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Regulation of enteric neuron migration by the gaseous messenger molecules CO and NO

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The enteric nervous system (ENS) of insects is a useful model to study cell motility. Using small-molecule compounds to activate or inactivate biosynthetic enzymes, we demonstrate that the gaseous messenger molecules carbon monoxide (CO) and nitric oxide (NO) regulate neuron migration in the locust ENS. CO is produced by heme oxygenase (HO) enzymes and has the potential to signal via the sGC/cGMP pathway. While migrating on the midgut, the enteric neurons express immunoreactivity for HO. Here, we show that inhibition of HO by metalloporphyrins promotes enteric neuron migration in intact locust embryos. Thus, the blocking of enzyme activity results in a gain of function. The suppression of migratory behavior by activation of HO or application of a CO donor strongly implicates the release of CO as an inhibitory signal for neuron migration in vivo. Conversely, inhibition of nitric oxide synthase or application of the extracellular gaseous molecule scavenger hemoglobin reduces cell migration. The cellular distribution of NO and CO biosynthetic enzymes, together with the results of the chemical manipulations in whole embryo culture suggest CO as a modulator of transcellular NO signals during neuronal migration. Thus, we provide the first evidence that CO regulates embryonic nervous system development in a rather simple invertebrate model.

KEY WORDS: Carbon monoxide, Nitric oxide, Insect nervous system, Stomatogastric, Grasshopper embryo, Cyclic GMP

INTRODUCTION

The formation of the insect enteric nervous system (ENS) provides a useful model with which to study the cell biology of neuronal migration. Neuronal precursors emerge from proliferative zones in the foregut epithelium and perform extensive cell migrations before they assemble into discrete peripheral ganglia and gastric nerve plexus. The genetic and cellular bases of neuronal specification, guidance cues for directed cell migration, and differentiation towards neuronal phenotypes have been particularly well investigated in the two holometabolous insects *Drosophila* and *Manduca* (Hartenstein, 1997; Copenhaver, 2007). In the hemimetabolous grasshopper *Schistocerca*, the enteric midgut neurons of the grasshopper embryo migrate posteriorly from caudal pockets of the ingluvial ganglia towards the foregut-midgut boundary of the embryo (Ganfornina et al., 1996). Subsequently, they undergo a rapid phase of migration and move in four migratory pathways posteriorly on the midgut surface. At the completion of migration, the enteric neurons invade the space between the four migratory pathways and extend terminal branches on the midgut musculature (Ganfornina et al., 1996). Neuronal motility along the migratory pathways of the grasshopper midgut depends crucially on the nitric oxide/cyclic GMP (NO/cGMP) signaling cascade, including nitric oxide synthase (NOS) and the target receptor protein soluble guanylyl cyclase (sGC) (Haase and Bicker, 2003).

NO has been reported to be a key signaling molecule during nervous system development (Peunova et al., 2001; Chen et al., 2004; Bicker, 2005; Krumenacker and Murad, 2006; Godfrey et al., 2007). In particular, the downstream acting cyclic nucleotide cGMP is implicated as intracellular mediator of growth cone behavior both in vertebrate and invertebrate nervous systems (Gibbs and Truman, 1998; Polleux et al., 2000; Seidel and Bicker, 2000; Song and Poo, 2001; Van Wagenen and Rehder, 2001; Schmidt et al., 2002; Demyanenko et al., 2005; Welshhans and Rehder, 2005; Gutierrez-Mecinas et al., 2007; Stern and Bicker, 2008).

In addition to NO, there is also increasing evidence for carbon monoxide (CO) as another gaseous messenger of neural tissues (Boehning and Snyder, 2003). CO is generated by heme oxygenase enzymes (HO) during oxidative degradation of heme to biliverdin-IX and ferrous iron (Tenhunen et al., 1968; Maines, 1997). Similar to NO, CO is able to activate the cGMP-synthesizing enzyme sGC, albeit about 100-fold less effective (Kharitonov et al., 1995; Denninger and Marletta, 1999; Baranano and Snyder, 2001; Koesling et al., 2004). In vertebrate nervous systems, the constitutive isoform HO-2 is the predominant heme oxygenase (Sun et al., 1990; Verma et al., 1993). In the majority of brain regions, HO-2 mRNA appears to be colocalized with that of sGC, whereas NOS and sGC transcripts show hardly any overlap (Verma et al., 1993). Although NO is the major activator of sGC, CO seems to reduce cGMP levels by modulating the effect of NO on sGC (Ingi et al., 1996a). At the mechanistic level, CO has been suggested to mediate long-term adaptation in amphibian olfactory receptor neurons (Zufall and Leinders-Zufall, 1997), to serve as a neurotransmitter in nonadrenergic/noncholinergic (NANC)-dependent smooth muscle relaxation (Boehning and Snyder, 2003), and to increase field potential oscillations in an invertebrate olfactory system (Gelperin et al., 2000). Exogenous CO application or administration of hemin influences human neutrophil migration and platelet aggregation via cGMP (Brüne and Ullrich, 1987; VanUffelen et al., 1996; Andersson et al., 2002; Freitas et al., 2006). In the vertebrate enteric nervous system, NOS and HO-2 are either co-expressed in a subset of myenteric neurons, or localized in separate, but nearby, cells. NO and CO may function here as co-neurotransmitters, with CO modulating the NO signaling pathway (Maines, 1997; Xue et al., 2000; Miller et al., 2001; Colpaert et al., 2002; Boehning and Snyder, 2003).

There are comparatively few studies about the distribution of HO enzymes and the functions of CO as messenger molecule in invertebrate nervous systems. Immunoreactivity to HO-2 has been

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described in the olfactory system of a mollusc (Gelperin et al., 2000) and the stomatogastric system of crayfish (Christie et al., 2003). A HO gene is expressed in the honeybee brain (Watanabe et al., 2007) and the catalytic properties of a recombinant *Drosophila* HO protein have been analyzed (Zhang et al., 2004). Unlike for NO signaling in arthropods, which could be linked to discrete developmental processes such as cell proliferation (Kuzin et al., 1996; Champlin and Truman, 2000; Benton et al., 2007), neurite outgrowth (Seidel and Bicker, 2000), patterning of synaptic connectivity (Ball and Truman, 1998; Gibbs and Truman, 1998; Wright et al., 1998) and axonal regeneration (Stern and Bicker, 2008), no neurodevelopmental functions of CO have so far emerged.

To investigate whether CO is a cellular messenger molecule in insect development, we use immunochemical techniques for the localization of HO in comparison with NOS during the formation of the grasshopper enteric nervous system. With the concept in mind that CO may interact with NO/cGMP signaling (Ingi et al., 1996a; Artinian et al., 2001), we focus on the NO-dependent cell migration of the midgut plexus neurons. Application of enzyme substrates, chemical inhibitors, activators, messenger releasing compounds and scavengers in whole-embryo culture implicate CO as an intracellular messenger molecule that modulates transcellular NO signaling during neuronal migration.

MATERIALS AND METHODS

Locust eggs (*Locusta migratoria*) were collected from our crowded animal culture, reared under standard conditions and kept in moist Petri dishes at 30°C prior to use. Embryos were staged by percentage of development (% E) according to Bentley et al. (Bentley et al., 1979) with additional criteria for later embryos (Ball and Truman, 1998). All chemicals were purchased from Sigma (St Louis, MO) unless stated otherwise.

Immunocytochemistry

All steps of immunocytochemistry were performed at room temperature and with smooth agitation unless stated otherwise. Embryonic guts were dissected and collected as whole-mount preparations in cooled Leibowitz 15 medium (L15, Gibco Life Technologies, Paisley, UK). To reduce the background caused by the yolk inside guts, some whole guts were dissected and transferred to a Petri-dish containing a poly-D-lysine-coated coverslip with L15 culture medium. Subsequently, guts were carefully rolled over the coverslips. During this 'tissue blotting' procedure, cells on the gut surface, including neurons of the plexus, muscle fibers and ganglia of the ENS adhered to the coated surface, while the intact epithelium and yolk could be removed. To ensure sufficient adherence of the nerve cells, preparations were allowed to settle for about 30 minutes at room temperature ahead of fixation.

Preincubation for NO-dependent cGMP immunocytochemistry was carried out as already described (De Vente et al., 1987; Haase and Bicker, 2003) but with adding YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzyl Indazole, 25 μM], a sensitizer of sGC, immediately after preparation (Ott et al., 2004). Omitting the NO-source from the cGMP-preincubation solution or adding sGC inhibitors, revealed no cGMP-IR at all. All specimens were fixed in 4% paraformaldehyde (PFA) dissolved in phosphate-buffered saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4) overnight at 4°C. Preparations were permeabilized in 0.3% saponin in PBS for 1 hour, rinsed in PBS containing 0.5% Triton X-100 (PBS-T) and blocked for at least one hour in 5% normal serum/PBS-T (serum of animal in which secondary antibody was raised). The primary antibody was diluted in blocking solution and applied overnight at 4°C. Used primary antibodies and concentrations were: sheep anti-cGMP (1:10,000-1:20,000) (Tanaka et al., 1997), monoclonal mouse anti-acetylated α-tubulin (1:500-1:1000) and polyclonal rabbit anti-heme oxygenase 2 (1:400-1:1000, Stressgen, Victoria, BC, Canada). After rinsing in PBS-T, guts were exposed for at least 2 hours at room temperature or overnight at 4°C to biotinylated (Vector, Burlingame, CA) or AlexaFluor488-coupled (Molecular Probes, Eugene, OR) secondary antibodies in blocking solution. Biotinylated secondary antibodies were visualized using fluorescent streptavidin-coupled dyes (Sigma, Molecular Probes). After washing in PBS-T and PBS, preparations were cleared in 50% glycerol (Roth, Karlsruhe, Germany)/PBS and mounted in 90% glycerol/PBS with 4% n-propyl-gallate. Control preparations incubated with 5% normal serum instead of primary antibodies and subsequent detection system revealed absolutely no staining. For double labeling, ENSs were stained first for cGMP followed by acetylated α-tubulin immunocytochemistry.

Western blotting

To obtain tissue homogenates of embryonic CNS, whole brains and ventral nerve cords were dissected. For homogenates of the ENS, we used complete gut tissue with the yolk removed. Tissue was dissected in cooled PBS, collected and homogenized in ice-cold 0.3% Saponin-PBS containing 1% protease inhibitor cocktail (HALT, Pierce, Rockford, IL). Samples of 20 (ENS) or 10 (CNS) embryos were collected per 200 μl lysis buffer. Homogenization was carried out using a Kontes Duall tissue grinder with PTFE pestle (Landgraf Laborsysteme, Langenhagen, Germany). Homogenates were centrifuged for 10 minutes at 6000 ***g*** to allow a crude separation of cytosolic soluble proteins from membrane-bound protein fractions and cellular debris. For HO-2 immunoblots, proteins were precipitated with acetone for 16-48 hours at –20°C. Protein pellets were re-dissolved in 2× Laemmli-buffer (100 mM Tris-HCl, pH 6.8, with 4% SDS, 20% glycerol, 0.02% bromophenol blue) after centrifugation and ethanol washing. For universal NO synthase (uNOS) immunoblots, homogenates were used without any precipitation. Prior to SDS-PAGE, samples were denaturated at 95°C for 3 minutes in loading buffer (2× Laemmli buffer with 2% SDS, 10% 1 M DTT). Proteins were separated either on 8% (NOS) or 15% (HO-2) PAGE and transferred to a PVDF-membrane (Roth). Membranes were equilibrated in PBS, blocked for at least 1 hour at room temperature and incubated overnight at 4°C with the antibody dissolved in blocking solution. The following antibodies and blocking solutions were used: polyclonal rabbit anti-uNOS (1:400, Affinity Bioreagents, Golden, CO) in 5% low-fat milk powder (Humana, Herford, Germany) in PBS containing 0.05% TWEEN (PBS-TW); polyclonal rabbit anti-HO-2 (1:400-1:1000), blocked with 1% bovine serum albumin (BSA) in PBS-TW. Membranes were then rinsed with PBS-TW and incubated with biotinylated secondary antibody in the appropriate blocking solution for 2 hours at room temperature. After washing with PBS-TW, bound antibodies were visualized by standard peroxidase staining techniques using the Vectastain ABC Kit (Vector). To estimate total cell mass in immunoblots from different tissues, some blots were stained additionally for α-tubulin. Stained membranes were dried, scanned, and after reactivation of the membrane with methanol, the blot was probed but with anti-acetylated α-tubulin diluted 1:10,000 in PBS-TW containing 5% low-fat milk powder.

For a specificity control of the HO-2 antiserum, purified recombinant rat HO-2 (rHO-2, Stressgen) was applied to a 15% SDS-PAGE in parallel with locust homogenates. Homogenates were divided in two aliquots and separated on the identical gel. After blotting, half of the membrane was incubated with antibody, while a pre-adsorbed antibody was applied to the other half. For pre-adsorption, recombinant rat HO-2 was added to the anti-HO-2 solution (75 μg protein/ml) and pre-incubated overnight at 4°C. Western blot analysis was repeated at least three times for each protein with homogenates from several preparations and developmental stages.

In vivo culture experiments

Embryos were staged between 60 and 65% E. An optimal stage for in vivo chemical manipulation of cell migration on the midgut is 63% E. At this stage, which is indicated by the first appearance of brownish pigmentation at the tips of the antennae, midgut neurons have just started their cell migration (Fig. 1A).

Eggs of one clutch were sterilized in 70% ethanol and dissected in sterile L15 medium. Subsequently embryos were randomly divided into groups that were exposed to the pharmacological compounds or the control media, respectively. Embryos were immobilized in Sylgard embedded Petri dishes and covered with cell culture medium, supplemented with 1% penicillin-streptomycin solution. A small incision in the dorsal epidermis above the foregut allowed access of pharmacological agents to the developing ENS

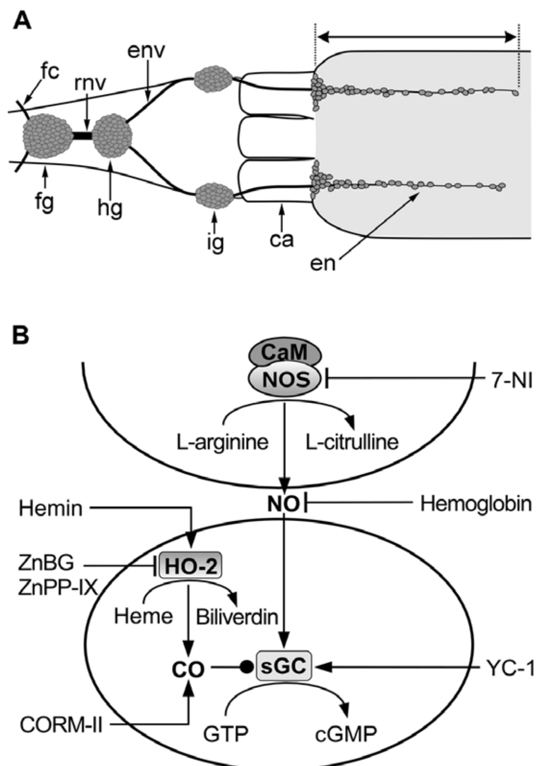

Fig. 1. Cellular organization of embryonic enteric nervous system and proposed signal transduction cascades regulating neuronal motility. (**A**) Schematic drawing of locust embryonic foregut and anterior midgut at 65% E in dorsal view. Midgut is shaded light gray, enteric ganglia and neurons are dark gray. Two of the four migratory pathways are visible on the dorsal midgut. Already developing foregut plexus is omitted for sake of clarity. Double-headed arrow indicates measured distance of enteric neuron migration after 24 hours in vivo culture incubation. ca, cecum; en, enteric neuron; env, esophageal nerve; fc, frontal connective; fg, frontal ganglion; hg, hypocerebral ganglion; ig, ingluvial ganglion; rnv, recurrent nerve. Anterior is towards the left as in following figures. (**B**) Schematic diagram of CO and NO/cGMP signaling transduction influencing enteric neuron migration. Ca^{2+}-calmodulin (CaM)-activated NOS catalyses the conversion of L-arginine into L-citrulline, thereby releasing NO. NOS activity can be stimulated by applying an excess of arginine or blocked by the inhibitor 7-NI. The diffusible NO binds to the heme moiety in soluble guanylyl cyclase (sGC), thus stimulating synthesis of cGMP. Intercellular diffusing NO can be trapped by the extracellularly acting scavenger hemoglobin. The stimulation of sGC with YC-1 artificially amplifies cGMP production. Heme oxygenase enzymes (HO), such as the HO-2-immunoreactive constitutive isoform, release CO as a by-product during heme degradation. The enzyme activity can be manipulated by its substrate analog hemin or metalloporphyrin inhibitors such as ZnBG and ZnPP-IX. CORM-II is an exogenous CO donor. CO competes with NO for binding to sGC (blunt tip), leading only to a rather modest increase in the cGMP level.

during the in vivo culturing period. Following an incubation for 24 hours at 30°C, guts were dissected and prepared for anti-cGMP and anti-acetylated α-tubulin double staining.

To quantify the cell migration on the midgut surface, we measured the distance from the foregut-midgut boundary to the position of the leading enteric neuron (Fig. 1A, indicated by a double arrow) (Wright et al., 1998) using NIH ImageJ (v. 1.35-1.39). The obtained values were normalized with respect to the mean distance of migration of the control group. A Wilcoxon Mann-Whitney test was employed for statistical comparisons of experimental and control groups using KyPlot (version 2.0 beta15). All significance levels are two-sided. Bar graphs display mean values±s.e.m. as percentage of the matched control values of each experiment.

Fig. 1B displays the mode of action of chemical agents we used to manipulate NO and CO signal transduction cascades. L-arginine and hemoglobin were predissolved in L15. Hemin and zinc protoporphyrin-IX (ZnPP-IX, Alexis, San Diego, CA) were predissolved in 0.1 M NaOH resulting in a final concentration less than 0.5% NaOH in the culture medium. 7-nitroindazole (7-NI), YC-1, tricarbonyldichlororuthenium (II) dimer (CORM II) and zinc deuteroporphyrin-IX 2,4 bis glycol (ZnBG, Alexis) were predissolved in DMSO, resulting in less than 0.5% DMSO in the culture medium.

Cell viability and cytotoxicity test

To exclude neurotoxic side effects of hemin and CO-donor applications, a cell viability assay (Live/Dead Viability/Cytotoxicity Kit for animal cells, Molecular Probes) was carried out. This assay allows for a clear simultaneous discrimination between living and dead cells using two different fluorescent probes that indicate distinct parameters of cell viability: intracellular esterase activity and plasma membrane integrity. Immediately after in vivo culturing, guts were dissected and the tissue was blotted on poly-D-lysine-coated coverslips. Subsequently tissue blots were incubated in the assay reagents for 30 minutes at room temperature in the dark, followed by image acquisition. After counting of living and dead enteric neurons, the percentage of living neurons was calculated.

Image acquisition and processing

Preparations were analyzed and photographed using a Zeiss Axioscope equipped with an Axiocam3900 digital camera linked to a Zeiss image acquisition system (Zeiss Axiovision) or a Zeiss Axiovert 200 equipped with a Photometrics Cool Snap digital camera and associated MetaFluor Imaging software. Confocal images of selected preparations were taken with a Leica TCS SP2 confocal microscope using Leica LCS software. Image processing, including arrangement, conversion to grayscale, inversion and contrast enhancement, were carried out using Adobe Photoshop or NIH ImageJ (W. S. Rasband, ImageJ, US National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/).

RESULTS

Localization of NOS and cGMP on the developing midgut

During the phase of directed cell movement along the four midgut migratory pathways (Fig. 1A) (Ganfornina et al., 1996), the enteric neurons express NO-induced cGMP immunoreactivity (Haase and Bicker, 2003). To obtain a high resolution image of the cellular distribution of cGMP, we probed tissue blots of the midgut for acetylated α-tubulin and cGMP. Confocal microscopy showed an evenly distributed cGMP-IR throughout the entire cell bodies of enteric neurons, growth cones and the trailing neurites (Fig. 2A,C). Neither the underlying gut epithelium nor the developing musculature show any cGMP-IR, whereas these tissues are clearly stained with an antibody against α-tubulin, indicative for stable microtubules (Fig. 2B,C). Whereas filopodia and thinner cell processes of the migratory neurons typically are lacking immunoreactivity for acetylated α-tubulin, these cell structures show strong cGMP-IR (Fig. 2B,C). This NO-dependent cGMP synthesis suggests a source of NO in the adjoining tissue, that might stimulate the formation of cGMP in the enteric neurons, including their highly motile filopodia. As cytochemical markers for NOS did not label the motile neurons but indicated staining in the midgut epithelium (Haase and Bicker, 2003), we used an additional biochemical approach. Immunoblots of CNS homogenates from 65% E locust embryos revealed a protein of about 135-140 kDa that was clearly recognized by the universal NOS antibody (Fig. 2D, CNS). This immunostained protein is also present in homogenates

DEVELOPMENT

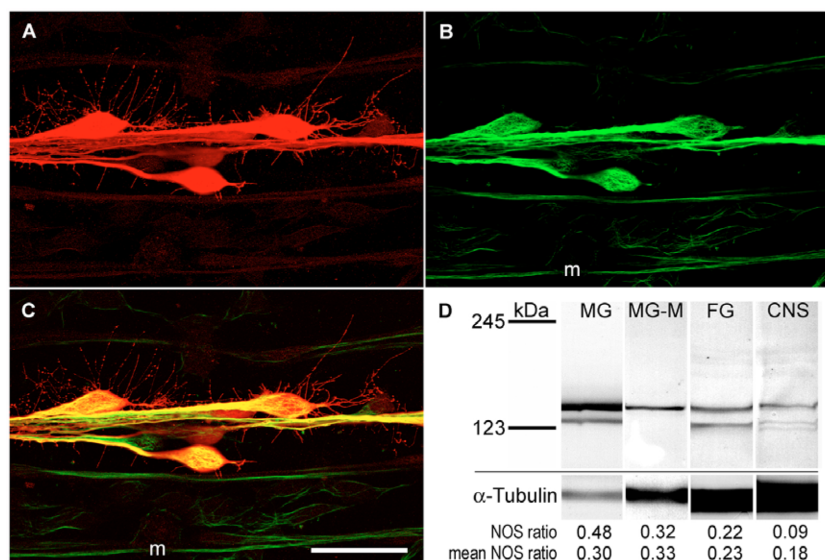

Fig. 2. Immunochemical investigation of cGMP formation and the expression of NOS in the embryonic enteric nervous system. (**A**,**B**) Confocal fluorescence images of migrating enteric neurons on the midgut at 65% E in a so-called 'tissue blot' preparation. Neurons were marked immunocytochemically for anti-cGMP (red) and anti-acetylated α-tubulin (green). (**C**) Merged image of A and B. m, gut musculature. Scale bar: 50 μm. (**D**) Immunoblot analysis with an antibody against universal NOS in homogenates of different parts of embryonic gut and in the CNS (65-70% E). The antibody recognizes a protein of ~135-140 kDa and a second, somewhat lighter protein band in the fore- and midgut cytosolic fraction. Bottom lanes provide an acetylated α-tubulin band as loading control. For quantification, the ratio of the NOS signal to the α-tubulin signal was calculated and averaged for all probed blots (mean NOS ratios, n=4, s.e.m. of the ratios are: ±0.07 MG, ±0.04 MG-M, ±0.01 FG, ±0.08 CNS). The NOS ratio directly below the lanes corresponds to the actual signals of the shown western blot. MG, midgut; MG-M, membranous part of midgut (both midgut homogenates included adjacent hindgut); FG, foregut; CNS, central nervous system, including ventral nerve cord.

of the embryonic foregut (FG) as well as of the midgut (MG, MG-M, midgut homogenates do also include the adjacent hindgut) (Fig. 2D). An additional, somewhat smaller, protein of ~128 kDa was labeled that is missing in the membrane fraction of midgut (MG-M) and is only very faintly visible in the CNS lane by the uNOS antibody. This double band appearance of NOS may be due to post-translational modifications, with the smaller one representing pre-processed or degraded proteins. To normalize for total protein content, some immunoblots were additionally marked for α-tubulin (Fig. 2D, bottom lane). We estimate from the ratio of the loading control to the NOS band, that the midgut contains in average a two times higher NOS concentration than the CNS probe (midgut cytosol 0.30 and midgut membrane 0.33 versus 0.18, n=4).

Transcellular NO signaling regulates enteric neuron migration

As NO/cGMP signal transduction is a positive regulator of enteric neuron migration, pharmacological inhibition of NOS leads to a reduction of cell motility (Haase and Bicker, 2003). We could confirm that bath application of the NOS inhibitor 7-NI results in a significant reduction of enteric neuron migration (Fig. 3A). Moreover, in vivo incubation of 63% E embryos with the extracellular NO scavenger hemoglobin revealed a similarly strong reduction in the migrated distance (Fig. 3A). Most likely, NO released by cells of the gut (Fig. 2D) serves as a transcellular regulator of neuronal cell migration.

To test whether enteric neuron migration could be enhanced by a stimulation of NO/cGMP signal transduction, embryos of 63 to 65% E were allowed to develop in culture with an excess of the NOS substrate L-arginine (2 mM). The substrate activation of NOS did not result in any significant increase of migrated distance (Fig. 3B). NO donors, such as sodium nitroprusside or S-nitroso-N-acetyl-l,l-penicillamine, also failed to enhance enteric neuron migration (data not shown). Stimulation of sGC by application of 65 μM YC-1 (Ko et al., 1994; Evgenov et al., 2006) appeared to slightly increase the migrated distance; however, this result was not statistically significant (Fig. 3B).

Carbon monoxide acts antagonistical to nitric oxide in enteric neuron migration

Heme oxygenase activity can be inhibited with low concentrations of metalloporphyrins such as ZnBG or ZnPP-IX (Maines, 1981; Verma et al., 1993; Ingi et al., 1996a; Ingi et al., 1996b; Appelton et al., 1999; Labbe et al., 1999). Embryos at stages 63-65% E were exposed in culture to 5 μM ZnBG, leading to a highly significant increase of the average migrated distance up to 131% (Fig. 3C). Figs 4A,B illustrate that HO inhibition by ZnBG enhances enteric neuron migration without affecting the precision of pathfinding on the migratory routes. Using ZnPP-IX (10 μM) as another HO inhibitor, we obtained a similar gain-of-function result (Fig. 3C).

By stimulating the production of CO, we performed a complementary experiment and applied 100 μM hemin as a substrate analogue for HO to the cell culture medium. Activation of HO indeed resulted in an opposite effect, as enteric neuron migration was significantly reduced (Fig. 3C, Fig. 4A,C). Moreover, exogenous application of carbon monoxide by using the CO releasing compound CORM-II (Motterlini et al., 2002) reduced enteric neuron migration in a similar way to stimulation of HO enzymes (Fig. 3C, Fig. 4A,D). In an additional control experiment, we tested whether the carbon monoxide exhausted compound might account for the slowing down of enteric neuron migration. Therefore, CORM-II was dissolved in DMSO and kept for 24-48 hours at room temperature and used afterwards. This exhausted CORM-II (iCORM) did not affect enteric neuron

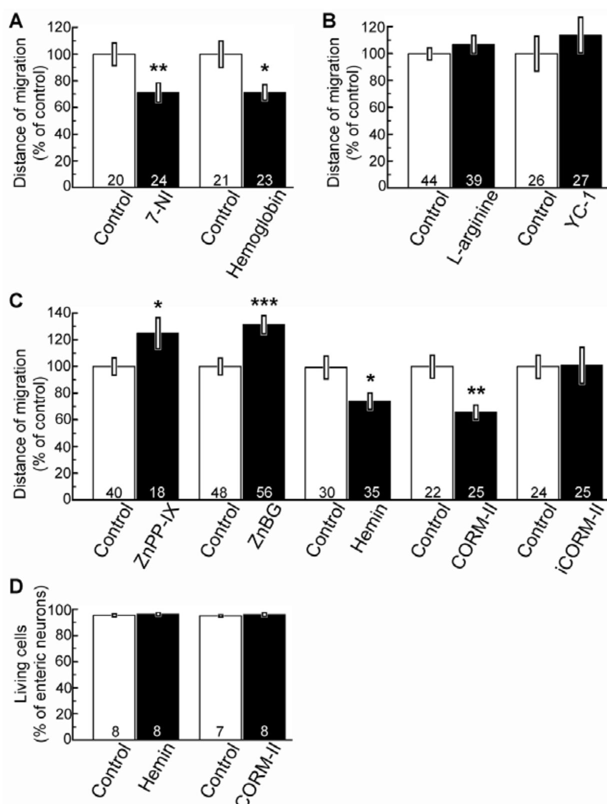

Fig. 3. Quantitative evaluation of enteric neuron migration after chemical manipulation of the NO/cGMP pathway and HO enzymes. Bar plots show average migration distance covered by the leading enteric neurons during 24 hours of in vivo culture. Data result from at least two independent experiments, each normalized to the mean of the corresponding control. (**A**) Inhibition of NOS with 500 μM 7-NI or scavenging NO with 500 μM hemoglobin result in a significant reduction of migration on dorsal migratory pathways. (**B**) Excess of NOS substrate L-arginine (2 mM) or stimulation of sGC with 65 μM YC-1 revealed no difference of migration compared with the control. (**C**) Inhibition of CO releasing HO enzymes with 10 μM ZnPP-IX or 5 μM ZnBG lead to a significant acceleration of enteric neuron migration. Activating HO with 100 μM hemin or applying CORM-II (20 μM) resulted in a significant reduction of average migrated distance. Application of inactivated CORM-II (iCORM-II, 20 μM) did not affect migration. (**D**) Enteric neuron viability after 24 hours of in vivo culture with 100 μM hemin or 200 μM CORM-II. 100% represents total number of counted enteric neurons. Error bars are ±s.e.m. The numbers of experimental gut preparations are indicated in the bars. A Wilcoxon Mann-Whitney test was employed for statistical comparisons. ***P<0.001; **P<0.005; *P<0.05.

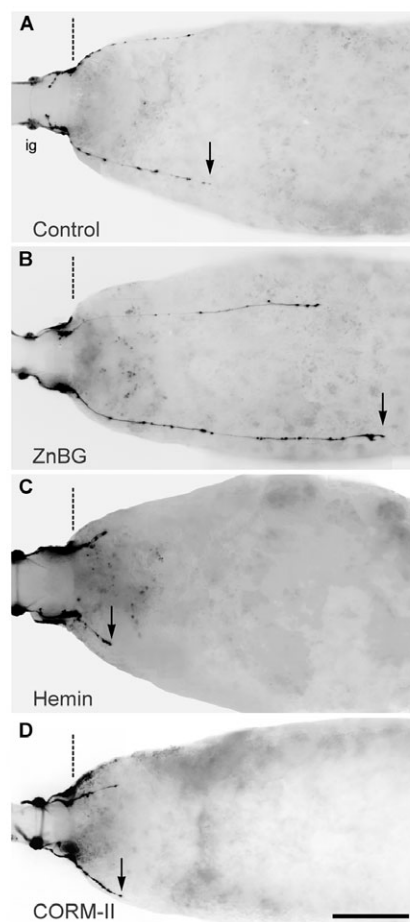

Fig. 4. Enteric neuron migration under influence of neurochemicals affecting heme oxygenase catalyzed CO production. Immunofluorescence images of enteric neuron migration on individual guts after 24 hours in vivo culture. Dissected guts were stained with cGMP antiserum. Composed images are resulting from microphotographs of several focal planes, that were converted to gray scale, inverted for fluorescence intensity and arranged in Adobe Photoshop. (**A**) Control conditions. (**B**) Inhibition of HO enzymes by 5 μM ZnBG. (**C**) Activation of HO with excess of its substrate analogue hemin (100 μM). (**D**) Exogenous carbon monoxide (CORM-II, 20 μM). Broken lines indicate foregut-midgut boundary. Arrows indicate furthest migrated enteric neuron. ig, ingluvial ganglion. Scale bar: 500 μm.

migration (Fig. 3C). Neither hemin nor the CO-donor caused any decrease in cell viability at used (hemin) or even higher (CORM-II) concentrations (Fig. 3D). All of these findings are in line with a negative regulatory role of HO activity and CO signaling on enteric neuron migration.

Localization of HO-positive neurons in the developing ENS

We used homogenates of whole guts, including ENS, gut epithelium and musculature (ENS in Fig. 5), or central nervous systems (CNS in Fig. 5) of 65% embryos for immunoblot analysis of HO. An antibody to HO-2 strongly recognized proteins of ~30-35 kDa (Fig. 5A). These proteins are comparable in size with recombinant rat HO-2 (rHO-2), labeled with the same antibody (Fig. 5B, first two lanes). Remarkably, anti-HO-2 labeled proteins are mainly present in the membrane fraction of homogenates of gut and CNS (Fig. 5A, left lanes). This is in line with reports about an association of vertebrate HO-2 with the endoplasmic reticulum and nuclear outer membrane (Maines, 1988; Ma et al., 2004). The multiple band appearance and the slightly heavier proteins between 35 and 45 kDa that is visible in cytosolic portions may be a result of post-translational modifications. Diverse cleavage of the C terminus of the protein has also been often observed during separation of HOs on SDS gels (Ding et al., 1999).

To test the specificity of the antibody against vertebrate HO-2 on invertebrate tissues, we used pre-adsorbed antibody-solution (75 μg rHO-2/ml). Pre-adsorption leads to a complete loss of labeling of the 30-35 kDa protein bands from locust homogenates (Fig. 5B,

arrowhead). Additional labeled proteins around 60 kDa become visible, when rHO-2 is applied in high quantity (1 μg) to the gel (Fig. 5B, left side/HO-2). As they are not recognized by the pre-adsorbed antibody, these bands are probably due to aggregates of HO-2 (Fig. 5B, right side/HO-2). Such high protein concentrations may also account for some of the larger molecular weight protein bands in homogenates. However, as pre-adsorption abolished both the labeling of 30-35 kDa protein bands derived from recombinant HO-2 and embryonic locust, we can safely infer the presence of HO in the developing CNS and gut tissue.

We also performed immunocytochemical stainings for HO-positive cells on the midgut. At the onset of neuronal migration at 63-65% E, virtually all migrating enteric neurons on the midgut exhibit a clear immunoreactivity to HO (Fig. 6A,B). HO-IR can be detected in soma and main neurites. Although underlying tissues of the gut show mild background staining, it is easily possible to discern the signal intensity between enteric neurons and non-neuronal cells. The gut musculature (m) is only faintly stained in whole mount (Fig. 6A) and also in tissue blot preparations (Fig. 6B). When enteric neurons start to form the midgut plexus by leaving the anterior-posterior migratory pathways around 80% E, HO-IR is still apparent (data not shown). At a later embryonic stage, around 95% E, the staining intensity of neuronal HO-IR approaches the level of the underlying tissues (Fig. 6C). However, the enteric neurons of the nearly established midgut remain clearly identifiable by their α-tubulin-IR (Fig. 6D). In summary, the results of western blotting and immunocytochemistry indicate the presence of HO in embryonic guts and the migrating enteric neurons.

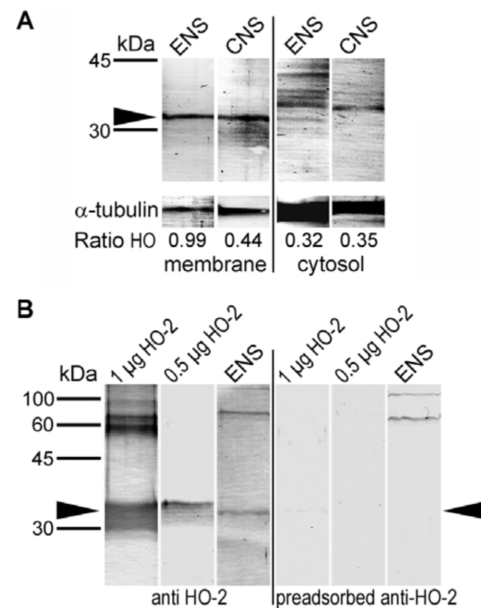

Fig. 5. Western blot analysis of HO in the enteric nervous system. (**A**) Immunoblots obtained from whole guts at 65% E with enteric nervous system attached (ENS) and from dissected CNS. HO-2 antibody recognizes proteins of ~30-35 kDa. In CNS and cytosolic fractions an additional, slightly heavier protein band is enriched in the membrane fraction of homogenates. Both membrane (left) and cytosolic (right) fractions derive from the same preparation. Bottom lanes provide an acetylated α-tubulin band as loading control. For quantification, the ratio of the HO-2 signal to the α-tubulin signal was calculated for each lane (*n*=3, s.e.m. of the ratios are: ±0.29 membrane ENS, ±0.17 membrane CNS, ±0.08 cytosol ENS, ±0.03 cytosol CNS). (**B**) Immunoblotting of recombinant rat HO-2 protein and membrane fraction of ENS at 65% E. The blots were probed either with the antibody against HO-2 (left, anti-HO-2) or corresponding antibody solution pre-adsorbed with HO-2 protein (right, anti-HO-2, pre-adsorbed). Arrowheads in A and B indicate distinct HO bands.

DISCUSSION

In this study, we show that inhibition of HO with metalloporphyrins promotes enteric neuron migration in an intact locust embryo. This gain of function in conjunction with the suppression of migratory behavior by chemical activation of HO or application of exogenous CO strongly implicates the release of the messenger CO as inhibitory signal for neuron migration (Fig. 3C, Fig. 4). Further support for this concept comes from the immunoblotting of HO in dissected gut preparations (Fig. 5A) and the direct immunocytochemical localization of HO in the enteric neurons (Fig. 6A-C). All these data indicate an autoregulatory role of CO signaling during enteric neuron migration. The application of small molecule compounds to activate or inactivate proteins in real time is a powerful approach for the study of complex signal transduction systems (Yeh and Crews, 2003). Although a genetic approach would be very useful to reveal additional downstream molecular pathways of NO and CO signaling during insect embryonic development, the application of chemical agents at precise time intervals is more suited to detect subtle modulatory influences of the gaseous messengers.

NOS expression provides for a transcellular NO signal during formation of enteric midgut plexus

The uNOS antibody stains NADPH-diaphorase-positive interneurons in the adult locust antennal lobe (Bicker, 2001) that express Ca^{2+}/calmodulin sensitive NOS (Müller and Bicker, 1994; Elphick et al., 1995). In the absence of molecular sequence data for the NOS of locusts, we used a universal NOS antibody that recognizes a highly conserved sequence of the three mammalian NOS isoforms and also detects arthropod NOS (Bicker, 2001; Bullerjahn and Pflüger, 2003; Christie et al., 2003; Settembrini et al., 2007). Western blotting of homogenates from embryonic fore- and midguts revealed the presence of NOS-like 135-140 kDa proteins at the onset of enteric neuron migration (Fig. 2D). The approximate size of NOS has been independently determined as 135 kDa in the locust brain (Elphick et al., 1995), between 116 and 180 kDa in the locust abdominal nervous system (Bullerjahn and Pflüger, 2003) and between 130 and 150 kDa in other insects (Gibson and Nighorn, 2000; Watanabe et al., 2007; Settembrini et al., 2007). The genome of *Drosophila* contains only a single NOS gene (Regulsky and Tully, 1995; Enikopolov et al., 1999) and immunoblotting showed a band of the corresponding protein at about 150 kDa (Regulski et al., 2004). Our results indicate highly levels of NOS proteins in the midgut, compared with the CNS (Fig. 2D). Most likely, this is caused by high levels of NOS expression in a substantial fraction of the epithelial gut cells. Using quantitative PCR, NOS expression has also been found in the midgut epithelium of *Anopheles* (Akman-Anderson et al., 2007). NADPHd-diaphorase histochemistry detected staining in a subset of the locust embryonic midgut cells (Haase and Bicker, 2003) and hemocytes (data not shown) as potential endogenous NO-producing cells. However, as the diaphorase reaction depends on fixation conditions (Ott and Burrows, 1999), it may quite often not completely resolve the expression pattern of NOS. As application of hemoglobin as an extracellular scavenger of gaseous molecules suppressed enteric neuron migration, a transcellularly diffusing NO signal derived from the gut cells may stimulate cGMP-mediated enteric neuron migration. This concept is in line with the

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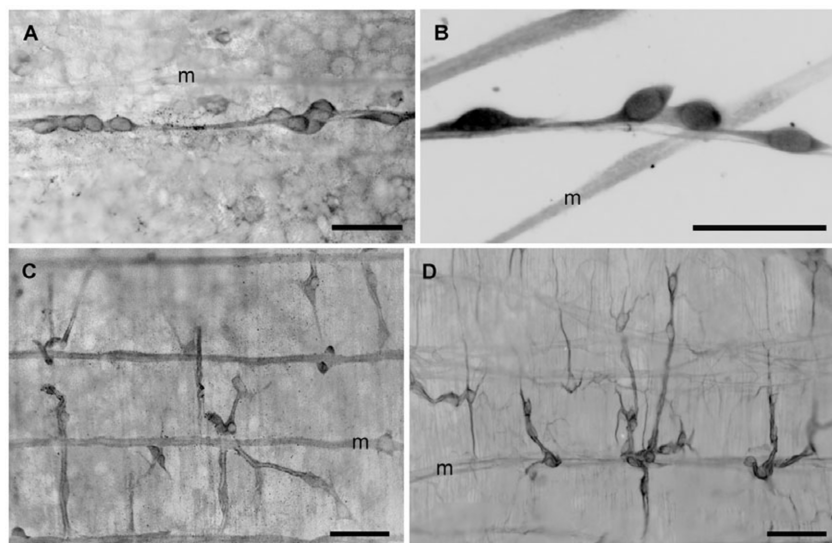

Fig. 6. Immunocytochemical detection of HO in the enteric nervous system. (**A**,**B**) Immunocytochemical labeling of the enteric neurons migrating on the midgut at 65% E, using the antiserum in a whole-mount (A) and tissue blot (B) preparation. 1% BSA was added to the blocking solution in B. (**C**) HO-2 immunocytochemistry at 95% E on a whole-mount midgut. Enteric neurons have left the four migratory pathways and established the midgut plexus. At this stage, other tissues such as gut musculature show similar labeling intensity. (**D**) ENS at the same developmental stage as in C but stained for acetylated α-tubulin. A, C and D are composed images from microphotographs of several focal planes. m, gut musculature. Scale bars: 50 μm.

immunocytochemical localization of cGMP in the cell bodies and filopodia after exogenous application of NO and the sGC sensitizer YC-1 (Fig. 1B, Fig. 2A,C) (Haase and Bicker, 2003).

NOS proteins are present in an even higher amount in the particulate fraction than in the cytosolic fraction of embryonic midgut homogenates (Fig. 2D,MG-M), suggesting that they may be partly membrane associated. In vertebrates a significant amount of NOS isoforms is present in the particulate cell fraction (Hiki et al., 1992; Watanabe et al., 1998; Oess et al., 2006). Therefore, the double band appearance of NOS proteins that we observed in homogenates of locust embryos (Fig. 2D) might be due to post-translational modifications similar to vertebrate NOS modifications accompanying protein membrane association.

CO is an inhibitor of enteric neuron migration

In a gain-of-function experiment we could show that inhibition of HO with low concentrations of ZnBG and ZnPP-IX resulted in a significant acceleration of migration on the midgut (Fig. 3C, Fig. 4A,B). Conversely, we could significantly delay enteric neuron migration by stimulating CO production with the HO substrate analogue hemin or with exogenous CO application (Fig. 3C, Fig. 4A,C,D). This confirms CO as a likely effector molecule of HO activation on the regulation of enteric neuron migration.

Unlike to the locust, application of 10 μM ZnPP-IX to the developing *Manduca* ENS caused no significant enhancement of enteric neuron motility (Wright et al., 1998). However, despite the common developmental origin from neuroepithelial parts of the foregut, enteric nervous systems of insects exhibit extensive variations in the detailed pattern of cell migration and underlying molecular guidance cues (Hartenstein, 1997; Ganfornina et al., 1996; Copenhaver, 2007). For example, whereas in *Manduca* specific sets of visceral muscle bands support migration of the enteric neurons on the midgut (Copenhaver and Taghert, 1989; Copenhaver et al., 1996; Wright et al., 1999) no morphologically distinct muscle bands can be recognized along the migratory pathways in the grasshopper embryo (Ganfornina et al., 1996). Instead, the migratory neurons move parallel to the longitudinal muscle bands directly on the surface of the midgut.

Specificity of chemical manipulations

At high concentrations, some metalloporphyrins have been reported not only to block HOs but also to inhibit sGC or NOS activity (Luo and Vincent, 1994; Grundemar and Ny, 1997; Serfass and Burstyn, 1998). An example is the potent inhibitor of HOs ZnPP-IX (Maines, 1981), which was shown to cause such unspecific side effects later on. However, the low concentrations of ZnBG (5 μM) or ZnPP-IX (10 μM) that we applied have been demonstrated to be highly effective for the inhibition of HO enzyme activity without influencing NOS or sGC (Ingi et al., 1996a; Appleton et al., 1999). Moreover, the direction of observed motility changes, rules out an additional inhibitory effect on sGC or NOS. As shown by Fig. 3A, a direct inhibition of NOS with 7-NI or sGC with ODQ (Haase and Bicker, 2003) revealed a significant retardation in enteric neuron migration. Thus, an additional inhibition of NOS/sGC enzymes by ZnBG or ZnPP-IX would have slowed down neuronal migration. As the application of metalloporphyrins caused a significant acceleration of migration (Fig. 3C), our results provide no evidence for unspecific side effects.

Application of the extracellular scavenger hemoglobin delayed migration (Fig. 3A). As hemoglobin binds both NO and CO, the net effect of a reduced motility can therefore be caused by a reduced concentration of both compounds in the extracellular space. Surprisingly, the reduced motility after incubation with hemoglobin matches the reduction after inhibition of NOS, whereas a lack of CO leads to an opposite effect of accelerating neuron migration (Fig. 3A,C). This result would suggest that the net effect of hemoglobin could be mainly due to scavenging extracellular NO. Moreover, the presence of the HO enzyme in enteric neurons and the lack of NOS (Fig. 2D, Fig. 6A-C) argue for an intracellular CO and transcellular NO signal transduction mechanism. However, in the absence of real concentration measurements of intra- and extracellular CO/NO levels before and after hemoglobin application, it is difficult to draw any firm conclusions. It remains a distinct possibility that CO produced in an enteric neuron may not only act intracellularly but that some of the CO escaping in the extracellular space may also downregulate the motility of neighboring cells.

Regulation of cell motility by gaseous messengers

The most straightforward functional explanation for all our reported data is a scenario in which the gut cells provide a transcellular NO signal (Fig. 2D, Fig. 3A) for stimulating sGC in the enteric midgut neurons (Fig. 2A-C). NO-induced cGMP synthesis is in turn a permissive, but essential prerequisite for enteric neuron migration (Haase and Bicker, 2003). An additional CO pathway within and between the HO-positive midgut neurons provides for an auto /paracrine signal that downregulates neuronal motility along the chain

of migrating enteric neurons (Fig. 3C, Fig. 4). We propose that both gaseous messenger molecules interact via sGC to organize the timing of the posterior directed cell migration along the midgut. As NO and CO can both bind to sGC, but CO with less efficiency to stimulate cGMP formation (Kharitonov et al., 1995; Denninger and Marletta, 1999; Baranano and Snyder, 2001; Koesling et al., 2004), a simple competition mechanism between the two messengers may regulate cGMP levels in the migratory neurons (Fig. 1B). In the presence of NO and CO, part of sGC enzymes would bind CO resulting in a suboptimal cGMP production. By decreasing CO concentrations, an increasing number of sGC enzymes would be available for efficient NO activation. A decrease in CO concentration by blocking HO (Fig. 1B) would thus be reflected by the increased cellular motility (Fig. 3C). It is also conceivable that CO binding causes conformational changes of sGC affecting its efficiency (Hernandez-Viadel et al., 2004) or perhaps induces allosteric effects arising from interactions among sGCs or their regulatory proteins (Ingi et al., 1996a) that cause a downregulation of the NO stimulated cGMP production. Currently, we cannot exclude an sGC/cGMP-independent pathway for the effect of HO/CO signaling on enteric neuron migration, as CO can have other cellular effects apart from modulating cGMP levels (Boehning and Snyder, 2003; Kim et al., 2006).

Even though the filopodial tips of migrating enteric neurons can upregulate cGMP (Fig. 2A,C), neither the chemical manipulations of NO/cGMP- nor CO-dependent signaling pathways causes misrouting of enteric neurons. Thus, a permissive effect of gaseous messengers on motility appears to be independent from growth cone steering. A quantitative evaluation of pathfinding errors (data not shown) provided no evidence for enhanced misrouting of migrating enteric neurons after the application of metalloporphyrins, hemin or CORM-II. This result is illustrated in examples of migratory pathways under the chemical manipulation of HO (Fig. 4).

To fully appreciate the role of HO/CO signaling in cell migration, it remains insufficient to define the cellular sources and targets of CO. Technology has yet to be developed to resolve the temporal pattern of CO production. Although neuronal production of NO is a tightly Ca^{2+}/calmodulin regulated process in locusts and vertebrates (Müller and Bicker, 1994; Elphick et al., 1995; Boehning and Snyder, 2003), regulation of HO activity is thought to depend mainly on the availability of heme. However, in response to neuronal stimulation a modest regulation of HO-2 has been demonstrated by casein kinase 2 (CK2) (Boehning et al., 2003) and in vitro studies have uncovered an additional mode of activation by binding of Ca^{2+}/calmodulin (Boehning et al., 2004). Thus, there is increasing evidence for an activity-dependent release of CO, one of the necessary criteria for a role as neural messenger molecule.

Using a rather simple invertebrate model, we have uncovered an antagonistic role of CO versus NO as gaseous messenger molecules regulating nerve cell migration. As many signaling mechanisms of neuronal and cellular guidance are strikingly conserved among vertebrate and invertebrate animals, it will be interesting to examine whether CO signaling plays also a vital role during vertebrate brain development.

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