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Nitric Oxide and Cyclic GMP Regulate Retinal Patterning in the Optic Lobe of *Drosophila*

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Summary

The photoreceptors of *Drosophila* express a nitric oxide–sensitive guanylate cyclase during the first half of metamorphosis, when postsynaptic elements in the optic lobe are being selected. Throughout this period, the optic lobes show NADPH-diaphorase activity and stain with an antibody to nitric oxide synthase (NOS). The NOS inhibitor L-NAME, the NO scavenger PTIO, the sGC inhibitor ODQ, and methylene blue, which inhibits NOS and guanylate cyclase, each caused the disorganization of retinal projections and extension of photoreceptor axons beyond their normal synaptic layers in vitro. The disruptive effects of L-NAME were prevented with the addition of 8-bromo-cGMP. These results suggest NO and cGMP act to stabilize retinal growth cones at the start of synaptic assembly.

Introduction

Development of the adult visual system in *Drosophila melanogaster* requires the establishment of precise retinotopic connections between the photoreceptors and their synaptic partners in the optic lobe of the CNS. The repetitive organization of the photoreceptor terminals facilitates the detection of abnormalities in the projection pattern, therefore offering several advantages for studying the regulation of synaptic organization. The compound eye of the adult fly consists of approximately 800 repeating units called ommatidia, arranged in a highly ordered fashion (reviewed by Wolff and Ready, 1993). The eight photoreceptor neurons comprising each ommatidium project to the optic lobes in a stereotyped manner during the last larval instar and beginning of pupal development (reviewed by Meinertzhagen and Hanson, 1993). Axons from the outer photoreceptor cells R1–R6 terminate in the first optic ganglion, the lamina. The inner photoreceptors, R7 and R8, make connections with two neuropilar layers in a deeper portion of the optic lobe, the medulla. The construction of this retinotopic projection pattern requires that individual photoreceptors undergo termination of axon outgrowth at the appropriate time and place, a process that is not well understood. Previous work has shown that pathfinding by retinal axons is independent of neighboring photoreceptors (Kunes et al., 1993) and that the formation of connections with target cells in the optic lobe is active and position-dependent (Ashley and Katz, 1994). Many components of the optic ganglia require retinal innervation for proper development (Fischbach and Technau, 1984; Steller et al., 1987; Selleck and Steller, 1991). For example, completion of neurogenesis and differentiation in the developing lamina is induced by the secretion of Hedgehog from the terminals of newly arrived retinal axons (Huang and Kunes, 1996). In addition, the photoreceptors themselves may depend on signals from target cells for survival (Campos et al., 1992; Xiong and Montell, 1995). These findings have led to speculation that molecular cues originating in the optic ganglia contribute to the construction of the retinotopic map (Kunes et al., 1993; Ashley and Katz, 1994; Xiong and Montell, 1995).

Nitric oxide (NO) displays properties that make it attractive as a candidate signal involved in the establishment of the retinal projection pattern. A free radical produced by nitric oxide synthase (NOS) through the conversion of arginine to citrulline, NO can diffuse across cell membranes to activate soluble guanylate cyclase (sGC). This interaction results in the production of a second messenger molecule, 3′,5′-cyclic guanosine monophosphate (cGMP) (Arnold et al., 1977). cGMP can alter cell physiology by acting on several potential targets, including ion channels (Johnson et al., 1986; Nawy and Jahr, 1990), protein kinases (Farber et al., 1979; Paupardin-Tritsch et al., 1986), and phosphodiesterases (Hartzell and Fischmeister, 1986). NO is short-lived in biological tissue (Meulemans, 1994); thus, it can rapidly effect local cellular changes. The role of the NO/cGMP pathway in regulating long-term changes in synaptic function has been demonstrated in the hippocampus and cerebellum of vertebrates (Schuman and Madison, 1991; Shibuki and Okada, 1991; Haley et al., 1992; Zhou et al., 1993), in the mushroom bodies of honeybees (Müller, 1996), and in modifying the output from neural circuits in terrestrial mollusks (Gelperin, 1994). NOS activity has been described in developing vertebrate tissues (Kalb and Agostini, 1993; Ientile et al., 1996), and theoretical models predict mediation of developing synapses by a short-lived, diffusible molecule (Gally et al., 1990).

Direct evidence supporting a role for NO in neural development is scarce but provocative. NO is involved in the pruning of retinotectal synapses in the developing chick visual system (Wu et al., 1994) and the patterning of retinogeniculate connections in postnatal ferrets (Cramer et al., 1996). NO has also been shown to regulate activity-dependent suppression at the developing neuromuscular junction in *Xenopus laevis* (Wang et al., 1995) and to stimulate the formation of synaptic connections in developing and regenerating rat olfactory neurons (Roskams et al., 1994). In arthropods, nervous system development is associated with the appearance of an NO-sensitive guanylate cyclase in subsets of neurons prior to synaptogenesis (Truman et al., 1996; Scholz et al., 1998). NOS activity is present in the adult nervous system of *Drosophila* (Müller and Buchner, 1993) and in the visual system of locusts (Elphick et al., 1996). A *Drosophila* homolog of neuronal NOS has been cloned

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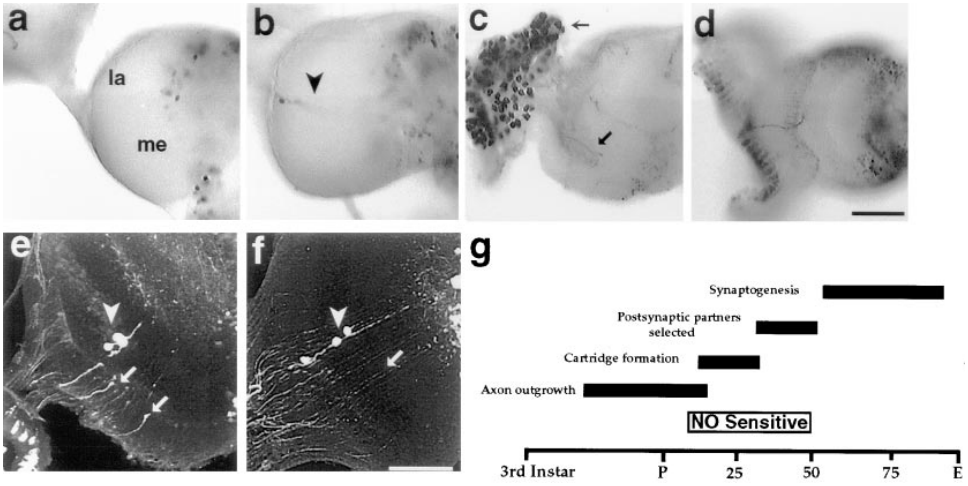

Figure 1. Photoreceptors Display Developmentally Regulated, NO-Sensitive Guanylate Cyclase Activity during Metamorphosis

(a–d) cGMP production in the visual system in response to 100 μM SNP and 1 mM IBMX as visualized by anti-cGMP immunoreactivity (IR).

(a) No cGMP IR was observed in the eye imaginal disc or optic lobes at 0 hr after puparium formation (APF). la, lamina; me, medulla.

(b) At 10 hr APF, Bolwig's nerve (arrowhead) shows cGMP IR.

(c) cGMP production in the photoreceptors at 16 hr APF. cGMP IR was observed in photoreceptor cell bodies in the developing eye (small arrow) as well as their axons in the lamina (large arrow).

(d) Photoreceptors continue to respond to SNP/IBMX treatment with increases in cGMP IR at 31 hr APF. Cell bodies of some medulla interneurons are also NO-responsive by this stage. Scale bar, 50 μm.

(e) Confocal fluorescent image of cGMP production in R1–R6 (arrows) and Bolwig's nerve (arrowhead) in response to 100 μM SNP and 1 mM IBMX at 24 hr APF.

(f) cGMP production in axons of R7/8 (arrow) and Bolwig's nerve (arrowhead) in response to 1 mM SNP and 1 mM IBMX at 24 hr APF. Scale bar, 50 μm.

(g) A time line of visual system development in *Drosophila*, emphasizing photoreceptor behaviors. Numbers indicate hours of development after pupariation (P) to adult eclosion (E). The photoreceptors displayed an increase in cGMP IR in response to SNP/IBMX treatment for approximately 40 hr during the first half of metamorphosis (white bar). Time line is adapted from Meinertzhagen and Hanson (1993).

(Regulski and Tully, 1995), and NO has been shown to influence cell proliferation in the leg imaginal discs, which display NOS activity during development (Kuzin et al., 1996). However, as of yet no developmental function has been attributed to NO in the fruit fly nervous system.

In the present study, we have investigated the role of the NO/cGMP signaling pathway in the development of the retinal projection pattern. We show that developing photoreceptors respond to NO stimulation with the production of cGMP during a discrete temporal window following axon outgrowth but preceding the appearance of functional connections with optic lobe interneurons. During this same period, neurons in the lamina and medulla display NADPH-diaphorase activity correlated with the presence of NOS (Bredt et al., 1991) and also stain with an antibody to a conserved region of the NOS protein. Using an in vitro culture system, we demonstrate that pharmacological inhibition of NOS, NO, and the sGC results in disorganization of the overall pattern of photoreceptor terminals in the medulla and the growth of individual retinal axons beyond their appropriate target layers. In addition, the disruption observed with NOS inhibition can be rescued by the presence of a cGMP analog. These results suggest that NO and cGMP are important components of a signaling pathway used to maintain initial contacts between the retinal axons and their postsynaptic targets.

Results

The Photoreceptors Express an NO-Sensitive Guanylate Cyclase during Metamorphosis

NO-sensitive sGC activity is present in subsets of photoreceptors during metamorphosis. The isolated CNS with intact eye imaginal discs was incubated with an NO donor, sodium nitroprusside (SNP), and an inhibitor of phosphodiesterases, isomethylbutylxanthine (IBMX). After a 15 min incubation, the tissues were fixed and processed for immunocytochemistry using a polyclonal antiserum against cGMP (De Vente et al., 1987). As seen in Figure 1, many neurons within the brain and optic lobes showed the appearance of cGMP immunoreactivity (IR) after the SNP/IBMX treatment. At the onset of metamorphosis (0 hr after puparium formation, APF) and through at least 10 hr APF, this response was limited to the central brain and did not include the photoreceptor axons (Figure 1a). The axons comprising Bolwig's nerve (the larval optic nerve) and the associated three larval optic lobe interneurons were variable in their response to SNP/IBMX treatment (Figure 1b), but cGMP IR was typically observed in Bolwig's nerve throughout metamorphosis. Photoreceptors began to respond to SNP/IBMX shortly after 10 hr APF. At 16 hr APF, cGMP IR was evident in the photoreceptor cell bodies and along the total length of their axons (Figure 1c). The photoreceptors continued to respond to SNP/IBMX treatment

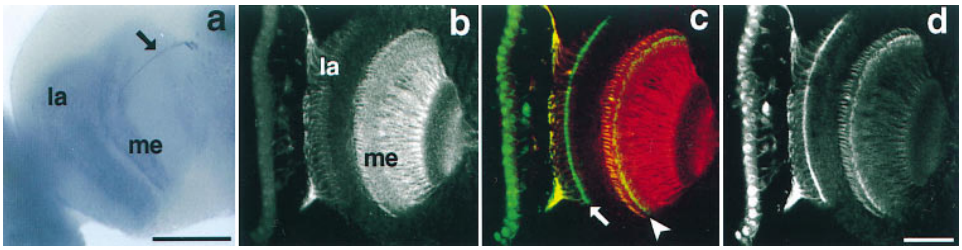

Figure 2. Localization of Nitric Oxide Synthase in the Optic Lobe

(a) Frontal view of the optic lobe showing NADPH-diaphorase staining at 24 hr APF. Intense dark blue staining is present in the region of the lamina (la) and medulla (me). Bolwig's nerve (arrow) also shows diaphorase staining. Scale bar, 50 μm.

(b–d) UNOS and chaoptin immunostaining in the visual system at 26 hr APF. Orientation is as in (a).

(b) The UNOS antibody labels fibers in both the lamina (la) and medulla (me), with the strongest staining in the medulla.

(c) When merged with the chaoptin staining seen in (d), the terminals of R1–R6 in the lamina (arrow) and R7 and R8 in two distinct layers of the medulla (arrowhead) are directly adjacent to or within areas that show UNOS IR. Scale bar, 50 μm.

through 36 hr APF (Figure 1d); however, after about 50 hr APF, they became unresponsive (data not shown). cGMP synthesis was most dramatic in photoreceptors R1–R6 that project to the lamina. The cGMP IR of R7 and R8, the photoreceptors that extend to the medulla, was somewhat weaker, and slightly higher concentrations of SNP were required to elicit their response. R1–R6 produced high levels of cGMP in response to 100 μM SNP (Figure 1e), whereas R7 and R8 responded best in the presence of 1 mM SNP (Figure 1f). cGMP IR in the photoreceptors was not seen in the absence of SNP and IBMX or with SNP or IBMX alone (data not shown).

The onset of NO sensitivity, which we define here as a visible increase in cGMP IR after exposure to SNP and IBMX, commenced around 10 hr APF and persisted until approximately 50 hr APF. Although the photoreceptors were no longer responsive to SNP/IBMX after this time, many interneurons in the optic lobe remained responsive, along with Bolwig's nerve (data not shown). This 40 hr temporal window began after the arrival of photoreceptor axons in the optic lobes and ended as the photoreceptors were starting to form synapses with neurons of the lamina and medulla (Figure 1g; adapted from Meinertzhagen and Hanson, 1993).

The Optic Ganglia Exhibit NADPH-Diaphorase Staining and NOS Immunoreactivity

NOS produces NO through the conversion of arginine to citrulline, using NADPH as a cofactor. This "diaphorase" activity is not sensitive to fixation and thus can be exploited histochemically to identify cells containing NOS (Bredt et al., 1991; Hope et al., 1991). At 24 hr APF, *Drosophila* brains showed strong diaphorase staining in the regions of the lamina and medulla as well as in Bolwig's nerve (Figure 2a). This pattern of diaphorase activity was present at the onset of metamorphosis (0 hr APF) and continued through about 40 hr APF, when it appeared to diminish in the optic ganglia, although Bolwig's nerve remained diaphorase-positive at 40 hr APF (data not shown). This period of most intense diaphorase activity in the lamina and medulla overlapped with the observed window of NO sensitivity in the photoreceptor axons (Figure 1g).

To examine further the distribution of NOS, we used a "universal" NOS (UNOS) antibody that recognizes a conserved region of vertebrate NOS isoforms that is also found in the *Drosophila* NOS (Regulski and Tully, 1995). In the visual system at 24 hr APF, this antibody labeled projections in the neuropilar regions of both the lamina and the medulla (Figure 2b), although the staining was more intense in the medulla. No staining was evident in the cell body layers. Double labeling with the UNOS antibody and an antibody to chaoptin that recognizes photoreceptor cell bodies and axons (Zipursky et al., 1984) showed that the axons of R7 and R8 terminate within a region of the medulla containing processes stained strongly with the UNOS antibody (Figure 2c). The UNOS staining in the optic lobe was present from 0 to 75 hr APF and was strongest between 24 and 50 hr APF (data not shown). The patterns of diaphorase staining and UNOS localization strongly suggest that NOS is present in retinal targets during the period of NO sensitivity displayed by the photoreceptors.

Axon Pathfinding and Retinal Patterning Proceed In Vitro

Components of the NO/cGMP pathway were manipulated in vitro to examine further the role of this pathway in visual system development. The isolated CNS of *Drosophila* undergoes morphological changes in culture mirroring those observed during metamorphosis (Awad, 1995). When the eye imaginal discs are left intact, the visual system continues to develop as well. Intact eye–brain complexes from early wild-type prepupae (0 hr APF) were placed in culture. After the first 24 hr in vitro, the hormone 20-hydroxyecdysone (20E; 1 μg/ml) was added to simulate the rise in ecdysteroids normally occurring in the intact animal during the first part of metamorphosis (Riddiford, 1993). The tissue remained in culture for a further 72 hr, for a total time of 96 hr. Visual system development in vitro was slower than in vivo, and after 96 hr in culture, the tissue displayed morphological features characteristic of about 50 hr APF. These included expansion of the central brain and optic ganglia as well as the beginnings of pigmentation in the eye imaginal discs (Figure 3a, arrow; also see Li and Meinertzhagen, 1995). When stained with the chaoptin antibody, the segregation of R1–R6 and R7/8 into the lamina and

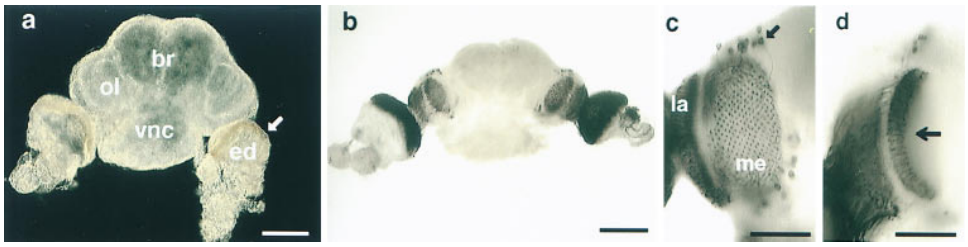

Figure 3. Development of the Visual System In Vitro

(a) Phase contrast image of a *Drosophila* CNS cultured from 0 hr APF for 96 hr. 20E (1 μg/ml) was present for the last 72 hr of culture. Notable developmental changes included expansion of the central brain and optic lobes and the onset of pigmentation in the eye imaginal disc (arrow). br, brain; ol, optic lobe; ed, eye disc; vnc, ventral nerve cord. Scale bar, 100 μm.

(b) The same CNS as in (a), after being processed for immunocytochemistry with MAb 24B10, which recognizes the chaoptin antigen expressed by the photoreceptors. Scale bar, 100 μm.

(c) A closer view of the optic lobe in a cultured nervous system after chaoptin immunocytochemistry, showing the separation and pattering of the photoreceptors in the lamina (la) and medulla (me). A subset of optic lobe interneurons also expresses the chaoptin antigen (arrow). Scale bar, 75 μm.

(d) R7 and R8 have segregated into two layers of the medulla after 96 hr in vitro (arrow). Scale bar, 75 μm.

medulla, respectively, was observed, as was the distinctive retinotopic patterning of R7 and R8 terminals in the medulla (Figures 3b and 3c). After 96 hr in vitro, R7 and R8 had also segregated into two separate layers of the medulla (Figure 3d). As is the case in vivo, a set of medulla interneurons also expressed the chaoptin antigen during in vitro development (3c, arrow). Although the retinal axons have arrived at their approximate targets and cartridge assembly in the optic lobe has commenced by 50 hr APF, the formation of synaptic connections between the photoreceptors and optic lobe neurons occurs during the second half of metamorphosis (Meinertzhagen and Hanson, 1993). Thus, synapse formation has most likely not occurred after 96 hr in vitro. However, our culture period did encompass presynaptogenic events and the time when the visual system shows maximal NOS expression and NO sensitivity.

All the photoreceptors responded to NO stimulation with increases in cGMP production; however, the density of laminar innervation by R1–R6 made it very difficult to discriminate changes in retinal patterning using light microscopy. Consequently, we focused on the projection pattern of R7 and R8 in the medulla, where fewer axons and the distinct segregation of R7 and R8 facilitate analysis of individual and populations of retinal projections. While we could not definitively quantitate the arrangement of R1–R6 projections in these experiments, it did appear that R1–R6 axons grew beyond their normal laminar targets and terminated in the medulla in several cases. The overgrowth of R1–R6 is most likely responsible for the appearance of fasciculated bundles of retinal axons extending beyond the lamina and "crowding" of terminals in the medulla, as seen in Figure 4 (panel 2).

A scoring system was developed to quantitate the results of in vitro manipulations (Figure 4). Parameters for assigning a score were as follows: 0, normal retinal pattern in the medulla; 1, slight disorganization in pattern, crowding of photoreceptor terminals; 2, further disorganization, gaps appearing in pattern; 3, normal patterning no longer discernible, photoreceptor axons extending beyond medulla but not beyond the lobula; 4, severe disruption, no evidence of normal pattern in medulla, photoreceptor axons extending beyond lobula. Each optic lobe was scored separately. A small percentage of nervous systems was excluded from analysis. These included tissues in which the eye discs had been removed or damaged prior to culture, or the optic lobe had degenerated such that no chaoptin-expressing optic lobe interneurons were observed. Each experiment was repeated at least three times, and all eye–brain complexes were scored blind. A mean score was then calculated for each treatment and presented as an index of projection pattern disruption in the medulla, hereafter referred to as the disruption index.

Inhibition of NO Production Disrupts Formation of the Retinal Projection Pattern In Vitro

Nervous systems cultured in the presence of the competitive NOS inhibitor L-nitro-arginine-methyl ester (L-NAME) displayed a disrupted pattern of photoreceptor projections in the medulla (Figure 5). Nervous systems cultured with no inhibitor showed normal, undisturbed patterning of R7 and R8 in the medulla (Figure 5a). When treated with low levels of L-NAME (10 μM), the projection pattern appeared less crystalline, with interruptions in the regular spacing of terminals (Figure 5b). At higher doses (100 μM), this disruption was more severe (Figure 5c), and individual axons were observed growing beyond the borders of the medulla. Nervous systems cultured in 1 mM L-NAME had the most severely disorganized projection pattern, often characterized by retinal axons extending beyond the medulla and lobula and into the brain (Figure 5d, arrow). This effect was not observed in the presence of 1 mM D-NAME (Figure 5e), the biologically inactive isomer of L-NAME, and the disruption indexes for D-NAME-treated nervous systems were not significantly different from controls at any concentration (Figure 5g). At concentrations over 10 μM, the disruption indexes for L-NAME-treated visual systems were significantly higher than those of controls or visual systems exposed to equivalent concentrations of D-NAME. The addition of L-arginine to cultures containing L-NAME blocked the disorganization of the retinal pattern observed with L-NAME alone (Figure 5f), thus demonstrating the specific effects of L-NAME on visual system development. Nervous systems cultured with L-NAME plus 10-fold excess L-arginine had disruption indexes significantly lower than those treated only with L-NAME but not significantly different from controls (Figure 5h).

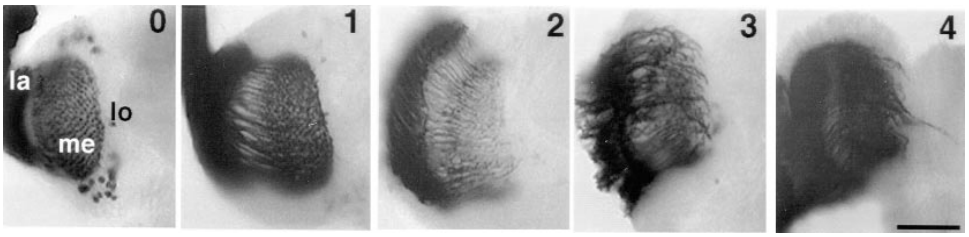

Figure 4. Examples of Optic Lobes Illustrating the Scoring System Used for Quantitating the In Vitro Disruption of Visual System Development

Each image is a frontal view of the optic lobe after culturing, immunostained with the chaoptin antibody to show the pattern of photoreceptor projections in the lamina (la) and medulla (me) as well as the approximate location of the lobula (lo). Numbers refer to the scoring system described in the text. Scale bar, 100 μm.

The disruption of retinal axon projections observed with L-NAME treatment does not appear to be a result of severe degeneration or disorganization of targets in the medulla. Nervous systems cultured with and without 100 μM L-NAME were stained with the chaoptin antibody to label the photoreceptors and an antibody to synaptotagmin to visualize the organization of the optic neuropils (Figures 6a–6d). In a single horizontal optical section through the optic lobe, the organization of R7 and R8 axons in the posterior medulla can be seen in relation to the overall condition of the neuropil. Nervous systems cultured under control conditions showed normal segregation and patterning of R7 and R8 (Figure 6a), and the medulla had undergone the rotation and expansion associated with the normal progression of development (Figure 6b). In the presence of 100 μM L-NAME (Figure 6d), the medulla neuropil was somewhat smaller but still had expanded and rotated, and synaptotagmin staining showed the beginnings of stratification. Many photoreceptor axons, though, did not segregate appropriately, and a few projected into the deeper regions of the medulla (Figure 6c, arrow). Gaps in the synaptotagmin staining were occasionally observed in the medulla and lobula complex in both control and L-NAME-treated nervous systems. The cause of these "holes" in the neuropil is unknown; however, they did not appear to correspond spatially with abnormalities in the retinal projection pattern.

PTIO is an imidazolineoxyl N-oxide derivative that scavenges NO by reacting with it to generate nitrite and nitrate (Akaike et al., 1993). Nervous systems were cultured in the presence of PTIO to reduce the levels of endogenous NO during the period of NO sensitivity. As with L-NAME, the presence of PTIO resulted in a disorganized projection pattern of R7 and R8. One millimolar PTIO was most effective in this regard, but concentrations as low as 10 μM produced nervous systems with a disruption index significantly greater than controls (Figure 6e).

The Effects of NOS Inhibition Can Be Prevented with 8-Bromo-cGMP

Having examined the effects of inhibiting NO or NO synthesis in the visual system during development, we

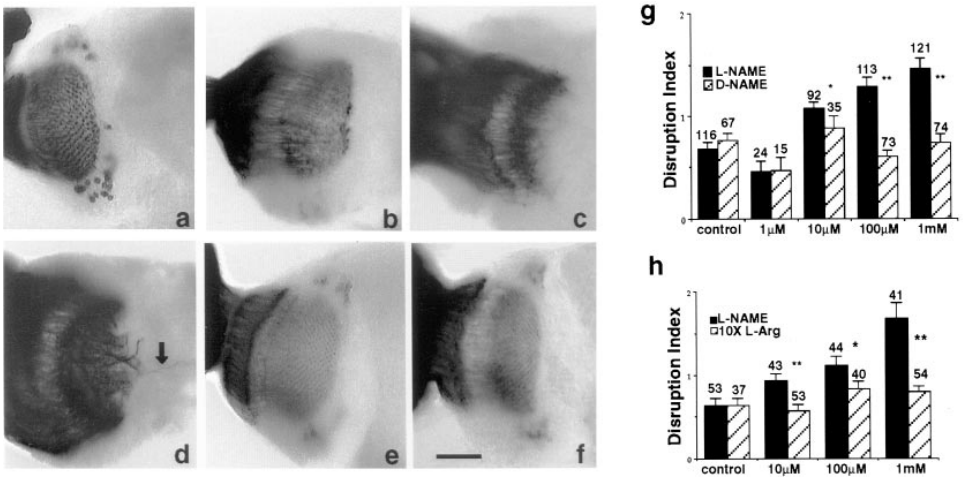

Figure 5. The Competitive NOS Inhibitor L-NAME Disrupts Retinal Patterning In Vitro

(a–f) Frontal views of optic lobes from nervous systems cultured 96 hr and processed for chaoptin immunocytochemistry.

(a) Control, cultured without L-NAME. (b) 10 μM L-NAME. (c) 100 μM L-NAME. (d) 1 mM L-NAME. One axon has grown past the medulla and lobula and into the central brain (arrow). (e) 1 mM D-NAME. (f) 1 mM L-NAME plus 10 mM L-arginine. Scale bar, 50 μm.

(g) Quantitative results from in vitro inhibition of NOS: L-NAME but not D-NAME disrupted retinal patterning in a manner that was significantly greater than controls.

(h) The effects of L-NAME were rescued by the addition of 10-fold greater L-arginine. (Asterisk, $p < 0.05$, double asterisks, $p < 0.001$, unpaired Student's t test). Numbers above standard error bars represent total number of optic lobes scored for each treatment.

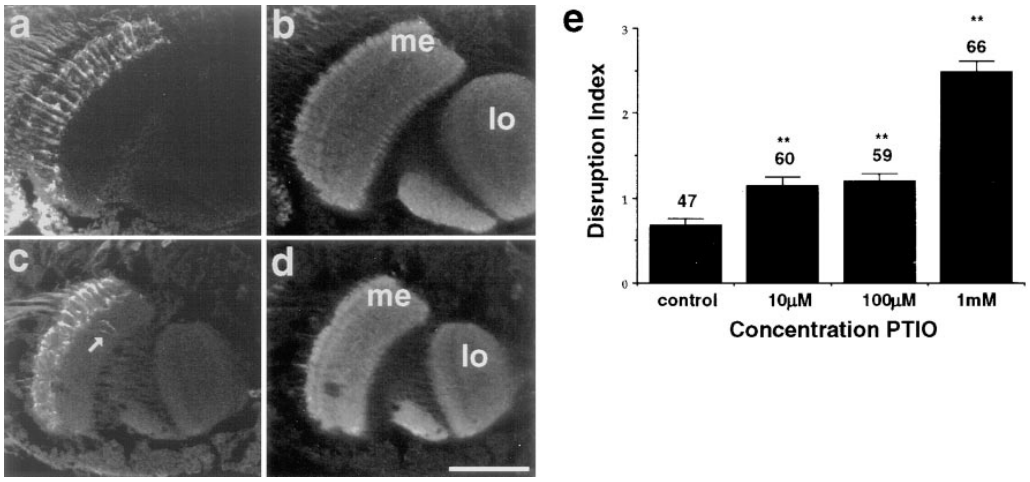

Figure 6. Effects of NOS Inhibition and NO Scavengers on Retinal Projections and the Structure of the Optic Neuropils

(a–d) Optical sections through the posterior optic lobes of cultured nervous systems stained with antibodies to chaoptin and synaptotagmin. (a and b) Control, cultured without L-NAME; (a) the chaoptin-stained pattern of R7 and R8, and (b) synaptotagmin staining showing the location and shape of the medulla (me) and lobula (lo).

(c and d) 100 μM L-NAME-treated nervous system showing (c) chaoptin staining, in which the pattern of R7 and R8 is disorganized and two retinal axons have projected beyond the posterior border of the medulla (arrow), and (d) synaptotagmin staining showing that the medulla is acquiring its layered organization. Scale bar, 25 μm.

(e) The NO scavenger PTIO significantly disrupts retinal patterning in vitro when compared to controls (double asterisks, $p < 0.001$, unpaired Student's t test). Numbers above standard error bars are total number of optic lobes scored for each treatment.

then attempted to draw a functional connection between NO and the NO-induced rise in cGMP production observed in the photoreceptors. Nervous systems cultured with 1 mM L-NAME showed severe disorganization in the medulla, including the overgrowth of retinal axons (Figure 7a). However, low levels (100 nM) of the membrane-permeable cGMP analog 8-bromo-cGMP effectively antagonized the disruptive effects of 1 mM L-NAME (Figure 7b). In nervous systems treated with 1 mM L-NAME alone, 45% of the optic lobes showed photoreceptor axons projecting beyond the medulla. In contrast, none of the nervous systems cultured with 1 mM L-NAME plus 100 nM 8-bromo-cGMP showed retinal axon overgrowth. These nervous systems consistently showed undisturbed patterning in the medulla and had an overall disruption index that was not significantly different from controls (Figure 7c).

Methylene Blue Disrupts Posterior but Not Anterior Retinal Patterning in the Medulla

Methylene blue inhibits sGC by binding to the heme group (Miki et al., 1977) and also has recently been reported to inhibit NOS activity through a similar mechanism (Mayer et al., 1993; Luo et al., 1995). Methylene blue affects retinal patterning in the medulla, producing nervous systems with significantly greater disruption

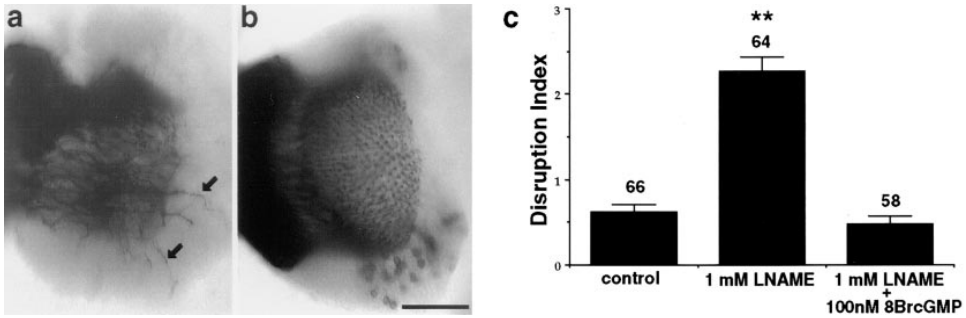

Figure 7. The Disruptive Effects of L-NAME Were Blocked with the Addition of 8-Bromo-cGMP

(a and b) Frontal view of optic lobes from brain–eye disc complexes that were maintained in vitro for 96 hr in the presence of (a) 1 mM L-NAME or (b) 1 mM L-NAME plus 100 nM 8-bromo-cGMP and then stained with the chaoptin antibody. With L-NAME alone, the projection pattern of photoreceptor axons is severely disorganized, and several retinal axons have grown beyond their targets in the medulla (arrows). When 100 nM 8-bromo-cGMP is included with the 1 mM L-NAME, the retinal patterning in the optic lobe is undisturbed.

(c) Nervous systems treated with 1 mM L-NAME were significantly more disrupted than controls or those treated with both L-NAME and 8-bromo-cGMP (double asterisks, $p < 0.001$, unpaired Student's t test). The disruption indexes for controls and 1 mM L-NAME plus 100 nM 8-bromo-cGMP were not significantly different. Numbers above standard error bars represent total number of optic lobes scored for each treatment. Scale bar, 75 μm.

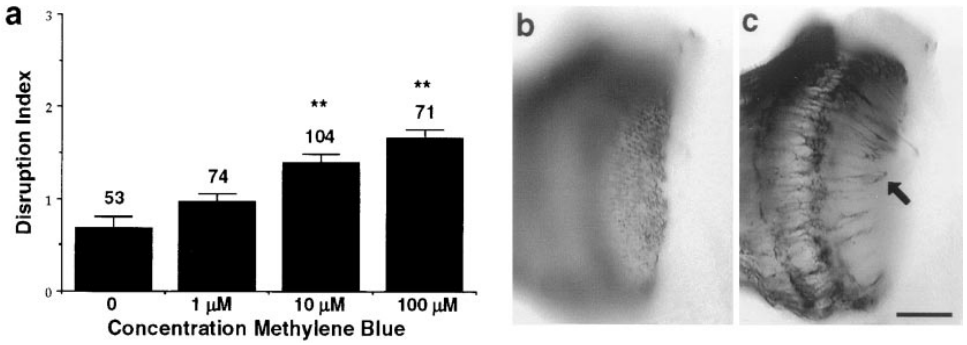

Figure 8. Methylene Blue Disrupts Retinal Patterning In Vitro

(a) Quantitative results showing that the presence of methylene blue caused a significant disruption in the retinal pattern when compared to controls. (Double asterisks, $p < 0.001$, unpaired Student's t test). Numbers above standard error bars represent total number of optic lobes scored for each treatment.

(b) Retinal patterning in the anterior medulla shows little disruption after treatment with 10 μM methylene blue.

(c) The same optic lobe as in (b), focusing on the posterior medulla and showing individual photoreceptor axons extending past the border of the medulla (arrow). Scale bar, 75 μm.

indexes when compared to controls (Figure 8a). In general, the disruption indexes resulting from methylene blue treatment were higher than those for L-NAME. However, there was also an aspect of this disruption that was specific to a subset of the photoreceptor population.

During normal development, the projection pattern of R7 and R8 is established in an anterior-to-posterior manner in the medulla, reflecting a temporal/developmental gradient. With the lowest level of methylene blue (1 μM), disruption of the terminal array was slight, with little difference observed between the anterior and posterior regions of the medulla (data not shown). By contrast, with higher levels (100 μM), disorganization of retinal axon projections was observed in both regions (data not shown). Of interest was the differential effects of 10 μM methylene blue. Anterior patterning appeared slightly disorganized, but there was no evidence of photoreceptor axons growing beyond the medulla (Figure 8b). However, in the most posterior region of the same optic lobe, many retinal axons were seen extending beyond their normal target layer in the medulla (Figure 8c). This effect on the posterior, and developmentally youngest, retinal axons was frequently observed in nervous systems treated with 10 μM methylene blue (43% of total scored) when compared to controls (8% of total scored).

Although methylene blue was effective at disrupting the retinal projection pattern, the results observed could not be attributed solely to inhibition of the sGC. ODQ is a more selective inhibitor of the sGC (Garthwaite et al., 1995), and incubating the CNS in 100 μM ODQ abolishes the SNP-induced increase in cGMP immunoreactivity in the photoreceptors (data not shown). Treatment with ODQ also produced a disorganization of the projection pattern in vitro. The disruption index for control cultures without ODQ was 0.43 ± 0.09 ([SEM]; $n = 56$). For nervous systems cultured with 10 μM ODQ, the disruption index was 1.90 ± 0.14 ($n = 67$), and with 1 μM ODQ, the disruption index was 1.52 ± 0.11 ($n = 68$). The disruption indexes for both 10 μM and 1 μM ODQ were significantly greater than controls ($p < 0.001$, Student's unpaired t test).

Discussion

Recent work in developing vertebrate systems has provided evidence that NO is necessary for proper synaptic patterning. Pharmacological inhibition of NOS activity prevents the pruning of ipsilateral retinal axons in the developing chick optic tectum in vivo (Wu et al., 1994) and has also been shown to disrupt the segregation of retinal ganglion cells into appropriate layers of the lateral geniculate nucleus of ferrets (Cramer et al., 1996). The in vitro application of NOS inhibitors, NO scavengers, and cGMP analogs to embryonic *Xenopus* nerve–muscle cultures showed that this pathway is involved in activity-dependent synaptic suppression (Wang et al., 1995). However, until now, the hypothesis that NO and cGMP work together to facilitate neuronal patterning during invertebrate development has not been tested.

We have shown that the photoreceptors of *Drosophila* express an sGC that can be stimulated to produce cGMP by exposure to an NO donor. The NO response can only be detected with cGMP immunocytochemistry during a discrete temporal window, beginning after the photoreceptor growth cones have arrived at their appropriate neuropils and terminating prior to the time at which the first postsynaptic currents are evident in the optic ganglia (Meinertzhagen and Hanson, 1993). Motor neurons in the embryonic grasshopper show a similar timing of NO sensitivity that commences when individual neurons reach their muscle targets and wanes as motor endplates begin to form (Truman et al., 1996). Identified neurons in larval lobsters also display transient NO sensitivity as neural circuits are reorganized during metamorphic development (Scholz et al., 1998). In each of these invertebrate examples, the sensitivity of the guanylate cyclase to NO appears to encompass the period of time following the arrival of axons at their targets and preceding the appearance of mature synapses.

NADPH-diaphorase activity is present in the optic lobe of *Drosophila* during the period when photoreceptor axons are responsive to NO treatment. A role for NOS in the insect visual system has been implicated for adult locusts, where diaphorase activity has been observed

in the laminar monopolar cell bodies and axons contributing to the first optic chiasm as well as neurons of the medulla and lobula complex (Elphick et al., 1996). *Drosophila* imaginal discs, including the eye-antennal disc, show diaphorase staining during the third larval instar (Kuzin et al., 1996). As NO appears to inhibit cell division in leg imaginal discs (Kuzin et al., 1996), the expression of NOS in the eye disc may be functionally correlated with proliferation associated with movement of the morphogenetic furrow. However, these proliferative events have been completed by the time an sGC response to NO can first be evoked in the maturing photoreceptors. The presence of NOS in *Drosophila* was proven by cloning of the *dNOS* gene from adult flies (Regulski and Tully, 1995). *dNOS* is expressed in adult fly heads, and its protein sequence bears strong identity to rat neuronal NOS (nNOS; Regulski and Tully, 1995). Similar to vertebrate neuronal NOS isoforms, DNOS activity in transfected cells is calcium/calmodulin-dependent and can be blocked with arginine analogs such as L-NAME (Regulski and Tully, 1995). As DNOS contains the epitope against which the UNOS antibody was raised, the pattern of UNOS immunoreactivity we observed is likely to reflect that of DNOS. More importantly, the region of the medulla where R7 and R8 terminate is positive for both diaphorase and UNOS staining. Thus, there is a potential source of NO present in the target at the time the photoreceptors begin to display NO sensitivity.

Using an in vitro culture system, we have examined the effects of inhibiting various components of the NO/cGMP pathway on the development of retinal projections in the medulla. Blocking the NO/cGMP signaling system reproducibly disrupted the proper positioning of photoreceptor terminals within their stereotypical crystalline array. Often we observed the growth of individual photoreceptor axons beyond their appropriate layers in the medulla. These aberrant projections were present after exposure to L-NAME and the NO scavenger PTIO, suggesting that NO is required as an intercellular signaling molecule in the developing visual system. The retinal disorganization and overgrowth associated with L-NAME treatment did not appear to arise from gross abnormalities in the medulla itself. Importantly, the disruption in patterning caused by high levels of L-NAME could be prevented by adding 8-bromo-cGMP. This result is paramount, as it establishes a functional link between NOS staining in the optic lobe neuropils and the NO-sensitive guanylate cyclase expressed by the photoreceptors. It also makes it unlikely that NO is acting through a target other than guanylate cyclase, as has been shown in other systems (Lei et al., 1992; Meffert et al., 1994), or that other molecular intermediates are activating the cyclase (Brune and Ullrich, 1987; Morton and Giunta, 1992; Zhou et al., 1993). The selective sGC inhibitor ODQ also disrupted the retinal projection pattern in a manner similar to that seen with L-NAME and PTIO, further evidence that sGC is part of this signaling pathway.

It is clear that the visual system has the components necessary for NO production and response. Moreover, the pharmacological inhibition of the signaling pathway disrupts development of the retinal projection pattern in the optic lobe. The question, thus, is what is the exact role of NO and cGMP in this process? Photoreceptors grow into the optic neuropil over a period of about 40 hr, beginning in the third larval instar. Here, the axons arrange themselves retinotopically within their appropriate neuropils but do not immediately begin the process of cartridge assembly. The first axons to reach their targets may remain in this condition for over a day. At ~10 hr APF, presumably in response to the rising steroid titers driving metamorphosis, this dormant period ends, and the photoreceptor axons begin to seek their synaptic partners (Meinertzhagen and Hanson, 1993). Subsequent growth cone spreading and activity thus necessitate a mechanism permitting maintenance and flexibility of the growth cone while preventing outgrowth of the axon beyond the target. It is during this time that the retinal axons become responsive to NO. More importantly, when we pharmacologically disrupt NO/cGMP signaling during this period, the retinal axons, rather than expanding to associate with their final targets, resume longitudinal growth to deeper layers of the medulla or in extreme cases beyond the medulla and into the brain.

This aberrant growth suggests that NO provides an arrest signal that is required once the axons emerge from their dormant period. At this time, they are switching their behavior from responding to cues important for pathfinding to interactive associations with potential synaptic partners. During the early phases of cartridge assembly, the strength of the contacts between the axons and these incipient targets is likely to be weak. We propose that NO may serve as a stabilizing influence on the maneuvering growth cone, preventing further extension of the axon beyond the vicinity of appropriate postsynaptic neurons during this period when more permanent connections have yet to be established. The subsequent selection of cartridge components and assembly of synaptic machinery may strengthen the contacts between the retinal axon and its targets, thus obviating the need for additional stabilization. At this time, the axons lose their responsiveness to NO, possibly by down-regulating expression of the sGC. Our interpretation of these results is consistent with observations on cultured *Xenopus* retinal ganglion neurons, in which NO induced growth cone collapse (Renteria and Constantine-Patton, 1996). In addition, in the developing visual system of postnatal ferrets, inhibition of NO production prevented the proper segregation of ON/OFF retinal ganglion cell projections into distinct layers of the lateral geniculate nucleus (Cramer et al., 1996). In the developing ferret, as we find in *Drosophila*, NO seems to be regulating events occurring after axon pathfinding but prior to the establishment of permanent synaptic connections.

When cultures were exposed to intermediate levels of methylene blue, axon overgrowth was most frequently observed in the posterior region of the optic lobe, while the anterior regions appeared normal (Figures 8b and 8c). The photoreceptors in the retina are determined in a posterior-to-anterior gradient (Wolff and Ready, 1993). As they project from the retina to the medulla, the axons of R7 and R8 extend through a chiasm, such that the youngest and anterior-most photoreceptors project to

the posterior margin of the medulla (Meinertzhagen and Hanson, 1993). In the context of our proposal that NO acts to prevent growth cone extension when neurons begin searching for their final synaptic partners, we suggest that the cellular machinery required for this arrest may not be complete when an axon first arrives in the medulla. This machinery may be augmented as the axons are waiting for the metamorphic cues to commence cartridge assembly; thus, the developmentally older axons in the anterior medulla may have a greater overall capacity for growth cone stabilization. The youngest axons have just arrived at the medulla when cartridge formation begins, however, and would thus have had less time to assemble the components required for growth arrest. It is these axons that are the most sensitive to treatment with 10 μM methylene blue, while all the retinal axons are affected by higher concentrations of the inhibitor. The effect of intermediate levels of methylene blue on the youngest cohort of photoreceptors may reflect that methylene blue can inhibit NOS as well as sGC (Mayer et al., 1993; Luo et al., 1995). Thus, while the actions of methylene blue remain specific to the NO/cGMP signaling cascade, a synergism may arise through inhibition of more than one component of the pathway.

Although there is abundant UNOS immunoreactivity in the medulla, we have not resolved whether it is due to neuronal processes, glial processes, or both. Also, we do not know if the production of NO from 10–40 hr APF is due to activity-dependent mechanisms or is a constitutive release coordinated by the hormones that trigger metamorphic development. In models of synaptic strengthening in the vertebrate brain, a signal from the presynaptic cell, generally glutamate, causes calcium influx and activation of NO synthesis postsynaptically (Garthwaite et al., 1988). Moreover, membrane turnover may result in spontaneous release of neurotransmitter from the tips of active growth cones (Young and Poo, 1983), thus a true "synapse" is not necessarily required for communication between a developing neuron and its environment. It remains to be determined if a dialog of this type exists in the *Drosophila* visual system, but we propose that the NO/cGMP signaling pathway provides a novel mechanism to maintain retinal axons in the vicinity of their targets until permanent connections are established.

Experimental Procedures

Fly Stocks

Drosophila of the wild-type Canton-S strain were grown at room temperature on standard cornmeal-molasses medium.

NO Stimulation and cGMP Immunocytochemistry

Tissue was dissected in phosphate-buffered saline (PBS) and placed in a solution of PBS containing 1 mM or 100 μM SNP and 1 mM IBMX for 15 min at room temperature. Tissues were then rinsed one to two times in PBS and fixed in 4% paraformaldehyde in PBS at room temperature for 2 hr or overnight at 4°C. After fixation, the tissue was rinsed three times in PBS-TX (PBS containing 0.3% Triton X-100) and blocked for 30 min in 5% normal donkey serum (NDS) in PBS-TX. An anti-cyclic GMP antiserum raised in rabbit (De Vente et al., 1987) was used at a final dilution of 1:4,000 in 1% NDS in PBS-TX. Primary incubation was overnight at 4°C. After removal of the primary solution, the tissue was rinsed for at least 1 hr with several changes of PBS-TX and incubated overnight at 4°C in a 1:1000 dilution of a donkey anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs). After rinsing for at least 1 hr with several changes of PBS-TX, localization of the antibody complexes was achieved by incubating the tissue in PBS-TX containing 0.5 mg/ml diaminobenzedine (DAB), 2 mg/ml b-D-glucose, 0.4 mg/ml ammonium chloride, and 0.2 units glucose oxidase. In most cases, 0.03% nickel chloride was added to the reaction to generate a black histochemical product. The tissue was arranged on poly-L-lysine-coated coverslips, dehydrated through an ethanol series, cleared in methyl salicylate or xylene, and mounted in DPX Mountant (Fluka). For fluorescent microscopy, tissue was prepared as above, with the secondary being a 1:1000 dilution of Texas red–conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Labs). After overnight incubation in secondary, tissue was rinsed, dehydrated, cleared in xylene, and mounted in DPX.

All reagents were from Sigma Chemical Company unless otherwise noted.

CNS Culture

The culture protocol outlined here was adapted from Awad (1995). Central nervous system/eye-antennal imaginal disc complexes were dissected from white prepupae (ring glands were removed) and cultured in a 100 μl droplet of D22 *Drosophila* medium (Sigma). All cultures were supplemented with 7.5% heat-inactivated fetal calf serum and contained a 1% final concentration of an antibiotic/antimycotic cocktail comprised of 10,000 units/ml penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B. Cultures were kept in a 25°C humidified culture incubator and aerated with a mixture of 95% oxygen, 5% carbon dioxide. After 24 hr, CNS–eye disc complexes were transferred into fresh media containing 1 μg/ml 20E. Cultures were continued in the presence of 20E for 72 hr, for a total culture time of 96 hr, at which point the CNS complexes were fixed in 4% paraformaldehyde in PBS in preparation for immunocytochemistry. All cultures containing inhibitors were accompanied by control cultures, which contained only the hormone and no inhibitor. Each experiment was repeated at least three times, and tissues were scored blind as to treatment. A mean score was calculated for each group, and results were analyzed using StatWorks and CricketGraph.

Pharmacological Agents

All pharmacological agents were diluted to working concentrations from sterile stock solutions that were made in tissue culture grade water (Sigma) and filtered. Dilutions were adjusted such that no more than 10 μl of any given inhibitor was added to the culture medium (total volume 1 ml); the same amount of tissue culture grade water was added to control cultures. The competitive NOS inhibitor L-NAME and its biologically inactive isomer D-NAME (both from Sigma) were made as 1 M stock solutions and diluted to final concentrations of 1 mM, 100 μM, 10 μM, and 1 μM. The amino acid L-arginine (Sigma) was also made as a 1 M stock and diluted to working concentrations of 10 mM, 1 mM, 100 μM, and 10 μM. 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; CalBiochem), an NO scavenger, was made as a 100 mM stock solution and diluted to final concentrations of 1 mM, 100 μM, and 10 μM. Methylene blue (Sigma) inhibits sGC and NOS and was made as a stock solution of 100 mM and diluted to working concentrations of 100 μM, 10 μM, and 1 μM. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, CalBiochem) was made as a 100 mM stock in DMSO and diluted to 1 mM in sterile water, then to working concentrations of 10 μM and 1 μM in D22. The final concentration of DMSO in these experiments was 0.01% and 0.001%, respectively. The control cultures for the ODQ experiments contained 0.01% DMSO. 8-Bromo-guanosine 3′,5′-cyclic monophosphate (8-bromo-cGMP, Sigma) was made as a stock solution of 100 μM and diluted to a working concentration of 100 nM.

NADPH-Diaphorase Staining

The NADPH-diaphorase staining protocol was adapted from Hope et al. (1991). In brief, the CNS was fixed in 4% paraformaldehyde in PBS at room temperature for 2 hr. After two rinses in PBS, the tissue was incubated in a solution of PBS with 0.1% Triton X-100 containing 1 mM NADPH (Sigma) and 0.2 mM nitroblue tetrazolium

(NBT, Sigma). Incubation was carried out at either 37°C or room temperature until a blue precipitate appeared. The tissue was then rinsed in PBS and mounted in 80% glycerol.

Chaoptin and NOS/Synaptotagmin Immunocytochemistry

After an overnight fixation at 4°C in 4% paraformaldehyde in PBS, the tissue was rinsed three times in PBS-TX (PBS containing 0.3% Triton X-100) and blocked for 30 min in 5% normal goat serum in PBS. MAb 24B10 (anti-chaoptin; Zipursky et al., 1984) was diluted 1:1000. Incubation in the primary antibody was carried out overnight at 4°C. After the primary solution was removed and the tissues rinsed through several changes of PBS-TX for at least 1 hr, the nervous systems were incubated overnight at 4°C in a 1:1000 dilution of a horseradish peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs). The antibody complexes were visualized using DAB as described previously.

In preparing tissue for NOS/chaoptin or synaptotagmin/chaoptin double labels and confocal microscopy, the same protocol was used with the following modifications: a 1:100 dilution of the universal NOS antiserum (anti-UNOS, Affinity Bioreagents) or a 1:1000 dilution of the synaptotagmin antibody (a gift from Dr. H. Bellen) and 1:500 dilution of MAb 24B10 were included together in the primary incubation. A Texas red–conjugated goat anti-rabbit IgG and an FITC-conjugated goat anti-mouse IgG were both used at a 1:500 dilution for the secondary incubation. After dehydration, the tissue was cleared in xylene and mounted in DPX mountant. Fluorescent images were taken on a BioRad MRC 600 confocal microscope and merged using Adobe Photoshop.

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