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Review

Nitric oxide and *Drosophila* development

Grigori Enikolopov*[,1], Julian Banerji[1] and Boris Kuzin[1]

[1] Cold Spring Harbor Laboratory Cold Spring Harbor, NY 11724, USA
* corresponding author: Grigori Enikolopov, Ph.D., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA. tel: (516) 367-8316; fax: (516) 367-6805; e-mail: enik@cshl.org

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Abstract

Mechanisms controlling the transition of precursor cells from proliferation to differentiation during organism development determine the distinct anatomical features of tissues and organs. NO may mediate such a transition since it can suppress DNA synthesis and cell proliferation. Inhibition of NOS activity in the imaginal discs of *Drosophila* larvae results in hypertrophy of tissues and organs of the adult fly, whereas ectopic overexpression of NOS has the reciprocal, hypotrophic, effect. Furthermore, NO production is crucial for the establishment of ordered neuronal connections in the visual system of the fly, indicating that NO affects the acquisition of the differentiated phenotype by the neural tissue. Increasing evidence points to a broad role that NO may play in animal development by acting as an essential negative regulator of precursor cell proliferation during tissue and organ morphogenesis.

Keywords: nitric oxide; drosophila; differentiation; synaptogenesis; organ development

Abbreviations: APF, after puparium formation; BrdU, 5-bromodeoxyuridine; cGMP, 3′<′ψθψλιψ γυανωσινε μωνωπℏωσπℏατε∴ ΔΠ∇∵ νιψωτιναμιδε αδενινε δινυψλεωτιδε πℏωσπℏατε∴ ΩΣ∵ νιτριψ ωξιδε σθντℏασε∴ ϱβ∵ ρετινωβλαστωμα

Organ and tissue development require a tightly controlled program of cell proliferation followed by growth arrest and differentiation and, often, programmed cell death. The balance between the number of cell divisions and the extent of subsequent programmed cell death determines the final size of a tissue or an organ (reviewed in[1–3]). Although much of the cellular machinery that controls cell division *per se* is well understood (reviewed in[4–8]) less is known about the signals that cause discrete groups of cells within organs to stop dividing upon reaching an appropriate cell number. These signals should relate cell cycle progression to information about the size of a domain of adjacent cells, and they probably involve some as yet undetermined inter- and intracellular second messenger molecules. These messengers might include multifunctional signaling molecules that would couple together several events, such as cessation of cell cycle progression, implementation of programmed cell death, and acquisition of specific traits that define a differentiated tissue.

Nitric oxide (NO) is a versatile diffusible second messenger implicated in numerous physiological functions in mammals, ranging from dilation of blood vessels and muscle relaxation to immune responses and potentiation of synaptic transmission (reviewed in[9–12]). It has also been shown to affect gene expression at the level of transcription[13–16] or translation.[17–19] NO is produced by nitric oxide synthase (NOS) in almost all cell types. Various isoforms of NOS are expressed throughout animal development, marking characteristic steps in tissue differentiation.[20] Recent evidence indicates that NO may be an important player in the program of development, directing the transition out of the proliferating state to differentiation and affecting the acquisition of a differentiated phenotype.

Studies on *Drosophila* in particular have been useful in revealing the importance of NO in organ development and tissue differentiation. NO is produced at high levels in imaginal discs at the end of the larval stage and acts as an essential negative regulator of cell proliferation: manipulation of NOS activity in the developing larvae affects the size of the adult fly's organs.[21] The morphogenic regulation by NO can be further evidenced at later points in development, being necessary for the proper development of the visual system of the fly, affecting synaptogenesis and the formation of retinal projection pattern.[22]

This review describes the NOS locus of *Drosophila* and the expression pattern of DNOS, discusses the data on the role of NO in cell proliferation and synapse formation in the developing fly, and considers evidence that NO has a conserved function in other developmental systems.

DNOS locus

The gene for *Drosophila* NO synthase is located on the second chromosome at cytological position 32B. This locus codes for a family of transcripts which may produce several NOS-related proteins. DNOS1 cDNA was originally cloned from a *Drosophila* head cDNA library using a fragment of the rat neuronal NOS gene as a probe.[23] Since that time, several attempts to clone putative orthologs of mammalian endothelial and inducible forms of NOS from cDNA or genomic libraries have not revealed any other loci that would code for NOS-like enzymes (Stasiv, Regulski, Tully and Enikolopov, unpublished). However, an extended family of transcripts within the DNOS locus has been identified. These transcripts have different 5′-ends, use alternative splice sites, and code for a family of NOS-related proteins. DNOS1 represents the major RNA isoform of NOS in *Drosophila* cells and codes for protein of 1350 amino acids which bears a strong resemblance to all

three NOS isoforms from mammals (43, 40 and 39% amino acid identity to rat neuronal, bovine endothelial, and mouse inducible isoforms of NOS, respectively) with a central portion showing 61% identity to the rat neuronal isoform.

The deduced DNOS1 protein contains all determinants which are crucial for NOS activity, such as regions for binding FMN, FAD, NADPH, tetrahydrobiopterin, heme, and calmodulin, and a consensus site for phosphorylation by cAMP-dependent protein kinase. At the same time, *Drosophila* NOS protein lacks the PDZ domain which is crucial for association of mammalian neuronal NOS with PDZ domains of other proteins such as PSD-95, α1-synthropin[24] and CAPON.[25] The first 200 amino acids of DNOS1 show no obvious similarity to any of the known forms of NOS. This region contains a long stretch of glutamine residues which are found in many *Drosophila*, and some vertebrate, proteins and have been implicated in the formation of protein–protein contacts.[26,27] DNOS1 cDNA, when expressed in cultured cells, produces a 150 kD polypeptide which can efficiently generate NO. This production of NO is dependent on calcium, calmodulin, and terahydrobiopterin, as reported for the neuronal NOS of mammals.

Molecular characterization of the DNOS locus shows that transcribed sequences are dispersed over a minimum of 40 kb of DNA and consist of at least 20 exons (Stasiv, Regulski, Tully and Enikolopov, unpublished). The locus gives rise to at least eight isoforms of RNA and four different forms of proteins, one of which is DNOS1 protein, the major enzymatically active form of NOS in the fly. Interestingly, two of these alternate isoforms encode truncated proteins having no apparent enzymatic activity but which can inhibit the activity of DNOS1 in cotransfection assays (Stasiv and Enikolopov, unpublished). These proteins may act as dominant negative regulators of NOS activity *in vivo*, perhaps by disrupting homodimerization of DNOS1. Transgenic flies which express these various isoforms have been generated (Regulski, Stasiv, Tully and Enikolopov, unpublished) and may provide additional insights into the developmental role of different DNOS-related proteins.

Expression of DNOS during development

Most of what we know about the expression and distribution of DNOS in developing *Drosophila* comes from the studies of NADPH-diaphorase staining of embryos and larvae. The biochemical properties of NOS and the NADPH-diaphorase activity have been well characterized in *Drosophila*, honeybee and locust.[28–31] These studies suggest that in insects, as has been rigorously demonstrated in mammalian systems, the identical enzyme is responsible for both NOS activity and NADPH-diaphorase staining after fixation. These data are corroborated by *in situ* hybridization of imaginal discs with the DNOS1 probe (Kuzin, Regulski, Stasiv and Enikolopov, unpublished).

There is no indication of cellular NADPH-diaphorase staining in embryos prior to stage 15.[31] In stage 15 embryos, a row of segmentally repeated NADPH-diaphorase-positive cells, presumably of neuronal origin, appear near the midline of the ventral nerve cord (VNC); however, this staining disappears at later stages. The first larval instar shows NADPH-diaphorase-positive interneurons in the brain and motorneurons in the VNC (Figure 1). As the larvae progress to the second instar stage, the staining intensity and the number of NADPH-diaphorase-positive interneurons in the brain and motoneurons in the VNC increase and, in addition, staining appears in the interneurons of the VNC and in the cellular projections from the brain into the VNC. In the third instar, the number and intensity of NADPH-diaphorase-positive neurons in the brain continue to rise. In addition, cells along the midline of the VNC which is composed of both neurons and glia, are strongly stained. Very strong NADPH-diaphorase staining

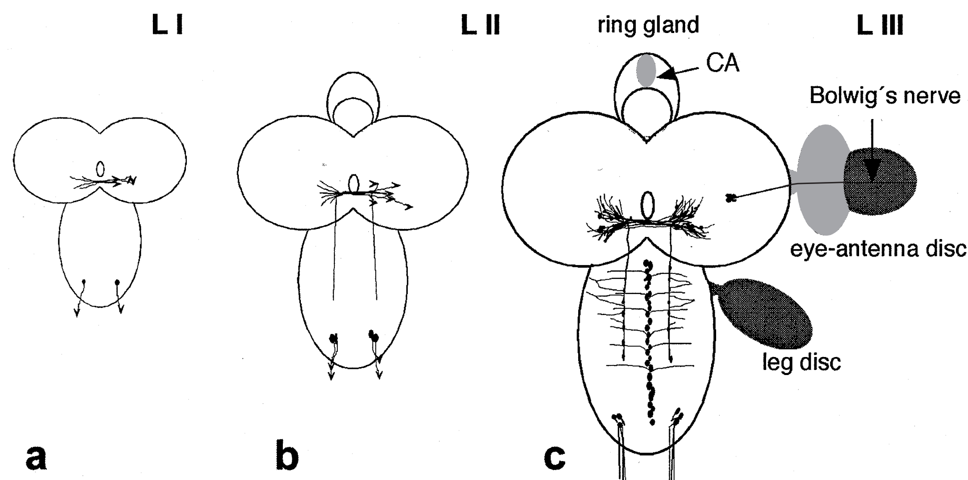

Figure 1 Schematic drawing of NADPH-diaphorase-positive cells of development *Drosophila* larvae (from Wildemann and Bicker).[31] (**a**) First larval instar showing NADPH-diaphorase-positive interneurons in the brain and motoneurons in the VNC. (**b**) In the second instar, the staining intensity and the number of interneurons stained in the brain increase. Cellular projections from the brain into the VNC are also stained. (**c**) NADPH-diaphorase staining in the CNS and imaginal discs of the third instar larvae. Strong staining is present in the Bolwig's nerve (the larval optic nerve) and in the region of the ring gland corresponding to the corpus allatum (CA)

can also be observed in the Bolwig's nerve, the optic nerve of the larvae, and in the region of the ring gland, a component of the endocrine system which regulates metamorphosis.

A dramatic increase in NADPH-diaphorase staining is observed in imaginal discs of the third instar. Imaginal discs, specialized groups of undifferentiated epithelial cells which later give rise to the epidermal structures of the adult fly, are formed in the first larval instar as integuments of the larval epidermis.[32] Disc cells divide rapidly throughout larval development, cease proliferating at the end of the third instar period and in early pupae, and undergo dramatic changes during metamorphosis, eventually differentiating into the organs of the adult fly (legs, wings, eyes, halteres, genitalia). NADPH-diaphorase staining is quite weak in the young imaginal discs of the third instar and gradually increases as development proceeds. In late third instar larvae and early pupae, a highly specific pattern of very intense staining is evident in all imaginal discs and also in the imaginal rings and histoblasts.[21,31] For instance, in the leg imaginal disc, NADPH-diaphorase staining is seen at the very beginning of the third instar and is confined to the center of the disc. As the discs mature, diaphorase staining is more apparent and at the end of the third instar period, it is present in all of the concentric rings of epithelial folding of the disc. Later in development, when the discs begin to evert in the prepupae, diaphorase staining of the forming leg becomes less intense. At 2–4 h after puparium formation, NADPH-diaphorase staining is seen in the presumptive tibia, first and second tarsal segments, and the proximal part of the fifth tarsal segment of the forming leg (see also Figure 3). Staining is much weaker in the third and fourth segments, and areas of staining are distributed throughout the regions of presumptive femur, coxa, and body wall. Similarly, highly reproducible patterns of intense staining are seen at the end of the third instar in the wing, the eye, the haltere, and the genital imaginal discs, as well as in the imaginal rings and histoblasts. This staining gradually decreases in a specific spatial pattern during pupal development.

After pupariation, very strong NADPH-diaphorase staining is observed in the brain in the regions of lamina and medulla of the optic lobe, where the synapses of the visual system are being formed.[22] In the adult *Drosophila*, NOS expression is mostly confined to the brain,[33–35] with weak NADPH-diaphorase staining being evident throughout the organs of the fly. Both NADPH-diaphorase and antibody staining suggest various levels of DNOS expression in different parts of the brain. The most abundant expression appears in (1) the antennal lobes of the olfactory system, (2) the fan-shaped body of the central complex and (3) the layer of medulla in the visual system. Intermediate to low levels of NOS expression include large parts of the neuropil of the central brain as well as the lobula and lamina of the visual system. The mushroom bodies, which are implicated in certain forms of learning and memory, display very low levels of NADPH-diaphorase activity and NOS immunoreactivity[34] (and Regulski and Tully, unpublished).

Together, the NADPH-diaphorase staining patterns of developing *Drosophila* indicate that in many cases NOS expression marks tissues that are differentiating, and neurons that are ready to establish synaptic contacts, suggesting that NO production may be involved both in the exit of cells from the proliferative phase, and in synapotogenesis in the developing nervous system.

NO and the transition from proliferation to differentiation

NO is a very effective inhibitor of cell proliferation. When chemical compounds that can release NO are added to cultured mammalian cells, DNA synthesis and cell division are suppressed.[36–38] This inhibition may affect various phases of the cell cycle: NO donors inhibit progression through S-phase,[39] suppress phosphorylation of pRb by cycle dependent kinases,[40] induce p53[41–43] and lead to an accumulation of a fraction of cells in the G2 phase.[44,45] Thus, exogenously added NO acts as a potent antiproliferative agent. Recent data indicate that the antiproliferative properties of endogenously produced NO are employed by the developing organism to control cell proliferation during tissue differentiation and organ development.

Developing *Drosophila* provides a good example of the importance of NO for growth regulation. The observation of high levels of NOS seen in mature imaginal discs combined with the observed potential of NO to block cell proliferation suggest that endogenously produced NO might act as a growth arrest agent during the transition to metamorphosis by inhibiting DNA synthesis and supporting cytostasis. This hypothesis is supported by experiments where NOS activity was manipulated in the developing larvae and the resulting changes were monitored in the adult fly.[21]

Suppression of NOS activity in imaginal discs by injection of NOS inhibitors into the developing larvae several hours before metamorphosis, at the end of the third instar, results in a dramatic enlargement of many adult structures: legs, wings, genital structures, tergites and sternites, etc. The changes are most profound in the legs, where the diameter of certain segments increases 3–4-fold (Figure 2). The first and second tarsal segments, tibia and femur, whose primordia had the highest levels of NOS at the larval and prepupal stages are the four leg segments most strongly affected by the inhibition of NOS. The use of

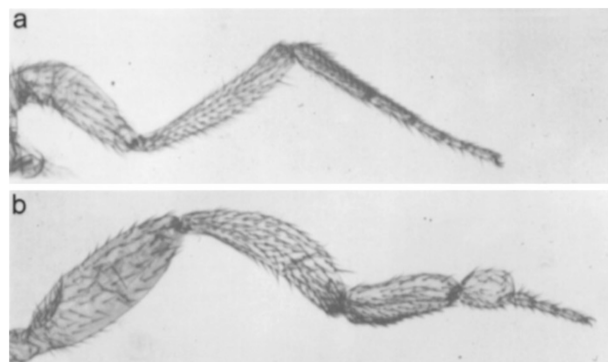

Figure 2 Overgrowth of adult leg segments after inhibition of NOS. (**a**) Third leg from a control untreated fly. (**b**) Third leg from a fly treated with NOS inhibitor L-nitroarginine methyl ester at the third instar stage

structurally unrelated inhibitors of NOS produced similar effects, indicating that the observed changes resulted specifically from blocking NOS activity. When the discs are labeled with 5-bromo-deoxyuridine (BrdU) after inhibition of NOS activity, the number of BrdU-labeled nuclei is significantly increased, suggesting that changing the NO levels directly affects DNA synthesis in the imaginal discs.

In a complementary series of experiments, ectopic expression of NOS was induced in transformed larvae carrying a mouse NOS cDNA transgene under the control of the heat–shock promoter. Changes seen upon treatment of transgenic larvae with heat shock included a reduction in the size of the limbs of the adult fly, supporting the hypothesis that NO acts to suppress cell proliferation in imaginal discs. In extreme cases, the length of the tarsus was halved and several segments were fused together with poorly defined boundaries. The segments of the adult leg most often affected by the overexpression of NOS (third, fourth, and fifth tarsal segments) were those whose precursors exhibited particularly low levels of NOS in the early prepupal stages. BrdU-labeling experiments with treated transgenic larvae support the notion that ectopic expression of NOS affects DNA synthesis: there were markedly fewer BrdU-labeled cells in imaginal discs of treated transgenic larvae, than in untreated controls, in contrast to augmented BrdU-labeling in experiments using NOS inhibitors. Interestingly, the segments of the leg whose size was most often affected when NOS activity was inhibited and the segments that were most often affected when NOS activity was ectopically induced were nonoverlapping and complementary (Figure 3). This reciprocal distribution of enlargement and shortening of leg segments in adult flies in response to a decrease or increase in NO matched the distribution of NOS within the leg imaginal discs, thereby supporting the hypothesis that NO plays a causative role in cell cycle arrest in normal development of the fly.

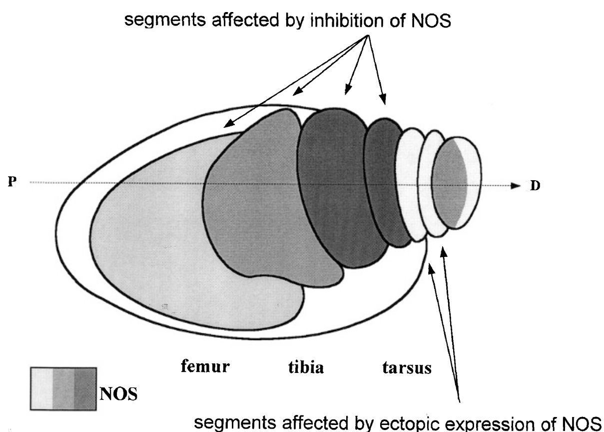

Figure 3 Changes of the leg segments in adult fly correspond to the distribution of NOS in the imaginal discs of the larvae. Segments that were most often and most strongly affected in adults (enlarged after NOS inhibition and reduced after ectopic NOS expression in larvae) are indicated. 'P-D' marks the proximodistal axis. Shading of the segments on the scheme corresponds approximately to the intensity of NADPH-diaphorase staining

Not all organs of the adult fly are equally affected by inhibition of NOS activity in the larval imaginal discs – for instance, in the eye imaginal disc, there is an increase in the number of cells in S-phase after inhibition of NOS, but in contrast to the leg, the adult eye is apparently unaffected. This is mainly due to programmed cell death counteracting the excessive cell proliferation induced by NOS inhibition: When NOS is inhibited in larvae in which apoptosis in the developing eye is largely prevented by expression of recombinant baculovirus p35 protein under an eye-specific promoter (GMR-P35 flies),[46] then the number of ommatidia, the repeating unit of the compound *Drosophila* eye, increases from the nearly invariant complement of 750 in wild-type or untreated GMR-P35 flies, to nearly 820 after NOS inhibition in GMR-P35 flies. In addition, the number of secondary and tertiary pigment cells, cone cells, and bristles, which is increased in GMR-P35 flies, is further augmented by inhibition of NOS activity. These experiments show that prevention of apoptosis in the developing *Drosophila* eyes reveals excessive proliferation of various cell types after NOS inhibition, which is otherwise masked by programmed cell death in the larvae and pupae. These results are remarkable since most of the screens for eye mutations in *Drosophila* have yielded mutants with a decreased number of cells and ommatidia in the compound eye. This suggests that there is a strict upper limit, which can apparently be exceeded when NOS activity is blocked on the background of suppressed apoptosis. Thus, NO can be involved in affecting the balance between cell proliferation and programmed cell death which determines the final size of an organ.

Although inhibition of NOS leads to hyperproliferation of cells, in most cases no duplications of larger structures (e.g., segments of the legs or wings) is detected. This indicates that the extra proliferation of cells under the influence of NOS inhibitors occurs after determination of the developmental fate of most of the cells in the imaginal disc. This suggests that NO may be more important for the induction of cell cycle arrest and subsequent differentiation of already committed cells than for the initial patterning events involving developmental commitment and establishment of cell identity in the embryo or larvae.

The molecular mechanisms by which NO exerts its antiproliferative activity are uncharacterized. NO is a highly reactive molecule which may interact with and modify a number of potential targets. In addition to guanylate cyclase and ribonucleotide reductase,[9,11,12] two direct targets of NO, the genes and proteins which mediate the antiproliferative activity of NO may include the components of the retinoblastoma (Rb) pathway such as p21,[47] or cell cycle-dependent kinases (cdks).[40] In the developing *Drosophila* eye the NO and Rb pathways interact to control the number of cells in the ommatidia, such that manipulations of NOS activity can enhance or suppress the effect of the Rb and E2F components of the pathway (Kuzin *et al.* submitted). These data suggest that NO controls precursor cell division in the developing discs by regulating entry into S-phase of the cell cycle. A recent screen has revealed that a number of genes which are known to participate in cell cycle control are transcription-

ally activated after exposure to NO (Nakaya and Enikolopov, unpublished).

NO and synaptogenesis in the developing visual system

NO signaling is employed at yet another stage of *Drosophila* development, during the establishment of ordered neuronal connections in the visual system of the fly. During the late larval and early pupal stages of visual system development, the photoreceptor neurons of each ommatidia project to the optic lobe of the brain, with the axons from the outer photoreceptors (R1 – R6) terminating in the first optic ganglion, the lamina, and with the axons from the inner photoreceptors (R7 and R8) terminating in the deeper layers of the optic lobe, the medulla.[48] Numerous observations indicate that the construction of the retinotopic map is dependent on retrograde signaling in the optic ganglia.[49 – 51] The nature of this retrograde signaling is not well characterized, but recent evidence points to a role for NO in the establishment of ordered connections by developing neurons.

Throughout the first half of the *Drosophila* pupal development there is a strong presence of NADPH-diaphorase activity and NOS immunostaining in the neurons of the lamina and medulla[22,52] (similar strong staining is also seen in lamina and medulla of the locust).[53] The staining, which is present in the projections but not in the cell bodies, is strongest between 24 and 50 h after puparium formation (APF). This period corresponds to a discrete temporal window of visual system development when active axon outgrowth from photoreceptors has begun but functional connections with optic lobe interneurons have not yet been established.

Importantly, this period of NOS expression in the optic lobe overlaps with the period when the photoreceptors become sensitive to NO and can respond to treatment with NO donors by displaying 3′,5′-cyclic guanosine monophosphate (cGMP) immunoreactivity[22] (when challenged with NO donors, soluble guanylate cyclase of the photoreceptor cells is activated and produces cGMP which can be detected using specific antibodies). cGMP immunoreactivity is evident in the photoreceptor cell bodies and along the total length of their axons. Different photoreceptors differ in their response to NO, with the axons of the outer cells which terminate in the lamina (R1 – R6) being more sensitive and producing stronger cGMP responses to NO than the axons of the inner photoreceptors which terminate in the medulla (R7 and R8). NO sensitivity lasts from 10 to 50 h APF, after which the photoreceptors no longer respond to the application of NO donors by synthesizing cGMP. This time window corresponds to the period between the arrival of the photoreceptor axons in the optic lobes and the beginning of the synapse formation between the axons and neuronal projections of the lamina and medulla (Figure 4). Thus, there is a correlation between the time when the photoreceptors' axons respond to NO by synthesizing cGMP and the time when the target neurons in the optic lobe express NOS. Similarly, in the embryonic grasshopper, synaptogenesis correlates with a phase when many identifiable nerve-cell types respond to NO by producing cGMP.[54] In *Drosophila*, locust, and grasshopper nervous system preparations, it has been observed that there is very little overlap between the distribution of NADPH-diaphorase-positive cells and cells which display cGMP immunoreactivity in response to NO,[22,31,35,53,54] suggesting that NO can act as a transcellular signaling molecule in these insect systems.

The crucial role for NO signals in the formation of a correct retinal projection pattern in *Drosophila* was demonstrated *in vitro* using isolated CNS of the pupae. These preparations are capable of undergoing morphological changes mirroring those observed during metamorphosis. When the isolated brain is cultured in the presence of NOS inhibitors or NO scavengers, the optic lobe displays a severely disorganized projection pattern, with retinal axons extending beyond the medulla and into the brain.

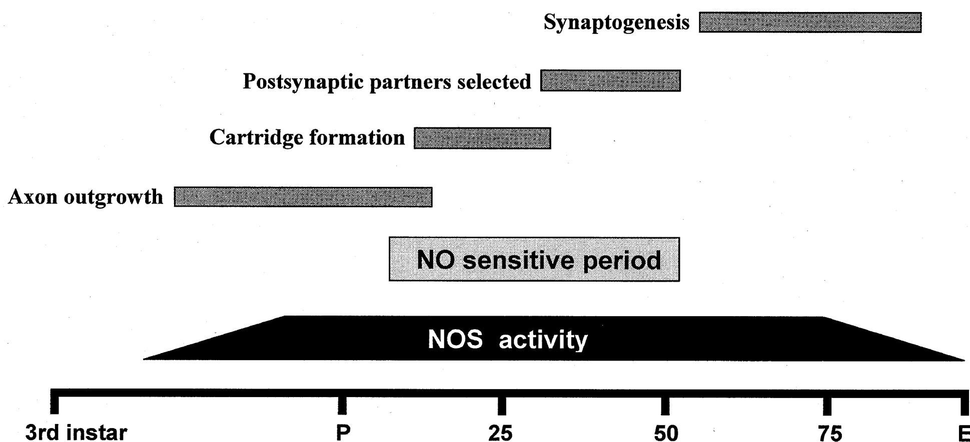

Figure 4 A time line of visual system development in *Drosophila*. Numbers indicate hours of development after pupariation (P) to adult eclosion (E). The photoreceptors displayed an increase in cGMP immunoreactivity in response to treatment with NO donors for approximately 40 h during the first half of metamorphosis (light shaded bar). NOS expression is strongly induced at the end of the third instar (black bar); in the optic lobe it is present until 75 h APF and is strongest between 24 and 50 h APF. Time line and scheme are adapted from Gibbs and Truman.[22]

Importantly, addition of a cGMP analog suppressed the disruptive effects of NOS inhibitor, suggesting a functional connection between the presence of NOS in the optic lobe neurons and the rise of NO-induced cGMP immunoreactivity in the photoreceptor cells. Furthermore, treatment with inhibitors of guanylate cyclase activity disrupted the retinal projection pattern in a manner similar to the action of NOS inhibitor. This suggests that a NO-cGMP retrograde signaling system is crucial for the proper development of the *Drosophila* visual system. NOS expression in the lamina may in turn depend on proper retinal innervation, since in the mutants *sine oculis* and *eyes absent* which have reduced or absent retinal innervation, NADPH-diaphorase staining of the lamina is significantly suppressed.[52] In summary, these observations provide strong evidence that NO and cGMP signals act in tandem to stabilize retinal growth cones at the start of synaptic assembly.

The NO sensitivity that occurs during synaptogenesis and synaptic maturation during *Drosophila* visual system development appears to be conserved among a number of species, suggesting a broad role for NO in the regulation of process outgrowth and synaptic remodeling during neural development. NO is involved in the pruning of retinotectal synapses in the developing chick visual system.[55] NO has been also shown to regulate activity-dependent suppression at the developing neuromuscular junction in *Xenopus*[56] and to stimulate the formation of synaptic connections in developing and regenerating rat olfactory neurons.[57] In the developing visual system of ferrets, inhibition of NO production prevents the proper segregation of retinal ganglion cell projections into distinct layers of the lateral geniculate nucleus, thus suggesting that NO regulates events occurring after axon pathfinding but prior to the establishment of permanent synaptic connections.[58] These activities of NO during nervous system development may be based on a common property of NO to induce growth cone collapse which has been demonstrated *in vitro* for rat dorsal root ganglion neurons[59] and cultured *Xenopus* retinal ganglion neurons.[60]

Concluding remarks

An increasing number of developmental systems provide evidence that the ability of NO to prevent DNA synthesis and cell division is exploited as a part of a developmental program in a variety of tissues. NOS expression and NO synthesis are strongly induced in cultured cells treated with growth factors, and there is increasing evidence that many of the pleiotropic effects of growth factors may be mediated through NO.[45,47,61–68] NOS levels are also transiently increased during the development of many tissues and organs in mammals, where this transient elevation often coincides with the cessation of division of committed precursor cells.[69–73] Furthermore, NOS activity is greatly elevated in regenerating tissues.[70,71,74–77] Together, these studies suggest that in various tissues and cell types, NO may regulate cell differentiation and tissue morphogenesis by acting as endogenous antiproliferative factor. Indeed, NO is crucial for the differentiation of cultured neuronal cells,[45,62] endothelial cells,[63,64,66] adipocytes,[67] osteoblasts,[65] and myoblasts.[78,79] Attempts to directly test the function of NOS in mouse development by studying the effects of nNOS gene disruption have so far yielded an incomplete picture: the use of alternate promoters and splice sites leading to the generation of multiple nNOS RNA isoforms during embryonic development[24,80–82] mask the effect of nNOS gene mutation in the knockout mouse lines. However, it was demonstrated that NO synthesis is essential for the transition from cell proliferation to cell cycle arrest during organ development in *Drosophila*,[21] hematopoiesis in mouse (Michurina *et al*. submitted) and brain development in *Xenopus* (Peunova *et al*. submitted). This suggests that NO may act as an essential negative regulator of cell proliferation during tissue differentiation and organ development, controlling the balance between cell proliferation and differentiation in a developing tissue. It may also induce or affect specific traits that characterize the differentiated tissue, thus coupling the exit from the cell cycle to the acquisition of the differentiated phenotype.

NO is easily diffusible and may therefore exert its antiproliferative effects both in the cell that produces it and its neighbors. Thus, it is possible that NO may induce synchronized changes in a group of adjacent cells and contribute to their coordinated development. Moreover, several adjacent cells producing easily diffusible antiproliferative molecules may share the total pool of these signaling molecules. If a particular threshold level of a signal is needed to initiate a cascade that eventually leads to cell cycle arrest, then the cells in this group could stop dividing when a certain number of cells, and, therefore, a certain local concentration of messenger molecules is reached. This way, by organizing groups of cells into functional clusters, NO may instruct the cells to coordinately terminate their proliferation, when the clusters attain the appropriate size and shape.

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