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Embryonic expression and multifunctional actions of the natriuretic peptides and receptors in the developing nervous system

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Abstract

Atrial natriuretic peptide (ANP) binding sites have been detected in the embryonic brain, but the specific receptor subtypes and biological functions for ANP family ligands therein remain undefined. We now characterize the patterns of gene expression for the natriuretic peptides [ANP, brain natriuretic peptide (BNP), type-C natriuretic peptide (CNP)] and their receptors (NPR-A, NPR-B, NPR-C) at several early stages in the embryonic mouse nervous system by in situ hybridization, and begin to define the potential developmental actions using cell culture models of peripheral (PNS) and central nervous systems (CNS). In the CNS, gene transcripts for CNP were present at the onset of neurogenesis, embryonic day 10.5 (E10.5), primarily in the dorsal part of the ventricular zone (VZ) throughout the hindbrain and spinal cord. On E14.5, new CNP signals were observed in the ventrolateral spinal cord where motor neurons reside, and in bands of cells surrounding the spinal cord and hindbrain, localized to dura and/or cartilage primordia. ANP and BNP gene transcripts were not detected in embryonic brain, but were highly abundant in the heart. The CNP-specific receptor (NPR-B) gene was expressed in cells just outside the VZ, in regions where post-mitotic neurons are differentiating. Gene expression for NPR-C, which recognizes all natriuretic peptides, was present in the roof plate of the hindbrain and spinal cord and in bilateral stripes just dorsolateral to the floor plate at E12.5. In the PNS, NPR-B and NPR-C transcripts were highly expressed in dorsal root sensory (DRG) and cranial ganglia beginning at E10.5, with NPR-C signal also prominent in adjoining nerves, consistent with Schwann cell localization. In contrast, NPR-A gene expression was undetectable in neural tissues.

To define ontogenetic functions, we employed embryonic DRG and hindbrain cell cultures. The natriuretic peptides potently stimulated DNA synthesis in neuron-depleted as well as neuron-containing Schwann cell cultures and differentially inhibited neurite outgrowth in DRG sensory neuron cultures. CNP also exhibited modest survival-promoting effects for sensory neurons. In marked contrast to PNS effects, the peptides inhibited proliferation of neural precursor cells of the E10.5 hindbrain. Moreover, CNP, alone and in combination with sonic hedgehog (Shh), induced the expression of the Shh target gene gli-1 in hindbrain cultures, suggesting that natriuretic peptides may also modify patterning events in the embryonic brain. These studies reveal widespread, but discrete patterns of natriuretic peptide and receptor gene expression in the early embryonic nervous system, and suggest that the peptides play region- and stage-specific roles during the development of the peripheral and central nervous systems.

Keywords: Natriuretic peptides; Receptor expression; Mouse development; In situ hybridization; Proliferation; Embryonic DRG and hindbrain progenitors

Introduction

Natriuretic peptides constitute a family of three structurally related hormones: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and the type-C natriuretic peptide (CNP) (Anand-Srivastava and Trachte, 1994; Espiner et al., 1995; Nakao et al., 1992; Needleman et al., 1989). Natriuretic peptides were first discovered as hormones produced

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primarily by the heart that regulate vascular tone, sodium and water homeostasis, and other cardiovascular functions through actions on the kidney and vascular smooth muscle cells. There are three known mammalian receptors for peptides in the ANP family (Anand-Srivastava and Trachte, 1994; Nakao et al., 1992). Two of these, types A and B (NPR-A and NPR-B, respectively), are single transmembrane-spanning proteins that contain guanylyl cyclase (GC) activity in their intracellular domain. In contrast, while the type C receptor (NPR-C) also spans the plasma membrane once, it contains only a short 37-amino-acid intracellular domain that lacks GC activity. Because NPR-C is devoid of GC activity and is internalized after peptide binding, it has been referred to as the ''clearance'' receptor. However, more recent data obtained using specific receptor agonists indicate that NPR-C can indeed transmit intracellular signals, leading to inhibition of cAMP formation, stimulation of intracellular calcium levels, and/or reduction in the MAPK signaling pathway (Prins et al., 1996; reviewed in Anand-Srivastava and Trachte, 1994). Members of this receptor family differ in their relative affinities for the natriuretic peptides. NPR-A binds ANP and BNP with high affinity and CNP with very low affinity. On the other hand, NPR-B is relatively selective for CNP, whereas NPR-C binds all natriuretic peptides with relatively high affinity.

Recent data suggest that natriuretic peptides regulate the development and function of several organ systems (reviewed in Appel, 1992). For example, natriuretic peptides regulate longitudinal growth of bones in explant assays, and transgenic mice that overexpress BNP, or carry targeted mutations in the NPR-C gene, exhibit pronounced skeletal overgrowth (Matsukawa et al., 1999; Suda et al., 1998). Natriuretic peptides may also play roles in the developing brain: ^{125}I-ANP binding sites were detected in embryonic mouse and rat brains (Brown and Zuo, 1995; Scott and Jennes, 1991; Tong and Pelletier, 1990; Zorad et al., 1993), and CNP and ANP mRNA transcripts were detected in embryonic brain and dorsal root ganglia (DRG), respectively (Cameron et al., 1996). Finally, an NPR-C specific analog was shown to inhibit DNA synthesis in mitogen-treated cultures of rat glial cells from rat brain (Prins et al., 1996), and Simpson et al. (2002) recently showed that CNP inhibited proliferation and promoted survival of postnatal mouse olfactory precursors.

We previously showed the neural crest-derived sympathetic neuroblastoma cell line Neuro2 A expresses NPR-A and NPR-B receptors, and that ANP and CNP stimulated proliferation at low concentrations (Lelièvre et al., 2001). Higher concentrations inhibited proliferation by another mechanism, which seemed to involve a NPR-C-like receptor. These, and the above data, suggest that natriuretic peptides perform growth factor-like functions in the developing brain. To characterize ontogenetic expression of natriuretic peptides and receptors in mice, and identify potential sites of peptide action, we performed in situ hybridization on embryonic mice using probes specific for each member of the ligand and receptor families. Then, to explore potential developmental functions, we employed embryonic DRG and hindbrain cultures to define peptide effects. The natriuretic peptides regulated precursor cell proliferation, neuronal survival, and process outgrowth in these culture models, suggesting that natriuretic peptide function contributes to region-specific nervous system development.

Methods

In situ hybridization

ND4 mice were mated overnight and checked the following morning for a vaginal plug. If a plug was present, the time was designated as embryonic day E0.5. Mice at E10.5, E12.5, and E14.5 were immersion fixed in 4% paraformaldehyde in PBS overnight at 4 °C. After cryoprotection in 30% sucrose in PBS, embryos were frozen in OCT embedding compound (Tissuetek, Miles Inc.). Transverse or sagittal sections (10–16 μm) were mounted on slides (Superfrost Plus, Fisher Sci.), then stored at −20 °C. Subsequent processing of slides and in situ hybridization conditions were as described (Waschek et al., 1998). The templates for receptor riboprobe synthesis were generated using RT-PCR as previously described (Lelièvre et al., 2001). The templates for the ligands were obtained by RT-PCR using total RNA from mouse brain as template. Primers (BRL/Life Technology) were designed using the online Primer3 software based on mouse or rat sequences published in NCBI database (GenBank). Sense and antisense primers were 5′-CATCAGATCGTGCCCCGACCC-3′ and 5′-AGGGGTGAGGATCTACTATAA-3′, respectively for ANP, 5′-CCGATCCCTTCTGCA GCATGG-3′ and 5′-AAAGGTGGTCCCAGAGCTGGGG-3′ for BNP, and 5′-CAGCAGTAGGACCCGTGCTCGC-3′ and 5′-CCTCCTTTGTATTTGCGCGC-3′ for CNP. Amplifications were carried out for 35 cycles of denaturation (94 °C, 50 s), annealing (54 °C, 45 s), and extension (72 °C, 45 s). PCR was finished by an incubation for 5 min at 72 °C. Amplified sequences were cloned into PCRII-Topo (Clontech), sequenced to confirm identity, and then cloned into pBluescriptII-SK (Stratagene). The sizes of the amplified cDNAs were 765, 475, and 431 bp for ANP, BNP, and CNP, respectively. Antisense riboprobes were made using *Not*I and Sp6, *Xho*I and Sp6, *Bam*HI and T7, *Bam*HI and T7, *Apa*I and T3, and *Pst*I and T7 for ANP, BNP, CNP, NPR-A, NPR-B, and NPR-C, respectively. Sense probes were made with *Sac*I and T7, *Sac*I and T7, *Xho*I and Sp6, *Hin*dIII and T3, *Hin*dIII and T7, and *Kpn*I and T3 for ANP, BNP, CNP, NPR-A, NPR-B, and NPR-C, respectively.

Dorsal root ganglion (DRG) cell cultures

For each experiment, DRG from four to five E14.5 rats were dissected and incubated with 0.25% trypsin at 37 °C for 20 min. After exposure to trypsin inhibitor (1 mg/ml) and

rinse with saline and Ca^{+}-, Mg^{+}-free solution, DRG were mechanically dissociated and cells were plated (10^5 cells) on poly-D-lysine (0.1 mg/ml)-coated 24-multi-well plates (Nunc, Denmark). Culture medium was composed of a 1:1 (v/v) mixture of Ham's F-12 and DMEM (Gibco, Grand Island, NY) supplemented with transferrin (100 μg/ml; Calbiochem, La Jolla, CA), putrescine (100 μM), progesterone (20 nM), selenium (30 nM), glutamine (2 mM), glucose (6 mg/ml), bovine serum albumin (10 mg/ml), penicillin (50 U/ml), and streptomycin (50 μg/ml) as previously reported (Lu and DiCicco-Bloom, 1997), with products from Sigma unless otherwise indicated. The natriuretic peptides (Peninsula) were diluted from 10^{-4} M stocks (dissolved in water) and added directly in culture media. Dose response analyses of peptide effects on DNA synthesis and survival were performed in the absence of added neuronal trophic factors. Under these conditions, approximately 10–15% of cells observed at 24 h incubation were neurons by morphological and immunocytochemical characteristics, yielding a glial-enriched culture. However, in other experiments, active peptide doses were assessed for mitotic activity in cultures containing 3 ng/ml of NGF, conditions promoting sensory neuron survival and neuron–glial interactions and modeling normal ganglion tissue composition.

In contrast, to assess effects of natriuretic peptides on neuronal processes, cells were incubated with the addition of insulin (10 μg/ml) and NGF (3 ng/ml) to enhance neuron survival (see Results).

DNA synthesis in DRG cultures

Incorporation of [^{3}H]thymidine ([^{3}H]dT) into cellular precipitates was used to assay DNA synthesis in DRG cultures. Cells were treated with various doses of ANP, CNP, and des-[Gln(18),Ser(19),Gly(20),Leu(21),Gly(22)]-ANP(4-23)-NH(2) ($desANP_{4\text{-}23}$) for 24 h on poly-D-lysine-coated 24-multi-well plates, in the absence and presence of NGF (3 ng/ml). [^{3}H]dT was added for the final 4 h of incubation, and incorporation was analyzed by scintillation spectroscopy, as previously described (Lu and DiCicco-Bloom, 1997). To quantify and characterize mitotically responsive cells, we assessed nuclear incorporation of thymidine analog, bromodeoxyuridine (BrdU), using double immunocytochemistry. Dissociated DRG cells plated on poly-D-lysine-coated 35-mm dishes at a cell density of 3×10^5 cells/dish were incubated with various concentrations of natriuretic peptides and BrdU (10 μM) using several paradigms. To assess peptide mitotic effects, cells were incubated in control and CNP-containing medium for 1, 2, and 3 days (peptides were refreshed at 48 h), and cultures were fixed after a 4-h terminal BrdU pulse. Following immunocytochemical staining (see below), the mitotic labeling index (LI) was determined as the ratio of BrdU-positive cells to total cells visualized under phase microscopy, assessing 2–3% of the dish surface area as reported (Carey et al., 2002; DiCicco-Bloom et al., 2000; Lu and DiCicco-Bloom, 1997).

Immunocytochemistry

To characterize mitotic cells in DRG cultures, dissociated cells (3×10^5 cells) were incubated continuously with BrdU in 35-mm dishes for 24 and 72 h, fixed with 4% paraformaldehyde, and stained with antibodies to BrdU (1:50; Beckton Dickinson); Schwann cell markers, S100 (1:200; Sigma) and p75 (1:100; Sigma); astrocyte marker, glial fibrillary acidic protein (GFAP, 1:1000; Sigma), and neuronal marker, βIII tubulin (TuJ1, 1:500; clone TU-20, Biogenesis, Poole, UK). To identify BrdU-positive cells for assessment of the labeling index (LI), nuclear immunoreactivity was detected using the VectorStain ABC kit, as previously reported (Carey et al., 2002; DiCicco-Bloom et al., 2000; Lu and DiCicco-Bloom, 1997). BrdU-expressing cells bearing Schwann cell markers were assessed using double immunofluorescent markers. The rabbit glial marker polyclonal antibodies were detected using goat anti-rabbit antibodies conjugated to Alexafluor 488 (1:200, green). Following 4% paraformaldehyde fixation, BrdU labeling was performed as above, detected using goat anti-mouse Alexafluor 594 (1:200, red). The Alexafluor products were from Molecular Probes, Inc (Eugene, OR). Double labeling was assessed using a Leica DM IRB inverted fluorescent microscope equipped with a dual red/green filter system. To capture cells for presentation, an Optronics Digital camera imported images to a PC computer, fluorescent signal was recorded in black and white and subsequently pseudo-colored as red or green and overlaid, using Adobe Photoshop software, as previously reported (Nicot and DiCicco-Bloom, 2001).

Neuronal survival and neurite outgrowth

To characterize peptide effects on neuronal survival, dissociated DRG cells (3×10^5 cells) were incubated in 35-mm dishes for 24 h in defined medium (see Dorsal root ganglion (DRG) cell cultures section above) without added trophic factors. Under these conditions, approximately 10–15% of cells in control medium were neurons by morphological and immunocytochemical characteristics. Three culture dishes were examined for each group under phase microscopy in two separate experiments, yielding $N = 6$. The number of neurons, identified as round cells bearing uniform processes >2 cell diameters in length and staining with TuJ1, was counted in two to three randomly selected, nonoverlapping 1-cm strips (3% of the culture dish area) as previously described (DiCicco-Bloom et al., 1993, 2000).

To characterize natriuretic peptide effects on neuronal process outgrowth, DRG cells were cultured in the presence of 10 μg/ml insulin and 3 ng/ml of NGF to enhance cell survival. To optimize the measurement of neurite length without cellular overlap, we plated 5×10^4 cells per 35-mm dish. Cells were fixed at 24 h incubation and stained with TuJ1 antibody to enhance detection of long neurites and growth cones. Data were obtained from three separate experiments, each consisting of two to three dishes per

group, yielding $N = 6–8$ dishes. Cell fields at 20× magnification were randomly photographed and the length in micrometers of the longest neurite was measured for each cell using NIH Image (object-image) software. After combining measurements from all fields from the three experiments, the total number of cells assessed for each group was: Control, 349; ANP, 221; CNP, 151; and dANP, 264.

DNA synthesis in E10.5 hindbrain neural tube progenitors

E10.5 mouse hindbrain cells were isolated as described (Waschek et al., 1998), plated at 60,000 cells/well in 96-well tissue culture plates in Neurobasal™ medium supplemented with 1%FBS and 1 ng/ml FGF-2 (Invitrogen/GibcoBRL), and treated on the following day for 24 h with natriuretic peptides at concentrations from 10^{-12} to 10^{-6} M as previously described (Lelièvre et al., 2002). [^{3}H]thymidine was added along with fresh drug for the last 6 h of treatment, after which cells were extracted in 0.5 M NaOH. Incorporated [^{3}H]thymidine was precipitated by TCA and assayed as previously described (Lelièvre et al., 1998).

Analysis of gli-1 gene expression in E10.5 hindbrain neural tube progenitors by real-time PCR

Cells were isolated from E10.5 mouse hindbrain as described above and treated for 8 h with 10 nM CNP (Peninsula), 700 ng/ml Shh (R&D Systems), or the combination of CNP and Shh. Total RNA was isolated, reverse transcribed, and analyzed for gli-1 and β_2-microglobin mRNA by real-time PCR. To obtain primers, cDNA encoding Gli-1 was first analyzed for secondary structures using M-fold software (BioRad). Portions of sequence lacking secondary structure were imported into Oligo6 software (Molecular Biology Insights) to design highly stringent primer sets. For Gli1, we chose the following oligonucleotides 5′-ATCTCTCTTTCCTCCTCCTCC-OH and 5′-CGAGCCTGGCATCAGAA, as sense and antisense primers, respectively. PCR amplification resulted in the generation of a single band at 95 bp, corresponding to the regions 356–449 of the previously published sequence of mouse Gli-1 mRNA (NM010296). To standardize the experiments, we designed, using the same approach, a primer set (5-CCGGCTTGTATGCTATC and 5-AGTTCATGTTCGGCTTC, as sense and antisense, respectively), for the mouse β2-microglobulin gene. These primers amplified an 87-bp region encoding the nucleotides 99–185 of the published sequence (MM2BMR) of the mouse mRNA. Amplified gli-1 and β2-microglobulin bands were cloned into PCRII and sequenced to confirm identity. Real-time PCR was set up using sybergreen-containing supermix from Biorad, for 50 cycles of a three-step procedure including a 30-s denaturation at 96 °C, a 30-s annealing at 60 °C, followed by a 30-s extension at 72°C. Amplification specificity was assessed by melting curve. Quantification utilized standard curves made from serial dilutions of control RNA sample or of the corresponding cDNA cloned into PCRII vector. Differences between samples were calculated as the difference between the specific ratios (gli-1/β2-microglobulin) calculated for each individual sample.

Results

Localization of natriuretic peptide and receptor gene expression

NPR-A and NPR-B receptor gene expression

NPR-A gene expression was not detected within the brain or any ganglia at E10.5 or E12.5, although transcripts were observed in many blood vessels (data not shown). In contrast, intense NPR-B expression was primarily localized to the nervous system from E10.5 to E12.5, and was not detectable in vascular tissue. The NPR-B gene was expressed in the brain at E10.5 in cells just outside the ventricular zone (VZ) of the hindbrain, a region where post-

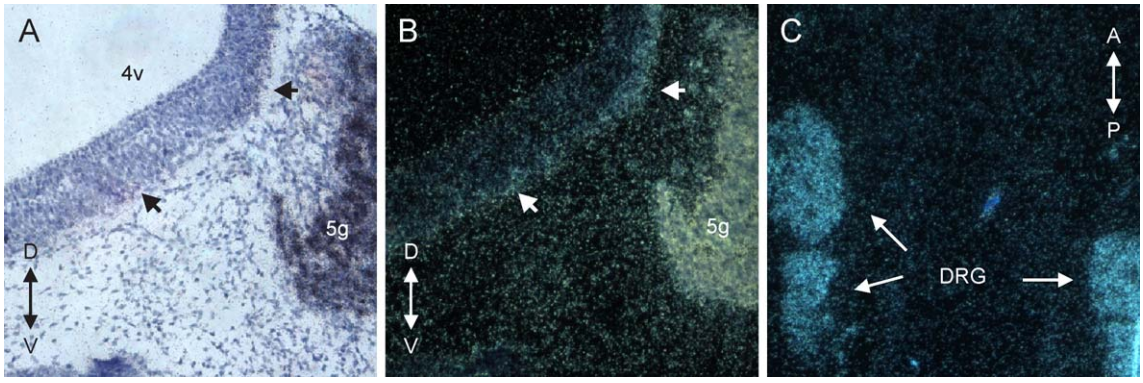

Fig. 1. NPR-B receptor gene expression in E10.5 mouse embryos, detected by in situ hybridization using a ^{33}P-labeled riboprobe. A and B are bright-and dark-field views, respectively, of a coronal section of the hindbrain (dorsal/ventral orientation is indicated). Dark-field photos are shown to better visualize silver grains, which are black in bright field but white in dark field. NPR-B transcripts appear to be localized to a region just outside of the VZ, where post-mitotic neurons undergo differentiation (arrows). Intense signal is present in the cranial trigeminal ganglion. C is a dark-field view of a section parallel to the spinal cord that shows NPR-B expression in bilateral segmental DRG. 4V = fourth ventricle; 5g = trigeminal ganglia; DRG = dorsal root ganglia.

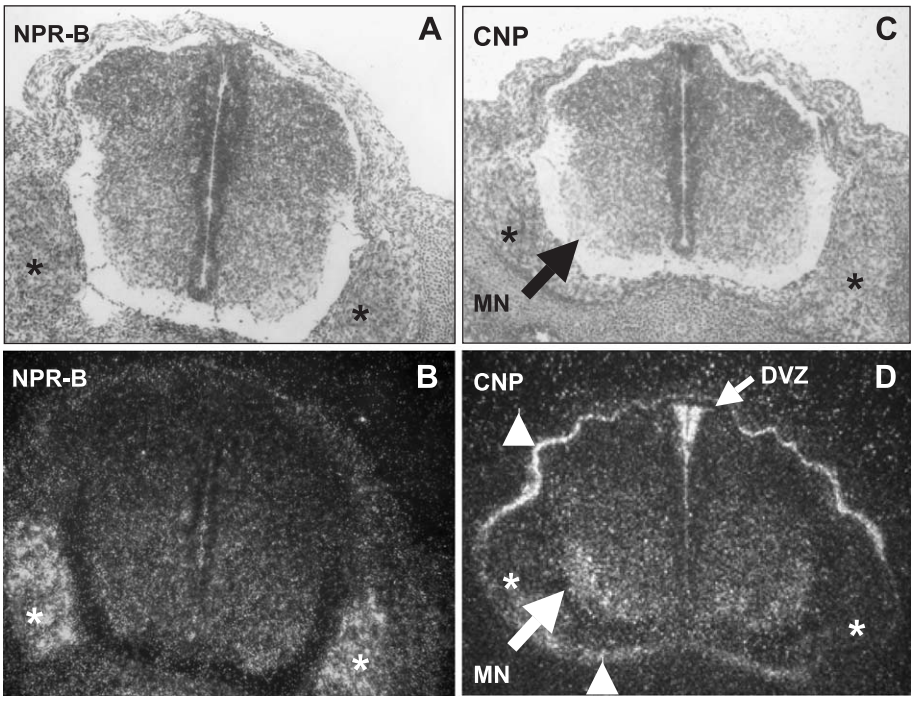

Fig. 2. NPR-B (A, B) and CNP (C, D) gene expression in the area of the spinal cord in E14.5 mice. A and C are bright-field micrographs; B and D are the same sections shown in dark field. DRG are indicated by asterisks (*) in all panels, and are strongly labeled by the NPR-B riboprobe in A and B. A population of CNP-mRNA-hybridizing motor neurons is indicated by large arrows in C and D. Arrowheads in D point to a band of CNP mRNA-positive cells that surrounds the spinal cord and DRG and may correspond to dura and/or chondrocytes in the growth plate of primordial bone (see text). Small arrow in D points to an area of intense CNP gene expression in the dorsal VZ (DVZ) of the spinal cord.

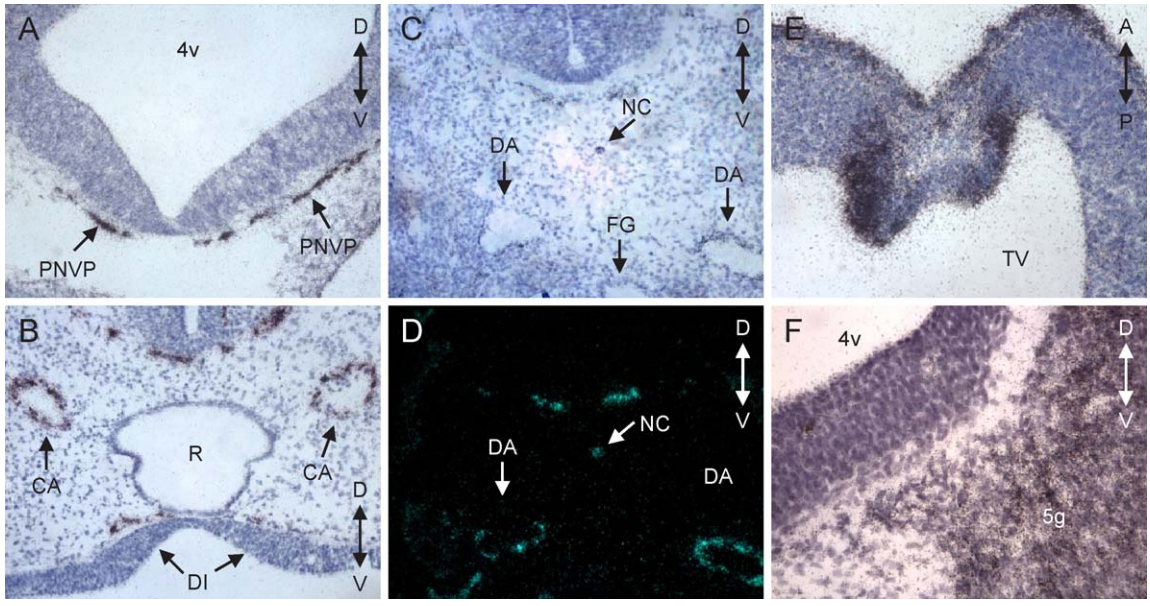

Fig. 3. NPR-C receptor gene expression in E10.5 mouse embryos. All sections are coronal, with dorsal/ventral and anterior/posterior orientations indicated. Images are bright-field photos at different levels of the embryo, except for panel D, which is a dark-field view of C. Sections at the level of the pons (A), diencephalon (B), and spinal cord (C, D) demonstrate NPR-C transcript expression in perivascular plexuses and major blood vessels. Intense signal is present in or near the forebrain lamina terminalis or emerging choroid plexus (E) and the pontine trigeminal ganglion (F). 4V = fourth ventricle; PNVP = perineural vascular plexus; CA = carotid artery; R = opening to Rathke's pouch; NC = notochord; DA = dorsal aorta; FG = pharyngeal region of the foregut; TV = telencephalic vesicle; 5g = trigeminal ganglia.

mitotic neurons are undergoing differentiation (Figs. 1A, B). Very high NPR-B expression was observed in several developing ganglia at E10.5, including the cranial trigeminal ganglion (Figs. 1A, B) and the segmental dorsal root ganglia (DRG) (Fig. 1C). This intense gene expression in sensory ganglia was maintained until at least E14.5 (Figs. 2A, B).

NPR-C receptor gene expression

The NPR-C gene was highly expressed at E10.5 in the area surrounding the neural tube (Fig. 3A), presumably the perineural vascular plexus, in the major arteries (Figs. 3B–D) and to a lesser extent in the veins (not shown). Expression was also observed in the notochord (Figs. 3C, D), a structure well known to produce diffusable factors, such as sonic hedgehog, responsible for dorsal/ventral pattering of neurons and glia in the neural tube. NPR-C expression was also observed in the heart and surface ectoderm (not shown).

In the E10.5 nervous system, NPR-C expression was detected in (or near) the lamina terminalis or emerging choroid plexus (Fig. 3E), and in several cranial ganglia, such as the trigeminal (Fig. 3F). At E12.5, NPR-C gene expression continued to be observed in the perineural vascular plexus and surface ectoderm (Figs. 4A, B), the segmental DRG (Figs. 4C, D), and the trigeminal ganglia (Figs. 4E, F). Analysis of sagittal sections surrounding the trigeminal ganglia also revealed intense signal in the ophthalmic, maxillary, and mandibular nerves, very likely reflecting Schwann cell expression (Figs. 4E, F). New sites of CNS gene expression at E12.5 included the roof plate and two bilateral stripes that surrounded the floor plate (Figs. 5A, B), and the choroid plexus (not shown). The bilateral stripes of signal surrounding the floor plate may represent NPR-C gene expression in glial precursors (Pringle and Richardson, 1993), or one of the NKX or other homeobox gene expression domains that give rise to specific subclasses of ventral neurons (Tanabe and Jessell, 1996). Small discrete clusters of NPR-C gene transcripts were observed within the forebrain neuroepithelium, presumably over invading capillaries, whereas broad expression was seen in overlying surface ectoderm (Figs. 5C, D). At E14.5, NPR-C gene expression was still high in the DRG, but transcripts in the spinal cord roof plate and surrounding the floor plate could no longer de detected (data not shown).

Natriuretic peptide gene expression

Sagittal sections at E12.5 revealed CNP peptide gene transcripts at all levels of the CNS caudal to the mesencephalon (Figs. 6A, B), with more restricted dorsal localization in the spinal cord. A very similar pattern of expression was observed at E10.5 (data not shown). Transverse sections of spinal cord revealed that CNP transcripts were localized in the dorsal and intermediate ventricular zone (VZ) and possibly in more lateral regions (Figs. 6C, D). Later, at E14.5, CNP expression was still observed at high levels in the VZ, although new signal was seen laterally in cells in the dorsal hindbrain, reflecting either

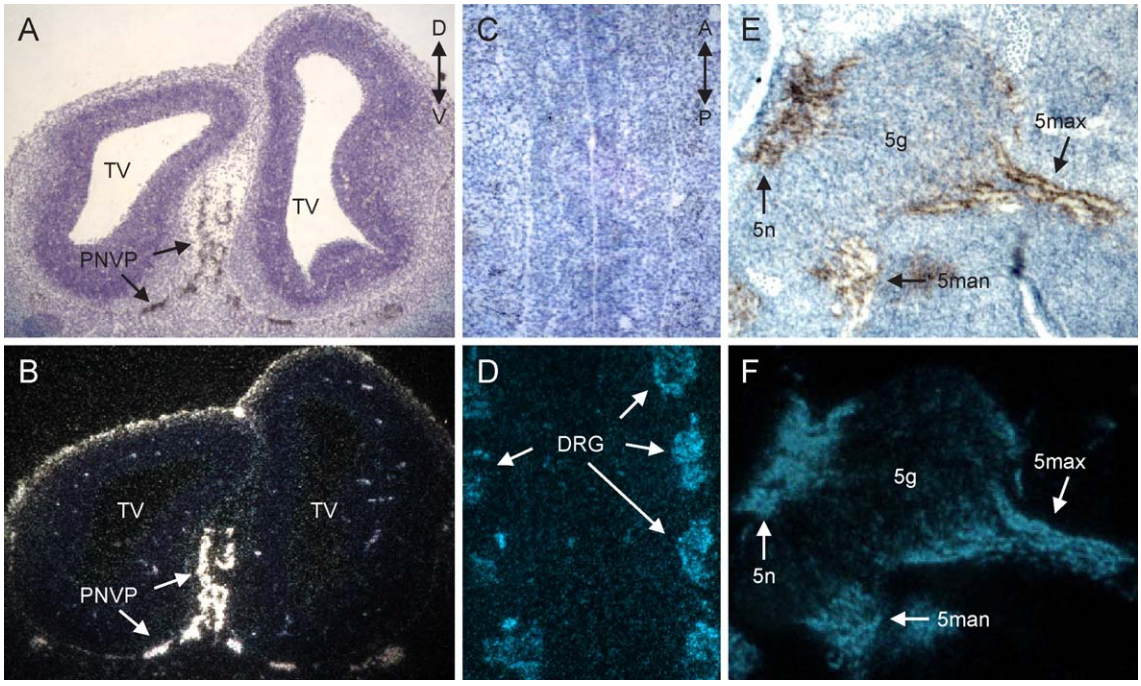

Fig. 4. NPR-C receptor gene expression in E12.5 mouse embryos. Panels A and B are coronal sections; C and D are in a plane parallel to the spinal cord. B, D, and F are dark-field views of A, C, and E, respectively. NPR-C transcripts are intensely expressed in the vascular plexus (A, B), in segmental DRG (C, D), and trigeminal nerve branches, including the mandibular and maxillary (E, F). Dorsal/ventral (D/V) and anterior/posterior (A/P) orientations are indicated. TV = telencephalic vesicle; PNVP = perineural vascular plexus; DRG = dorsal root ganglia; 5n = trigeminal nerve; 5g = trigeminal ganglia; 5max = maxillary nerve; 5man = mandibular nerve.

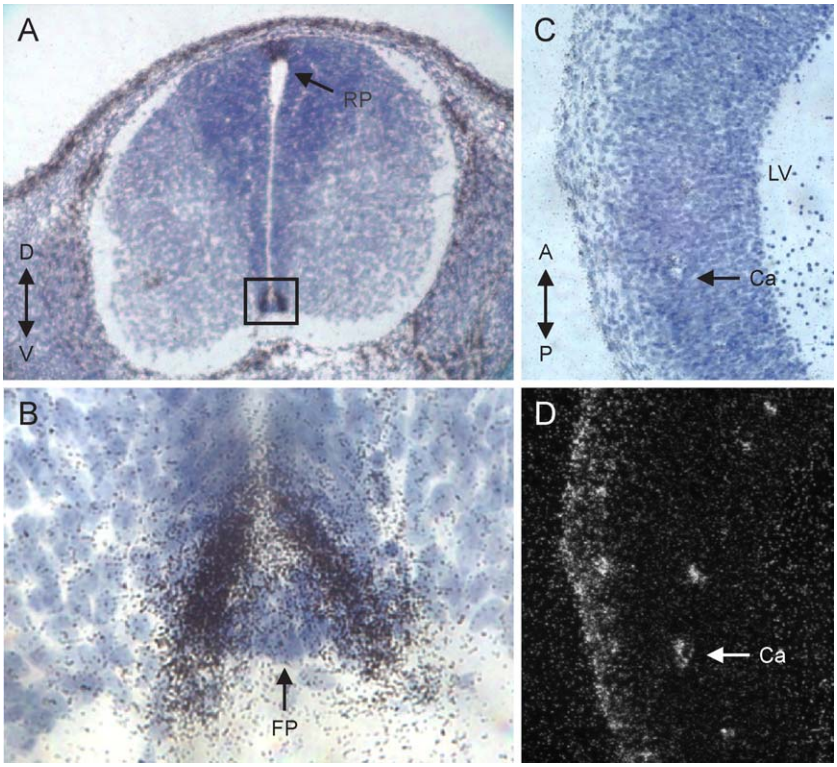

Fig. 5. NPR-C receptor gene expression in the spinal cord and telencephalon in E12.5 mouse embryos. All panels are transverse sections. New gene expression is localized to the roof plate and in stripes surrounding the floor plate (A, B). B is a magnification of the area within the rectangle in A. Signal is also present in invading forebrain capillaries, and in overlying surface ectoderm (C, D). D is a dark-field view of C. RP = roof plate; FP = floor plate; LV = lateral ventricle. Arrows in C and D point to one of several capillaries (Ca) shown in this section of the telencephalon.

new cellular expression or radial cell migration (Figs. 6E, F). Specific hybridization signals were also observed in cells surrounding the neural tube, apparently the dural layer of the meninges. In the area of the spinal cord, CNP mRNA was again detected in the dorsal VZ, but now also in the ventrolateral cord, where motor neurons are localized (Figs. 2C, D). CNP mRNA was also detected in a band of cells surrounding the spinal cord, possibly the dura as observed in the hindbrain (Fig. 6F), and adjacent to the DRG which express both NPR-B and NPR-C receptor mRNAs (see above). Alternatively, this cellular band may represent the perichondrial lining of the spinal canal, which at this stage, participates in appositional growth of the vertebral column. In this regard, CNP is known to be expressed in chondrocytes in the growth plate of developing skeletal bones (Chusho et al., 2001). In contrast to CNP, ANP and BNP gene transcripts were not detected in the nervous system, but were expressed at high levels in the heart (data not shown).

Action of natriuretic peptides on neural cells in culture

Effects of natriuretic peptides on DNA synthesis in DRG cell cultures

The foregoing expression studies indicated that both NPR-B and NPR-C receptor transcripts are present in cranial and dorsal root sensory ganglia, raising the possibility that the natriuretic peptides elicit ontogenetic effects. To begin defining activities, E14.5 rat DRG were dissociated and plated in fully defined medium containing various peptide concentrations, and assessed for effects on DNA synthesis. Cells were incubated in the absence of the mitogen/survival factor, insulin, and the neurotrophin, nerve growth factor (NGF), to enhance our detection of possible stimulatory activity which may be masked by other mitogens. Significantly, insulin family members are potent mitogens for peripheral ganglion cells, whereas axons of NGF-dependent sensory neurons possess well-characterized Schwann cell mitogenic activity (Ratner et al., 1985; Recio-Pinto et al., 1986). Incorporation of [^{3}H]thymidine during a terminal 4 h of a 24-h incubation was assessed. CNP elicited a 2-fold increase in DNA synthesis, with an EC_{50} of 10^{-9} M and peak activity at 10^{-8} M (Fig. 7A), indicating that DRG cells possess functional receptors. Significantly, $desANP_{4\text{-}23}$, a ligand relatively selective for NPR-C receptors, exhibited far greater potency, with an EC_{50} of approximately 10^{-10} M and a peak effect at 10^{-9} M (Fig. 7B). In contrast, while ANP also increased DNA synthesis, effects were not observed until a dose of 10^{-7} M (revealed by ANOVA; Fig. 7C) with an EC_{50} of approximately 10^{-8} M (compared to Control using Student's *t* test). The high potency of both CNP and $desANP_{4\text{-}23}$ in the mitogenic stimulation of DRG cells suggests actions at both NPR-B and NPR-C receptors, consistent with their expression in the embryonic DRG (Figs. 1C, 2A, B, 4C).

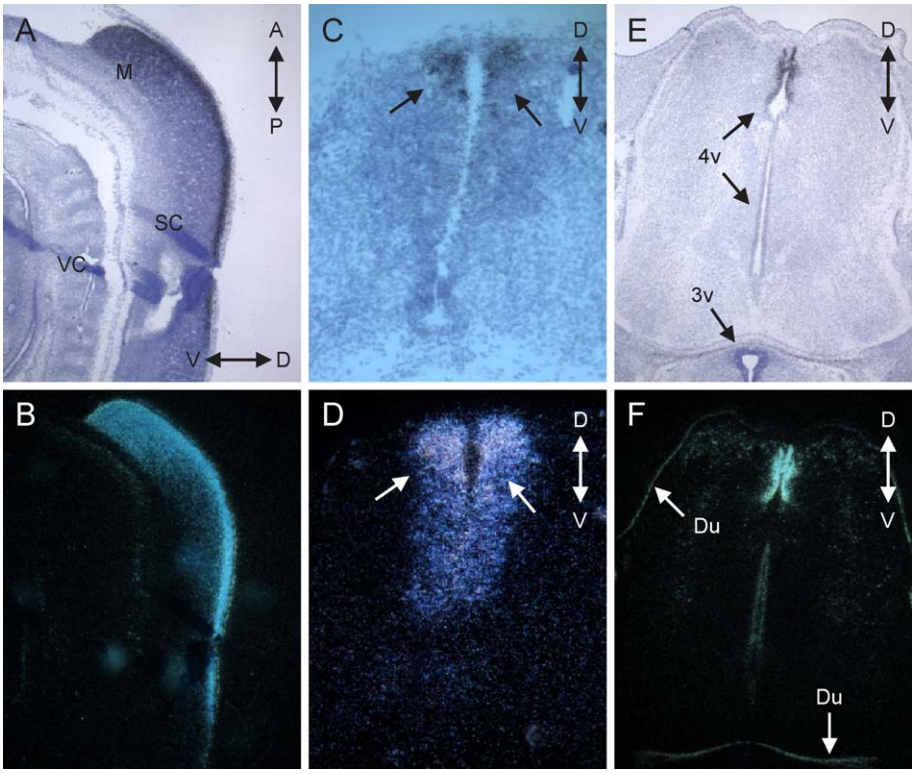

Fig. 6. CNP gene expression in E12.5 (A–D) and E14.5 (E–F) mouse embryos. CNP gene transcripts at E12.5 are present from the mesencephalon to the caudal spinal cord (A–D). CNP mRNA is particularly abundant in the dorsal region, including the VZ, especially in the caudal portions of the spinal cord (C, D). At E14.5 (E, F), CNP expression is maintained in the VZ and is present in scattered cells of the dorsolateral tegmentum. B, D, and E are bright-field views of A, C, and E, respectively. A and B are sagittal sections. C–F are coronal sections. M = medulla; SC = spinal cord; 4v = fourth ventricle; 3v = third ventricle. Du = cells associated with the dura. Arrows in C point to abundant hybridizing transcripts in the VZ of the dorsal part of the spinal cord.

In light of natriuretic peptide stimulatory activity, we next determined whether 10^{-7} M CNP, the most effective peptide and concentration in the above cultures, elicited mitogenic effects in the presence of neurons, a condition more relevant to neuron–glial interactions occurring in vivo. Neuron survival was maintained by adding the neurotrophin, NGF, at 3 ng/ml. CNP stimulated DNA synthesis by approximately ~40% in DRG cultures maintained in the presence of NGF (Con = 5896 ± 680; CNP = 8274 ± 96; mean cpm ± SEM; $P < 0.02$; $N = 7$). Furthermore, when neuron survival promotion was diminished by reducing NGF levels 3-fold, less neurons were present (data not shown) and CNP elicited a 2-fold increase in DNA synthesis (Con = 2510 ± 30; CNP = 5134 ± 65; $P < 0.001$), consistent with previous reports that neurons provide a mitogenic stimulus for Schwann cells in vitro (Ratner et al., 1985). In previous work, we have found that the presence of one mitogen can mask effects of another, apparently due to limited numbers of precursors available to enter the cell cycle (DiCicco-Bloom et al., 1993, 2000). Regardless, in the presence of neuron–glial interactions that occur in vivo, CNP may serve a mitogenic function.

Characterization of mitotic cells in DRG cultures

To characterize cells responsive to the natriuretic peptides, we used immunocytochemistry to detect glial and neuronal markers, as well as nuclear BrdU mitotic labeling, in two incubation paradigms. Cells were incubated in the presence of NGF in control or CNP-containing medium for 1–3 days, and fixed after a 4-h BrdU pulse to define the mitotic labeling index (LI). Alternatively, cells were incubated for 3 days in the continuous presence of BrdU to characterize the neural traits expressed by cells undergoing DNA synthesis. In preliminary studies performed without NGF addition, a condition yielding few neurons (approximately 10%) based on morphology and TuJ1 expression, we observed bipolar cells at 24 h expressing glial markers, including the low-affinity NGF receptor, p75, as well as S100, as reported previously (Jessen and Mirsky, 1991; Lemke and Chao, 1988; Mata et al., 1990; Taniuchi et al., 1988). When incubated for 3 days, many GFAP-positive cells were found (data not shown), consistent with Schwann cell maturation in the absence of neurons (Jessen and Mirsky, 1984).

To characterize mitogenic effects, DRG cells were incubated in NGF-containing medium, in the absence (Control) or presence of CNP (10^{-7} M) and the LI was determined. CNP elicited a 2-fold increase in mitotic cells at 24 h, increasing the LI from 10% in control to 21% in the presence of the peptide (Fig. 8). The labeling index increase elicited by CNP was consistent with the peptide's effects on [^{3}H]thymidine incorporation (Fig. 7). Furthermore, there was a pro-

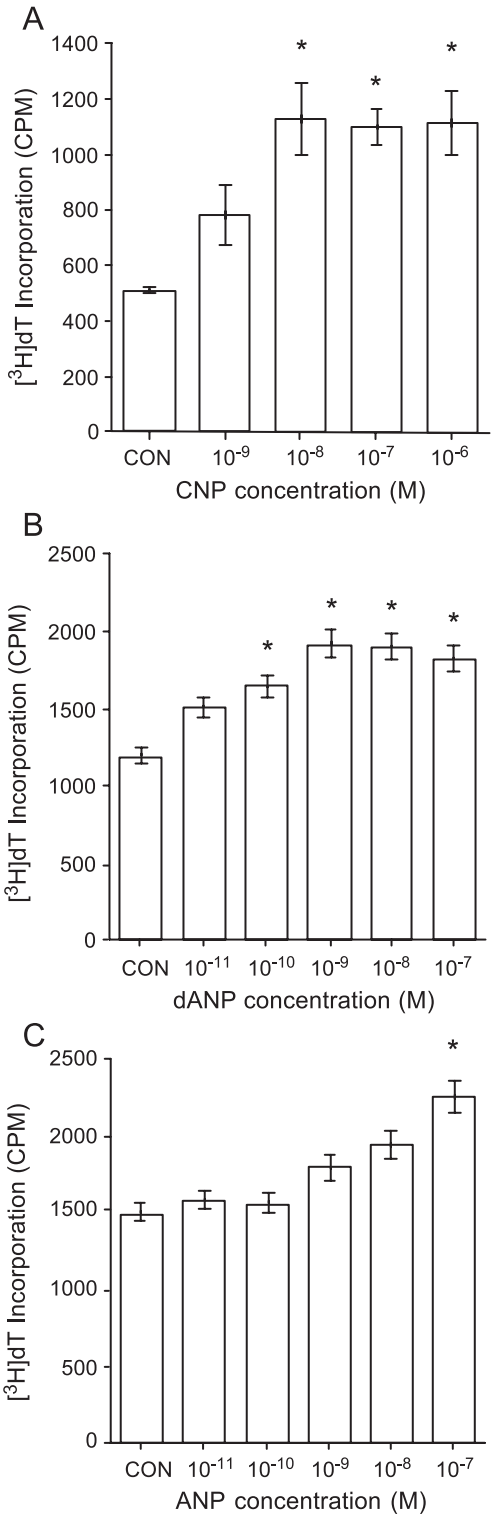

Fig. 7. Effects of natriuretic peptides on [^{3}H]thymidine incorporation in E14.5 rat DRG cell cultures. Dissociated DRG cells were plated in fully defined medium in the absence of insulin and NGF. Cells were incubated for 24 h in control medium or medium containing various peptide concentrations and incorporation of [^{3}H]thymidine during a 4-h terminal pulse was assessed. Data represent incorporation into six to eight culture wells per group from two to three separate experiments and are expressed as cpm ± SEM. *$P < 0.05$. dANP = des ANP_{4-23}.

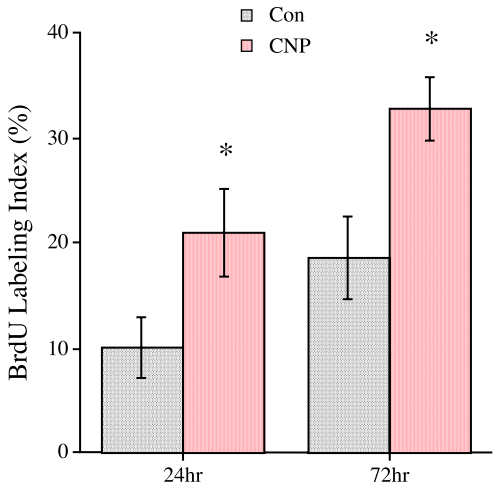

Fig. 8. Effects of CNP on the mitotic labeling index (LI) in E14.5 rat DRG cell cultures. Cells were incubated in medium containing NGF (3 ng/ml) in the absence (Control) or presence of CNP (10^{-7} M) for 1–3 days and fixed after a terminal 4-h pulse with BrdU and assessed by immunocytochemistry. Data are expressed as the percentage ± SEM of cells exhibiting BrdU nuclear labeling. It should be noted that BrdU labeling was not observed in cells exhibiting a neuronal morphology or TuJ1 expression, consistent with previous evidence that neurogenesis is complete in rat DRG by E13 (Lawson et al., 1974).

gressive expansion of the mitotic populations by 3 days, as the LI was 19% in control and 33% in CNP-treated cultures (Fig. 8), suggesting sustained peptide mitogenic activity.

To identify cells undergoing mitosis, we double labeled cells exposed continuously to BrdU for 3 days for several glial markers, including p75, S100 and GFAP. The majority of mitotic cells at 3 days, identified by nuclear BrdU labeling, also expressed glial marker GFAP (90 ± 4.0%). As shown at low magnification (Figs. 9A–C), many of the cells identified by phase microscopy at 3 days (Fig. 9A) exhibit BrdU (+) nuclei (Fig. 9B) which co-label with cellular GFAP signal (Fig. 9C). At higher magnification (Figs. 9D–F), BrdU-positive nuclei frequently associate with GFAP signal, which, on close inspection, appear as extended cytoplasmic filaments in these elongated cells. Extended BrdU-labeled cells also co-label with glial marker S100 which appears in either cytoplasmic or nuclear compartments (Figs. 9G–L). Double labeling analysis indicated that the majority of cells incorporating BrdU during 3 days of incubation expressed the Schwann cell markers, p75 (77 ± 4.9%) and S100 (67 ± 2.8%) along with the GFAP described above. These observations suggest that CNP exhibits mitogenic activity in mixed DRG cell cultures, stimulating proliferation of Schwann cells over several days. In contrast, no neuronal cell nuclei labeled with BrdU in these studies, consistent with previous evidence that neurogenesis in rat DRG is complete by E13 (Lawson et al., 1974).

Natriuretic peptide effects on DRG neuron survival

To examine potential trophic activity, we assessed the effects of natriuretic peptides on survival of neuronal cells,

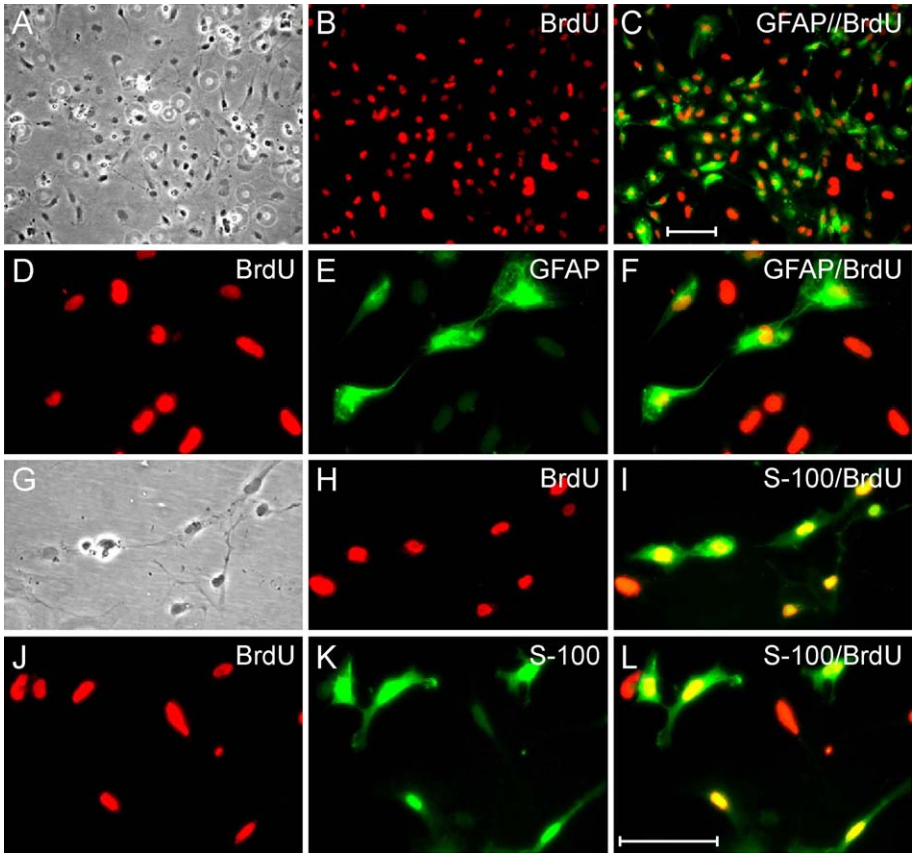

Fig. 9. Immunocytochemical characterization of mitotic cells in DRG cultures. DRG cells were incubated for 3 days in medium containing NGF (3 ng/ml), CNP (10^{-7} M), and BrdU (10 μM), and then fixed and processed for glial markers (GFAP, S100; green) and BrdU (red) double immunocytochemistry. (A–C) At low magnification, numerous cells observed under phase microscopy (A) exhibit nuclear BrdU labeling (B) that also co-localizes with GFAP (C). At higher magnification, BrdU-positive nuclei (D) occur in extended cells with GFAP-positive cytoplasmic filaments (E), with co-localization shown in F. (G) A series of flat, extended cells observed under phase (G) exhibit nuclear BrdU (H) that co-localizes with Schwann cell marker, S100 (I), which is found in both cytoplasmic and nuclear compartments. (J–L) Another series of cells exhibit typical bipolar and extended morphologies of Schwann cells that double label for BrdU and S100, similar to that seen for GFAP (A–C). Scale bar = 100 μm in A–C, and 50 μm in D–L.

defined by morphology and βIII tubulin (TuJ1) expression in the absence of the known trophic factors, insulin and NGF. CNP had modest effects on neuron survival at 24 h, increasing neuron number by only 50%, whether expressed in absolute terms (Control = 23 ± 2.7, CNP = 37 ± 3.7, mean cell number/3% dish area ± SEM; $N = 6$; $P < 0.0042$) or as a percentage of total cells in the dish (Control = 11 ± 1.1%, CNP = 16 ± 1.0%, mean percentage ± SEM; $N = 6$; $P < 0.0015$). This effect is relatively small compared to the robust changes elicited by insulin and NGF, conditions in which neurons comprised 51 ± 3.3% of the cells in the dish (mean percentage ± SEM; two experiments, $N = 8$), consistent with previously reported neurotrophic activities (Recio-Pinto et al., 1986).

Natriuretic peptide effects on process outgrowth of DRG sensory neurons

The foregoing mRNA expression studies suggest that DRG neurons express NPR-B (Figs. 1C, 2A, B), while the receptor's preferred ligand, CNP, is present in regions where sensory neuron terminals make targeting decisions, including the dura/developing vertebral column, the VZ of the dorsal spinal cord, and the ventrolateral spinal cord motoneuron pool (Figs. 2C, D). We thus hypothesized that CNP acts as an axon guidance signal, eliciting terminal extension, repulsion or attraction. In this respect, there is strong evidence that cGMP, the primary second messenger for NPR-B, is an important mediator or modulator of the effects of guidance molecules such as netrin and semaphorins (Nishiyama et al., 2003; Song et al., 1998). To examine the effects of natriuretic peptides on DRG neuron process elaboration, we cultured cells in the presence of insulin and NGF, which markedly enhanced neuron survival, and employed low-cell culture density (5×10^4 per 35-mm dish) to allow single cell analysis. The addition of the natriuretic peptides in the presence of NGF had no effects on neuronal survival (data not shown). To define effects on neurite outgrowth, we measured the lengths of the longest process, visualized by TuJ1 staining, on each neuron. The overall effects of natriuretic peptides on process growth in the culture population are depicted in Fig. 10A, whereas statistical significance was determined using the population means (Fig. 10B).

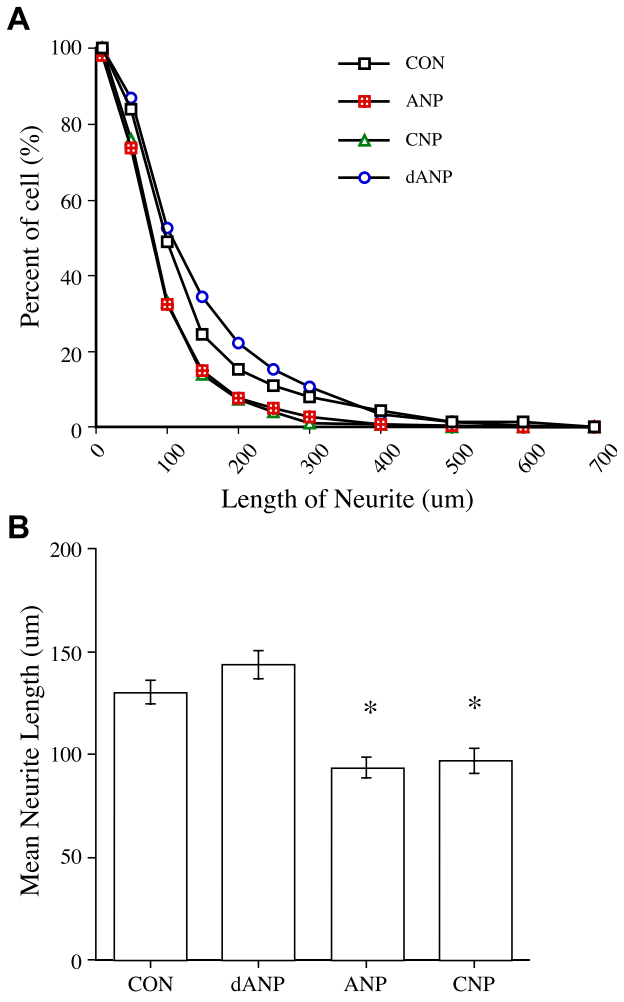

Fig. 10. Effects of the natriuretic peptides on the length of sensory neuron processes. (A) The overall effects of the peptides on process growth in the population are shown. Low-density DRG cultures containing trophic factors, insulin (10 μg/ml) and NGF (10 ng/ml), were incubated in control medium or medium containing natriuretic peptides (10^{-7} M), and fixed at 24 h for TuJ1 immunocytochemistry and process length assessment. Data were obtained from three separate experiments, each consisting of two to three dishes per group ($N = 6–8$ dishes), and 150–350 neurons in total for each condition were assessed. Cell fields at 20× magnification were randomly photographed and length (μm) of the longest neurite was measured using NIH Image (object-image) software. Data are expressed as the percentage of the cell population with a neurite equal to or greater than a given length. Both ANP and CNP reduced neuronal process length, appearing to shift the curves to the left. The curves for these peptides are almost superimposed. The apparent right shift induced by dANP = des ANP_{4-23} (dANP) was not statistically significant (see B). (B) The mean length of the longest neurite was reduced by approximately 30% in the presence of 10^{-7}M ANP or CNP. *$P < 0.001$. ANOVA followed by Scheffe F test.

$DesANP_{4-23}$ had a tendency to increase neurite outgrowth (Fig. 10A), though changes were not statistically significant (Fig. 10B). In contrast, both ANP and CNP appeared to induce a general shift to shorter processes on the cells (Fig. 10A), yielding a significant reduction in mean process length in the population (Fig. 10B). Thus, in the presence of either ANP or CNP, only approximately 30% of cells had processes greater than 100 μm, whereas 50% extended this distance in control or $desANP_{4-23}$-treated cultures. Conversely, twice as many cells had processes of ≥200 μm in controls as in the presence of ANP or CNP, whereas few cells extended to 300 μm when exposed to the peptides. Overall, ANP and CNP reduced mean neurite length by 25–30% (Fig. 10B). The neurite inhibitory effects of both ANP and CNP, without effect of the NPR-C selective agonist, $desANP_{4-23}$, suggest that the peptides elicited effects via NPR-B receptor, which is highly expressed in the DRG at this age (Figs. 2A, B).

Natriuretic peptide effects on DNA synthesis in E10.5 mouse hindbrain neuroblast cultures

Our expression studies indicate that CNP is expressed in the dorsal VZ of the hindbrain and spinal cord (Figs. 2C, D, 6A–F), whereas NPR-B gene is expressed transiently at E10.5 in cells just outside the proliferative zone (Fig. 1). This complementary localization pattern raises the possibility that VZ-derived peptide plays a role in controlling precursor proliferation and/or differentiation. To determine the effect of natriuretic peptides on neuroblast proliferation, we measured [^{3}H]thymidine incorporation in dispersed cell cultures from this region in E10.5 embryos using methods we reported previously (Lelièvre et al., 2002; Waschek et al., 1998). These proliferating cultures consist predominantly of neural progenitors over the culture period, with approximately 1–5% of cells staining for TuJ1 (β-tubulin), and no cells staining for glial markers (A2B5, CNPase, GFAP, GalC, NG2) or vimentin (data not shown). CNP potently inhibited DNA synthesis in these cultures, with an EC50 of approximately 0.1 nM (Fig. 11). ANP was clearly less potent than CNP, whereas $desANP_{4-23}$ exhibited activity between CNP and ANP. The potent inhibitory action of

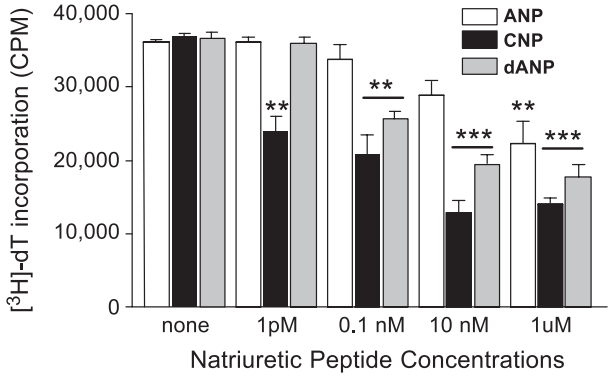

Fig. 11. Inhibition of neuroblast proliferation by natriuretic peptides. Cells were isolated from the hindbrain portions of the E10.5 mouse neural tubes and cultured in Neurobasal™ medium containing 1% FBS, 1× N2 supplement, and 1 ng/ml FGF-2, and treated on the following day for 24 h with natriuretic peptides at concentrations from 10^{-12} to 10^{-6} M. [^{3}H]thymidine was added along with fresh drug for the last 6 h of treatment. Mean [^{3}H]thymidine incorporation values (±SEM, $n = 3$ determinations) at various concentration of added peptides are shown. Values were fit using Graphpad Prism™ (ISI software). Data shown are representative of three independent experiments. Statistical analyses using analysis of variance (ANOVA) followed by Newman–Keul's test revealed significant differences between controls and treatments at ***$P < 0.001$ or **$P < 0.05$.

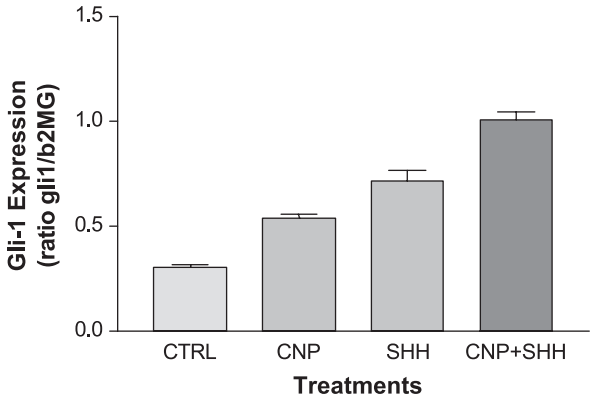

Fig. 12. Shh and CNP regulation of gli-1 gene expression in E10.5 hindbrain cultures. Cells were isolated from E10.5 mouse neural tubes and cultured as described in the previous figure and were treated for 8 h with 10 nM CNP, 700 ng/ml Shh, or the combination of CNP and Shh. Total RNA was isolated, reverse transcribed, and analyzed for gli-1 and β_2-microglobin (b2MG) mRNA by real-time PCR. Samples were measured in triplicate, and the experiment was repeated twice. Statistical analysis (ANOVA) revealed treatment effects are significantly different than control at $P < 0.05$.

CNP (which acts efficiently only on NPR-B and NPR-C) is consistent with in situ hybridization studies showing the presence of NPR-B gene expression in this embryonic brain region. The fact that the NPR-C-specific analog desANP$_{4\text{-}23}$ also inhibited DNA synthesis indicates that at least some of the antiproliferative actions may be mediated through a NPR-C- or NPR-C-like receptor. Although we could not localize NPR-C mRNA in the embryonic hindbrain by in situ hybridization, we previously detected its expression in this region by RT-PCR (Lelièvre et al., 2001). The data are consistent with the possibility that CNP acts as an antimitotic agent and/or promotes the differentiation of E10.5 hindbrain precursors.

Another potential activity of CNP at early stages of neural development would be to regulate the differentiation of cells by modulating the activity of patterning factors such as sonic hedgehog (Shh). There is precedence for factor interactions in that "chick" natriuretic peptide was able to enhance the ability of sonic hedgehog (Shh) to induce ventral phenotypes in chick dorsal neural plate explants (Robertson et al., 2001). We thus determined if Shh and CNP interact in mouse embryonic hindbrain cultures by analyzing the expression of the Shh target gene, gli-1. We found that both CNP and Shh increased the expression of gli-1, and that the combined effect was additive (Fig. 12). Thus, CNP may modify proliferation and patterning events in the E10.5 mouse hindbrain, acting across adjacent developmental compartments.

Discussion

Our observations indicate that the natriuretic peptides and their receptor subtypes exhibit region-specific expression patterns in the embryonic central and peripheral nervous systems, suggesting that the peptide systems play roles in regulating neural development. Based on these expression patterns, we employed both peripheral (PNS) and central nervous system (CNS) cell culture models to study effects on precursor cell mitosis, survival, neurite outgrowth, and gene expression. The peptides elicited both stimulatory and inhibitory mitogenic effects in both neuronal and glial precursors, as well as altered neuronal survival and process elaboration. The actions were natriuretic peptide subtype-selective and region-specific.

Our localization studies provide detailed information on the location as well as subtypes of natriuretic peptide receptors expressed in the early embryo. In general, NPR-B and NPR-C receptor gene expression patterns in the hindbrain, spinal cord, and peripheral ganglia were consistent with earlier reports of ligand binding during development. Four different groups previously characterized ANP binding sites using autoradiography, demonstrating high-affinity natriuretic peptide binding sites in the developing rat brain and associated blood vessels (Brown and Zuo, 1995; Scott and Jennes, 1991; Tong and Pelletier, 1990; Zorad et al., 1993). In the most comprehensive report, Scott and Jennes (1991) observed that ^{125}I-ANP binding sites at E14 were localized to the developing blood vessels and the vascular plexus around the developing brain and in the meningeal layer. In the spinal cord, bilateral accumulations of silver grains were observed adjacent to (or over) the floor plate and in the roof plate. Radioligand binding sites were also observed over the dorsal roots, the DRG and peripheral nerves, leading investigators to conclude that receptors were localized to Schwann and satellite cells, and possibly sensory neurons. ^{125}I-ANP binds primarily to NPR-A and NPR-C with high affinity, and thus may not detect NPR-B in receptor autoradiographic studies. Because we detected intense NPR-C gene expression (and not NPR-A mRNA) in all these sites, it seems likely that the ^{125}I-ANP binding sites reported in that study corresponded to NPR-C. However, the current in situ hybridization studies also demonstrated NPR-B transcripts within the developing brain in addition to peripheral ganglia.

The natriuretic peptides elicited diverse ontogenetic effects in PNS and CNS cultures, including bidirectional control of proliferation, survival promotion, and inhibition of neurotrophin-induced neurite outgrowth. In E14.5 DRG cultures, both CNP and NPR-C selective ligand, desANP$_{4\text{-}23}$, stimulated Schwann cell mitosis, identified by double immunocytochemistry, consistent with the expression of NPR-C receptors in developing ganglia and peripheral nerves. The intense CNP gene expression surrounding the spinal cord and DRG, apparently localized to the dura and/or vertebral perichondrium, suggests that the encoded peptide may be released locally to influence PNS development. Previously defined Schwann cell mitogens include PDGF, FGF, VEGF, and neuregulins (Eccleston et al., 1990; Sondell et al., 1999; Tabernero et al., 1998; Verdi et al., 1996), which generally employ tyrosine kinase cascades to enhance proliferation.

A different profile of natriuretic peptides, ANP and CNP, but not desANP$_{4-23}$, inhibited process outgrowth of dorsal root sensory neurons. The fact that this effect was resistant to the NPR-C specific analog desANP$_{4-23}$, and that we detected NPR-B transcripts in sensory ganglia, suggests that this action is mediated by NPR-B. The finding that CNP inhibited sensory neurite outgrowth suggests that the peptide may serve as a repulsive guidance cue under some circumstances, an activity it may share with other known signaling molecules. It is notable that CNP gene transcripts in the embryo were detected in areas which do not receive major sensory neuron innervation, including the developing vertebral bone and/or dura, the dorsal spinal cord VZ, and the ventral motor horn. Moreover, while further characterization is necessary, this activity may be involved in regulating growth of sensory axon terminals into blood vessel walls (which might contain natriuretic peptides from the blood or in endothelial cells). In this regard, cGMP, which may be induced by natriuretic peptides either directly via NPR-A or NPR-B (Anand-Srivastava and Trachte, 1994), or indirectly via NPR-C (Murthy et al., 2000), may have a role in growth cone guidance (Song et al., 1998).

In contrast to the mitogenic action of natriuretic peptides on Schwann cells, which appeared to be mediated primarily by NPR-C, natriuretic peptides appeared to inhibit the proliferation of cultured E10.5 mouse hindbrain neural precursors via NPR-B. The evidence for this is that CNP was more potent than desANP$_{4-23}$, and that NPR-B and not other receptor mRNA was detected in the E10.5 hindbrain. Moreover, the finding that NPR-B gene transcripts were detected in cells just outside the VZ suggests that this receptor gene becomes expressed just as cells leave the cell cycle. We speculate that CNP, produced in the actively proliferating cells in the VZ, reinforces primary signals or mechanisms that induce cells to leave the cell cycle.

We also found that CNP synergized with Shh to induce the expression of the Shh target gene gli-1 in hindbrain cultures. Robertson et al. (2001) recently reported that chick natriuretic peptide promoted the ventralizing action of Shh in chick dorsal spinal cord explants, an effect that could be mimicked by cGMP analogs. Conversely, Shh signaling is well known to be antagonized by cAMP and protein kinase A in numerous developmental processes. Based on this and results in mouse hindbrain cultures and chick explants, we propose that cGMP and cAMP constitute opposing signals that modulate Shh signaling. In this regard, another neuropeptide, pituitary adenylyl cyclase activating peptide (PACAP) and its PAC$_1$ receptor are expressed in the mouse E10.5 hindbrain, and PACAP activation of cAMP inhibits expression of gli-1 in these neural precursors (Waschek et al., 1998).

The developmental action of natriuretic peptides appears to be pleiotropic, reminiscent of that of several growth factors, neurotrophins, and cytokines, as well as PACAP (reviewed in Waschek, 2002). The pleiotropic activity of the natriuretic factors is further indicated by their cell type-specific actions, stimulating and inhibiting DNA synthesis in Schwann cells and embryonic hindbrain precursors, respectively (shown herein), and inhibiting proliferation of olfactory precursors (Simpson et al., 2002) and astrocytes (Levin and Frank, 1991). Natriuretic peptides have also been reported to stimulate DNA synthesis in embryonic cardiomyocytes (Koide et al., 1996), and to inhibit the proliferation of several other non-neural cell types, including vascular smooth muscle cells (Cahill and Hassid, 1991), kidney mesangial cells (Appel, 1990), chondrocytes (Hagiwara et al., 1996), and osteoblast-like cells (Hagiwara et al., 1994).

Given that NPR-C is classically known for its role in clearing excess natriuretic peptides, it was of interest that this receptor appeared to mediate the mitogenic action of natriuretic peptides in Schwann cells. This provides further support for the idea that this receptor has a signaling function (Anand-Srivastava and Trachte, 1994; Murthy et al., 2000). Of additional interest was that the same NPR-C-specific agonist that stimulated Schwann cell proliferation in our studies was found to potently inhibit the proliferation of astrocytes (Prins et al., 1996). Further study may reveal how this receptor can mediate opposing proliferative actions on these two glial phenotypes.

The detection of NPR-C and NPR-A in blood vessels in the early embryonic brain may also be significant from a developmental perspective. As discussed above, natriuretic peptides regulate the proliferation of vascular smooth muscle, and may be involved in other aspects of angiogenesis in the brain, such as recruitment of vascular smooth muscle cells (Ikeda et al., 1997; Kohno et al., 1997), and choroid plexus formation. The peptides are also potentially involved in establishment of blood–brain and blood–nerve barriers.

One puzzling result reported here is that we could not detect gene expression for any of the known natriuretic peptide receptors in the embryonic forebrain, nor did we detect peptide effects in our well-characterized embryonic cortical precursor culture model (unpublished results; Lu and DiCicco-Bloom, 1997; Nicot and DiCicco-Bloom, 2001), though further study is warranted. Three other groups focused specifically on rat brain and reported a highly abundant presence of ^{125}I-ANP binding sites in the telencephalon, localized to the VZ (Brown and Zuo, 1995; Tong and Pelletier, 1990; Zorad et al., 1993). These sites could be detected as early as E13 (Tong and Pelletier, 1990), corresponding approximately to E12 in mice. Radioligand displacement studies indicated that these sites appeared to be "NPR-C-like" receptors (Brown and Zuo, 1995; Zorad et al., 1993). One potential explanation for the apparent discrepancy would be that ^{125}I-ANP binding sites in the embryonic telencephalon correspond to a natriuretic receptor that has not yet been cloned or characterized. Regardless, our expression and functional studies now identify the natriuretic peptide ligand/receptor systems as potential participants in development of the peripheral and central nervous systems.

Acknowledgments

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References

Anand-Srivastava, M.B., Trachte, G.T., 1994. Atrial natriuretic factor receptors and signal transduction mechanisms. Pharmacol. Rev. 45, 455–497.

Appel, R.G., 1990. Mechanism of atrial natriuretic factor-induced inhibition of rat mesangial cell mitogenesis. Am. J. Physiol. 259, E312–E318.

Appel, R.G., 1992. Growth-regulatory properties of atrial natriuretic factor. Am. J. Physiol. 262, F911–F918.

Brown, J., Zuo, Z., 1995. Natriuretic peptide receptors are expressed during cerebral growth in the fetal rat. Am. J. Physiol. 269, R261–R273.

Cahill, P.A., Hassid, A., 1991. Clearance receptor-binding atrial natriuretic peptides inhibit mitogenesis and proliferation of rat aortic smooth muscle cells. Biochem. Biophys. Res. Commun. 179, 1606–1613.

Cameron, V.A., Aitken, G.D., Ellmers, L.J., Kennedy, M.A., Espiner, E.A., 1996. The sites of gene expression of atrial, brain, and C-type natriuretic peptides in mouse fetal development: temporal changes in embryos and placenta. Endocrinology 137, 817–824.

Carey, R.G., Li, B., DiCicco-Bloom, E., 2002. Pituitary adenylate cyclase activating polypeptide anti-mitogenic signaling in cerebral cortical progenitors is regulated by $p57^{Kip2}$-dependent CDK2 activity. J. Neurosci. 22, 1583–1591.

Chusho, H., Tamura, N., Ogawa, Y., Yasoda, A., Suda, M., Miyazawa, T., Nakamura, K., Nakao, K., Kurihara, T., Komatsu, Y., Itoh, H., Tanaka, K., Saito, Y., Katsuki, M., Nakao, K., 2001. Dwarfism and early death in mice lacking C-type natriuretic peptide. Proc. Natl. Acad. Sci. U. S. A. 98, 4016–4021.

DiCicco-Bloom, E., Friedman, W.J., Black, I.B., 1993. NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor survival. Neuron 11, 1101–1111.

DiCicco-Bloom, E., Deutsch, P.J., Maltzman, J., Zhang, J., Pintar, J.E., Zheng, J., Friedman, W.F., Zaremba, T., 2000. Autocrine expression and ontogenetic functions of the PACAP ligand/receptor system during sympathetic development. Dev. Biol. 219, 197–213.

Eccleston, P.A., Collarini, E.J., Jessen, K.R., Mirsky, R., Richardson, W.D., 1990. Schwann Cells Secrete a PDGF-like Factor: evidence for an autocrine growth mechanism involving PDGF. Eur. J. Neurosci. 2, 985–992.

Espiner, E.A., Richards, A.M., Yandle, T.G., Nicholls, M.G., 1995. Natriuretic hormones. Endocrinol. Metab. Clin. North Am. 24, 481–509.

Hagiwara, H., Sakaguchi, H., Itakura, M., Yoshimoto, T., Furuya, M., Tanaka, S., Hirose, S., 1994. Autocrine regulation of rat chondrocyte proliferation by natriuretic peptide C and its receptor, natriuretic peptide receptor-B. J. Biol. Chem. 269, 10729–10733.

Hagiwara, H., Inoue, A., Yamaguchi, A., Yokose, S., Furuya, M., Tanaka, S., Hirose, S., 1996. cGMP produced in response to ANP and CNP regulates proliferation and differentiation of osteoblastic cells. Am. J. Physiol. 270, C1311–C1318.

Ikeda, M., Kohno, M., Yasunari, K., Yokokawa, K., Horio, T., Ueda, M., Morisaki, N., Yoshikawa, J., 1997. Natriuretic peptide family as a novel antimigration factor of vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 17, 731–736.

Jessen, K.R., Mirsky, R., 1984. Nonmyelin-forming Schwann cells coexpress surface proteins and intermediate filaments not found in myelin-forming cells: a study of Ran-2, A5E3 antigen and glial fibrillary acidic protein. J. Neurocytol. 13, 923–934.

Jessen, K.R., Mirsky, R., 1991. Schwann cell precursors and their development. Glia 4, 185–194.

Kohno, M., Yokokawa, K., Yasunari, K., Kano, H., Minami, M., Ueda, M., Yoshikawa, J., 1997. Effect of natriuretic peptide family on the oxidized LDL-induced migration of human coronary artery smooth muscle cells. Circ. Res. 81, 585–590.

Koide, M., Akins, R.E., Harayama, H., Yasui, K., Yokota, M., Tuan, R.S., 1996. Atrial natriuretic peptide accelerates proliferation of chick embryonic cardiomyocytes in vitro. Differentiation 61, 1–11.

Lawson, S.N., Caddy, K.W.T., Biscoe, T.J., 1974. Development of rat dorsal root ganglion neurons: studies of cell birthdays and changes in mean cell diameter. Cell Tissue Res. 153, 399–413.

Lelièvre, V., Pineau, N., Du, J., Wen, C.-H., Nguyen, T.B., Janet, T., Muller, J.-M., Waschek, J.A., 1998. Differential effects of peptide histidine isoleucine (PHI) and related peptides on stimulation and suppression of neuroblastoma cell proliferation. J. Biol. Chem. 273, 19685–19690.

Lelièvre, V., Pineau, N., Hu, Z., Ioffe, Y., Byun, J.-Y., Muller, J.-M., Waschek, J.A., 2001. Proliferative actions of natriuretic peptides on neuroblastoma cells. Involvement of guanylyl cyclase and non-guanylyl cyclase pathways. J. Biol. Chem. 276, 43668–43676.

Lelièvre, V., Hu, Z., Byun, J.-Y., Ioffe, Y., Waschek, J.A., 2002. Fibroblast growth factor-2 converts PACAP growth action on embryonic hindbrain precursors from stimulation to inhibition. J. Neurosci. Res. 67, 566–573.

Lemke, G., Chao, M., 1988. Axons regulate Schwann cell expression of the major myelin and NGF receptor genes. Development 102, 499–504.

Levin, E.R., Frank, H.J., 1991. Natriuretic peptides inhibit rat astroglial proliferation: mediation by C receptor. Am. J. Physiol. 261, R453–R457.

Lu, N., DiCicco-Bloom, E., 1997. Pituitary adenylate cyclase activating polypeptide is an autocrine inhibitor of mitosis in cultured cortical precursor cells. Proc. Natl. Acad. Sci. U. S. A. 94, 3357–3362.

Mata, M., Alessi, D., Fink, D.J., 1990. S100 is preferentially distributed in myelin-forming Schwann cells. J. Neurocytol. 19, 432–442.

Matsukawa, N., Grzesik, W.J., Takahashi, N., Pandey, K.N., Pang, S., Yamauchi, M., Smithies, O., 1999. The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide system. Proc. Natl. Acad. Sci. U. S. A. 96, 7403–7408.

Murthy, K.S., Teng, B.Q., Zhou, H., Jin, J.G., Grider, J.R., Makhlouf, G.M., 2000. G(i-1)/G(i-2)-dependent signaling by single-transmembrane natriuretic peptide clearance receptor. Am. J. Physiol. 278, G974–G980.

Nakao, K., Ogawa, Y., Suga, S.I., Imura, H., 1992. Molecular biology and biochemistry of the natriuretic peptide system: II. Natriuretic peptide receptors. J. Hypertens. 10, 1111–1114.

Needleman, P., Blaine, E.H., Greenwald, J.E., Michener, M.L., Saper, C.B., Stockmann, P.T., Tolunay, H.E., 1989. The biochemical pharmacology of atrial peptides. Annu. Rev. Pharmacol. Toxicol. 29, 23–54.

Nicot, A., DiCicco-Bloom, E., 2001. Regulation of neuroblast mitosis is determined by PACAP receptor isoform expression. Proc. Natl. Acad. Sci. U. S. A. 98, 4758–4763.

Nishiyama, M., Hoshino, A., Tsai, L., Henley, J.R., Goshima, Y., Tessier-Lavigne, M., Poo, M.M., Hong, K., 2003. Cyclic AMP/GMP-dependent modulation of Ca2+ channels sets the polarity of nerve growth-cone turning. Nature 424, 990–995.

Pringle, N.P., Richardson, W.D., 1993. A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage. Development 117, 525–533.

Prins, B.A., Weber, M.J., Hu, R.M., Pedram, A., Daniels, M., Levin, E.R., 1996. Atrial natriuretic peptide inhibits mitogen-activated protein kinase through the clearance receptor. Potential role in the inhibition of astrocyte proliferation. J. Biol. Chem. 271, 14156–14162.

Ratner, N., Bunge, R.P., Glaser, L., 1985. A neuronal cell surface heparan

sulfate proteoglycan is required for dorsal root ganglion neuron stimulation of Schwann cell proliferation. J. Cell Biol. 101, 744–754.

Recio-Pinto, E., Rechler, M.M., Ishii, D.N., 1986. Effects of insulin, insulin-like growth factor-II, and nerve growth factor on neurite formation and survival in cultured sympathetic and sensory neurons. J. Neurosci. 6, 1211–1219.

Robertson, C.P., Gibbs, S.M., Roelink, H., 2001. cGMP enhances the Shh response in neural plate cells. Dev. Biol. 238, 157–167.

Scott, J.N., Jennes, L., 1991. Localization of ^{125}I-atrial natriuretic peptide (ANP) in the rat fetus. Anat. Embryol. (Berl.) 183, 245–249.

Simpson, P.J., Miller, I., Moon, C., Hanlon, A.L., Liebl, D.J., Ronnett, G.V., 2002. Atrial natriuretic peptide type C induces a cell-cycle switch from proliferation to differentiation in brain-derived neurotrophic factor- or nerve growth factor-primed olfactory receptor neurons. J. Neurosci. 22, 5536–5551.

Sondell, M., Lundborg, G., Kanje, M., 1999. Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J. Neurosci. 19, 5731–5740.

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

Suda, M., Ogawa, Y., Tanaka, K., Tamura, N., Yasoda, A., Takigawa, T., Uehira, M., Nishimoto, H., Itoh, H., Saito, Y., Shiota, K., Nakao, K., 1998. Skeletal overgrowth in transgenic mice that overexpress brain natriuretic peptide. Proc. Natl. Acad. Sci. U. S. A. 95, 2337–2342.

Tabernero, A., Stewart, H.J.S., Jessen, K.R., Mirsky, R., 1998. The neuron-glia signal beta neuregulin induces sustained CREB phosphorylation on Ser-133 in cultured rat Schwann cells. Mol. Cell. Neurosci. 5/6, 309–322.

Tanabe, Y., Jessell, T.M., 1996. Diversity and pattern in the developing spinal cord. Science 274, 115–123.

Taniuchi, M., Clark, H.B., Schweitzer, J.B., Johnson Jr., E.M., 1988. Expression of nerve growth factor receptors by Schwann cells of axotomized peripheral nerves: ultrastructural location, suppression by axonal contact, and binding properties. J. Neurosci. 8, 664–681.

Tong, Y., Pelletier, G., 1990. Ontogeny of atrial natriuretic factor (ANF) binding in various areas of the rat brain. Neuropeptides 16, 63–68.

Verdi, J.M., Groves, A.K., Farinas, I., Jones, K., Marchionni, M.A., Reichardt, L.F., Anderson, D.J., 1996. A reciprocal cell–cell interaction mediated by NT-3 and neuregulins controls the early survival and development of sympathetic neuroblasts. Neuron 16, 515–527.

Waschek, J.A., 2002. Multiple actions of pituitary adenylyl cyclase activating peptide in nervous system development and regeneration. Dev. Neurosci. 24, 14–23.

Waschek, J.A., Casillas, R.A., Nguyen, T.B., DiCicco-Bloom, E.M., Carpenter, E.M., Rodriguez, W.I., 1998. Neural tube expression of pituitary adenylate cyclase-activating peptide (PACAP) and receptor: potential role in patterning and neurogenesis. Proc. Natl. Acad. Sci. U. S. A. 95, 9602–9607.

Zorad, S., Tsutsumi, K., Bhatia, A.J., Saavedra, J.M., 1993. Localization and characteristics of atrial natriuretic peptide receptors in prenatal and postnatal rat brain. Eur. J. Pharmacol. 241, 195–200.