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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 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 diffusable 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 Ca^{2+}/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 controling 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). Thus, insects, with their relatively simpler nervous system and identified cells, offer convenient preparations for the analysis of NO/cGMP signaling during development.

The body appendages of grasshopper embryos have provided a useful model system with which to study a precise set of pathfinding processes during growth cone navigation (reviewed in Bentley and O'Connor, 1992). As an embryonic limb begins to emerge from the body wall, a pair of pioneer neurons arises within the distal tip of limb epithelium, which establish the first neuronal pathway to the CNS. En route, the growth cones of the pioneering axons recognize guidance cues of the limb-bud epithelium, including the segment borders, and pre-positioned guidepost cells. Similar to the appendages of the thoracic segments, the first neural pathways in the antenna of the grasshopper are established by identified pairs of pioneer neurons (Bate, 1976; Ho and Goodman, 1982).

In the present study, we examine in detail the pattern of axonal growth in the antenna during the first half of embryonic development and show that the pioneer neurons of the antenna synthesize cGMP in response to exogenous NO treatment. Using an embryo culture system we demonstrate that pharmacological inhibition of endogenous GC and of NOS activity results in a perturbation of pioneering pathway in the antenna. This pharmacological disruption of pioneering pathways can be rescued by supplementing with membrane-permeant cGMP and protoporphyrin IX free acid, an activator of sGC. Our investigation reveals an accessible *in vivo* system in which the role of NO/cGMP signaling during embryonic axonogenesis can be analyzed at the level of identified nerve cells.

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, we used neurochemicals that are known as inhibitors or activators of this signal transduction system. 7-nitroindazole (7NI), and 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Alexis, San Diego, CA) were dissolved in dimethyl sulfoxide (DMSO) to provide a final concentration of 5 mM DMSO in the culture medium. Protoporphyrin IX free acid (Alexis) was dissolved in chloroform such that the final concentration of chloroform in the culture medium was 10mM. Control culture plates contained the same concentration of either DMSO or chloroform. 8Br-cGMP was dissolved in L15 medium.

In pilot experiments, a range of concentrations (from 1 μM to 1 mM) of 7NI, ODQ, 8Br-cGMP and protoporphyrin IX free acid was tested to determine a minimum dose that produced a maximal response level. Culture plates were incubated for 24-30 hours at 34°C. Embryos of one clutch were randomly divided into groups that were exposed to the substances described above. Additionally, three embryos of the same clutch were immediately fixed following dissection to determine the locations of the pioneer neuron growth cones at the start of the culture period. Except where noted, all reagents were purchased from Sigma.

The evaluation of each experiment in embryo culture was performed by a second observer in a double-blind procedure. The results presented here are based on examination of 12-29 embryos for each treatment. The anatomical nomenclature of the antennal pioneer neurons established by Ho and Goodman (1982) is used with respect to the body axis of the embryo.

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% H_2O_2 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 anti-cGMP (Courtesy of 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 used at a dilution of 1:200 in PBT for 4 hours at room temperature. Subsequently, after several rinses in PBT we used Cy3-conjugated 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-alumn/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

captured with a Kontron camera mounted on a Zeiss Axioskop microscope.

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)

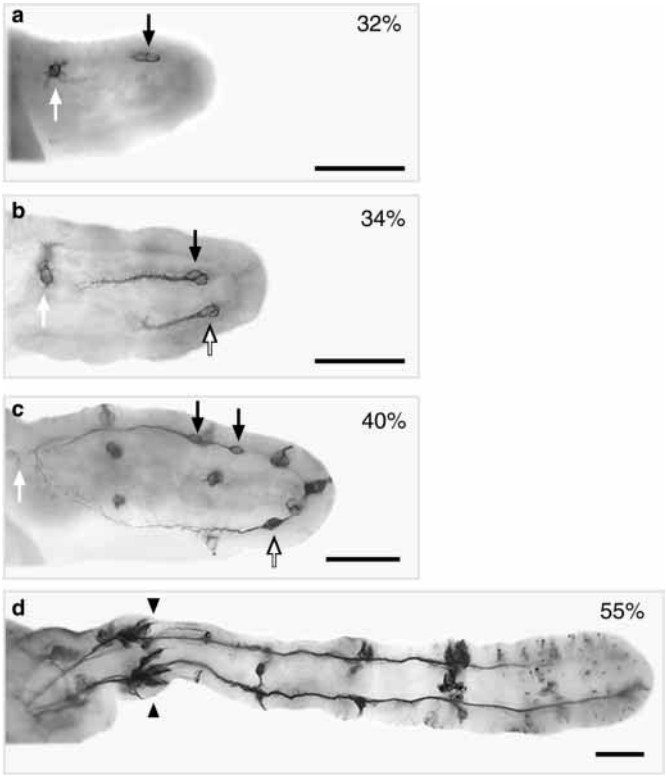

Fig. 1. Development of pioneer neurons and sensory cells in the antenna of the grasshopper embryo revealed by anti-HRP immunocytochemistry. Description of the anatomy is with respect to the embryo axis. In this and all subsequent photomicrographs ventral is upwards, dorsal is downwards and proximal is leftwards. (a) Antenna of a 32% stage embryo. A pair of cell bodies appear at the ventral inner epithelium at the tip of the antenna that stain for anti-HRP (ventral pioneer neurons, black arrow). At the same time, a single neuron, the base pioneer, is visible at the base of the antenna (white arrow). Filopodia are extending distally and proximally from both the pioneer neurons and the base pioneer. A few hours later axonal growth cones emerge from the proximal side of the cells. (b) At 34% of embryogenesis, the ventral pioneer neurons (black arrow) and the more dorsal laying dorsal pioneer neurons (open arrow) extend growth cones towards the base pioneer (white arrow). (c) At 40% of embryogenesis both the ventral (black arrows) and dorsal pioneer neurons (open arrow) succeed in contacting the base pioneer that looses its HRP-IR (white arrow). Note that in some preparations the cell bodies of the pioneer neurons are in separate positions. Sensory cells appear at the tip, in the middle, and near the base (black arrowheads) of the antenna. (d) Antenna of a stage 55% embryo. Numerous sensory cells appear during subsequent development arranged in annular segments. Pioneer neurons are no longer stained. Note the Johnston's organ (black arrowheads) at the base of the antenna. Scale bars: 50 μm in a-c; 20 μm in d.

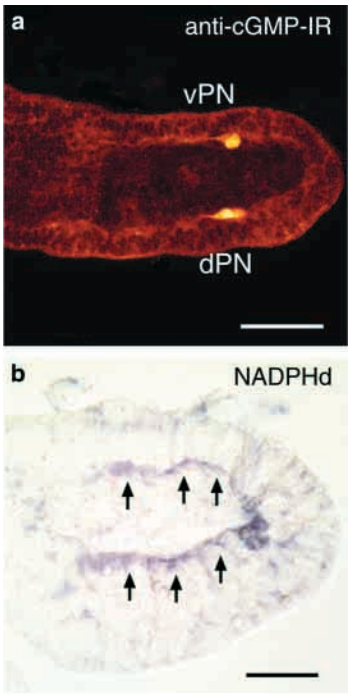

Fig. 2. Different cell types of the early embryonic tissue of the antenna stain for NADPH-diaphorase and NO induced cGMP-immunoreactivity. (a) NO-induced cGMP-IR of the ventral (vPN) and dorsal pioneer neurons (dPN) within the antenna of a stage 38% embryo. The confocal image shows a high level of cGMP-IR in the nuclei of the pioneers. (b) Parasagittal section of antenna of a stage 32% embryo. NADPH-diaphorase staining is concentrated at membranes that face the basal lamina (arrows). Scale bars: 50 μm in a; 20 μm in b.

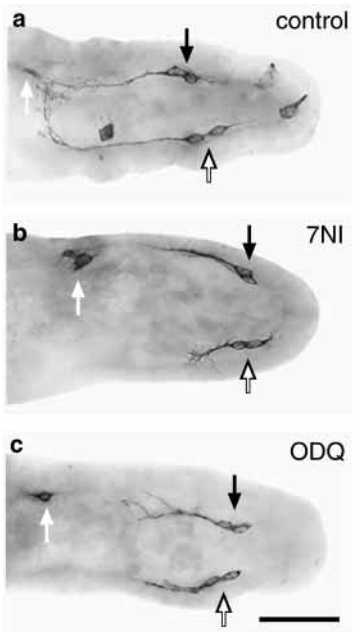

Fig. 3. Effects of pharmacological agents on antennal pioneer neurons that affect the NO/cGMP pathway during early embryogenesis. Antennae of embryos after a culture period of 24-30 hours under different treatment conditions. Embryos were taken in culture at developmental stage 31%-32% when antennal pioneer neurons initiated axonal outgrowth. (a) Normal outgrowth pattern of ventral (black arrow) and dorsal pioneer neurons (open arrow) of an embryo cultured under control conditions in which the culture medium contains the vehicle DMSO. Axons of both siblings succeeded in contacting the base pioneer (white arrow, HRP-IR has disappeared) and extended towards the CNS. (b) Ventral (black arrow) and dorsal pioneer neurons (open arrow) of an embryo that was exposed to 500 μM 7NI just after initiating axonogenesis showed a retardation in axon elongation and failed to reach the base pioneer (white arrow). Anti-HRP staining of the base pioneer is still visible. (c) Pioneer neurons (black and open arrow) of an embryo that was treated with 200 μM ODQ showed a retardation in axon elongation and failed to reach the base pioneer (white arrow). Anti-HRP staining of the base pioneer is still visible. Scale bar: 50 μm.

(Ochieng et al., 1998). 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 in parts of the epithelial cells facing the basal lamina (Fig. 2b). 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

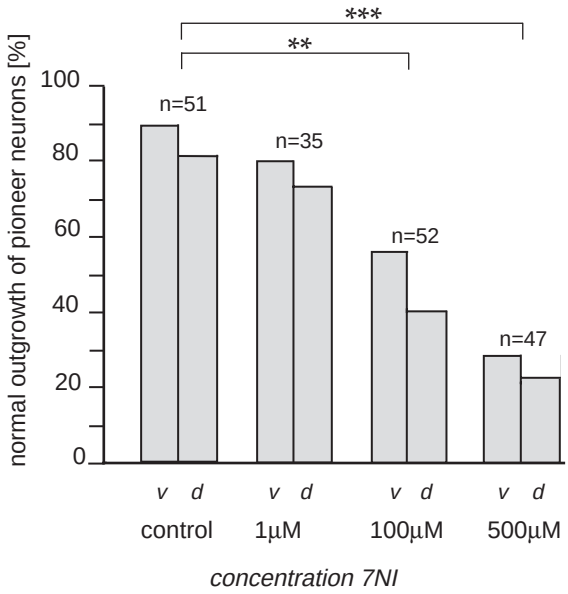

Fig. 4. Quantification of NOS inhibition by 7NI. 7NI prevents pioneer neurons from normal axon elongation in a manner that was significantly greater than controls. The number of antennae in each group is indicated as *n* above the bars. d dorsal pioneer neurons; v, ventral pioneer neurons; ***P*<0.005; ****P*<0.001, χ^2 test.

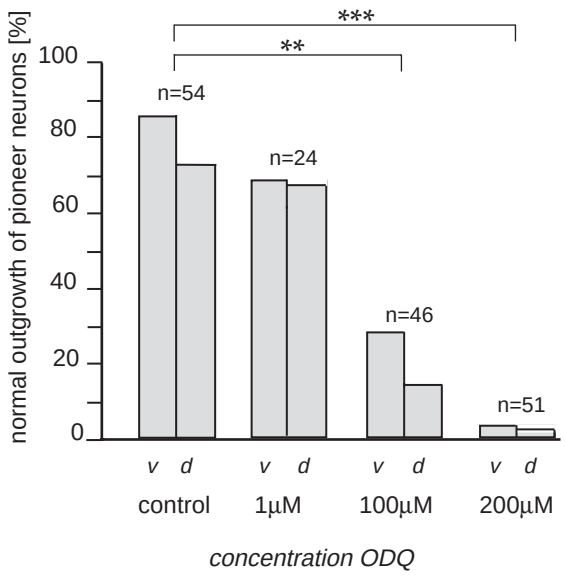

Fig. 5. Quantification of sGC inhibition by ODQ. Normal outgrowth of pioneer neurons was disturbed in the presence of ODQ. The number of antennae in each group is indicated as *n* above the bars. d, dorsal pioneer neurons; v, ventral pioneer neurons; ***P*<0.005; ****P*<0.001, χ^2 test.

lamina. Diaphorase staining of the epithelial cells became visible during a developmental period ranging from about 32-35% and disappeared at later stages.

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 at 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

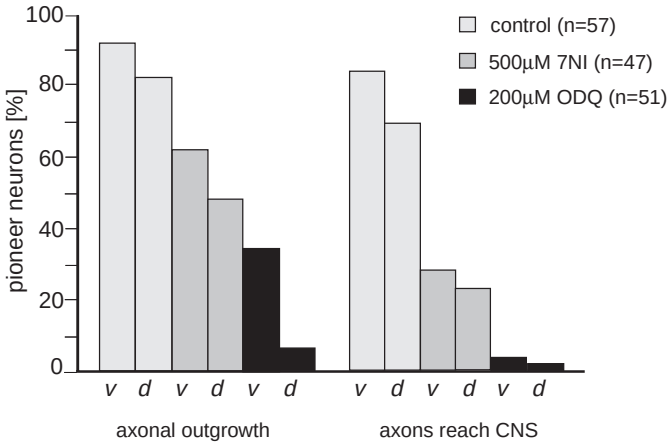

Fig. 6. Effects of compounds that can inhibit endogenous NO or cGMP levels on axonogenesis of pioneer neurons. Histograms are presented in a cumulative manner, showing the percentage of pioneer axons that extended an axon over a distance of at least 20 µm (axonal outgrowth) and the group of pioneer axons 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; $P<0.001$ (χ^2 test).

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 (Fig. 6). Perturbation of growth-cone extension was observed in 96% (vPN)/98% (dPN) with a proportion of 65% (vPN)/92% (dPN) showing no axonogenesis and 31% (vPN)/6% (dPN) that extend axons of at least 20 µm length (Fig. 6).

NO- and cGMP-analogs rescue disruptive effects of NOS and sGC inhibitors

Both 7NI and ODQ produced similar effects in disrupting antennal pioneer axonogenesis. In order to test if the disruptive effects of both enzyme inhibitors can be reversed by adding activators of NO/cGMP signaling we treated embryos in culture with 7NI plus protoporphyrin IX free acid (Fig. 7) as well as with ODQ plus 8-Br-cGMP (Fig. 8). Embryos cultured in the presence of 500 µM 7NI showed a distinct perturbation in normal outgrowth of the antennal pioneer neurons (Figs 3a, 4) at a percentage of 29% (vPN)/23% (dPN). However, addition of the sGC-activating protoporphyrin IX free acid (1 mM) reversed the disruptive effects of 7NI and resulted in a rate of 72% (vPN)/52% (dPN) normal outgrowing axons (Fig. 7a,b).

Normal outgrowth of the pioneer neurons was more effectively disturbed in the presence of 200 µM ODQ, which allows only 4% (vPN)/2% (dPN) of the axons to reach the CNS (Fig. 5) However, adding 500 µM of the membrane-permeable 8Br-cGMP lead to a recovery of the normal outgrowth in 46% (vPN)/53% (dPN) of all cases (Figs 8a, b). These results show that application of an NO-analog and membrane-permeant cGMP rescue the defects caused by inhibiting endogenous NO/cGMP signaling.

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

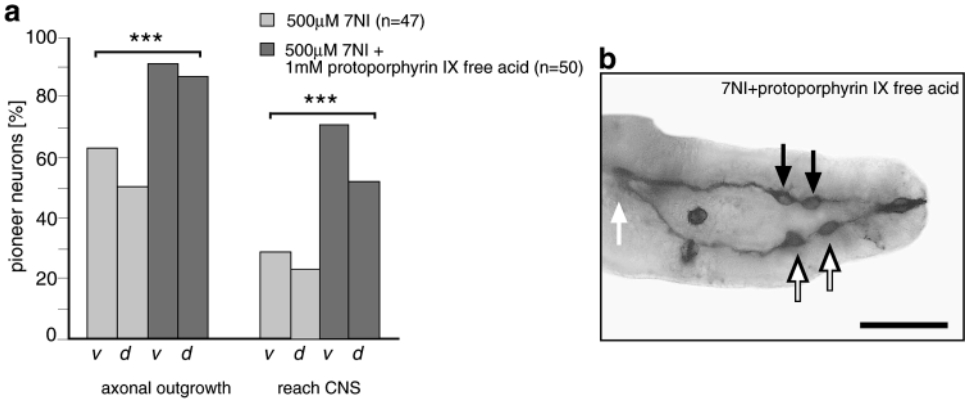

Fig. 7. The disruptive effects of 7NI were rescued by the addition of protoporphyrin IX free acid.
(a) Histograms show the percentage of axons that show axonal outgrowth of at least 20 µm (axonal outgrowth) or passed the base pioneer neuron and succeeded in entering the CNS (reach CNS). Embryonic antennae were treated in culture with listed compounds. Control experiments in which both of the carriers (DMSO, chloroform) were added at appropriate dilution, showed a normal outgrowth (about 80%) which was indistinguishable from the controls in regular culture medium. For each group the number of examined antennae is given in the key. d dorsal pioneer neurons; v ventral pioneer neurons. For 7NI/7NI+ protoporphyrin IX free acid of either ventral or dorsal pioneer neurons experimental values were significantly different; $P<0.005$ (χ^2 test). (b) Embryonic antenna after a culture period of 24 hours treated with 7NI in the presence of protoporphyrin IX free acid showing ventral (black arrow) and dorsal pioneer neurons (open arrows) that succeeded to reach the CNS. Staining of the base pioneer (white arrow) is lost. Scale bar: 50 µm.

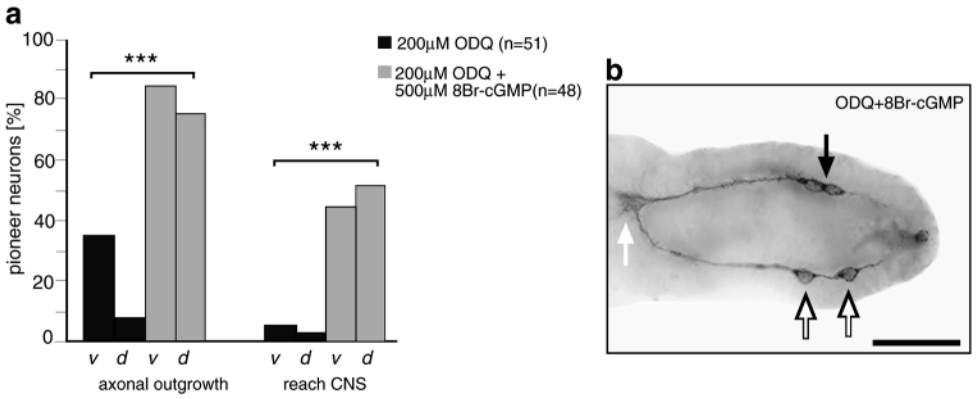

Fig. 8. The disruptive effects of ODQ were rescued by the addition of 8Br-cGMP. (a) Histograms show the percentage of axons that show axonal outgrowth of at least 20 µm (axonal outgrowth) or passed the base pioneer neuron and succeeded in entering the CNS (reach CNS). For each group the number of examined antennae is given in the key. d, dorsal pioneer neurons; v, ventral pioneer neurons. For ODQ/ODQ+8Br-cGMP of either ventral or dorsal pioneer neurons, experimental values were significantly different; $P<0.001$ (χ^2 test). (b) Embryonic antenna treated with ODQ in the presence of 8Br-cGMP during a culture period of 26 hours. The histological staining reveals the complete recovery to the normal outgrowth pattern of ventral (black arrows) and dorsal pioneer neurons (open arrows). Anti-HRP-staining of the base pioneer (white arrow) is lost. Scale bar: 50 µm.

(Isbister et al., 1999). In this paper, we have provided evidence that in addition, NO-induced cGMP synthesis 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 embryo culture, 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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