ABSTRACT: Nitric oxide (NO) is a membrane-permeant signaling molecule which activates soluble guanylyl cyclase and leads to the formation of cyclic GMP (cGMP). The NO/cGMP signaling system is thought to play essential roles during the development of vertebrate and invertebrate animals. Here, we analyzed the cellular expression of this signaling pathway during the development of the *Drosophila melanogaster* nervous system. Using NADPH diaphorase histochemistry as a marker for NO synthase, we identified several neuronal and glial cell types as potential NO donor cells. To label NO-responsive target cells, we used the detection of cGMP by an immunocytochemical technique. Incubation of tissue in an NO donor induced cGMP immuno-reactivity (cGMP-IR) in individual motoneurons, sensory neurons, and groups of interneurons of the brain and ventral nerve cord. A dynamic pattern of the cellular expression of NADPHd staining and cGMP-IR was observed during embryonic, larval, and prepupal phases. The expression of NADPH diaphorase and cGMP-IR in distinct neuronal populations of the larval central nervous system (CNS) indicates a role of NO in transcellular signaling within the CNS and as potential retrograde messenger across the neuromuscular junction. In addition, the presence of NADPH diaphorase-positive imaginal discs containing NO-responsive sensory neurons suggests that a transcellular NO/cGMP messenger system can operate between cells of epithelial and neuronal phenotype. The discrete cellular resolution of donor and NO-responsive target cells in identifiable cell types will facilitate the genetic, pharmacological, and physiological analysis of NO/cGMP signal transduction in the developing nervous system of *Drosophila*. © 1999 John Wiley & Sons, Inc. J Neurobiol 38: 1–15, 1999

**Keywords:** midline glia; imaginal disc; NADPH diaphorase; neuromuscular junction; retrograde messenger

Nitric oxide (NO) is a short-lived signaling molecule which readily diffuses across membranes. In the nervous system, NO is generated by Ca\(^{2+}\)/calmodulin-stimulated NO synthases (NOS) and serves as an activator of soluble guanylyl cyclase (sGC), although other signal transduction mechanisms are possible (reviewed in Bredt and Snyder, 1992; Dawson and Snyder, 1994; Garthwaite and Boulton, 1995). The dynamic regulation of NOS during the formation and regeneration of the nervous system (Bredt and Snyder, 1994; Roskams et al., 1994) has led to the suggestion that NO functions in developmental processes. Experimental manipulations of NO signal transduction provided evidence that NO mediates the...
refinement of retinotectal projections (Wu et al., 1994) and activity-dependent synaptic suppression at developing neuromuscular synapses (Wang et al., 1995). Moreover, in the insect nervous system of the embryonic grasshopper, synaptogenesis correlates with a phase when many identifiable nerve cell types respond to NO by producing cGMP (Truman et al., 1996).

The fruit fly *Drosophila melanogaster* has emerged as a useful model system for the genetic analysis of the developing nervous system. There is compelling evidence that the nervous system of *Drosophila* employs NO/cGMP signaling. Biochemical measurements in the brain of adult *Drosophila* (Müller, 1994) have demonstrated a Ca\(^{2+}\)-stimulated NOS activity. Using NADPH diaphorase (NADPHd) staining as a marker for NOS in fixed tissue, NO expression has been localized to olfactory and other central neuropiles of the adult brain of *Drosophila* (Müller and Buchner, 1993; Müller, 1994). Meanwhile, the genes encoding a Ca\(^{2+}\)/calmodulin-dependent NOS (Regulski and Tully, 1995) and the two subunits of sGC (Liu et al., 1995; Shah and Hyde, 1995) have been cloned. Moreover, further downstream acting components of cGMP signaling pathways have also been identified. Two genes for cGMP-dependent protein kinases have been cloned and the biochemical properties of one cGMP-dependent kinase have been characterized (Foster et al., 1988, 1996; Kalderon et al., 1989).

Two recent investigations have been devoted to NO signaling during *Drosophila* development. NO synthesis in imaginal discs appears to regulate cell proliferation, thus controlling morphogenesis of body structures (Kuzin et al., 1996). During the development of the optic lobe, NO and cGMP regulate the formation of the retinal projection pattern (Gibbs and Truman, 1998). To identify further cell types employing NO signaling during *Drosophila* development, we have evaluated the nervous system of embryonic and larval stages for expression of NADPHd staining. Since the immunocytochemical detection of cGMP is a powerful tool to localize NO-receptive cells, we used well-defined antisera against cGMP (De Vente et al., 1987) to label potential target cells of the NO/cGMP messenger system. In insects, this method has also been successfully applied to embryonic grasshoppers (Truman et al., 1996) and to the adult locust nervous system (Bicker et al., 1996; Bicker and Schmachtenberg, 1997). In this study, we report the dynamic expression pattern of the two markers for the NO/cGMP signaling pathway in neuronal and glial cells in the central nervous system (CNS) and epithelial cells of the imaginal discs. Using neuronal and glial markers, we will identify the phenotype of potential NO-releasing and -responsive cell types. Thus, our cytochemical survey will provide a basis for studies on the effects of disrupting NO/cGMP signaling on the development of individually identified cells. The discovery of identified NO-sensitive motoneurons that have been used extensively in developmental, electrophysiological, and genetic studies (Broadie et al., 1993; Keshishian et al., 1996; Petersen et al., 1997; Davis and Goodman, 1998; Davis et al., 1998) will facilitate the understanding of NO/cGMP signal transduction at the neuromuscular junction of *Drosophila*.

**MATERIALS AND METHODS**

In this study, we used the wild-type strain Oregon-R, the enhancer trap line 3400 as a marker of midline glia (Klambt et al., 1991; Ito et al., 1995; Callahan and Thomas, 1994), and the line 2138 repo4/lacZ (Xiong et al., 1994) which expresses the lacZ fusion protein in all glial cells except midline glia. *Drosophila melanogaster* larvae were reared on a standard agar corn meal medium at 25°C in a 12:12 h light/dark photoperiod.

**Staging**

The embryos were staged according to Campos-Ortega and Hartenstein (1985). The staging of the larvae was based on the days after hatching and on the number of teeth of the mouth hooks (Ashburner, 1989).

**NADPHd Staining**

The embryonic and larval CNS including attached imaginal discs were dissected in ice-cold L-15 medium (Gibco), fixed for 30 min (embryo) or 1 h (larva) in 4% formaldehyde dissolved in phosphate-buffered saline (PBS) (0.01 M, pH 7.4), and permeabilized overnight in 1% Saponin/PBT (PBS containing 0.1% Triton X-100) at 4°C. For visualization of the fixation-insensitive NADPHd activity, the tissue was incubated for 2–3 h with PBT, pH 7.4, and 0.1 mM nitroblue tetrazolium (NBT; Sigma) in the presence of 0.1 mM β-NADPH (Sigma). Incubations in the absence of β-NADPH showed no staining. After termination of the staining, the tissue was washed with PBT and mounted in glycerol.

**cGMP Immunocytochemistry**

The embryonic and larval CNS including attached imaginal discs were dissected and incubated for 30 min in ice-cold L-15 medium containing 1 mM 3-iso-butyl-1-methylxanthine (IBMX). After the addition of sodium nitroprusside (SNP) to a final concentration of 100 μM, the tissue was incubated
for 15 min at room temperature. The tissue was fixed in 4% phosphate-buffered formaldehyde for 2 h on ice and rinsed in PBT. The primary rabbit cGMP antiserum (De Vente et al., 1987; De Vente and Steinbusch, 1993) was applied at 4°C overnight in a dilution of 1:1000 in PBT. For visualization of cGMP immunoreactivity (cGMP-IR), we used an ABC-kit (Vector Laboratories) according to the manufacturer’s instructions. After washing, the tissue was reacted with DAB (PBT containing 0.25 mg/mL diaminobenzidine) and H2O2 in the presence of 0.08% nickelchloride yielding a black reaction product and mounted in glycerol. A different sheep cGMP antiserum, courtesy of J. De Vente, used at a dilution of 1:5000, gave identical staining results. Details of the generation and tests for specificity of the employed anti-cGMP sera in a gelatine model system, nitrocellulose blots, mammalian, and insect tissue are given elsewhere (De Vente et al., 1987; De Vente, personal communication; Ewer et al., 1994; Bicker et al., 1996). Control incubations of the various developmental stages in the absence of both IBMX and SNP showed no immunoreactivity. Control incubation in the presence of either IBMX or SNP gave occasionally slight staining in unidentified cell types of the CNS. This article reports only NO-induced immunoreactivity that required treatment with both SNP and IBMX. Whole mounts were viewed and photographed with a Polyvar microscope (Reichert-Jung).

**cGMP/22C10 Double Labeling**

The larval CNS including attached imaginal discs were processed for cGMP immunocytochemistry as described above. The DAB staining occurred in the presence of 0.08% nickelchloride yielding a black reaction product. Subsequent, a mouse 22C10 antibody (Fujita et al., 1982) was applied overnight at 4°C in a dilution of 1:5 in PBT. Immunoreactivity was visualized with a secondary horse-radish peroxidase–coupled goat anti-mouse immunoglobulin G (IgG) (Sigma) diluted 1:200 in PBT and incubation at room temperature for 3 h. The DAB staining resulted in a brown reaction product. The tissue was either mounted in glycerol or embedded in plastic (Durcupan) and cut into 4-μm sections. Color micrographs were generated on an Olympus Provis compound microscope. Images were captured electronically using a Sony CCD color videocamera and transferred to a Power Macintosh. Figures were arranged and labeled using Adobe Photoshop.

**NADPHd and β-Galactosidase Double Labeling**

The animals of the strain 3400 or 2138 were prepared for NADPHd staining as described above and incubated overnight at 4°C with a rabbit antibody against β-galactosidase (1:2000; Aurion). Binding of the rabbit antibody was detected with a CY3-coupled secondary antibody (Jackson ImmunoResearch) diluted 1:500 in PBT and incubated overnight at 4°C.

**RESULTS**

**NADPHd Staining during Development**

To determine the pattern of potential NO-synthezing nerve cells in the CNS, we followed the expression of NADPHd during embryonic and larval phases of development. In this study, we focused on examples of the most prominent staining of soma clusters in the brain and the segmental ventral nerve cord (VNC). However, since an NO signaling system has already been implicated in the development of imaginal discs (Kuzin et al., 1996), we also considered the staining in imaginal discs connected to the nervous system.

Prior to stage 15, there was no indication of discrete cellular NADPHd expression. The first NADPHd staining appeared in the embryos of stage 15 [Fig. 1(a)]. Filleted preparations of embryos revealed NADPHd-expressing cells of neuronal morphology in the ventral nerve cord [Fig. 1(a)]. A conspicuous pair of large NADPHd-positive cells was segmentally repeated near the midline of the VNC. At later developmental stages, this pair of cells was no longer detectable. To distinguish between neuronal and non-neuronal cells, we stained for repo, a glial cell-specific molecular marker (Xiong et al., 1994; Halter et al., 1995; Campbell et al., 1994). The repo lacZ line 2138 expresses the β-galactosidase fusion protein in all glial cells except the midline glia. Double labelings showed no colocalization of the NADPHd and anti-β-galactosidase staining. Thus, we have to assume that the paired NADPHd-positive cells are of neuronal phenotype. At stage 15, no staining was visible in the peripheral nervous system. The brain of filleted embryos showed indications of the presence of the blue diaphorase reaction product, but no discrete cellular resolution was obtained. In the VNC of later embryonic stages, the NADPHd staining was reminiscent of stage 15; however, discrete cells became gradually visible in the brain.

At the first larval stage, discrete cellular staining could also be detected in the brain. NADPHd expression was found in a bilaterally symmetrical cluster of interneurons which projected from posterior parts of the brain below the esophagus into the contralateral hemisphere [Fig. 1(b,e)]. In the first larval instar, the staining appeared rather weak [Fig. 1(b)], with an increase in intensity during subsequent development. Some of these interneurons sent out descending projections into the neuromeres of the ventral nerve cord [Fig. 1(c,d,f,g)] which could be clearly resolved at larval stages 2 and 3. The most posterior abdominal neuromeres contain NADPHd-positive motoneurons which exit the ventral nerve cord through peripheral
nerves [Figs. 1(b–g) and 2(d)]. Again, in the first larval instar, this staining was initially rather weak with an increase in intensity at later developmental stages. In the third larval stage, a group of neuronal fibers became visible which left the brain lobe and accompanied Bolwig’s nerve toward the eye imaginal disc [Fig. 1(d,g)]. The terminal arborizations could not be resolved. NADPHd staining was also found in endocrine tissue. The region of the ring gland corresponding to the corpus allatum contained NADPHd-stained glandular cells [Figs. 1(g) and 2(c)].

The two hemispheres of the segmented VNC are separated by a distinctive strip of midline cells composed of neurons and glial cells (Jacobs and Goodman, 1989; Klambt et al. 1991; Bossing and Technau, 1994). In the neuromeres of the VNC of late third-instar larvae, strong NADPHd staining was expressed in cell bodies and processes originating in the midline region [Figs. 1(d) and 2(d)]. Most likely, both glial cells and the ventral unpaired median neurons (VUM) contributed to the staining of the midline. Evidence for NADPHd staining in the midline glia will be presented in a separate section. The development of the ventral unpaired median cells was extensively analyzed in the embryo (Jacobs and Goodman, 1989; Klambt et al. 1991; Bossing and Technau, 1994). In the VNC of late third-instar larvae, the neurites of the VUM-neurons stained for NADPHd [Fig. 2(d)], whereas the peripheral projections onto the body-wall muscles were not stained. The developmental sequence of NADPHd expression in the nervous system is summarized in Table 1. An overview of the NADPHd pattern in the CNS and attached imaginal discs during larval development is schematically depicted in Figure 1(e–g).

**Figure 1** NADPH-diaphorase staining. (a) Filleted preparation of a stage 15 embryo. Stained cells are visible in the ventral nerve cord. Segmentally repeated paired cells near the midline show NADPHd-positive staining (arrows). (b) CNS of a first instar larva. Weak NADPHd staining is visible in interneurons of the brain (arrowheads) and in motoneurons of the ventral nerve cord (arrow). (c) Increase in NADPHd staining in the second larval instar. Interneurons which project below the esophagus into the contralateral hemisphere of the brain (arrowheads), interneurons of the ventral nerve cord (open triangle), and motoneurons (arrow) of the posterior abdominal neuromeres are labeled. Scale bar = 50 µm (a–c). (d) Whole mount of CNS and attached imaginal discs of third-instar larva. The intensively stained neurons corresponding to the second instar are clearly visible. In addition, cells along the midline of the ventral nerve cord are stained (small arrowheads). The cells of the attached pro- and mesothoracic leg discs (stars) and of the antennal disc (asterisk) express intense staining. The small arrow indicates NADPHd-positive cells of the Bolwig’s nerve. Scale bar = 100 µm. Schematic drawing of NADPHd-positive cells of developing larva. (e) First larval instar showing NADPHd-positive interneurons in the brain and motoneurons in the VNC. (f) In the second instar, the staining intensity and number of interneurons stained in the brain increase. Cellular projections from the brain into the VNC are also stained. (g) Drawing of NADPHd staining in the CNS and attached imaginal discs of the third larval instar. Strong staining was also found in the region of the ring gland corresponding to the corpus allatum (CA).

**cGMP Staining during Development**

Since NO functions as an activator of the cGMP-synthesizing enzyme sGC, we used antisera against cGMP to detect potential target cells of the NO/cGMP messenger system. Under conditions where the tissue was exposed to the NO donor SNP and breakdown of cGMP was blocked by the phosphodiesterase inhibitor IBMX, cGMP-IR could be visualized in distinct neurons. This technique did not resolve any stained cell prior to stage 15. In embryos of stage 16 and later, segmentally repeated cGMP-IR somata could be resolved in the VNC [Fig. 3(a)]. Because of the characteristic dorsal soma localization close to the midline and near the anterior commissure (Broadie et al., 1993, 1996) these cells are most likely the RP3 motoneurons. The sensory neurons of the developing peripheral nervous system were not stained except for one cGMP-IR cell in the eighth abdominal segment [Fig. 3(a)]. The morphology of this cell type resembles the dorsal bipolar dendrite (BD) neuron (Jones et al., 1995). In the body wall of third larval stages, such cGMP-IR sensory neurons were serially repeated in all segments.

The number of cGMP-IR cells increased during the development of the CNS in first and second larval stages (Table 1). In the ventral nerve cord, cGMP-IR was expressed in segmentally arranged cell clusters lateral to the midline and more dispersed cells at the outer margins of the neuromeres [Fig. 3(b,c,e,f)]. The clusters near the midline matched the soma position of the RP2, RP3, RP4, and aCC motoneurons. These clusters also expressed their NO-induced cGMP-IR during the third larval instar [Fig. 3(d,g)]. In the brain, one cluster of cGMP-IR neurons originated in the pars
intercerebralis innervating the ring gland [Figs. 2(b) and 3(g)]. In the region of the ring gland corresponding to the corpora cardiaca, intrinsic immunoreactive cell bodies were found among unlabeled cells [Figs. 2(a) and 3(g)]. Anterior parts of the brain lobe contained a neuronal cluster which projected descending neurites via the contralateral hemisphere into the ventral nerve cord. An overview of the cGMP-IR in the CNS and attached imaginal discs is schematically depicted in Figure 3(e–g). A comparison to the NADPHd staining [Figs. 1(e–g) and 3(e–g)] indicates that in the larval CNS both neurochemical markers were localized in separate cell types.

Figure 2 Innervation of glandular and peripheral structures in third-instar larvae. (a) Cells of the corpora cardiaca (CC) showing cGMP-IR (stars). (b) Whole mount of brain area showing cGMP-IR in somata and looping neurites innervating the ring gland (arrows). Scale bar = 80 μm. (c) NADPH diaphorase staining of the corpus allatum (CA) of the ring gland (asterisk). Scale bar = 50 μm (a,c). (d) NADPHd staining in ventral nerve cord. Note the stained cells along the midline (arrows), the axons of the VUM motoneurons (small arrows), and posterior motoneurons exiting via peripheral nerves (arrowheads). Scale bar = 30 μm.

Presynaptic Boutons of Distinct Motoneurons Are cGMP-IR

In the embryonic grasshopper, synaptogenesis at the neuromuscular junction correlates to a phase when certain identifiable motoneurons respond to NO by producing cGMP (Truman et al., 1996). To examine whether this occurs also in *Drosophila*, we prepared embryonic fillets from successiveley older stages of development. Even though in late embryos cGMP-IR was found in cell bodies which most likely belong to the RP3 motoneurons [Fig. 3(a)], no staining could be detected in the peripheral projections to the muscles.
Synaptic boutons on the corresponding muscle fibers 6/7, which receive their innervation from the two motorneurons RP3 and 6/7b, began to express NO induced cGMP-IR during the first larval stage. In the third larval instar, the boutons of the prominent motoneurons RP2, RP3, RP4, and aCC were clearly stained. The identification of these cells was based on the characteristic anatomical arrangement of immunoreactive cell bodies in the CNS and the staining of synaptic terminals on the corresponding muscle fibers 6/7 (RP3 and 6/7b), muscle fiber 1 (aCC), muscle fiber 2 (RP2), and muscle fiber 13 (RP4), respectively. An example of immunoreactive boutons on muscle 6/7 is shown in Figure 4(b). The diagram in Figure 4(c) provides a summary of the cGMP-IR synaptic terminals in third-instar larvae which could also be detected on the muscles 5/8, 9, 10, 11, 14.1, 14.2, 15/16, 19, 21, and 22, and in the posterior segments on muscle 12. The NO sensitivity of synaptic terminals and their central cell bodies lasted up to the prepupal stages.

Table 1: NADPHd Staining and cGMP-IR during Different Developmental Stages

<table>
<thead>
<tr>
<th>NADPHd Staining</th>
<th>Developmental Stages</th>
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<th>LII</th>
<th>LIII</th>
<th>Prepupa</th>
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<td>CNS</td>
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<tr>
<td>PNS</td>
<td>Imaginal discs of body appendages</td>
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<td>Corpus allatum</td>
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Table 1: NADPHd Staining and cGMP-IR during Different Developmental Stages (with SNP and IBMX) and Control without:

<table>
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<th>VNC</th>
<th>Motoneuronal somata</th>
<th>PNS</th>
<th>NMI, boutons</th>
<th>Sensory neurons of leg disc</th>
<th>Corpora cardiaca</th>
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<td>Corpora cardiaca</td>
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† Subjective ratings of intensity and number of stained cells.

Midline Glial Cells Express NADPHd

A prominent example of developmentally regulated NADPHd expression was provided by midline cells of the segmented ventral nerve cord. Whereas midline cells neither stained at embryonic, first, and second larval stages, they began to express NADPHd activity in the late third larval instar [Figs. 1(d), 2(d), and 5(a,c)]. The midline is organized as a mixed population of distinct neuronal and glial cell types (Jacobs and Goodman, 1989; Klämbt et al. 1991; Bossing and Technau, 1994; Callahan and Thomas, 1994). To determine glial contributions to the NADPHd staining in the dorsal midline, we performed double labelings using the tau lacZ line 3400 as a specific marker for midline glia (Klämbt et al., 1991; Ito et al., 1995). This line is advantageous because midline glia expresses the tau-β-galactosidase fusion protein in all parts of the cell during embryonic and larval development (Callahan and Thomas, 1994). As shown in Figure 5(a,b), the midline cells coexpress NADPHd...
NADPHd and cGMP Staining of the Imaginal Discs

Conspicuous NADPHd staining was found in the leg discs (Kuzin et al., 1996), and, as shown in Figure 1(d), the antennal part of the eye-antennal disc was also strongly stained. Sections cut through a leg disc showed that the expression of NADPHd was confined to the imaginal disc cells without labeling of sensory nerves (data not shown). Remarkably, the epithelial cells of the leg discs did not express cGMP-IR [Fig. 6(a)]. However, cells of the larval sensory organs (Tix et al., 1989) were immunoreactive. The cGMP-IR was expressed in the cell bodies and processes [Fig. 6(a,b)] running through the stalk of the disc into the CNS. During the pupal differentiation of the imaginal disc to the leg, continuous staining of the neuronal somata and their processes was detectable. Because control incubations in the absence of IBMX, SNP, or both did not reveal immunoreactivity in the leg discs, the expression of cGMP-IR depends entirely on NO stimulation.

The larval leg discs contain several sensory organs composed of neurons and nonneuronal support cells (Tix et al., 1989). To identify unambiguously the neuronal identity of the cGMP-IR cells, we performed a double staining using an axonal marker (Fujita et al., 1982) in combination with cGMP immunocytochemistry. As shown in Figure 6(b), the cell bodies expressed cGMP-IR which were superimposed by the 22C10 marker in the axonal projection to the CNS.

DISCUSSION

The biochemical properties of NO synthase have been characterized in Drosophila, Apis, and Schistocerca, suggesting that NOS activity and NADPHd staining after fixation are caused by identical enzymes (Müller, 1994; Müller and Bicker, 1994; Elphick et al., 1995). As has already been described for mammalian tissue (Matsumoto et al., 1993), the selectivity of the histochemical staining is presumably caused by the resistance of NOS to paraformaldehyde fixation. Thus, it is likely that the cytoplasmic NADPHd staining of neuronal, glial, and imaginal disc cells in Drosophila reflects NOS expression. To reveal potential target cells for endogenous NO signals, we resolved the cellular accumulation of cGMP by immunocytochemistry (De Vente et al., 1987). In this article, we focused on NO-induced immunoreactivity. All the developmental stages that were examined required treatment with both the NO donor SNP and the phosphodiesterase inhibitor IBMX for expression of cGMP-IR. The two cGMP antisera raised in different animals gave essentially identical staining patterns. Neuropeptide-induced increases in cGMP-IR that occur in insect development during the stages of ecdysis (Ewer et al., 1994) were not considered.
Similar to studies performed on the vertebrate CNS (De Vente et al., 1990; Bredt and Snyder, 1994; Roskams et al., 1994; Wu et al., 1994; Wang et al., 1995), there is increasing evidence for a developmental regulation of the NO/cGMP system of insects. For example, the developing photoreceptors of pupal *Drosophila* respond to NO stimulation with the formation of cGMP during a specific temporal window, while the postsynaptic optical ganglia express NADPHd (Gibbs and Truman, 1998). Based on pharmacological manipulations of NO and cGMP levels which caused a disorganization of the photoreceptor terminals, Gibbs and Truman (1998) suggested that NO and cGMP act to stabilize photoreceptor growth cones at the start of synaptic assembly.

This article presents additional examples of cellular staining showing developmental regulation of NADPHd expression (Table 1). One example is provided by the paired NADPHd-positive neurons of the ventral nerve cord of *Drosophila* [Fig. 1(a)] which either disappear or lose their staining at later stages. Other examples are the midline cells of the segmented ventral nerve cord. Whereas midline glial cells do not stain at embryonic and the first or second larval stages, they express NADPHd activity in the late third larval instar [Figs. 1(d), 2(d), and 5(a,c)]. At larval

![Figure 4](image)

**Figure 4** NO-induced cGMP staining of motoneurons. (a) High magnification of abdominal segments of the ventral nerve cord. Paired clusters of four to five cGMP-IR cells are visible near the midline. Arrows point to cells in the position of the RP2, RP3, and aCC motoneurons. (b) High magnification of cGMP-IR boutons on muscles 6 and 7. The arrow indicates a large bouton, and the arrowhead, a small one. Scale bar = 20 μm (a,b). (c) cGMP-IR innervation of body-wall muscles in one abdominal segment. The diagram of the body wall muscle pattern was modified after Landgraf et al. (1997). The ventral midline is on the left side and anterior is upside. Immunoreactive synaptic terminals are indicated in black. The arrow emphasizes the neuromuscular junction on muscles 6/7 shown above in (b).
stages, midline glia cells undergo various cellular reconfigurations including growth in cellular size, endomitotic DNA replication, cell divisions, and fragmentation of giant midline glia into a variable number of small midline glial cells (Stollewerk et al., 1996; Awad and Truman, 1997). Later in the pupa, midline glia enter apoptotic pathways. Expression of NADPHd activity in the late third larva (Figs. 1(d) and 5(a,c)) coincides with the phase of cell proliferation and fragmentation (Stollewerk et al., 1996; Awad and Truman, 1997). To decide whether NO formation is causally involved in any of these cellular fates requires experimental manipulations of NO levels in the midline glia. Whereas VUM neurons of the midline express NADPHd staining at third larval stage, they do not stain during earlier developmental stages. It is puzzling that we could not detect NADPHd staining in the neuromuscular release sites of the VUM cells. However, this enzymatic staining pattern may be indicative of a requirement of NO release in the larval CNS and not at the peripheral terminals.

A clear demonstration that NO regulates morphogenesis by controlling the balance between cell proliferation and cell differentiation has been obtained in imaginal discs of Drosophila. Imaginal discs are sac-like clusters of dividing cells which differentiate into the epidermal structures of the adult fly (reviewed in Cohen, 1993). The detection of NADPHd-expressing epithelial cells and genetic manipulations of NOS activity suggest the use of NO as messenger molecule regulating morphogenesis in the tissue of the discs (Kuzin et al., 1996). Our investigation found no evidence for NO-induced cGMP-IR in the epithelial cells of the leg discs (Fig. 6(a,b)). Since NO has several molecular mechanisms of action (Dawson and Snyder, 1994; Garthwaite and Boulton, 1995), the anti-proliferative properties of NO (Kuzin et al., 1996) may not be mediated via cGMP signal transduction.

Interestingly, we detected cGMP-IR sensory neurons embedded in the epithelial parts of the disc (Fig. 6(a,b)). These NO-responsive neurons are candidate target cells for NO released from the epithelium. The close anatomical proximity indicates a potential role of NO as a transcellular messenger between epithelial and neuronal cells. Imaginal discs have been a fertile source of information about developmental processes (Cohen, 1993) and might also provide insight into a role of NO/cGMP signal transduction during differentiation of distinct cellular phenotypes in a tissue.

The findings of cGMP-IR and NADPHd staining (Fig. 2(a,c)) in intrinsic cells of endocrine tissue, together with the detection of the cGMP-IR innervation of the ring gland (Fig. 2(b)), add to the arguments supporting the involvement of NO/cGMP signaling during insect development. In particular, the NADPHd-positive tissue region of the ring gland (Fig. 2(c)) corresponding to the corpus allatum is a prominent player of the endocrine system regulating metamorphosis via the release of juvenile hormone (Riddiford, 1993).

The dynamic regulation of cGMP accumulation in NO-responsive cells during insect development is striking. In the embryonic grasshopper, synaptogenesis correlates to a phase when many identifiable nerve cell types respond to NO by producing cGMP.

Figure 5 NADPHd staining of midline cells. Double staining of the tau lacZ line 3400 for NADPHd and anti-β-galactosidase (a,b) in the ventral nerve cord. (a) NADPHd-stained midline composed of glial and nerve cells. The arrows point to examples of NADPHd expressing midline glia (arrows). Additional neuronal cells contribute to NADPHd labeling. Note the absence of NADPHd staining in the nucleus of the glia cell, indicated by upper arrow. (b) The same preparation showing anti-β-galactosidase immunofluorescence staining of midline glia (arrows). Note that fluorescence is quenched by NADPHd staining. Scale bar = 60 µm (a,b). (c) Cross section (4 µm) of the ventral nerve cord stained for NADPHd. Between the neuropile of the commissures stained midline glial cells are visible (arrows). The large nuclei of the midline glial cells are not stained (arrowhead). Scale bar = 20 µm.
(Truman et al., 1996). Some of these are identifiable motoneurons whose growth cone can be visualized by cGMP-IR. The NO sensitivity appears after the growth cone has arrived at its target, but before branches have started to explore the target muscles. Moreover, certain sensory and interneurons also become NO sensitive as they change from axonal outgrowth to synaptogenesis and maturation. Remarkably, we could not detect NO-induced cGMP-IR accompanying synaptogenesis of motoneurons in embryonic *Drosophila* [Fig. 3(a)]. Rather, the phase of NO sensitivity in individual motoneurons appeared to be shifted to larval stages [Figs. 3(b–d) and 4(a,b)], where a considerable number of the motoneuronal pool innervating the body wall synthesized cGMP. For example, in *Drosophila* cGMP accumulations were found in prominent mononeurons such as RP2, RP3, 6/7b, RP4, and aCC [Fig. 4(a–c)]. Although the neuromuscular transmission is fully differentiated by the end of embryogenesis (Bate and Broadie, 1995), the muscles grow substantially during larval life. This developmental process requires the adjustment of the morphological size and physiological properties of neuromuscular junctions (Keshishian et al., 1996). As has been hypothesized before (Truman et al., 1996), synaptic maturation may use a retrograde signal from the postsynaptic muscle fiber to stimulate the cGMP accumulation in the presynaptic boutons. Intriguingly, genetic manipulations in the expression of a postsynaptic glutamate receptor type, the cell-surface molecule fasciclin II, and a constitutively active catalytic subunit of protein kinase A have provided compelling evidence that a retrograde signal regulates presynaptic transmitter release at the neuromuscular junction of *Drosophila* (Petersen et al., 1997; Davis and Goodman, 1998; Davis et al., 1998). Since the focus of these investigations has been on muscles 6/7, our discovery of NO-induced cGMP-IR in the boutons of this neuromuscular junction implicates NO as potential synaptic signal.

However, as in the grasshopper embryo (Truman et al., 1996), we could present no histochemical evidence for the presence of NOS in the postsynaptic muscle fibers. Neither embryonic nor larval muscle

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**Figure 6** Double labeling for cGMP and 22C10 immunoreactivity of the prothoracic imaginal disc. Note the absence of cGMP-IR in epithelial cells of the imaginal disc. (a) Whole mount of paired leg discs. Neuronal somata (arrowhead) show cGMP-IR (black) and axons (arrows) are stained for 22C10-IR (brown). Axonal projections running into the CNS are out of focus. The stained projections on top of the figure are running peripherally. Scale bar = 40 μm. (b) Section through a leg disc showing the cGMP-IR cell bodies (arrowhead) of the sensory neurons in black and the 22C10-IR axons (arrows) in brown. Scale bar = 15 μm.
fibers of *Drosophila* stain for NADPHd. Nonetheless, it remains a distinct possibility that the muscles contain an NOS isofrom that does not stain for NADPHd after formaldehyde fixation. For example, mammalian hippocampal pyramid cells that contain the neuronal and endothelial isoforms of NOS (O'Dell et al., 1994) are reportedly difficult to stain for the NADPHd reaction (e.g., Vincent and Kimura, 1992; but see also Southam and Garthwaite, 1993). Alternatively, the motoneurons may receive another sGC-stimulating signal from the muscles, such as carbon monoxide (Verma et al., 1993; Dawson and Snyder, 1994). A third possibility is that in the intact larva the cGMP synthesis is not induced by an NO signal released from the muscle fibers onto their synaptic terminals, but rather from central sources in the VNC and rising cGMP levels that extend peripherally along the axons.

Using a fluorescence assay, we obtained physiological evidence that NO donors and membrane-permeant cGMP analogues can induce vesicle release at the neuromuscular junction of muscle fibers 6/7 (Wildemann and Bicker, 1998). Moreover, we found that surgically isolated neuromuscular synapses stain for cGMP after application of NO donors. The immunocytochemical staining for cGMP in the presynaptic terminal, together with the demonstration of an NO-induced vesicle release, suggests that a chemical compound related to NO acts as retrograde messenger at the neuromuscular junction.

In summary, our cytochemical study provides an anatomical survey for the localization of NO-synthesizing and -responsive cells in the developing nervous system of *Drosophila*. Despite the fact that NADPHd stainings were performed in filleted preparations where penetration of staining solutions should cause no problems, few cell types were stained in the embryonic nervous system [Fig.1(a)]. With the caveat in mind that the NADPHd histochemistry may not reveal all enzymatic isoforms of NOS, our results suggest a significant increase in NO-releasing cellular structures during postembryonic development (Table 1). In contrast to the olfactory system of the locust (Bicker et al., 1996, 1997), there is no indication for a colocalization of NADPHd and cGMP-IR in the embryonic and larval CNS. Thus, the cellular organization of the NO/cGMP messenger system in *Drosophila* resembles more closely the distribution of NOS and cGMP-IR in separate cell types described in the vertebrate nervous system (Southam and Garthwaite, 1993; Hopkins et al., 1996), which is in line with the concept of NO as a transcellular messenger (Garthwaite and Boulton, 1995). The finding that NADPHd histochemistry and cGMP immunocytochemistry can reveal identifiable cell morphologies provides a background for ongoing investigations into how NO/cGMP signaling is incorporated into the function and formation of the nervous system.

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