronics, Palatine, IL) with an 8° emittance angle positioned 1 cm in front of each cornea. One LED was encased in an opaque tube, the lumen of which covered the eye socket. The tube exit was lined externally with black tape to further block the escape of light. The LEDs had a peak wavelength of 520 nm and peak output of 2100 cd/m² measured with a calibrated photometer (UDT Instruments Inc., Baltimore, MD). Animals were dark adapted for 4 hours prior to data collection (Behn et al., 2003). Full-field scotopic ERGs were then recorded for a series of 200 brief (10ms) flashes (1.32 log cd·s/m²) delivered to one or both eyes. After scotopic recording, animals were exposed for 30 min to ambient room light (15 cd/m²) from a LED strip that circumscribed the booth ceiling, and full-field photopic ERGs were recorded from the uncovered light-adapted eye while the tube covered eye remained in darkness. All flash sequences had an interstimulus interval of 3 sec, which provided sufficient time for full recovery of ERG responses under photopic conditions and represented a tradeoff between data collection time and response recovery time under scotopic conditions. The animal and experiment were monitored from outside the booth with an infrared camera. Recordings were stable
over the duration of anesthesia and were terminated upon completion of data collection or deterioration of animal health.

Pharmacological injections

Baseline ERG data were collected for a series of 200 full-field flashes delivered separately to each eye of 15 animals in darkness or room light. One eye was then injected intravitreally with a 5-µL bolus of tetrodotoxin (TTX, Abcam Biochemicals, Toronto, ON), or physiological saline as a control. TTX was dissolved in saline to produce an estimated concentration of 5 µM based on a rat vitreous volume of 40 µL (Hughes, 1979). Solutions were injected 2 mm behind the limbus with a 10-µL microsyringe (Hamilton Company, Reno, NV) at a 45-degree angle to avoid contact with the lens. They were administered to dark-adapted animals under dim red illumination. After injection, conventional ERGs were recorded every 10 min for a series of 30 flashes delivered to both eyes. Once signal amplitude stabilized in the injected eye (~30 min), full-field ERGs were recorded for a series of 200 flashes delivered to one eye and then the other. Data were collected every 30 min for up to 5 hours, during which effects of TTX partially or completely washed out in some animals.

Intraocular pressure (IOP) manipulations

The anterior chamber of one eye of 6 animals was cannulated with a 33-gauge needle connected to a pressure transducer and a reservoir of physiological saline via a three-way stopcock and plastic tubing. The pressure transducer was calibrated before data collection with a mercury manometer. Animals were dark-adapted for 4 hours, after which baseline ERG data were collected for a series of 200 full-field flashes delivered separately to each eye at the resting IOP level. The IOP of the cannulated eye was then elevated in 10 mmHg steps to the mean arterial blood pressure by varying the height of the saline reservoir. After each IOP step, conventional ERGs were recorded every 10 min for a series of 30 flashes delivered to both eyes. Once signal amplitude stabilized in the cannulated eye (~30 min), full-field
ERGs were recorded for a series of 200 flashes delivered to one eye and then the other. The process was repeated as IOP was lowered in 10 mmHg steps back to the resting level.

**Compound action potential (CAP) recordings**

The optic nerves of 3 animals were transected behind the globe, both eyes were removed, and the orbits were filled with artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 3 mM KCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 1.2 mM NaH$_2$PO$_4$, 26 mM NaHCO$_3$, and 10 mM glucose (pH: 7.45). ACSF-filled suction electrodes were positioned in each orbit using micromanipulators, and optic nerve stumps were gently aspirated into the glass tip of the electrodes. The tips were shaped by heat to snugly accommodate the nerves and coiled with silver chloride wire that served as a reference electrode. One pair of electrode leads was connected to the bioamplifier and the other pair was connected to an electrical stimulator (S48, Grass Instruments, Warwick, RI). Bipolar current pulses (0.1 ms) of increasing amplitude were applied to one nerve until a compound action potential was recorded in the opposite nerve. Once CAP threshold was identified, a series of 100 current pulses were delivered at 1 Hz for one subthreshold and several suprathreshold strengths. Evoked signals were digitized at 1000 Hz to computer and processed offline by averaging with respect to the stimulus onset and response onset. The latter was specified by the time-of-crossing of the leading edge of the CAP above a criterion level.

**Retinal ganglion cell labeling**

One eye of 7 animals was injected with a 10μL bolus of fluorescent dye (70k MW, rhodamine dextran B, Life Technologies, Eugene, OR) in distilled water for the purpose of retrograde ganglion cell labelling. The dye was injected intravitreally with a 25-gauge needle and Hamilton microsyringe in rats that were temporarily anesthetized with isoflurane. After 5-7 days the animal was euthanized, and both eyes were enucleated. Retinas were whole mounted on slides, coverslipped, and immediately viewed through a rhodamine filter with a fluorescent microscope (EVOS, Life Technologies, Grand Island, NY).
The whole-mounted retinas of 3 animals were counter-stained in Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) prior to fluorescence imaging.

**Data analysis**

Light-evoked signals were quantified in terms of amplitude, duration, and time-to-peak. Amplitude was measured from the peak-to-trough, duration from the width at half-peak amplitude, and time-to-peak from the stimulus onset. Statistical analysis was performed with SigmaPlot software (San Jose, CA) using a two-sample $t$-test that assumed unequal variance for pairwise comparisons of raw measurements, one-sided $t$-test for ratio measurements, and one-way ANOVA for comparisons of multiple datasets. Bonferroni’s multiple-comparisons test was used to determine which ANOVA datasets were significantly different. Statistical significance was assessed at alpha level of 0.05.

**RESULTS**

**Anatomical confirmation of retino-retinal connections**

Existence of retinal ganglion cells that send axons to the opposite eye of adult rats was confirmed by injecting the dye into the vitreous chamber of one eye (n = 7). The dye was uptaken by numerous retinal gangion cells in the injected eye (Figure 2A, top), and after several days retrogradely labeled a small number (<10) of ganglion cells in the opposite retina of every animal (Figure 2A, bottom). The staining pattern was punctate and confined to the cell body, implying that the dye was encapsulated in vesicles carried by axonal transport from the injected eye. If intracellular diffusion were involved, the dye would be expected to label dendritic and axonal arbors as well. The soma diameter of labeled cells in the non-injected eye was between 8-12 µm, which falls on the low end for rat retinal ganglion cells (Danias et al. 2002). The retino-retinal ganglion cells tended to reside in nasal portion of the eye (Figure 2B). These
findings are consistent with prior descriptions of tracer-labelled retino-retinal ganglion cells in adult rats (Mullander and Hollander 1988; Avellaneda-Chevrier et al. 2015; Nadal-Nicholas et al. 2015).

Physiological demonstration of retino-retinal signal transmission

The functional status of retino-retinal efferent fibers in adult rats was assessed by excising both eyes of anesthetized animals and applying current pulses to one optic nerve stump while recording from the other nerve stump. No bioelectrical activity was observed at low current strengths, but higher strengths elicited a spike-like signal in the non-stimulated nerve (Figure 3A), which we named “crossed CAP” (xCAP). xCAP latency ranged from 10-13 ms relative to pulse onset across stimulus applications and animals (n = 3). Averaging responses to a series of suprathreshold current pulses yielded a multi-peaked waveform (Figure 3B), which would normally imply the spike discharges of multiple types of ganglion cells with different conduction velocities combined to produce the signal (Sefton and Swinburn 1964). However, this is probably not the case for the xCAP due to the variability in signal latency. Averaging responses aligned by their leading edge instead of current onset reveals that the waveform is biphasic in shape and scales in amplitude with current strength (Figure 3C), indicating that the xCAP reflects the summed activity of a single homogeneous population of efferent optic nerve fibers and that the number of active fibers and the synchrony of their discharges increased with stimulus intensity. The xCAP is not explained by volume conduction of afferent optic nerve activity to the recording electrode because the CAP has a complex multimodal waveform (Sefton and Swinburn 1964; Hale et al. 1979).

A novel light-evoked interocular signal of the eye

The full-field ERG evoked by a flash of light delivered to the dark-adapted rat eye has a stereotypic waveform, consisting of a brief negative a-wave followed by a larger positive b-wave (Figures 4A and 4B). Small OPs are often seen riding on the b-wave as well. If a single flash is presented to one eye, no
bioelectrical activity is apparent in the other eye. However, when responses to a large number (>100) of monocular flashes are averaged, noise in the records decreases and a small light-evoked signal becomes detectable even in the non-flashed eye (Figure 4C and 4D). We named this signal the “crossed ERG” (xERG) since it is evoked by illumination of the opposite eye. It is not a stray-light response as the flash was confined to the illuminated eye by an opaque tube (Figure 1). Moreover, the xERG was recorded under both scotopic and photopic lighting conditions. Full-field flashes to the dark-adapted eye evoked a multi-peaked xERG waveform when the non-flashed eye was exposed to room light (Figure 4E), and full-field flashes to a light-adapted eye evoked a xERG of similar shape and size in the dark-adapted non-flashed eye (Figure 4F). Any stray light that reached the non-flashed eye under photopic conditions would be orders of magnitude dimmer than the ambient illumination and much too small to evoke a light response.

Several control experiments were performed to further eliminate a stray-light explanation for the xERG. Figure 5A shows that no light-evoked activity is recorded in either eye if the flash is delivered through the opaque tube elsewhere on the head. Figure 5B shows that an opaque patch placed against the cornea and over the tube exit blocked not only the xERG of the non-flashed eye but also the ERG of the flashed eye, confirming that no light escaped the tube. Figure 5C shows that cutting the optic nerve of the flashed eye also eliminated the xERG in the non-flashed eye, demonstrating that the signal was not evoked by light passing through the skull to the opposite retina. Taken together, the data imply that the xERG derives from interocular signal transfer through the optic nerve, presumably via the retino-retinal projections that underlie the xCAP.

Characterization of the xERG waveform

The xERG has at least three components under dark-adapted conditions that were named xP1, xN1, and xP2 (Figure 6). The signal begins with a positive wave (xP1) followed by a longer negative wave
(xN1) and ends with a broad positive wave (xP2). Mean amplitude, time-to-peak, and duration of the xERG components and the dark-adapted ERG a- and b-waves were measured and compared. xP1, xN1, and xP2 amplitudes (6 ± 1, 11 ± 4, and 11 ± 5 μV, respectively; n = 16) were all measurably larger than the noise level after response averaging (Figure 4; RMS = 2.1 ± 0.2; n = 16) and much smaller than the a-wave (80 ± 12 μV, F(3,60) = 404, p < 0.001) and b-wave (197 ± 45 μV, F(3,60) = 261, p < 0.001).

xP1, xN1, and xP2 peak times (72 ± 9, 152 ± 25, and 288 ± 39 ms) were all much later than the a-wave (20 ± 2 ms, F(3,60) = 374, p < 0.001). xP1 coincided in time with the b-wave (83 ± 11 ms, p > 0.999) while xN1 and xP2 peaked much later (F(3,60) = 258, p < 0.001). xP1, xN1, and xP2 durations (44 ± 12, 99 ± 13, and 150 ± 33 ms) were all much longer than the a-wave (13 ± 2 ms, F(3,60) = 154, p < 0.001). xP1 and xN1 were also longer in duration than the b-wave (138 ± 19 ms, F(3,60) = 79, p < 0.001), while xP2 was not (p = 0.308).

The xERG has a slightly different shape under light-adapted conditions (Figure 7). The signal begins and ends with positive components but the negative component is not apparent in the population-average waveform. The mean amplitude, time-to-peak, and duration of the xERG components and light-adapted ERG b-wave were measured and compared. xP1 and xP2 amplitudes (7 ± 3 and 8 ± 6 μV, respectively; n = 11) were both larger than the 2-μV noise level after response averaging and much smaller than the b-wave (128 ± 26 μV, F(3,36) = 242, p < 0.001). xP1 and xP2 peak times (87 ± 7 and 325 ± 28 ms) were both later than the b-wave (63 ± 4 ms, F(3,20) = 546, p < 0.001). xP1 duration (52 ± 10 ms) was not significantly different from b-wave duration (66 ± 11 ms, p = 0.308), while xP2 duration was much longer (169 ± 21 ms, F(3,20) = 110, p < 0.001).

Since monocular illumination evokes bioelectrical activity in both eyes, binocular stimulation should induce both an ERG and xERG in each eye. The signal interaction was examined by delivering full-field flashes to one or both eyes simultaneously. Monocular and binocular ERGs looked virtually identical but subtraction of the two records yielded a difference signal (Figure 8). The signal resembled the xERG
of the non-illuminated eye for monocular flashes to a first approximation. The goodness-of-fit between
the two records ($R^2$) averaged $0.52 \pm 0.16$ across animals ($n = 6$). This is significantly greater than the
level expected by chance ($R^2 = 0.03 \pm 0.02$) given by the goodness-of-fit between the difference signal
and average noise records (Figure 5). The similarity of waveforms suggests that the xERG contributes a
very small but measurable component to the flash ERG of rats.

Effect of TTX on the flash ERG and xERG

To confirm that the xERG is neural in origin, a bolus of TTX was injected in one eye of anesthetized
rats. TTX is a potent blocker of voltage-gated sodium channels and thereby arrests action potential
transmission in and out of the retina. In every animal ($n = 15$) TTX completely eliminated the xERG in
the non-flashed eye regardless of whether the flash was delivered to the injected eye (Figure 9A) or non-
injected eye (Figure 9B, +30min). The xERG returned after a few hours to the injected eye (Figure 9B,
+90min) of some animals ($n = 3$) as TTX was slowly cleared from the eye, but not to the non-injected
eye at applied concentrations. Intravitreal injection of an equivalent bolus of physiological saline had
little-to-no effect on bioelectrical activity in either eye (Figures 9C and 9D). Effects of TTX were not
limited to the xERG. The flash ERG of the injected eye was altered under dark-adapted and light-adapted
conditions (Figure 10A-C, left). The b-wave was particularly affected. On average, the scotopic
amplitude was reduced by $60 \pm 8\% (p < 0.001; n = 6)$, delayed by $20 \pm 17$ ms ($p = 0.047$), and prolonged
by $45 \pm 23$ ms ($p = 0.020$), while the photopic amplitude was reduced by $40 \pm 15\% (p < 0.001; n = 5)$
with time-to-peak and duration remaining unchanged (Figure 11). Similar effects of TTX on the flash
ERG have been reported previously (Bui and Fortune 2004; Mojumder et al. 2008). More interestingly,
the flash ERG of the non-injected eye was also altered (Figure 10A-C, right). The changes were specific
to the contralateral b-wave, which was prolonged in duration by $40 \pm 8$ ms ($p < 0.001; n = 6$) under
dark-adapted conditions and reduced in amplitude by $33 \pm 8\% (p = 0.011; n = 5)$ under light-adapted
conditions (Figure 11). The ERG returned to normal after several hours in the non-injected eye of some animals (n = 2, Figure 10B) but not the injected eye. Effects of TTX on the contralateral ERG suggest that interocular signals communicated by efferent optic nerve fibers modify the response dynamics of retinal neurons.

Effect of elevated IOP on the ERG and xERG

It is known that ganglion cells cease firing at high IOP levels due to onset of retinal ischemia (Grehn et al. 1984). To further confirm that the xERG derives from efferent optic nerve activity, the anterior chamber of one eye of anesthetized rats was cannulated with a needle connected to a variable-height reservoir of saline. The dark-adapted ERG b-wave progressively decreased in amplitude and eventually disappeared (Figure 12A, left) as IOP was increased from baseline to near the arterial blood pressure (22 and 80 mmHg, respectively, for this animal). The a-wave was relatively insensitive to IOP elevation, in agreement with prior findings in rat (Bui et al. 2013). Light flashes evoked a xERG in the opposite eye (Figure 12A, right) that also disappeared at high IOP in every animal tested (n = 3). The disappearance of the xERG in the non-cannulated eye coincided with the loss of the ERG b-wave in the cannulated eye. Both signals re-appeared when IOP was lowered back to baseline. The pressure effect was unidirectional; in that, a xERG was still recorded in the cannulated eye at IOP levels that eliminated the ERG b-wave in that eye (Figure 12B). This is more evidence against a stray light explanation of the xERG. The data imply that acute ocular hypertension eliminated the xERG in the non-cannulated eye because it blocked inner retinal signaling and afferent optic nerve spiking in the cannulated eye, while the non-cannulated eye was not subjected to the pressure block so efferent optic nerve signals from still produced a xERG in the cannulated eye. Moreover, IOP elevation altered the contralateral flash ERG (Figure 12C). Similar to the effect of TTX, the dark-adapted b-wave of the non-cannulated eye lengthened in duration by 45 ± 15 ms (p = 0.035) at high IOP and returned to normal when baseline IOP was restored (n = 3 of 4).
DISCUSSION

The existence of direct retino-retinal projections in mammals has long been known but evidence that the projections are retained into adulthood has been scant. Using modern immunohistochemical methods two recent studies demonstrated that a small group (5-25) of retinal ganglion cells send axon collaterals to the contralateral retina in adult mice and rats (Avellaneda-Chevrier et al. 2015; Nadal-Nicolas et al. 2015). Since the number of retino-retinal ganglion cells was reported to vary between animals and rat strains (Nadal-Nicolas et al. 2015), we investigated our cohort of animals and confirmed that retrograde tracers label ganglion cell bodies (<10) in the contralateral retina of Brown-Norway rats at age P180 and above, which is late adulthood and beyond the age span of previous studies. Our results give a lower bound on retino-retinal ganglion cell counts in old-age rats because intravitreal tracer injection does not guarantee that all efferent fiber terminals sequester and transport tracer at sufficient level for visual detection of efferent cell bodies in the opposite eye. The existence of these cells means that not all retinopetal fibers come from the brain, as commonly implied (Gastinger et al. 2006, Reperant et al. 2006). Information about axonal and dendritic tree morphology of retino-retinal ganglion cells is sorely needed though to move beyond mere soma sightings and begin dissecting the connectivity pattern of interocular circuits.

The functional integrity of retino-retinal projections was demonstrated here for the first time in mammals. We electrically stimulated one optic nerve of adult rats and observed in the other non-stimulated optic nerve a biphasic compound action potential (xCAP). The xCAP must come from efferent optic nerve fibers because the CAP generated by afferent optic nerve fibers is known to have a different waveform. We investigated whether retino-retinal fibers can communicate visual information by recording the bioelectrical activity evoked in one eye by flashes delivered to the other eye. Since retinal efferents are few in number, many records were averaged in order to detect a putative intraocular
signal buried in the noise. Our results show that monocular light flashes elicit a small multi-peaked
signal in the non-flashed eye of adult rats (xERG). The novel signal has presumably escaped notice
because it is several-fold smaller than the conventional flash ERG. Our results also show that
experimental treatments which eliminated the xERG in one eye altered the ERG of the non-treated eye.
The binocular effects suggest that retino-retinal projections serve a presently unknown purpose and are
not just an anomaly of visual system development.

There are two neural routes by which light-evoked signals in one eye could be communicated to the
other eye of mammals: directly via retinal-retinal efferent fibers and indirectly via efferent fibers from
cortical or subcortical neurons that receive visual input. There could be a non-neural route if the eyes are
connected by an interorbital communicating artery (Ruskell 1962), although hormonal transmission
would seem much too slow to explain xERG response times. Based on rat optic nerve length and optic
chiasm width (~23 mm eye-to-eye), the xCAP latency translates to a conduction velocity of 2-3 m/s,
which is below the range of X- and Y-type ganglion cells but within the range of W-type cells (Hale et
al. 1979). The anatomical and physiological properties of W cells are diverse and not known for every
subtype, so it is plausible that a subpopulation could project directly to the opposite eye. Alternatively,
the faster-conducting X- and Y-type fibers could perhaps relay afferent signals to cortical or subcortical
circuits, which then send back efferent signals in time to produce a xCAP in the opposite eye. The extra
synaptic processing might explain the observed dispersion in xCAP timing. However, measurements of
cortico-retinal transmission time do not appear to support such an explanation. Travel from the raphe
nucleus to the optic chiasm of rats was clocked around 9 ms (Lorincz et al. 2008), which is comparable
to xCAP latency without accounting for signal transmission from the eye to the brainstem, synaptic
processing by target neurons, and transmission down the contralateral optic nerve. Hence, the simplest
interpretation of our results is that retino-retinal (W-type) optic nerve fibers produce the xCAP and, by
inference, the early components of the xERG.
xERG – interocular signal or methodological artifact?

The xERG was investigated from multiple independent directions to guard against possible sources of methodological error. The most often cited and suspected source of possible error is stray light. Our control experiments demonstrate that this is not the case in our experimental setup as: i) an opaque tube confined flashes to the illuminated eye, ii) the signal disappears when the eyes are covered or when the optic nerve is transected, and iii) the signal disappears after injecting TTX or increasing IOP in the non-flashed eye. Similarly, a visuomotor reflex can be excluded as a potential source as: i) the animal was continuously infused with paralytics, ii) pupil movements were pharmacologically blocked, iii) the xP1 time-to-peak is much shorter than the latency of pupil contractions and dilations (290-490 ms) in rats (Clarke 2007), and iv) the signal is affected by IOP manipulations. Finally, volume conduction of bioelectrical activity from the flashed eye to the contralateral eye electrode is unlikely as: i) the waveform is different from the measured ERG, ii) largely unaffected by light adaptation, and iii) disappears after sectioning the optic nerve or injecting TTX in the non-flashed eye. The latter observation also implies that it is not a visual signal passively conducted from cortical or subcortical circuits, although the waveform looks strikingly similar to field potentials that were recorded in the rat optic chiasm following light flashes (Galambos et al. 2000, 2005) and brainstem stimulation (Lorincz et al. 2008). Together, our results collectively point to the conclusion that the xERG is an interocular signal communicated from one retina to the other via the optic nerve.

One methodological limitation of the present work should be noted. The opaque tube used to prevent light leakage restricted LED illumination to the central retina of the stimulated eye, which would lower ERG amplitudes relative to studies that employ true Ganzfeld illumination. This preferential stimulation of the central retina might explain the stronger than usual effect of TTX on a- and b-wave amplitudes for dark-adapted conditions. Under such a hypothesis some LED light is scattered into peripheral retina and
acts as background stimulation similar to ambient illumination, for which TTX application is reported to
decrease ERG amplitude (Mojumder et al. 2008). Ganzfeld stimulation of the ipsilateral eye may also
alter the amplitude, timing, and waveform of the xERG, a possibility that would be investigated in future
studies.

**Origins of ERG and xERG waveforms**

Our results do not directly address the origin of the different peaks of the xERG waveform. However,
it is presumed that the xP1 component, and perhaps xN1 and xP2 components too, derives from efferent
spike trains coming from retino-retinal ganglion cells in the illuminated eye. The time-to-peak of optic
tract responses to a bright spot flashed over ganglion cell receptive field centers ranges from 50-150 ms
depending on cell type and stimulus condition (Heine and Passaglia 2011), which fits with xP1 time-to-
peak. The xP1 and xN1 amplitude and waveform are also comparable to the scotopic threshold response
(STR), which is a small positive-then-negative ERG signal evoked by very dim flashes in darkness that
is attributed to ganglion cell spike activity in rats (Bui and Fortune 2004; Naarendorp et al. 2001). Since
efferent fibers are few in number and branch widely across the retina, the xERG probably reflects spike-
evoked currents in retinal neurons of the non-illuminated eye more than efferent spikes themselves. The
contralateral effects of TTX and IOP on the ERG b-wave suggests that bipolar cells may be one efferent
target. Alternatively, the latter components of the xERG could reflect, in whole or part, efferent signals
from cortical and subcortical circuits. Determining the precise origin of the various xERG peaks requires
surgical dissection of visual pathways and pharmacological blockade of specific retinal circuits, which
were beyond the scope of this study.

The origin of the conventional full-field flash ERG waveforms is well defined and characterized
(Weymouth and Vingrys 2008; Frishman and Wang 2011). The leading edge of the a-wave reflects
photoreceptor activity, the ascending shoulder of the b-wave derives mainly from on-type bipolar cells,
and the OPs are thought to reflect amacrine-ganglion cell interactions in the inner retina. We observed that TTX significantly reduced scotopic a- and b-wave amplitude and increased b-wave time-to-peak and duration. TTX also reduced photopic b-wave amplitude. The amplitude reductions could be due to the injected volume or a shift in adaptation state caused by the injection procedure, but control injections of saline argue against this. Prior studies found that TTX had negligible effect on the dark-adapted a-wave in rats under fully dark-adapted conditions (Bui and Fortune 2004; Mojumder et al. 2008). This study employed different stimulation parameters (e.g., non-Ganzfeld, non-broadband), which may have also contributed to observed a-wave changes. Reported effects of TTX on the b-wave have been mixed, but a similar reduction, delay, and prolongation were seen in rats (Bui and Fortune 2004; Mojumder et al. 2008). The b-wave changes can be attributed to the presence of voltage-gated sodium channels in cone bipolar cells (Pan and Hu 2000; Mojumder et al. 2008) since the flash intensity used in this study likely stimulated the cone system. In addition, we observed that TTX significantly increased the scotopic b-wave duration and decreased the photopic b-wave amplitude of the non-injected eye. The contralateral ERG changes could perhaps signify that rats have an interorbital artery. However, the scotopic b-wave amplitude and time-to-peak were not altered and b-wave duration was similarly prolonged even though any TTX transported to the opposite eye would be much lower in concentration. Furthermore, IOP elevation not only eliminated the ERG b-wave in the cannulated eye, as described previously (Bui et al. 2005, 2013; Kong et al. 2009), but also eliminated the xERG and altered the ERG in the non-cannulated eye. The contralateral ERG changes resembled those of TTX, in that the b-wave was longer in duration under scotopic conditions and smaller in amplitude under photopic conditions. Interestingly, a decrease in photopic b-wave amplitude was noted at high IOP levels in the non-cannulated eye of rats in a prior study (Tsai et al. 2014). We conclude that TTX injection and IOP elevation blocked optic nerve output from the illuminated eye and the resultant loss of interocular communication altered retinal processing in the other eye.
The function of efferent optic nerve fibers is poorly understood in mammals. What is known about their physiological properties comes primarily from research on the isthmo-optic nucleus of birds, which sends a sizeable projection to the eyes (~10,000 nerve fibers) that is well studied as a result. It has been suggested that efferent feedback mediates a dynamic adaptation process that adjusts retinal sensitivity in conjunction with neural command signals for eye and head movements (Miles 1972). Other suggestions are that efferent feedback serves to stabilize the retinal image (Woodson et al. 1995), highlight retinal activity of visual interest in one or both eyes (Uchiyama 1999), or initiate tracking circuits that follow shadows cast by objects in the scene (Wilson and Lindstrom 2011). These functions all require a fairly coordinated feedback system that appears unique to birds. Based on immunohistochemical staining and tract tracing, it has been suggested that efferent feedback in mammals may be part of a circadian arousal system (Gastinger et al. 2006). This is because neurons that project to the retina reside in the areas of the hypothalamus and brainstem that vary in activity level according to the sleep/wake cycle and efferent nerve terminals in the retina stain for histamine and serotonin, which are neurotransmitters that modulate the firing rates of retinal ganglion cells. Perhaps retino-retinal efferent fibers act in concert with cortico-retinal efferent fibers while the animal is awake to tune the visual system for ambient lighting conditions, providing information about environmental illumination that allows retinal neurons to anticipate changes in adaptation state as the eyes and head move.

Interocular communication might play a role in human vision as well. There are some physiological processes and phenomena that still await detailed understanding for which retino-retinal fibers can offer a viable mechanism. These phenomena include binocular interactions in dark-adaptation (Makous et al. 1975), interocular transfer of dark- and light-adaptation (Auerbach and Peachey 1984, Auerbach et al. 1992), interocular transfer of motion aftereffects (Wade et al. 1993), interocular suppression and contrast...
gain control (Denny et al. 1991, Eysteinsson et al. 1993, Baker and Meese 2007, Baker et al. 2007a,b),
binocular capture (Raghunandan 2011), and bilateral ERG changes following monocular IOP elevation
(Lovasik et al. 2005) and eye manipulation (Francis et al. 2013). More research on retinal efferents in a
variety of mammalian species is clearly needed, and the discovery of the xERG provides a much-needed
tool for investigating this sparse subpopulation of optic nerve fibers that is otherwise difficult to access
electrophysiologically.

ACKNOWLEDGEMENTS

We thank Geoffrey Arden, John Dowling, and Neal Peachey for helpful comments. Kayla Ficarrotta and
Sarah Davis provided assistance with data processing.

GRANTS

The work partially supported by NIH grant R21 EY023376 and by a Thomas R. Lee Award from the
Bright Focus Foundation.
REFERENCES

Auerbach E, Beller AJ, Henkes HE, Goldhaber G. Electric potential of retina and cortex of cats evoked

Auerbach E, Feinsod M. Centrifugal effects on the cat electroretinogram after section of one optic nerve.

Auerbach E, Peachey N. Intercocular transfer and dark adaptation to long-wave test lights. Vision Res

Auerbach E, Dörrenhaus A, Cavonius CR. Changes in sensitivity of the dark-adapted eye during

Avellaneda-Chevrier VK, Wang X, Hooper ML, Chauhan BC. The retino-retinal projection: Tracing

Baker DH, Meese TS. Binocular contrast interactions: dichoptic masking is not a single process. Vision

Baker DH, Meese, TS, Georgeson MA. Binocular interaction: Contrast matching and contrast
discrimination are predicted by the same model. Spatial Vision 20:397–413, 2007

Baker DH, Meese TS, Summers RJ. Psychophysical evidence for two routes to suppression before

Behn D, Doke A, Racine J, Casanova C, Chemtob S, Lachapelle P. Dark adaptation is faster in

Bohn RC, Stelzner DJ. Aberrant retino-retinal pathway during early stages of regeneration in adult Rana

Bohn RC, Stelzner DJ. The aberrant retino-retinal projection during optic nerve regeneration in the frog.

Brindley GS, Hamasaki DI. Evidence that the cat’s electroretinogram is not influenced by impulses

Brindley GS, Hamasaki DI. Histological evidence against the view that the cat's optic nerve contains

Bui BV, Fortune B. Ganglion cell contributions to the rat full-field electroretinogram. J Physiol 555:

Bui BV, Edmunds B, Cioffi GA, Fortune B. The gradient of retinal functional changes during acute

Bui BV, He Z, Vingrys AJ, Nguyen CTO, Wong VH, Fortune B. Using the electroretinogram to


Lovasik JV, Kergoat H, Gagnon M. Experimentally reduced perfusion of one eye impairs retinal...


Peachey NS, Sokol S, Moskowitz A. Recording the contralateral PERG: effect of different electrodes.


FIGURE LEGENDS

Figure 1. Electroretinography setup. (A) Ring electrodes were rested on the corneas and needle reference electrodes were inserted in the cheeks of an anesthetized paralyzed rat, while LEDs delivered a series of 10ms flashes (interflash interval: 3s) to one or both eyes. One of the LEDs was housed in an opaque tube which blocked stray light from entering that eye. Flashes to the other non-covered eye were presented in darkness or roomlight. (B) Diagram of the stimulus configuration in A. The star marks the flashed eye. The dark and bright borders respectively indicate the eye was dark or light adapted. Similar diagrams are provided in subsequent figures to illustrate the stimulus configuration.

Figure 2. Interocular connections in adult rats. (A) Fluorescence image of retinal ganglion cells labelled in the ipsilateral eye (top) and contralateral eye (bottom) with dye injected intravitreally in the ipsilateral eye. The dye was uptaken by thousands of cells in the ipsilateral retina and four cells in the contralateral retina. Cell nuclei were countstained blue with DAPI. (B) Topographical map of retino-retinal ganglion cell locations across animals (1-7). Central circle is the optic disc.

Figure 3. Electrical stimulation evokes signals that cross to opposite nerve. (A) Superposition of traces recorded in vivo from one optic nerve of a rat while a sequence of 50 current pulses (top: 1mA, middle: 2mA, bottom: 4mA) was applied to the other optic nerve. Larger current amplitudes evoked compound action potentials (triangle) in the non-stimulated nerve. Asterisk indicates the current pulse as recorded by the bioamplification system. (B) Signal waveform when triggered averaged on the current pulses. (C) Signal waveform when triggered averaged on the leading edge of the signal itself.

Figure 4. A novel light-evoked signal of the eye. (A) Dark-adapted ERG traces of an anesthetized rat evoked by a single flash (10 ms) delivered to one eye while recording from the flashed (left) and non-
flashed (right) eyes. (B) Dark-adapted ERG traces for a single flash delivered to the opposite eye. (C) Average dark-adapted ERG traces from the flashed (left) and non-flashed (right) eye of the same animal for 200 stimuli. A small “crossed ERG” (xERG) signal is seen in the non-flashed eye. (D) Average dark-adapted ERG traces for a flash sequence delivered to the opposite eye. A xERG signal can be recorded in either eye. (E) Average dark-adapted ERG trace of the flashed eye (left) of another rat, and the xERG trace of the non-flashed eye (right) in ambient room light. (F) Average light-adapted ERG trace of the flashed eye (right) of the animal, and the xERG trace of the non-flashed eye (right) in darkness.

Figure 5. Control experiments. (A) Average bioelectrical activity recorded from each eye for 200 flashes delivered through an opaque tube to the forehead of a rat. (B) Average dark-adapted ERG of the flashed eye (left) and xERG of the non-flashed eye (right) of a rat before (top traces) and after (bottom traces) covering the flashed eye with an opaque patch. (C) Average light-adapted ERG of the flashed eye (left) and xERG of the non-flashed eye (right) of another rat before (top traces) and after (bottom traces) severing the optic nerve behind the globe of the flashed eye.

Figure 6. xERG properties under dark-adapted conditions. (A) Scotopic xERG population-average (n = 16) waveform has three components: xP1, xN1, and xP2. Dashed lines are ±1 SD confidence levels. (B) Example dark-adapted ERG (top) and dark-adapted xERG (bottom) traces from a representative animal. Arrows illustrate how the amplitude, time-to-peak, and duration were measured for the xP1, xN1 and xP2 components of the xERG and the a-wave and b-wave of the ERG (amplitude: peak-to-trough, time-to-peak: latency from stimulus onset, duration: width at half maximum amplitude). Arrowheads mark flash onset. (C) Boxplots showing the mean, lower and upper quartiles, and minimum and maximum amplitude (left), time-to-peak (middle), and duration (right) of the ERG and xERG components.
Figure 7. xERG properties under light-adapted conditions. (A) Photopic xERG population-average (n = 11) waveform has two components: xp1 and xp2. Dashed lines are ±1 SD confidence levels. (B) Example light-adapted ERG (top) and light-adapted xERG (bottom) traces from a representative animal. Arrows illustrate how the amplitude, time-to-peak, and duration were measured for the xp1 and xp2 components of the xERG and the b-wave of the ERG (amplitude: peak-to-trough, time-to-peak: latency from stimulus onset, duration: width at half maximum amplitude). Arrowheads mark flash onset. (C) Boxplots showing the mean, lower and upper quartiles, and minimum and maximum amplitude (left), time-to-peak (middle), and duration (right) of the ERG and xERG components.

Figure 8. xERG contribution to the full-field flash ERG. (A) ERG recorded from one eye for full-field flashes to both eyes of a rat. The recorded eye was exposed to room light and the other eye to darkness. (B) ERG recorded from the same eye as panel A for flashes delivered only to that eye. (C) Comparison of the xERG (thin trace) recorded from the same eye as panel A for flashes delivered to the other eye and the signal produced by subtracting the monocular and binocular ERGs in panels A and B (thick trace). The records were normalized in amplitude to facilitate comparison of waveforms.

Figure 9. TTX abolishes the xERG. (A) Full-field flash ERG (left) and xERG (right) recorded before (top), 1.5 hrs after (middle), and 4 hrs after (bottom) intravitreal injection of TTX in the flashed eye of a rat. (B) Full-field flash ERG (right) and xERG (left) recorded before (top), 0.5 hrs after (middle), and 1.5 hrs after (bottom) injection of TTX in the non-flashed eye of another rat. (C) Flash ERG (left) and xERG (right) recorded before (top) and 0.5 hrs after (bottom) equivalent intravitreal injection of saline in the flashed eye. (D) Flash ERG (left) and xERG (right) recorded before (top) and 0.5 hrs after (bottom) equivalent intravitreal injection of saline in the non-flashed eye.
Figure 10. TTX alters the ERG in both eyes. (A) Dark-adapted ERGs recorded for full-field flashes to both eyes of a rat before (thin trace) and 2 hrs after (thick trace) injecting TTX in one eye. (B) Flash ERGs recorded from both eyes of another rat before (thin trace), 1hr after (thicker trace), and 2hrs after (thickest trace) injecting TTX in one eye. The injected eye was light adapted, while the non-injected eye was dark-adapted. (C) Flash ERGs recorded from both eyes of another rat before (thin trace) and 1hr after (thick trace) injecting TTX in one eye. The injected eye was dark adapted, while the non-injected eye was light-adapted.

Figure 11. Summary of TTX effects on the ipsilateral and contralateral ERG. (A) Normalized amplitude, time-to-peak, and duration of the dark-adapted (DA) a-wave of saline-injected, TTX-injected, and TTX non-injected eyes. Maximum post-injection values were normalized by pre-injection values. (B) Normalized amplitude, time-to-peak, and duration of the dark-adapted b-wave of injected and non-injected eyes. (C) Normalized amplitude, time-to-peak, and duration of the light-adapted (LA) b-wave of injected and non-injected eyes. Bars give group mean ± standard deviation. Asterisks indicate datasets for which pre- and post-injection measurements were statistically different.

Figure 12. Acute IOP elevation alters the ERG and xERG. (A) Full-field flash ERGs (left) and xERGs (right) recorded simultaneously from a rat in darkness as the IOP of the flashed eye was raised from the resting level of 22 mm Hg (top trace) to near mean arterial pressure of 80 mm Hg and lowered back to the resting level (bottom trace). IOP level is indicated next to each trace. (B) Full-field dark-adapted ERG (right) and xERG (left) recorded from the same rat for flashes delivered to the non-cannulated eye with the cannulated eye at an IOP of 72 mmHg. A xERG was recorded at all IOP levels, including one shown for which the flash ERG of that eye lacked a b-wave in A. (C) Dark-adapted flash ERGs recorded
from both eyes of another rat with IOP at the resting level of 15 mmHg (thin trace), increased to 70 mmHg (thicker trace), and returned back to the resting level (thickest trace).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

ERG and xERG components under dark adapted conditions
Figure 7
Figure 8
Figure 9
Figure 10
Figure 11
Figure 12