The Journal of Experimental Biology 207, 923-935
Published by The Company of Biologists 2004
doi:10.1242/jeb.00845

The early ontogeny of neuronal nitric oxide synthase systems in the zebrafish

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Accepted 17 December 2003

Summary

To examine a putative role for neuronal nitric oxide synthase (nNOS) in early vertebrate development we investigated nNOS mRNA expression and cGMP production during development of the zebrafish *Danio rerio*. The nNOS mRNA expression in the central nervous system (CNS) and periphery showed a distinct spatio–temporal pattern in developing zebrafish embryo and young larvae. nNOS mRNA expression was first detected at 19 h postfertilisation (h.p.f.), in a bilateral subpopulation of the embryonic ventrorostral cell cluster in the forebrain. The number of nNOS mRNA-expressing cells in the brain slowly increased, also appearing in the ventrocaudal cell cluster from about 26 h.p.f., and in the dorsorostral and hindbrain cell cluster and in the medulla at 30 h.p.f. A major increase in nNOS mRNA expression started at about 40 h.p.f., and by 55 h.p.f. the expression constituted cell populations in differentiated central nuclei and in association with the proliferation zones of the brain, and in the medulla and retina. In parts of the skin, nNOS mRNA expression started at 20 h.p.f. and ended at 55 h.p.f. Between 40 and 55 h.p.f., nNOS mRNA expression started in peripheral organs, forming distinct populations after hatching within or in the vicinity of the presumptive swim bladder, enteric ganglia, and along the alimentary tract and nephritic ducts. Expression of nNOS mRNA correlated with the neuronal differentiation pattern and with the timing and degree of cGMP production.

These studies indicate spatio–temporal actions by NO during embryogenesis in the formation of the central and peripheral nervous system, with possible involvement in processes such as neurogenesis, organogenesis and early physiology.

Key words: morphogenesis, zebrafish, *Danio rerio*, brain, retina, gut, intestine, hybridisation, *in situ*, development, regeneration, neuronal differentiation.

Introduction

Nitric oxide (NO) has recently been shown to play a fundamental role in the development and plasticity of the central nervous system (CNS), during both embryonic and post-embryonic life stages. However, in vertebrates little is known about the detailed ontogeny of the NO-producing systems or their individual functional significances. Since NO-producing systems have been partially characterized in teleosts, and the zebrafish is well suited as a model species for early embryogenesis in vertebrates, we investigated the detailed spatio–temporal expression of an endogenous NO-producing enzyme during early development of the zebrafish, the neuronal nitric oxide synthase (nNOS) isoform.

NO is a free radical molecule that is formed in biological tissues from L-arginine by three major nitric oxide synthase isoforms, nNOS, endothelial NOS (eNOS) and inducible NOS (iNOS), using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor (see Alderton et al., 2001). In addition to NO's multifunctional properties in various normal and pathophysiological events (see Bredt and Snyder, 1994; Moncada et al., 1991, 1998; Vincent, 1994), recent studies have also emphasized important roles for NO in early life processes (Gouge et al., 1998; Jablonka-Shariff et al., 1999; Kuo et al., 2000). One proposed mechanism for the effects of NO in developmental processes is a suppressive influence on DNA synthesis, whereby NO acts as a negative regulator on precursor cells and thereby affects the balance of cell proliferation, differentiation and apoptosis (Enikolopov et al., 1999; Puenova et al., 2001; Puenova and Enikolopov, 1995). In the developing nervous system, studies performed in different species have implicated NO in mechanisms such as neural differentiation, pathfinding and synapse formation (Kuzin et al., 2000; Mize et al., 1998; Ogura et al., 1996; Shoham et al., 1997). In developing insects (Enikolopov et al., 1999; Gibbs and Truman, 2000; Kuzin et al., 1996, 2000) and amphibians (tadpoles; Puenova et al., 2001), NO participates in the regulation of cell proliferation, differentiation and apoptosis, and in developing gastropods (snails), nitrergic neurons have been demonstrated to participate in both behavioural and physiological functions (Serfözö and Elekes, 2002). In mammals, the presence of NO-producing systems

and NO-mediated action in developmental processes of the CNS have preferentially been studied during early postnatal stages (see Mize et al., 1998). In different species, nNOS or nNOS-like isoforms may be the major source of NO-mediated action in developmental and plastic processes (Mize et al., 1998; Puenova et al., 2001), including in restricted brain areas with ongoing neurogenesis and neural plasticity in adult mammals (Islam et al., 1998; Moreno-Lopez et al., 2000). In lower vertebrates such as teleosts, NO has been emphasised to play a versatile role in the development of the central nervous system during both embryonic and post-embryonic life stages (Devadas et al., 2001; Fritsche et al., 2000; Gibbs et al., 2001; Ribera et al., 1998). In the brain of adult zebrafish, nNOS mRNA-expressing populations are closely associated with the proliferation zones (Holmqvist et al., 2000a) that generate new cells throughout life (see Ekström et al., 2001; Wulliman and Knipp, 2000). A role for NO in cell proliferation zones of different brain areas was recently demonstrated in tadpoles (Puenova et al., 2001), and has been indicated in more restricted ongoing neurogenesis of the subventricular zone in adult mammals (Islam et al., 1998; Moreno-Lopez et al., 2000).

All three major NOS isoforms are expressed during early development. The differentiated expression of NOS isoforms in certain tissues at different developmental stages indicates that temporal and spatial NO-mediated activities may be regulated by different NOS-producing systems (see Alderton et al., 2001; Eliasson et al., 1997; Lee et al., 1997; Wang et al., 1999). In the developing brain, NOS enzyme activity, NOS proteins and mRNAs of the NOS isoforms have been detected in different mammalian species; however, few species have been studied in detail, and there has been little discrimination between the specific NOS isoforms (see Judas et al., 1999). To date, detailed analysis of NOS systems during early embryonic development have mainly been limited to the presence of the nNOS protein in the brain of rat and mouse. In teleosts, NOS proteins and their activity have been characterized, and the molecular identity demonstrated for nNOS and iNOS (Cox et al., 2001; Holmqvist et al., 2000a; Øyan et al., 2000; Saeij et al., 2000). In zebrafish and salmon, preliminary studies have shown an early expression of nNOS mRNA in the CNS (Holmqvist et al., 1998, 2000b). In adult teleosts, peripheral organs contain nNOS systems homologous to mammals (see Brüning et al., 1996), whereas the NOS isoform identities of different developing NADPHd active peripheral systems (Villani, 1999b) are unknown. Spatial documentation of specific NOS mRNA expression in a whole organ has been reported for nNOS in brains of adult rat (Iwase et al., 1998) and zebrafish (Holmqvist et al., 2000a).

Guanylate cyclase is proposed to be the major target for NO, causing an increase in intracellular guanosine 3′,5′ cyclic monophosphate (cGMP), a second messenger affecting multiple molecular targets (see Denninger and Marletta, 1999; McDonald and Murad, 1996). Data accumulated so far suggest that NO and cGMP together may play an important role in the development of specific pathways in the CNS and peripheral nervous system (PNS) of both vertebrates and invertebrates (Gibbs et al., 2001; Gibbs and Truman, 2000; Giulli et al., 1994; Serfözö and Elekes, 2002).

NO is indicated to be an important factor in early developmental processes throughout the vertebrate phylogeny. However, little is known about the detailed ontogeny of NOS-isoforms during vertebrate embryogenesis. We therefore investigated the morphological basis for putative NO-mediated actions derived from nNOS during early development of the zebrafish. The spatio–temporal expression of nNOS mRNA was investigated in the whole developing body using *in situ* hybridisation techniques, and was related to the neural differentiation pattern and temporal cGMP expression.

Materials and methods

Zebrafish *Danio rerio* H. embryos, treated as whole mounts or cryosections, were used for detection of nNOS mRNA by *in situ* hybridisation. Embryos bred in our own zebrafish facility (from wild-type stock) were maintained at 28.5°C in Petri dishes containing embryo medium. Embryonic development stages were confirmed according to descriptions by Kimmel et al. (1995). Embryos were sampled for *in situ* hybridisation every hour from the time of fertilization (h.p.f.) to 24 h.p.f., and then every second hour until hatching (55 h.p.f.). One post-hatching sampling was made at 72 h.p.f. Embryos were de-chorionated and immersed in cold 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 mol l^{-1}, pH 7.2) for 16 h. For whole-mount *in situ* hybridisation, embryos were rinsed in PBS, immersed in methanol (100%), and stored at –20°C until use. For cryosectioning, embryos were rinsed in PBS and then immersed in PBS containing sucrose (25%) and embedding medium (20%; Tissue-Tek, Miles Inc., Eikhart, IN, USA) at 8°C. They were then frozen in embedding medium (100%). Cryosections were cut at a thickness of 10 μm and collected on slides (Super Frost, Merck, Germany) in parallel series for *in situ* hybridisation and immunocytochemistry. Animal experiments were approved by the local animal welfare committee (Lund, Sweden).

For *in situ* hybridisation, cDNA constituting the 621 bp gene sequence encoding zebrafish nNOS mRNA (GenBank, accession number AF219519) was used to make RNA probes (Holmqvist et al., 2000a). Antisense and sense probes were made from cDNA inserted into pGEM-T Easy vectors (Promega, Madison, USA) and linearized with BSP 120I (anti-sense) or *Sal*I (sense), respectively. Digoxigenin (DIG) labelling of probes was performed using T7 and SP6 RNA polymerase, respectively, according to the manufacturer's instructions (Boehringer Mannheim, Germany). Whole mounts from life stages that achieved pigmentation were treated with hydrogen peroxide (0.1% in methanol) for 1–3 h. Whole mounts were permeabilized with Triton X-100 (1% in PBS) for 24–72 h, depending on their developmental stage. Whole mounts and sections were postfixed in 4% paraformaldehyde in PBS, and then further permeabilized with proteinase-K (0.25 mg ml^{-1}, 5–10 min at room temperature). After

immersion in fixative (4% paraformaldehyde for 10 min) and treatment with acetic anhydride (0.25% for 10 min), whole mounts and sections were rinsed in 5× sodium citrate buffer (SSC) and incubated for 1–3 h at room temperature in hybridisation buffer [50% formamide + 5× SSC + 5× Denhardt's solution (Sigma) + 250 μg ml^{-1} MRE 600 tRNA (Roche, Darmstadt, Germany) + 500 μg ml^{-1} denatured and sheared salmon testes DNA (Sigma)]. For cryosections, 10% dextran sulphate was added to the hybridisation buffer. Hybridisation with 600–800 ng ml^{-1} probe was performed in hybridisation buffer for 16 h at 65°C. Post-hybridisation rinses were performed in 5× SSC for 2× 15 min or 30 min at room temperature, in 3.5× SSC containing 30% formamide for 30 min at 65°C, in 0.2× SSC for 2× 30 min at 65°C, and in 0.2× SSC for 2× 15 min or 2× 5 min at room temperature. Visualization of hybridised transcripts was performed *via* sequential incubation with a goat anti-DIG, alkaline phosphatase-conjugated antibody for 16 h at 8°C (1:2000; Roche) and alkaline phosphatase reaction solution containing 3.4 μl ml^{-1} nitro-blue tetrazolium, 3.5 μl ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Roche) and 0.001 mol l^{-1} Levamisole (Sigma). The reaction was performed for 6–32 h at room temperature, and was sometimes continued up to 72 h at room temperature or at 8°C. The reaction was stopped in Tris-EDTA (TE, 0.01 mol l^{-1}). Whole mounts were immersed in TE containing 50% glycerol and mounted between coverslips. Cryosections were mounted directly in Kaiser's glycerol gelatin (Merck, Penzberg, Germany), or were dehydrated in an alcohol series ending with xylol, and mounted in Histomount (Histolab, Gothenburg, Sweden).

For correlation of nNOS mRNA expression with the general neuronal differentiation pattern, parallel sections to those used for *in situ* hybridisation were labelled with monoclonal mouse antibodies against acetylated α-tubulin (AT; Incstar USA, diluted 1:1000), which specifically detects newly differentiated neuronal structures in the zebrafish (Chitnis and Kuwada, 1990; Ross et al., 1992; Wilson et al., 1990). Sections were incubated in the AT antiserum for 48–72 h at 8°C, and then in swine anti-mouse IgG antiserum (1:50; DAKO, Denmark) and mouse peroxidase anti-peroxidase (PAP, 1:50; DAKO) for 30 min each at room temperature). Tissue sections were then incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB; 0.01–0.05%) containing H_2O_2 (0.0125%) and $NiSO_4$ (0.025%) in Tris-HCl (0.1 mol l^{-1}, pH 7.6) for 5–10 min.

Sections and whole mounts were analyzed using a light microscope equipped with interference Nomarski optics (Olympus AX60, Tokyo, Japan), and digital images were collected with a digital camera (Olympus DP50-CU). Images were corrected for brightness, contrast and colour balance, and were mounted as plates using Adobe Photoshop (version 5.0 for Macintosh, Apple).

For cGMP analyses, 60 fertilized eggs per sample (of wild type used for nNOS studies, or the Tübingen strain) were sampled at 8, 14, 20, 24, 30, 34, 40 and 55 h.p.f. (these time points were also used for nNOS mRNA *in situ* hybridisation). Eggs were placed in a 1 ml Eppendorf tube, the medium removed, and the tube immediately placed in liquid nitrogen. Samples were stored at –80°C until the extraction procedure. As controls for cGMP levels present in the egg yolk soon after fertilization, egg samples (Tübingen strain only) were also collected at the 1–2 cell stage and processed in the same way.

Prior to the cGMP assay, frozen tissue was homogenized and sonicated in cold 6% trichloroacetic acid to give 10% w/v homogenate. The sample was then centrifuged at 2000 ***g*** for 15 min at 4°C, and the supernatant was then recovered and washed 4× with 5 volumes of water-saturated diethyl ether. The aqueous extract was dried in a vacuum drier (Savant, Newington, USA) and then reconstituted in assay buffer. cGMP concentrations in the embryos were measured in duplicate using ^{125}I-cGMP radio-immunoassay kits (Amersham International, England), according to a standard acetylation protocol. In addition to the standard curve, standard cyclic nucleotide concentrations were repeatedly measured throughout the assay procedure (two different standards after every four duplicate samples) in order to ensure the stable performance of the assay. The intra-assay coefficient of variation was 4.1%. All the samples collected were analyzed for cGMP concentrations within the same extraction and assay procedure. The data were processed for statistical evaluations using an unpaired Student's *t*-test.

Results

In situ hybridisation

Using the anti-sense probes, the spatio–temporal expression of nNOS mRNA was followed throughout the embryonic development until hatching, and at one early larval stage (72 h.p.f.). A schematic representation of the nNOS mRNA expression pattern is shown in Fig. 1. nNOS mRNA transcripts were restricted to the cytoplasm of cell bodies (see Fig. 2D–G), and the use of a high hybridisation temperature (65°C) followed by stringent post-hybridisation procedures produced virtually no background labelling (see Figs 2–4), even after longer reaction times (up to 72 h) of the alkaline phosphatase in older embryos (see Figs 4–6). Low labelling intensities detected in cryosections, such as at the onset of mRNA transcription by most cell groups in the CNS or in peripheral organs, were not visualized in whole-mount preparations. The pre-treatment and post-clearing after hybridisation for whole-mount preparations virtually abolished labelling in the skin of the embryos (see Fig. 5A,B). By contrast, nNOS mRNA transcripts in the skin were intensely labelled in cryosections, distributed in the anterior body between 20–55 h.p.f. As previously shown in the adult zebrafish brain (Holmqvist et al., 2000a), in embryo tissue the anti-sense probe produced a specific hybridisation to nNOS mRNA transcripts in the skin, retina and other peripheral organs, confirmed by the lack of hybridisation when excluding the anti-sense probe or using the sense probe (Fig. 5E). The cellular labelling of nNOS mRNA transcripts corresponded with that obtained in the study of the adult zebrafish brain (Holmqvist et al., 2000a), in which the specific hybridisation of the probe was confirmed further

by correlation with NADPHd histochemical and nNOS immunocytochemical labelling. In the CNS, AT-immunoreactive (AT-IR) elements comprised both labelled perikarya and projections (Figs 2H–J, 3B, 4B,D,G). The temporal and spatial pattern of AT-IR elements corresponded to that described previously in the developing CNS of the zebrafish (Chitnis and Kuwada, 1990; Ross et al., 1992; Wilson et al., 1990), depicting the spatial and temporal differentiation of neuronal perikarya, axonal fibres and finer arborisations (Figs 2–4). Thus, nNOS mRNA is indicated to be expressed by early differentiated and mature neurons in both the CNS and PNS, and transiently in skin epithelial cells.

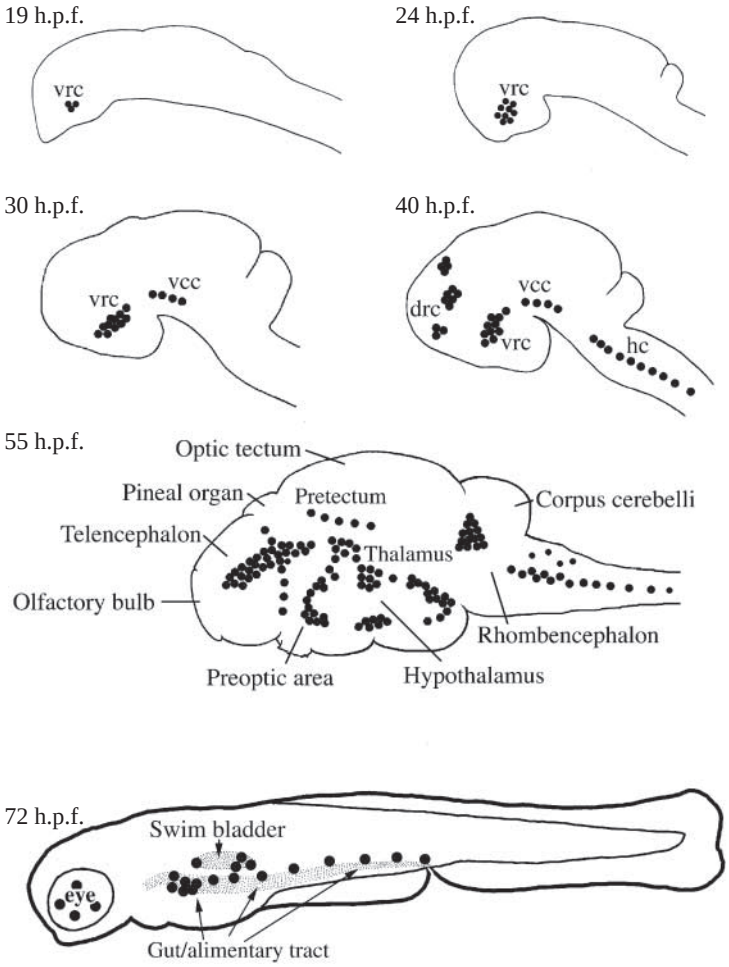

Fig. 1. Schematic representation of the spatial and temporal distribution of nNOS mRNA-expressing cell populations (filled circles) in embryonic zebrafish during representative developmental life stages (brain at 19, 24, 30, 40 and 55 h.p.f. and eye and peripheral organs at 72 h.p.f.). Note the initial expression in the brain restricted to the ventrorostral cell cluster (vrc), the subsequent expression in ventrocaudal cell cluster (vcc), dorsorostral cell cluster (drc) and hindbrain cell clusters (hc), followed by the major increase in expression from around 40 h.p.f. and the presence of different nNOS mRNA-expressing cell populations in all major parts of the brain at 55 h.p.f. (hatching). Around hatching, the first nNOS mRNA expression in the eye and peripheral body organs appears, represented in the image of the 72 h.p.f. larvae.

Temporal and spatial expression of nNOS mRNA transcripts in the CNS

Expression of nNOS mRNA transcripts was first detected in the forebrain at 19 h.p.f., after which the labelling intensity increased, both in terms of the number of expressing cells and their anatomical distribution. The first labelled cells (Fig. 2A,B) were located bilaterally in the ventral forebrain close to the neuroepithelium in the most ventrolateral position adjacent to the eye primordium, i.e. corresponding to the ventrorostral cell cluster (vrc). Between 22 and 24 h.p.f., additional strongly labelled cells appeared in the vrc, constituting around 5–8 cell bodies at these stages (Fig. 2C,D). At this stage, AT-IR structures, perikarya and axonal projections, had increased significantly (Fig. 2H–J).

Between 26 and 30 h.p.f., the number of strongly labelled nNOS mRNA-expressing cells in vrc increased. At this time, additional labelled cells appeared more caudal, in a position corresponding to the ventrocaudal cell cluster (vcc; Fig. 2E).

At 34 h.p.f., relatively weak labelling of nNOS mRNA transcripts was also visualized in the dorsorostral embryonic cell cluster (drc), in hindbrain cell clusters (hc) and in the medulla (Fig. 2F,G). Between 19 and 34 h.p.f., nNOS mRNA-expressing cells had appeared in different cell populations in vrc, drc, vcc, hc and in the medulla, preceded by AT-IR populations (Fig. 2H–J).

Between 40 and 55 h.p.f., nNOS mRNA-expressing populations increased most significantly with respect to the number of labelled cell bodies, a wide distribution in all major brain areas, and to labelling intensity of most populations (Figs 3, 4). At 55 h.p.f., several cell populations were intensely labelled and were distributed in all major parts of the brain. nNOS mRNA-expressing populations in areas that contained AT-IR cells appeared to be nNOS subpopulations of not yet differentiated (presumptive) brain nuclei, expressing nNOS in the adult brain (Holmqvist et al., 2000a), which have been anatomically defined in the adult brain (Wulliman et al., 1996). In addition, large nNOS mRNA-expressing populations were present in areas that did not have any AT-IR perikarya (Figs 3, 4), distributed along the proliferation zones as described in larval zebrafish (Wulliman and Knipp, 2000). AT-IR neuronal projections, axons and fine fibre arborisations had increased significantly at this time (Fig. 3A,B). In the telencephalon, a large nNOS mRNA-expressing cell population appeared in the central area (Figs 3A, 4A), of which a smaller portion reached into the forming rostral thalamic portion of the diencephalon. In the ventrorostral diencephalon, relatively small to large cell populations expressing nNOS mRNA were located in the presumptive preoptic (and suprachiasmatic) and rostral thalamic area (Fig. 4C). A distinct nNOS cell cluster was present in the dorsal diencephalon, located in the presumptive pretectal area (Figs 3A, 4E). Relatively intensely labelled nNOS cell populations were located in the presumptive

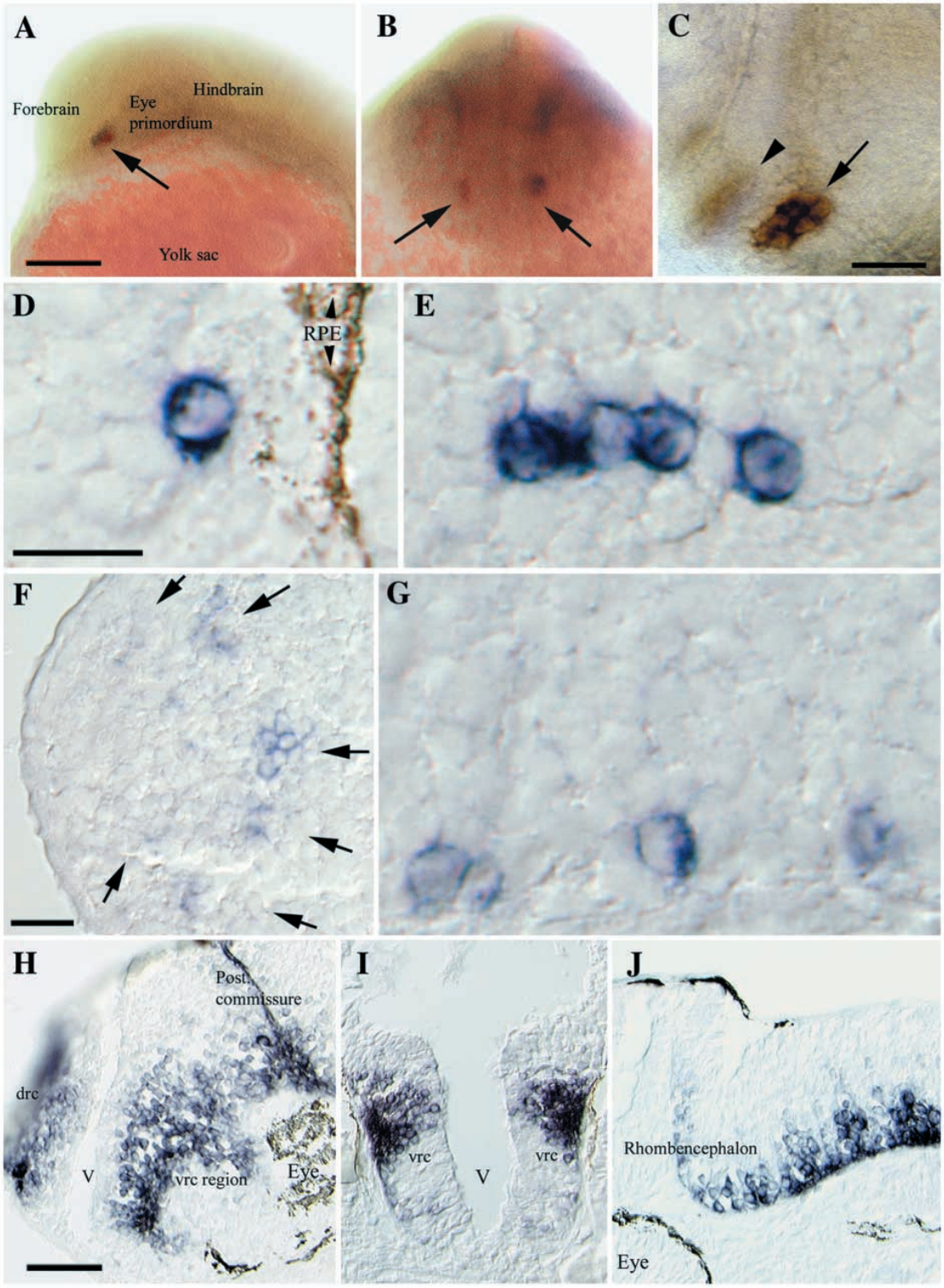

Fig. 2. nNOS mRNA expression in the brain of embryonic zebrafish at different developmental stages. (A-C) Whole mounts; (D–J) cryosections; (H–J) comparison with AT immunoreactive neurons in corresponding regions at a representative stage (24 h.p.f., cryosections). (A,B) 19 h.p.f.; nNOS mRNA expression in the forebrain, in two bilateral cell populations (arrows) as part of vrc (sagittal view in A and frontal view in B). (C) 23 h.p.f.; the bilateral vrc cell clusters (arrow and arrowhead in whole-mount preparation) are seen in a semi-sagittal view. (D) 24–25 h.p.f.; one nNOS mRNA-expressing vrc cell (blue) is shown adjacent to the initial pigmentation of the retinal epithelium (brown; RPE, arrowheads) from a frontal view. (E,F) 30 h.p.f.; nNOS mRNA-expressing cell bodies in vcc (E; sagittal view) and nNOS mRNA-expressing cell populations in drc (F; sagittal view). (G) 34 h.p.f.; nNOS mRNA-expressing cells in hc (sagittal view). (H–J) AT immunoreactivity at 24 h.p.f. in brain regions corresponding to sites of the nNOS mRNA expression in vrc and vcc (H, sagittal view; I, frontal view), and in hc and medulla (J; sagittal view). Scale bars: in A, 100 μm (A,B); 30 μm (C,F); in D, 20 μm (D,E,G); in H, 50 μm (H–J). V, ventricle.

posterior tuberal, caudal and lateral portions of the forming hypothalamus (Figs 3A, 4E). A large cell cluster was located dorsal to the hindbrain, in the mesencephalon, on the border between the caudal portion of the optic tectum and corpus cerebelli (Figs 3A, 4E). Intensely labelled cells were located along the ventral spinal cord close to the central canal (Fig. 4F,H), coinciding with the extensive AT-IR neuronal network (Fig. 4G). Scattered nNOS mRNA-expressing cells were present in the central rhombencephalon, in areas corresponding to the facial and vagus lobes. In the brain, most nNOS-expressing populations present in adults (Guido et al., 1997) had appeared at 55 h.p.f., with no noticeable increase observed at 72 h.p.f.

In the retina, nNOS mRNA transcripts were expressed first between 45–55 h.p.f., with no noticeable increase in larva, as represented by a few weakly labelled cells located in the morphologically undifferentiated inner nuclear layer (Fig. 4I).

Temporal and spatial expression of nNOS mRNA transcripts in peripheral organs

In peripheral organs (Figs 5, 6), nNOS mRNA expression was first detected in the skin, at 20 h.p.f., predominantly in the posterior two thirds of the animal (Fig. 5A,B). The labelling was restricted to epithelial cells (Fig. 5C,D). Expression in the skin decreased during embryonic development, and only a few cells with relatively low labelling intensity were detected at 55 h.p.f. By 72 h.p.f. there was no labelling in the skin.

In body organs, nNOS expression was first detected at 55 h.p.f. and was associated with the forming alimentary tract (Fig. 5F). There was a significant increase in nNOS expression just after hatching, and at 72 h.p.f. widespread nNOS-expressing cell populations were detected in the vicinity of the presumptive swimbladder, gut and nephritic ducts (Figs 5G–I, 6A–G). Rostrally, nNOS-expressing cells were located bilaterally in the mesenchyme of the swim bladder and in the dorsolateral portion of the gut (Fig. 5G–I). Larger populations of nNOS-expressing cells associated with the swim bladder were preferentially detected in the caudal portion (Fig. 6A,B). Larger clusters of nNOS-expressing cells were located at the rostral level of the alimentary tract (Fig. 6A,C,D), in presumptive enteric ganglia. More caudal nNOS-expressing populations were fewer (Fig. 6E), and cells located in the mesenchyme were evenly distributed uniformly throughout the length of the alimentary tract and nephritic duct (Fig. 6G).

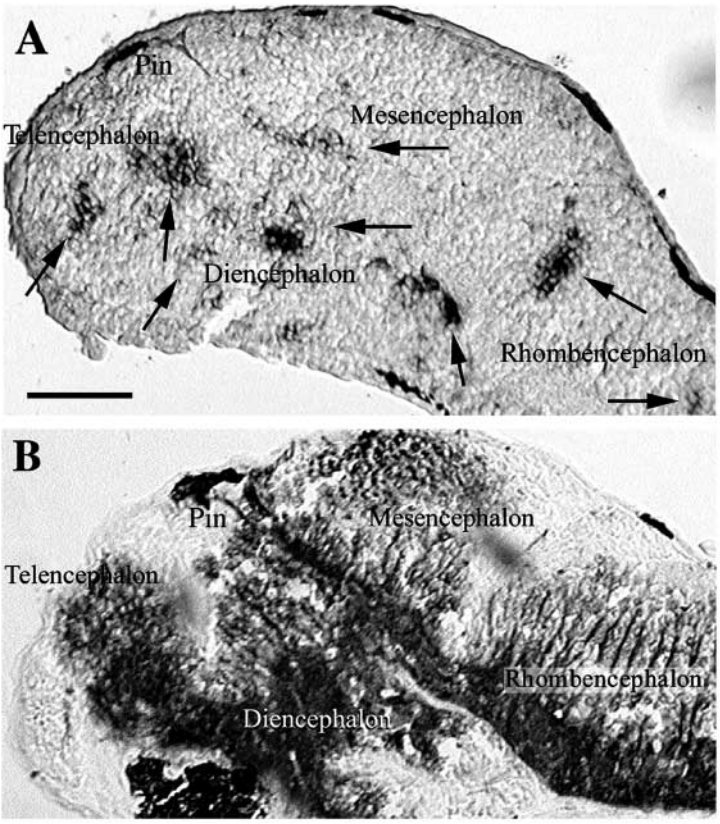

Fig. 3. Distribution of nNOS mRNA expression (A) and AT immunoreactivity (B) in the zebrafish brain at 55 h.p.f. (parallel cryosections). At this stage the nNOS mRNA-expressing cell populations are present in all major brain areas, coinciding with the differentiation of neuronal structures, mainly represented by AT immunoreactive axons, axon arbors and dense fibre nets at putative termination areas. Scale bar, 100 μm. Pin, pineal organ.

Temporal cGMP expression

The production of cGMP (Fig. 7) at the early stages of zebrafish development (8 h.p.f.) corresponded to that in 1–2 cell eggs (0.8–1.2 fmol/egg), and was thus considered to be of extra-embryonic origin. The same temporal patterns and absolute levels of cGMP production were observed in the two wild-type strains of zebrafish used, both strains showing a correlated increase in cGMP level with age. Distinct temporal changes in cGMP levels during zebrafish embryogenesis were characterized by the rapid raise in cGMP levels between 20 and 24 h.p.f. (Δ0.47 fmol h^{-1}) and between 40 and 55 h.p.f. (Δ0.38 fmol h^{-1}; $P<0.05$), and by the lack of significant and slow increase in cGMP levels between 8 and 20 h.p.f. (Δ0.09 fmol h^{-1}) and 24–40 h.p.f. (Δ0.03 fmol h^{-1}).

Discussion

The present study demonstrates a distinct spatio–temporal pattern of formation of nNOS systems in developing zebrafish, revealed by the temporal increase in number and labelling intensity of nNOS mRNA-expressing cells, and in their distribution and formation of cell clusters and distinct populations. The onset of nNOS mRNA expression in distinct cell populations of the forebrain is closely followed by expression in the skin, and subsequently throughout the brain (associated with presumptive brain nuclei and differentiation zones), in the medulla and retina, and in distinct populations in peripheral organs. Correlated with the general neuronal

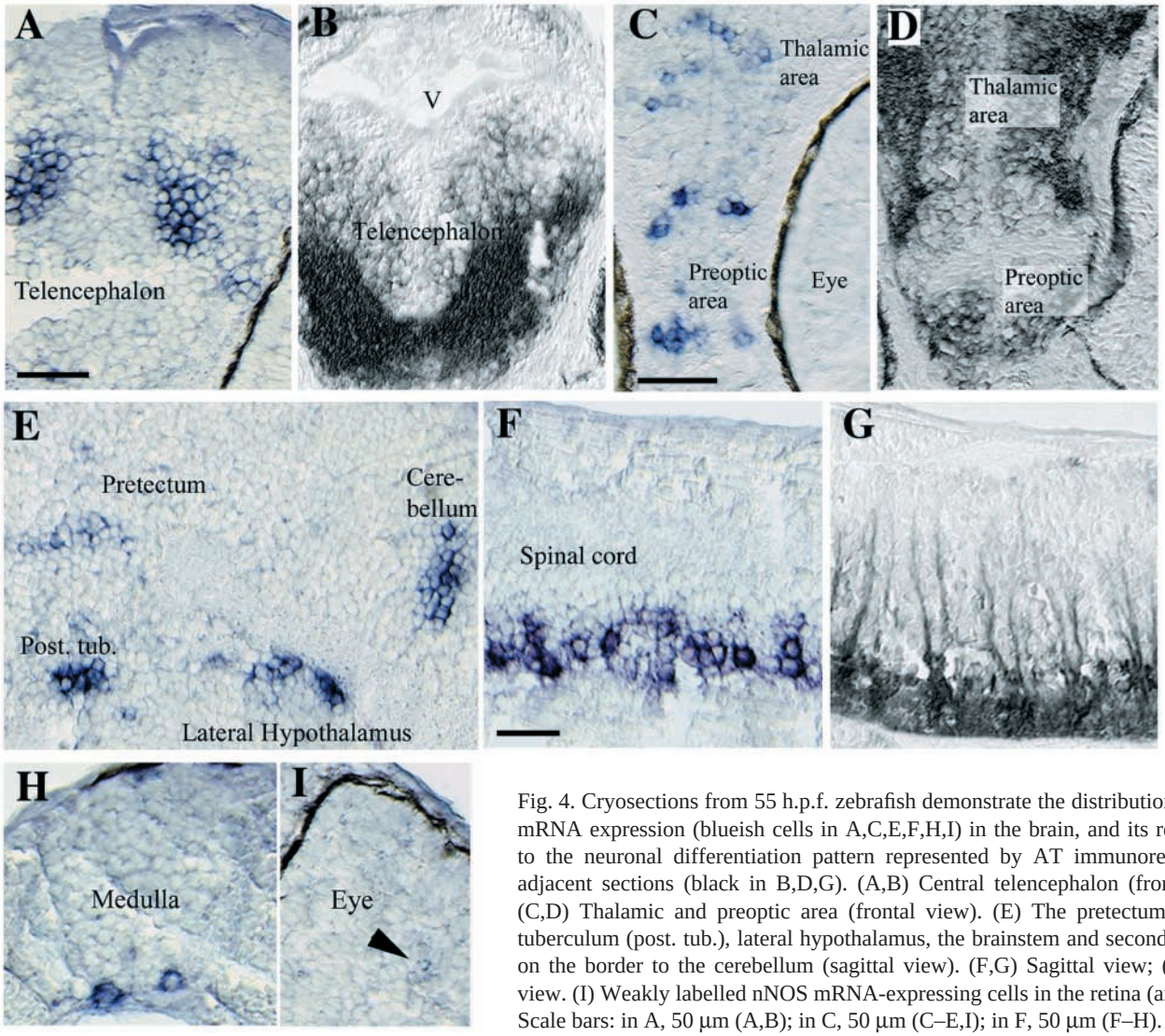

Fig. 4. Cryosections from 55 h.p.f. zebrafish demonstrate the distribution of nNOS mRNA expression (blueish cells in A,C,E,F,H,I) in the brain, and its relationship to the neuronal differentiation pattern represented by AT immunoreactivity in adjacent sections (black in B,D,G). (A,B) Central telencephalon (frontal view). (C,D) Thalamic and preoptic area (frontal view). (E) The pretectum, posterior tuberculum (post. tub.), lateral hypothalamus, the brainstem and secondary matrix on the border to the cerebellum (sagittal view). (F,G) Sagittal view; (H) frontal view. (I) Weakly labelled nNOS mRNA-expressing cells in the retina (arrowhead). Scale bars: in A, 50 μm (A,B); in C, 50 μm (C–E,I); in F, 50 μm (F–H).

differentiation pattern, the vast majority of nNOS mRNA-expressing cells are indicated to be neurons, possibly both early differentiating and mature neurons, of both the CNS and the PNS. The temporal pattern of nNOS expression and cGMP production were found to coincide, indicating early NO-mediated cGMP action. Together with data from other species, the nNOS mRNA expression pattern in the zebrafish confirms nNOS/NO-mediated action in a specific spatio–temporal manner of the developing CNS and peripheral organs of the vertebrate body.

Methodological considerations and nNOS/cGMP activity

To our knowledge, in embryonic teleost species NOS has so far only been detected using NADPHd enzyme histochemical techniques (Villani, 1999a,b). The specific detection, hybridisation and visualization of zebrafish nNOS mRNA in embryonic tissue by the anti-sense probe used and *in situ* hybridisation technique is demonstrated by the lack of labelling using the sense probe, the stringent hybridisation conditions used, and the restricted labelling of the cytoplasm (see Fig. 2D,E,G), which comply with that shown previously in tissue from adults (Holmqvist et al., 2000a). Importantly, whole-mount preparations could not be used since whole mounts provided a lower signal of nNOS mRNA expression, which did not detect initial expression in newly differentiated cells within the embryo, and also the pre- and post-treatment (de-pigmentation and clearing) of whole mounts diminished detection of transcripts in the skin (see Figs 2, 5). A corresponding, relatively low expression of new nNOS cells in embryo was previously noted in cells located in association with the brain proliferation zones in adult zebrafish (Holmqvist et al., 2000a). Thus, for the detection of nNOS mRNA expression, cryosections yielded better preservation, detection and visualization of transcripts, which together with the higher morphological resolution on microscopical analysis, provided reliable and detailed spatial, cellular and anatomical analysis of the expression.

The initial nNOS expression appeared in areas with newly differentiated neuronal populations, previously indicated to be NADPHd-positive in another teleost species (Villani,

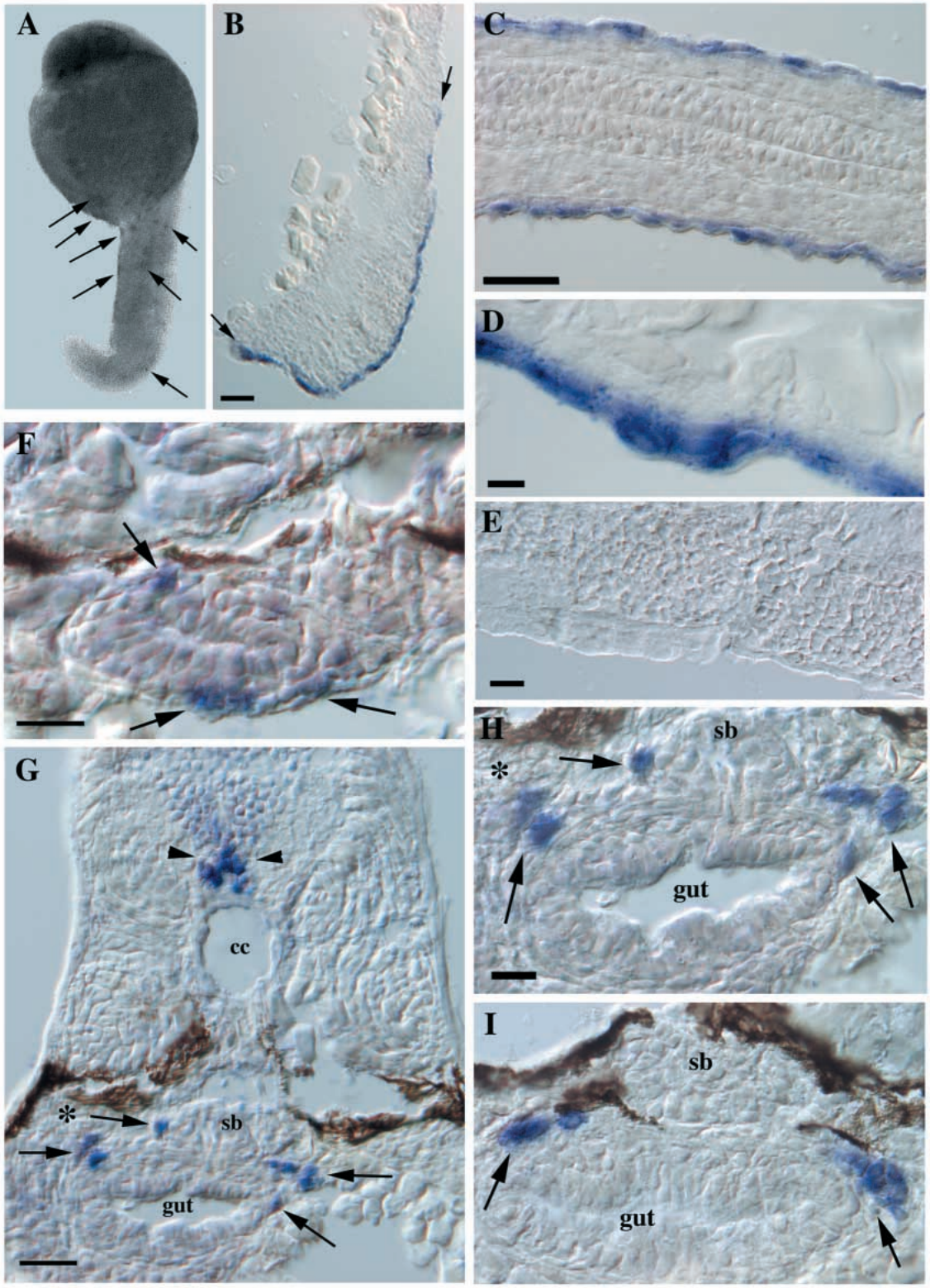

Fig. 5. nNOS mRNA expression in skin and peripheral organs of the developing zebrafish. (A) Weak labelling of nNOS mRNA (arrows) in the skin of a 20 h.p.f. embryo, treated as a whole-mount preparation before clearing, compared to the strong labelling in the same region of a cryosection from another embryo (B). In the skin, labelling is preferentially present in epithelial cells of the tail (C) and around the yolk sac (D). (E) Absence of labelling after incubation with the sense probe. (F) The initial nNOS mRNA expression in body organs (at 55 h.p.f.), in cells located in the rostral portion of the forming gut (arrows). (G–I) Strong labelling of expression in transversal sections of nNOS mRNA in cells (arrows) located bilateral to the swim bladder (sb) and gut (gut), in relation to the pro-nephritic duct (asterisks in G and H) and to the nNOS-expressing cells in the medulla (arrowheads in G). Brownish structures are pigments. Scale bars: 50 μm (B,C,G); 5 μm (D); 10 μm (E,F,H,I).

1999a,b). At late embryonic stages, nNOS mRNA-expressing cells formed distinct populations in differentiating presumptive brain nuclei, possessing nNOS protein and NADPHd activity in adult zebrafish (Holmqvist et al., 2000a), and in peripheral clusters and ganglia identified as nNOS immunoreactive in another adult teleost species (Brüning et al., 1996). The early nNOS mRNA-expressing cells may thus comprise both mature and early differentiating neurons (see below). The lack of nNOS mRNA expression in areas that are NADPHd positive or NOS immunoreactive in adults, such as the olfactory system, brain, retina and pineal organ, agrees with previous indications of as-yet-unknown NOS isoforms in these systems (Holmqvist et al. 1994, 2000a; Östholm et al., 1994; Shin et al., 2000). Whether teleost iNOS isoforms, possessing corresponding molecular structure and induced expression to those in mammals (Saeij et al., 2000), are expressed during development is not known. The zebrafish nNOS mRNA fragment detected here may also be part of an alternatively spliced nNOS mRNA variant (see Eliasson et al., 1997; Lee et al., 1997; Wang et al., 1999), indicated previously in a teleost species (Øyan et al., 2000). In mammals, spliced nNOS mRNA variants have been shown to participate in the differentiated developmental pattern (Eliasson et al., 1997; Lee et al., 1997; Northington et al., 1996; Oermann et al., 1999). Also, gene duplication (see Van de Peer et al., 2002) may be considered for zebrafish nNOS. Further investigations are needed to elucidate whether a specific nNOS isoform or splice variant is preferentially engaged in the developmental processes.

The 621 bp fragment of zebrafish nNOS mRNA detected in this study has a relatively close homology in sequence identities/similarities with the corresponding region of nNOS in mammals. It corresponds to positions that hold the

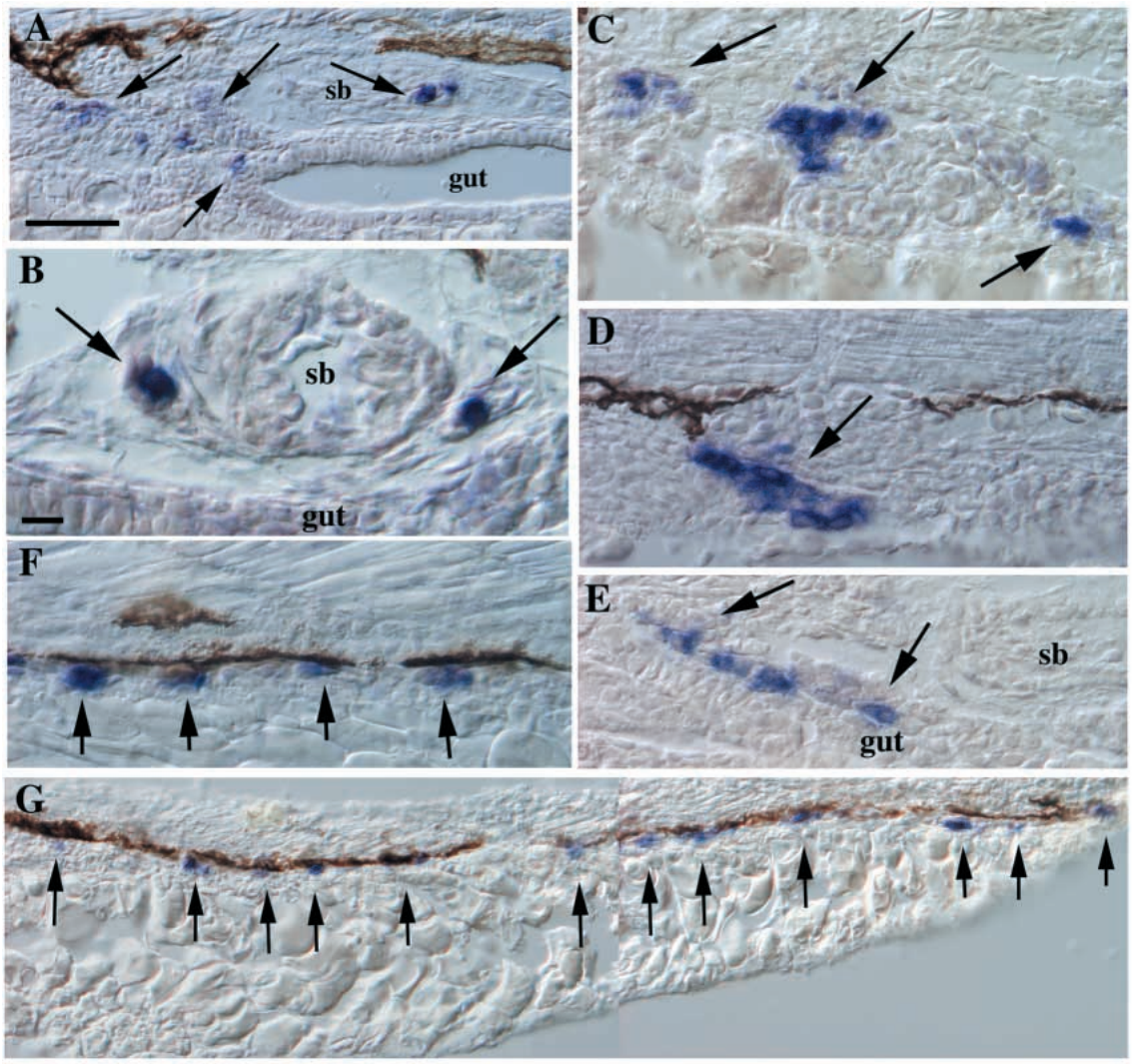

Fig. 6. nNOS mRNA expression in peripheral organs of developing zebrafish (at 72 h.p.f.). (A–D) (A) Sagittal section, low magnification, demonstrating nNOS-expressing cell clusters in the posterior portion of the swim bladder (sb; see transversal section in B), and in the rostral portion of the alimentary system (putative enteric ganglia shown in C,D). (E–G) Sagittal sections demonstrating how the rostral nNOS-expressing cell clusters become a population with evenly distributed cells in the mesenchyme along the alimentary tract and nephritic duct. Brownish structures are pigments. Scale bars: in A, 100 μm (A,G); in B, 10 μm (B–E). sb, swim bladder.

conserved calmodulin and monoflavin binding sites, designating its NO-producing character, and thus its functional capacity (see Holmqvist et al., 2000a). The spatial distribution of NOS activity by the identified nNOS systems is supported by the corresponding cell populations expressing nNOS mRNA in embryonic zebrafish and NOS-like activity (i.e. NADPHd activity) in embryonic *Tilapia* (Villani, 1999a,b). The spatial expression of nNOS mRNA, together with the temporal correlation between the pattern of nNOS mRNA expression and cGMP levels (Figs 1, 7), may support previously reported NO-cGMP action in developmental processes (Giulli et al., 1994; Gibbs et al., 2001; Gibbs and Truman, 2000; Kuzin et al., 2000). Although guanylyl cyclase may be the major target for NO, however, NO has other molecular targets, and the ability to alter gene expression at different levels and *via* modifications of gene products (Bogdan et al., 2001). Furthermore, cGMP signalling is involved as a second messenger in multiple systems that do not involve NO (Denninger and Marletta, 1999; McDonald and Murad, 1996), which in our measurements may constitute an unknown portion of the whole body cGMP (see also below).

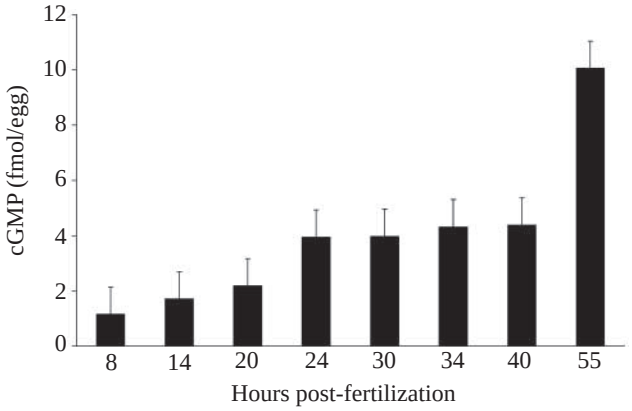

Fig. 7. cGMP levels in zebrafish embryos of different age, 8–55 h.p.f. Values are means ± S.D. of two groups of wild-type *Danio rerio* (Tubingen and local), *N*=60 eggs per group.

Ontogeny of nNOS, and temporal correlations with cGMP

The initial expression of nNOS mRNA in the brain of the zebrafish follows the formation of specific neuronal populations, the vrc and vcc embryonic clusters, and corresponds to the first neurotransmitter differentiation in these cell clusters. The initial neuronal differentiation in the zebrafish development (see Kimmel et al., 1995) begins from cellular precursors in the basal plate at 10–12 h.p.f., just prior to the completion of the neural tube. The differentiation of the specific embryonic neuronal cell clusters and axonal scaffolds occurs around 16–18 h.p.f., comprising the primary cell clusters in the brain termed the drc, vrc, vcc, hindbrain cell cluster, the epiphyseal and pituitary cell clusters (Ross et al., 1992). The embryonic cell clusters contain the first transmitter phenotypic cells, and the vrc and vcc in zebrafish comprise subpopulations of cell clusters holding different primary transmitter differentiated cells, i.e. catecholaminergic, serotonergic and gamma amino butyric acid expressing (GABA) cells (Doldan et al., 1999; Ellingsen et al., 1998; Holzschuh et al., 2001). GABAergic cells are part of all embryonic cell clusters from an early stage, whereas nNOS and the primary catecholaminergic and serotoninergic cells are initially restricted to the vrc and vcc.

The corresponding temporal and spatial patterns for NADPHd activity in developing *Tilapia* sp. (Villani, 1999a) and nNOS mRNA expression in developing zebrafish, stress a common differentiation of nNOS cells and formation pattern of homologous nNOS systems in the brain of teleosts. The nNOS vrc cells identified in the zebrafish correspond to some of the first NADPHd positive cells, described in the diencephalon of *Tilapia* sp. (at 20 h.p.f.). During subsequent development, other nNOS mRNA-expressing cell populations corresponding to NADPHd positive populations in *Tilapia* appear in a similar sequence in brain areas such as the telencephalon, hypothalamus and hindbrain, octavolateral and vagus region, and in the ventral spinal cord. At later life stages, correlating nNOS mRNA-expressing and NADPHd positive cell populations are those in the differentiating optic tectum and within the cerebellum. Species differences in the labelling pattern of nNOS mRNA expression and NADPHd labelling, such as in the olfactory placodes and parts of the hindbrain, may be due to non-specific NADPHd labelling of other NOS-like enzymes (as discussed above).

In the developing brain, data concerning NOS enzyme activity, NOS proteins or mRNAs of the NOS isoforms have been reported in different mammalian species (Derer and Derer, 1993; Gorbatyuk et al., 1997; Keilhoff et al., 1996; Kimura et al., 1999; Lizasoain et al., 1996; Northington et al., 1996; Oerman et al., 1999; Takemura et al., 1996; Terada et al., 1996, 2001; Töpel et al., 1998; Wang et al., 1998), including human (Downen et al., 1999; Ohyu and Takashima, 1998; Yan and Ribak, 1997). The detailed early ontogeny of specific nNOS immunoreactive systems has been reported in the rat brain (Terada et al., 1996). Together with recent preliminary data from combined immunocytochemical, *in situ* hybridisation and NADPHd histochemical studies in mouse (Holmqvist et al., 2001), these studies emphasize that the nNOS expression in rodents starts in brain regions preceding the forming hypothalamus and pons. This corresponds to the distribution of the initial nNOS populations in the zebrafish vrc and vcc, which will form preoptic/hypothalamic regions, and in rostral hc, which will form the rhombencephalon. In addition, in spite of the temporal differences between rodents and zebrafish, corresponding nNOS populations appearing during embryonic and postnatal development are present in homologous brain regions, such as the telencephalon, thalamus, collicular/tectal regions, cerebellum and spinal cord. In the retina of embryonic zebrafish, relatively few and weakly labelled nNOS mRNA-expressing cells were detected just prior to hatching, and were located in the presumptive inner nuclear layer. In the retina of *Tilapia*, the first NOS active (NADPHd positive) cell bodies appear at a similar

developmental stage. Correspondingly, in the rat, the first nNOS immunoreactive cells appear in the inner neuroblast layer, at postnatal day 5 (Kim et al., 2000). In late zebrafish embryos, in addition to the nNOS populations in central brain areas, presumptive nuclei, nNOS populations are present in the brain regions associated with the proliferation zones (Ekström et al., 2001; Wulliman and Knipp, 2000), shown in adult zebrafish to possess retained nNOS expression (Holmqvist et al., 2000a). The close morphological relation of nNOS or nNOS-like enzymes with proliferation zones has been noted in different brain areas of tadpoles (Puenova et al., 2001), and in the more restricted brain regions exhibiting ongoing neurogenesis in adult mammals (Islam et al., 1998; Moreno-Lopez et al., 2000). Thus, similarities in the spatial formation of specific nNOS systems in the brain are indicated in vertebrate phylogeny, including a retained expression throughout life in restricted regions.

The nNOS expression in peripheral organs also followed a specific spatio–temporal pattern in developing zebrafish. Similarly, temporal differences in expression of NOS isoforms, including nNOS, have been noted in different tissues during development of mammals, reflecting involvement by specific NOS isoforms or splice variants in organogenesis (Eliasson et al., 1997; Lee et al., 1997; Northington et al., 1996; Oermann et al., 1999). In developing zebrafish, nNOS mRNA expression was present transiently in skin epithelial cells, from 20 h.p.f. and until just after hatching. NO produced by constitutive NOS plays a role in growth and remodelling of the skin, and NO-mediated pathological conditions are preferentially reported to be related to iNOS and eNOS expression (Dippel et al., 1994; Stallmeyer et al., 2002). In body organs of the zebrafish, we found that the initial expression was associated with the forming alimentary tract. The onset of nNOS expression in peripheral organs at hatching may also contribute to the rapid increase in cGMP expression levels recorded at this time. After hatching there was an increase in number of nNOS cells and presumptive neurons located in close vicinity to the swim bladder, in enteric ganglia, and in the mesenchyme along the alimentary tract and nephritic duct. These peripheral nNOS mRNA-expressing cell populations in zebrafish embryo are reported as NADPHd active in developing *Tilapia* (Villani, 1999b). Corresponding populations are both NADPHd active and NOS immunoreactive in adult goldfish (Brüning et al., 1996), indicating expression by identified nNOS populations in peripheral organs through adulthood. Peripheral organs lacking nNOS mRNA expression but with reported NADPHd activity include the olfactory placodes, neuromasts, otic vesicle during development, and the sensory vagal and glossopharyngeal ganglia in adults. NOS immunoreactive but nNOS mRNA-lacking cells include intracardiac cells, previously indicated in adult teleosts (Brüning et al., 1996). Further studies of later developmental stages in zebrafish, after 72 h.p.f., are needed to elucidate the developmental pattern of specific nNOS in indicated nitrergic sensory systems of peripheral organs, and/or whether they possess a low (or undetectable) expression at the stages studied here.

Early expressed nNOS may be involved in a broad range of functions *via* NO-mediated actions. The participation of NO in different cellular processes has been documented throughout the whole animal phylogeny, indicating its influence on different cellular processes such as mitosis and apoptosis, neuronal pathfinding, refinement and maturation of neuronal circuits (see Mize et al., 1998; Moncada et al., 1998). In invertebrates, NO appears to be central for morphogenesis and neurogenesis during early development, as well as for early behavior and physiology (Enikolopov et al., 1999; Serfözö and Elekes, 2002). Corresponding roles for NO in developmental processes in lower vertebrates are supported by recent experimental data on NO manipulation in tadpoles (Puenova et al., 2001). The effects of NO can be widely distributed well beyond the site of its origin, and beyond the classical neuronal targets, due to its diffusive properties, thereby reaching various cellular and molecular targets. The ontogeny of nNOS expression in zebrafish leads us to propose that NO, produced by nNOS systems specifically, may participate in early physiology as well as in a spatio–temporal pattern in developmental processes of different body organs, including brain, eyes, gut, alimentary tracts and the skin.

The influence of NO on cGMP activity related to developmental processes is one pathway for early NO-mediated action. The coincident temporal development of these systems in developing zebrafish, i.e. the cGMP levels accompanying the pattern of nNOS expression (see Figs 1, 7), support this. The timing was shown by the initial nNOS mRNA expression and the high increase in number of nNOS-positive cells between 19 h.p.f. and 26 h.p.f., which was accompanied by an initial increase in cGMP production (4.8% per hour) and a subsequent major surge in cGMP expression (19% per hour), respectively. Furthermore, the slow increase in the number of nNOS-expressing cells between 24 h.p.f. and 40 h.p.f. was accompanied by a low rise in cGMP production (0.8% per hour), whereas the dramatic increase of nNOS-expressing cells between 40 h.p.f. and 55 h.p.f. was accompanied by a significant increase in cGMP production (8.5% per hour). In the zebrafish, NO-cGMP actions influence the floor plate proliferation in the spinal cord, proposed to be mediated by NADPHd active fibres present at 24–48 h.p.f. (Gibbs et al., 2001). The spatial expression of nNOS mRNA indicates that early spinal NO-mediated cGMP action initially originates from fibres developing from the nNOS populations in the vrc and/or vcc, whereas at later developmental stages NO-mediated cGMP actions may occur *via* the nNOS-expressing cell populations located in hc and/or in local cells in the spinal cord. NO-cGMP systems have been found to play an essential role in the development of the visual system in *Drosophila* (Gibbs and Truman, 2000; Gibbs et al., 2001; Kuzin et al., 2000) and in the maturation of central visual circuits in mammals (Giulli et al., 1994). In teleosts, different NOS-like isoforms (Östholm et al., 1994; Shin et al., 2000) and guanylyl cyclase forms (Hisatomi et al., 1999; Seimiya et al., 1997) are present in the retina and photosensory pineal organ of teleosts and may participate in NO-cGMP functions,

including axogenesis and synaptogenesis (Devadas et al., 2001; Villani, 1999a), or photoreceptor light/dark adaptation (Angotzi et al., 2002; Zemel et al., 1996). The detailed spatial relationship between the actual nNOS enzyme activity (or NO) and cGMP-expressing target cells needs to be elucidated to determine the cGMP-mediated functional role of the specific nNOS mRNA-expressing populations identified in the different body organs.

We thank Lillemor Turesson for technical assistance. This study was supported by the Swedish Medical research council (MFR #1125), Experimental animal centre (Centrala Försöksdjursnämnden, CFN), the Royal Physiographic Society, the Crafoord foundation and Chaikin–Wile foundation.

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