Nitric oxide and cGMP influence axonogenesis of antennal pioneer neurons

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SUMMARY
The grasshopper embryo has been used as a convenient system with which to investigate mechanisms of axonal navigation and pathway formation at the level of individual nerve cells. Here, we focus on the developing antenna of the grasshopper embryo (Schistocerca gregaria) where two siblings of pioneer neurons establish the first two axonal pathways to the CNS. Using immunocytochemistry we detected nitric oxide (NO)-induced synthesis of cGMP in the pioneer neurons of the embryonic antenna. A potential source of NO are NADPH-diaphorase-stained epithelial cells close to the basal lamina. To investigate the role of the NO/cGMP signaling system during pathfinding, we examined the pattern of outgrowing pioneer neurons in embryo culture. Pharmacological inhibition of soluble guanylyl cyclase (sGC) and of NO synthase (NOS) resulted in an abnormal pattern of pathway formation in the antenna. Axonogenesis of both pairs of pioneers was inhibited when specific NOS or sGC inhibitors were added to the culture medium; the observed effects include the loss of axon emergence as well as retardation of outgrowth, such that growth cones do not reach the CNS. The addition of membrane-permeant cGMP or a direct activator of the sGC enzyme to the culture medium completely rescued the phenotype resulting from the block of NO/cGMP signaling. These results indicate that NO/cGMP signaling is involved in axonal elongation of pioneer neurons in the antenna of the grasshopper.

Key words: Nitric oxide, cGMP, Pathfinding, Growth cone, Schistocerca gregaria, Grasshopper

INTRODUCTION
A basic question in developmental neurobiology is how do developing neurons extend processes to find their correct targets cells, so as to create a functional nervous system? To establish the network of interconnections between nerve cells, neuronal cell bodies send out axonal growth cones that navigate from their site of origin to the appropriate target site. A large amount of progress has been made in the analysis of the molecular mechanisms of axon guidance and synapse formation (Goodman and Shatz, 1993; Tessier-Lavigne and Goodman, 1996; Goodman, 1996; Mueller 1999). Axon guidance has been shown to be mediated by contact with substrate-bound molecular guidance cues and by diffusible substances that give instructions that can either attract or repel the growth cone.

A novel aspect of cellular signaling during the formation of the nervous system is the involvement of the membrane-permeant messenger molecule nitric oxide (NO). In nerve cells, NO is generated by Ca2+/calmodulin stimulated NO synthases (NOS) and serves as a short-lived 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; Brüning and Mayer, 1996) has lead to the suggestion that NO functions in developmental processes. Experimental manipulations of NO signal transduction provided evidence that NO mediates the refinement of projections in the visual system (Wu et al., 1994; Cramer et al., 1996) and activity-dependent synaptic suppression at developing neuromuscular synapses (Wang et al., 1995).

NO is also a messenger molecule in insect nervous systems (reviewed in Bicker, 1998) and there is mounting evidence that NO/cGMP signaling participates in developmental processes. In embryonic grasshoppers, synaptogenesis correlates with a phase when many identifiable nerve cell types respond to NO by producing cGMP (Truman et al., 1996; Ball and Truman, 1998). Inhibition of NOS and sGC results in a reduction of terminal synaptic branch formation in a migratory population of embryonic Manduca neurons (Wright et al., 1998). During larval development, a set of subepidermal plexus neurons of Manduca express a persistent NO-induced cGMP-immunoreactivity (cGMP-IR) (Grueber and Truman, 1999). In Drosophila larvae, differentiating sensory cells of the imaginal leg discs express NO-induced cGMP-IR (Wildemann and Bicker, 1999a).

Two experimental investigations have shown that NO signaling is essential during certain periods of 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...
Materials and Methods

Embryo culture
Embryos were obtained from a Schistocerca gregaria colony kept in H. J. Pflügers laboratory and staged according to the criteria established by Bentley et al. (1979). Embryos for culture experiments were carefully staged to between 31% and 32% of development. During this stage, the pioneer neurons in the antenna could be stained with anti-HRP and the length of the outgrowing axons was not more than 20 μm.

Eggs were sterilized in 0.02% benzethonium chloride in 70% ethanol and dissected in sterile cell culture medium (L15, Gibco, Life Technologies). The entire amnion and dorsal membrane were removed from the embryo to ensure access of the reagents during culturing. The culture medium was L15 supplemented with 1% penicillin-streptomycin solution (10,000 units/ml). To test for a possible developmental function of the NO/cGMP signaling during embryonic axonogenesis, we analyzed at the level of identified nerve cells.

Histochemical procedures

Anti-HRP immunocytochemistry
To visualize the developing nervous system, neurons were labeled with an antiserum against horseradish peroxidase (anti-HRP) that selectively binds to insect neurons (Jan and Jan, 1982). Embryos were fixed in 4% formaldehyde in PBT (0.01M phosphate buffered saline, pH 7.4 and 0.1% Triton X-100) for 12 hours at 4°C. After washing several times in PBT, the tissue was blocked in 5% normal rabbit serum. The primary HRP antiserum (Jackson Immunoresearch) was applied at 4°C overnight in a dilution of 1:500 in PBT/3% normal rabbit serum. For visualization of anti-HRP immunoreactivity (HRP-IR) we used a peroxidase-conjugated anti-goat antiserum diluted 1:200 in PBT for 4 hours at 25°C. Immunoreactivity was visualized using 0.2 mg/ml diaminobenzidine, 0.08% NiCl and 0.015% H2O2 as chromogen. Embryos were cleared in a glycerol series (25%, 50%, 90% in PBS), mounted and viewed under a Zeiss Axioskop microscope. Optical sections of whole-mount antenna were captured with a Kontron camera. For reconstruction of neurons, optical sections were layered in Adobe Photoshop 4.0 and combined to make one image.

Anti-cGMP immunocytochemistry
Embryos of stages 30-55% were dissected in ice-cold L15 medium (Gibco). The entire amnion and dorsal membrane was removed to allow access of the reagents. To induce activity of the sGC, embryos were exposed to sodium nitroprusside (SNP, 100 μM) in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 1 mM) for 20 minutes at room temperature (De Vente et al., 1987). The embryos were fixed in 4% formaldehyde in PBT for 2 hours on ice and rinsed in PBT. The primary sheep antiserum (Dr Jan De Vente; see Tanaka et al., 1997; Wildemann and Bicker, 1999a,b) was applied at a dilution of 1:5000 in PBT at 4°C. Overnight. For visualization of cGMP immunoreactivity, we applied an anti-sheep IgG antiserum conjugated with biotin followed by streptavidin (1:200 in PBT, 4 hours at room temperature) for fluorescence staining. Embryos were mounted in glycerol and immunofluorescence was imaged using a Leica TCS 4D confocal microscope.

NADPH-diaphorase staining of antennal epithelium
For NADPH-diaphorase histochemistry embryos of stages between 30% and 45% were dissected in sterile cell culture medium. The tissue was fixed in 4% formaldehyde in PBT for 2 hours at 4°C. After fixation, the tissue was rinsed in PBT and cryoprotected in 30% sucrose/PBT overnight at 4°C. Subsequently, the embryos were embedded in Tissue Tek II (Miles, USA). Serial frozen sections (6 μm) were cut using a Jung CM 3000 Cryostat and collected on chrome-alum/gelatine-coated slides. After washing in 50 mmol Tris-HCl (pH 7.8), the tissue was incubated in 0.1 mmol β-NADPH/0.1 mmol Nitro Blue Tetrazolium in Tris-HCl at room temperature (in the dark) for 2 hours, or, alternatively, at 4°C overnight. After washing in Tris-HCl the tissue was mounted in a glycerol series. Pictures were
RESULTS

Development of peripheral pathways in the embryonic antenna

As the embryonic antennae begin to evaginate from the head, they are initially devoid of sensory neurons. Unlike in the thoracic and gnathal segments (Bentley and O’Connor, 1992; Meier and Reichert, 1991), where a single sibling pair of sensory cells serve as pioneers to the CNS, in the antenna, two sibling pairs of pioneer neurons are born (Bate, 1976; Ho and Goodman, 1982). These are termed ventral and dorsal pioneers (Ho and Goodman, 1982), terminology that we will follow in this paper. To examine neurogenesis and peripheral pathfinding in the antenna, we labeled the differentiating pioneer neurons at various stages with a neuron-specific anti-HRP antiserum (Jan and Jan, 1982; Snow et al., 1987). This method allowed the reliable identification of the pioneer neurons. Using the staging criteria of Bentley et al. (1979), we present the detailed timecourse of early neuronal differentiation in the antennal segments.

The first cells that expressed HRP-IR at 32% of embryonic development were the pair of ventral pioneer neurons (vPN, Fig. 1a) at the distal tip of the antenna. At the same time a single immunoreactive neuronal cell body appeared at the base of the antenna (Fig. 1a). The axon of this base pioneer (BP, Ho and Goodman, 1982) is the first peripheral process to reach the CNS from the antenna. During the next few hours two additional dorsal pioneer neurons (dPN) also became immunoreactive. Subsequently, the growth cones of the vPN extended along the ventral inner surface of the antennal epithelium with a migration path that runs straight to the BP. At 34%, the growth cones come into filopodial contact with the BP. Compared with the axons of the vPN the processes of the dPN initiated their outgrowth slightly later (Fig. 1b), navigating along the dorsal inner surface of the antennal lumen. The dorsal pioneer neurons did not follow a straight trajectory towards the CNS. Rather, after growing for a distance of 100 μm in proximal direction, the axons of the dorsal pioneers performed a prominent turn of about 90°, extending ventrally towards the BP. Remarkably, the BP lost its HRP immunoreactivity after the growth cones of the vPN and the dPN made contact (Fig. 1c). Subsequently, the processes of vPN and dPN fasciculated with the axon of the base pioneer, which, meanwhile, has established the earliest afferent pathway towards the brain (Bate, 1976; Ho and Goodman, 1982). The axonal projections of the pioneers entered the HRP-stained CNS at a distance of about 50-80 μm from the base pioneer.

At 38% of development, additional HRP-IR neurons differentiated in the antenna. These neurons elaborated prominent apical dendrites indicative of their phenotype as sensory cells. During the period 38-45% of embryogenesis, the regions of sensory cell formation appeared restricted to three circumferential bands (Fig. 1c). Initially sensory neurons were born at the tip of the antenna. A few hours later another two other regions that initiated sensory cell formation could be discerned. One region was positioned approximately in the middle of the antenna slightly more distal to the pioneer neurons. The third region was found near the base of the antenna but proximally to the BP cell. In this zone, sensory cells of the later pedicellar chordotonal organ (Johnston’s organ, Gewecke, 1979) were generated (Fig. 1d). Neurogenesis of numerous sensory cells continued within the three initiating zones. Parallel to the onset of segmentation at 45%, additional sensory cell initiating zones differentiated within the frame of the developing annular segments of the antenna (Fig. 1d).
Taken together, these results indicated that the vPN and dPN axons prefigure two axonal fascicles to the brain (Fig. 1d), which are joined by later-born sensory neurons to form the bipartite antennal nerve of larval and adult stages.

NO-induced cGMP-immunoreactivity and NADPH-diaphorase staining of developing antenna

About midway through insect embryogenesis, the appearance of NO-induced cGMP synthesis in selective neuronal cell types appears to be a common developmental phenomenon (Truman et al., 1996; Ball and Truman, 1998; Wright et al., 1998; Wildemann and Bicker, 1999a; Grueber and Truman, 1999). The peripheral neurons of the antenna express cGMP-IR in a rather early stage of development. After stimulation with the NO donor SNP, cGMP-IR could be induced in both pairs of the pioneer neurons in the antenna (Fig. 2a). The onset of the cGMP-IR became visible in the 38% stage, and immunoreactivity persisted during the following developmental period. As has been reported for other embryonic grasshopper neurons (Truman et al., 1996) strong cGMP-IR was also found in the nuclei of the antennal pioneers.

At the 55% developmental stage, a second embryonic cuticle is secreted (Shankland and Bentley, 1983) that prevents the access of compounds like SNP or IBMX in embryonic whole mounts. Therefore, with our experimental approach we were not able to determine how long the NO-induced cGMP-IR persisted during embryogenesis. Occasionally, we noticed an additional occurrence of NO-induced cGMP-IR in some distal sensory neurons in preparations of later stages (50-55%, data not shown). Embryos that were exposed to 200 μM ODQ for at least 20 minutes or during a culture period of 24-30 hours failed to express cGMP-IR after stimulation with an NO donor (data not shown). These observations provide evidence that the inhibitors used to study blocking effects of the cGMP pathway are suitable to reduce the sGC activity in situ effectively.

To search for potential cellular sources of NO, we used NADPH-diaphorase staining of formalin-fixed embryonic whole mounts as a histochemical marker for NOS. On transverse sections through the antenna, we found the blue precipitate of the diaphorase reaction concentrated at membranes that face the basal lamina (arrows). The staining of the basal parts of epithelial cells was not very pronounced but there is a striking contrast in staining intensity compared with the mesodermal tissue bordering the basal
Nitric oxide/cGMP signaling is essential for axonal outgrowth of the pioneer neurons

The appearance of inducible cGMP-IR within both the vPN and dPN in an early stage of embryogenesis suggested that the activation of NO-sensitive sGC might play a role in axon initiation or outgrowth of peripheral neurons to the CNS. To examine a potential role of the NO/cGMP pathway during the early embryonic development of the antenna, we cultured whole grasshopper embryos for 24-30 hours in neurochemicals known to affect this signaling pathway. Special care was taken to use embryos of 31-32% development, a stage when antennal pioneer initiated axonal outgrowth. Considering that vPN start axonogenesis a few hours before the dPN, we evaluated the growth pattern of both types of pioneer neurons separately.

Cultured embryos were exposed to neurochemicals that elevated cGMP levels. We used protoporphyrin IX free acid which stimulates sGC independently of NO (Wollin et al., 1982). The membrane-permeable 8-Br-cGMP was used to raise cGMP levels directly. Alternatively, embryos were treated with compounds that inhibit the enzyme activity of NOS or sGC. Selective inhibition of NOS was provided by the exposure to 7NI (Doyle et al., 1996) and blocking of sGC activity was achieved by using ODQ (Garthwaite et al., 1995). Normal outgrowth of pioneer neurons was defined as axons that pass the base pioneer either in a direct trajectory (vPN) or in the characteristic turns (dPN) and then extend into the developing CNS. Neither protoporphyrin IX free acid nor 8-Br-cGMP had any effect on the normal outgrowth pattern of both the pioneer neurons and receptor cells. In contrast, neurochemicals that inhibited the NO/cGMP pathway affected significantly axon elongation of antennal pioneers. Normal outgrowth was perturbed in pioneer neurons that were exposed to 7NI or ODQ just after initiating axonogenesis (Figs 3b,c, 4 and 5). Moreover, the number of antennal sensory cells that differentiated from the epithelium appeared to be reduced in the perturbation experiments. However, this effect was not pursued in the present investigation.

When embryos at 31-32% of development, a stage at which the pioneer neurons initiate axonogenesis, were exposed to the NOS inhibitor 7NI, 71% (vPN)/77% (dPN) of the outgrowing axons failed to reach the BP. Conversely, normal outgrowth was observed only in 29% (vPN) / 23% (dPN) (Fig. 6). In these cases, axons of the pioneer neurons succeeded to contact the BP and migrate into the CNS. We observed that within the 7NI-treated group that did not succeed in reaching the CNS, growth cones of both vPN and dPN grew for different distances (Fig. 3b). Therefore we distinguished between pioneer neurons with very short projections (maximum length 20 μm, no true axonal growth) and pioneer neurons that extended axons of at least 20 μm but without reaching the BP. In general, the axon length of the vPN was longer than the axon length of dPN. As already discussed above, this observation may derive from the fact that vPN initiate axonogenesis earlier than dPN.

In contrast, 84% (vPN)/70% (dPN) of growth cones in control-treated antennae followed their stereotyped pathway of migration in culture, maintaining their proximal growth orientation and contacting the BP before the axons entered the CNS (Fig. 6). Consequently, only 16% (vPN)/30% (dPN) were not able to reach the BP during the time in culture. We conclude from this result that an inhibition of NO synthesis leads to a perturbation of axon elongation during outgrowth of the pioneer neurons.

To test for an involvement of the target enzyme sGC in axonal outgrowth of pioneer neurons, embryos were incubated...
in the specific inhibitor ODQ. Embryo culture in the presence of ODQ caused similar types of axonal perturbations in pioneer neurons as 7NI treatment, but with a markedly higher probability. Here, we found only 4% (vPN)/2% (dPN) of ODQ-treated embryos that succeeded in migrating into the CNS (axons reach CNS). Embryonic antennae were treated in culture with listed compounds. For each group the number of examined antennae is given in the legend. d, dorsal pioneer neurons; v, ventral pioneer neurons. For control/7NI and control/ODQ of either ventral or dorsal pioneer neurons experimental values were significantly different; P<0.001 ($\chi^2$ test).

**DISCUSSION**

Studies of axonal navigation in the grasshopper embryo suggest that the growth cones of the pioneer neurons use a precise set of pathfinding processes to reach the CNS. Pathfinding seems to involve selective adhesion of the growth cones to substrate-bound guidance cues provided by the epithelial cells and partly by recognition of guide post cells (reviewed in Bentley and O’Connor, 1992). Moreover, a recent investigation has identified a novel semaphorin (gSema 2a), that is expressed in a gradient in the developing limb bud epithelium and acts as chemorepulsive guidance molecule.

**Fig. 6.** Effects of compounds that can inhibit endogenous NO or cGMP levels on axogenesis of pioneer neurons. Histograms are presented in a cumulative manner, showing the percentage of pioneer axons that extended an axon over a distance of at least 20 μm (axonal outgrowth) and the group of pioneer axons who succeeded to enter the CNS (axons reach CNS). Embryonic antennae were treated in culture with listed compounds. For each group the number of examined antennae is given in the legend. d, dorsal pioneer neurons; v, ventral pioneer neurons. For control/7NI and control/ODQ of either ventral or dorsal pioneer neurons experimental values were significantly different; $P<0.001$ ($\chi^2$ test).
In embryonic and adult stages (Müller and Bicker, 1994; Elphick et al., 1995), the selectivity of this histochemical staining is already been described for mammalian tissue (Matsumoto et al., 1993), the selectivity of this histochemical staining is already been described for mammalian tissue (Matsumoto et al., 1993), suggesting that NOS activity and NADPH-diaphorase may also contribute to growth cone extension of the antennal pioneers. This proposal is based on the following pharmacological experiments performed in embryo culture. Blocking of endogenous NO synthesis by the NOS inhibitor 7NI disrupts outgrowth of the ventral and dorsal pioneers in a dose dependent manner. Treatment with ODQ, a specific inhibitor of sGC (Boulton et al., 1995) also prevented the growth cones from reaching the base pioneer. Immunocytochemical experiments have demonstrated that ODQ is indeed effective in insects inhibiting NO-induced cGMP synthesis (Gibbs and Truman, 1998; Ball and Truman, 1998; Wildemann and Bicker, 1999b). It is rather unlikely that the pharmacological effects on axonal outgrowth are an artefact of the embryonic pioneers, since in controls about 80% of the pioneer growth cones reached their correct targets. Intriguingly, the disruption of axonal outgrowth caused by inhibiting cGMP synthesis could be rescued by exogenous application of membrane-permeant cGMP and pharmacological stimulation of sGC, suggesting that in vivo a certain level of cGMP is necessary for axonal outgrowth.

The hypothesis that a NO/cGMP pathway is involved in the axonogenesis of the antennal pioneers receives additional support from cytochemical stainings. Treatment with NO donors induced cGMP-IR both in the ventral and dorsal pioneer neurons, showing that both cell types contain sGC. Moreover, there is some indication that parts of epithelial cells in the embryonic antenna stain for NADPH-diaphorase, a histochemical marker for NOS. This method is based on the requirement of NADPH as cofactor of the NOS enzyme. The biochemical properties of NOS have been characterized in the CNS of the locust (Müller and Bicker, 1994; Elphick et al., 1995), suggesting that NOS activity and NADPH-diaphorase staining after fixation are caused by identical enzymes. As has already been described for mammalian tissue (Matsumoto et al., 1993), the selectivity of this histochemical staining is presumably caused by the resistance of NOS to formaldehyde fixation. Clearly, the staining of the epithelial cells was not very pronounced as opposed to the homogeneous cytoplasmic stainings of insect neurons that have meanwhile been reported in embryonic and adult stages (Müller and Bicker, 1994; Elphick et al., 1995; Bicker et al., 1996; Truman et al., 1996; Wildemann and Bicker, 1999a). However, a close inspection of the sections through the antenna revealed that the blue precipitate of the histochemical diaphorase reaction was concentrated at the basal lamina of the epithelium close to the mesodermal tissue. Such a staining pattern can be explained by a membrane targeting of the enzyme that is responsible for the diaphorase reaction. Whether the epithelial NADPH-diaphorase staining pattern reflects true NOS activity remains to be confirmed, but it is attractive to assume that the axons of the pioneers that grow on the basal lamina receive NO stimulation from a membrane-bound NOS of the inner epithelial cells. The diaphorase staining of epithelial cells was visible at about 32-35% and faded during subsequent development. This developmental period is crucial for the outgrowing pioneer neurons. Although the staining intensity of NO-inducible cGMP-IR of the pioneer neurons was optimal at about the 38% stage, the pioneer neurons proved already sensitive to pharmacological manipulation of the NO/cGMP pathway during earlier developmental stages when NADPH-diaphorase activity in epithelial cells was apparent. It is also possible that the pioneer neurons in vivo may receive other sGC-stimulating signals such as carbon monoxide (Verma et al., 1993; Dawson and Snyder, 1994), from yet unidentified tissue sources.

Although we cannot exclude other functions of NO/cGMP signalling during the development of the peripheral nervous system, here, we have focussed on its role in axonal outgrowth of pioneer neurons. There are indeed two observation that indicate other potential roles. The first refers to the high nuclear levels of cGMP-IR in the pioneer neurons (Fig. 2a). The presence of cGMP-IR in the nucleus of certain groups of differentiating insect neurons has also been observed by Truman et al. (1996), suggesting that some neurons may have nuclear cGMP-binding proteins that could function in a yet unknown developmental context. The second observation appears to be a reduction in number of sensory cells that have differentiated under the inhibition of NO/cGMP signalling (Fig. 3). However, it should be emphasized that the perturbation experiments did not affect the number of antennal pioneer neurons whose cell bodies had clearly differentiated from epithelial tissue.
Our experimental manipulation of NO and cGMP levels in the embryo cultures has provided evidence that in vivo NO/cGMP signaling might be involved in axonal outgrowth of the antennal pioneers. The reported pharmacological manipulations and substitutions of NO and cGMP levels in embryo culture occur on a timescale of about 24 hours and therefore do not allow any conclusions with respect to a role of NO/cGMP signaling in rapid cellular decisions affecting growth cone guidance. However, an increasing number of investigations in several animal species has reported that NO affects a multitude of growth cone behaviors both in vivo and in vitro. Some of these studies show NO-induced growth cone collapse (Hess, et al., 1993; Renteria and Constantine-Paton, 1995), others report enhanced neurite extension (Hindley et al., 1997; Poluha et al., 1997), enhanced filopodial elongation (Van Wagenen and Rehder, 1999), and both cGMP-dependent and -independent effects have been found. To this end, there appears to be no single, species-independent cellular mechanism mediating growth cone behavior in response to NO.

If NO influences growth cone extension, then what might be the role of a NO/cGMP system in axonogenesis of the pioneer neurons of the grasshopper? Our data from the antennal pioneers suggest that the growth cone may receive a NO signal from the epithelial cells and that the elevated cGMP levels are permissive for the ability of axonogenesis. Cell culture experiments with dissociated Xenopus spinal neurons have shown that cyclic nucleotide second messenger levels in growth cones switch repulsive guidance cues to attractive cues (Song et al., 1998). In the Xenopus culture system, elevated levels of cGMP can convert the response of growth cones to a semaphorin from repulsion to attraction. Remarkably, an asymmetric cellular localization of sGC to the dendrites of pyramidal cells is thought to confer the oppositely directed outgrowth to dendrites and axons in a semaphorin (Sema 3A) gradient of the cerebral cortex (Polleux et al., 2000). Thus, intracellular cyclic nucleotide levels may be involved in modulating the directional behavior of growth cones in response to other extracellular guidance cues.

To detect NO-induced cGMP-IR in the pioneer neurons, we have blocked the degradation of cGMP by a phosphodiesterase inhibitor. Our immunocytochemical method does therefore not allow us to infer the detailed subcellular localization of sGC. Nevertheless, it might be rewarding to compare the immunocytochemical distribution of sGC in the sensory and motoneurons that grow in opposite fashion along the body appendages of the grasshopper. Embryo culture allows for experimental manipulations of cyclic nucleotide signal transduction during the development of the antennal pathway. This accessible system will thus allow to elucidate further cellular mechanisms of axonogenesis in pioneer neurons.

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