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Nitric oxide and cyclic nucleotides are regulators of neuronal migration in an insect embryo

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SUMMARY

The dynamic regulation of nitric oxide synthase (NOS) activity and cGMP levels suggests a functional role in the development of nervous systems. We report evidence for a key role of the NO/cGMP signalling cascade on migration of postmitotic neurons in the enteric nervous system of the embryonic grasshopper. During embryonic development, a population of enteric neurons migrates several hundred micrometers on the surface of the midgut. These midgut neurons (MG neurons) exhibit nitric oxide-induced cGMP-immunoreactivity coinciding with the migratory phase. Using a histochemical marker for NOS, we identified potential sources of NO in subsets of the midgut cells below the migrating MG neurons. Pharmacological inhibition of endogenous NOS, soluble guanylyl cyclase (sGC) and protein kinase G (PKG) activity in whole embryo culture significantly blocks MG neuron migration. This pharmacological inhibition can be rescued by supplementing with protoporphyrin IX free acid, an activator of sGC, and membrane-permeant cGMP, indicating that NO/cGMP signalling is essential for MG neuron migration. Conversely, the stimulation of the cAMP/protein kinase A signalling cascade results in an inhibition of cell migration. Activation of either the cGMP or the cAMP cascade influences the cellular distribution of F-actin in neuronal somata in a complementary fashion. The cytochemical stainings and experimental manipulations of cyclic nucleotide levels provide clear evidence that NO/cGMP/PKG signalling is permissive for MG neuron migration, whereas the cAMP/PKA cascade may be a negative regulator. These findings reveal an accessible invertebrate model in which the role of the NO and cyclic nucleotide signalling in neuronal migration can be analyzed in a natural setting.

Key words: Nitric oxide, cGMP, cAMP, Enteric nervous system, PKG, *Locusta migratoria*

INTRODUCTION

Neuronal migration is central to the development of nervous systems, as neurons are usually born in specialized proliferative zones but finally reside in distinct locations of the mature nervous system. Thus, directed cell migration is necessary for these neurons to reach their ultimate destination (Hatten, 1999; Nadarajah and Parnavelas, 2002).

An emerging theme in neuronal navigation is that extracellular axon guidance cues can also guide directed migration of nerve cell bodies (Hedgecock et al., 1990; Serafini et al., 1996; Mitchell et al., 1996; Wu et al., 1999; Zhu et al., 1999; Song and Poo, 2001; Causeret et al., 2002). An increasing number of investigations have implicated the gaseous signalling molecule nitric oxide (NO) (reviewed by Bredt and Snyder, 1992; Dawson and Snyder, 1994; Garthwaite and Boulton, 1995) and its main effector, the cGMP synthesizing enzyme soluble guanylyl cyclase, in mechanisms of neurite growth. Investigations on cultured neurons have revealed that NO affects a multitude of growth cone behaviors (Hess et al., 1993; Renteria and Constantine-Paton, 1995; He et al., 2002; Hindley et al., 1997; Poluha et al., 1997; Van Wagenen and Rehder, 1999). Evidence from developmental studies also suggests an involvement of NO/cGMP signalling in neurite growth, synaptogenesis and synaptic maturation processes (Wu et al., 1994; Wang et al., 1995; Cramer et al., 1996; Truman et al., 1996; Ball and Truman, 1998; Gibbs and Truman, 1998; Wildemann and Bicker, 1999; Seidel and Bicker, 2000; Leamey et al., 2001). Moreover, the recent analysis of cGMP-dependent protein kinase I deficient mice has revealed axon guidance defects of sensory neurons (Schmidt et al., 2002). So far, NO/cGMP signalling has not been implicated in the migration of postmitotic neurons.

The formation of the insect enteric nervous system (ENS) (reviewed by Hartenstein, 1997) provides a well established model to study the cell biology of neuronal migration. In this paper, we focus on the directed migration of enteric neurons in the grasshopper, which populate a nerve plexus that spans the midgut (Ganfornina et al., 1996). The midgut plexus neurons (MG) neurons arise in a neurogenic zone in the foregut, forming a packet of postmitotic but immature neurons at the foregut-midgut boundary. Subsequently, they undergo a rapid phase of migration, during which the neurons cross the foregut-midgut boundary and migrate several hundred micrometers posteriorly on the midgut surface. At the completion of migration, the MG neurons invade the space between the four

migratory pathways and extend terminal synaptic branches on the midgut musculature (Ganfornina et al., 1996). In the hawkmoth *Manduca*, migrating enteric plexus neurons synthesize cGMP in response to the application of exogenous NO (Wright et al., 1998). In this study, the pharmacological blocking of NO/cGMP signalling effects only post migratory synaptic branch formation, but not the actual process of cell migration (Wright et al., 1998).

We have examined the role of NOS and cyclic nucleotide signalling in the regulation of neuronal migration in the ENS of the hemimetabolous grasshopper (*Locusta migratoria*). We find that the MG neurons of the grasshopper exhibit inducible cGMP-immunoreactivity (cGMP-IR) throughout the phase of migration and continue to show high levels of anti-cGMP staining in the phase of lateral axon branching and the formation of terminal processes. When the midgut plexus acquires its mature configuration, the cGMP-IR decreases. Using NADPH-diaphorase staining as a histochemical marker for NOS, we identify potential sources of NO in subsets of the midgut cells below the migrating MG neurons.

Pharmacological inhibition of endogenous NOS, sGC and PKG activity in embryo culture results in a significant delay of MG neuron migration. This pharmacological perturbation of MG neuron migration can be rescued by supplementing with protoporphyrin IX free acid, an activator of sGC and membrane-permeant cGMP. Whereas NO/cGMP/PKG signalling is a positive regulator of MG neuron translocation, the stimulation of the cAMP/PKA signalling cascade results in an inhibition of cell migration. Correspondingly, activation of the cGMP and cAMP cascade appears to influence the cellular distribution of F-actin in the MG neurons in an antagonistic fashion. Together, these findings represent the first evidence for a functional role of NO/cGMP/PKG signalling during migration of postmitotic neurons.

MATERIALS AND METHODS

Locusta migratoria eggs were collected from a crowded laboratory culture and kept in moist petri dishes at 30°C. Staging of the embryos was based on the system of Bentley et al. (Bentley et al., 1979) with additional criteria for later embryos (Ball and Truman, 1998).

In vivo culturing experiments

Embryos used for culture experiments were staged between 60% and 63% of embryonic development (% E). During these stages, the MG neurons had not migrated more than 40 μm along the midgut surface. An optimal stage for in vivo culturing and pharmacological manipulation was indicated by the first appearance of brownish pigmentation at the tips of the antennae.

Eggs were sterilized in 70% ethanol and dissected in sterile cell culture medium (L15, Gibco, Life Technologies), supplemented with 1% penicillin-streptomycin solution (10,000 units/ml). A small incision in the dorsal epidermis was created directly above the foregut. Embryos were then allowed to develop for 24 hours at 30°C in the presence of pharmacological substances. The incision did not reseal and thus, the developing ENS was exposed to the pharmacological agents during the whole culturing period. Finally, guts were dissected out of the embryos and fixed in PIPES-FA (100 mM PIPES, 2.0 mM EGTA, 1 mM $MgSO_4$, 4% paraformaldehyde, pH 7.4) overnight at 4°C.

The NOS inhibitor 7-nitroindazole (7NI, Alexis), the sGC activator protoporphyrin IX free acid (Alexis), and the sGC inhibtor 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 culture medium. Control cultures contained the same concentration of DMSO. The NO-donor sodium nitroprusside (SNP), the phosphodiesterase inhibitor 3-isobutyl-methylxanthine (IBMX), the cGMP analogon 8-Bromo-cGMP (8Br-cGMP) and the PKG inhibitor 8-Bromo-guanosine 3′,5′-cyclic monohosphothioate Rp-Isomer (RPcGMPS, Alexis) were dissolved directly in L15 medium.

Anti-acetylated α-tubulin immunocytochemistry

To visualize the migrating neurons on the midgut, the preparations were labeled with an antiserum against acetylated α-tubulin (Sigma), a characteristic feature of stable microtubular arrays in axons. This antiserum stains somata and neurites of the MG neurons (Ganfornina et al., 1996).

The fixed guts were permeablized in 0.3% Saponin-PBS (phosphate-buffered saline, pH 7.4) for 1 hour and subsequently blocked in 5% normal goat serum in PBT (PBS + 0.3% BSA + 0.5% Triton) for 1 hour. The polyclonal mouse antiserum against acetylated α-tubulin was applied at a dilution of 1:250 in PBT/5% normal horse serum at 4°C overnight. After washing in PBT, the guts were exposed to a biotinylated horse anti-mouse antibody (Vector, diluted 1:250). For visualization of anti α-tubulin immunoreactivity we used Streptavidin-Cy3 (Sigma, diluted 1:250) as fluorescent marker. Guts were cleared in a glycerol series (50%, 90% in PBS) and mounted in Vectashield (Vector).

Analysis of MG neuron migration

Preparations were observed using a Zeiss Axiovert microscope. Pictures were captured with an AxioCam HRc linked to a Zeiss image processing system. We measured the distance from the foregut-midgut boundary to the position of the leading MG neuron (Wright et al., 1998). A Mann-Whitney U-test was employed for statistical comparisons of the means of the experimental and the control groups. Histogram values indicate the mean values±s.e.m. as percentage of the matched control values of each experiment, using $n \geq 20$ embryos for each experimental group. All significance levels are two-tailed. For visualization of the migration pattern on the midgut, captured images of the immunolabelled MG neurons were traced and arranged as drawings.

Analysis of neurite branching

The extent of neurite branching was measured similar to the approach used for *Manduca* (Wright et al., 1998) according to the method of Weeks and Truman (Weeks and Truman, 1986). Digital images of the enteric plexus were stored and processed using Photoshop (Adobe). The image was marked with a grid of 4 cm^2 (corresponding to a 3.8 mm^2 area within the embryo) divided into 64 squares. The number of squares containing neurite processes on the midgut were counted and averaged for each gut. The mean values were calculated from four grids per gut using six to eight guts for each experimental group. Statistical analyses were performed using a Mann-Whitney *U*-test.

Anti-cGMP immunocytochemistry

Embryos of stages 55-95% E were opened dorsally and guts were dissected out in ice-cold L15 medium. To induce activity of sGC, guts were exposed to SNP (100 μM) in the presence of IBMX (1 mM) for 20 minutes at room temperature (De Vente et al., 1987; Seidel and Bicker, 2000). The guts were fixed in PIPES-FA overnight at 4°C and permeablized in 0.3% Saponin-PBS for 1 hour. After blocking in PBT/5% normal rabbit serum, the primary sheep anti-cGMP antiserum (courtesy of Dr Jan De Vente) (see Tanaka et al., 1997) was applied (dilution 1:5000) at 4°C overnight. Subsequently, the guts were exposed to a biotinylated rabbit anti-sheep antibody (Vector, diluted 1:250). Immunoreactivity was visualized by standard peroxidase staining techniques using the Vector ABC kit. For

quantification of cGMP-IR neurons at different embryonic stages, the guts were counterstained with anti-acetylated α-tubulin, as described above. For the stages 55% to 95% E, the total number of α-tubulin and cGMP-positive MG neurons was calculated for each preparation. The mean values and s.e.m. were calculated for each developmental stage (n=5).

NADPH-diaphorase staining

For NADPH-diaphorase histochemistry, guts of stages between 50% and 95% were dissected in L15 medium. The guts were fixed in 4% PIPES-FA with 10% methanol for 1 hour on ice. Subsequently, the preparations were permeablized in 0.3% Saponin-PBS for 30 minutes. After rinsing in 50 mM Tris-HCl (pH 7.8), the guts were incubated in 0.1 mM β-NADPH/0.1 mM Nitro Blue Tetrazolium in Tris-HCl at room temperature (in the dark) for 1 hour. Subsequently, the tissue was washed in PBS and cleared in glycerol series.

Phalloidin staining of actin cytoskeleton in isolated MG neurons

A cell blotting procedure was chosen for better visualization of the actin cytoskeleton. The embryos were cultured in the pharmacological compounds leaving the natural tissue environment intact. Subsequently, the gut was dissected out, the yolk was removed by forceps and the gut was transferred to a petri dish containing 3 ml L15 medium and a poly-L-Lysine (10 μg/ml) coated coverslip at the bottom. Using a dissecting microscope for visual control, the gut was carefully rolled on the coverslip. During the blotting procedure, the MG neurons adhered to the coated surface, while the intact gut epithelium was finally removed from the petri dish. The blotted cells were immediately fixed with PIPES-FA for 15-30 minutes on ice. The immediate access of the fixative ensured the rapid preservation of F-actin organization in the region of the cell body.

For visualizing the actin cytoskeleton in MG neurons, F-actin was stained with AlexaFluor 568 phalloidin (Molecular Probes) according to the instructions of the supplier. The neuronal identity (Jan and Jan, 1982) of the blotted cells was confirmed using an antiserum against horseradish peroxidase (anti-HRP, Jackson Immunoresearch). The primary goat anti-HRP antiserum was applied at a dilution of 1:5000 in blocking solution for 30 minutes. Subsequently, the neurons were rinsed in PBS and exposed to a biotinylated secondary antibody (Vector, diluted 1:250). Immunoreactivity was visualized using Streptavidin Alexa Fluor 488 conjugate (Molecular Probes).

To analyze cytoskeletal rearrangements, we examined for each blotted gut 50 neurons from randomly chosen visual fields on the coverslip. For each experiment, five guts were evaluated under the fluorescence microscope. Depending on the F-actin distribution, two phenotypes could be readily assigned to the cell bodies. One was characterized by a dense network of F-actin bundles spanning the soma, whereas in the other discrete F-actin bundles were almost completely absent. To quantify these results, we assigned somata with more than three bundles to one group (here called the stationary phenotype) and somata with equal or less than three discrete fiber bundles to the other group (the migratory phenotype). A Mann-Whitney U-test was employed for statistical comparisons. Histogram values indicate the mean values±s.e.m. as percentage of the matched control values of each experiment. All significance levels are two-tailed.

Time-lapse video microscopy

For in situ video microscopy of living MG neurons we used embryos staged between 64% and 68% E. To trace cell migration with a lipophilic dye, guts were dissected in 3 ml L15 medium in an incubation chamber with a glass cover slip as bottom. Then, 20 μl of DiO (1 μg 3,3′-dioctadecycloxacarbocyanine perchlorate, Molecular Probes, dissolved in 0.5 ml 100% Ethanol) were applied to the incubation chamber, so as to form a thin DiO layer on the surface of the L15 medium. The anterior parts of the guts were carefully raised

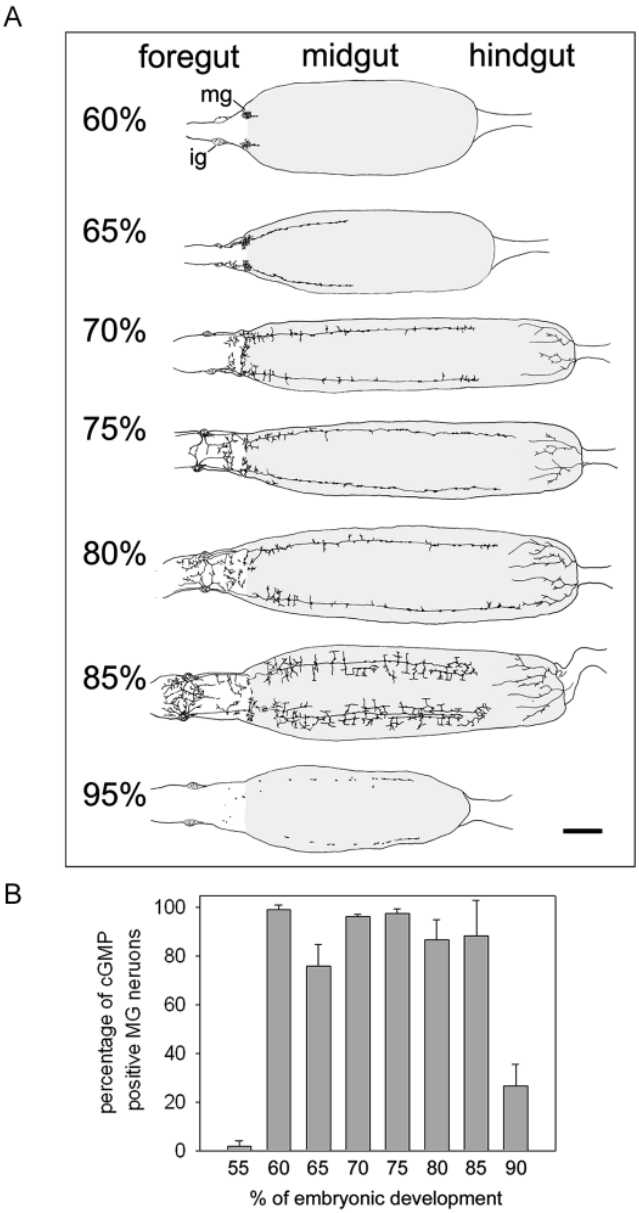

Fig. 1. NO induced cGMP-immunoreactivity of MG neurons during midgut plexus development. Guts were incubated with SNP and then immunostained with an anti-cGMP antiserum. (A) Images were drawn from individual preparations. Each panel shows a dorsal view of the embryonic gut: ingluvial ganglion (ig), the midgut is marked in gray; mg indicates midgut neurons. For the sake of clarity, the caeca are not shown. Scale bar: 200 μm. (B) Appearance of NO-induced cGMP-IR in MG neurons. The total number of MG neurons was calculated from anti-acetylated α-tubulin staining which labels all MG neurons. The percentages of cGMP-positive MG neurons during different embryonic stages are shown. The mean values and s.e.m. were calculated for each developmental stage (n=5). At 55% E, almost no cGMP-IR was found in the premigratory population of MG neurons. When migration started at about 60% E, all migrating MG neurons showed strong levels of anti-cGMP staining. The MG neurons exhibited cGMP-IR throughout the phase of migration (60-70% E) and continued to show high levels of anti- cGMP staining in the phase of lateral axon branching and the formation of terminal processes on the midgut musculature (70-85% E). When the midgut plexus acquired a mature innervation pattern (90 and 95% E), there was a rapid decrease of cGMP-IR.

several times to the level of this layer, which resulted in intense staining of the MG neurons and parts of the midgut cells. The DiO layer on the surface was removed with filter paper and the guts were washed several times in L15 medium.

Each preparation was placed in a PTC-10npi warming heater (33°C) on the stage of a Zeiss Axiovert 35 fluorescence microscope equipped with FITC filters. A 10% neutral density filter was used to attenuate the excitation light emitted by the Xenon lamp. Images were captured at 20 minute intervals for a minimum of 2 hours with a Hamamatsu 2400 SIT camera linked to Simple PCI computer software (Hamamatsu, Hamamatsu City, Japan) which also controlled the opening of the shutter in the light path of the fluorescence excitation. Brightness and contrast of the photos were enhanced with Adobe Photoshop 6.0.

RESULTS

NO-induced cGMP-immunoreactivity and NADPH-diaphorase staining of the developing enteric midgut plexus

During the formation of the midgut plexus of the grasshopper enteric nervous system, postmitotic neurons arise in a neurogenic zone on the foregut and then migrate laterally to accumulate at the posterior edge of the ingluvial ganglia. (Ganfornina et al., 1996). The migrating neurons are of bipolar morphology with short processes running posteriorly. The processes that are trailing behind establish the four midgut nerves, two derived from each ingluvial ganglion (Ganfornina et al., 1996). Our results revealed that sGC activity in the neuritic branches and the cell bodies (Fig. 1A,B) of the MG neurons is developmentally regulated, coincident with their movement on the midgut surface. Prior to the onset of migration (up to 60% E) we could not detect NO-induced cGMP-IR in MG neurons. At 62% of embryonic development, the MG neurons accumulate near the foregut-midgut boundary and start to migrate posteriorly (Fig. 1A, Fig. 2A,B). At this stage, first cGMP-IR became visible and persisted during the following stages. The onset of detectable levels of cGMP-IR within the MG neurons occurred rapidly (Fig. 1B). Between 60% and 65% E, all migrating MG neurons acquired substantial levels of immunoreactivity (Fig. 1, Fig. 2F). The MG neurons exhibited cGMP-IR throughout the phase of migration (Fig. 1, Fig. 2G) and continued to show high levels of anti-cGMP staining in the phase of lateral axon branching and the formation of terminal processes on the midgut (Fig. 1, Fig. 2H). The immunoreactivity was visible in the cell bodies

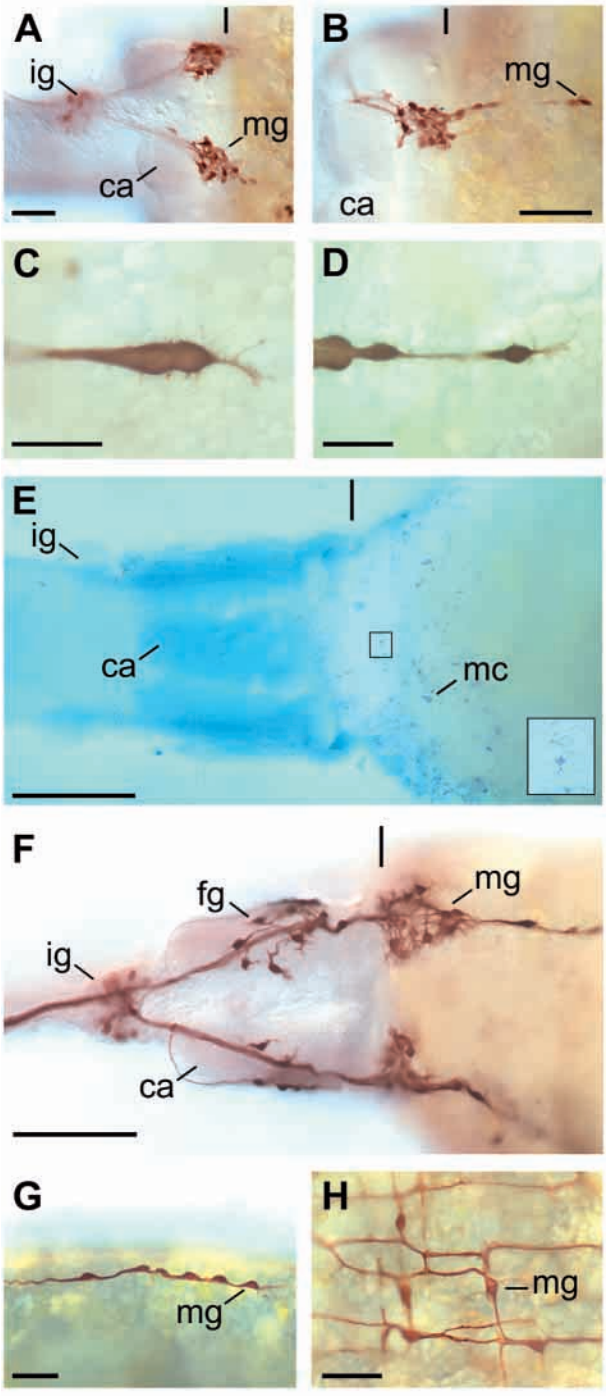

Fig. 2. Developmental expression of NO induced cGMP-IR in MG neurons and NADPH diaphorase staining of the midgut epithelium. (A) At about 60% of embryonic development (% E), the MG neurons (mg) formed a cellular packet at the foregut-midgut boundary (vertical line indicates boundary). Caeca (ca) were not stained. At this stage, all MG neurons began to show strong anti-cGMP staining. In the ingluvial ganglion (ig), some neurons expressed sGC activity (lateral view). (B) Between 60 and 65% E, the first MG neurons started to migrate posteriorly on the midgut surface. All MG neurons showed high levels of anti-cGMP staining. (C,D) Between 60 and 65% E, NO-sensitive sGC activity was expressed in the cell body and the advancing processes of the leading MG neurons. (E) At 60% E, first NADPH-diaphorase staining was present in distinct cells of the midgut. The inset of the designated area shows an example of NADPH-diaphorase-positive cellular staining. The first appearance of the diaphorase staining was coincident with the onset of MG neuron migration (compare to A with B). (F) Anti-cGMP-IR at 65% E; lateral view. At this stage, anti-cGMP IR was present in cells of the ingluvial ganglion, the enteric nerves and the foregut neurons (fg). Some of the midgut neurons migrated laterally to form a nerve ring near the foregut-midgut boundary. (G) At 70% E, the MG neurons were still migrating posteriorly. The leading as well as the following neurons of one migratory pathway showed strong cGMP-IR. (H) During the phase of lateral neurite branching and the formation of terminal processes on the midgut musculature, the MG neurons continued to exhibit strong cGMP-IR. These micrographs were compiled from several focal planes. Scale bars: 50 μm in A,B; 20 μm in C,D; 200 μm in E,F; 25 μm in G,H.

and processes of the neurons (Fig. 2C,D). At 80% E, some MG neurons leave the main migratory routes to spread out between the midgut nerves (Ganfornina et al., 1996). Between 90% and 95% E the midgut plexus acquires a mature branching pattern (Ganfornina et al., 1996), accompanied by a rapid decrease in the percentage of cGMP expressing neurons (Fig. 1).

NO-sensitive sGC activity was also present in the branches of the hindgut nerves (Fig. 1A). The leading processes of these abdominal neurons exhibited inducible cGMP-IR at the time of their initial growth onto the hindgut and remained immunopositive throughout their subsequent elaboration of processes on the hindgut and posterior midgut musculature.

Moreover, we identified potential sources of NO near the MG neurons, using NADPH-diaphorase staining of formalin-fixed embryonic guts as a histochemical marker for NOS. We found the blue precipitate of the diaphorase staining concentrated in a subset of the midgut cells (Fig. 2E). Remarkably, the NADPH-diaphorase staining was also developmentally regulated. Diaphorase staining of the midgut cells was visible during a developmental period ranging from about 60% to 95% of development. Between 60% to 65% E, diaphorase activity was restricted to an intense band of staining on the midgut surface, adjacent to the foregut-midgut boundary, which corresponded to the position of the migrating MG neurons. At later stages, discrete NADPH-diaphorase-positive cells were found evenly distributed over all areas of the midgut epithelium.

NO/cGMP/PKG signalling is a positive regulator of MG neuron migration

Neurochemicals that inhibited the NO/cGMP pathway significantly affected the migration of MG neurons (Figs 3, 4). When embryos at 62% of development, a stage at which the MG neurons initiate their migration, were allowed to develop in culture for 24 hours, we observed that MG neuron migration proceeded normally (Figs 3, 4). By contrast, migration was significantly reduced in embryos that were exposed to 500 μM NOS inhibitor 7NI (Doyle et al., 1996) (Fig. 3, Fig. 4A).

To test for an involvement of the target enzyme sGC in MG neuron migration, embryos were cultured with the specific inhibitor ODQ (Boulton et al., 1995). This treatment reduced MG neuron migration in a concentration-dependent manner (Fig. 3, Fig. 4B). A potential downstream effector protein for cGMP signalling are cGMP-dependent protein kinases (Wang and Robinson, 1997). In embryos treated with the specific inhibitor RPcGMPS, which irreversibly binds to PKG (Layland et al., 2002), MG neuron migration was significantly reduced compared with control-treated animals (Fig. 4A). In addition, cultured embryos were also exposed to neurochemicals that elevated cGMP levels. We applied protoporphyrin IX free acid which stimulates sGC independently of NO (Wollin et al., 1982). The membrane-permeable 8Br-cGMP was used to raise cGMP levels directly. Neither SNP, protoporphyrin IX free acid nor 8Br-cGMP had any effect on the normal migration of the MG neurons (data not shown).

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

To test if the disruptive effects of 7NI and ODQ can be reversed by adding activators of NO/cGMP signalling, we treated embryos in culture with 7NI plus protoporphyrin IX free acid (Fig. 3, Fig. 4C) as well as with ODQ plus 8Br-cGMP (Fig. 3, Fig. 4D). Embryos cultured in the presence of 7NI showed a significant reduction of migration. However, adding 1 mM protoporphyrin IX free acid led to a complete recovery of the

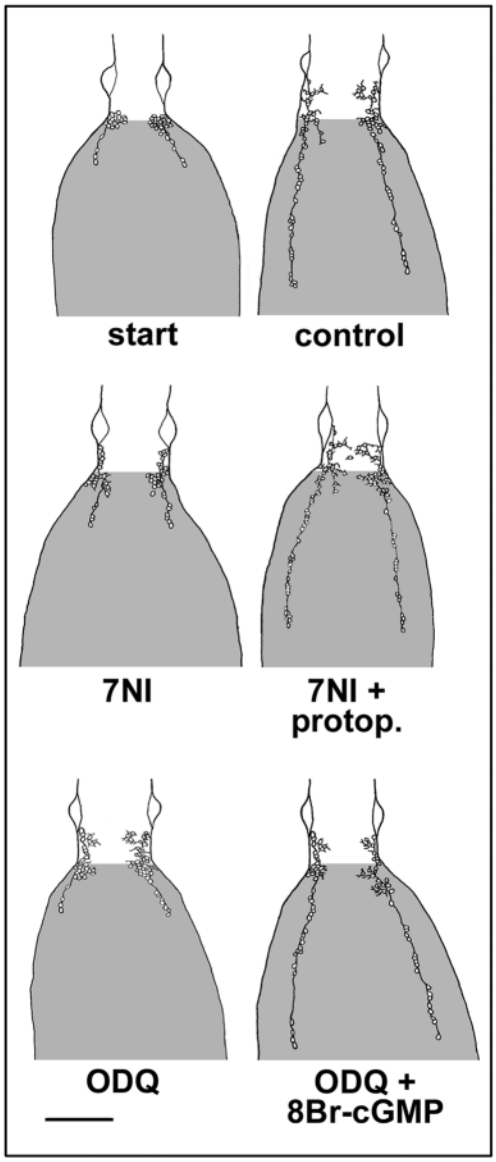

Fig. 3. MG neuron migration under the influence of neurochemicals that affect the NO/cGMP signalling pathway. Images were drawn from individual guts that were stained with anti-α-tubulin antiserum. At the beginning of the experiment, MG neurons migrated not more than 40 μm on the midgut surface (start). The next drawing shows normal MG neuron migration after 24 hours incubation under control culture conditions (control). MG neuron migration was inhibited in an embryo that was exposed to 500 μM 7NI (7NI). This disruptive effect of 7NI could be rescued by the addition of 1 mM protoporphyrin IX free acid so that the MG neurons again covered the normal distance (7NI + protop.). MG neuron migration was reduced in an embryo that was exposed to 200 μM ODQ (ODQ). The inhibitory effect of ODQ was rescued by the addition of membrane permeable 8Br-cGMP (ODQ 200 μM + 8Br-cGMP). Scale bar: 200 μm.

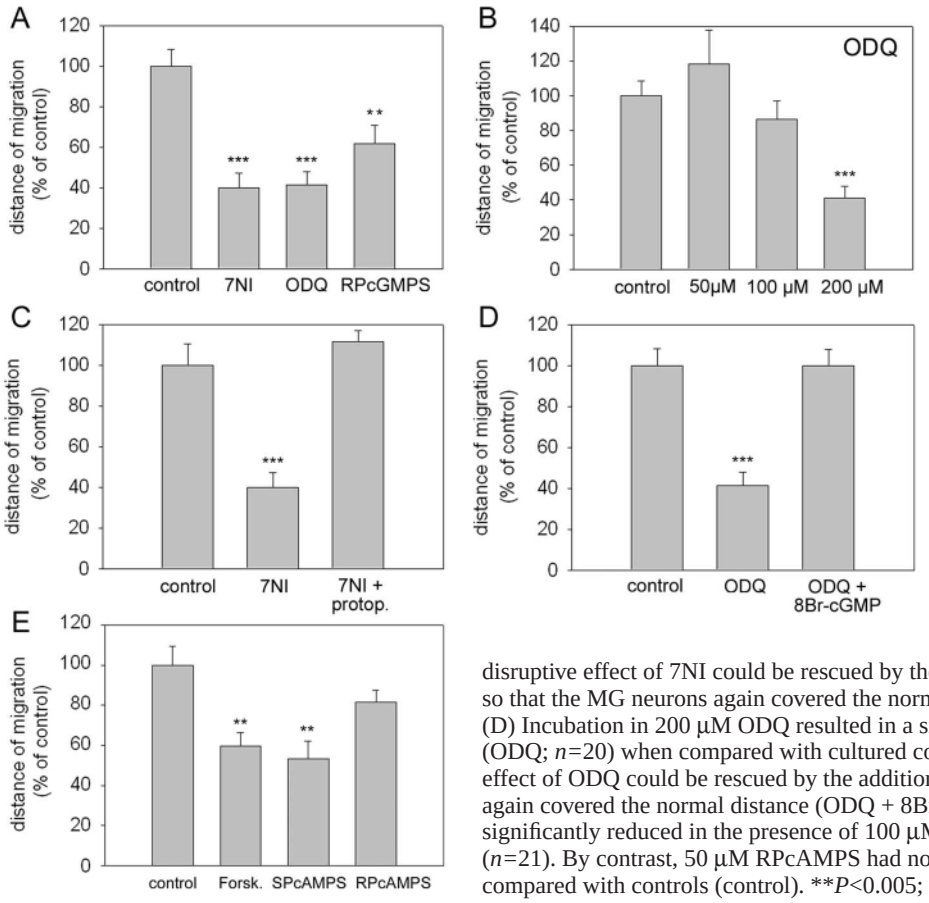

Fig. 4. Blocking of MG neuron migration by enzyme inhibitors of the NO/cGMP/PKG pathway and the cAMP/PKA cascade. Histograms show the distance migrated by the leading MG neuron on the midgut. (A) After 24 hours, normal migration of MG neurons was found in cultured control embryos (*n*=21). MG neuron migration was significantly reduced in the presence of 500 µM 7NI (*n*=21), 200 µM ODQ (*n*=20) and 50 µM RPcGMPS (*n*=21). (B) ODQ reduced MG neuron migration in a concentration-dependent manner. Normal migration of MG neurons was highly significantly inhibited in embryos that were cultured in the presence of 200 µM ODQ (control, *n*=21; 200 µM, *n*=20; 100 µM, *n*=20; 50 µM, *n*=24). (C) Histogram shows that incubation in 500 µM 7NI resulted in a significant reduction of the migratory distance (7NI; *n*=21) when compared with cultured control embryos (control). This disruptive effect of 7NI could be rescued by the addition of 1 mM protoporphyrin IX free acid so that the MG neurons again covered the normal distance (7NI + protoporphyrin; *n*=20). (D) Incubation in 200 µM ODQ resulted in a significant reduction of the migratory distance (ODQ; *n*=20) when compared with cultured control embryos (control; *n*=21). This disruptive effect of ODQ could be rescued by the addition of 8Br-cGMP such that the MG neurons again covered the normal distance (ODQ + 8Br-cGMP; *n*=20). (E) MG neuron migration was significantly reduced in the presence of 100 µM forskolin (*n*=20) and 50 µM SPcAMPS (*n*=21). By contrast, 50 µM RPcAMPS had no significant effect on migration (*n*=21) when compared with controls (control). ***P*<0.005; ****P*<0.001.

normal MG neuron migration (Fig. 3, Fig. 4C). Embryos cultured in the presence of 200 µM ODQ showed a significant inhibition of normal MG neuron migration. Again, addition of 500 µM of the membrane permeable 8Br-cGMP rescued the inhibitory effect of 200 µM ODQ on cell migration and resulted in a normal migration pattern (Fig. 3, Fig. 4D).

Inhibition of sGC and NOS activity perturbs neurite branching

Similar to the development of the enteric plexus in *Manduca* (Wright et al., 1998), inhibitors of both NOS and sGC activity applied to cultured embryos (80% E) influenced neurite branch formation substantially. We found that neurite growth proceeded normally in control cultures. By contrast, treatment with either 500 µM 7NI or 200 µM ODQ resulted in a significant reduction in terminal branch formation. The percentage of terminal arbor density was reduced by 7NI to 44% (s.e.m.=3.439; *n*=6; *P*<0.005) and by ODQ to 46% (s.e.m.=2.146; *n*=6; *P*<0.0059) compared with control values (100%±4.532; *n*=8).

cAMP signalling is a negative regulator of MG neuron migration

As the cyclic nucleotide cGMP is permissive for normal MG neuron migration, we examined also a potential contribution of cAMP signalling on cellular motility. To elevate cAMP levels, we cultured embryos in forskolin (100 µM), which stimulates the adenylate cyclase (AC). MG migration was significantly reduced in embryos that were exposed to 100 µM forskolin in the presence of the phosphodiesterase inhibitor IBMX (Fig. 4E). When the membrane permeable ligand SPcAMPS (50 µM) was used to activate PKA directly, MG neuron migration was also significantly reduced (Fig. 4E), whereas exposure to the specific PKA inhibitor RPcAMPS (50 µM) had no effect on the normal migration of the MG neurons (Fig. 4E).

Organization of F-actin cytoskeleton in MG neurons

Cellular movement results from the reorganization of the cytoskeleton. To investigate changes in the cytoskeletal organization caused by NO/cyclic nucleotide signalling, we examined the pattern of phalloidin-labeled F-actin in isolated MG neurons. In control experiments, we found F-actin staining predominantly in the neurites. In these cells, distinct bundles of F-actin fibers were almost absent from the somata. Fig. 5A shows a representative example with only a single F-actin bundle extending from the neurite into the cell body. This cytoskeletal arrangement is indicative for a motile cellular phenotype. Quantification of the stainings showed that under these control conditions where neuronal migration proceeded normally, 70% of the MG neurons showed this motile phenotype (Fig. 5B,C). Conversely, in MG neurons treated with the sGC inhibitor ODQ, prominent F-actin staining was concentrated in the somata. The F-actin fibers formed a dense net of multiple actin-bundles in all parts of the cell body (Fig. 5A). This phenotype, which is likely to reflect a stationary cell was shown by 90% of the neurons. Adding the membrane-

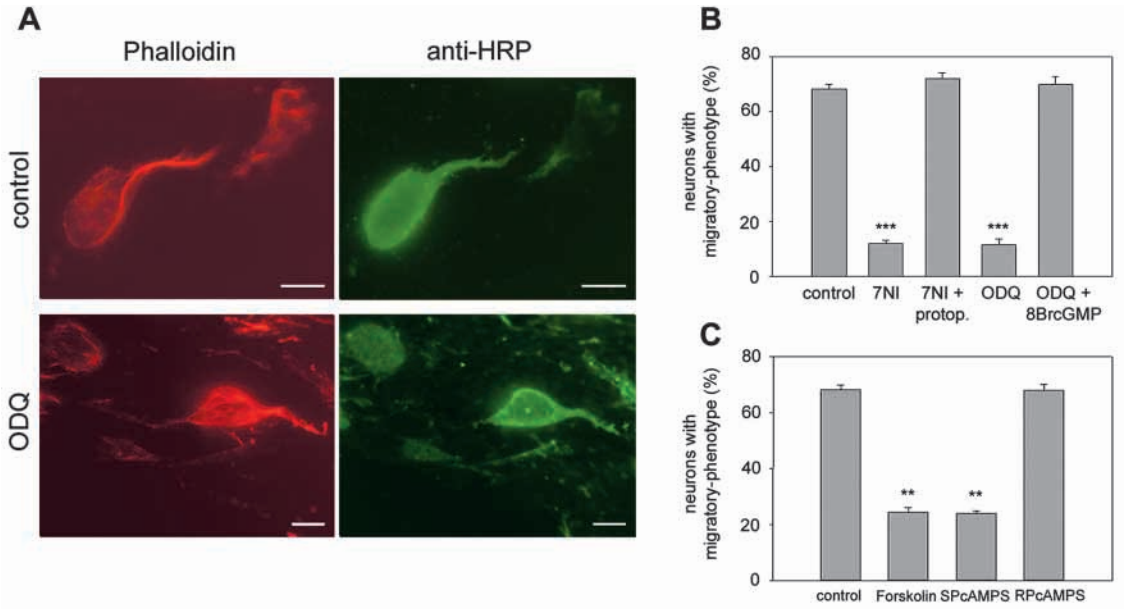

Fig. 5. F-actin staining in isolated MG neurons. (A) The control shows a representative example of the so called migratory-phenotype, with F-actin staining in the neurite and only a single fiber bundle in the soma (red). When the embryos were cultured in ODQ, the MG neurons showed a stationary-phenotype with multiple actin fiber bundles in the somata (ODQ). Co-staining with anti-HRP antiserum (green) reveals the neuronal identity of the cell (anti-HRP), whereas non-neuronal cells are unlabelled. Scale bars: 10 μm. (B) Quantitative evaluation of the actin cytoskeleton. After incubation in neurochemicals for 24 hours, a high percentage of MG neurons show the normal migratory phenotype in cultured control embryos (control). Conversely, a significantly smaller number of neurons with migratory phenotype was found in the presence of NOS inhibitor 7NI (500 μM). The disruptive effect of 7NI on the actin cytoskeleton could be rescued by the addition of 1 mM protoporphyrin IX free acid (7NI + protop.). Similarly, the number of MG neurons showing the migratory phenotype was reduced after incubation in the sGC inhibitor ODQ (200 μM). This disruptive effect of ODQ on the actin cytoskeleton organization could be rescued by the addition of 500 μM 8Br-cGMP (ODQ + 8Br-cGMP). (B) Neurochemicals that affect the cAMP/PKA pathway also had an effect on the actin organization. A significantly smaller number of neurons from embryos cultured in the AC activator forskolin (100 μM) or the PKA activator SPcAMPS (50 μM) showed the migratory phenotype as compared with controls. The PKA inhibitor RPcAMPS (50 μM) had no significant effect on the actin cytoskeleton organization. $^{**}P<0.005$; $^{***}P<0.001$.

permeable 8Br-cGMP to the culture medium completely reversed the effect of ODQ. Similar to the expression level of control conditions, 94% of the MG neurons showed the motile phenotype in these rescue experiments (Fig. 5B). 8Br-cGMP which had no effect on normal migration of the MG neurons did not affect the distribution of F-actin (data not shown). Blocking of endogenous NO synthesis with the inhibitor 7NI did also reduce the expression of the motile phenotype to 12%, which could be rescued again by activating sGC with protoporphyrin to control levels (Fig. 5B).

Neurochemicals that activated the cAMP/PKA pathway significantly affected the F-actin distribution in MG neurons. When the MG neurons were exposed to forskolin (100 μM) in the presence of the phosphodiesterase inhibitor IBMX (500 μM) only 20% of the MG neurons showed the motile phenotype (Fig. 5C). Forskolin without IBMX had no significant effect on the actin cytoskeleton (data not shown). Embryo culture in the PKA activator SPcAMPS (50 μM) did also reduce the expression of the motile phenotype (Fig. 5B), whereas inhibition of PKA by RPcAMPS (50 μM) resulted in no differences in the F-actin distribution compared with controls (Fig. 5C).

Time-lapse video microscopy of living MG neurons

To investigate the normal migratory behavior of individual cells in situ, the motility of fluorescence-labeled MG neurons was analyzed under time-lapse video microscopy (Fig. 6). The movements of DiO-stained MG neurons were followed on intact midguts for 2 hours, showing that the average velocity migrated by each cell (n=5) was ~12 μm/hour (Fig. 6). When the migrating neurons were exposed to 200 μM ODQ, all neurons immediately stopped migration (n=5) (Fig. 6). This shows that the pharmacological treatment does not cause any misrouting or reversion of the movement direction.

DISCUSSION

NO/cGMP signalling is permissive for MG neuron migration

In this paper, we have provided evidence that NO/cGMP signalling is essential for the regulation of neuronal migration in the developing ENS of the grasshopper. We found that blocking of endogenous NO synthesis by the NOS inhibitor 7NI disrupts migration of the MG neurons (Fig. 4A). Treatment with ODQ, a specific inhibitor of sGC also prevented the MG neuron migration in a dose-dependent manner (Fig. 4B). In embryos treated with the specific PKG inhibitor RPcGMPS, MG neuron migration was significantly reduced (Fig. 4A). This effect suggests that cGMP might influence migration via activating PKG.

It is rather unlikely that the pharmacological effects on MG

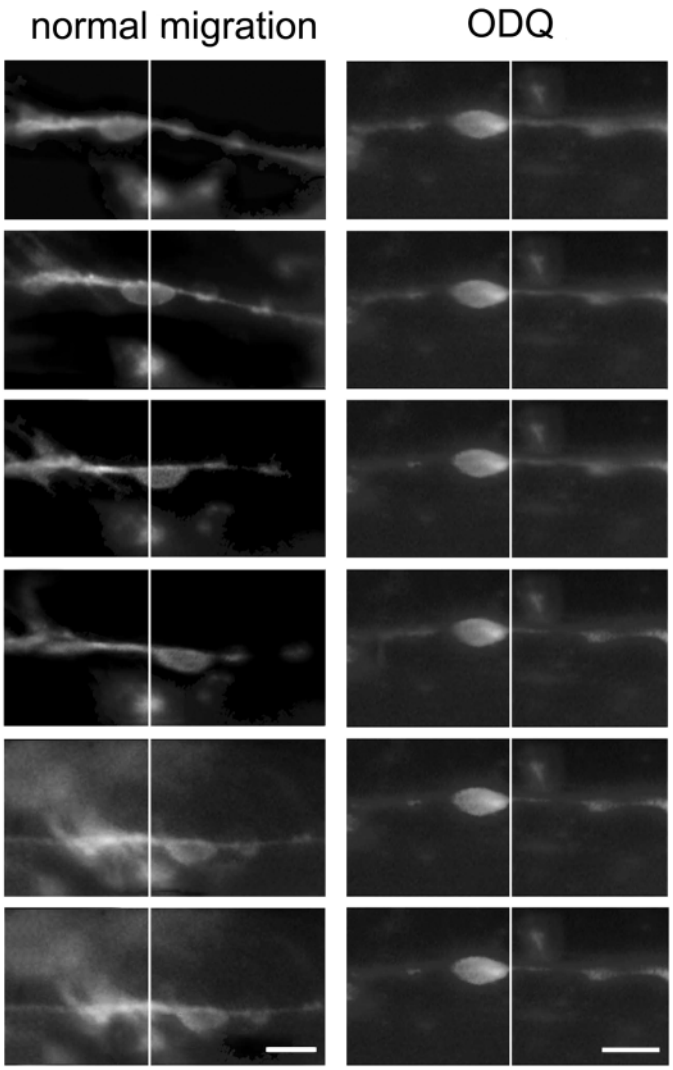

Fig. 6. Time-lapse video microscopy of living MG neurons migrating on the midgut. Examples show DiO-labeled MG neurons from embryos staged between 64% and 68% E. Time intervals between the images are 20 minutes. On average, MG neurons migrated 12 μm/hour under normal conditions. To examine the effects of inhibiting sGC on individual neurons, the preparation was incubated in culture medium containing 200 μM ODQ (right). Under these conditions, there was no misrouting of the neurons and they completely stopped migrating. Scale bars: 10 μm.

neuron migration are an artifact of the embryo culture, because in control cultured embryos, migration proceeded normally. Intriguingly, the disruption of MG neuron migration caused by inhibiting NO production or cGMP synthesis could be rescued by exogenous application of membrane-permeant cGMP (Fig. 4D) and pharmacological stimulation of sGC (Fig. 4C), suggesting that in vivo a certain level of cGMP is necessary for MG neuron migration.

The experimental findings that NO/cGMP signalling is necessary for MG neuron migration receive additional support from cytochemical stainings. Treatment with a NO donor induced cGMP-IR in the MG neurons in the developing ENS. Moreover, the timing of sGC activity coincides exactly with periods of neuronal motility as well as axonal outgrowth (Figs 1, 2). At the onset of their migration on the midgut surface, first detectable levels of cGMP-IR within the MG neurons became apparent. The MG neurons exhibited cGMP-IR throughout the phase of migration and continued to show high levels of anti-cGMP staining in the phase of lateral axon branching and the formation of terminal processes on the midgut musculature (Figs 1, 2). When the midgut plexus acquired a mature morphology, a rapid decrease in the amount of cGMP-positive MG neurons occurred (Figs 1, 2). Thus, NO-induced sGC activity in MG neurons is developmentally regulated and correlates with their migratory phase.

In *Manduca* development, the migrating enteric neurons do also show NO-sensitive sGC expression. The inhibition of NOS and sGC causes a reduction of terminal branch formation in a later phase of development both in *Manduca* (Wright et al., 1998) and also in our experiments with *Locusta*. However, in contrast to the grasshopper, inhibition of NO/cGMP signalling in *Manduca* does not affect neuronal migration and there was no detectable NO source near the migrating EP cells (Wright et al., 1998). In the grasshopper, however, we identified a potential source of endogenous NO in immediate vicinity to the migrating MG neurons. A subpopulation of the midgut cells stain for NADPH-diaphorase, a histochemical marker for NOS (Dawson et al., 1991; Vincent and Kimura, 1992) (Fig. 2E). Similar to the cGMP-IR, the first appearance of NADPH-diaphorase activity in the midgut epithelium exactly coincides with the onset of MG neuron migration. The diaphorase staining persisted throughout the phase of MG neuron migration and the phase of terminal branch formation. The conflicting data obtained from neuronal migration experiments might be due to species-specific differences in the development of holometabolous versus hemimetabolous insects. Differences in the experimental procedures of animal culture, or most likely the concentration of the pharmacological agents may have also contributed to the different outcome of the cell migration experiments. For example, we found a strong inhibitory effect on cell migration of the sGC blocker ODQ at 200 μM (Fig. 4B), a concentration that has not been used in *Manduca*. Nonetheless, it should be stressed that in the grasshopper the ODQ effect could be fully rescued to normal migratory behavior by a cGMP analogue.

NADPH-diaphorase histochemistry after formaldehyde fixation is considered to be diagnostic for the presence of NOS containing cells (Dawson et al., 1991; Vincent and Kimura, 1992). Measurements of NOS activity in cell homogenates of the grasshopper nervous system do indeed correlate well with the biochemical determination of NADPH-diaphorase activity and the histochemical staining pattern of NADPH-diaphorase-positive cells (reviewed by Bicker, 1998). Nevertheless, the results of the diaphorase method are subject to variations due to the fixation protocols and may even lead to false positive results (Ott and Burrows, 1999). However, using an antiserum that recognizes a highly conserved sequence of the different mammalian NOS isoforms, it has been shown that NOS-IR does indeed co-localize with a NADPH-diaphorase stainings of the antennal lobe (Bicker, 2001b). This finding supports the molecular identity of NADPH diaphorase and NOS enzymes in grasshopper tissue fixed according to the histological protocol of this paper. In the intact embryo, there may be additional messenger molecules apart from NO that could activate sGC. For example the MG neurons may receive a

carbon monoxide signal (Baranano and Snyder, 2001) from yet unidentified tissue sources.

Another line of evidence that indicates that NO/cGMP and the cAMP/PKA cascade directly act in the migrating MG neurons comes from the investigation of the cytoskeleton. Cell migration depends on forces generated by the polymerization of actin in cellular protrusions (Lauffenburger and Horwitz, 1996). When we examined the distribution of F-actin in migratory MG neurons, we found indeed prominent F-actin based structures mainly in the cellular processes but not in the cell bodies. Under condition where migration was blocked by inhibitors of the NO/cGMP/PKG cascade, we found a cytoskeletal organization with a dense network of F-actin bundles spanning the cell body. Correspondingly, activation of cAMP/PKA cascade resulted in an inhibition of MG neuron migration that was also accompanied by a cytoskeletal rearrangement. This distribution of actin fibers would be expected in stationary cells (Brown et al., 1999). The experimental perturbations of the signalling cascades revealed that NO/cGMP signalling appears to act antagonistically to cAMP/PKA signalling at the level of the MG neurons. To support these findings, it would be helpful to obtain data on the in vivo production of cAMP and PKA activity in the MG neurons, similar to the cytochemical localization of the NO/cGMP pathway

Cyclic nucleotide levels and neuronal motility

Dynamic regulation of cyclic nucleotide levels play a key role in modulating neuronal motility. First evidence for a discrete role the involvement of the cAMP cascade in insect growth cone motility came from primary cultured neurons of the *Drosophila* memory mutants dunce and rutabaga, which have oppositely altered intracellular cAMP levels (Kim and Wu, 1996). Cyclic nucleotides have also regulatory effects on growth cone responses in vertebrates. Cell culture experiments with dissociated *Xenopus* spinal neurons have shown that growth cone responses to netrin 1 could be converted between attraction and repulsion by altering the cAMP level in the growth cone (Ming et al., 1997). Elevated levels of cGMP can also change the response of growth cones to a semaphorin from repulsion to attraction (Song et al., 1998). Remarkably, an asymmetric cellular localization of sGC to the dendrite of pyramidal cells is thought to confer the opposite directional outgrowth to dendrites and axons in a semaphorin gradient of the cerebral cortex (Polleux et al., 2000). Thus, intracellular cyclic nucleotide levels can be the crucial factors that govern process extension to the same chemotropic guidance cue. Similarly, the cGMP or cAMP cascade may modulate the transduction of extracellular guidance cues governing the migration of the MG neurons. One candidate link between extracellular guidance signals and actin-associated proteins is the Rho family of small GTPases, which are modulated by cyclic nucleotide levels (Song and Poo, 2001). In *Drosophila*, Rho and Rac GTPases have recently been implicated in actin cytoskeletal dynamics during the migration of peripheral glia migration (Sepp and Auld, 2003).

Our data indicate that the MG neurons may receive a NO signal from the visceral midgut cells and that elevated cGMP levels are essential for the ability of migration. Could a tissue-intrinsic NO signal play a role as a guidance factor for the cell migration? Diaphorase staining appears to be distributed in all areas of the midgut surface (Fig. 2E) and is not exclusively confined to cells in the vicinity of the four migratory pathways. Therefore, we view it as rather unlikely that NO release can prefigure the migratory routes on the midgut.

Alternatively, the midgut cells may synthesize NO in an anterior/posterior gradient which might provide directional information. As we have not found any significant misrouting by the MG neurons after pharmacological inhibition of NOS or sGC (Fig. 6), there is no evidence for a directional guidance function of NO. This view receives support from transplantation experiments in the ENS of *Manduca* showing that EP cells are capable of migrating in both directions along the host muscle band (Copenhaver et al., 1996).

To fully appreciate the role of NO signalling in cell migration, it is essential not only to investigate the spatial distribution of NO synthesizing cells, but also to monitor the temporal pattern of NO/cGMP formation and breakdown. Similar to the vertebrate nervous system, neuronal production of NO in the locust is a tightly Ca^{2+}/calmodulin regulated process (reviewed by Bicker, 1998). Thus, increases in cytosolic Ca^{2+} levels may provide a developmental timing signal for the production of NO.

The initial appearance of inducible sGC activity in the MG neurons just at the onset of migration suggests that NO/cGMP signalling might be required for the initiation of migratory behavior. In primary cultured aortic smooth muscle cells, NO induces changes in cell shape, reorganization of the actin cytoskeleton and reduction of adhesion (Brown et al., 1999). Correspondingly, in the grasshopper ENS, NO might be crucial as a permissive factor for the initiation and maintenance of MG neuron migration.

Both pharmacological and genetic approaches have contributed towards an analysis of NO signalling during the development of insect nervous systems (reviewed by Enikolopov et al., 1999; Bicker, 2001a). Whereas the genetic approach is likely to reveal additional downstream molecular components of NO signal transduction, the application of pharmacological agents during precise time intervals of embryogenesis is helpful to detect subtle modulatory interactions in the functioning of the signalling network. The findings of this paper together with the study of pioneer neuron outgrowth (Seidel and Bicker, 2000) implicate NO/cGMP signalling both in axon elongation as well as in neuronal migration in the grasshopper. To our knowledge, this is the first experimental evidence that the NO/cGMP/PKG signalling pathway is a positive regulator for the migration of postmitotic nerve cells in vivo. Whereas in insects the migration of postmitotic nerve cells is mainly confined to the enteric nervous system, neuronal migration is an almost universal feature during the development of the more complex vertebrate nervous system (Hatten, 1999; Nadarajah and Parnavelas, 2002). As several signal transduction pathways that regulate axon guidance mechanisms in simple invertebrate and vertebrate animals are strikingly conserved in function (Tessier-Lavigne and Goodman, 1996; Dickson, 2002) it is conceivable that NO signalling may also play a role during neuronal migration in the vertebrate brain.

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REFERENCES

Ball, E. E. and Truman, J. W. (1998). Developing grasshopper neurons show variable levels of guanylyl cyclase activity on arrival at their targets. *J. Comp. Neurol.* **394**, 1-13.

Baranano, D. E. and Snyder, S. H. (2001) Neural roles for heme oxygenase: contrasts to nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **98**, 10996-11002.

Bentley, D., Keshishian, H., Shankland, M. and Toroian-Raymond, A. (1979). Quantitative staging of embryonic development of the grasshopper, Schistocerca nitens. *J. Embryol. Exp. Morphol.* **54**, 47-74.

Bicker, G. (1998). NO news from insect brains. *Trends Neurosci.* **21**, 349-355.

Bicker, G. (2001a). Sources and targets of nitric oxide signalling in insect nervous systems. *Cell Tissue Res.* **303**, 137-146.

Bicker, G. (2001b). Nitric oxide: an unconventional messenger in the nervous system of an orthopteroid insect. *Arch. Insect Biochem. Physiol.* **48**, 100-110.

Boulton, C. L., Southam, E. and Garthwaite, J. (1995). Nitric oxide-dependent long-term potentiation is blocked by a specific inhibitor of soluble guanylyl cyclase. *Neuroscience* **69**, 699-703.

Bredt, D. S. and Snyder, S. H. (1992). Nitric oxide, a novel neuronal messenger. *Neuron* **8**, 3-11.

Brown, C., Pan, X. and Hassid, A. (1999). Nitric oxide and C-type atrial natriuretic peptide stimulate primary aortic smooth muscle cell migration via a cGMP-dependent mechanism: relationship to microfilament dissociation and altered cell morphology. *Circ. Res.* **84**, 655-667.

Causeret, F., Danne, F., Ezan, F., Sotelo, C. and Bloch-Gallego, E. (2002). Slit antagonizes netrin-1 attractive effects during the migration of inferior olivary neurons. *Dev. Biol.* **246**, 429-440.

Copenhaver, P. F., Horgan, A. M. and Combes, S. (1996). An identified set of visceral muscle bands is essential for the guidance of migratory neurons in the enteric nervous system of Manduca sexta. *Dev. Biol.* **179**, 412-426.

Cramer, K. S., Angelucci, A., Hahm, J. O., Bogdanov, M. B. and Sur, M. (1996). A role for nitric oxide in the development of the ferret retinogeniculate projection. *J. Neurosci.* **16**, 7995-8004.

Dawson, T. M. and Snyder, S. H. (1994). Gases as biological messengers: nitric oxide and carbon monoxide in the brain. *J. Neurosci.* **14**, 5147-5159.

Dawson, T. M., Bredt, D. S., Fotuhi, M., Hwang, P. M. and Snyder, S. H. (1991). Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc. Natl. Acad. Sci. USA* **88**, 7797-7801.

De Vente, J., Steinbusch, H. W. M. and Schipper, J. (1987). A new approach to immunocytochemistry of 3′,5′-cyclic guanosine monophosphate: preparation, specificity and initial application of a new antiserum against formaldehyde-fixed 3′,5′-cyclic guanosine monophosphate. *Neuroscience* **22**, 361-373.

Dickson, B. J. (2002). Molecular mechanisms of axon guidance. *Science* **298**, 959-964.

Doyle, C., Hölscher, C., Rowan, M. J. and Anwyl, R. (1996). The selective neuronal NO synthase inhibitor 7-Nitro-Indazole blocks both long-term potentiation and depotentation of field EPSPs in rat hippocampal CA1 *in vivo*. *J. Neurosci.* **16**, 418-424.

Enikolopov, G., Banerji, J. and Kuzin, B. (1999). Nitric oxide and *Drosophila* development. *Cell Death Differ* **6**, 956-963.

Ganfornina, M. D., Sanchez, D. and Bastiani, M. J. (1996). Embryonic development of the enteric nervous system of the grasshopper *Schistocerca americana*. *J. Comp. Neurol.* **372**, 581-596.

Garthwaite, J. and Boulton, C. L. (1995). Nitric oxide signaling in the central nervous system. *Annu. Rev. Physiol* **57**, 683-706.

Gibbs, S. M. and Truman, J. W. (1998). Nitric oxide and cyclic GMP regulate retinal patterning in the optic lobe of Drosophila. *Neuron* **20**, 83-93.

Hartenstein, V. (1997). Development of the insect stomatogastric nervous system. *Trends Neurosci.* **20**, 421-427.

Hatten, M. E. (1999). Central nervous system neuronal migration. *Annu. Rev. Neurosci.* **22**, 511-539.

He, Y., Yu, W. and Baas, P. W. (2002). Microtubule reconfiguration during axonal retraction induced by nitric oxide. *J. Neurosci.* **22**, 5982-5991.

Hedgecock, E. M., Culotti, J. G. and Hall, D. H. (1990). The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. *Neuron* **4**, 61-85.

Hess, D. T., Patterson, S. I., Smith, D. S. and Skene, J. H. P. (1993). Neuronal growth cone collapse and inhibition of protein fatty acylation by nitric oxide. *Nature* **366**, 562-565.

Hindley, S., Juurlink, B. H., Gysbers, J. W., Middlemiss, P. J., Herman, M. A. and Rathbone, M. P. (1997). Nitric oxide donors enhance neurotrophin-induced neurite outgrowth through a cGMP-dependent mechanism. *J. Neurosci. Res.* **47**, 427-439.

Jan, L. Y. and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in Drosophila and in grasshopper embryos. *Proc. Natl. Acad. Sci. USA* **79**, 2700-2704.

Kim, Y. T. and Wu, C. F. (1996). Reduced growth cone motility in cultured neurons from Drosophila memory mutants with a defective cAMP cascade. *J. Neurosci.* **16**, 5593-5602.

Lauffenburger, D. A. and Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.

Layland, J., Li, J. M. and Shah, A. M. (2002). Role of cyclic GMP-dependent protein kinase in the contractile response to exogenous nitric oxide in rat cardiac myocytes. *J. Physiol.* **540**, 457-467.

Leamey, C. A., Ho-Pao, C. L. and Sur, M. (2001). Disruption of retinogeniculate pattern formation by inhibition of soluble guanylyl cyclase. *J. Neurosci.* **21**, 3871-3880.

Ming, G. L., Song, H. J., Berninger, B., Holt, C. E., Tessier-Lavigne, M. and Poo, M. M. (1997). cAMP-dependent growth cone guidance by netrin-1. *Neuron* **19**, 1225-1235.

Mitchell, K. J., Doyle, J. L., Serafini, T., Kennedy, T. E., Tessier-Lavigne, M., Goodman, C. S. and Dickson, B. J. (1996). Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron* **17**, 203-215.

Nadarajah, B. and Parnavelas, J. G. (2002). Modes of neuronal migration in the developing cerebral cortex. *Nat. Rev. Neurosci.* **3**, 423-432.

Ott, S. R. and Burrows, M. (1999). NADPH diaphorase histochemistry in the thoracic ganglia of locusts, crickets, and cockroaches: species differences and the impact of fixation. *J. Comp. Neurol.* **410**, 387-397.

Polleux, F., Morrow, T. and Ghosh, A. (2000). Semaphorin 3A is a chemoattractant for cortical apical dendrites. *Nature* **404**, 557-573.

Poluha, W., Schonhoff, C. M., Harrington, K. S., Lachyankar, M. B., Crosbie, N. E., Bulseco, D. A. and Ross, A. H. (1997). A novel, nerve growth factor-activated pathway involving nitric oxide, p53, and p21WAF1 regulates neuronal differentiation of PC12 cells. *J. Biol. Chem.* **272**, 24002-24007.

Renteria, R. C. and Constantine-Paton, M. (1995). Exogenous nitric oxide causes collapse of retinal ganglion cell axonal growth cones *in vitro*. *J. Neurobiol.* **29**, 415-428.

Schmidt, H., Werner, M., Heppenstall, P. A., Henning, M., More, M. I., Kuhbandner, S., Lewin, G. R., Hofmann, F., Feil, R. and Rathjen, F. G. (2002). cGMP-mediated signaling via cGKIalpha is required for the guidance and connectivity of sensory axons. *J. Cell Biol.* **159**, 489-498.

Seidel, C. and Bicker, G. (2000). Nitric oxide and cGMP influence axonogenesis of antennal pioneer neurons. *Development* **127**, 4541-4549.

Sepp, K. J. and Auld, V. J. (2003). RhoA and Rac1 GTPases mediate the dynamic rearrangement of actin in peripheral glia. *Development* **130**, 1825-1835.

Serafini, T., Colamarino, S. A., Leonardo, E. D., Wang, H., Beddington, R., Skarnes, W. C. and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* **87**, 1001-1014.

Song, H. and Poo, M. (2001). The cell biology of neuronal navigation. *Nat. Cell Biol.* **3**, E81-E88.

Song, H., Ming, G., He, Z., Lehmann, M., McKerracher, L., Tessier-Lavigne, M. and Poo, M. (1998). Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* **281**, 1515-1518.

Tanaka, J., Markering-van Ittersum, M., Steinbusch, H. W. M. and de Vente, J. (1997). Nitric oxide-mediated cGMP synthesis in oligodendrocytes in the developing rat brain. *Glia* **19**, 286-297.

Tessier-Lavigne, M. and Goodman, C. S. (1996). The molecular biology of axon guidance. *Science* **274**, 1123-1133.

Truman, J. W., De Vente, J. and Ball, E. E. (1996). Nitric oxide-sensitive guanylate cyclase activity is associated with the maturational phase of neuronal development of insects. *Development* **122**, 3949-3958.

Van Wagenen, S. and Rehder, V. (1999). Regulation of neuronal growth cone filopodia by nitric oxide. *J. Neurobiol.* **39**, 168-185.

Vincent, S. R. and Kimura, H. (1992). Histochemical mapping of nitric oxide synthase in the rat brain. *Neuroscience* **46**, 755-784.

Wang, T., Xie, Z. and Lu, B. (1995). Nitric oxide mediates activity-dependent synaptic suppression at developing neuromuscular synapses. *Nature* **347**, 262-266.

Wang, X. and Robinson, P. J. (1997). Cyclic GMP-dependent protein kinase and cellular signaling in the nervous system. *J. Neurochem.* **68**, 443-456.

Wildemann, B. and Bicker, G. (1999). Developmental expression of nitric

oxide/cyclic GMP synthesizing cells in the nervous system of *Drosophila melanogaster*. *J. Neurobiol.* **38**, 1-15.

Wollin, M. S., Wood, K. S. and Ignarro, L. J. (1982). Guanylate cyclase from bovine lung: a kinetic analysis of the regulation of the purified soluble enzyme by protoporphyrin IX, heme and nitrosyl-heme. *J. Biol. Chem*. **257**, 13312-13320.

Weeks, J. C. and Truman, J. W. (1986). Hormonally mediated reprogramming of muscles and motoneurones during the larval-pupal transformation of the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol*. **125**, 1-13.

Wright, J. W., Schwinof, K. M., Snyder, M. A. and Copenhaver, P. F. (1998). A delayed role for nitric oxide-sensitive guanylate cyclases in a migratory population of embryonic neurons. *Dev. Biol.* **204**, 15-33.

Wu, H. H., Williams, C. V. and McLoon, S. C. (1994). Involvement of nitric oxide in the elimination of a transient retinotectal projection in development. *Science* **265**, 1593-1596.

Wu, W., Wong, K., Chen, J., Jiang, Z., Dupuis, S., Wu, J. Y. and Rao, Y. (1999). Directional guidance of neuronal migration in the olfactory system by the protein Slit. *Nature* **400**, 331-336.

Zhu, Y., Li, H., Zhou, L., Wu, J. Y. and Rao, Y. (1999). Cellular and molecular guidance of GABAergic neuronal migration from an extracortical origin to the neocortex. *Neuron* **23**, 473-485.