The sensitivity of light-evoked responses of retinal ganglion cells is decreased in nitric oxide synthase gene knockout mice

Guo-Yong Wang

Department of Structural and Cellular Biology, School of Medicine, Tulane University, New Orleans, USA

Deborah A. van der List

Department of Neurobiology, Physiology & Behavior, Division of Biological Sciences, University of California, Davis, USA

Joseph P. Nemargut

Department of Structural and Cellular Biology, School of Medicine, Tulane University, New Orleans, USA

Julie L. Coombs

Department of Neurobiology, Physiology & Behavior, Division of Biological Sciences and Ophthalmology Department, School of Medicine, University of California, Davis, USA

Leo M. Chalupa

We have shown previously that increasing the production of nitric oxide (NO) results in a dampening of visual responses of retinal ganglion cells (G. Y. Wang, L. C. Liets, & L. M. Chalupa, 2003). To gain further insights into the role of NO in retinal function, we made whole-cell patch clamp recordings from ganglion cells of neural type nitric oxide synthase (nNOS) gene knockout mice. Here we show that in the dark-adapted state, the sensitivity of retinal ganglion cell to light stimulation is decreased in nNOS knockout animals. The lowest light intensities required to evoke optimal responses and the average intensities that evoked half-maximal responses were significantly higher in nNOS knockouts than in normal mice. Retinal histology and other features of light-evoked responses of ganglion cells in nNOS mice appeared to be indistinguishable from those of normal mice. Collectively, these results, in conjunction with our previous work, provide evidence that increasing levels of NO dampen visual responses of ganglion cells, while a lack of nNOS decreases the sensitivity of these neurons to light. Thus, NO levels in the retina are capable of modulating the information that ganglion cells convey to the visual centers of the brain.

Keywords: retinal ganglion cell, neural type nitric oxide synthase (nNOS), visual response, nitric oxide, patch clamp recording


Introduction

Nitric oxide (NO) is synthesized from l-arginine by nitric oxide synthase (NOS; Deguchi & Yoshioka, 1982; Palmer, Rees, Ashton, & Moncada, 1988), and in the retina NOS has been localized to different cell types, including pigment epithelium cells (Bredt, Hwang, & Snyder, 1990; Goureau, Lepoivre, Becquet, & Courtois, 1993), Müller cells (Liepe, Stone, Koistinaho, & Copenhagen, 1994), amacrine and ganglion cells (Dawson, Bredt, Fotuhi, Hwang, & Snyder, 1991; Koistinaho, Swanson, de Vente, & Sagar, 1993; Yamamoto, Bredt, Snyder, & Stone, 1993), as well as photoreceptors (Yamamoto et al., 1993). Given the diversity of cell types that appear capable of synthesizing NO, it might be expected that NO has diverse roles. The available evidence indicates that this is indeed the case. NO has been implicated in diverse retinal functions, ranging from transduction to the gating of the output signal (Goldstein, Ostwald, & Roth, 1996). Most studies have dealt with the modulation by NO of membrane conductances in dissociated retinal cells, so as yet little is known about the effects
of NO on light-evoked responses of retinal ganglion cells (RGCs) in the intact retina.

There is considerable evidence implicating NO in signal processing at different levels of the visual system (reviews: Cudeiro & Rivadulla, 1999; Goldstein et al., 1996). For instance, NO has been reported to modulate voltage-gated ion channels in rods and cyclic-nucleotide-gated channels in both rods and cones (Kurenny & Barnes, 1994; Rieke & Schwartz, 1994; Savchenko, Barnes, & Kramer, 1997). NO has also been found to decrease electrical coupling in horizontal cells (DeVries & Schwartz, 1989; Miyachi, Murakami, & Nakaki, 1990; Xin & Bloomfield, 2000), to modulate cGMP levels in bipolar cells (Nawy & Jahr, 1990, 1991; Shiells & Falk, 1990), and to reduce coupling between AII amacrine cells and On-cone bipolar cells (Mills & Massey, 1995). In ganglion cells, NO donors have been shown to modulate cGMP-gated conductances (Ahmad et al., 1994; Kawa & Sterling, 2002) and to increase the amplitude of N-type calcium currents (Hirooka, Kourennyi, & Barnes, 2000). A recent study has also demonstrated that NO can modulate the effects of GABA and glycine to impact retinal inhibitory interactions (Yu & Eldred, 2005).

Recently, we showed that increasing retinal NO production by bath application of L-arginine, the NO precursor, dampens visual responses of retinal ganglion cells by reducing the peak discharge rates of On responses and selectively blocking Off responses mediated by the APB-sensitive rod-Off pathway (Wang, Liets, & Chalupa, 2003). To further explore the physiological function of NO in the retina, the effects of reducing NO production on light-evoked responses of retinal ganglion cells need to be studied. The neural type nitric oxide synthase (nNOS) knockout mouse (Huang, Dawson, Bredt, Snyder, & Fishman, 1993) provides a unique model for addressing this issue. In the present study, we made whole-cell patch clamp recordings from RGCs in nNOS knockout and wild-type mice in order to assess how the lack of nNOS affects the light-evoked responses of these retinal output neurons. Our results provide evidence that a lack of nNOS decreases the light sensitivity of ganglion cells in the dark-adapted retina.

**Methods**

**Animals**

All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were in compliance with National Institutes of Health guidelines, and also were approved by the campus animal use committees of University of California, Davis, and Tulane University. Wild-type (C57/BL) and neural type nitric oxide synthase (nNOS) knockout mice were obtained from The Jackson Laboratory (Bar Harbor, Maine), ranging in age from 4 to 5 months.

The basic methods in this study were similar to those we employed in previous studies of ferret and mouse retinas (Wang, 2006; Wang, Liets, & Chalupa, 2001, 2003). Animals were dark-adapted overnight prior to the experiments and all procedures, including animal surgery, dissection of retinas, and recordings from cells were made in complete darkness. Infrared goggles were used to visualize the tissue on the dissecting and recording microscopes and to maneuver in the recording room. LEDs (850 nm) were used to provide light to the dissecting microscope while the illumination from the recording microscope was passed through an ≥850-nm cut-filter.

**Retinal preparation**

Following a lethal dose of barbiturate (Nembutal 200 mg/kg i.p.), the eyes were removed and placed in oxygenated L15 at 37°C for 12 min. The retinas were then carefully peeled from the eyecup and stored at room temperature in Eagle’s minimal essential medium (EMEM), continuously bubbled with 95% O2 and 5% CO2. A small piece of retina was placed ganglion cell layer up in the recording chamber and stabilized with an overlying piece of filter paper. A 2-mm hole in the filter paper provided access for the recording electrode. Cells were visualized through a 40× objective mounted on an upright epifluorescence microscope (Nikon).

During recording, the retina was perfused continuously with EMEM (1.5 ml/min) through a gravity fed line, heated with a Peltier device, and continuously bubbled with 95% O2 and 5% CO2. A calibrated thermocouple monitored the temperature in the recording chamber, maintained at 35°C. Recordings from individual cells usually lasted 30–120 min, and retinal segments from which recordings were made typically remained viable for 8–12 hours. Patch electrodes were filled with a solution containing (in mM): KCl, 140; HEPES, 10; EGTA, 0.5; 0.5 mg/ml Nystatin; 2.5 mg/ml Pluronic F-68; 0.5% Lucifer Yellow; pH 7.4. There were no differences in the results obtained with and without nystatin and pluronic, although the use of these chemicals permitted stable recordings for longer time periods (Robinson & Chalupa, 1997; Wang, Ratto, Bisti, & Chalupa, 1997). Usually within 5 min after the high-resistance seals were obtained, the cells were filled with Lucifer Yellow, indicating the whole-cell configuration was formed with Nystatin in the electrode solution. By the end of the experiment, the soma and the dendritic arboreations were usually completely filled. Once complete filling was achieved, the retina was removed and fixed in 4% paraformaldehyde for 6–8 hours at 4°C.

**Electrophysiology**

Whole-cell current clamp recordings were made from retinal ganglion cells in the dark-adapted retinas. Patch
pipettes with a tip resistance of 3 and 7 MΩ were pulled from thick-walled 1.5-mm OD borosilicate glass on a Sutter Instruments puller (P-97). Current clamp recordings were made with an Axopatch 200B patch clamp amplifier. The data were low-pass filtered at rates between 1 and 2 kHz and digitized at rates between 1 and 4 kHz before storage on a computer for subsequent off-line analysis. To attain whole-cell access, the vitreous and the outer limiting membrane overlying the recording area were removed by gently brushing the retinal surface with the tip of a glass pipette. Recordings were obtained by patching onto cells with clear, non-granular cytoplasm. High-resistance seals were obtained by moving the patch electrode onto the cell membrane and applying gentle suction. After formation of a high-resistance seal between the electrode and the cell membrane, transient currents caused by pipette capacitance were electronically compensated by the circuit of the Axopatch 200B amplifier. Recordings from cells with a seal resistance <1 GΩ were discarded. The series resistance was 7–16 MΩ. After attaining a whole-cell configuration the resting membrane potential was read off the amplifier. The value of the resting potential was monitored regularly throughout the recording, and if significant changes were observed, the recording was terminated. Input resistance was calculated by using Ohm’s law from the voltage obtained to hyperpolarizing current pulses (−10 to −20 pA) from a holding potential of −60 mV. There were no significant differences in the input resistances between the wild-type and the nNOS knockout animals. The mean value of the input resistance was 455 ± 170 (SD) MΩ (n = 24) and 470 ± 195 MΩ (n = 22) for the wild-type and the nNOS mice, respectively.

**Light stimulus**

Light-evoked responses were obtained by delivering spots of light to the retina from a 1-inch-diameter computer monitor, with a green (P43, 545 nm light) phosphor (Lucivid MR1-103; MicroBrightField, Colchester, VT), through the camera port of the microscope (Demb, Haarsma, Freed, & Sterling, 1999). A spot of light, ranging in diameter from 200 to 500 μm, was centered on the soma and flashed for 2 s. The “best” spot size for a given cell was the one that evoked the maximum number of discharges. Using the best spot size, different light intensities were used to test the functional properties of On, Off, and On–Off cells. Within the spot size range, we did not find significant differences in the best spot sizes between the wild-type and the nNOS knockout, nor were there significant differences between the two groups of animals in the spot sizes used for optimal responses of On, Off, and On–Off cells. It should be noted, however, that the diameters of the spot sizes we employed may not have covered the entire receptive field of some cells studied. Stimuli were delivered once every 20 s to limit light adaptation (Xin & Bloomfield, 1999). The stimuli were programmed in Matlab (Math Works, Natick, MA), using the Psychophysics Toolbox extensions (Brainard, 1997; Pelli, 1997). The intensity of a spot of light was calibrated with a spectroradiometer/photometer (Photo Research, PR703-A) and expressed in terms of photons per μm² per second (photons μm⁻² s⁻¹). The instrument was calibrated relative to standards of the National Institute of Standards and Technology. Recordings were made in complete darkness.

**Drug application**

DL-2-Amino-phosphonobutyric acid (APB, Calbiochem, 50 μM or 100 μM), L-arginine (1 mM), and nitro-L-arginine methyl ester (L-NAME 100 μM) were freshly dissolved in EMEM on the day of the experiment and administered through a gravity fed line. A six-position rotary valve (Western Analytical Products) was used to switch between bath and drugs solutions. The washout time for chemicals usually took 6 to 20 min.

**Immunohistochemistry**

Wild-type and nNOS knockout mice were euthanized by IP injection of sodium pentobarbital (Western Medical Supply Inc., Arcadia, CA), then perfused with 0.1 M PBS followed by 4% paraformaldehyde in PBS. After enucleation, the eyes were hemisected and post-fixed for 1 hour in 4% paraformaldehyde. The eyes were washed in PBS then cryoprotected in 25% sucrose solution overnight. Each eye was then embedded in OCT (Ted Pella, Torrence, CA) and sections were cut at 10 μm on a Leica (Deerfield, IL) cryostat. All sections were incubated in blocking solution containing 10% normal serum (donkey or goat), 2% bovine serum albumin, and 0.3% Triton X-100 in 0.1 M phosphate buffer for 1 hour at room temperature. The sections were then incubated with diluted primary antibodies overnight at 4°C. For control slides, primary antibodies were omitted. Following several changes of PBS, the sections were incubated with fluorescent secondary antibodies CY3 (1:500; Jackson Immuno Research, West Grove, PA) or Texas red (1:500; Molecular Probes, Eugene, OR) in PBS for 45 min. After several washes in PBS, the sections were counterstained with SYBR green II (0.5×, Molecular Probes) in PBS for 2 min or DAPI (1:500; KPL, Gaithersburg, MD), briefly washed in PBS, and then coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

**Retrograde labeling of ganglion cells**

Small crystals of DiI (D-282, Molecular Probes) were placed on the optic nerve stumps. Retinas were subsequently
isolated with the optic nerve stump still attached, flattened on a glass slide, and held in place by coverslips mounted on silicone grease posts. The slide was then placed in 4% PFA and incubated at 37°C for 1–2 days. The tissue was then blocked in 3% agar and cut at 50-µm sections on a vibratome mounted in PBS and imaged.

**Antibodies**

The following antibodies were used to identify different classes of retinal neurons: rabbit anti-NOS antibody (1:75, Chemicon, Temecula, CA), goat anti-choline acetyltransferase (ChAT, 1:50, Chemicon, Temecula, CA), monoclonal anti-calbindin (1:500, Swant, Bellinzona, Switzerland), rabbit anti-parvalbumin (1:1000, Swant, Bellinzona, Switzerland), rabbit anti-recoverin (1:1000, a gift from Dr. Alexander Dizhoor Wayne State University, Detroit, MI), and rabbit anti-protein kinase C (PKC 1:1000, Research Diagnostics Inc., Flanders, NJ).

**Imaging**

All images were acquired on Olympus FV300 and FV500 series confocal microscopes (Tokyo, Japan), equipped with an argon and krypton lasers and 405 diode. Image stacks were collected on the z axis and steps were made at the optimal voxel distance for plan apochromatic ×40 lens. Stack depths were from 5 to 8 µm using and a pixel resolution of 1024 × 1024. For enhanced clarity, DAPI was pseudo colored green using Olympus Fluoview software. The contrast and brightness were enhanced using Adobe Photoshop (Mountain View, CA).

**Results**

**Light-evoked response patterns are similar in nNOS knockout and wild-type RGCs**

On, Off, and On–Off response patterns observed in different retinal ganglion cells of wild-type mice were also found in the nNOS knockouts. Moreover, the incidence of RGCs that manifested each response pattern was also not appreciably different in the two groups of mice. Thus, in wild-type animals, recordings were obtained from 59 On cells, 31 Off cells, and 17 On–Off cells, while in the knockouts there were 57 On cells, 23 Off cells, and 20 On–Off cells. Examples of the three major response types from the wild-type and knockout retinas are shown in Figure 1.

The magnitude of On and Off responses to each light stimulation in the wild-type and knockout RGCs is...
depicted as average peak firing rates at light onset or offset (Figure 2, top panel). For each stimulus, the average peak firing rate was calculated by counting the number of spikes within a window that encompassed the highest firing rate and dividing the spike number by the duration of the window. The width of the window was determined by the time period that most clustered spikes occurred and varied from cell to cell (Wang, 2006; Wang et al., 2003). The window ending points generally corresponded to the time point where the frequency dropped by 15% of its highest frequency. By using different spot sizes and intensities of light stimuli, the optimal response of each cell was determined. The optimal response level was characterized as the highest average peak firing rate. As may be seen, the average peak firing rates of optimal On and Off responses in the nNOS knockouts are very similar to those of wild-type retinas. The average peak firing rates of On responses (from On cells and On–Off cells) were $220 \pm 35$ spikes/s (mean $\pm SD$, $n = 76$) and $183 \pm 44$ spikes/s ($n = 77$) for the wild-type and nNOS knockout retinas, respectively. The average peak firing rates of Off responses (from Off cells and On–Off cells) were $195 \pm 55$ spikes/s ($n = 48$) and $165 \pm 46$ spikes/s ($n = 43$) for the wild-type and nNOS knockout retinas, respectively.

The average peak firing rates of the optimal responses for the wild-type and nNOS knockout retinas were not significantly different ($p > 0.05$; Figure 2, top panel). Furthermore, the latency distributions of the optimal responses of the nNOS knockouts were found to be indistinguishable from those of the wild-type retinas (Figure 2, middle and bottom panels). Response latency was measured from the onset or offset of the light stimulus to the onset of membrane depolarization giving rise to the spike discharges of the response (indicated by arrows in Figure 1). Note that there appear to be two distributions of Off latencies (Figure 2, bottom panel). The peak of the first distribution is around $60–70$ ms, and the peak of the second distribution is about $100–150$ ms. These two latency distributions may reflect the APB-sensitive and -insensitive rod-Off pathways. We have previously shown that the Off response latencies of the APB-insensitive rod-Off pathways were shorter than those of the APB-sensitive rod-Off pathway (Wang, 2006).

**Differences in visual sensitivity**

The lowest light intensities required to evoke optimal responses were found to be significantly higher in nNOS knockout than in wild-type RGCs. This is evident in the distributions of On and Off responses as a function of light intensities shown in Figures 3A and 3B, respectively. The response curves for both On and Off cells obtained from the nNOS knockouts are shifted to the right of those obtained for the wild-type cells. The average lowest light intensity to evoke optimal On responses was $9 \pm 4.6 \times 10^2$ photons $\mu m^{-2} s^{-1}$ (mean $\pm SD$, $n = 21$) for wild-type and $60 \pm 32 \times 10^3$ photons $\mu m^{-2} s^{-1}$ ($n = 15$) for nNOS knockout retinas, respectively. For Off responses, it was $2.5 \pm 1.3 \times 10^3$ photons $\mu m^{-2} s^{-1}$ ($n = 18$) and $35 \pm 18 \times 10^3$ photons $\mu m^{-2} s^{-1}$ ($n = 12$) for the wild-type and nNOS knockout mice retinas. As may be seen, the average peak firing rates of the optimal On and Off responses in the nNOS knockouts are very similar to those in the wild-type retina. There is no significant difference of average peak firing rate in the optimal visual responses between the wild-type and nNOS knockout retina. *Middle panel:* The distributions of the optimal On responses as a function of the On latencies from the wild-type and nNOS knockout mice retinas. It is clearly showed that the distribution pattern between the wild-type and the nNOS knockout mice are indistinguishable. *Bottom panel:* The distributions of the optimal Off responses as a function of the Off latencies from the wild-type and nNOS knockout mice retinas. Again, the distribution pattern of the wild-type mice is indistinguishable from that of the nNOS knockout mice.
nNOS knockouts, respectively. These differences are statistically significant (Figure 3C, \( p < 0.05 \), two-tailed \( t \)-test).

To further analyze the intensity–response functions of different cells, the peak firing rates of each cell were normalized and plotted as a function of light intensity. The data points were then fitted with Michaelis–Menten equation (Baylor, Hodgkin, & Lamb, 1974; Naka & Rushton, 1966; Thibos & Werblin, 1978) as follows:

\[
R = \frac{R_{\text{max}} N}{I + \sigma^N}
\]

(1)

where \( R \) represents the measured response, \( R_{\text{max}} \) represents the maximum response (optimal response), \( I \) indicates stimulus intensity, \( \sigma \) indicates the intensity that evokes a half-maximal response, and \( N \) is the Hill coefficient. Two examples of the fitting from the cells of the wild-type and nNOS knockout are shown in Figure 4A.

The intensity \( (\sigma) \) that evoked a half-maximal response of each cell was obtained from the fitting. The average intensities that evoked half-maximal responses of On responses were 757.8 ± 265 photons \( \mu m^{-2} s^{-1} \) (mean ± SD, \( n = 21 \)) and 1740.4 ± 430 photons \( \mu m^{-2} s^{-1} \) (\( n = 15 \)) in the wild-type and nNOS knockout retinas, respectively. The average Hill coefficients were \( 1.91 \pm 0.6 \) (\( n = 21 \)) and \( 1.6 \pm 0.26 \) (\( n = 15 \)) for the On responses of the wild-type and nNOS knockout retinas, respectively. With Off responses, the average intensities that evoked half-maximal responses were 164 ± 20 photons \( \mu m^{-2} s^{-1} \) (\( n = 18 \)) and 268 ± 41 photons \( \mu m^{-2} s^{-1} \) (\( n = 12 \)) in the wild-type and nNOS knockout retinas, respectively. The average Hill coefficients were \( 2.57 \pm 0.58 \) (\( n = 18 \)) and \( 1.9 \pm 0.46 \) (\( n = 12 \)) for the Off responses of the wild-type and nNOS knockout retinas, respectively. The average intensities that evoked half-maximal responses were significantly higher in cells of the nNOS knockout retinas (Figure 4B, \( p < 0.05 \), two-tailed \( t \)-test).

**Functional state of different signal pathways in nNOS knockout mouse retina**

To test whether different rod signal pathways are functional in the nNOS knockout retinas, DL-2-amino-phosphonobutyric (APB) was used to differentiate different signal pathways. In the dark-adapted retina, two APB-sensitive pathways convey light increments. APB blocks these two pathways (reviewed by Sharpe & Stockman, 1999). By contrast, in the dark-adapted retina, one APB-sensitive and two APB-insensitive rod Off-pathways process light decrements. We found that the APB-sensitive rod-On pathways, the APB-sensitive rod-Off pathways, and the APB-insensitive rod-Off pathways were all functional in the dark-adapted nNOS knockout retina. All On responses (\( n = 34 \)) were blocked by APB (25 \( \mu M \)). This drug also blocked the majority of Off responses tested (22 of 39, the Off responses were from Off cells and On–Off cells). Examples of recordings from these pathways in the nNOS knockouts retinas are shown in Figure 5.
The effects of NO precursor and NOS inhibitor on the light-evoked responses of retinal ganglion cells in the dark-adapted wild-type mouse retina

The NO precursor, L-arginine (1 mM), decreased On responses of ganglion cells in the wild-type mice retinas, but L-arginine had little effect on the On responses of ganglion cells in the nNOS knockout retinas. In the wild-type retinas, L-arginine significantly decreased the peaking firing from 196 ± 53 to 112 ± 46 spikes/s (mean ± SD, n = 6, p < 0.05, two-tailed t-test). Examples of the effects of L-arginine on the light-evoked responses of ganglion cells from the wild-type and the nNOS knockout retinas are shown in Figure 6.

We also tested the effects of a NOS inhibitor on the light-evoked responses of wild-type mouse retinal ganglion cells. An example of the effect of nitro-L-arginine methyl ester (L-NAME), NOS inhibitor, on the light-evoked responses of a retinal ganglion cell in the dark-adapted wild-type retina is shown in Figure 4 (A).

Figure 4. (A) The response–intensity relations of two On cells from the wild-type and the nNOS knockout retina, respectively. The data sets were fitted with Michaelis–Menten equation. The curve of the On cell from nNOS knockout shifted to the right of the curve of the On cell from wild-type. (B) The average light intensities that evoke half-maximal response were much higher for On (left panel) and Off (right panel) responses in nNOS knockout retinas than that in wild-type retinas. The differences between the nNOS and the wild-type retinas were significant (*p < 0.05 compared with wild-type).

The effects of NO precursor and NOS inhibitor on the light-evoked responses of retinal ganglion cells in the dark-adapted wild-type mouse retina

The NO precursor, L-arginine (1 mM), decreased On responses of ganglion cells in the wild-type mice retinas, but L-arginine had little effect on the On responses of ganglion cells in the nNOS knockout retinas. In the wild-type retinas, L-arginine significantly decreased the peaking firing from 196 ± 53 to 112 ± 46 spikes/s (mean ± SD, n = 6, p < 0.05, two-tailed t-test). In the nNOS knockout retinas, the peak firing rates before and after L-arginine application were 177 ± 41 spikes/s and 163 ± 48 spikes/s, respectively (n = 5, p > 0.05, two-tailed t-test). Examples of the effects of L-arginine on the light-evoked responses of ganglion cells from the wild-type and the nNOS knockout retinas are shown in Figure 6.

We also tested the effects of a NOS inhibitor on the light-evoked responses of wild-type mouse retinal ganglion cells. An example of the effect of nitro-L-arginine methyl ester (L-NAME), NOS inhibitor, on the light-evoked responses of a retinal ganglion cell in the dark-adapted wild-type retina is shown in Figure 4 (A).

Figure 4. (A) The response–intensity relations of two On cells from the wild-type and the nNOS knockout retina, respectively. The data sets were fitted with Michaelis–Menten equation. The curve of the On cell from nNOS knockout shifted to the right of the curve of the On cell from wild-type. (B) The average light intensities that evoke half-maximal response were much higher for On (left panel) and Off (right panel) responses in nNOS knockout retinas than that in wild-type retinas. The differences between the nNOS and the wild-type retinas were significant (*p < 0.05 compared with wild-type).

Figure 5. Visual responses of three ganglion cells from the nNOS knockout mice show examples of the three rod signal pathways, APB-sensitive On, APB-sensitive Off, and APB-insensitive Off pathways, that are functional in the dark-adapted nNOS knockout retinas. (A) Recordings from an On cell, the On response was blocked by APB (50 μM) application, suggesting APB-sensitive On pathway was involved. (B) Off responses from an Off cell, the Off response was blocked by APB (50 μM) application, manifesting the APB-sensitive Off pathway underlie the Off response. (C) Visual responses from an Off retinal ganglion cell, APB (100 μM) application did not block the Off response, indicating that the responses were conveyed via the APB-insensitive rod Off pathway.
mouse retina is shown in Figure 7. After bath application of L-NAME (100 µM), the response–intensity curve of the ganglion cell was shifted to the right of its control curve. Similar results were found in 5 cells (3 On cells and 2 Off cells). These findings indicate that the L-NAME, NOS inhibitor, has similar effects as knockout nNOS on the light-evoked responses of ganglion cells.

Histology of the retinas from nNOS knockout mice

We also compared the structural organization of nNOS knockout and wild-type retinas. In transverse sections wild-type and nNOS knockout retinas were indistinguishable with respect to the thickness, appearance, and arrangement of cellular elements in the three retinal layers (Figure 8A, wild-type, and Figure 8B, nNOS knockout). nNOS was identified by immunostaining with antibody to nNOS. The staining demonstrated that in the wild-type retinas the anti-nNOS reactive amacrine cells were sparsely populated in the INL. Moreover, lightly immunoreactive dendritic processes were also found in the IPL of the wild-type retinas. These observations are similar to previous reports (Haverkamp & Wässle, 2000; May & Mittag, 2004). However, nNOS antibody reactivity was completely absent in the retinas of nNOS knockout mice (Figure 8C, wild-type, and Figure 8D, nNOS knockout).

To examine the structural details of the nNOS knockout retinas, different antibodies were employed. The goat anticholine acetyltransferase labeled starburst amacrine cells and processes. The results resembled those described previously (Haverkamp & Wässle, 2000; Kang, Ryu, Kim, Oh, & Chun, 2004; Schmidt, Wässle, & Humphrey, 1985; Tauchi & Masland, 1984; Voigt, 1986) with cell bodies distributed in the INL and displaced cells within the ganglion cell layer. Two distinct bands were evident within the IPL (Figure 8E, wild-type, and Figure 8F, nNOS knockout). The monoclonal antibody anti-calbindin (Swant, Bellinzona, Switzerland) was used to label horizontal cells (Figure 8G, wild-type, and Figure 8H, nNOS knockout). Antibodies to protein kinase C (PKC) labeled rod bipolar cells. Cell bodies within the INL, descending axons, and terminals within the innermost IPL were strongly labeled (Figure 9A, wild-type, and Figure 9B, nNOS knockout). Rabbit anti recoverin (a generous gift from Dr. Alexander Dizhoor Wayne State University, Detroit, MI) strongly labeled photoreceptors and OFF cone bipolar cells (Haverkamp, Ghosh, Hirano, & Wässle, 2003). Bushy terminals were seen in the outer third of the IPL with recoverin staining (Figure 9C, wild-type, and Figure 9D, nNOS knockout). Rabbit anti parvalbumin
(Swant, Bellinzona, Switzerland) stained AII amacrine cells and α-like ganglion cells with primary dendrites in the IPL of both the wild-type and nNOS knockout retinas (Casini, Rickman, & Brecha, 1995; Wässle, Grünert, & Röhrenbeck, 1993; Figure 9E, wild-type, and Figure 9F, nNOS knockout). Thus, for all the antibodies we employed

Figure 8. The nNOS knockout retinas (right column) and wild-type retinas (left column) were compared by light microscopy and by immunostaining. (A and B) The transverse sections show that the wild-type and nNOS knockout retinas were indistinguishable in the thickness, in the appearance, and arrangement of cellular elements in each of the three retinal layers. (C and D) The confocal images of retinal transverse sections staining with rabbit anti-nNOS antibody in wild-type (C) and nNOS knockout (D) retinas, respectively. SYBR-green II was used to stain the nuclei (green). The INL in wild-type retina is sparsely populated with anti-nNOS reactive amacrine cells (red). There are also immuno reactive dendritic processes in the IPL. These cells and processes are absent in the nNOS knockout retina. (E and F) The goat anti-choline acetyltransferase labeled starburst amacrine cells and processes. The cell bodies distributed in the INL and displaced cells within the ganglion cell layer. Two distinct bands are seen within the IPL. (G and H) The monoclonal antibody anti calbindin labeled horizontal cells. DAPI was used to stain the nuclei (pseudo colored green). The patterns of labeling were indistinguishable between the wild-type and nNOS knockout retinas. Scale bar: 50 μm.

Figure 9. Confocal images were taken from the transverse sections of the wild-type (left column) and nNOS knockout (right column) retinas, respectively. (A and B) Antibodies to protein kinase C (PKC) labeled rod bipolar cells. (C and D) Rabbit anti recoverin strongly labeled photoreceptors and Off cone bipolar cells. Bushy terminals were seen in the outer third of the IPL with recoverin staining. (E and F) Rabbit anti parvalbumin stained amacrine cells and α-like ganglion cells with primary dendrites seen in the IPL in both the wild-type (E) and nNOS knockout (F) retinas. (G and H) Dil was imbedded into optic nerves to retrograde label ganglion cells. The gross patterns of dendritic stratification of retinal ganglion cells in IPL were revealed in the wild-type (G) and nNOS knockout (H) retinas. The patterns of each label in nNOS knockout retinas appear normal. Scale bar: 50 μm.
the labeling patterns in the retinas of wild-type and nNOS knockout mice appeared not to be appreciably different.

The dendritic stratification of RGCs in the wild-type and nNOS knockout mice was also compared. As may be seen in Figure 9G, wild-type, and Figure 9H, in the nNOS knockout, the pattern of dendritic stratification of retinal ganglion cells was indistinguishable between the wild-type and nNOS knockout mice.

Discussion

In a previous study, we demonstrated that increasing NO production can have a powerful effect on the visual responses of retinal output neurons. In all but a small proportion of ganglion cells (86%) NO dampened visual responses, and the Off discharges in the APB-sensitive rod-Off pathway were completely blocked by the actions of NO in the ferret retina (Wang et al., 2003). Similar results were found in the wild-type mouse (Wang, 2006; Figure 6). Therefore, we surmised that reducing or eliminating NO production would enhance the visual responses of retinal ganglion cells. Contrary to this expectation, the results of the present study showed that lack of nNOS reduced the sensitivity of RGCs to light. The lowest light intensities required to evoke optimal responses and the average intensities that evoked half-maximal responses were found to be significantly higher in the nNOS knockouts than that in the wild-type retina. At the same time, the optimal responses of RGCs to optimal stimuli and rod signal pathways were found not to be disturbed in the nNOS knockouts.

Taken together these findings, in conjunction with the results of our earlier studies (Wang, 2006; Wang et al., 2003), indicate that the modulation by NO of visual signal processing in the retina is not linear. Rather, some optimal level of NO production appears to be required for elicitation of optimal visual responses of retinal ganglion cells. Either increases or decreases in NO from this presumed optimal level serves to dampen visual responses. This may also be the case at higher levels of the retino–geniculo–cortical pathway. For instance, in the dorsal lateral geniculate nucleus, blocking NO synthesis has been reported to reduce the visual responses of virtually all X and Y cells (Cudeiro et al., 1994). This effect could be reversed by application of L-arginine, but L-arginine administration alone had no appreciable effect on visual responses. In the cortex, manipulations of the NO system have been reported to produce reductions as well as enhancements of visual responses (Cudeiro et al., 1997).

It has been found that nNOS is not the only nitric oxide synthase expressed in retina; endothelial type nitric oxide synthase (eNOS) is also found in retina. How eNOS affects retinal function is unknown. It should be noted that in the present study, we focused on basic aspects of retinal function in dark-adapted nNOS knockout retina and its morphological structure. Other aspects of retinal function, such as light adaptation, direction selective, and receptive field, remain to be established in nNOS knockout retina. The optimal response properties, characterized with highest peak firing rate, were compared between the wild-type and nNOS knockout retina, because the optimal responses reflect the maximum capability of each ganglion cell to respond to visual input. In the dark-adapted retina, mixed rod/cone inputs have been found to be involved in signal processing of all amacrine cells (Xin & Bloomfield, 1999) and retinal ganglion cells (Pang, Gao, & Wu, 2003) when relative high levels of light intensities were used to stimulate the retina. The light intensities that evoked optimal responses in present study were within the range of 3–4 log unit of photons µm⁻² s⁻¹, indicating that mixed rod/cone inputs may be involved in the optimal responses.

The mechanisms that underlie the decreased sensitivity to light stimulation of RGCs in the nNOS knockouts are not known, but the available literature suggests several possibilities. For example, NO has been reported to be involved in visual signal transduction of photoreceptors (Goldstein et al., 1996). In particular, NO has been shown to modulate voltage-gated ion channels in rods and cyclic-nucleotide-gated channels, which are essential for light transduction, in both rods and cones (Kurenni & Barnes, 1994; Rieke & Schwartz, 1994; Savchenko et al., 1997). The lack of neuronal NO in the nNOS knockouts might alter the function of photoreceptors, thereby changing the sensitivity of rods to light stimulation. In the future, the functional state of rods in the nNOS knockout retinas needs to be studied.

Since NOS has been localized to neurons in all retinal layers (review: Goldstein et al., 1996), it is also possible that the effects described here in RGCs could reflect events at virtually all stages of synaptic transmission. If changes in the synaptic transmission of the signal pathways are the mechanisms for the reduced sensitivity of retinal ganglion cells to light in the nNOS knockout retinas, it would be expected that the sensitivity of selected signal pathways would be affected in the nNOS knockout retinas. However, the signal pathways of APB-sensitive rod-On, APB-sensitive rod-Off, and APB-insensitive rod-Off were found to be functionally normal in the nNOS knockout mice retinas. These results indicate that knockout of nNOS does not impair the basic functional organization of the retina.

The gross structural organization of the retina also appears normal in the nNOS animals. The thickness and the appearance as well as the arrangement of cellular elements in the three retinal layers of the nNOS knockout retinas were indistinguishable from those of the wild-type retina. The pattern of each immunohistochemical probe staining was also indistinguishable between the nNOS knockout and wild-type retinas. Moreover, dendritic stratification of retinal ganglion cells in the nNOS knockout retinas were similar to those of the wild-type retinas.
Thus, our results indicate that neural NO in the mammalian retina seems to have a surprisingly specific role in retinal signal processing. It remains for future studies to uncover the mechanism underlying the regulation of RGC response sensitivity by NO described here.

**Conclusion**

In summary, we found the histology of the retinas in the nNOS knockout mice to be normal. The light-evoked response patterns that were found in dark-adapted wildtype retinas were evident in nNOS knockout retinas. The rod signal pathways, including APB-sensitive On, APB-sensitive Off, and APB-insensitive Off, were also found to be functional in nNOS knockout retinas. However, the sensitivity of retinal ganglion cell to light stimulation was decreased in nNOS knockout retina. Collectively, these results, combined with our previous findings (Wang et al., 2003), provide evidence that increasing NO dampens visual responses of ganglion cells, while a lack of nNOS decreases the sensitivity of ganglion cell to light.

**Acknowledgments**

We thank Xianghong Shan for technical support. This research was supported by National Eye Institutes Grants EY-13301, EY-03991, and EY-012576.

Commercial relationships: none.

Corresponding author: Guoyong Wang.

Email: gwang@tulane.edu.

Address: Department of Structural and Cellular Biology, School of Medicine, Tulane University, 1430 Tulane Avenue, SL-49, New Orleans, LA 70112, USA.

**References**


of retinal ganglion cells. *Journal of Neurophysiology, 90*, 1304–1313. [PubMed] [Article]


