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Toluene Inhalation Exposure for 13 Weeks Causes Persistent Changes in Electroretinograms of Long-Evans Rats

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Abstract

Studies of humans chronically exposed to volatile organic solvents have reported impaired visual functions, including low contrast sensitivity and reduced color discrimination. These reports, however, lacked confirmation from controlled laboratory experiments. To address this question experimentally, we examined visual function by recording visual evoked potentials (VEP) and/or electroretinograms (ERG) from four sets of rats exposed repeatedly to toluene. In addition, eyes of the rats were examined with an ophthalmoscope and some of the retinal tissues were evaluated for rod and M-cone photoreceptor immunohistochemistry. The first study examined rats following exposure to 0, 10, 100 or 1000 ppm toluene by inhalation (6 hr/d, 5 d/wk) for 13 weeks. One week after the termination of exposure, the rats were implanted with chronically indwelling electrodes and the following week pattern-elicited VEPs were recorded. VEP amplitudes were not

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significantly changed by toluene exposure. Four to five weeks after completion of exposure, rats were dark-adapted overnight, anesthetized, and several sets of electroretinograms (ERG) were recorded. In dark-adapted ERGs recorded over a 5-log (cd-s/m^2) range of flash luminance, b-wave amplitudes were significantly reduced at high stimulus luminance values in rats previously exposed to 1000 ppm toluene. A second set of rats, exposed concurrently with the first set, was tested approximately one year after the termination of 13 weeks of exposure to toluene. Again, dark-adapted ERG b-wave amplitudes were reduced at high stimulus luminance values in rats previously exposed to 1000 ppm toluene. A third set of rats was exposed to the same concentrations of toluene for only 4 weeks, and a fourth set of rats exposed to 0 or 1000 ppm toluene for 4 weeks were tested approximately 1 year after the completion of exposure. No statistically significant reductions of ERG b-wave amplitude were observed in either set of rats exposed for 4 weeks. No significant changes were observed in ERG a-wave amplitude or latency, b-wave latency, UV- or green-flicker ERGs, or in photopic flash ERGs. There were no changes in the density of rod or M-cone photoreceptors. The ERG b-wave reflects the firing patterns of on-bipolar cells. The reductions of b-wave amplitude after 13 weeks of exposure and persisting for 1 year suggest that alterations may have occurred in the inner nuclear layer of the retina, where the bipolar cells reside, or the outer or inner plexiform layers where the bipolar cells make synaptic connections. These data provide experimental evidence that repeated exposure to toluene may lead to subtle persistent changes in visual function. The fact that toluene affected ERGs, but not VEPs, suggests that elements in the rat retina may be more sensitive to organic solvent exposure than the rat visual cortex.

Keywords

toluene; retina; electroretinograms; visual function

INTRODUCTION

Impaired visual function has been reported in human populations exposed to organic solvents, as described in several review articles (Benignus et al 2005, Fox 2015, Fox & Boyes 2013, Geller & Hudnell 1997, Gobba 2000, Gobba 2003, Gobba & Cavalleri 2003, Iregren et al 2002, Kishi et al 2000, Lomax et al 2004, Paramei et al 2004). The visual deficits reported have included impaired color discrimination, impaired visual contrast sensitivity, and several other measures. Color vision impairments have been reported following exposure to toluene (Campagna et al 2001, Cavalleri et al 2000, Muttray et al 1999, Muttray et al 1995)), benzene (Lee et al 2007), perchloroethylene (Cavalleri et al 1994, Gobba et al 1998), n-hexane (Issever et al 2002), styrene (Campagna et al 1996, Campagna et al 1995, Gong et al 2002, Iregren et al 2005, Kishi et al 2001), xylene (Lee et al 2013) and mixed organic solvents (Braun et al 1989, Gong et al 2003, Guest et al 2010, Paallysaho et al 2007). Visual contrast sensitivity, although investigated less frequently than color discrimination, also has been impaired following chronic exposure to styrene (Campagna et al 1996, Campagna et al 1995), (Castillo et al 2001, Mergler et al 1996), perchloroethylene (Schreiber et al 2002), and mixed organic solvents (Böckelmann et al 2005, Boeckelmann & Pfister 2003, Broadwell et al 1995, Donoghue et al 1995, Frenette et al 1991, Gong et al 2003, Mergler et al 1991). Prenatal exposure to organic solvents also has

been associated with impaired color vision and visual contrast sensitivity in offspring of solvent-exposed pregnant women (Till et al 2001, Till et al 2003, Till et al 2005).

Persistent effects of solvents on vision are not uniformly observed. Several studies have found no differences in visual function between solvent exposed workers and controls (Nakatsuka et al 1992, Schäper et al 2004, Seeber et al 2009, Seeber et al 2005), athough the dose levels in these studies were generally below those of the positive outcome studies. Some of the studies of solvent exposure and visual function have been limited by small sample sizes, control groups that were not well matched for potential confounding variables, inability to conduct single or double-blinded testing, and potential motivational issues related to workplace disability compensation (Lomax et al 2004). The small magnitude of the deficits and variability within and between studies has led to skepticism as to the reliability and severity of the impairments observed (Lomax et al 2004, Paramei et al 2004).

Acute exposure to toluene, trichloroethylene or perchloroethylene reliably reduces the amplitude of visual evoked potentials (VEPs) of rats (Boyes et al 2003, Boyes et al 2007, Boyes et al 2009, Dyer et al 1988, Rebert et al 1989a, Rebert et al 1989b). In contrast, there has been little work from controlled laboratory experiments using animal models to assess visual toxicity from chronic or sub-chronic organic solvent exposure. One exception is a study of female Sprague-Dawley rats exposed to 300 ppm styrene vapor daily for 12 weeks. Compared to controls, styrene-exposed rats showed lower retinal dopamine content and fewer tyrosine hydroxylase immuno-reactive retinal amacrine cells (Vettori et al 2000). In other studies, repeated inhalation exposure of rats to toluene, trichloroethylene, perchloroethylene, or 1,1,1-trichloroethane revealed only minor changes or no changes in flash-elicited VEPs (Dyer et al 1984, Mattsson et al 1993, Mattsson et al 1998, Rebert et al 1991) unless the dose levels were extremely high simulating inhalant abuse (Mattsson et al 1990). New Zealand albino rabbits exposed (4h/d, 4d/w) to trichloroethylene vapors for 12 weeks showed dose-dependent changes in VEP (Blain et al 1992), and increases of electroretinograms (ERG) a-wave and b-wave amplitudes (Blain et al 1994) that returned to baseline levels after termination of exposure. In summary, the experimental evidence to date has not identified a toxicological basis of chronic occupational solvent-induced visual deficits.

Toluene is one of the most commonly used substances in industry and commerce and is also one of the organic solvents for which there have been reports of visual impairments in exposed workers, including impaired color discrimination (Campagna et al 2001, Cavalleri et al 2000, Zavalic et al 1998a, Zavalic et al 1998b, Zavalic et al 1998c) and decreased contrast sensitivity (Donoghue et al 1995). The U.S. Environmental Protection Agency evaluated the scientific literature regarding toluene exposure as a component of setting a reference concentration (RfC) value intended to be protective over a lifetime of exposure EPA ((EPA) 2005). As a component of that analysis, a set of studies was identified which evaluated occupationally exposed populations and evaluated a series of neurological outcomes including visual function (their Table 1). From among the studies evaluating visual functions, those designated as a providing No Observable Effects Level (NOAEL) included Eller et al (Eller et al 1999) (duration 1–12 years, 20 ppm), Nakatsuka et al., (Nakatsuka et al 1992) (duration unspecified, 44–48 ppm), Neubert et al., (Neubert et al 2001) (duration

unspecified, 39 ppm); and Zavalic et al., (Zavalic et al 1998a) (16.21 ±6.1 years, 32 ppm). The IRIS document also identified studies with visual function assessments that provided Low Observable Effects Level (LOAEL) including: Eller et al., (Eller et al 1999) (>12 years exposure, > 100 ppm), Neubert et al., (Neubert et al 2001) (duration unspecified, 81 ppm), Vrca et al, (Vrca et al 1995) duration 21.4 ± 7.4 years, 40–60 ppm), and Zavalic et al., (Zavalic et al 1998a) (18.34 ± 6.03 years, 132 ppm). Therefore, occupational NOAEL values for visual deficits in workers exposed to toluene ranged from 20 – 39 ppm, or approximately 240 – 500 ppm-years when expressed as cumulative exposure. Similarly, the levels of toluene exposure reported as LOAEL values for visual deficits ranged from 40 – 132 ppm, or approximately 850 – 2400 ppm-years.

The current studies sought to characterize persistent changes in visual functions following repeated inhalation exposure to toluene. These studies were conducted as a component of a larger integrated evaluation of potential neurotoxic effects of repeated toluene inhalation, which has been reported separately. Rats exposed to these atmospheres of toluene for 4 weeks were found to have attentional deficits (Beasley et al 2012), and when exposed for 13 weeks, to be delayed in acquisition of an appetitive lever-press response (Beasley et al 2010). Other rats from the 13-week exposure cohort had increased markers of oxidative stress in frontal cortex, hippocampus, and cerebellum (Kodavanti et al 2015). In addition, gene expression profiles in the brains of rats exposed to toluene for 13 weeks differed from those of rats after acute exposure, and included differentially expressed genes in canonical pathways participating in oxidative phosphorylation, mitochondrial function and EIF2 (Eukaryotic Translation Initiation Factor 2) signaling pathways (Hester et al 2011, Hester et al 2012).

The goals of the studies reported here were first: to investigate visual function in rats repeatedly exposed to toluene vapors in order to determine if the reports of persistent visual dysfunction observed in occupationally-exposed populations could be replicated in an animal model under experimentally-controlled conditions; second, to explore the dose-response relationships, exposure-duration relationships and persistence of any deficits observed; and third, to try to identify the locus and nature of any deficits observed. In these studies, pigmented rats were exposed by inhalation to 0, 10, 100 or 1000 ppm toluene for either 4 or 13 weeks duration, and evaluated 2–6 weeks after the end of exposure, or approximately one year later. A pigmented strain of rats was used, rather than an albino strain as commonly employed in general toxicity testing, because albino rats lack ocular melanin making them vulnerable to light-induced retinal pathologies under standard laboratory conditions, and because albino animals have a number of visual system abnormalities and show relatively poor pattern-elicited VEPs (Boyes & Dyer 1983). In initial studies VEPs elicited with patterned stimuli were recorded. In all the studies retinal function was evaluated using electroretinograms (ERGs). Samples of retinal tissue were evaluated for immunohistochemical staining and quantification of retinal rod and M-cone photoreceptor densities. The results of these studies suggest that toluene exposure for 13 weeks caused a lasting decrease in the amplitude of ERG b-waves, suggestive of impaired function of the inner nuclear layer of the retina which includes retinal on-bipolar cells. The ERG deficits were observed after only the highest concentration tested (1000 ppm) and the longest duration exposure (13 weeks), but persisted as long as one year after the termination

of exposure. These results provide experimental evidence confirming human observational studies that chronic exposure to high concentrations of organic solvents may be associated with persistent visual dysfunction.

METHODS

The experiments described here were conducted as a component of a larger evaluation of the neurotoxic effects of repeated exposure to toluene. The experimental materials and procedures for animal housing and exposure are described in detail elsewhere (Beasley et al 2012, Beasley et al in preparation, Beasley et al 2010). Therefore, those methods will be summarized only briefly here.

Compound

Toluene (99.5% pure, ACS grade) (CAS # 108-88-3) was obtained from Sigma-Aldrich Chemical Co., St Louis, MO.

Animals

The experimental subjects were male Long-Evans rats (LE) (Crl:(LE)BR) obtained from Charles River Laboratories (Portage, MI) at 64 or 67 days of age. Male rats were selected for study because there was no a priori reason to expect that there would be a gender-based difference in sensitivity of the visual system to toluene exposure, and it was thought to be preferable, given limited testing resources, to increase the sample size and statistical power of detecting changes in one sex, rather than diluting the power and increasing variability by studying both sexes. Animals were allowed to acclimate to the animal colony for 12 days prior to the beginning of the exposures. Exposures were conducted in four Hazelton 2000 inhalation chambers, one for each of three toluene concentrations plus a control chamber for clean air. While in the inhalation chambers, rats were housed in individual wire mesh cages containing a stainless steel loft for environmental enrichment and to allow the rats an opportunity to move off of the wire mesh cage bottoms. Rats remained inside the chambers from Monday morning through Friday afternoon during exposure sessions, and were returned to polycarbonate individual cages with heat-treated pine chip bedding over the weekends. Rats had free access to drinking water through an automatic watering system at all times. Standard rat chow (PMI Nutrition International, Richmond, IN) was available *ad libitum* in the inhalation chamber during the times when there was no ongoing toluene exposure, but was removed during the exposure periods. Each rat received 20 grams of food per day on the weekends to control weight gain. After the completion of the exposure phase, rats were relocated to the animal colony and housed individually in polycarbonate cages with kiln-dried pine shaving bedding (Northeastern Products, Warrensburg, New York), and had *ad libitum* access to tap water and rat chow. The ambient temperature and relative humidity of the animal colony were 22±2°C and 50±10%, respectively, with a 12:12 hour light:dark cycle (lights on at 6:00 am). Illumination in the animal colony was maintained at approximately 325 lux measured 1 meter above the floor, well below the level of illumination associated with retinal pathology in albino rats. Because these experiments employed a pigmented strain of rats that is less susceptible to light-induced retinal degeneration than are albino strains, the level of ambient illumination was not expected to

influence the experimental results. Illumination of the interior of the exposure chambers was not measured directly, but was dimmer than that of the animal colony rooms.

The animal facility followed the guidelines of the National Institutes for Health for animal care, and was fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the USEPA National Health and Environmental Effects Research Laboratory (NHEERL), which ensured conformance with the 2004 National Research Council "Guide for the Care and Use of Laboratory Animals, Eighth Edition", the Animal Welfare Act and Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Inhalation Exposure

The inhalation facilities and procedures are described in detail elsewhere (Beasley et al 2012, Beasley et al 2010) and will be summarized here briefly. The nominal concentrations of toluene in the four exposure chambers were 0, 10, 100 and 1000 ppm. Rats were randomly assigned to one of the groups and were exposed 6 hours/day, 5 days/week. In the first experiment the exposures were conducted for 13 weeks. In the second experiment the exposures were conducted for 4 weeks. All experimental dose groups were color coded and provided to the electrophysiology testing lab in a blinded fashion. The dose-color code was not broken until after all data were recorded and scored.

Experimental Designs and Sample Sizes

Experimental Set 1—Immediately following the 13 weeks of exposure, 10 male rats/dose group were made available for visual function testing. The mean body weights of those rats were 473±7.6, 475±12.7, 485±8.1 and 478±7.5 g for groups of rats exposed to 0, 10, 100 or 1000 ppm, respectively. These rats were subject to survival surgery for implantation of electrodes for VEP testing and subsequently tested in the VEP and ERG procedures. The VEP testing occurred 2–3 weeks after termination of exposure and the ERG testing occurred 5–6 weeks after termination of exposure. Following ERG testing these rats were examined with an ophthalmoscope, sacrificed, and their eyes collected for immunohistochemistry as described below.

Experimental Set 2—Approximately 1 year after the termination of the 13 weeks of exposure, a second set of rats (n=8–10/dose group) became available after they had completed a series of appetitive behavioral tasks as described by Beasley and co-workers (Beasley et al 2010). During behavioral testing, these animals were kept on a weight maintenance schedule with a target mean body weight of 85% (±10 g) of their weight measured on the last day of exposure. After completion of behavioral evaluations they were switched to *ad libitum* feed and at the time of visual testing had mean body weights of 619.4±8.6, 614.9±15.1, 610.31.4, and 622.7±22.9 g for dose groups previously exposed to 0, 10, 100 or 1000 ppm toluene, respectively. Because the VEP testing of the first set of rats showed no effects of toluene treatment, only ERGs assessments were conducted on this and subsequent sets of rats.

Experimental Set 3—A third set of 10 rats per dose group was available for testing at the completion of 4 weeks of exposure, 5 days/week. The nominal dose levels were again 0, 10, 100 or 1000 ppm toluene and all aspects of the housing and care of the animals were as described above for the 13-week experiments. The ERG testing occurred 2–3 weeks after the completion of exposure. At the time of testing, rats had mean body weights of 529±7.5, 526±12.4, 551±12.6, and 529±13.2 g, for dose groups treated with 0, 10, 100, or 1000 ppm toluene, respectively. After ERG testing was completed the rats were sacrificed and their eyes collected for immunohistochemistry as described below.

Experimental Set 4—Finally, a fourth set of rats (n=9–10/dose group) was evaluated 1 year after the 4 weeks of exposure at the completion of their behavioral assessments (Beasley et al 2012). As before, these rats were weight maintained during the behavioral evaluations, but returned to *ad libitum* feeding prior to ERG evaluation. Only controls and rats previously exposed to 1000 ppm toluene were evaluated in this set because only this dose level was affected in the group mean data of the preceding evaluations. The rats had mean body weights of 433±15.0 and 451±4.1 g, respectively at the time of ERG testing.

Surgery

The first set of animals underwent survival surgery to implant indwelling electrodes for VEP recording. After the completion of the 13 weeks of exposure, and after rats were returned to the general animal colony housing, the rats designated for VEP testing were surgically equipped with chronic indwelling cranial electrodes as described previously (Boyes et al 2003, Herr et al 1992). Briefly, rats were anesthetized (sodium pentobarbital; 50 mg/kg i.p.), placed in a stereotaxic device, and the skull was exposed for electrode implantation. The electrodes, constructed from stainless steel screws (00–90 × 1/16″) soldered to Nichrome wires, were implanted into the skull in following locations: 1 mm anterior and 4 mm lateral left of lambda over the primary visual cortex; and 2 mm anterior and 2 mm lateral left and right of bregma for ground and reference electrodes, respectively. The electrode assembly was encased in dental acrylic and the wound closed. Approximately one week was allowed for surgical recovery before VEP testing. The subsequent sets of rats did not experience surgery or VEP testing.

VEP Stimuli

The stimuli to elicit pattern VEPs were presented on a video monitor located outside of a Faraday cage. The stimuli were generated with the green gun of the video monitor using a computer-based system described elsewhere (Boyes et al 2003, Hamm et al 2000). The stimulus generation system was calibrated to achieve a linear relationship between video input signal voltage and screen luminance. Mean stimulus luminance was calibrated to be 50 lux measured at the position of the rat's eye. Other than the stimulus screen, the recording chamber was dark. The stimulus patterns were vertical gratings with a sinusoidal spatial luminance profile and a spatial frequency of 0.16 cycles per degree visual angle. The stimulus temporal modulation was either pattern on/off (appearance / disappearance) or pattern reversal (phase alternation) modulated at 4.55 Hz. Stimulus peak contrast values included 0, 2, 4, 6, 8, 12, 16, 24, 32, 48 and 64 %. Thus, a series of 22 VEP waveforms was recorded from each subject (2 temporal modulation modes multiplied by 11 levels of

contrast). The different stimulus conditions were presented in a random order with a 1 minute adaptation period between tests during which the non-patterned mean luminance screen was presented. A complete recording session for an individual rat took approximately 60 min. Testing commenced from 8:00 am until approximately 4:00 pm daily.

Evoked Potential Recording

Electrophysiological potentials were recorded using a system described in Hamm et al., (2000): amplified (10K), bandpass filtered (0.1–1000 Hz), and sampled at a rate of 1.2 KHz in 5 sec epochs. Awake rats were restrained in a flexible plastic tube with their head exposed, secured to a rigid plastic tray, and placed inside the Faraday cage. Body temperature was monitored during the procedure. Pupils were not dilated in order to maintain optimal refraction for pattern perception. The electrode headsets were connected to a cable leading to the amplifiers, and the rats were placed facing the screen of the video monitor with their eyes approximately 15 cm from the monitor. The Faraday cage and the video monitor were covered with a black felt cloth so that the only source of light for the rat was the video monitor screen. Each rat was given a few minutes to adapt while viewing a mean luminance non-patterned screen, after which the VEP recordings began. Each VEP was constructed from the average of 20 5-sec epochs. Each averaged waveform was submitted to a spectral analysis. The spectral amplitude was measured at the rate of the visual pattern modulation (F1) and twice that rate (F2). For pattern reversal modulation only F2 was analyzed because this stimulus modulation yields no F1 response. The waveforms recorded to the non-patterned mean luminance screen (0 % contrast) were used to indicate the recording noise levels.

Contrast Amplitude Functions

A linear relationship has been reported between the amplitude of pattern VEPs and the log of visual pattern contrast (Silveira et al 1987). The zero-amplitude intercept of this function has been interpreted as equivalent to perceptual contrast threshold, and the slope of the function as contrast-gain (Bobak et al 1988). Contrast sensitivity is typically expressed as the reciprocal of contrast threshold, and VEP amplitude log-contrast functions have been used to define contrast sensitivity functions in rats (Silveira et al 1987). Because reduced visual contrast sensitivity has been reported in humans exposed to organic solvent vapors, we hypothesized that the intercept of the contrast amplitude functions would be increased after repeated exposure to toluene.

In the experiment reported here VEP amplitude log-contrast functions were generated from the F2 amplitudes for each rat. Amplitude values that were less than the mean noise level + 2 SEMs were considered indistinguishable from noise and were omitted from the linear regression determinations. Linear regressions were fit to the remaining data from each individual rat using SigmaPlot (SigmaPlot for Windows Version 11.0, Systat Software, Inc., San Jose, California), and the slope and intercept values were defined.

Electroretinograms

Electroretinograms were recorded using an Espion E2 ColorDome™ system (Diagnosys, LLC, Lowell, MA) which was custom modified by the manufacturer to replace the standard

blue LED stimulus lights with UV LEDs that were better matched to the spectral sensitivity of rat Scone photoreceptors, which are responsive to UV wavelengths of radiation (Jacobs et al 2001). The system retained the other standard stimulus capabilities. Visible wavelengths of light were calibrated using a photometer (model DR-2550-1, Silicon Detector Probe Model and Calibration Module model DR-2250-2B, photometric filter, Gamma Scientific, San Diego, CA). The UV radiation was calibrated with a radiometer (model IL1700, SED005 detector, WBS320 filter, International Light Technologies, Newbury, MA). The spectral power distribution of both visible and UV wavelengths was measured with a spectroradiometer (model RPS900, International Light Technologies, Newbury, MA). All the photometric and radiometric instruments were calibrated according to the manufacturer's specifications.

The ERG recording session for Experimental Set 1 included collecting a dark-adapted (scotopic) flash series, a UV flicker series, and a green flicker series. After Set 1 was completed, a light-adapted photopic flash series was included in subsequent testing. The dark-adapted flash series measured the function of rod photoreceptors (a-wave) and retinal on-bipolar neurons (b-wave) (Jamison et al 2001, Karwoski & Xu 1999, Robson et al 2004, Stockton & Slaughter 1989, Tian & Slaughter 1995). The UV flicker series reflected the function of S-cones, and the green flicker series reflected the function of M-cones (Eells et al 2003, Viswanathan et al 2002). The light-adapted flash series reflected cone photoreceptors (a-wave) and on-bipolar cells (b-wave) (Bush & Sieving 1994, Rangaswamy et al 2004, Wu 2010).

Scotopic Flash ERG—Dark-adapted flash ERGs were recorded first in the series of ERGs. Rats were housed overnight prior to ERG recording in the ERG testing laboratory, which was designated as a satellite animal housing facility and inspected and approved for overnight housing by the NHEERL Institutional Animal Care and Use Committee. The testing room was painted black, sources of stray light eliminated, and the room kept dark overnight. Animals were housed overnight in individual plastic cages with standard bedding and *ad libitum* access to food and water. All animal handling and preparations for recording the next day were accomplished under dim red darkroom safelights. Each rat was anesthetized with an i.p. injection of a combined solution of ketamine (80 mg/kg) and xylazine (8 mg/kg), with supplemental dosing as needed based on responses to a mild tail pinch. Once the rat was anesthetized, the left eye was given eye drops of ophthalmic solutions of proparacaine HCl (0.5%), tropicamide (0.75%) and phenylephrine HCl (2.5%). The right eye was taped closed and whiskers were trimmed to prevent interference with the electrodes. The animal was placed in a flexible plastic cone with the head exposed, equipped with a rectal thermometer to monitor body temperature, placed on a circulating warm water blanket and secured to a rigid plastic tray which was located under the ganzfeld of the ColorDome™. Subdermal needle electrodes were placed in the back of the neck for grounding and at the lateral canthus of the left eye for reference. The active electrode was a loop of wire (10% iridium/90% platinum) placed in contact with the cornea of the left eye using a micromanipulator. The cornea was kept moist throughout the session with drops of an ophthalmic solution of Isotears™ (Allergan, Inc., Irvine, CA). Suitable recording conditions were verified with the impedance meter of the system control computer, which

was located in the adjacent room. Beginning with the dimmest flash, single flash ERGs were recorded at incrementing half-log steps over a range of 5 log luminance values. The dark adaptation time between dim flashes of light was 30 s and 1 min between flashes greater than 0.1 cd*s/m^2. ERG signals were band-pass filtered (1.25 – 300 Hz) and sampled at a rate of 1000 Hz. Each sweep included 10 ms pre-stimulus baseline and 300 ms post-stimulus time. Peaks were scored automatically for a-waves and b-waves, with subsequent manual inspection and verification. Parameters analyzed included the peak latencies of the a-wave and b-wave, the a-wave amplitude (measured baseline to peak) and the b-wave amplitude (measured trough-to-peak from a-wave to b-wave). In addition, an alternative measurement of a-wave amplitude was obtained at 10 ms after the flash, which was thought to better reflect the function of only rod photoreceptors prior to the onset of neural activity from other retinal cells (Fox et al 2008, Jamison et al 2001).

For the ERG b-wave amplitude values, Naka-Rushton functions (Evans et al 1993, Gangadhar et al 1989, Severns & Johnson 1993) were fit to the amplitude vs log flash luminance data using the nonlinear regression sigmoidal 3-parameter Hill function of SigmaPlot (Systat Software, Inc., San Jose, CA), which is equivalent to the Naka-Rushton Equation. The general form of the Naka-Rushton Equation is:

$$\mathrm{R(I)=R_{max}\,I^n/(I^n+K^n)},$$

in which I refers to stimulus luminance (cd*s/m^2); R refers to the ERG amplitude (μV); R_{max} refers to the maximum asymptotic amplitude (interpreted to reflect the total area of functioning retina); K refers to the half saturation constant (interpreted as retinal sensitivity), and n is a constant controlling the slope of the function.

UV Flicker ERGs—After the dark flash protocol was completed, a UV flicker series was conducted. The stimuli were generated by UV LEDs and had a sharp uni-modal spectrum between about 340–400 nm with a peak wavelength of 370 nm. The ERG procedures were modeled after those of Eells and co-workers (Eells et al 2003). The UV stimuli were flickered at 21 Hz, which is above the temporal response limit of rod photoreceptors (Goto et al 1998, Moses et al 1987). In Experimental Set 1, four levels of flicker irradiance were used. These were reduced in subsequent experiments to three UV stimulus levels, presented in dim-to-bright order, which were calibrated with the spectroradiometer to provide peak irradiance at 370 nm of 0.7, 2.4 and 7.8 μW/cm^2, respectively. Concurrently, a steady green background light (515 nm, 0.93 μW/cm^2) was delivered to suppress potential contribution of M-cones. A 30 s adaptation time to the steady green stimulus alone was provided prior to each recording. Electrophysiological data were bandpass filtered (10–30 Hz) and sampled at 1000 Hz. Each sweep included 2000 ms of data, and ERG recordings were averaged over 20 consecutive sweeps. The resulting waveforms had a sinusoidal shape with the primary frequency at the stimulus rate (i.e. 21 Hz). The data were transferred to a central computer network where they were subjected to spectral analysis (PROC Spectra, SAS Institute Inc., Cary NC) and the spectral amplitude at 21 Hz was taken as the dependent measure for statistical analysis.

Green Flicker ERGs—The procedures to record green flicker ERGs were analogous to the UV flicker series, except that the green LEDs were flickered while the UV stimulus was held steady. The green LEDs had a uni-modal spectrum between approximately 480–560 nm with a peak of 520 nm. In Experimental Set 1, nine levels of green flicker were used, but this was reduced in subsequent experiments to 4 levels of half log steps between 1 and 30 cd/m^2 because the 4 levels gave a suitable intensity response function. The steady UV background was 10 $\mu W/cm^2/nm$ at 370 nm. Other aspects of the green flicker ERG recording and analysis were identical to those for the UV flicker.

Photopic ERG—For the Experimental Set 2 and afterward, a photopic ERG series was added at the conclusion of the green flicker ERG recordings. To record photopic ERGs a steady green adaptation light of 40 cd/m^2 was turned on to saturate rod photoreceptors, and a series of white flashes of 0.3, 1, 3 and 10 $cd*s/m^2$ was presented in sequence. An adaptation time of 30 sec was provided prior to the two dimmer flashes and of 1 min prior to the two brighter flashes. The other aspects of the recording and data scoring were identical to the dark-adapted (scotopic) flash series.

Ophthalmoscopic Examination

At the conclusion of ERG recordings, while the pupils were dilated, both eyes of each rat were examined by an ophthalmologist (JCM) using an indirect ophthalmoscope. The observer was not aware of the treatment group of any subject at the time of assessment. Any clinically evident abnormalities in the posterior segment of the eye were noted. Due to scheduling conflicts, only the rats from Experimental Set 1 and Experimental Set 4 were examined.

Immunohistological Evaluation of Photoreceptors

Forty rats exposed to control air or to toluene for either 4 or 13 weeks were subject to immunohistochemical assessment. One eye was enucleated and placed in 4% paraformaldehyde fixative at 4°C for approximately 24 hrs and then transferred to 0.1M phosphate buffer prior to transfer to the University of Wisconsin School of Medicine and Public Health. Just prior to enucleation, 39 of the 40 eyes had a small cautery mark placed near the corneal limbus at the 12:00 o-clock meridian for purposes of orientation. The eyes were cut open coronally with the aid of a dissecting microscope. The crystalline lens was taken out of the eye and a 2 mm trephine was used to remove a disk of inferior retina. The sampling location was based on the observations of Szél and Röhlich (Szél & Röhlich 1992) who found similar populations of S- and M-cones in 4 mid-peripheral regions of the retina. The density of S-cones was virtually the same in all areas and there was a slightly higher density of M-cones in the inferior region. The superior-most part of the trephined retinal disk was located approximately 0.5–1 mm inferior to the optic nerve. Another 2 mm disk of retina was removed from an unknown retinal location in the single eye that was not marked. Processing difficulties were encountered in preparing another eye and, so, a second 2 mm disk of retina was removed from the superior retina and used for data analysis.

The retina sections were processed in the University of Wisconsin Electron Microscopy Laboratory. Samples were dehydrated in a graded ethanol series, 30%, 50%, 70%, 80%,

90%, 95%, 100% for 10 min each on a rotator (100% - 3X-10 min). The samples were then transferred to a 50/50 mixture of unaccelerated (omitting part “C”) Durcupan epoxy resin and 100% ethanol for 12 hrs. Next, the samples were transferred to 100% unaccelerated Durcupan epoxy resin and placed in a 60°C oven on a rotator for 4 hrs. The samples were then transferred to accelerated Durcupan epoxy resin and infiltrated in a 60°C oven on a rotator. The accelerated resin was exchanged 3X every 45 min. Finally, the samples were flat embedded between two glass microscope slides pre-treated with a releasing agent (Rain-X™ windshield treatment) and polymerized in a 60°C oven for 24 hrs. After polymerization, one of the glass slides was removed and the embedded specimens were viewed under a light microscope for orientation. Following orientation, the specimens were glued to a cylinder of polymerized Durcupan resin and cut with a Leica EM UC6 ultramicrotome (Leica; Wetzlar, Germany) into 1 μm sections using a Diatome diamond histology knife (Diatome; Hatfield, PA). The sections were placed on glass slides and dried on a hot plate. One tissue section on each slide was stained with Richardson’s stain for examination of the tissue before immunohistochemistry (IHC) was performed.

The IHC procedure was performed on all 40 eyes using antibodies to rods and M- cones. Prior to immunoreaction, the Durcupan epoxy resin was removed according to a method described previously (Mayor et al 1961). Once re-hydrated, the sections were incubated in a trypsin digest at 37°C for 8 min. After cold double-deionized H_2O rinses, the sections were incubated with a mouse monoclonal antibody for rhodopsin staining or a rabbit polyclonal antibody for M/L cone opsin staining (both at 1:2000, catalog numbers MAB5316 and AB5405, respectively, Chemicon/Millipore; Billerica, MA) overnight at 4°C. After washing, the sections were incubated with biotinylated anti-mouse IgG and biotinylated anti-rabbit IgG, respectively, for 30 min and then incubated for 1 h at room temperature with avidin-biotin complexes (Vectastain ABC Kit Elite PK-6102 with the rhodopsin primary and Vectastain ABC Kit Elite PK-6101 with the cone opsin primary, Vector Laboratories; Burlingame, CA). Following washes in phosphate-buffered saline and Tris buffer, the bound antibodies were revealed for both rhodopsin and M-cones by incubation with 3, 3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich; St Louis, MO) for 2 and 6 min, respectively. Tissue sections stained for M-cones were counterstained with Richardson’s stain (30 s at approximately 60°C) prior to cover slipping.

The tissue sections were viewed with light microscopy (Olympus BH-2; Olympus Corp; Lake Success, NY). One tissue section from each slide stained with Richardson’s stain was chosen and then photographed at 100x (oil immersion) magnification with a Nikon E5000 digital camera (Nikon; Chiyoda-ku, Tokyo, Japan). Four photographs from each tissue section chosen were uploaded to NIH ImageJ software. Rods and M-cones were hand counted in ImageJ. Rods were counted from one of the four pictures for each tissue and M-cones were counted from all four photos and added. In all but one sample, at least 2000 photoreceptors were counted. Staining on slides was scored subjectively (scale range from 0+ to 3+) using a light microscope (Olympus CH-2; Olympus Corp; Lake Success, NY) at 10x magnification for analysis.

Statistical Analysis

Electrophysiological data were analyzed statistically using the GLM procedure of SAS (SAS, Software release 9.1, Cary NC). For the data from VEP experiments, statistical analysis was conducted on VEP F2 amplitudes using the dose as a between-subjects factor, and the pattern temporal modulation and contrast as within-subjects repeated measures. In addition, the slope and intercept of contrast amplitude functions fit to the data from individual subjects were analyzed separately using dose as a between-subjects factor.

For scotopic and photopic ERG data the amplitude and latency of both a-waves and b-waves were analyzed using dose as a between-subjects factor and flash luminance as a within-subjects repeated factor. In addition, the R_{max} and K parameters derived from Naka-Rushton functions fit to the scotopic ERG data from individual rats were combined across all the data sets and analyzed using dose as a between-subjects factor.

For UV and green flicker ERG data, the amplitude derived from the spectral analysis at the stimulation rate (F1) was used as a dependent measure. The independent variables were dose group, a between-subjects factor, and stimulus luminance or radiance, within-subjects factors.

It is important to consider the problem of multiple dependent measures artificially inflating the probability of detecting a statistically significant difference between the treatment groups by chance alone. For the electrophysiological measures, where the ability to collect multiple dependent measures make this issue particularly important, the issue was addressed by designating the initial data set from the 13 week study as a hypothesis generation experiment, in which all dependent variables were evaluated at a probability level of α=0.05, but none of the conclusions were considered definitive until replicated (Muller et al 1984). The subsequent experiments involved independent sets of test subjects, and were designated for replication of the findings of the first set (even though the exposure durations and times after exposure differed between sets). Positive results in the hypothesis generation experiment were thereby confirmed in the subsequent sets of data for which only previously significant results were assessed. The probability level for replication was designated as α=0.05.

RESULTS

Exposure and general state of the rats

Data regarding the actual toluene concentrations and other conditions monitored in the exposure chambers are presented in Beasley et al. (Beasley et al 2010). Briefly, the mean (±SD) concentrations measured during the 13 week study were 10 (±1.4), 97 (±7), and 995 (±43) ppm for nominal concentrations of 10, 100 or 1000 ppm respectively. During the 4-week study the mean (±SD) measured concentrations in the inhalation chambers were 9.9 (±1.6), 100 (±5), and 1005 (±43) ppm for nominal concentrations of 10, 100 or 1000 ppm, respectively (Beasley et al 2012). No animals were observed to show signs of intoxication or distress during the exposures, and there were no significant differences in body weight between the treatment groups during or after exposure in any of the experiments.

Visual Evoked Potentials and Contrast Sensitivity Functions

Experimental Set 1—Two rats died prior to testing for reasons unrelated to toluene exposure (one exposed to 0 ppm, and one exposed to 1000 ppm group). In addition, the data were eliminated from 6 rats for reasons that included problems with the electrodes, headsets or excessively noisy recordings (n=1 exposed to 0 ppm, n=2 exposed to 10 ppm; n=2 exposed to 100 ppm), and failure to produce VEP amplitudes above twice the measured noise level (n=1 exposed to 0 ppm). The resultant sample sizes for VEP analyses were n = 7 (0 ppm), n = 8 (10 ppm), n = 8 (100 ppm) and n = 9 (1000 ppm). The overall shape of the resulting visual evoked potential waveforms and the spectral analysis of those waveforms appeared qualitatively similar to those reported previously (Boyes et al 2007). VEP F2 amplitude data were analyzed using analysis of variance with one between subjects factor (dose) and two repeated within-subjects factors: stimulation type, (*i.e.* on/off or pattern reversal) and contrast. There was no statistically significant effect of dose ($F(3,28) = 1.44$, $p = 0.2528$), no significant stimulation type by dose interaction ($F(3,28) = 0.83$, $p=0.4873$), no significant contrast by dose interaction ($F(30,280) = 1.17$, $p=0.3374$), and no significant interaction of dose by stimulation type by contrast ($F(30,280) = 1.10$, $p=0.3571$).

The amplitude of the pattern reversal VEP F2 was plotted as a function of log contrast for the different treatment groups receiving control air or toluene for 13 weeks in order to assess contrast thresholds and contrast gain (Figure 1). The amplitude/log-contrast functions were approximately linear, as has been reported previously (Silveira et al 1987). The zero-amplitude intercept values of these functions were 2.24 ± 0.11, 2.43 ± 0.26, 2.21 ± 0.26, and 2.27 ± 0.20 (mean % contrast ± SEM) for groups treated with 0, 10, 100 or 1000 ppm, respectively. These intercept determinations corresponded to contrast sensitivity values of 45.4 ± 2.2, 45.3 ± 5.9, 50.8 ± 7.3, and 47.8 ± 5.6 (mean ± SEM) (units are 1/contrast), respectively. The slope values for these functions were 10.0 ± 1.7, 10.4 ± 1.4, 6.3 ± 1.0 and 8.7 ± 1.9 (μV/log(% contrast)), respectively. There were no statistically significant changes between the dose groups for either contrast thresholds ($F(3,28) = 0.2$, $p=0.8951$) or slope ($F(3,28) = 1.41$, $p = 0.2617$) of the VEP amplitude-log contrast functions.

Electroretinograms: Scotopic Flash Series

Experimental Set 1—A sample ERG series recorded from one control rat and one rat treated with 1000 ppm toluene for 13 weeks is shown in Figure 2. Both the a-wave and b-wave were identified. The waveforms grew in amplitude and the peaks decreased in latency with increasing flash luminance. The mean b-wave amplitude of each of the dose groups is presented in Figure 3A. Naka-Rushton functions fit to the group mean data are also presented. The group treated with 1000 ppm toluene had lower maximal b-wave amplitudes at the higher flash luminance values than did the other dose groups. Statistical analysis of the ERG data revealed no significant changes in a-wave peak latency or amplitude, a-wave amplitude measured at 10 ms, or b-wave latency. There was no statistically significant main effect of treatment on b-wave amplitude ($F(3,33)=2.22$, $p=0.1044$). There was, however, a statistically significant interaction between flash illuminance and toluene treatment on the b-wave amplitude ($F(30,330)=1.92$ $p=0.0034$ (unadjusted); $p=0.0453$ (Huynh-Feldt Epsilon adjustment). Step-down contrast comparisons showed significant differences between the dose groups at the two highest values of flash illuminance (both $F(3,33) > 3.0$, both $p<$

0.03). The reduction of maximal b-wave amplitude observed in Experiment 1 was considered as an exploratory observation until confirmed in a second experiment.

Experimental Set 2—The ERG b-wave amplitudes of rats treated with toluene for 13 weeks and tested approximately one year after the termination of testing are presented in Figure 3B. Naka-Rushton functions fit to the group mean data are also presented. As noted in Experimental Set 1, the b-wave amplitudes of animals treated with 1000 ppm toluene were lower than those of the other dose groups at the higher values of stimulus luminance. There was no statistically significant main effect of toluene treatment ($F(3,33)=1.36$ $p=0.2721$), but again there was a statistically significant interaction between flash illuminance and toluene treatment ($F(30,330)=2.22$ $p=0.004$ (unadjusted); $p=0.0520$ (Huynh-Feldt episilon adjusted). Step down contrasts showed no statistically significant differences among the 4 dose groups at individual flash illuminance values. An analysis comparing only the groups exposed to control air and 1000 ppm toluene showed significant differences of the b-wave amplitudes between groups at the highest five values of flash illuminance (all $F(1,17) > 6.0$ all $p < 0.02$). The reduction of b-wave amplitude at higher levels of flash illuminance following 1000 ppm toluene exposure in Experimental Set 2 was considered to be confirmation of the result observed in Experimental Set 1.

Experimental Set 3—The ERG b-wave amplitudes recorded from rats exposed to toluene for 4 weeks and tested two weeks later are presented in Figure 4A. As observed in the data from Experimental Sets 1 and 2, the ERG b-wave amplitudes of rats treated with 1000 ppm toluene were lower than those of the other dose groups at the higher values of stimulus luminance. The apparent difference was not statistically significant, however. There was no significant main effect of toluene treatment ($F(3,34) = 0.98$ $p=0.4115$) and no significant interaction between toluene treatment and flash luminance ($F(30,340) = 0.73$ $p=0.8464$ (unadjusted).

Experimental Set 4—The ERG b-wave amplitudes recorded from rats treated with toluene for 4 weeks and recorded approximately 1 year later showed a pattern of results different from the previous assessments. Animals observed to have cataracts were removed from the ERG dataset, yielding n=7, for both groups. ERG b-wave amplitudes of the 1000 ppm dose group were not statistically different from control (Figure 4B). The main effect of dose was not statistically significant ($F(1,12) = 2.43$ $p=0.1448$). The statistical test of the dose by flash illuminance interaction was not statistically significant after adjusting for the non-sphericity of the interaction matrix ($F(10,120) = 0.93$ $p=0.5067$ (unadjusted), $p=0.4072$ (Huyng-Feldt epsilon adjusted).

Electroretinograms: UV and Green Flicker and Photopic Flash ERG

There were no significant effects of exposure to toluene observed in the UV or Green flicker data or the photopic flash data from the experimental data sets evaluated (data not shown).

Ophthalmoscopy

The eyes examined ophthalmoscopically were scored as normal or abnormal and any abnormalities were described. Among the lesions described in the 1,000 ppm group of

Experimental Set 1 were bilateral optic nerve atrophy (n=1), and slight or pronounced degeneration of the retinal pigment epithelium (RPE) (n=3). Rats treated with 100 ppm for 13 weeks also showed slight bilateral RPE degeneration (n=2). In the rats from Experimental Set 4, who were approximately 1 year older than the first set, both controls (n=2) and high dose rats (n=3) were judged to have early stage or fully developed cataracts, but no retinal lesions were noted. The ERG data from subjects observed to have cataracts were removed from the ERG data set.

Immunohistological Evaluation of Photoreceptors

An example of 3+ anti-rhodopsin staining is shown in Figure 5A (note that this is an oblique section used to demonstrate the contrast between the positive outer segments and the negatively stained inner segments). Evaluation of the inner and outer segments of photoreceptors was selected, instead of counting photoreceptor nuclei in outer nuclear layer (ONL), because the antibodies used here only label the outer segments. The inner nuclear layer was not examined. The density of rods is plotted in Figure 5B. Figure 6A illustrates 3+ anti-M-cone staining. Counterstaining with Richardson's (blue) shows the rods. Such sections were employed for the quantitative photoreceptor density assessments. The IHC sections were graded by an observer unaware of the dose condition using a scale of 0 (no IHC staining activity) to 3+ (marked IHC staining activity). All of the sections except one (19–7, 13 week, 0 ppm toluene) had strong (3+) staining with anti-rhodopsin antibody. A 2+ staining was noted in the one exception. Similarly, for the anti-M-cone antibody reaction, nearly all of the retinas were graded as having 3+ staining. The only exceptions to the latter were animals numbered 19-Y, 13 week, 0 ppm (2+ staining); 20-Y, 13 week, 0 ppm (1+ staining); 11-G, 4 week, 100 ppm (1+ staining); 17-G, 4 week, 100 ppm (2+ staining); and 12-R, 4-week, 1000 ppm (2+ staining). The density of M-cones is plotted in Figure 6B. No dose-related changes in the density of rod (Figure 5B) or M-cone (Figure 6B) photoreceptors were observed

DISCUSSION

The studies reported here observed dose-related and persistent reduction of the ERG b-wave maximum amplitude following 13 weeks of toluene inhalation. The b-wave reduction was observed in Experiment 1 for rats exposed to 1000 ppm toluene for 13 weeks and tested a few weeks after termination of exposure. This observation of a reduced b-wave amplitude at high levels of flash luminance was confirmed in Experiment 2 in rats exposed to 1000 ppm toluene for 13 weeks and tested 1 year after exposure. This result was observed in an exploratory study in which all the dependent variables were analyzed at the level of α=0.05, and then repeated in a second confirmatory study.

Rats exposed for 4 weeks and tested a few weeks after showed a trend in the same direction of reduced ERG amplitudes, but the reductions were not statistically significant. Statistically significant changes in ERG b-wave amplitude were not observed one year after 4 weeks of toluene exposure. The shorter duration exposure (4 weeks) was apparently insufficient to produce the retinal dysfunction that was observed after longer duration exposure (13 weeks). The deficits were only observed after exposure to 1000 ppm toluene and not after exposure

to lower concentrations. Therefore, the persistent ERG deficits were dependent on both the concentration and duration of toluene exposure.

In occupational studies, populations of workers that have been reported to have visual deficits were exposed typically to higher concentrations than were those in negative studies ((EPA) 2005), although there have been reports of impairments in populations with exposures below the then current occupational limits (Iregren et al 2005). Analysis of occupational exposure to styrene (Benignus et al 2009), or toluene (Cavalleri et al 2000) showed a concentration-duration relationship in which the magnitude color vision discrimination errors increased as a function of the product of concentration x duration of exposure. As discussed previously, NOAEL values for visual deficits in workers exposed to toluene ranged from 20 – 39 ppm, or 240 – 500 ppm-years, and LOAEL values ranged from 40–132 ppm, or approximately 850 – 2400 ppm-years ((EPA) 2005). The occupational literature in many cases is compromised, however, by limitations including limited sample sizes, control groups inadequately matched for potential confounding variables, lack of longitudinal measures of exposure, lack of baseline measures prior to exposure, small effect sizes, and high divergence across studies of color confusion scores using the Lanthony D-15d color test panel (Geller & Hudnell 1997, Paramei et al 2004). These factors have led some to question the relationship between exposure to organic solvents and visual impairments (Lomax et al 2004). The current studies in experimental animals under controlled laboratory conditions alleviate concerns for mismatched control groups, undocumented prior exposure levels and other uncontrolled potential confounding factors that are typically present in cross sectional occupational studies. Exposure concentrations in the current study without effect included those up to 100 ppm, while 1000 ppm was the only concentration producing a measurable deficit. The rats were exposed for a maximum duration of only 13 weeks, however, compared to multiple years of exposure in human occupational studies. When expressed as a cumulative exposure metric, rats exposed to 1000 ppm toluene in the current experiments for 4 or 13 weeks (assuming 6 hours/day, 5 days/week is approximately equivalent to one occupational work week) would show NOAELs and LOAELs of approximately 77 or 250 ppm-years, respectively, which are below the ranges of cumulative exposures reported to cause comparable effects in human occupational studies. The results of the current laboratory experiments therefore support the veracity of occupational reports by demonstrating that repeated exposure to the organic solvent toluene is associated with the development of persistent deficits in visual function.

The Naka-Rushton parameter Rmax was reduced by toluene, and reflects the asymptotic maximal response amplitude of the ERG, which has been interpreted to reflect the total functional area of the retina (Gangadhar et al 1989) (Gangadhar et al 1989). In mammalian retinas, rod photoreceptors make synaptic contact with only one type of retinal bipolar cell, the depolarizing rod bipolar cell (DBC_R), which depolarizes in response to light spots (Wu et al., 2005). The dark-adapted ERG b-wave amplitude is thought to reflect the firing patterns of DBC_R cells (Gurevich & Slaughter 1993, Robson et al 2004, Stockton & Slaughter 1989, Tian & Slaughter 1995). The DBC_R cells synapse onto AII amacrine cells. The cell bodies of AII amacrine cells receive multiple inputs from dopaminergic amacrine cells that are involved in modulation of response sensitivity to changing levels of background illumination (Farsaii & Connaughton 2007). Thus, the report of a loss of

dopaminergic amacrine cells following experimental exposure to styrene (Vettori et al 2000) and the current finding of lower ERG b-wave amplitudes (presumably reflecting lower DBC_R activation) in response to toluene may have in common the altered function of AII amacrine cells. Dopaminergic amacrine cells are involved in feedback modulation of the antagonistic center-surround receptive fields of retinal bipolar and ganglion cells (Wu 2010). The antagonistic center surround receptive field structure is the primary functional organization that first encodes both luminance contrast and color contrast. Therefore, these feedback mechanisms are critical for both visual contrast sensitivity and for color discrimination. The altered B-wave amplitudes could reflect damage or dysfunction of the inner nuclear retinal layer where the cell bodies of the bipolar cells and the dopaminergic amacrine cells reside. On the other hand, we did not observe changes in visual contrast sensitivity. In addition, behavioral testing of some of the same rats used in the current experiments, or other rats from the same exposure chambers, did not reveal altered sensitivity to challenges with dopaminergic agonists or antagonists (Beasley et al 2010) suggesting that there was not a general dopaminergic toxicity across the CNS from this set of toluene treatments.

Although the reduction of b-wave amplitude observed in this study was persistent and dose-related, the data available were not sufficient to definitively identify any particular cell type or region of the retina as the locus of damage. We previously reported that acute exposure to 1000 ppm toluene altered gene expression profiles in rat cortex and striatum that were associated with changes in synaptic plasticity, long term depression, GABA receptor signaling and mitochondrial function (Hester et al 2011). After subchronic exposure, however, far fewer differentially expressed genes were observed, suggesting adaption to most of the effects of toluene exposure (Hester et al 2012). Of the few differentially expressed genes in brain tissue after subchronic toluene exposure, there were no genes related to long term depression, and only one of 24 genes associated with GABA receptor signaling. Mice treated acutely with intravitreal injections of GABA to suppress activity in ganglion, amacrine and horizontal cells showed reduced b-wave amplitudes at low levels of stimulus luminance and increased b-wave amplitudes at high levels of stimulus luminance (Robson et al 2004), the opposite of the pattern observed here. A mutant strains of mouse, termed the nob (no b-wave), have mutations in the Nyx gene, which encodes a protein nyctaopin that is strongly expressed in the inner nuclear layer of the retina (Gregg et al 2003, Pesch et al 2003). The nob mice have a defect in synaptic transmission between photoreceptors and depolarizing bipolar cells which is associated with a lack of a b-wave in the ERG. Among the genes altered after acute exposure (Hester et al 2011) that were associated with long term depression were genes involved in pathways for two metabotropic glutamate receptors (mGluRs) including mGluR1 (type I) and mGluR7 (type III). Interestingly, both retinal rod and cone pathways include mGluR6, a retinal variant of the Type III metabotropic glutamate receptor. Blockage of the mGluR6 receptor with the drug l-2-amino-4-phosphonobutyric acid (APB) leads to ERG responses with no b-wave (Knapp & Schiller 1984). In addition, Riegal and co-workers reported that lesions of dopaminergic pathways or blockage of metabotropic glutamate receptors in the nucleus accumbens blocked acute toluene increases in locomotor activity (Riegel et al 2003). Therefore, another possibility is that acute toluene exposure leads to altered expression of mGluR6 in the retina,

and longer term toluene exposure causes the differential gene expression to subside, but leaving the inner nuclear synaptic function at a sub-optimal level causing less than maximal b-wave amplitudes to be achieved even with very bright stimuli.

Rats exposed to toluene for 13 weeks did not show changes in pattern-elicited VEPs recorded from visual cortex. Similarly, there were no significant effects of toluene treatment on the slope or intercept for VEP amplitude/log contrast functions. The intercept of the VEP contrast amplitude function has been interpreted to be equivalent to the perceptual contrast threshold (Silveira et al 1987). We hypothesized that the VEP intercept contrast threshold would be increased in rats exposed to toluene, but this was not the case. The lack of change in visual contrast sensitivity in these toluene exposed rats, as opposed to occupationally-exposed humans (Donoghue et al 1995), might be attributed to many factors such as differences between the dose and duration of exposure, or differences between sensitivity of the visual systems of rats and humans. The fact that we observed changes in ERGs but not VEPs from subchronic toluene exposure suggests that elements in the rat retina may be more sensitive to organic solvent exposure than is the rat visual cortex.

Several lines of evidence suggest that subchronic toluene exposure did not impair retinal rod or cone photoreceptors. There were no changes in scotopic and photopic ERG a-waves measured either at the peak or at 10 ms. The latter measure is thought to reflect the function of the photoreceptors without the contribution of other retinal cells (Fox et al 2008, Jamison et al 2001). The flicker ERG amplitudes recorded with UV or green lights were unchanged, suggesting that the function of S-cones and M-cones was not altered by toluene. Finally, no treatment effects could be identified either by non-parametric grading of IHC staining reaction or by quantitative assessment of rod and M-cone densities in rats exposed to various levels of toluene for either 4 or 13 weeks. These data together suggest that the photoreceptor population was not impaired by toluene exposure.

The negative IHC result in this study should not be interpreted as sufficient to show a lack of photoreceptor toxicity. Both methods of analysis (IHC and photoreceptor density) are somewhat insensitive measures of cellular injury. For example, IHC is excellent for demonstrating the presence of a protein, but not how much of it is contained within a cell or how rapidly it is being turned over. It may be that cells have a lowered metabolic activity as a result of the injury. This is especially relevant for the opsin proteins evaluated in this study, since the rod and cone outer segments are normally turned over at a rapid rate. If both the rate of production and turn-over are simultaneously reduced, the total protein in the cells would remain unchanged. A better approach might be to look at opsin-specific mRNA production. Likewise, photoreceptor density analysis would reveal an effect only if the toxic insult was sufficient to actually cause cell death. Although photoreceptors can be easily killed by some agents (e.g., iodate salts), they are generally quite robust in that they have the ability to greatly reduce their metabolic activity in response to hypoxia, such as they do in retinal detachment.

It was interesting to note that in all cases in which damage was observed, both optic nerve atrophy and cataract were bilateral, suggesting that these lesions might be of toxic origin. However, since the rats were not examined with an ophthalmoscope prior to toluene

exposure, we cannot conclude with certainty that this is the case, particularly for optic atrophy. In addition, the bilateral cataracts may have been congenital or simply age-related. The extent to which the degeneration of the RPE observed in some rats was related to toluene exposure is unknown, as this lesion can be observed in normal subjects, *e.g.* those with longer ocular axis, or again as the result of normal aging.

In summary, 13 weeks of toluene exposure to 1000 ppm toluene caused a persistent reduction of ERG b-wave amplitudes, suggestive of changes in the function of retinal on-bipolar cells, which are located in the inner nuclear layer of the retina with synaptic connections in adjacent inner and outer plexiform layers. The deficits in ERG b-wave amplitude were still observed one year after termination of the 13 weeks of exposure, indicating that there were long lasting changes in retinal function with no observed recovery. Exposure for 4 weeks was insufficient to cause this result. The other electrophysiological and immunohistochemical evidence collected did not reveal additional sites of damage in the visual pathway including the rod or cone photoreceptors or the visual cortex.

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Highlights

Rats were exposed to either control air or one of three concentrations of toluene vapors for either 4 or 13 weeks and their visual function was measured either shortly after termination of exposure or approximately 1 year afterward. Rats exposed to 1000 ppm toluene for 13 showed a reduction of the electroretinogram b-wave amplitude at higher flash illumination values when tested shortly after termination of exposure and also one year later. Rats exposed to lower concentrations (10 or 100 ppm) or for a shorter duration (4 weeks) did not show significant ERG changes. The results confirm previous observational studies of persistent visual deficits in workers exposed to organic solvent vapors in a controlled experimental setting that avoids many of the potential confounding and interpretational factors associated with cross-sectional occupational studies. When expressed as cumulative exposure (ppm-years), the deficits of visual function observed in rats occurred at levels of exposure lower than those reported to impair visual function in occupational studies.

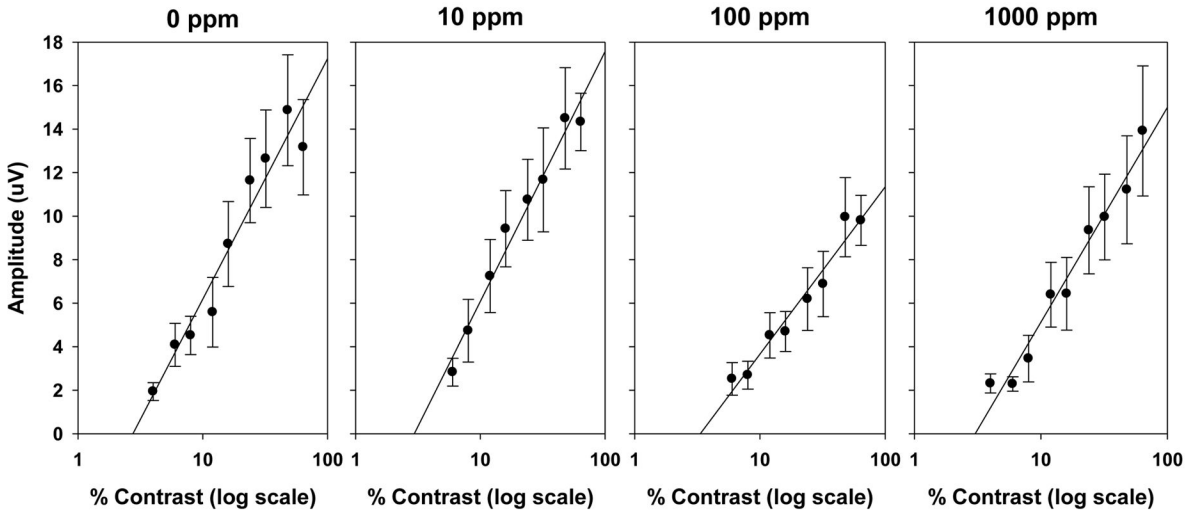

FIGURE 1.

Contrast-amplitude functions for pattern-reversal elicited visual evoked potentials recorded from rats approximately 2 weeks after termination of 13 weeks of exposure to 0, 10, 100 or 1000 ppm toluene. The contrast of the visual pattern is expressed in percent and plotted on a log scale. The lines are linear regressions fitted to the F2 amplitude-log contrast data. Amplitude values that were less than the mean noise level +2 SEM were omitted. There were no statistically significant effects of toluene treatment on the VEP amplitudes or either the slope or intercept of the contrast amplitude functions. Plotted values reflect the mean +/− SEM.

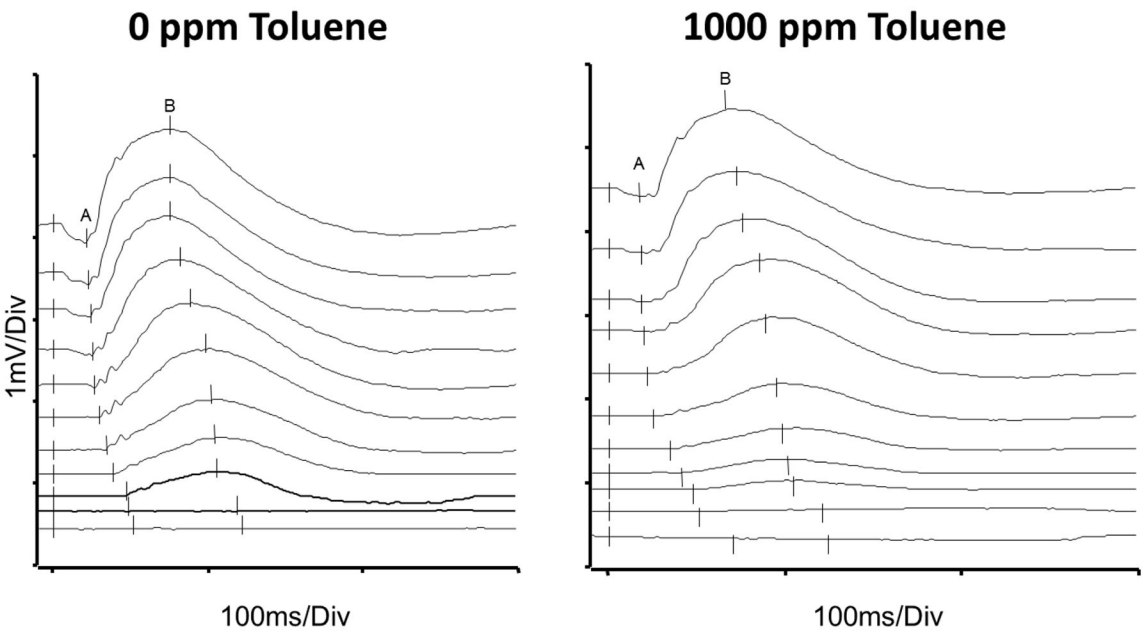

FIGURE 2.

Sample electroretinograms from two individual rats treated for 13 weeks with 0 ppm (left panel) or 1000 ppm (right panel) toluene. ERGs are shown from the dark-adapted intensity series progressing from dim (bottom trace) to bright (top trace) flash stimuli. The a and b-waves are identified on the top trace. The latencies of both peaks decreased and the amplitudes increased with progressively brighter stimuli.

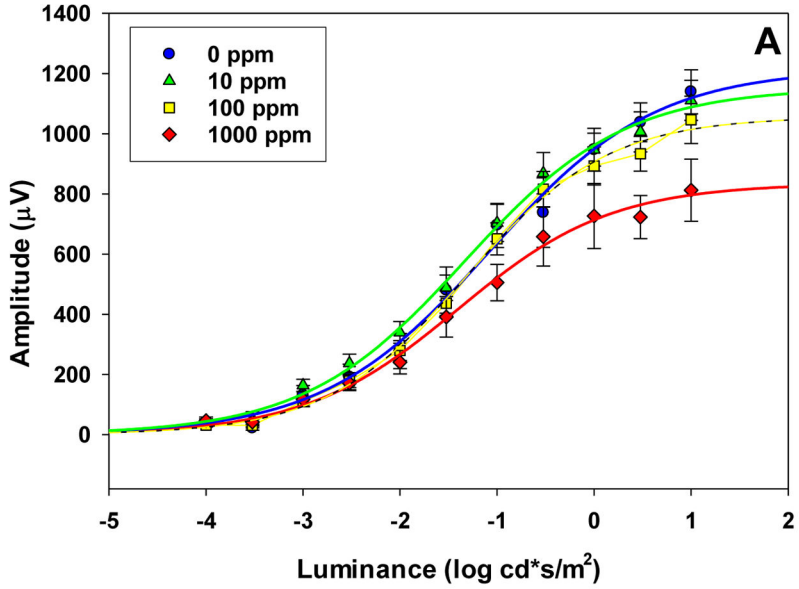

Figure 3a

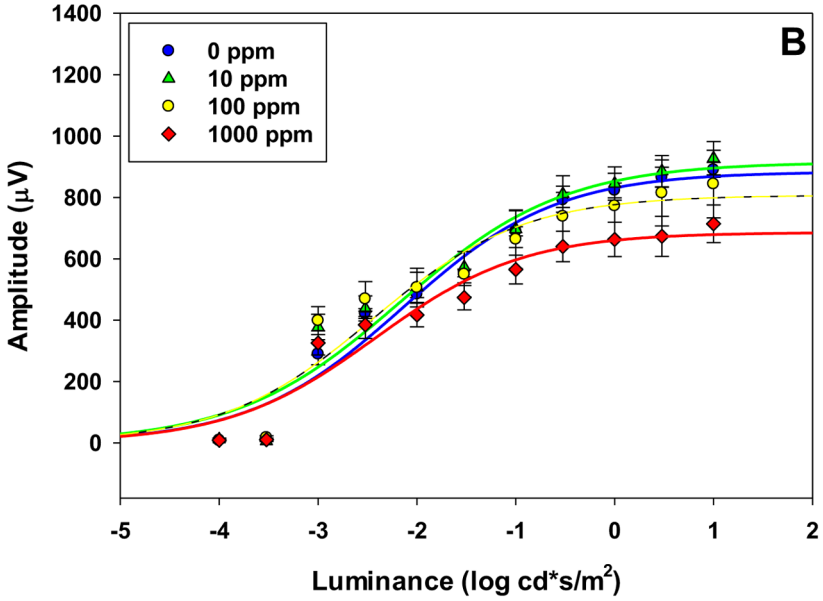

Figure 3b

FIGURE 3.

Panel A Dark-adapted b-wave ERG amplitudes as a function of log flash luminance in groups of rats treated for 13 weeks with 0 (n=9), 10 (n=10), 100 (n=9) or 1000 (n=9) ppm toluene. ERGs were recorded 5–6 weeks after the termination of exposure. There was a statistically significant reduction of ERG amplitudes at the higher flash luminance values in rats treated with 1000 ppm toluene. **Panel B**. Dark-adapted b-wave ERG amplitudes in groups of rats treated for 13 weeks with 0, 10, 100 or 1000 ppm toluene and recorded approximately 1 year after the termination of exposure. There was a statistically significant

reduction of ERG amplitudes at the higher flash luminance values in rats treated with 1000 ppm toluene.

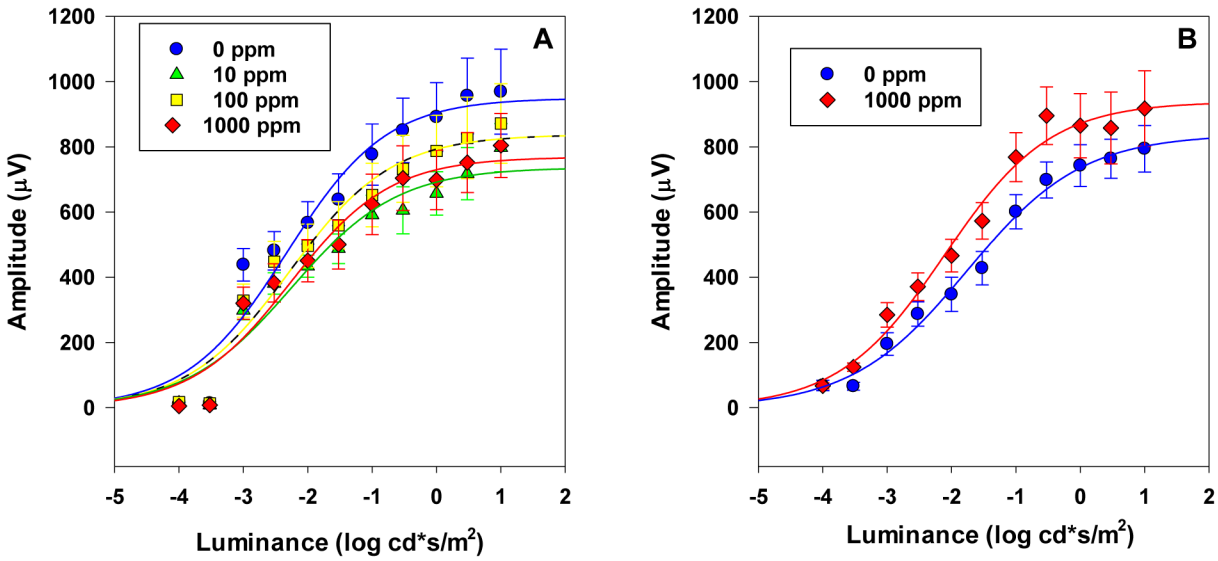

Figure 4.

Panel A Dark-adapted b-wave ERG amplitudes as a function of log flash luminance in groups of rats treated for 4 weeks with 1, 10, 100 or 1000 ppm toluene and recorded approximately 2–3 weeks after the termination of exposure. There was no statistically significant reduction of ERG amplitudes. **Panel B.** Dark-adapted b-wave amplitudes in groups of rats treated for 4 weeks with 0 or 1000 ppm toluene and recorded approximately 1 year after the termination of exposure. No significant difference between ERG amplitudes of toluene-exposed and control rats was seen. Plotted values are +/− SEM. The lines are Naka-Rushton functions fit to the group mean data as described in the Methods Section.

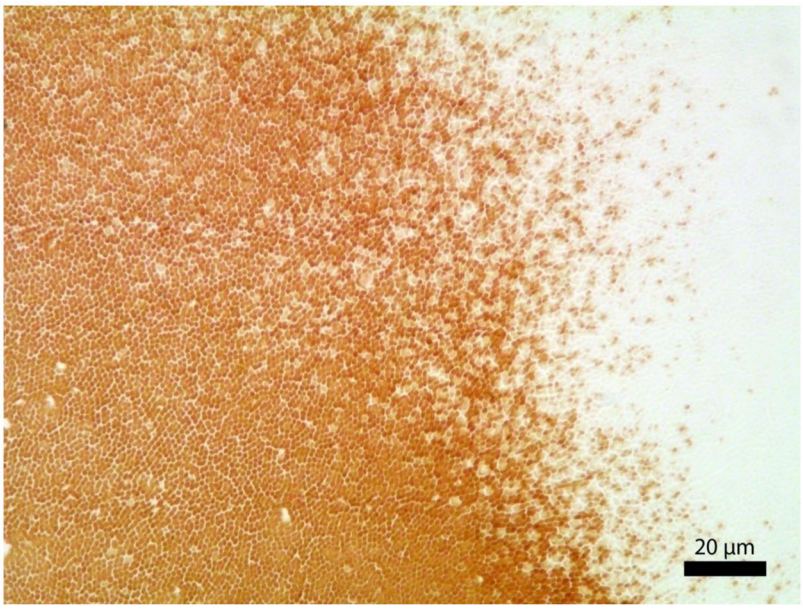

FIGURE 5A

Figure 5a

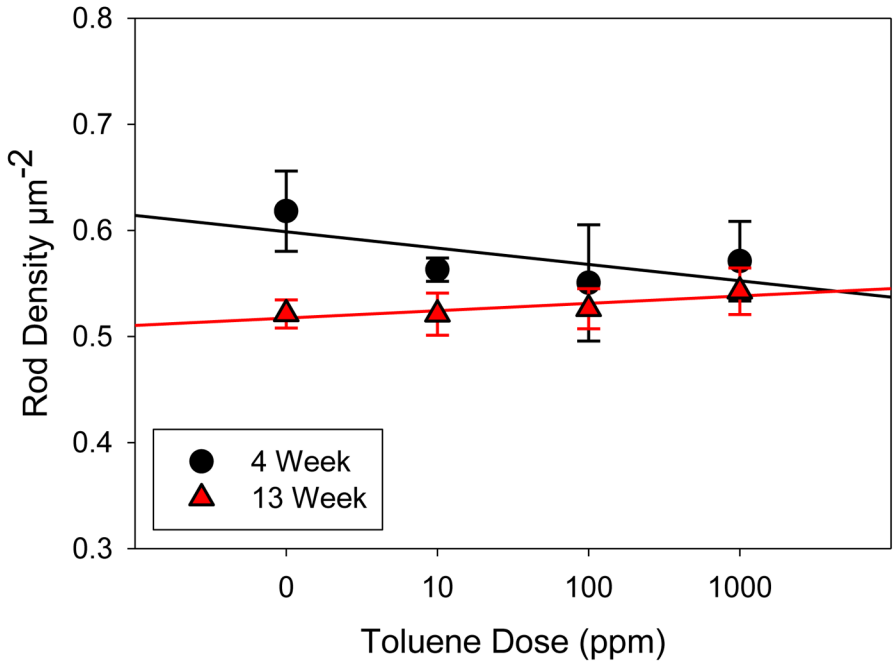

FIGURE 5B

Figure 5b

Figure 5.

FIGURE 5A. Transverse section of the retina of one representative eye stained with an antibody for rhodopsin that labeled the outer segments of rod photoreceptors. The photoreceptor inner segments (lower left) are labeled with diaminobenzidine (DAB, brown

reaction product). The photoreceptor inner segments (upper right) are unlabeled. Scale bar = 10 μm.

FIGURE 5B. *Rod density vs. toluene dose.* The mean number of retinal rods / micron^2 was not significantly altered by treatment with toluene. Bars reflect mean +/− S.E.M.

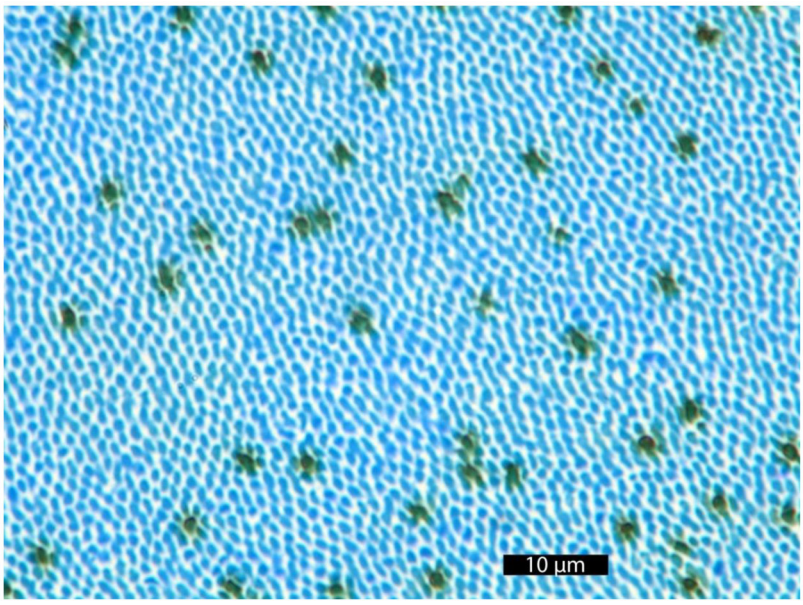

FIGURE 6A

Figure 6a

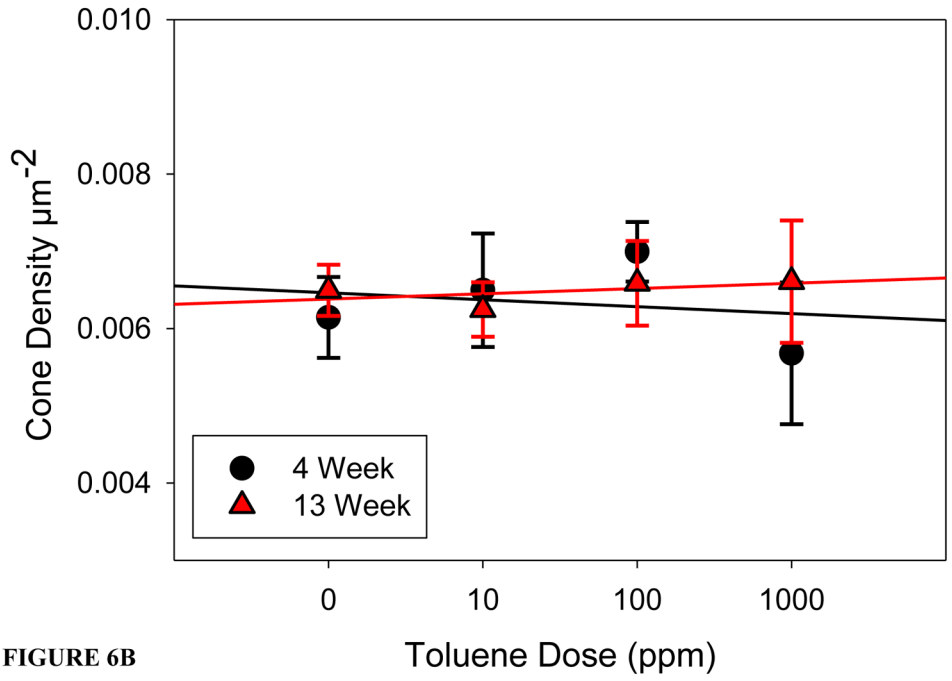

FIGURE 6B

Figure 6b

Figure 6.

FIGURE 6A. Transverse section at the level of the photoreceptor outer segments of the retina of one representative eye stained with an antibody for M-cone opsin (diaminobenzidine, DAB, brown reaction product). The rods are counterstained with toluidine blue. Scale bar = 10 µm.

FIGURE 6B. *M-cone density vs. toluene dose.* The mean number of retinal m-cones / micron^2 was not significantly altered by treatment with toluene. Bars reflect mean +/− S.E.M