Development 122, 3949-3958 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV8336

Nitric oxide-sensitive guanylate cyclase activity is associated with the maturational phase of neuronal development in insects

James W. Truman[1,*], Jan De Vente[2] and Eldon E. Ball[1]

[1]Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra, ACT, 2601, Australia
[2]Department of Psychiatry and Neuropsychology, Section of Neuropsychology, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands

*Author for correspondence at permanent address: Department of Zoology, University of Washington, Box 351800, Seattle, WA 98195, USA (e-mail: trumanj@zoology.washington.edu)

SUMMARY

Many developing insect neurones pass through a phase when they respond to nitric oxide (NO) by producing cyclic GMP. Studies on identified grasshopper motoneurones show that this NO sensitivity appears after the growth cone has arrived at its target but before it has started to send out branches. NO sensitivity typically ends as synaptogenesis is nearing completion. Data from interneurones and sensory neurones are also consistent with the hypothesis that NO sensitivity appears as a developing neurone changes from axonal outgrowth to maturation and synaptogenesis. Cyclic GMP likely constitutes part of a retrograde signalling pathway between a neurone and its synaptic partner. NO sensitivity also appears in some mature neurones at times when they may be undergoing synaptic rearrangement. Comparative studies on other insects indicate that the association between an NO-sensitive guanylate cyclase and synaptogenesis is an ancient one, as evidenced by its presence in both ancient and more recently evolved insect groups.

Key words: guanylate cyclase, cyclic GMP, nitric oxide, synaptogenesis, *Locusta migratoria*

INTRODUCTION

During its ontogeny a neurone passes through two discrete developmental phases, the first involving axonal outgrowth and navigation, and the second encompassing maturation and synaptogenesis. The transition between these phases occurs within a short time after an axonal growth cone reaches its target. Besides obvious changes in synthetic programs, this transition likely also involves a changeover in signalling systems used by the developing neurone. Systems appropriate for navigation involve contact or short distance attraction and repulsion of growth cones (Goodman and Shatz, 1993; Baier and Bonhoeffer, 1994), whereas once a target has been reached, bidirectional signalling is often required to establish the appropriate number and strength of synaptic contacts (Jessell and Kandel, 1993).

An effective dialog between pre- and postsynaptic cells requires retrograde as well as anterograde signalling. A retrograde pathway of increasingly recognized significance involves nitric oxide (NO) and cyclic 3′,5′ guanosine monophosphate (cGMP). In both the cerebellum and hippocampus, NO and cGMP are part of a retrograde pathway involved in long-term changes in synaptic function (Garthwaite, 1991; Zhuo et al., 1994; Hawkins et al., 1994). On theoretical grounds, NO signalling has been proposed as a possible mediator of the role of neural activity in refining synaptic connections during development (Gally et al., 1990). While experimental support for such an involvement has been slow to emerge, NO synthase (NOS), as indicated by diaphorase staining, shows pronounced developmental regulation in various brain regions (e.g., Williams et al., 1994; Samama et al., 1995). The use of NOS inhibitors suggests that NO is involved in activity-dependent synaptic suppression at the developing neuromuscular junction in *Xenopus* (Wang et al., 1995) and the pruning of ipsilateral projections in the developing visual system of the chick (Wu et al., 1994).

Besides the developmental modulation of the distribution of NOS in the nervous system, one of the key targets of NO, the soluble guanylate cyclase (sGC), also shows developmental modulation. The activity of this enzyme can be investigated in situ by treating nervous systems or brain slices with NO donor compounds and then assessing by immunocytochemistry the levels of cGMP produced (De Vente et al., 1987). In the rat, comparisons of regions of the brain from immature versus mature animals reveal dramatic differences in the capacity of cells to respond to NO depending on their developmental stage (De Vente et al., 1990; De Vente and Steinbusch, 1992). A similar developmental modulation of NO sensitivity is also evident in invertebrates. In larval lobsters, for example, identified neurones show transient NO sensitivity during the time that their neural circuits are being organized to accomodate post-metamorphic behaviors (Scholz et al., 1995). Similarly, our studies on embryonic development of the nervous system of the locust, *Locusta migratoria*, show that transient NO sensitivity is a feature of a wide variety of developing neurones. In these cells NO sensitivity becomes manifest as the neurone

switches from axon extension to maturation and synaptogenesis. A preliminary report of these findings was presented in Truman and Ball (1994).

MATERIALS AND METHODS

Experimental animals

A laboratory colony of *Locusta migratoria* was maintained on a diet of wheat germ and sprouted wheat seedlings. Breeding females were provided cups of moist sand into which they deposited their egg pods. The oviposition cups were removed weekly and kept at 30°C. Intact individual egg pods were removed from the sand and maintained in Petri dishes between layers of moist cheesecloth. Individual eggs were dissected at intervals to determine the developmental stage of each clutch. Staging of embryos was based on the system of Bentley et al. (1979), with additional criteria added for late embryos (Ball and Truman, unpublished data).

Silverfish (*Ctenolepisma longicaudata*) were kept in rolls of cardboard and fed on dried powdered high protein cereal. Eggs were laid in balls of cotton placed within the cardboard and were collected at regular intervals.

Fruit flies (*Drosophila melanogaster*) were raised using standard techniques.

Fixation and immunocytochemistry

Staged embryos were dissected in phosphate-buffered saline (PBS). They were stripped out of the chorion and opened mid-dorsally. Following removal of the gut, the body wall was pinned out flat. For observations of 'spontaneous' expression of cGMP the animals were then flooded with fixative (4% formaldehyde (Sigma) in PBS).

Soluble guanylate cyclase was activated in situ by exposing filleted embryos to sodium nitroprusside (SNP, Sigma) or hydroxylamine (Sigma). These compounds were made up in PBS containing 0.1 mM isomethylbutylxanthine (IBMX). Embryos were routinely exposed to these reagents for 15 minutes prior to fixation. Fixation was for about 2 hours at room temperature or overnight at 4°C.

Cyclic GMP levels were determined in situ using the immunocytochemical methods developed by De Vente et al. (1987). After repeated washes, fixed tissues were preblocked in 5% normal goat serum (NGS) in PBS with 0.3% Triton X-100 (PBS-TX), and then incubated with a 1:4000 dilution of a rabbit anti-cGMP antiserum in PBS-TX with 1% NGS. After 12-36 hours at 4°C the tissues were repeatedly washed and then incubated overnight with a peroxidase-conjugated goat anti-rabbit IgG (Kierkegaard and Perry Labs) in PBS-TX with 1% NGS. After additional rinses, the location of the antibody complexes was revealed by reaction with diaminobenzidine (DAB, Sigma) and glucose oxidase (Watson and Burrows 1981) or H_2O_2. The developing solution contained 0.03% nickel chloride to yield a black reaction product. Tissues were then dehydrated, cleared in methyl salicylate and mounted in Permount (Fisher Labs).

For tissues that were double labeled for cGMP and with the muscle-specific monoclonal antibody Mes3 (Kotrla and Goodman, 1984), the preparations were first immunostained for cGMP using a peroxidase-conjugated secondary plus $NiCl_2$ to stain the expressing neurones black, washed, placed in the Mes3 monoclonal overnight, washed, incubated with a peroxidase-conjugated goat anti-mouse IgG and reacted with DAB without nickel to stain the muscles brown.

Diaphorase staining

We used the technique described by Hope and Vincent (1989) for the diaphorase reaction. Tissues were fixed for 1 hour in 4% formaldehyde in PBS and then incubated in a solution containing 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 0.2 mM nitroblue tetrazolium in PBS containing 0.2% Triton X-100. Incubations were typically for 1-2 hours at RT in the dark. Tissues were subsequently mounted in 100% glycerol after being taken through a glycerol series.

Image processing

Figures were assembled either from scanned 35 mm slides or from video images captured using a Sony DXC-960MD video camera mounted on a Nikon Optiphot microscope. Two-dimensional projections of neurones within a ganglion were constructed from stacks of optical sections that were imported into Adobe Photoshop 3.0. The stack was assembled using the layering palettes with out-of-focus elements being deleted.

RESULTS

Expression of cGMP-immunoreactivity in the embryonic CNS of locusts in the absence of pharmacological treatment

Approximately 200 grasshopper embryos ranging from 16% to 100% of embryonic development (%E) were dissected in PBS, fixed and stained for cGMP-immunoreactivity (cGMP-IR). No cGMP staining was found in embryos younger than 55% of embryonic development (%E). Of the older embryos, about 70% had neurones that showed detectable cGMP-IR.

Many of the neurones that expressed cGMP-IR could be repeatably identified in a number of embryos. One prominent set of neurones was the segmentally repeated neurones that contain crustacean cardioactive peptide (CCAP, Dircksen et al. 1991). These cells transiently produce cGMP-IR at every ecdysis in a wide variety of insects including locusts (Ewer et al., 1994; Truman et al., 1996). Some late stage (>75%E) embryos initiated weak ecdysis/hatching movements after they were removed from the chorion, and these were the animals whose CCAP cells were cGMP-immunoreactive (Truman et

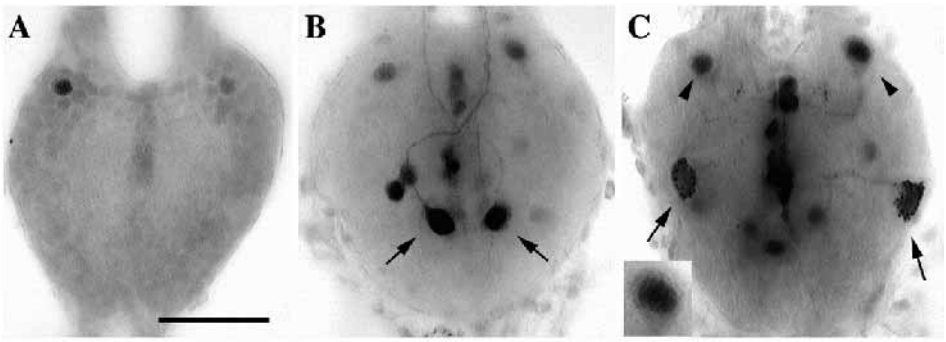

Fig. 1. Abdominal ganglia of locust embryos showing neurons expressing 'spontaneous' cGMP immunoreactivity. (A) Ganglion A6 of a 63%E embryo showing moderate cGMP-IR in the cell body of the left AVL neurone. (B) Ganglion A6 of an 85%E embryo showing strong cGMP expression in the cell body and processes of the PVM neurones (arrows). (C) Ganglion A6 from an embryo at 87%E that had started ecdysis movements before fixation. cGMP-IR in the ecdysis-related CCAP cells (arrows) was in granular inclusions in the cytoplasm; the development-related cGMP of other neurones was primarily nuclear (arrowheads; also inset). In B and C, the midline expression of cGMP is in glial cells. Scale bar is 100 μm.

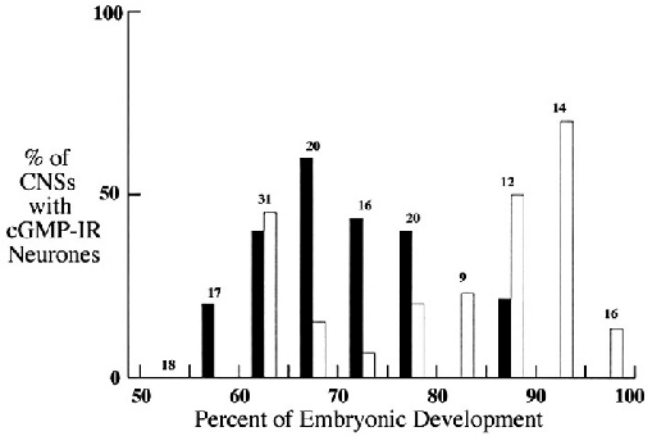

Fig. 2. Relationship between the stage of embryonic development with the occurrence of cGMP-IR in the AVL neurones (black bars) and the PVM neurones (white bars). Numbers are the number of embryos examined within each 5% block of development.

al., 1996). We term this 'ecdysis-related' cGMP expression. The cGMP expression in other neurones was not associated with overt behaviors and we refer to it as 'development-related' cGMP expression. Typically, any given embryo showed only a few neurones that showed cGMP-IR. The neurones that were most frequently observed depended on the stage of embryonic development (Fig. 1). For example, an interneurone in the anteroventrolateral (AVL) region of the ganglion was frequently observed to be cGMP immunoreactive in embryos ranging from 55 through 80%E (Figs 1A, 2). By contrast, posteroventromedial (PVM) neurones were more frequently seen in embryos ranging from 80 through 95%E (Figs 1B, 2).

The ecdysis-related cGMP expression in the CCAP neurones typically included the axon as well as the cell body. Within the cell body, the cGMP-IR was confined to the cytoplasm (Fig. 1C). These features are consistent with the pattern of cGMP production seen in these cells during the ecdysis of postembryonic stages (Truman et al., 1996). For the neurones that showed the development-related expression, by contrast, cGMP staining was most frequently seen in the cell body. Only rarely were cGMP levels high enough to reveal stained processes. Moreover, the cGMP staining in the cell body was more intense in the nucleus, as compared to the cytoplasm (Fig. 1C).

Response of neurones to agents that generate nitric oxide

When late-stage embryos (ca. 75-95%E) were removed from the chorion and placed in saline, the ecdysis-related cGMP response in the CCAP neurones appeared and strengthened through time if the animals attempted ecdysis, but the development-related response typically disappeared within 10 minutes. We exposed dissected embryos to a number of transmitters and drugs to try to restore cGMP production in the latter cells. NO donor compounds, such as hydroxylamine or sodium nitroprusside (SNP), were effective in inducing cGMP-IR in these embryonic neurones. Inclusion of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX; Beavo et al., 1970) further enhanced the intensity of the cGMP response. Among the neurones responding to NO donor compounds were all of the identified neurones that showed development-related cGMP expression. In contrast, neurones involved in the ecdysis-related response were unresponsive to SNP or hydroxylamine treatment. This lack of response in the latter cells is consistent with results from other insects showing that their ecdysial cGMP production is not mediated via NO (Ewer et al., 1994). The cGMP immunostaining was blocked by preincubation of the antiserum with 10^{-5} M cGMP but only slightly reduced by preincubation with 10^{-3} M cAMP (data not shown). Hence the antiserum appears to recognize bone fide cGMP (see also De Vente et al., 1987).

A few neurones were sensitive to levels of SNP as low as 10^{-7} M, but maximal responses required 10^{-3} to 10^{-2} M SNP with IBMX. Fig. 3 shows the time course of response to 10^{-2} M SNP with 0.1 mM IBMX. When embryos were flooded with fixative 1 minute after treatment with SNP, a weak response was detected in the AVL neurones. Cyclic GMP was detectable in many other neurones by 5 minutes, with a maximal response by 15 minutes. For the studies below, we routinely used 15 minutes exposure to 10^{-2} M SNP with 0.1 mM IBMX.

The onset of NO sensitivity in embryonic neurones

Neurogenesis begins in abdominal ganglia at about 25-30%E and is essentially complete by about 60%E (Shepherd and Bate, 1990). As seen in Fig. 4, the sensitivity of neurones to NO donors was confined to the later stages of embryonic development. A few neurones became responsive to SNP treatment as early as 45-50%E but the majority began to show responsiveness between 60 and 80%E. For most neurones, however, the sensitivity to SNP treatment was only transient. By 2 days after hatching, only a few neurones (25-30 per ganglion) were still able to produce cGMP when challenged with SNP plus IBMX. These neurones maintained their competence through at least the first few instars.

To determine the relationship between NO sensitivity and the progression through neuronal development, we followed a variety of identified neurones through embryogenesis. Locust embryos are especially favorable for such studies since the

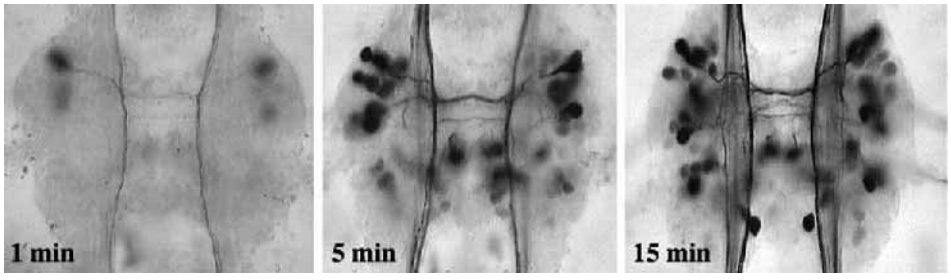

Fig. 3. Videomontages of abdominal ganglion A4 from embryos at 60%E showing the time course of cGMP induction after treatment with 10^{-2}M SNP and 0.1 mM IBMX. The embryos were fixed at the times indicated and processed for cGMP immunoreactivity.

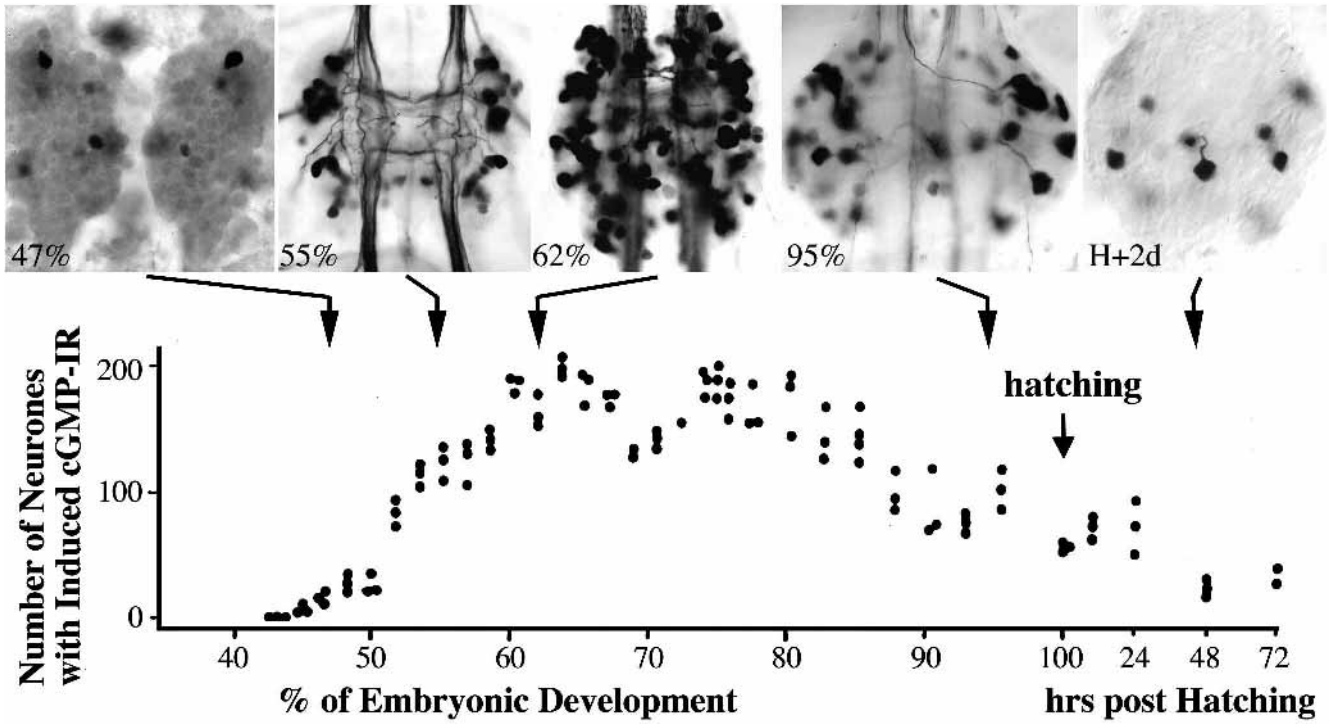

Fig. 4. Changes in the ability of neurones in ganglion A4 to produce cGMP in response to treatment with SNP and IBMX during embryogenesis. Top: dorsal views of immunostained ganglia showing the arrays of neurones that respond when treated at various times during embryonic development or after hatching. Bottom: counts through embryonic development of the number of cGMP immunoreactive cell bodies in ganglion A4 after SNP treatment. H+2d, 2 days after hatching. Each dot represents counts from a single embryo.

developmental stage of a given abdominal segment significantly lags its anterior neighbor (e.g., Ho et al., 1983). Hence, by moving caudally down the abdomen one moves to neurones that are progressively earlier in their developmental program.

Motoneurones

Our most detailed observations are for motoneurones, which could be identified by the path of their peripheral axon as well as by cell body position. Motoneurons were among the first neurones to become NO responsive. Consequently, during the initial stages of their response, they could be seen with little interference from other reacting cells. Also, since many are segmentally reiterated, one could move down the length of an embryo to see the response of the cell at successively earlier stages in its development. Fig. 5 shows two abdominal segments, the more anterior of which has a motoneurone that is NO responsive. This motoneurone is RP2 (Thomas et al., 1984) and it was consistently the first motoneurone in a given abdominal segment to become responsive to SNP treatment. At the time RP2 became responsive, its axon was already at its target muscle (Fig. 5). Initially, the cGMP response in the neurone was relatively weak but uniform throughout the cytoplasm from the cell body to the growth cone. As the neurones aged, the cGMP response became progressively stronger but, again, uniform throughout the cell's cytoplasm. Within a given cell, there was no consistent gradient of response to bath applied SNP. The only heterogeneity that we observed was in the cell body where the cGMP-IR in the nucleus was consistently stronger than that in the cytoplasm.

Within a given segment, the motoneurones showed a stereotyped order in which they became NO responsive (Fig. 6). For example, in segment A4, RP2 was followed 1-2%E later by the acquisition of competence in the aCC motoneurone and the motor axons that innervate muscles 187, 188 and 191. By 57-59%E, the motoneurones to muscle 205 and the spiracular muscles had also become responsive to SNP treatment. The

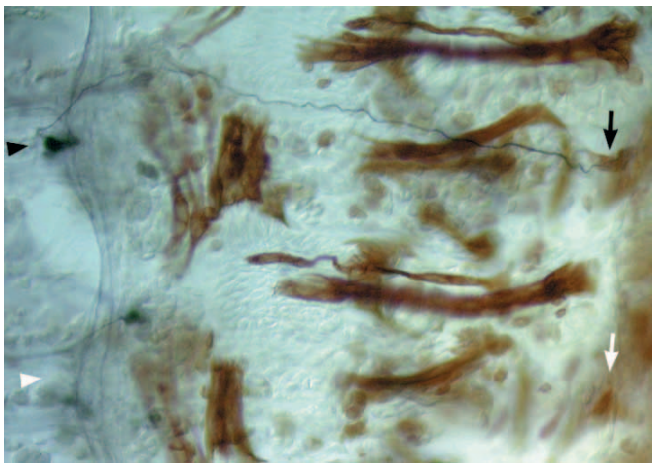

Fig. 5. Lateral body wall of segments A4 and A5 from an approximately 50%E embryo that had been treated with SNP and IBMX to activate guanylate cyclase. The embryo was double-stained with the Mes3 mAb to show the location of embryonic muscles (brown), and with anti-cGMP antiserum to show NO-responsive neurones (black). RP2 in segment A4 was developmentally old enough to respond to the SNP but RP2 in A5 was not. The location of the cell body (arrowhead) and muscle target (arrow) for RP2 in A4 (black symbols) and A5 (white symbols) are indicated.

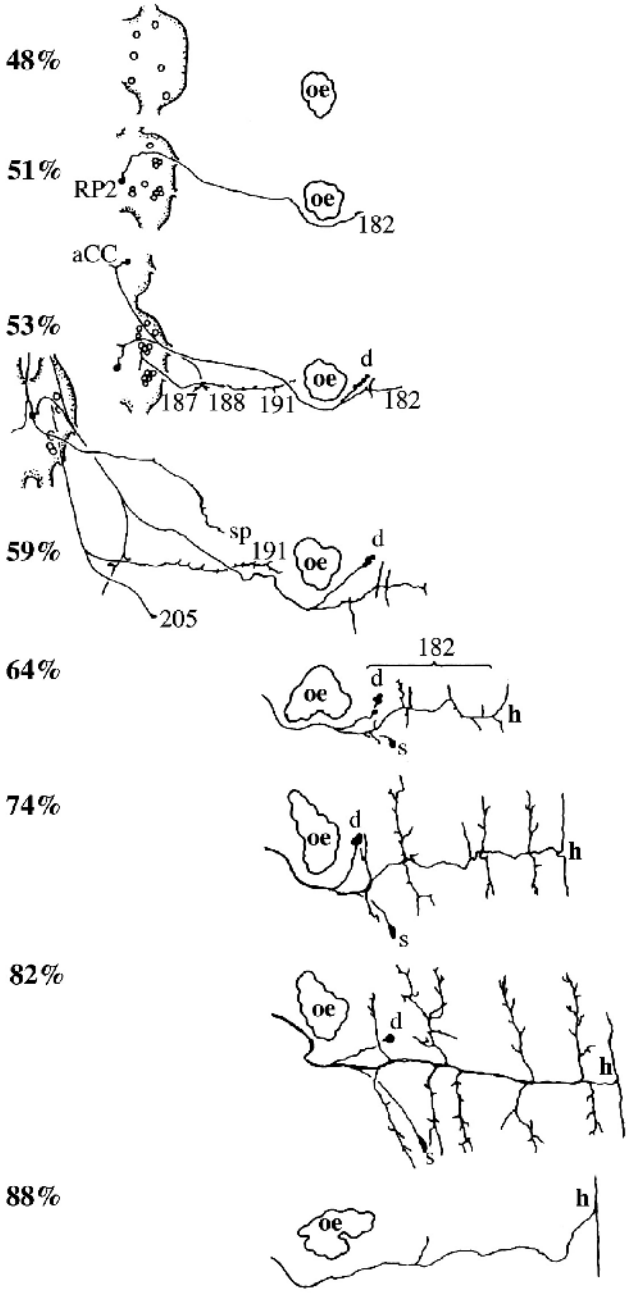

Fig. 6. Camera lucida drawings of the 4th abdominal hemisegment showing the array of motor axons that show cGMP-IR after treatment with SNP and IBMX from 48 to 88% of embryogenesis. 48%: no A4 motoneurones show increases in cGMP although a small number of interneurones have acquired responsiveness (open circles); oe, a cluster of oenocytes in the dorsolateral portion of the segment is a useful marker for successive drawings. 51%: RP2 is the first motoneurone to become responsive to SNP treatment; its growth cone is at its target muscle (group 182) when it first responds. 53%: responding neurones now include motoneurone aCC, whose muscle target is distal to that of RP2, and the motoneurones innervating the ventral longitudinal muscles (187,188) and muscle 191; sensory neurone cell bodies of the dBw (d) chordotonal organ also are responsive. 59%: Responsiveness is now shown by a motoneurone innervating the spiracular muscle (sp) and the axon to muscle 205. 64%-88% shows only the portion of the intersegmental nerve dorsal to the oenocytes. Drawings are the combined response from the axons and terminal arbors of RP2, aCC and a cardioregulatory axon that extends along the heart (h). 64%: axon arbors spreading over the dorsal longitudinal muscles; dorsal stretch receptor neurone (s) becomes responsive. 74%, 82%: motor axons continuing to branch over their targets. 88%: all motor axons have lost responsiveness except for the cardioregulatory axon. Muscle designations from Xie et al. (1992) and Hustert (1974).

last motoneurone to acquire NO sensitivity was a cardioregulatory neurone that innervates the heart (by 64%E). In all of these cases, the motor axon was at its target muscle when a first response to SNP treatment was observed. During the following hours of development, the terminals of these motor axons branched and spread over their muscles (Fig. 6). To the best of our knowledge, every muscle group in the abdomen received innervation from an axon that was NO responsive. Importantly, however, most of these muscles also received innervation from other motor axons that did not exhibit NO sensitivity (Ball and Truman, unpublished).

Interneurones

The density of responding neurones and processes within the CNS made it difficult to assess the location of the growth cones of many identifiable interneurones at the time they became NO responsive. A favorable cell in this regard, though, is the 'H' cell, a large interneurone whose development has been described in detail (Goodman et al., 1981). It is born at about 25-30%E and extends a contralateral axon that pioneers part of the longitudinal connectives. The cell then initiates a second round of axonogenesis and elaborates its characteristic 'H'-shaped axon (Fig. 7A) as the original contralateral axon is removed. By 50-55%E, axon production is finished and the neurone begins maturation as characterized by soma enlargement, changes in membrane currents and elaboration of dendritic arbors. We found that the H cell acquired NO sensitivity at 53-55%E (Fig. 7B) coincident with this transition.

Interneurones constituted the vast majority of central neurones that became responsive to SNP treatment. Peak numbers of responsive cells were evident by about 60%E (Fig. 4). We do not have detailed developmental information on other identified interneurons but the timing of their acquisition of NO sensitivity is appropriate for when we would expect them to be entering their maturational phase.

Sensory neurones

A wide variety of sensory neurones also responded to SNP treatment during embryogenesis. Mechanoreceptor neurones that became NO sensitive included those of the dorsal body wall (dBw) chordotonal organs (Figs 6, 7D), the segmental and wing-hinge stretch receptors (Fig. 7E), the abdominal 'ear', campaniform sensilla and mechanosensory bristles (Fig. 8). The photoreceptor neurones of the compound eye (Fig. 7F) also became responsive to SNP. The manner by which we exposed embryos to SNP did not provide ready access of the chemical to the antennal lumen so we do not know if chemoreceptor neurones also became responsive to SNP treatment during their development.

Our most detailed observations of cGMP production by sensory neurones are on the mechanoreceptors of the body

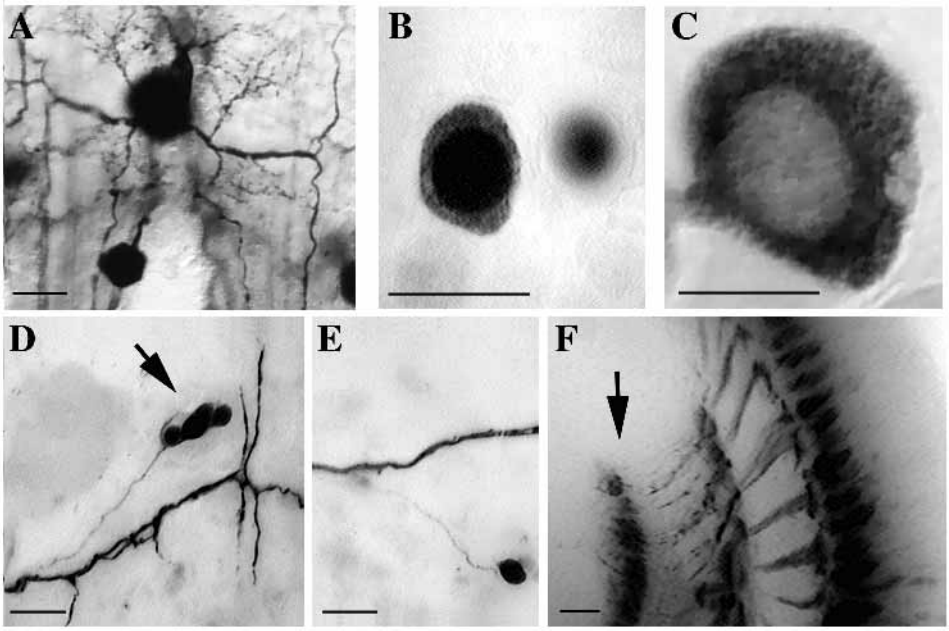

Fig. 7. Immunocytochemical detection of cGMP in the nervous system of embryonic locusts after embryos were treated with 10 mM SNP plus 0.1 mM IBMX. (A) Dorsal midline of ganglion T2 showing the cell body and 'H'-shaped arbor of the H cell. (B,C) cGMP distribution in the soma of the H cell at 55%E (B) when it first becomes NO responsive and (C) in a 2 day old larva after the cell has matured. (D) Cluster of cell bodies of the abdominal dBW sensory neurones (arrow) at about 65%E. (E) The wing hinge stretch receptor at 60%E. (F) NO responsive photoreceptors and their axons in the developing eye, arrow shows their synaptic terminations in the lamina. Scale bars are 20 μm.

wall. Proprioceptors, like the dBw chordotonal neurones (Fig. 7D) and the wing-hinge stretch receptors (Fig. 7E) were the first mechanoreceptors to become responsive to SNP treatment. In both cases NO sensitivity was evident by 50-55%E. For the wing-hinge stretch receptor this time coincides with expansion of its terminal axonal arbor within the CNS (Heathcote, 1981). The dBw neurones begin axonal outgrowth shortly after 37%E and have reached the CNS prior to 50%E (Meier et al., 1991). Their period of SNP responsiveness likely coincides with the elaboration of their central arbors but the details of this elaboration are not known.

In contrast to proprioceptors, the afferents that supply external sensory organs, such as bristle and dome sensilla, are born relatively late in development (55-60%) as the cuticle for the first stage hopper is being formed. These neurones finally become responsive to SNP treatment at about 80%E (Fig. 8). The associated glial cell also showed a weak cGMP-IR after SNP treatment. The timing of synaptogenesis for the bristle afferents of the body wall has not been determined, but it is likely similar to their counterparts on the terminal cerci. The latter begin elaborating their terminal arbors at about 80%E (Shankland, 1981), the same time that the bristle afferents of the body wall became NO sensitive.

Termination of sensitivity to SNP

Most of the motoneurones, interneurones and sensory neurones showed only a transient sensitivity to SNP. RP2, for example, was responsive from about 51%E to 85%E. During this time, its axonal arbor was spreading over its target muscle. Centrally, RP2 was also elaborating its dendritic arbor during this period. By 70-75%E the response of the cell body had waned although that in the axonal arbor was still strong. The terminal arbor, though, ceased being responsive to SNP treatment at about the time that obvious endplates were being elaborated (Fig. 6).

A few neurones continued to show NO responsiveness as mature cells. These included the heart regulatory neurones, the PVM cells and the H cell. In the mature H cell (from late embryogenesis onward) SNP treatment resulted in a cGMP distribution that was primarily cytoplasmic (Fig. 7C). This distribution is in marked contrast to that seen in the same cell at the start of its maturational phase; in the latter case, the cGMP produced after SNP treatment was seen concentrated in the nucleus (Fig. 7B). A cytoplasmic distribution of cGMP was characteristic of the response of all mature neurones that we observed in postembryonic stages. The nuclear concentration of cGMP-IR, by contrast, was a characteristic feature of immature neurones as they acquired their sensitivity to SNP treatment.

The bristle sensory neurones were unusual because they showed recurring periods of NO sensitivity. They ceased responding to SNP by about 95%E, but their responsiveness returned after hatching and its accompanying ecdysis. By 24 to 48 hours later, the neurones were again refractory to SNP treatment, a condition that persisted through the molt to the second instar (Fig. 8). At that ecdysis, though, responsiveness to SNP treatment reappeared. New sensory hairs are added at each molt but the NO sensitivity that we observed included the old sensory neurones as well as the new ones. This pattern of recurring NO sensitivity was not observed in proprioceptor neurones such as the stretch receptors or the chordotonal neurones.

Phylogenetic distribution of the NO/cGMP response

In order to assess the generality of the ability of neurones to respond to NO with cGMP production, we chose the silverfish, *Ctenolepisma longicaudata*, as a representative of the primitive apterygote orders and *Drosophila* as a representative of the more recent holometabolous orders. Embryos of silverfish showed responses to SNP treatment that were similar to those described above for *Locusta*. In contrast, cGMP responses were relatively poorly developed in embryos of *Drosophila*. A cGMP response to SNP treatment, though, was very prominent when the larval nervous system went through its second developmental period at metamorphosis (J. Truman, S. Gibbs, D. Currie and E. Ball, unpublished data).

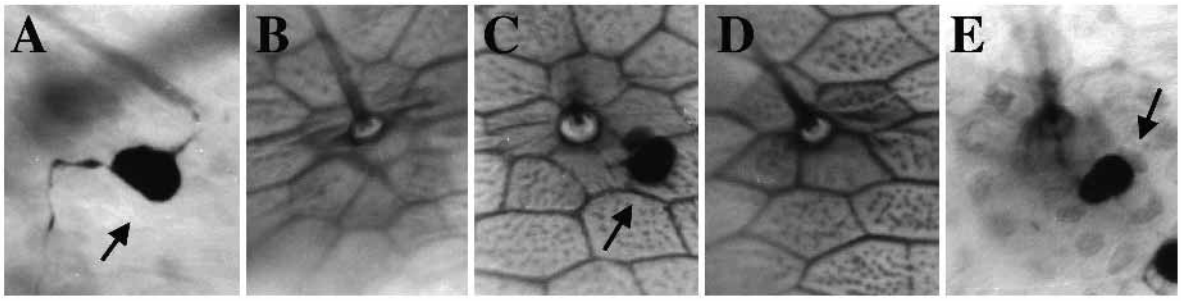

Fig. 8. Changes through embryogenesis and larval life in the ability of SNP and IBMX to induce intracellular cGMP increases in bristle sensory neurones. (A) At 80%E the neurone shows a strong induction of cGMP immunoreactivity that includes the axon (left) and the dendrite, which inserts into the forming bristle shaft (b). (B) By 95%E the bristle is fully formed and covered with cuticle as is the epidermal pavement; the sensory neurone no longer responds to SNP. (C) 10 hours after hatching and ecdysis of the embryonic cuticle, responsiveness has returned. (D) By 2 days after hatching, the neurone is refractory. (E) Responsiveness reappears after ecdysis to the 2nd larval stage; bristle socket is out of focus. Arrow: reacting cell body of sensory neurone.

Development of diaphorase activity

We stained whole mounts of embryonic locusts for diaphorase activity to determine the potential distribution of nitric oxide synthase (NOS) activity. Studies by Elphick et al. (1995) show that diaphorase staining matches the distribution of NOS enzymatic activity in the CNS. The ventral CNS showed no diaphorase staining until about 45%E. Initially, each abdominal ganglion showed 2 pairs of diaphorase-positive cell bodies along with more extensive staining in the neuropil (Fig. 9). As embryogenesis progressed, 6-10 cells eventually became diaphorase positive accompanied by intense staining in the neuropil. We did not observe a dramatic modulation in diaphorase staining, as we observed for NO responsiveness. Double staining of the nervous systems for cGMP (after SNP stimulation) followed by diaphorase staining showed that most, but not all, of the cell bodies that were diaphorase positive were not responsive to SNP. We did not detect reliable diaphorase staining in the periphery at any time during development.

DISCUSSION

NO sensitivity as a characteristic of maturing neurones

The NO/cGMP pathway has roles in regulating both physiological (Garthwaite, 1991) and developmental processes. Evidence for its role in neuronal development comes from the chick visual system in which pharmacological blockade of NOS activity interferes with elimination of ipsilateral axonal projections (Wu et al., 1994). Likewise, in *Xenopus*, inhibition of NOS blocks activity-dependent synaptic suppression at the developing neuromuscular junction (Wang et al., 1995). Besides these experimental data, a developmental role for NO signaling is suggested by the transient appearance of diaphorase staining, indicative of NOS activity, in selected regions of the developing brain and spinal cord (e.g., Bredt and Synder, 1992; Williams et al., 1994; Samama et al., 1995).

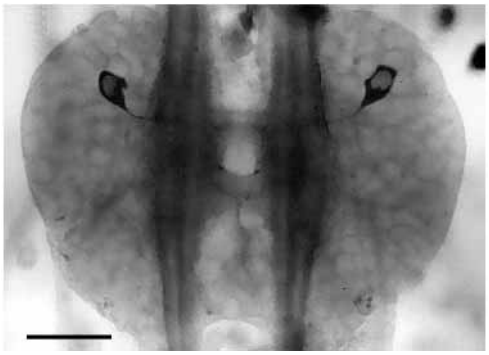

Fig. 9. Staining of ganglion A4 at 60%E for NADPH-diaphorase activity. Staining is observed in fibers in the neuropil and in a pair of cell bodies. Scale bar is 50 μm.

Less is known about the developmental modulation of the guanylate cyclase part of the pathway. Studies on rat cerebellar slices indicate that N-methyl-D-asparate (NMDA) is effective in inducing cGMP production in immature tissue but much less so in brain slices from adults (Southam et al., 1991). Moreover, basal soluble guanylate cyclase activity is prominent in the immature rat brain but then declines after 10 days postpartum (De Vente et al., 1990; De Vente and Steinbusch, 1992). In this study, we find that NO-sensitive guanylate cyclase activity is likewise prominent in insect embryos and then wanes as development finishes. The embryonic expression of this activity was confined primarily to the nervous system. The only non-neural cells that responded to SNP treatment were certain glial cells associated with peripheral sensory neurones. Although not all neurones became NO responsive, it was a characteristic feature of the development of a wide spectrum of neurones, including motoneurones, sensory neurones and interneurones.

Locust neurones varied widely in the time during embryonic development when they became NO-responsive. Motoneurones were among the first cells to respond whereas mechanoreceptor neurones were among the last, reflecting differences in the time when the various classes of neurones were born. We found no instances of motoneurones or sensory neurones that became NO responsive while their axons were still navigating through the periphery. Their axons were either at their target muscle or well within the CNS, respectively, when these neurones first responded. Therefore, their acquisition of NO sensitivity occurs at a consistent time in development that marks the neurone's transition from the phase of axonal outgrowth to that of maturation. For interneurones, their

complex patterns of axonal growth makes it difficult to define anatomically when this transition occurs. However, in the H cell, which is the one interneurone that we could examine whose transition time is known (Goodman et al., 1981), the time of appearance of NO sensitivity coincided with the shift to maturation. Thus, for all neurones that show it, the appearance of NO sensitivity appears to mark the cell's transition into its maturational phase of development.

We do not know what causes the abrupt appearance of NO responsiveness in these neurones, although the most obvious possibility is contact with the target. The pattern by which this sensitivity appears, though, is interesting. In the case of RP2, for example, when one first sees a weak response to bath applied SNP, the cGMP immunoreactivity appears uniformly from the cell body to the axon terminal. This pattern suggests that the guanylate cyclase is already uniformly distributed throughout the neurone when the cell first becomes responsive. Why, then, cannot the cyclase be stimulated by NO at an earlier time? An intriguing feature of sGC is that it is inhibited by elevated levels of intracellular Ca^{2+} (Knowles et al., 1989). Ca^{2+} transients and Ca^{2+} spikes are characteristic of extending axons and growth cones in many types of developing neurones (Bentley et al., 1991; Gu et al., 1994; Gu and Spitzer, 1995) and a reduction in Ca^{2+} currents often accompanies the transition from axonal outgrowth phase into the maturational phase (Spitzer, 1994). This reduction in intracellular Ca^{2+} might serve to 'unmask' the guanylate cyclase, thereby allowing it to respond to extracellular signals.

The major question presented by these observations is: what role does cGMP play during the development of these neurones? From the limited data on developing vertebrate systems (Wang et al., 1995; Wu et al., 1994) as well as its role in synaptic plasticity in mature systems (Garthwaite, 1991; Hawkins et al., 1994), the obvious hypothesis is that cGMP is part of a retrograde signaling pathway between the developing neurone and its target. In invertebrates (Davis and Murphey, 1994), as with vertebrates, retrograde signals from the postsynaptic cell appear to play an important role in determining the final distribution of synaptic contacts. The timing of appearance of NO sensitivity in locust neurones is obviously consistent with cGMP being involved in such a retrograde pathway. Since we cannot selectively stain the neurone prior its becoming NO sensitive, we do not know how long the axon may wait on its target before it becomes NO-responsive. We do know, though, that this responsiveness is in place prior to the spread of the axon over its target. Thus, the capacity for reception of retrograde signals is in place before the neurone starts to explore its target.

Further correlative support for the involvement of the sGC in retrograde signalling comes from the recurring periods of NO sensitivity seen in the bristle afferents (Fig. 8). NO sensitivity reappears transiently in these sensory neurones following each ecdysis. Physiological studies on these neurones are lacking in the locust but, in crickets, the bristle afferents on the cercus shift their central synapses as new sensilla are added and as the physical properties of the sensory bristles change due to growth (Chiba et al., 1988; Murphey and Chiba, 1990). Retrograde signals from sensory interneurones are apparently involved in this synaptic remodeling but the nature and exact timing of the signaling is unknown. The period following ecdysis is the first time that new sensory hairs can respond to external stimuli. The reappearance of NO sensitivity in the bristle afferents at this time makes NO and cGMP excellent candidates for this retrograde pathway.

Besides a potential role at the axon terminals, cGMP is likely involved in other aspects of neuronal development. Early in its maturation, a neurone's cGMP levels increase throughout the cell after bath application of SNP, but levels become especially high in the nucleus (Fig. 7). This nuclear concentration of cGMP suggests that immature neurones have a nuclear protein(s) that binds cGMP and, hence, keeps it in the nucleus. Of the known targets for cGMP (Waldman and Murad, 1987), the only reasonable option would be the cGMP-dependent protein kinase. Whether this putative nuclear binding protein is the translocated kinase or a novel cGMP-binding protein is not yet known. Nevertheless, it is intriguing that an intercellular signaling molecule, like NO, can rapidly evoke changes in nuclear concentrations of cGMP at the time that the neurone is undergoing a major change in transcriptional activity as it begins maturation. Cyclic GMP is not known to regulate transcription but such an action would obviously have parallels with the control of transcription by the cAMP-CREB pathway (Montminy et al., 1990; Delmas et al., 1994). In the latter pathway, though, cAMP stays in the cytoplasm and only the activated kinase goes to the nucleus. By contrast, in this instance cGMP itself appears to travel into the nucleus.

The nuclear distribution of cGMP appears confined to cells using cGMP in a developmental context. For example, in neurones, like the H cell, that continue to be NO responsive after they mature, the mature cell shows cGMP retained in the cytoplasm rather than moving into the nucleus. This change in subcellular distribution of the second messenger may reflect a shift in the role of cGMP from a development role to one involved in physiological adjustments in the cell. A similar, non-nuclear distribution of cGMP is seen in the CCAP cells during their ecdysis-related increases in cGMP (Fig. 1C; Truman et al., 1996). Studies of ecdysis-related cGMP increases in the CCAP neurones of the moth *Manduca sexta* show that the elevated cyclic nucleotide levels control membrane excitability (Gammie et al., 1994), a response consistent with a cytoplasmic or membrane localization at that stage.

At this time, we do not know why only a subset of neurones become NO sensitive during their development. One possibility is that neurones differ in the importance that retrograde signals have in their development and the cells that we see are the ones for which it is most important. Alternatively, there may be a number of retrograde pathways, only one of which might involve guanylate cyclase. Such a multiplicity of feedback systems might be important for reducing cross-talk in a rapidly developing nervous system.

The signal for sGC stimulation

In the case of the 'spontaneous' production of cGMP, the patterns that we observed may represent the picture of ongoing expression within the CNS or they may have been induced during the course of dissection or fixation. The fact that the neurones that we most commonly observed were the ones that were most responsive to SNP treatment suggests that the development-related increases were due to stimulation of sCG activity in those neurones. The relatively low level of the response, though, suggests that the normal signals in vivo do

not involve levels or durations of cGMP production close to those that we induced experimentally by bath application of SNP and IBMX.

We do not know the normal signaling molecule that stimulates the sGC in *Locusta* neurones but NO is a good candidate. NOS activity is present in the locust CNS (Elphick et al., 1993; Muller and Bicker, 1994) and, as with mammals (Bredt et al., 1991; Huang et al., 1993), the histochemical stain for NADPH diaphorase identifies areas rich in NOS activity in the locust (Muller and Bicker, 1994; Elphick et al., 1995). Moreover, a gene for NOS has been isolated in *Drosophila melanogaster* (Regulski and Tully, 1995). In the embryonic CNS, the onset of diaphorase staining coincides with the first appearance of NO responsiveness at about 45%E. Our failure to find diaphorase staining in the periphery, though, leaves an open question as to the molecule involved in signaling between motoneurone and muscle. In mammals, diaphorase staining after formaldehyde fixation does not reveal all forms of NOS, and this may be the situation in insect muscle. Alternatively, other signaling molecules, such as carbon monoxide or metabolites of arachidonic acid (Verma et al., 1993; Hawkins et al., 1994) may play a prominent role in the periphery.

The data presented here show that, in grasshoppers, the switch of many developing neurones from axonal pathfinding to synaptogenesis and maturation is associated with a biochemical change in the neurone as manifest by its ability to respond to NO. This feature is not only seen in grasshoppers but is also evident in very primitive insects such as the silverfish *Ctenolepisma longicaudata* during its embryonic development and in advanced insects such as *Drosophila melanogaster* during their metamorphic transition. Hence, the sensitivity of developing neurones to NO appears to be a widespread and ancient feature of neuronal maturation.

We thank I. Morgan, P. Miethke and J. Wellard for chemicals and advice, C. S. Goodman for the Mes-3 mAb, and M. Ball and N. Scholz for comments on the manuscript. The work was supported in part by a grant from the National Institutes of Health (NS-13079).

REFERENCES

Baier, H. and Bonhoeffer, F. (1994) Attractive axon guidance molecules. *Science* **265**, 1541-1542.

Beavo, J. A., Rogers, N. L., Crofford, O. B., Hardman, J. G., Sutherland, E. W. and Newman, E. V. (1970). Effects of xanthine derivatives on lipolysis and on adenosine 3′,5′-monophosphate phosphodiesterase activity. *Mol. Pharmacol.* **6**, 597-603.

Bentley, D., Gutherie, P. B. and Kater, S. B. (1991). Calcium ion distribution in nascent pioneer axons and coupled preaxonogenesis neurons in situ. *J. Neurosci.* **11**, 1300-1308.

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

Bredt, D. S., Glatt, C. E., Hwang, P. M., Fotuhi, M., Dawson, T. M. and Snyder, S. H. (1991). Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron* **7**, 615-624.

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

Chiba, A., Shepherd, D. and Murphey, R. K. (1988). Synaptic rearrangement during postembryonic development in crickets. *Science* **240**, 901-905.

Davis, G. and Murphey, R. K. (1994). Retrograde signaling and the development of transmitter release properties in the invertebrate nervous system. *J. Neurobiol.* **25**, 740-756.

De Vente, J. and Steinbusch, H. W. M. (1992). On the stimulation of soluble and particulate guanylate cyclase in the rat brain and the involvement of nitric oxide as studied by cGMP immunocytochemistry. *Acta Histochemica* **92**, 13-38.

De Vente, J., Bol, J. G. J. M., Berkelmans, H. S., Schipper, J. and Steinbusch, H. W. M. (1990). Immunocytochemistry of cGMP in the cerebellum of the immature, adult and aged rat: the involvement of nitric oxide. A micropharmacological study. *Eur. J. Neurosci.* **2**, 845-862.

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.

Delmas. V., Molina, C. A., Lalli, E., de Groot, R., Foulkes, N. S., Masquilier, D. and Sassone-Corsi, P. (1994). Complexity and versatility of the transcriptional response to cAMP. *Rev. Physiol.Biochem. Pharmacol.* **124**, 1-28.

Dircksen, H., Muller, A. and Keller, R. (1991). Crustacean cardioactive peptide in the nervous system of the locust, *Locusta migratoria*: an immunocytochemical study on the ventral nerve cord and peripheral innervation. *Cell Tissue Res.* **263**, 439-457.

Elphick, M. R., Green, I. C. and O'Shea, M. (1993). Nitric oxide synthesis and action in an invertebrate brain. *Brain Research* **619**, 344-346.

Elphick, M. R., Rayne, R. C., Riveros-Moreno, V., Moncada, S. and O'Shea,M. (1995) Nitric oxide synthesis in locust olfactory interneurones. *J. Exp. Biol.* **198**, 821-829.

Ewer, J., de Vente, J. and Truman, J. W. (1994). Neuropeptide induction of cyclic GMP increases in the insect CNS: resolution at the level of single identifiable neurons. *J. Neurosci.* **14**, 7704-7712.

Gally, J. A., Montague, P. R., Reeke, G. N. and Edelman, G. M. (1990) The NO hypothesis: possible effects of a short-lived, rapidly diffusible signal in the development and function of the nervous system. *Proc. Natl. Acad. Sci., USA* **87**, 3547-3551.

Gammie, S. C., Ewer, J. and Truman, J. W. (1994). An endogenous increase in cyclic GMP is associated with increased excitability in identified neurosecretory cells in *Manduca sexta*. *Soc. Neurosci. Abstr.* **20**, 1604.

Garthwaite, J. (1991). Glutamate, nitric oxide, and cell signaling in the nervous system. *Trends Neurosci.* **14**, 60-67.

Goodman, C. S. and Shatz, C. J. (1993) Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell/Neuron* **10** (Suppl.), 77-98.

Goodman, C. S., Bate, M. and Spitzer, N. C. (1981). Embryonic development of identified neurons: origin and transformation of the H cell. *J. Neurosci.* **1**, 94-102.

Gu, X. and Spitzer, N. C. (1995). Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. *Nature*, **375**, 783-787.

Gu, X., Olson, E. C. and Spitzer, N. C. (1994). Spontaneous neuronal calcium spikes and waves during early differentiation. *J. Neurosci.* **14**, 6325-6335.

Hawkins, R. D., Zhuo, M. and Arancio, O. (1994). Nitric oxide and carbon monoxide as possible retrograde messengers in hippocampal long-term potentiation. *J. Neurobiol.* **25**, 652-665.

Heathcote, R. D. (1981). Differentiation of an identified sensory neuron (SR) and associated structures (CTO) in grasshopper embryos. *J. Comp. Neurol.* **202**, 1-18.

Ho, R. K., Ball, E. E. and Goodman, C. S. (1983). Muscle pioneers:large mesodermal cells that erect a scaffold for developing muscles and motoneurones in grasshopper embryos. *Nature* **301**, 66-69.

Hope, B. T. and Vincent, S. R. J. (1989). Histochemical characterization of neuronal NADPH-diaphorase. *Histochem. Cytochem.* **37**, 653-661.

Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H. and Fishman, M. C. (1993). Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* **75**, 1273-1286.

Hustert, R. (1974). Morphologie und Atmungsbewegungen des 5. Abdominalsegmentes von *Locusta migratoria migratorioides*. *Zool. Jb. Physiol.* **78**, 157-174.

Jessell, T. M. and Kandel, E. R. (1993). Synaptic transmission: a bidirectional and self-modifiable form of cell-cell communication. *Cell/Neuron* **10** (Sup), 1-30.

Knowles, R. G., Palacios, M., Palmer, R. M. J. and Moncada, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. USA.* **86**, 5159-5162.

Kotrla, K. J. and Goodman, C. S. (1984). Transient expression of a surface antigen on a small subset of neurones during embryonic development. *Nature* **311**, 151-153.

Meier, T., Chabaud, F. and Reichert, H. (1991). Homologous patterns in the embryonic development of the peripheral nervous system in the grasshopper *Schistocerca gregaria* and the fly *Drosophila melanogaster*. *Development* **112**, 241-253

Montminy, M. R., Gonzalez, G. A. and Yamamoto, K. K. (1990). Regulation of cAMP-inducible genes by CREB. *Trends Neurosci.* **13**, 184-188

Muller, U. and Bicker, G. (1994). Calcium-activated release of nitric oxide and cellular distribution of nitric oxide synthesizing neurons in the nervous system of the locust. *J. Neurosci.* **14**, 7521-7528.

Murphey, R. K. and Chiba, A. (1990). Assembly of the cricket cercal sensory system: genetic and epigenetic control. *J. Neurobiol*. **21**, 120-137.

Regulski, M. and Tully, T. (1995). Molecular and biochemical characterization of dNOS: a *Drosophila* Ca^{2+}/calmodulin-dependent nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **92**,9072-9076.

Samama, B., Chateau, D. and Boehm, N. (1995). Expression of NADPH-diaphorase in the rat forebrain during development. *Neurosci. Let.* **184**, 204-207.

Scholz, N. L., Chang, E. S., Truman, J. W. and Graubard, K. (1995). Cellular distribution of nitric oxide-activated cGMP in the developing lobster nervous system. *Soc. Neurosci. Abstr.* **21**, 630.

Shankland, M. (1981). Development of a sensory afferent projection in the grasshopper embryo. II. Growth and branching of peripheral sensory axons within the central nervous system. *J. Embryol. Exp. Morphol.* **64**, 187-209.

Shepherd, D. and Bate, C. M. (1990). Spatial and temporal patterns of neurogenesis in the embryo of the locust (Schistocerca gregaria). *Development* **108**, 83-96.

Southam, E., East, S. J. and Garthwaite, J. (1991). Excitatory amino acid receptors coupled to the nitric oxide/cyclic GMP pathway in rat cerebellum during development. *J. Neurochem.* **56**, 2072-2081.

Spitzer, N. C. (1994). Spontaneous Ca^{2+} spikes and waves in embryonic neurons: signaling systems for differentiation. *Trends Neurosci.* **17**, 115-118.

Thomas, J. B., Bastiani, M. J., Bate, M. and Goodman, C. S. (1984). From grasshopper to Drosophila: a common plan for neuronal development. *Nature* **310**, 203-207.

Truman, J. W. and Ball, E. E. (1994). The nitric oxide/cyclic GMP system is involved in the maturation of insect neurons. *Soc. Neurosci. Abstr.* **20**, 1298

Truman. J. W.,Ewer, J. and Ball, E. E. (1996). Dynamics of cyclic GMP changes in identified neurones during ecdysis behaviour in the locust, ***Locusta migratoria***. *J. Exp. Biol.* **199,** 749-758.

Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V. and Snyder, S. H. (1993). Carbon monoxide: a putative neural messenger. *Science* **259**, 381-384.

Waldman, S. A. and Murad, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.* **39**, 163-196.

Wang, T., Xie, Z. and Lu, B. (1995). Nitric oxide mediates activity-dependent synaptic suppression at developing neuromuscular synapses. *Nature* **374**, 262-266.

Watson, A. H. D. and Burrows, M. (1981). Input and output synapses on identified motor neurones of a locust revealed by intracellular injection of horseradish peroxidase. *Cell Tissue Res.* **215**, 325-332.

Williams, C. V., Nordquist, D. and McLoon, S. C. (1994). Correlation of nitric oxide synthase expression with changing patterns of axonal projections in the developing visual system. *J. Neurosci.* **14**, 1746-1755.

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.

Xie, F., Meier, T. and Reichert,H. (1992). Embryonic development of muscle patterns in the body wall of the grasshopper. *Roux's Arch. Dev. Biol.* **201**, 301-311.

Zhuo, M., Hu, Y., Schultz, C., Kandel, E. R. and Hawkins, R. D. (1994). Role of guanylyl cyclase and cGMP-dependent protein kinase in long-term potentiation. *Nature* **368**, 635-639.

(Accepted 15 August 1996)