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Review

Developmental actions of natriuretic peptides in the brain and skeleton

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Abstract. The concept that atrial natriuretic peptide (ANP) and the closely related peptides BNP and CNP might be involved in the ontogeny of several organ systems emerged in the late 1980s. While many of the reported in vitro actions have not been examined in the context of organ development in vivo, recent studies demonstrate that mice which lack or overexpress natriuretic peptides or receptors exhibit pronounced skeletal growth defects. This article discusses how natriuretic peptides and other factors appear to regulate bone growth as an example of how natriuretic peptides might participate in the ontogeny of other organ systems. Evidence indicating that natriuretic peptides regulate neural development is then reviewed. Natriuretic peptides and receptors exhibit complex expression patterns in the developing nervous system, where they have been shown to act on neural cells as early as at the embryonic neural tube stage. Interestingly, both bone and brain growth appear to utilize primarily CNP and the CNP-specific type B receptor, and perhaps the type C receptor. In vitro data indicate that CNP may act on developing neurons, astrocytes and Schwann cells like a classical growth factor, regulating proliferation, patterning, phenotypic specification, survival and axonal pathfinding. Natriuretic peptides might also have roles in the vascularization of the embryonic brain, establishment of the blood-brain and blood-nerve barriers, and perhaps in nerve regeneration.

Key words. Atrial natriuretic peptide (ANP); BNP; CNP; receptor; embryo.

Introduction

Natriuretic peptides constitute a family of the three structurally related molecules: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and the type-C natriuretic peptide (CNP) [1–4]. The founding member of this peptide family, ANP, was first known as a hormone produced by the heart that regulated vascular tone, sodium and water balance, and other cardiovascular functions through action on the kidney and vascular smooth muscle cells (VSMCs). However, all three peptides are expressed in the adult brain, where one function appears to be central control of the cardiovascular system. There are three known mammalian receptors for peptides in the ANP family [3, 4]. Two of these, type A and B (NPR-A and NPR-B, respectively), are single transmembrane spanning proteins that contain guanylyl cyclase (GC) activity in their intracellular domain. The third, type C (NPR-C), is also a single transmembrane spanning protein, but contains only a short 37-amino acid intracellular domain, and lacks GC activity. Because NPR-C has a short intracellular domain devoid of GC activity, and is internalized after binding ANP, it has been referred to as the ‘clearance’ receptor. However, more recent data obtained with specific analogs for this receptor indicate NPR-C can transmit signals by inhibiting cyclic AMP (cAMP) formation, stimulating intracellular calcium levels, and/or by inhibiting a MAPK signaling pathway [5,

reviewed in 4]. Natriuretic peptide receptors differ in their relative affinity for ANP analogs. NPR-A binds to ANP and BNP with high affinity, and to CNP with very low affinity. NPR-B, on the other hand, is relatively selective for CNP. NPR-C binds all natriuretic peptides with high affinity. The molecular pathways purportedly utilized by natriuretic peptides to regulate growth have recently been reviewed [6]

This paper focuses on natriuretic peptide control of bone and brain development. Evidence that natriuretic peptides regulate cardiac development and fetal cardiovascular homeostasis have been reviewed elsewhere [7, 8].

Early studies on the proliferative actions of natriuretic peptides on cultured cells

Early data indicating that natriuretic peptides might perform regulatory actions in the development of several organ systems were reviewed in 1990 [9]. The first report suggesting that natriuretic peptides might have growth factor-like actions appeared in 1985, and showed that ANP potently stimulated DNA synthesis in cultures of bovine adrenal zona glomerulosa cells with a significant increase at 10^{-12} M [10]. This interesting finding seems to have received little subsequent attention. Three years later, two groups showed that ANP, at concentrations as low as 10^{-11} M, inhibited the proliferation of rat kidney mesangial cells [11, 12]. Among other early studies, it was demonstrated that natriuretic peptides exerted antiproliferative effects on cultured vascular smooth muscle [13, 14] and endothelial cells [15]. Whereas the significance of the effects on mesangial and vascular cells during development remains to be determined, in vivo studies in mature animals suggest that the antiproliferative effects observed in vitro may be important after renal and vascular injury, respectively. For example, transgenic mice overexpressing BNP in the liver were found to exhibit greatly reduced mesangial expansion and cell proliferation compared to wild-type mice in two different models of glomerulonephritis [16, 17]. In the vascular system, CNP expression was found to be induced in carotid arteries after balloon injury [18, 19], and adenoviral delivery of CNP (but not control vector expressing β-galactosidase) to injured carotid arteries reduced neointimal formation by 90% [20]. Moreover, ANP and NPR-A knockout mice exhibited increased muscularization of pulmonary arterioles under normal and hypoxic conditions [21, 22],

Natriuretic peptide regulation of skeletal growth

The regulation of longitudinal bone growth has become the most well documented developmental action of natriuretic peptides. This comes as a result of data obtained from classical approaches and from transgenic and knockout mouse models of natriuretic peptides, receptors and their downstream signaling components. These studies are discussed in some detail here because the deduced models may serve in some ways as an example for how natriuretic peptides might pattern the brain and other organ systems. To help explain the mechanisms by which natriuretic peptides regulate bone growth, some background on bone development in the embryo is presented here [23, 24]. Bone formation in vertebrates proceeds by two distinct mechanisms. One of these, termed intramembraneous ossification is utilized primarily to form the flat bones of the skull. In this process, mesenchymal cells differentiate directly into osteoblasts, which deposit bone matrix to form ossification centers. There is currently no evidence that natriuretic peptides are involved in this process, so it will not be discussed further. The other mechanism, called endochondrial ossification, is the primary mechanism used to form the long bones and vertebrae. In this case, mesenchymal cells initially condense to form primordial bone. Cells in the core differentiate into chondrocytes, which then, as discussed below, undergo a program of sequential proliferation, hypertrophy, calcification, apoptosis and finally replacement with bone marrow. Surrounding the core, mesenchymal cells differentiate to become the cells of the perichondrium, which later differentiate into osteoblasts. These eventually replace most of the cartilage with bone. The chondrocyte lineage is a key cellular component driving the longitudinal growth of bones. Chondrocytes at the center of the primordial bone first differentiate into prehypertrophic and then hypertrophic chondrocytes. In contrast, chondroctyes outside of the central zone continue to proliferate along future longitudinal bone axis. As bone development proceeds, chondrocytes near the central zone begin to differentiate into hypertrophic chondrocytes, whereas older hypertrophic chondrocytes lying closer to the center induce mineralization of the surrounding matrix and undergo cell death. Thus, fronts of proliferating and differentiating chondrocytes (together called the epiphyseal growth plates) advance in opposing directions as the bone develops.

Many aspects of long bone development are dictated by autocrine or paracrine signals in these growth plates. Among these signals are Indian hedgehog (IHH), parathyroid hormone-related peptide (PTHrP), and members of the bone morphogenetic protein (BMP) family [25–28]. IHH is produced in chondrocytes as they progress from proliferative to hypertrophic states (fig. 1). PTHrP is expressed in articular perichondrium and signals to receptors on proliferating and prehypertrophic chondrocytes. IHH and PTHrP signaling is necessary to maintain a normal proliferative zone of chondrocytes in the advancing epiphyseal plate, and to prevent premature

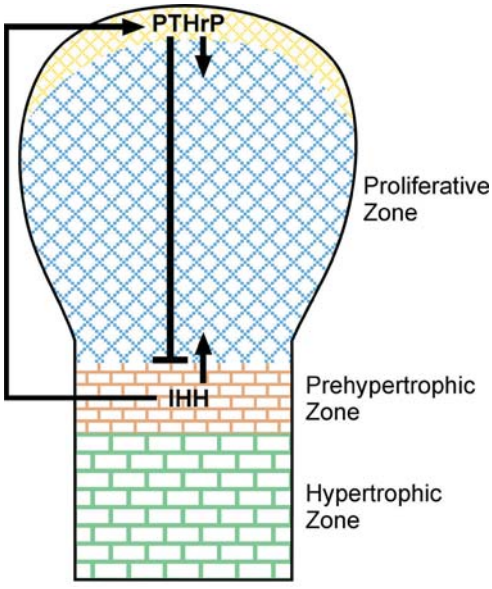

Figure 1. Paracrine regulation of long bone growth by Indian hedgehog (IHH) and parathyroid hormone related peptide (PTHrP). Only the end of the bone where growth is taking place is shown, i.e. the area of ossification underneath the hypertrophic zone is not shown. IHH is expressed in prehypertrophic chondrocytes (orange) as they progress from a proliferative (blue) to a hypertrophic (green) state. IHH acts directly on chondrocytes to positively regulate their proliferation, and indirectly by stimulating production of PTHrP in the articular perichondrium (yellow). PTHrP inhibits the differentiation of chondrocytes, thereby preventing hypertrophy from occurring in the growth zone near the articular surface. Thus, the range of PTHrP signaling determines the distance to the articular surface from which hypertrophy begins. Bone morphogenetic proteins (see text) as well as other secreted proteins also appear to have roles in long bone growth. As discussed in detail in this review, transgenic and knockout mouse experiments have confirmed that natriuretic peptides are important regulators of bone growth. CNP and its specific receptor (NPR-B) are expressed in both proliferating and prehypertrophic chondrocytes, but the way in which they interact with IHH/PTHrP signaling to regulate long bone growth is currently unknown.

differentiation of proliferating chondrocytes into hypertrophic chondrocytes. Both IHH [29] and PTHrP [30, 31] knockout mouse strains show a smaller zone of proliferating cells, resulting in hypertrophy occurring relatively close to the articular surface. In IHH knockout mice, PTHrP gene expression is absent in the articular perichondrium, suggesting that at least part of IHH action is mediated by PTHrP. Transgenic expression of a constitutively active form of the PTHrP receptor in chondrocytes prevented the premature chondrocyte hypertrophy in IHH knockout mice, but not the defects on chondrocyte proliferation and overall limb size [31]. Based on these and other functional studies and on gene expression patterns, a model has emerged (fig. 1) in which IHH expression in prehypertrophic chodrocytes positively regulates the proliferation of chondrocytes independent of PTHrP. At the same time, IHH signals to the articular perichondrium to induce expression of PTHrP, which in turn prevents hypertrophy from occurring in the growth zone near the articular surface. In this way, PTHrP maintains chondrocytes near the articular surface in a proliferative state. Moreover, its range of signaling determines the distance to the articular surface where hypertrophy begins. As chondrocytes proliferate, cells at the trailing edge of the growth plate fall out of range of the PTHrP signals, and thus are allowed to differentiate. BMPs appear to positively regulate IHH expression, but also appear to act in parallel with IHH and IHH/PTHrP to regulate chondrocyte proliferation and differentiation, respectively [28].

The first indication that natriuretic peptides might regulate bone growth was a report in 1988 demonstrating that ANP inhibited both basal and parathyroid hormone-stimulated DNA synthesis in cultures derived from avian tibia epiphyseal growth plates (presumably containing primarily cells of the chondrocyte lineage) [32]. Later, Hagiwara and co-workers showed that cultured rat chondrocytes expressed NPR-B, and that the preferred ligand for this receptor, CNP, was produced in these cultures and potently inhibited DNA synthesis [33]. Consistent with the antiproliferative effect being mediated by NPR-B, ANP was much less potent. Seemingly in conflict with these in vitro studies suggesting that natriuretic peptides might inhibit bone growth, transgenic mice which overexpressed BNP in the liver were found to exhibit skeletal overgrowth [34]. Investigators in the latter study felt that BNP acted promiscuously on NPR-B, because CNP was more potent than BNP in inducing growth of mouse tibial explants. They also argued against the involvement of NPR-C, because the actions in explant cultures were blocked by HS-142-1, an agent which appears to selectively block NPR-A and NPR-B. Consistent with a model in which CNP regulates bone growth via NPR-B, the CNP and NPR-B genes were found by Northern analysis to be expressed at high levels in embryonic mouse tibias, whereas no ANP, BNP, NPR-C and very little NPR-A gene expression were detected [35]. Moreover, in situ hybridization revealed that both CNP and NPR-B were localized in proliferative and prehypertrophic chondrocytes [36].

Further evidence that natriuretic peptides positively regulate bone growth in vivo came from reports indicating that three different strains of mice with spontaneous or chemical induced mutations in the NPR-C gene [37], and mice with a targeted deletion in the NPR-C gene [38] exhibited skeletal overgrowth. Because NPR-C functions to clear natriuretic peptides, the investigators proposed that the skeletal defects were due to elevated local levels of natriuretic peptides. Finally, CNP knockout mice showed a dwarf phenotype with a marked reduction in the heights of proliferative and hypertrophic chondrocyte zones [36]. The bone growth defect in CNP knockout mice was rescued by transgenic expression of CNP in chondrocytes, providing evidence that CNP acts locally on bone growth. Mice with targeted deletions of ANP, BNP and NPR-A

show no obvious skeletal defects, strengthening the notion that CNP, acting via the NPR-B receptor, is the primary natriuretic peptide regulator of longitudinal bone growth.

CNP appears to regulate bone growth in vivo primarily via cyclic GMP (cGMP)-dependent protein kinase II (cGK-II). Like CNP knockout animals, mice with a targeted mutation in the cGK-II gene had a dwarf phenotype which, in this case, could not be rescued by targeted expression of CNP in growth plate chondrocytes [39]. In summary, an abundance of data strongly support a model in which chondrocyte-derived CNP acts via NPR-B, cGMP and cGK-II within the epiphyseal growth plate to promote proliferation and/or delay differentiation of chondrocytes. However, despite knowledge gained from the pronounced skeletal phenotypes of natriuretic peptide ligand and receptor knockout and overexpressing mice, the relationships of this ligand receptor signaling system with other factors regulating bone growth such as IHH, PTHrP or BMPs are uncertain. Clearly these ligand receptors systems are non-redundant, because targeted deletion of each of the respective genes produces a strong phenotype. The interaction of these factors is likely to be addressed in the near future, with new epistasis information obtained by, for example, interbreeding of respective mice and/or analyses in other vertebrate models.

Natriuretic peptide action in the developing brain

The first indication that natriuretic peptides might have actions in brain development was a study published in 1990 in which Tong and Pelletier [40] used receptor autoradiography to show that high-affinity binding sites for ^{125}I-ANP were present in the embryonic day (E) 13 rat telencephalon ventricular zone (VZ). This zone is the specific area in the embryonic telencephalon where neuroblasts actively proliferate. Moreover, E13 is a time of peak neurogenesis in the rat. Subsequently, three other groups provided receptor autoradiographic evidence that high-affinity natriuretic peptide receptors are present in the developing rat nervous system, and associated blood vessels [41–43]. Two groups focused specifically within the rat brain and again reported their presence of ^{125}I-ANP binding sites in the telencephalon VZ [42, 43]. Radioligand displacement studies with different ANP analogs indicated that these latter sites exhibited a 'NPR-C-like' receptor peptide binding profile [42, 43], but were coupled to cGMP production [43].

In another more detailed study performed in embryonic day E14 mice, ^{125}I-ANP binding sites were observed over the developing blood vessels and the vascular plexus around the developing brain and in the meningeal layer [41]. In the area of 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 boundary caps, DRG and peripheral nerves. The investigators reported that these latter binding sites were in Schwann cell precursors, satellite cells and Schwann cells respectively.

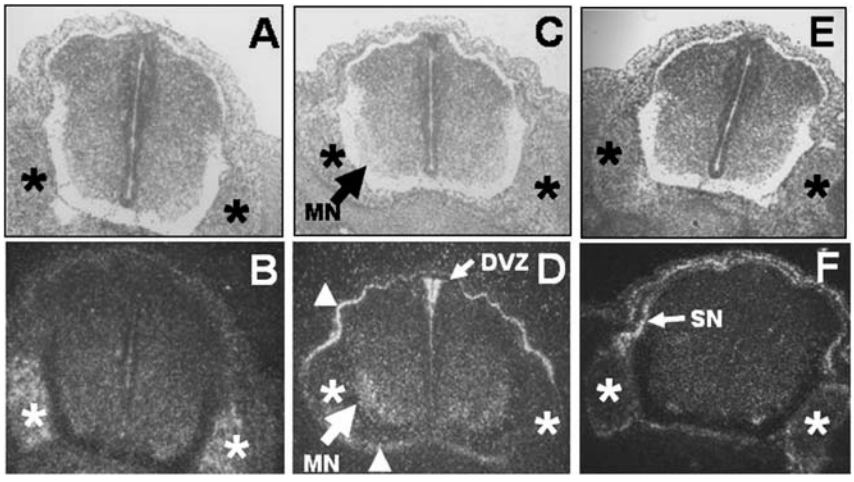

Figure 2. NPR-B (*A*, *B*), CNP (*C*, *D*) and NPR-C (*E*, *F*) gene expression in the area of the spinal cord in E14.5 mice. *A*, *C* and *E* are bright field micrographs; *B*, *D* and *F* are the same sections show in dark field. DRG are indicated by asterisks (*) in all panels, and are strongly labeled by the NPR-B riboprobe in *A* and *B*. A population of NPR-B-mRNA-hybridizing motor neurons is also indicated by large arrows in *C* and *D*. Arrowheads in *C* and *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. The small arrow in *D* points to an area of intense CNP gene expression in the dorsal VZ (DVZ) of the spinal cord. Among the sites of NPR-C gene expression were the DRG and spinal nerves (SN) (*E* and *F*) (panels *A*–*D* are taken from [44].

Localization of specific receptor and ligand subtypes in the developing nervous system by in situ hybridization

Using in situ hybridization, we recently examined the regional gene expression patterns for each of the specific natriuretic peptide receptors and ligands in the early embryonic mouse brain (E10.5 to E14.5) [44]. In the peripheral nervous system, NPR-B messenger RNA (mRNA) was abundant in sensory ganglia from E10.5 to E14.5 [fig. 2A, B show expression in E14.5 dorsal root ganglia (DRG)]. Tissue culture studies discussed below indicate that these receptors might mediate sensory neuron survival and/or neurite outgrowth. Within the brain, NPR-B mRNA was detected at E10.5 in cells just outside the VZ of the hindbrain [44], a region where post-mitotic neurons are undergoing differentiation. Receptors in this location may be involved in cell cycle exit, specification of cell lineage and/or patterning events (see below). NPR-C mRNA was found to be abundantly expressed at several sites within or near the nervous system. Among the prominent sites of expression were the trigeminal and DRG. Gene transcripts were localized within these ganglia, but also in the associated nerves, most likely in Schwann cells (fig. 2E, F, figs 3 and 4A). In vitro data discussed below suggest that natriuretic peptides might be a proliferative signal for these cells. Intense signals for NPR-C mRNA were also observed in the notochord (figs 3 and 4B), hinting that natriuretic peptides might play a role in spinal cord dorsal/ventral patterning. Moreover, signals were also observed in the roof plate of the spinal cord and in bilateral stripes in the VZ just lateral to the floor plate (fig. 3). The latter site of expression was reminiscent of Nkx and other homeobox transcription factor-expressing domains that give rise to oligodendrocytes or specific subclasses of ventral neurons [45, 46]. Given this complex pattern of NPR- C expression, it will be important to determine if this receptor serves in vivo just to clear or limit the diffusion of natriuretic peptides, or to signal in a direct way to regulate some aspect of development. In addition to expression in neuronal and glial cells, small discrete clusters of NPR-C gene transcripts were also observed within the CNS parenchyma, most likely over invading capillaries [44]. High levels of NPR-C expression were also observed in the perineural vascular plexus surrounding the brain [44]. NPR-A mRNA was not detected within the brain or any ganglia, although transcripts were observed in many large blood vessels.

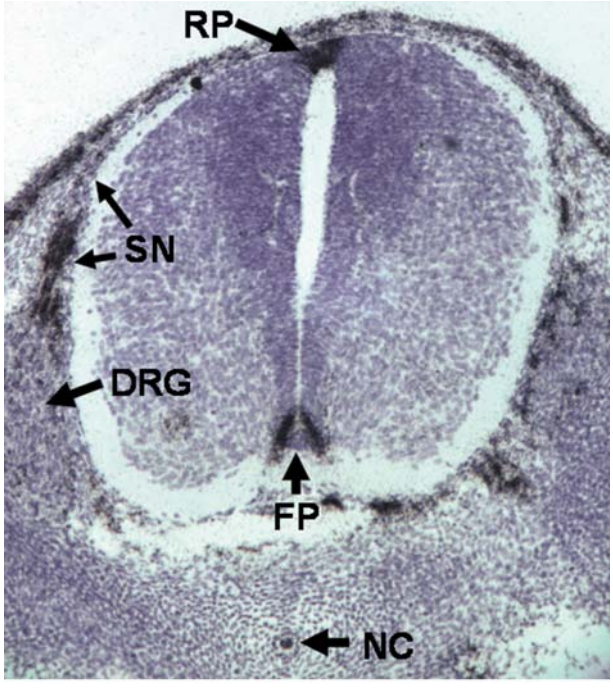

Figure 3. NPR-C receptor gene expression in the spinal cord in E12.5 mouse embryos. All panels are transverse sections. Intense gene expression is localized to the roof plate (RP), in stripes surrounding the floorplate (FP), in the spinal nerve (SN) and notochord (NC). A lower-level signal is present in the dorsal root ganglia (DRG). See figure 4 for higher magnification views (data adapted from [44].

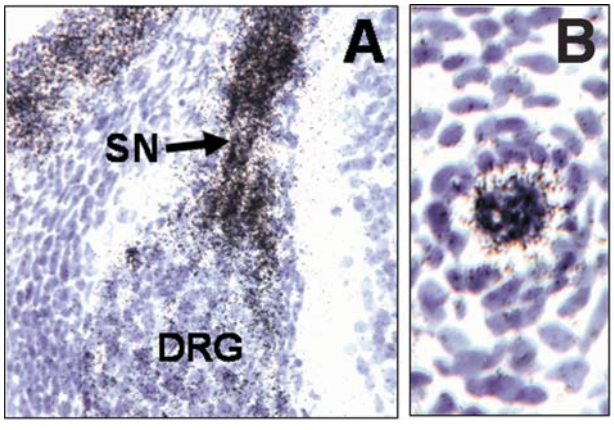

Figure 4. High magnification photos of selected areas of figure 3 showing NPR-C receptor gene expression in the area of spinal cord in E12.5 mouse embryos.

It may be instructive to compare the in situ hybridization results with the previously described receptor localizations by receptor autoradiography. Because ANP is a poor ligand for NPR-B, ^{125}I-ANP would not be expected to recognize, and did not localize to the NPR-B gene expression sites in the hindbrain that we observed by in situ hybridization. On the other hand, NPR-C gene transcripts showed an almost perfect one-to one spatial relationship with the ^{125}I-ANP binding sites reported by Scott and Jared [41, see above]. It is intriguing, however, that we were unable to detect gene expression for any of the receptors in the telencephalon VZ, whereas autoradiographic approaches by three different groups detected highly abundant ^{125}I-ANP binding sites at this location

[40, 42, 43]. One potential explanation for this apparent discrepancy is that these binding sites correspond to an unknown receptor. Binding and displacement profiles of natriuretic peptide analogs on both embryonic brain extracts and autoradiographic sections do suggest the existence of a novel high-affinity natriuretic peptide receptor [42, 43]. Moreover, an NPR-C specific analog potently inhibited the proliferation of a mouse neuroblastoma cell line in which no NPR-C gene transcripts could be detected by reverse transcription – polymerase chain reaction using multiple primer sets [47]. Because the human genome does not seem to contain other sequences with extensive homology to known natriuretic receptors, such an atypical receptor, if it exists, is likely to have a primary structure considerably different than NPR-A, NPR-B and NPR-C.

The above receptor localization data suggest that natriuretic peptides might regulate neural development via NPR-B, NPR-C and/or novel receptors, but what could be the source of the peptide? We detected high levels of CNP gene expression at all levels of the CNS caudal to the mesencephalon beginning at E10.5 [44], in agreement with a previous observation of Cameron [48]. Specifically, CNP transcripts were detected at E10.5 in the dorsal and intermediate VZ in these regions. Later, at E14.5, CNP expression was still observed at high levels in the dorsal VZ, although new signals were seen laterally in cells in the dorsal hindbrain, reflecting either new cellular expression or radial cell migration. Specific hybridization signals were also observed in cells surrounding the neural tube, apparently in the dura layer of the meninges (fig. 2C, D). CNP gene transcripts were also observed in the cartilage of the neural arch at E14.5, which is juxtaposed to Schwann cells in developing spinal nerves, and in a layer on the outside or just lateral to the DRG. We did not examine expression earlier than E10.5, but another group detected CNP mRNA in RNA isolated from embryonic mouse heads at E.9.5 [49].

In contrast to CNP, we did not detect ANP and BNP gene transcripts in the nervous system at E10.5 to E14.5, but they were expressed at high levels in the embryonic heart. Although we did not extend our experiments beyond E14.5, another group reported that ANP mRNA was present in the mouse DRG at E15.5 [48]. CNP is also reported to be expressed in vascular endothelial cells in adults [50], although we could not detect CNP gene transcripts in capillaries that invaded the developing brain. In any case, CNP may be available locally from several sources to regulate neural development in the embryo: in the dorsal CNS VZ and parenchyma, dura and cartilage adjacent to peripheral spinal nerves, and possibly in endothelial cells of capillaries invading the brain, peripheral nerves and ganglia. ANP produced in DRG cells and ANP and BNP from the heart might also be sources of natriuretic peptides that regulate neural development. The latter endocrine source might be especially important at early stages, before the development of blood-brain and blood-nerve barriers.

Postnatal time courses of ANP, BNP and CNP gene expression in the rat brain have also been reported [51]. Interestingly, ANP and CNP gene expression in numerous brain regions was transient or significantly higher between P4 and P13 than at P60, suggesting that natriuretic peptides may play one or more general roles in postnatal development, such as regulation of synaptogenesis or myelination.

Analysis of natriuretic peptide action using tissue culture models

Embryonic CNS cultures

On the basis of the above in situ hybridization data showing NPR-B gene expression in the E10.5 hindbrain, we established cultures of neural precursors isolated from this region and treated cells with different natriuretic peptides [44]. CNP potently inhibited DNA synthesis in these cultures, with an EC_{50} of approximately 10^{-10} M. ANP was much less potent than CNP, whereas the NPR-C specific agonist des-[Gln(18),Ser(19),Gly(20), Leu(21),Gly(22)]-ANP(4-23)-NH(2) ($desANP_{4-23}$) exhibited a level of potency between CNP and ANP. The potent inhibitory action of CNP was 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 $desANP_{4-23}$ also inhibited DNA synthesis indicated that at least some of the antiproliferative actions may have been 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 [47].

Using a chick neural plate explant model, it was recently reported that 'chick' natriuretic peptide was able to enhance the ability of the patterning factor sonic hedgehog (Shh) to induce ventral phenotypes in the dorsal explants [52]. To determine if a similar interaction between Shh and natriuretic peptides might occur in the mouse embryonic hindbrain, we studied the ability of Shh and CNP to regulate expression of the sonic Shh target gene gli-1 in our embryonic mouse hindbrain cultures. We found that both CNP and Shh induced the expression of gli-1, and that the combined induction was additive [44]. These data, plus the fact that NPR-B mRNA was detected in the early embryonic hindbrain and that NPR-C gene expression was localized to the notochord, bilateral stripes just above the anterior spinal cord floor plate and in the roof plate (see above), suggest that natriuretic peptides might have a role in dorsal/ventral patterning.

E14.5 rat DRG

The receptor localization studies showing that NPR-B and NPR-C genes were expressed in the embryonic DRG suggested that developing sensory neurons might also be a target of natriuretic peptide action during development. Thus, the actions of these peptides were examined using cells isolated from E14.5 rat DRG [44]. In cultures containing both neurons and Schwann cells, CNP elicited a twofold increase in DNA synthesis, with a peak activity at 10^{-8} M. Significantly, desANP$_{4-23}$, exhibited far greater potency, with an EC50 of approximately 10^{-10} M. In contrast, while ANP also increased DNA synthesis, effects were not observed until a dose of 10^{-7} M. The high potency of both CNP and desANP$_{4-23}$ in the mitogenic stimulation of DRG cells suggested that effects were mediated by both NPR-B and NPR-C receptors. Labeling with BrdU and cell-specific markers indicated that the cells undergoing proliferation in these cultures were Schwann cells. We also determined the ability of 10^{-7} M CNP to regulate neuron survival and neurite outgrowth in DRG cultures**.** CNP had modest positive effects on neuron survival and significantly inhibited neurite outgrowth. Thus natriuretic peptides appear to have the ability to regulate the proliferation of Schwann cells and sensory neuron survival, and might have a function in sensory neuron process elaboration. The latter might have significance in the regulation of sensory neuron axon pathfinding, an idea supported by the fact that cGMP exhibits potent action in growth cone tuning assays [53, 54]. Because CNP inhibits process elongation in vitro, CNP might function in vivo as a repulsive signal. The expression data discussed above suggest one possibility: as axons leave the DRG they encounter CNP in either the dura or cartilage surrounding the spinal cord (fig. 2C, D). Because of this they avoid entering into this tissue and instead traverse up towards and eventually into the cord. Sensory axons then terminate in the dorsal cord, a consequence potentially reinforced by inhibitory CNP signals emanating from the VZ and ventral cord (fig. 2C, D).

Cultured astrocytes

Although natriuretic peptide receptors (mRNA or protein) have not been demonstrated on astrocytes in vivo, several papers indicate that astrocyte proliferation can be regulated by natriuretic peptides via NPR-C. Levin and Frank first showed in 1991 that natriuretic peptides inhibit DNA synthesis in cultured astrocytes [55]. The antiproliferative action was produced with very low concentrations of an analog specific for NPR-C, and was associated with an inhibition of MAP kinase activity [5]. An NPR-C analog was also found to inhibit the proliferation of a mouse neuroblastoma cell line [47]. This antiproliferative effect was lost when MAP kinase activity was previously inhibited with the MEK1/2 antagonist PD98059. The data suggest that a MAP kinase growth factor pathway constitutively operated in these cells. In the presence of PD98059, this growth factor pathway may have been blocked, preventing any further inhibitory action of an NPR-C agonist. That a MAP kinase growth pathway was constitutively active in these cells was evidenced by the fact the PD98059 alone inhibited basal and serum-stimulated proliferation in these cells.

Cultured olfactory sensory neurons

Olfactory sensory neurons in rodents undergo continuous proliferation and turnover throughout adult life. Thus, the expression patterns and growth-related actions of natriuretic peptides and receptors were examined in the rat olfactory epithelium by Ronnett and co-workers [56]. NPR-B was localized to olfactory neurons in the upper one-half of the epithelium of the adult rat, where immature and maturing neurons reside. NPR-B was also expressed at and just above the interface of the olfactory epithelium and the lamina propria. The ligand CNP was associated with sustentacular cells, most prominently in the cell foot processes. CNP was also associated with olfactory sensory neuron axon bundles in the lamina propria leading into the bulb. In contrast to NPR-B, CNP was also expressed in the olfactory bulb, in periglomerular cells that surround the glomeruli and, in some cases, within the mitral cells deeper within the bulb.

The expression of CNP in sustentacular cells and in cells surrounding glomeruli and of NPR-B in olfactory neurons located in the different layers of the epithelium, suggested that CNP acts as a paracrine factor that could regulate olfactory sensory neuronal proliferation, differentiation or survival. To study the effect of natriuretic peptides on developing olfactory neurons, primary neonatal rat olfactory neuron cultures were utilized [56]. CNP by itself had no effect on BrdU incorporation into olfactory precursors, but strongly inhibited the proliferative effect of BDNF. CNP also induced differentiation in BDNF-treated cultures, as monitored by the percentage of cells containing processes and the expression of a marker of differentiated olfactory neurons. CNP also appeared to promote the survival of mature neurons. Taken with data obtained from other culture models, these results indicate that natriuretic peptides exhibit diverse action of neural cells that depend on cell type and developmental stage.

Vascularization of the brain

Although no published studies appear to have addressed the role of natriuretic peptides on angiogenesis specifically in the brain, considerable evidence indicates that natriuretic peptides regulate several general aspects of angiogenesis during development and/or after vascular in-

jury. Blood vessel formation in a particular organ or tissue can begin by either vasculogenesis (formation of blood vessels from mesodermal tissue de novo) or angiogenesis (generation of new vessels by extension or modification of existing vessels). The former process occurs by the differentiation of mesodermal cells into endothelial cells, expansion and the condensation of these cells into a primitive capillary tubelike network. Subsequent steps include recruitment of smooth muscle cells and pericytes, secretion of extracellular matrix proteins and vascular remodeling. The vascular system in the brain appears to occur exclusively by angiogenesis.

The molecular steps involved in the formation of blood vessels are beginning to be elucidated [57–60]. Vascular endothelial growth factor (VEGF) and its receptors lie at a center of a regulatory network controlling blood vessel growth and differentiation. In the process of vasculogenesis, VEGF is secreted by tissue in response to hypoxia, and acts on local mesoderm-derived angioblasts to induct their differentiation and proliferation. VEGF appears to also be important at later steps in vasculogenesis and angiogenesis (see below). Another set of important factors include the angiopoietins and their receptors (Tie1/2). Activation of Tie 2 receptors expressed on endothelial cells appears to result in release of factors that recruit primordial VSMCs. Gene knockout and transgenic overexpression studies indicate that the VEGF and angiopoietin ligand receptor systems are critical for blood vessel development, but the precise actions for these ligands and receptors have not yet been elucidated (reviewed in [59]). Other transgenic studies implicate PDGF-α and perhaps TGF-β in the recruitment/proliferation/differentiation of VSMCs. *Eph* receptors and their ligands, classically believed to direct migration of neurons and axons, may also direct vascular network assembly, affecting endothelial migration, capillary morphogenesis and angiogenesis [61].

Although certain fundamental processes in angiogenesis are probably used in the vascularization of all organs, each tissue must carefully regulate this process to suit its specific needs. One aspect of angiogenesis that is unique in the brain is that blood vessels must eventually develop and maintain a blood-brain barrier (BBB) [62]. Nerves leaving or entering the brain must also develop a barrier, in this case, called the blood-nerve barrier (BNB). These barriers include endothelial cells specialized to contain high-resistance tight junctions, specific transporters, metabolizing enzymes and other proteins [63]. In contact with these endothelial cells are pericytes and astrocyte foot pads, which appear to provide factors which contribute to the endothelial cell specialization [62–66]. The BBB and BNB develop gradually during development, and are not fully mature until the postnatal period [62, 67, 69].

Initial vascularization of the brain begins about the time of neural tube closure with the formation of a primitive endothelial capillary network over the surface of the brain (called the perineural vascular plexus) [66, 68–70]. The perineural vascular plexus arises in part from angioblasts that migrate from the splanchnopleuric mesoderm to the surface of the neural tube. New blood vessels invade the brain by a process called sprouting angiogenesis. Vascularization of the brain thus requires the proliferation and recruitment of both endothelial and VSMCs/pericytes derived from the perineural vascular plexus, and mechanisms which direct sprouting vessels into proper locations in the developing brain.

In embryonic mice, it has been shown that VEGF is transiently expressed in the ventricular zone (VZ) [71], while a VEGF receptor (VEGFR2/*flk-1*) is expressed in the perineural vascular plexus and in the endothelial cells that have invaded the developing brain tissue towards the VZ [66]. This has led to the suggestion that VEGF in the VZ stimulates the ingrowth of capillaries from the perineural vascular plexus.

Vascularization of the retina has recently been modeled in the rat, cat and human [72–75]. These studies suggest that VEGF, produced in retinal glial cells, guides capillary growth in neural tissue. A primary stimulus for angiogenesis during the development of the retina (like many other tissues) is believed to be hypoxia, and is mediated by VEGF and other angiogenic factors. In the retina, transient VEGF expression is observed specifically in glial cells just ahead, and at the leading edge, of advancing blood vessels. One of its receptors (VEGF2/flk-1), on the other hand, is expressed in the endothelial cells at the leading edge and just behind the advancing blood vessel. A short period of time after the primitive vessel is formed, pericytes from the established part of the vessel proliferate and migrate along the vessel sprout. The latter also appears to be a VEGF-mediated action, but may also involve PDGF-α Blood vessel formation and glial VEGF expression have been shown to be coordinately regulated in response to changes in atmospheric oxygen in rats and mice. Cultured glial cells exposed to hypoxic conditions respond with an increase in VEGF gene expression. Thus, glial cell appear to be a critical mediator of angiogenesis in the retina.

Natriuretic peptides and blood vessel formation

Although the role of natriuretic peptides in brain angiogenesis or BBB formation has not been specifically addressed, several potential roles of natriuretic peptide and their receptors in blood vessel formation have been proposed based on in vitro studies. As previously discussed, ANP and BNP are expressed early in the developing heart (E11 in the rat [76]), whereas CNP appears to be produced by vascular endothelial cells. Natriuretic peptides have been shown to inhibit the spontaneous as well as hypoxia- and endothelin-stimulated proliferation of puri-

fied VSMCs [9, 77–81], to inhibit the proliferation of endothelial cells [82] and to inhibit the recruitment of VSMCs [83, 84]. Natriuretic peptides were shown to inhibit the production and release of the VEGF from cultured VSMCs [77], suggesting one potential mechanism for natriuretic peptide regulation of angiogenesis. Functional receptors also appear to be present on pericytes [85].

As previously discussed, an early described action of ANP was inhibition of vascular endothelial cell proliferation. A recent study indicates that ANP can inhibit VEGF signaling in these cells [86]. Interestingly, an extended dose-response analysis showed that low concentrations of natriuretic peptides can increase, rather than decrease endothelial cell proliferation and migration in vitro [87]. The effects were significant at ANP concentrations as low as 10^{-13} M, and were blocked by inhibition of PKG and a MAP kinase pathway. This underscores the importance of considering a full concentration range of peptides in such studies.

Summary

Soon after the initial characterization of ANP about 20 years ago, it was proposed that natriuretic peptides play important roles in the development of certain organ systems. Recent reports indicating that natriuretic transgenic and knockout mice exhibited severe skeletal defects and showed impaired recovery after vascular and renal injury have added strong evidence that natriuretic peptides can act in growth-related capacities. With respect to brain development, there is abundant data indicating that high-affinity natriuretic binding sites and receptor gene transcripts are expressed in the early developing brain and associated blood vessels. Further, natriuretic peptides have been shown to potently regulate the proliferation, survival and neurite outgrowth of cultured neuronal and/or glial cells, and to regulate the proliferation and recruitment of blood vessel cell components. In vivo models should clarify the significance of in vitro studies in the next few years. In addition, given that natriuretic peptides appear to exert antiproliferative actions after renal and vascular injury, it will be of interest to determine if these peptides have roles in nerve injury and regeneration.

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