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Exploring the Regulation of Neuronal Nitric Oxide Synthase in Retinal Amacrine Cells

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Retinal amacrine cells express high levels of nitric oxide synthase (nNOS), capable of converting L-arginine to L-citrulline and nitric oxide (NO). NO in the inner retina has been shown to effect neurotransmitter release from GABAergic amacrine cells and to influence the sign of the postsynaptic response. This experiment was undertaken to investigate how different methods for elevating cytosolic calcium influence the productivity of calcium-dependent nNOS. High K^+ external solution was used to activate voltage-dependent calcium channels and 250 μM NMDA was used to activate NMDA receptors at the plasma membrane. Increases in fluorescence of a NO indicator dye in response to exposure to either stimulus were compared. Data show that both stimuli produce reliable increases in cytosolic NO levels. Results indicate that the efficacy of 250 μM NMDA over 100 mM K^+ for increasing NO production varies between cultures. Glutamatergic input to an amacrine cell from a bipolar cell of the retina can effectively increase NO levels via both voltage-gated calcium channel and NMDAR activation. *In vivo*, these mechanisms could potentially influence amacrine GABA release and the postsynaptic response.

I. INTRODUCTION

Amacrine cells are ubiquitous in the vertebrate retina. They can be found in the eyes of species throughout the animal kingdom, from *Homo sapiens sapiens* to *Gallus gallus domesticus*. Retinal amacrine cells have dendritic arbors that begin at the soma in the inner nuclear layer of the retina and extend laterally into the inner plexiform layer [for review, see 23]. Within that layer, amacrine cells form synaptic connections with bipolar cells, ganglion cells, and other amacrine cells [for review, see 23]. Amacrine cells receive glutamatergic input from bipolar cells [for review, see 23]. Furthermore, most amacrine cells express either γ-aminobutyric acid (GABA) or glycine, with the majority being GABAergic [1]. Yet relatively little is known about the purpose of these enigmatic cells. The secretion of these inhibitory neurotransmitters can serve to alter synaptic transmission between bipolar and ganglion cells. The nature and extent of this modulation varies based on a variety of factors such as neuronal excitability, the history of synaptic activity, competitive input, and nitric oxide present at the synapse [2].

Amacrine cells in culture express high levels of the neuronal isoform of nitric oxide synthase (nNOS) [Gleason, unpublished observation]. Active nitric oxide synthase converts L-arginine to L-citrulline and nitric oxide (NO) [3]. The neuronal isoform is calcium-dependent in its activation [4]. High levels of cytosolic calcium can bind calmodulin to form a complex that will bind and activate nNOS [4]. NO has been shown to have important roles in the vertebrate retina. Among other functions, NO blocks gap junctions between horizontal cells, inhibits gap junctions between amacrine and cone bipolar cells, and uncouples metabotropic glutamate receptor mGluR6 from its downstream signaling cascade [5–7].

NO is known to colocalize with the neurotransmitter GABA in the retina of several species. Via this relationship it has been shown that NO affects GABA output in a concentration- and location-specific manner [8]. In studies of the mouse hippocampus, high concentrations of NO produced by the exogenous addition of 1.00 mM L-arginine enhance GABA release from hippocampal cells [8]. In the same neurons, low levels of NO production achieved through N-methyl-D-aspartate receptor (NMDAR) stimulation inhibit GABA release [2]. In the chicken retina, it has been similarly observed that low and moderate NO levels produced by the addition of less than 1 μM L-arginine inhibit GABA release from amacrine cells [2]. However, high levels of NO produced by the addition of 1 mM L-arginine have no effect on GABA release from the same cells [2]. The difference in inhibitory regulation patterns observed with NO can be accounted for by the involvement of NO in multiple pathways within the cell.

In amacrine cells of the chicken retina it has also been shown that NO can convert normally inhibitory GABAergic or glycinergic synapses to excitatory synapses. This is seen as an enhancement of GABA-gated current [9]. The NO-dependent mechanism functions by releasing intracellular Cl^- stores and a consequently shifting E_{Cl^-} positive [9, 10]. Neuronal excitability in the retina, therefore, depends on the concentration of intracellular NO influencing amacrine cell function.

The varied effects of NO on multiple signal transduction pathways depending on cell type, function, and location, indicate that NO synthase can have varied effects within amacrine cells of the inner retina. The function of NO in the inner retina and within amacrine cells is largely unstudied.

This research was undertaken to investigate the functioning of nNOS in amacrine cells of the *Gallus gallus domesticus* retina. In the first phase of the project, three methods for increasing cytosolic calcium were tested for efficacy in amacrine cell cultures. The exogenous application of NMDA was used to activate NMDA receptors

at the plasma membrane. NMDA receptor activation allows calcium to move down its concentration gradient and into the cell. In additional experiments, caffeine was used to stimulate calcium-sensitive ryanodine receptors on the endoplasmic reticulum (ER) to move calcium ions from the ER into the cytoplasm. The last method tested was the use of a high K^+ solution to elevate intracellular calcium via the activation of voltage-dependent calcium channels at the plasma membrane. All three methods were tested for efficacy and experimental reproducibility.

The two most reliable methods for increasing intracellular calcium were then used in the second phase of the experiment to stimulate nNOS. Relative changes in NO levels within cultured amacrine cells between the two experimental conditions were compared. The purpose was to measure quantitatively how different types of calcium elevations affected the activity of nNOS in retinal amacrine cells.

II. MATERIALS AND METHODS

A. Amacrine cell cultures

Cultures of embryonic day eight White Leghorn chicken retinae (*Gallus gallus domesticus*, Animal Sciences Department, Louisiana State University) were plated in neurobasal medium (NBM) (Invitrogen, Carlsbad, CA) with B27 (Invitrogen), 1,000 U penicillin/ml, 100 μg streptomycin/ml, and 1 mM L-glutamine (Sigma, St. Louis, MO) on polystyrene dishes pre-treated with polyornithine (POLY-O) (0.1 mg/mL). Between embryonic day 14 and 19, cells were transferred from NBM to one milliliter of Hanks Balanced Salt Solution (Table I). Embryonic day one is considered to be 24 hours after fertilization of the egg. By embryonic day 14, amacrine cell cultures have developed mature receptors capable of responding to stimuli.

B. Calcium imaging

Oregon Green 488 fluorescence dye (2',7'- difluorofluorescein) (Invitrogen, Grand Island, NY) was used as a reporter of relative intracellular Ca^{2+} levels within individual cells. An Oregon Green stock solution was prepared in 2 mM dimethyl sulfoxide (DMSO). The Oregon Green stock was added to cells at a 1:1 ratio with Pluronic F127 acid (Invitrogen, 25 mg/ml DMSO) for a final concentration of 2 μM Oregon Green 488 in Hanks Balanced Salt Solution (Table I). Cultured cells were loaded with Oregon Green for one hour at room temperature in the dark.

TABLE I.

Hank's Balanced Salt Solution	
NaCl	8000.00 mg/L
KCl	400.00 mg/L
KH_2PO_4	60.00 mg/L
Glucose	1000.00 mg/L
NaH_2PO_4 $7H_2O$	90.00 mg/L
$NaHCO_3$	350.00 mg/L

C. Nitric oxide imaging

Changes in intracellular NO concentrations were monitored using DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate) (Invitrogen) fluorescent dye. A stock solution of DAF-FM diacetate prepared in 2 mM DMSO was added to each culture dish to achieve a final solution of 1 μM DAF-FM diacetate in Hanks Balanced Salt Solution (Table I). The dye was loaded for 30 minutes in the dark. DAF-FM fluorescent dye indicates relative changes in NO levels.

D. Real-time fluorescence imaging

Culture dishes were mounted on the stage of an Olympus IX-70 (Tokyo, Japan) inverted microscope and images acquired using SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO). Fluorescence measurements were taken from the largest diameter portion of the amacrine cell body. Data points were collected at 5 s intervals with a 400 ms exposure unless otherwise indicated. Background fluorescence was subtracted from all fluorescence values during the course of the recording with SlideBook software.

Both fluorescent dyes exhibited a stimulus-independent decay in fluorescence during recording. The fluorescence decay of DAF-FM and Oregon Green dyes was fit to a third-order polynomial and subtracted from the data using Origin 7.5 (OriginLab, Northampton, MA). Baseline data values from the original data were added back to all data points to avoid near-zero fluorescence intensity values.

In experiments using DAF-FM diacetate dye, a new baseline for recording was established by taking the mean of the three data points within the recording immediately prior to the stimulus response, during a perfusion of normal external solution. All data points were divided by the baseline to give a fold change in fluorescence (F/F_0). One was subtracted from all F/F_0 values and new data points were multiplied by 100 to give a percent change from the baseline values. The mean percent change from baseline was determined by pooling values from each culture tested for each condition tested. The analysis was done with Origin 7.5.

E. Solutions

1. External solutions

Cells were continuously perfused with normal external solution during recording and 10 minutes prior to the onset of recording unless otherwise indicated (Table II). The composition of external solution was altered for NMDA experiments by the addition of 250 μM NMDA, 1 mM arginine, and 10 μM glycine. The composition of external was altered for caffeine experiments by the addition of 2 mM caffeine. A normal external solution was also prepared containing 1 mM arginine. All external solutions, including a high K^+ solution (Table III), were brought to a physiological pH of 7.4.

2. Nitric oxide solution

NO solution was made by bubbling normal external solution with pure argon for 15 minutes to remove chemical species that would react with NO. The solution was then bubbled with pure NO that had been filtered through soda lime for 15 minutes to remove nitric dioxide. NO solution was injected into the perfusion line using a 50 μL Hamilton syringe.

III. RESULTS

A. 100 mM K^+ and 250 μM NMDA produce reliable increases in intracellular Ca^{2+} levels in cultured amacrine cells.

The intention of this experiment was to determine the extent to which different methods for increasing intracellular Ca^{2+} affected the NO production by nNOS in retinal amacrine cells. The first stage of experimentation sought to confirm that the selected methods were reliable means of increasing intracellular Ca^{2+}. Methods tested included the activation of NMDARs by a solution of 250 μM NMDA, the activation of voltage-gated calcium channels by a solution of 100 mM K^+, and the activation of ryanodine receptors by a solution of 2 mM caffeine. For these tests, amacrine cells were loaded with the Ca^{2+}-sensitive Oregon Green 488 fluorescent dye. An increase in the amplitude of fluorescence (arbitrary units) over the baseline value upon the addition of a stimulus was used to establish a positive response to stimulation. Cells in which no increase in fluorescence or a decrease in fluorescence from baseline was recorded were considered non-responsive (Fig 1.).

Increases in intracellular Ca^{2+} levels could be observed during the perfusion of either 100 mM K^+, 2 mM caffeine, or 250 μM NMDA. Each test solution produced different mean fluorescence increases above baseline. The number of positive responses for each condition was recorded and success rates for Ca^{2+} elevations were compared.

N-Methyl-D-aspartate receptors (NMDARs) are positioned along the plasma membrane in retinal amacrine cells[11]. Receptors contain a magnesium voltage-gated block that prevents the flow of cations through the channel. Depolarization of the membrane expels the Mg^{2+} ion [for review, see 13]. Once activated, NMDA receptors are highly permeable to Ca^{2+}, which will flow along a gradient from regions of high concentration to regions of lower concentration [for review, see 13]. My experiment utilized a normal external media free of $MgCl_2$. This removed the voltage-gated magnesium block from NMDARs without requiring that amacrine cells in culture be depolarized for the activation of NMDARs. Ca^{2+} could then flow through activated NMDARs down the established concentration gradient into the Ca^{2+}-poor cytoplasm of the cells. The NMDA-supplemented solution was enriched with glycine, required as a coagonist for the activation of NMDARs [for review, see 13].

In a culture of embryonic day 12, 85.7% of cells (n=14) measured showed elevations in cytosolic Ca^{2+} in response to a 30-second NMDA exposure. In a different culture of embryonic day 18, 82.6% of cells (n=23) expressed a positive reaction to a 30-second NMDA exposure. In a culture of embryonic day 17, 57.5% of cells (n=33) showed an increase in cytosolic Ca^{2+} or Ca^{2+} elevations to a 10-second NMDA exposure. For tests of a 10-second NMDA exposure, data points were collected at 2.5 s intervals with a 400 ms exposure during 5-minute recordings.

Calcium increases were sustained for the full length of NMDA exposure. Intracellular Ca^{2+} concentrations recovered and returned to resting levels after NMDA was removed from the bath. In total, NMDARs were successfully activated with a concomitant increase in intracellu-

TABLE II.

Normal External Solution	
NaCl	136.98 mM
KCl	5.36 mM
$MgCl_2$	0.41 mM
Glucose	5.60 mM
HEPES	10.00 mM
$CaCl_2$	3.00 mM

TABLE III.

High K^+ Solution	
KCl	0.100 M
NaCl	0.038 M
$MgCl_2$	0.410 mM
$CaCl_2$	3.000 mM
Glucose	5.600 mM
HEPES	3.000 mM
Arginine	1.000 mM

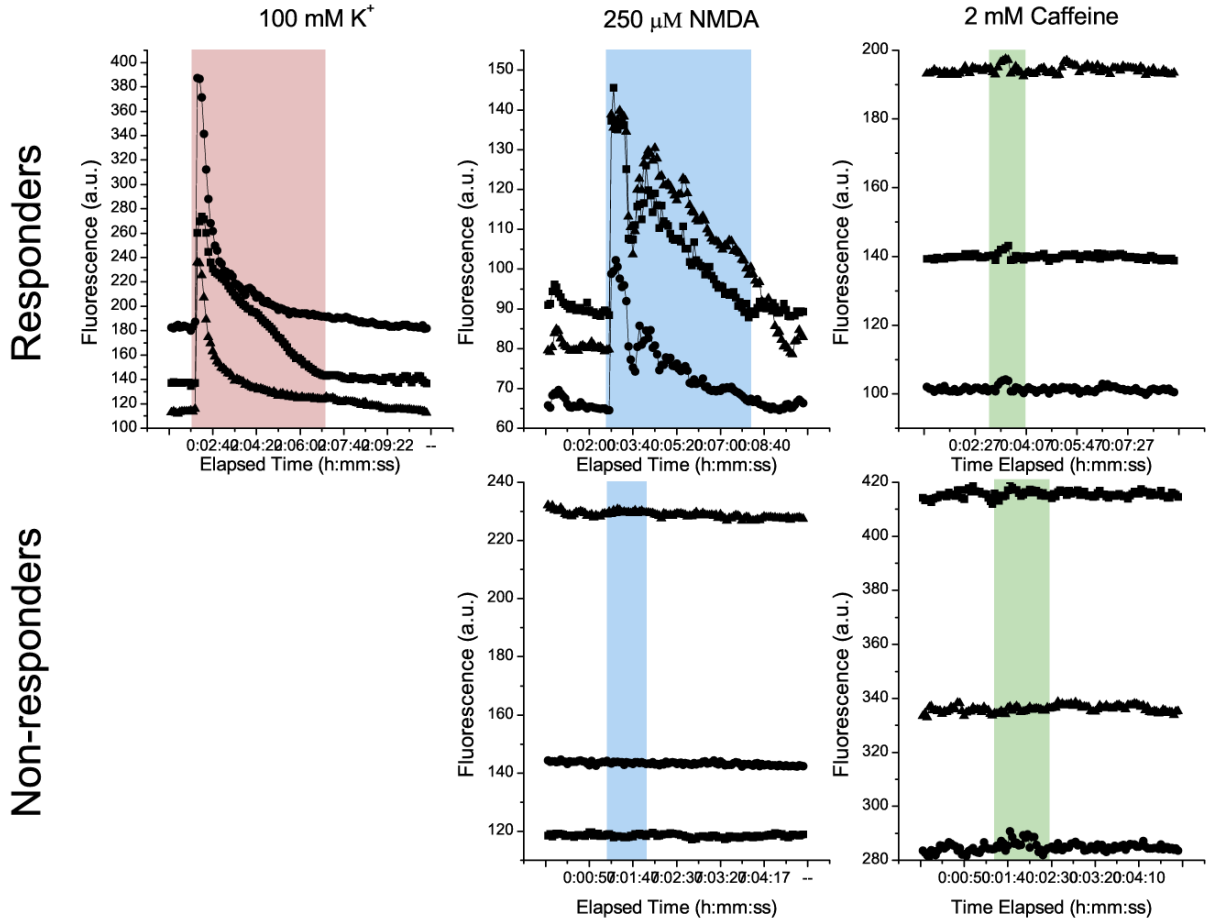

FIG. 1. The response of select amacrine cells to perfusions of 250 μM NMDA (blue), 100 mM K^+ (red), or 2 mM caffeine (green). The top row shows typical responses to each stimulus. Nonresponsive cells for each stimulus are shown in the lower row.

lar Ca^{2+} in 71.4% of cells tested (n=70). The observed 71.4% success rate for increasing cytoplasmic Ca^{2+} indicates that a solution of 250 μM NMDA is a reliable means of increasing Ca^{2+} within amacrine cells. The effectiveness of NMDA for elevating intracellular Ca^{2+} did not change with time in culture.

Ryanodine receptors are ligand-gated, calcium- sensitive Ca^{2+} channels located on ER and sarcoplasmic reticulum (SR) membranes. Treatment of ryanodine receptors with caffeine increases their sensitivity to Ca^{2+} [for review, see 13]. In the presence of caffeine, small increases in cytoplasmic Ca^{2+} concentrations evoke a large release of Ca^{2+} from internal stores via ryanodine receptors. Caffeine increases the probability that ryanodine receptors will open at resting Ca^{2+} levels in the cell [for review, see 13].

In one experiment, caffeine was perfused twice for a 30-second exposure each time after which normal external solution continued to be perfused. In between exposures, normal solution was perfused for 140 seconds. In a culture of embryonic day 18, 4.3% of cells (n=23) measured showed a positive reaction to the first caffeine exposure. 21.7% of cells, (n=23) showed a positive reaction to the second caffeine perfusion. 78.2% of cells (n=23) showed no reaction to either compound.

In a separate experiment, caffeine was perfused for 10 seconds. During the 10-second exposure, data points were collected at 2.5 s intervals with a 400 ms exposure. In cells of embryonic day 17, 16.6% of cells (n=78) showed a positive reaction to caffeine exposure.

In responding cells, Ca^{2+} increases were sustained for the full length of caffeine exposure. After caffeine was removed from the perfusion, intracellular Ca^{2+} concentrations returned to the baseline level established before

the addition of 2 mM caffeine. In total, caffeine evoked a positive increase in intracellular Ca^{2+} in just 17.8% of cells (n=101) from which recordings were collected. The data show that caffeine is capable of inducing the activation of ryanodine receptors in the cell but produces unreliable elevations in Ca^{2+}. Due to the low reproducibility of Ca^{2+} elevations, the 2 mM caffeine solution was not used to test the function of nNOS.

Normal external solution brought the extracellular concentration of K^+ to 5.36 mM KCl. The high K^+ solution utilized in our experiments elevated extracellular KCl concentrations to 100 mM. In normal conditions, the equilibrium potential for K^+, as calculated by the Nernst equation, is -78.4 mV. In the presence of the high K^+ solution, the equilibrium potential for K^+ shifted to -9.5 mV. The more positive equilibrium potential for K^+ caused the membrane potential of the cells in culture to shift positively. This is due to K^+ being the most permeant ion at rest. Depolarization opened voltage-gated calcium channels at the plasma membrane. The change in equilibrium potential for K^+ brought the membrane potential of the cells into the activation range of voltage-gated calcium channels expressed by amacrine cells [12].

In amacrine cell cultures between embryonic days 11 to 19, a 30-second perfusion of a 100 mM K^+ solution successfully increased intracellular Ca^{2+} in 94.3% of amacrine cells (n=53). The high success rate can be attributed to the responsiveness of calcium channels at the high degree of depolarization produced by 100 mM extracellular K^+.

Cumulatively, NMDA tests showed a success rate of 71.4%. The success rate for the 100 mM K^+ solution was 94.3%. Caffeine produced Ca^{2+} elevations 17.8% of the time (Fig. 1). Perfusion of either 250 μM NMDA or 100 mM K^+ were more reliable stimulators of intracellular Ca^{2+} elevations within amacrine cells over 2 mM caffeine.

B. 100 mM K^+ and 250 μM NMDA affect nNOS activity to different extents depending on the culture tested.

The second stage of experimentation sought to measure changes in intracellular NO concentrations in response to Ca^{2+} elevations engendered by activation of NMDARs or voltage-gated calcium channels (Fig. 2). The mean percent increase in DAF fluorescence levels in responsive cells in the presence of two Ca^{2+} channel activators were compared within the same cultures. The criteria for a NO response was established as a greater than 0.5% increase in mean fluorescence above baseline levels. A separate experiment was undertaken in order to confirm that evoked changes in NO levels were the result of the stimulus being tested and not produced by the presence of arginine in the perfused solution. For these tests, amacrine cells were loaded with DAF-FM diacetate fluorescent dye. Data are presented as the mean ± the standard deviation.

Freely diffusible NO is an important secondary messenger [for review, see 21]. The production of NO via nNOS in retinal amacrine cells can define the NO concentration of the inner retina. Understanding the mechanisms regulating nNOS activity in amacrine cells is therefore fundamental in understanding how amacrine cells affect the activity of the retina.

The nNOSa splice variant of nNOS in the brain is associated with the postsynaptic density. It forms a tight association with NMDARs via the postsynaptic density protein PSD-95 [13]. NMDARs bind PSD-95 with their cytosolic tail and nNOSa with its N-terminal PDZ domain [14]. The close spatial relationship enables quick, effective nNOSa activation in response to a Ca^{2+} influx through activated NMDARs [15]. NMDARs are therefore typically highly effective in triggering Ca^{2+}-dependent intracellular signaling cascades involved in the processes of plasticity and development [16]. A similar relationship between nNOS of the retina and NMDARs in amacrine cells has not yet been observed. Were there a close spatial relationship between NMDARs and nNOS in retinal amacrine cells, a significant increase in NO production might be observed in response to NMDAR activation above the NO production rate elicited from the activation of voltage-gated calcium channels.

Recordings were taken from a single culture on embryonic days 15 and 16 of development. On embryonic day 15, the largest mean percent increase in DAF fluorescence in response to NMDAR activation was 2.4±1.4% and 1.4±0.6% in response to voltage-gated calcium channel activation. On embryonic day 16, the largest mean percent increase in DAF fluorescence was measured at 8.0±3.1% in response to NMDAR activation and 4.0±3.2% in response to voltage-gated calcium channel activation (Fig. 2B).

In a different amacrine cell culture of embryonic day 16, the largest mean percent increase in DAF fluorescence levels was measured as 1.5±1.0% in response to NMDAR activation and 2.1±1.2% in response to the perfusion of 100 mM K^+ (Fig. 2A).

Given the conflicting results, the data cannot confirm or discount any advantageously spatial relationship that may exist between nNOS and NMDARs at the plasma membrane in amacrine cells. Data from two separate cultures were compared (Fig. 3). Within the culture of embryonic day 16, the activation of voltage-gated calcium channels increased NO levels within the amacrine cells above that of NMDAR activation. The change in mean fluorescence measured in response to K^+ was 142.7% greater than the response observed upon the addition of NMDA (Fig. 3). The heightened response to K^+ would suggest that the activation of voltage-gated calcium channels at the plasma membrane is a stronger stimulator of nNOS than the activation of NMDARs. The opposite response was observed in a second culture on both embryonic day 15 and 16 of its development. On embryonic day 15, the mean increase in DAF-FM diacetate dye fluores-

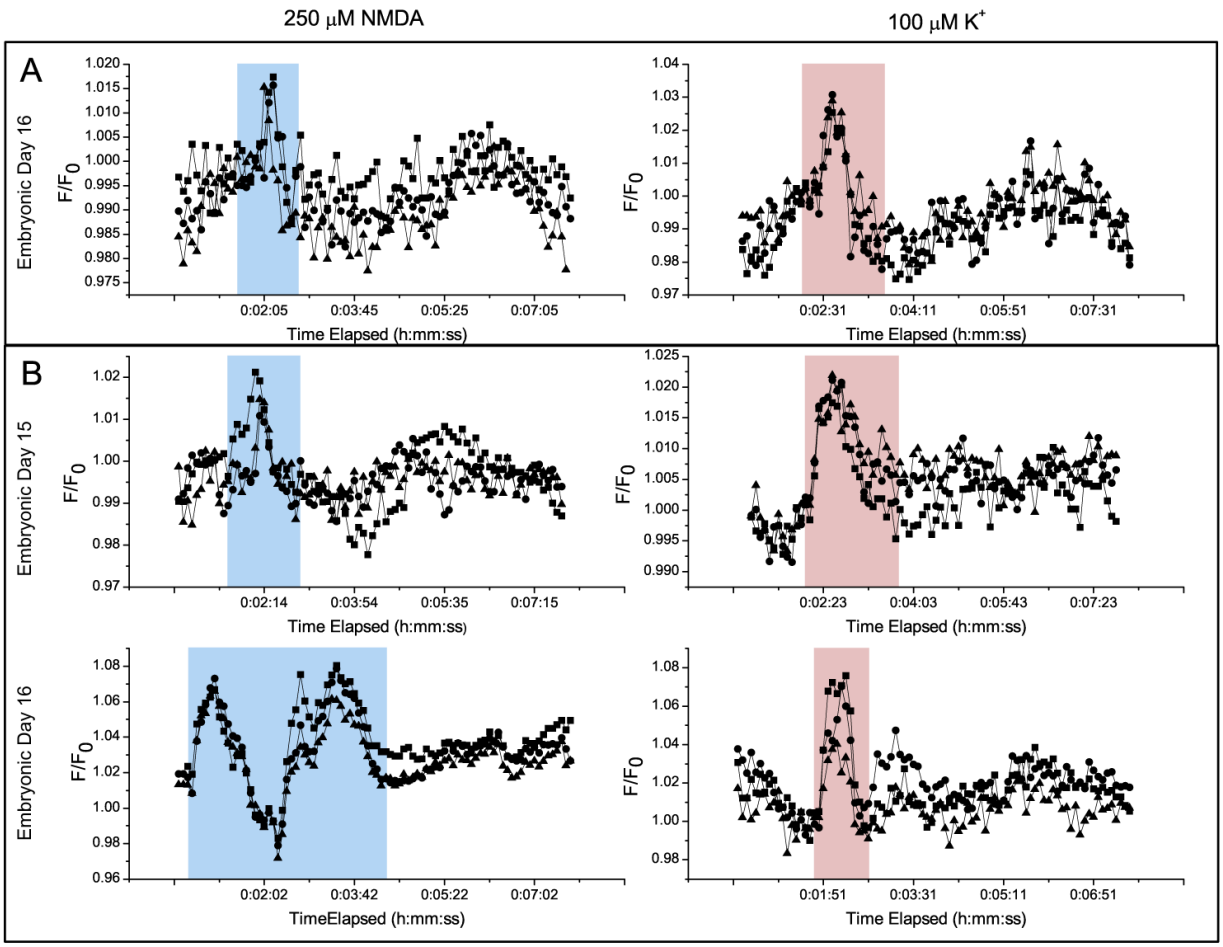

FIG. 2. A. Responses of select amacrine cells within a culture of embryonic day 16 to 30-second perfusions of 250 μM NMDA (blue) or 100 mM K^+ (red). B. The response of select amacrine cells within a separate culture on embryonic days 15 and 16.

cence in response to NMDA was 171.8% greater than the response from K^+. On embryonic day 16, the response to NMDA was 197.6% greater than the response measured to K^+ (Fig. 3). The strength of the NO response to NMDA grew slightly in comparison to the NO response to the K^+ stimulus as the culture matured. The heightened response to NMDA would suggest an advantageous functional relationship between NMDARs and nNOS. An unpaired t-test was performed on the data collected form the same culture on embryonic day 15 and embryonic day 16. The two-tailed p value was equal to 0.18, which indicates no statistically significant difference between the two sets of data.

The data show that both the activation of NMDARs and voltage-gated calcium channels can cause NO production. However, no overall conclusion about the nature of the relationship between nNOS and NMDARs can be reached because of the conflicting results between the two cultures tested.

In the culture that was tested on different days of development, on embryonic day 15 the NMDA solution increased NO more than 0.5% above baseline in 96.2% of cells tested (n=27). The 100 mM K^+ solution increased NO more than 0.5% above baseline in 81.2% of cells tested (n=32). In the same culture on embryonic day 16, the NMDA solution increased NO more than 0.5% above baseline in 100.0% of cells tested (n=28). The 100 mM K^+ solution increased NO more than 0.5% above baseline in 86.3% of cells tested (n=44).

In the culture tested only on embryonic day 16, the NMDA solution increased NO more than 0.5% above baseline in 90.4% of cells tested (n=21). The 100 mM K^+ solution increased NO more than 0.5% above baseline in 96.3% of cells tested (n=83).

Cumulatively, NMDAR activation produced a NO response in 96.0% of amacrine cells. The perfusion of

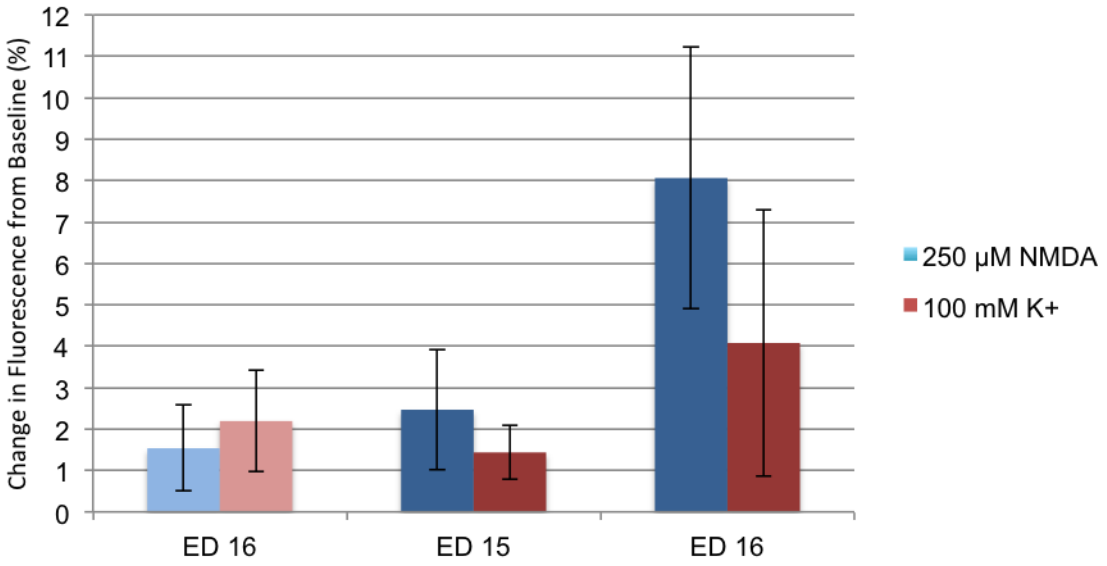

FIG. 3. Changes in DAF-FM diacetate dye fluorescence were measured in response to a 250 μM NMDA (blue) or a 100 mM K^+ (red) exposure. Results were compared within two cultures; one of embryonic day (ED) 16 (left) and another culture on embryonic days 15 and 16 of development (right). In the culture of embryonic day 16, the mean increase in fluorescence of the dye was a 0.6% greater increase from baseline values in response to K^+ than in response to NMDA. In the second culture, NMDA exposure produced the greatest increase from baseline values on both days of development. On embryonic day 15, NMDA exposure caused a 1.0% greater mean increase in fluorescence from baseline than K^+ and on embryonic day 16 NMDA caused a 3.9% greater increase in fluorescence from baseline than K^+.

the high K^+ solution produced a response in 90.5% of amacrine cells tested.

Both 100 mM K^+ and 250 μM NMDA were highly effective at producing any mean increase in fluorescence above baseline in both cultures. It was observed that the efficacy of both NMDA and K^+ increased from embryonic day 15 to embryonic day 16 within a single culture. On embryonic day 15, NMDA was 15.0% more effective at evoking a response from the amacrine cells than K^+. On embryonic day 16, NMDA was 16.6% more effective at eliciting a change in fluorescence when compared to K^+ efficacy. The separate culture of embryonic day 16 showed similarly high rates of efficacy for both K^+ and NMDA, however, K^+ was 5.9% more effective at increasing DAF-FM diacetate fluorescence than NMDA. The overall efficacy of NMDA for producing a NO response was higher than the overall efficacy of K^+ for producing an increase in NO within the amacrine cells by 5.5%.

To determine whether our protocols were saturating the DAF-FM dye, I compared the effect of 100 mM K^+ to the addition of NO itself. Two minutes after a 30-second 100 mM K^+ exposure, 25 μL of NO-bubbled external solution was injected into the perfusion line. In an amacrine cell culture of embryonic day 18, a 1.8±0.8% increase in mean fluorescence values from baseline was observed in response to the perfusion of 100 mM K^+. Two minutes following the exposure, the injection of 25 μL of NO-bubbled external solution into the culture produced a 256.3±175.3% increase in fluorescence from baseline values. Within the culture one out of 18 amacrine cells tested were not responsive to 100 mM K^+. 94.4% of the cells tested were responsive to the 100 mM K^+ solution and 100.0% of the same cells (n=18) were responsive to the NO-bubbled external solution injection. Results show that the DAF-FM dye was not saturated in response to a high K^+ perfusion. The mean increase in fluorescence over baseline in response to the NO injection was 142.3 times greater than that measured in response to the K^+ exposure (Fig. 4). This indicates that the similar results recorded for mean fluorescence changes in the presence of high K^+ and in the presence of NMDA are not the results of any limitations in DAF-FM dye dynamic range.

Amacrine cells in culture were subjected to a 30-second perfusion of 250 μM NMDA during a continuous perfusion of normal external solution containing 1 mM arginine (data not shown). This was done in order to show that the presence of 1 mM arginine in the NMDA-supplemented solution was not the sole source of NO increases in the cells. Under this condition, in a cell culture of embryonic day 19, a mean percent increase in fluorescence of 3.5±1.0% from baseline was observed during the 30-second perfusion of 250 μM NMDA. These data show that an increase in NO levels in response to NMDA perfusion is a result of the NMDAR activation and not a basal level of nNOS activity produced by the mere presence of its substrate in solution.

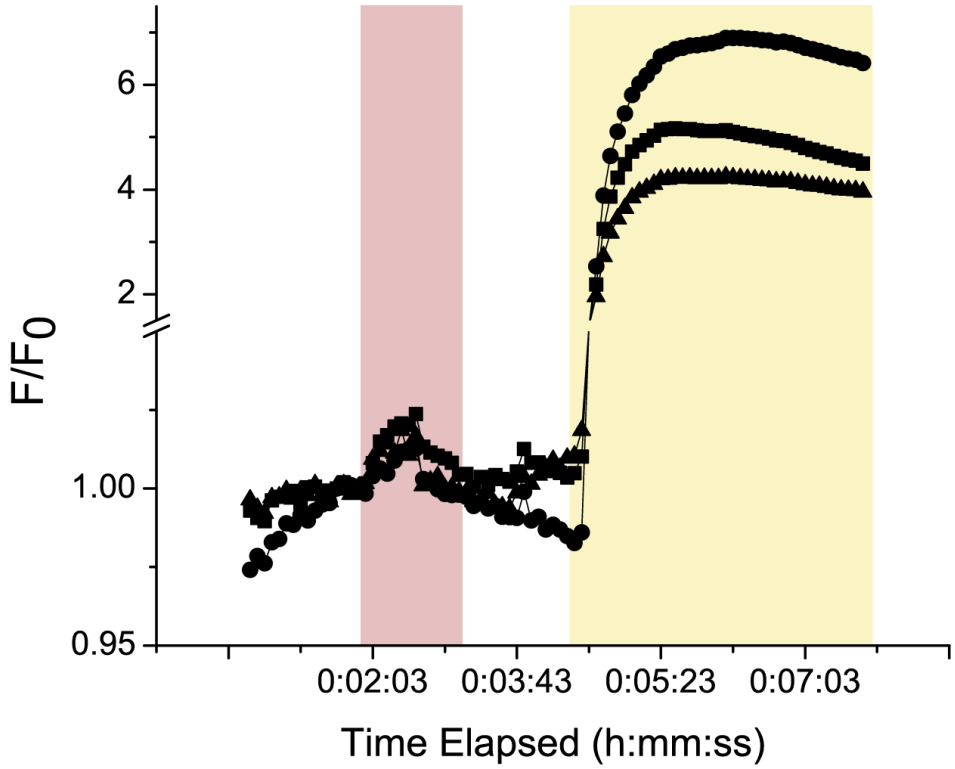

FIG. 4. High K^+ solution (red) increases the mean fluorescence 1.8±0.8% from baseline during a 30-second perfusion and an injection of 25 μL of NO-bubbled external solution (yellow) produced a 256.3±175.3% increase in fluorescence from baseline.

IV. DISCUSSION

In the intact retina, amacrine cells receive glutamatergic input from bipolar cells [for review, see 19]. NMDARs are glutamate receptors to which NMDA serves as a selective agonist [for review, see 19]. *In vivo*, glutamatergic input would activate NMDARs on the plasma membrane of postsynaptic amacrine cells. In culture, NMDAR activation stimulated nNOS. If NMDAR activation stimulates nNOS *in vivo*, then glutamatergic synaptic activity would elevate cytosolic NO in the postsynaptic cell.

Excitatory glutamatergic input depolarizes postsynaptic cells [for review, see 19]. NMDARs function as nonselective cation channels [for review, see 19]. Upon activation, Ca^{2+}, K^+, and Na^+ flow into the cell to depolarize the membrane [for review, see 19]. Depolarization via this mechanism has the same effect as the depolarization produced by an increase in external K^+. Voltage-gated calcium channels in the plasma membrane open and the cytosolic Ca^{2+} level of the cell increases. Data show that, in culture, the activation of voltage-gated calcium channels increases NO levels. *In vivo*, if voltage-gated calcium channels stimulate nNOS , the glutamatergic synaptic transmission could influence NO levels within amacrine cells.

Previous work illustrates that NO concentrations in GABAergic amacrine cells affect GABA output [8]. If the same results observed *in vitro* hold *in vivo*, then glutamatergic input from bipolar cells increase NO within postsynaptic cells. NO increases would then affect signaling through the retina via amacrine cells. Glutamatergic input to an amacrine cells may even convert the postsynaptic GABAergic synapse from inhibitory to excitatory by stimulating an increase in NO [9].

V. FUTURE EXPERIMENTS

In this study, different embryonic days of development were compared within the same culture. Measuring DAF-FM diacetate dye fluorescence from the same cells within the same culture in response to different stimuli would allow for stronger comparisons to be made. The data could then be subjected to a paired t-test. A paired t-test has greater statistical power and could limit the effects of confounding variables on the data.

In the brain, it has been observed that nNOSa colocalizes with NMDARs via a postsynaptic density protein

[13]. The cultured amacrine cells used in this study did not receive glutamatergic input and had not undergone synaptogenesis. The postsynaptic density was not established within those cells. The protein necessary for the association of NMDARs and nNOS in the retinal amacrine cells may, therefore, be present *in vivo* but not *in vitro*. To pursue the possibility of a synapse-dependent protein involved in the association of NMDARs with nNOS, experiments of the same nature can be conducted in retinal slices.

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[1] H. Sun and W. Crossland, "Quantitative assessment of localization and colocalization of glutamate, aspartate, glycine, and GABA immunoreactivity in the chick retina," Anat. Rec. **260**, 158 (2000).

[2] R. Maggesissib, P. Gardinob, E. Guimarães-Souzaa, R. P. de Carvalhoa, R. Silva, and K. Calaza, "Modulation of GABA release by nitric oxide in the chick retina: Different effects of nitric oxide depending on the cell population," Vis. Res. **49**, 2494 (2009).

[3] D. Stuehr, "Mammalian nitric oxide synthases," Biochim. Biophys. Acta, Bioenerg. **1411**, 217 (1999).

[4] I. Mungrue, D. Bredt, D. Stewart, and M. Husain, "From molecules to mammals: What's NOS got to do with it?" Acta. Physiol. Scand. **179** (2003).

[5] M. Pottek, L. Schultz, and R. Weiler, "Effects of nitric oxide on the horizontal cell network and dopamine release in the carp retina," Vis. Res. **37**, 1091 (1997).

[6] S. Mills and S. Massey, "Differential properties of two gap junctional pathways made by AII amacrine cells," Nature **377**, 734 (1995).

[7] J. Snellman and S. Nawy, "CGMP-dependent kinase regulates response sensitivity of the mouse on bipolar cell," J. Neurosci. **24** (2003).

[8] S. Getting, J. Segieth, S. Ahmad, C. Biggs, and P. Whitton, "Biphasic modulation of GABA release by nitric oxide in the hippocampus of freely moving rats *in vivo*," Brain Res. **17**, 196 (1996).

[9] B. Hoffpauir, E. McMains, and E. Gleason, "Nitric oxide transiently converts synaptic inhibition to excitation in retinal amacrine cells," J. Neurophysiol. **95**, 2866 (2006).

[10] E. McMains and E. Gleason, "Role of pH in nitric oxide-dependent increase in cytosolic Cl^- in retinal amacrine cells," J. Neurophysiol. **106**, 641 (2011).

[11] M. Slaughter and R. Miller, "The role of excitatory amino acid transmitters in the mudpuppy retina: An analysis with kainic acid and N-methyl aspartate," J. Neurosci. **3**, 1701 (1983).

[12] M. Tekmen and E. Gleason, "Multiple Ca^{2+}-dependent mechanisms regulate L-type Ca^{2+} current in retinal amacrine cells," J. Neurophysiol. **104**, 1849 (2010).

[13] J. Brenman, D. Chao, S. Gee, A. McGee, S. Craven, and D. Santillanno, "Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-synthropin mediated by PDZ domains," Cell **84**, 757 (1996).

[14] K. Christopherson, B. Hillier, W. Lim, and D. Bredt, "PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and bivalent neuronal NO synthase PDZ domain," J. Biol. Chem. **274**, 27467 (1999).

[15] R. Sattler, Z. Xiong, W. Lu, M. Hafner, J. MacDonald, and M. Tymianski, "Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein," Science **284**, 1845 (1999).

[16] I. Mungrue and D. Bredt, "nNOS at a glance: implications for brain and brawn," J. Cell Sci. **117**, 2627 (2004).

[17] C. Rousseaux, "A review of glutamate receptors: Current understanding of their biology," J. Toxicol. Pathol. **21**, 25 (2008).

[18] D. Bredt and S. Snyder, "Nitric oxide: a physiologic messenger molecule," Ann. Rev. Biochem. **63**, 175 (1994).

[19] R. Zucchi and S. Ronca-Testoni, "The sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor: Modulation of endogenous effectors, drugs and disease states," Pharm. Rev. **49**, 1 (1997).

[20] A. Fischer and W. Stell, "Nitric oxide synthase-containing cells in the retina, pigmented epithelium, choroid, and sclera of the chick eye," J. Comp. Neurol. **405** (1999).

[21] L. Langado, "Retinal processing: Amacrine cells keep it short and sweet," Curr. Biol. **8**, R593 (1998).

[22] Y. Shen, X.-L. Liu, and X.-L. Yang, "N-methyl-D-aspartate receptors in the retina," Mol. Neurobiol. **34**, 163 (2006).

[23] D. Friel, "Interplay between ER Ca^{2+} uptake and release fluxes in neurons and its impact on $[Ca^{2+}]$ dynamics," Biol. Res. **37**, 665 (2004).