Review

Natriuretic Peptides in Embryonic Stem Cell-Derived Cardiomyocytes and Their Receptors in the CNS.

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Abstract

The natriuretic peptides (NPs) are a family of related hormones that play important roles in the cardiovascular homeostasis, cell growth and neuroendocrine functions. Recently, they have emerged as potentially important clinical biomarkers in heart failure. The heart secretes two major natriuretic peptides: atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), while C-type natriuretic peptide (CNP) is mainly secreted from the brain and blood vessels. The physiological effects of NPs are initiated by binding to natriuretic peptide receptors (NPRs), which are widely distributed in several organs. This review describes: the expression of natriuretic peptides in the cardiomyocytes differentiated from ES cells and their role in the cardiomyocyte development. We also describe the detailed distribution of NPRs in the central nervous system and their possible functions in various brain regions.

Keywords: natriuretic peptides; ES cells; development; brain; neuroedocrine.

Introduction

The natriuretic peptides (NPs) are a family of three peptide hormones: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) [1-4]. NPs are released into the circulation from cardiac cells to act as hormones in the control of fluid volume homeostasis and blood pressure by causing natriuresis, diuresis, vasorelaxation and inhibition of the renin–angiotensin–aldosterone system [5]. In addition, cell-based studies have shown that ANP and BNP exhibit important autocrine and paracrine functions such as modulating myocyte growth, apoptosis and proliferation in smooth muscle cells [6] and cardiac myocytes [7], and suppress cardiac fibroblast proliferation [8] and extracellular matrix secretion [8-9]. Their local production or presence in various extracardiac tissues suggests additional activities, including roles as neuropeptides [10-21]. The physiological effects of natriuretic peptides are initiated by binding to cell surface receptors. These include natriuretic peptide receptor type A (NPR-A), which is sensitive to ANP and BNP [22], natriuretic peptide receptor type B (NPR-B), which is highly specific for CNP [23], and natriuretic peptide receptor type C (NPR-C), which may comprise up to 95% of the total NPR population [24] and is known to bind all the natriuretic peptides with similar affinity [5]. The hormone binding to NPR-A and NPR-B results in activation of guanylyl cyclase and production of cGMP, which is believed to be the second messenger for a number of biological responses associated with natriuretic peptides [22, 25, 26].The NPR-C has been involved in peptide clearance, removing natriuretic peptides [27], and in the mediation of natriuretic peptide-induced inhibition of cyclic adenosine monophosphate (cAMP) synthesis, and activation of phospholipase C without affecting cGMP levels [28-29].

In this review, we focus on the ability of ES cells to differentiate into functional cardiomyocytes, which have the ability to produce natriuretic peptides in vitro. In addition, we discuss the distribution of NPRs in the brain and their possible functions in various types of neurons.
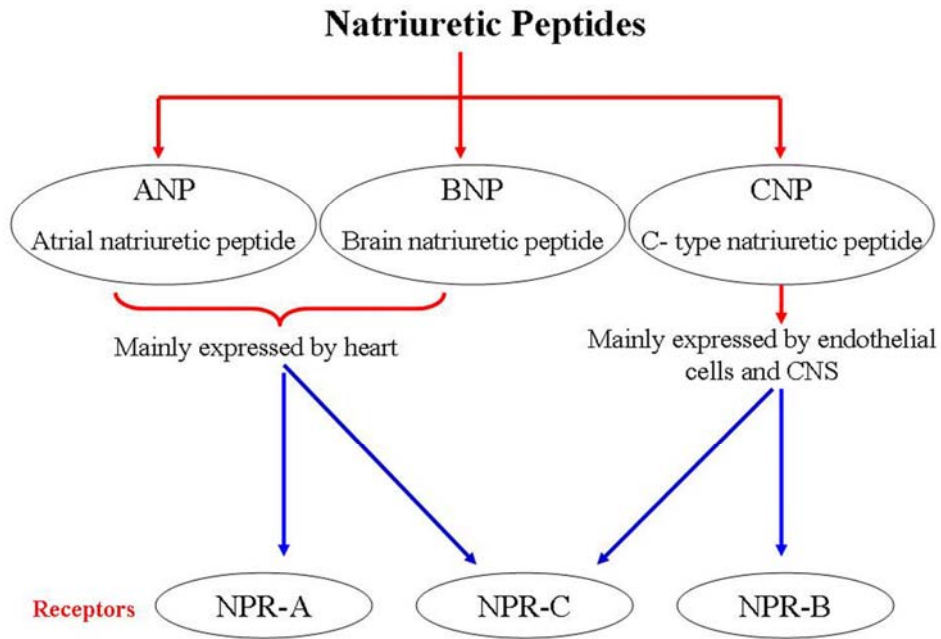
Figure 1. Schematic showing the types of natriuretic peptides and their receptors.
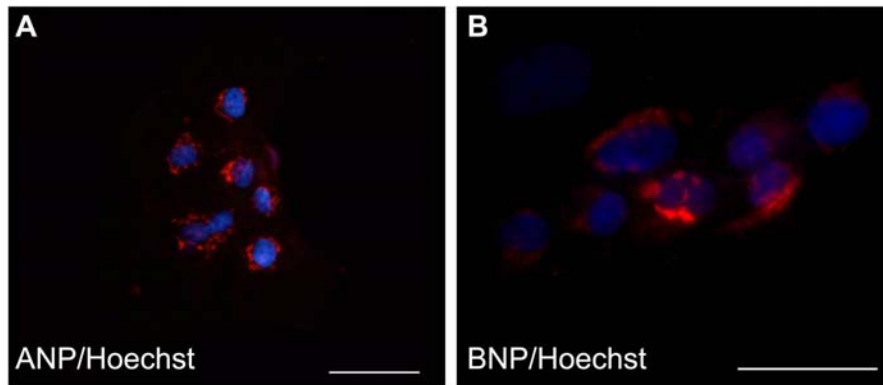

Figure 2. Expression of ANP and BNP in ES cell-derived cardiomyocytes. Note, the perinuclear localization of ANP and BNP. Bar = 50 µm.

Natriuretic peptides in ES cell-derived cardiomyocytes

The heart is the first functioning organ in the embryo and any impairment of its function leads to early lethality [30]. Cardiomyocytes do not regenerate after birth, and they respond to mitotic signals by increasing in the size (hypertrophy) rather than by cell division (hyperplasia) [31]. Recently, embryonic stem (ES) cells and adult cardiac stem cells have been

proposed as transplantable cell candidates that would avoid the problems caused by other types [32-35]. Although, adult cardiac stem cells are capable of differentiating into cardiomyocytes [34-35], and to proliferate to some extent *in vitro*, the rarity of cardiac-specific stem cells makes them difficult to isolate from biopsy specimens and poses a problem for their future clinical application [36]. On the other hand, transplanted ES cell-derived cardiomyocytes have been shown to survive, proliferate and connect with host myocardium [37]. Therefore, the generation of functional cardiomyocytes from ES cells has potential applications including myocardial repair through cell transplantation. One of the important functions of cardiomyocytes is their production of natriuretic peptides, which secreted into circulation to perform several functions in the body [5] (Fig. 2). Previously, we could differentiate monkey ES cells into functional cardiomyocytes that can produce natriuretic peptides [38, Fig. 2]. Furthermore, it has been reported that myocardial tissue produced after ectopic transplantation of mice ES cell-derived cardiomyocytes showed natriuretic peptides expression *in vivo* [39]. These data suggest that natriuretic peptides can be expressed after transplantation of ES cell-derived cardiomyocytes to perform its important *in vivo* function in maintaining the cardiovascular homeostasis [6].

The presence of natriuretic peptides at early stages of ES cell-derived cardiomyocyte development suggests the involvement of natriuretic peptides in cardiomyocyte development. *In vivo*, ANP and BNP levels in fetal ventricles have been reported to be greater than in the adult ventricle [41-42]. It is known that natriuretic peptide receptors are expressed in the heart itself [24]. It has been reported that ANP increases the proliferative activity, expression of contractile proteins and DNA synthesis of cultured chick embryo cardiomyocytes through receptor-mediated pathway [43]. Other studies using a locker of NPR-A and NPR-B showed inhibitory effect of endogenous natriuretic peptides on cardiomyocytes hypertrophy [7]. More recent data

suggested that ANP might antagonize cardiomyocyte hypertrophy-promoting effects of vasoactive peptides and / or growth promoting factors [44]. These studies suggest that ANP has mitogenic action on embryonic cardiomyocytes and inhibitory function on hypertrophy of adult cardiomyocytes.

Interestingly, mice with targeted deletion of BNP exhibit a different phenotype than ANP-deficient mice. Mice without BNP do not have hypertension or cardiac hypertrophy; instead they show focal ventricular fibrotic lesions with a remarkable increase in factors, which implicated in the generation and progression of ventricular fibrosis [45]. Therefore, the BNP may have a role as a local, paracrine antifibrotic factor within the heart. These findings suggest complementary roles of ANP and BNP in the regulation of myocardial structure. The precise reason remains unknown. However, a possibility is speculated that there may be a separate unknown receptor for BNP in cardiac fibroblasts [45]. Thus, the *in vitro* expression of natriuretic peptides observed in the ES cell– derived

cardiomyocytes, together with their elevated levels seen *in vivo* during normal heart formation [46], suggest a paracrine and/or autocrine function for natriuretic peptides during *in vivo* embryonic cardiac development as well as *in vitro* ES cell– derived cardiomyocyte development. These observations suggest that cardiac natriuretic peptides may play a role in the regulation of cardiomyocyte development *in vivo* and *in vitro.*

Cardiac gene expression of both ANP and BNP is increased in animal models of myocardial infarction [47], heart failure [48] and hypertrophy [49]. Therefore, the appearance of increased ANP expression in adult ventricles has become a marker for the embryonic gene program during the development of ventricular hypertrophy [50]. Overexpression of the NPR-A gene in the heart reduced cardiac myocyte size. Coincident with the reduction in myocyte size, ANP was reduced significantly at both mRNA and peptide levels by the overexpression of NPR-A. Thus, cardiac overexpression of NPR-A reduced cardiomyocyte size and

ventricular ANP expression, suggesting a role of NPR-A/cGMP signaling pathway in the regulation of cardiac myocyte
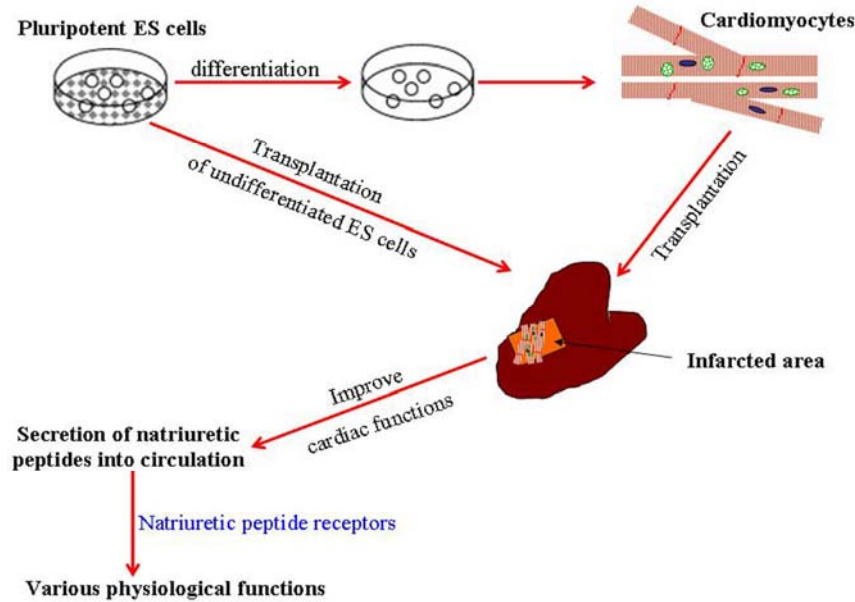
hypertrophy and ANP mRNA expression [51].

Figure 3. Proposed model for transplantation of ES cell-derived functional cardiomyocytes into infracted heart. Pluripotent ES cells differentiate into functional cardiomyocytes *in vitro* then transplanted into infracted region. Another method, undifferentiated ES cells are directly transplanted into infracted regions, and then they differentiate into functional cardiomycoytes *in vivo*. As a result of successful transplantation, cardiac functions are improved leading to secretion of natriuretic peptides into the circulation to regulate cardiovascular homeostasis.

The data presented here suggest that the production of fully functional cardiomyocytes, which can produce NP hormones from ES cell is important for proper development of cardiomyocytes and for their application in cell therapy (Fig. 3).

Natriuretic peptide receptors (NPRs) in the brain

We have reported that NPR-A (Fig. 4A) and NPR-C (Fig. 4B) were localized to neurons in specific nuclei of the brain stem [20-21]. In addition to our reports, the NPRs were found in various regions in the CNS (Table 1), and in several species including, rat, guinea pig, human, monkey, and cat [13-16]. Neuronal NPR-A mRNA

was observed in the mitral cell layer of the olfactory bulb, media; habenula, area postrema, and glia cells [14]. In rat, NPR-B mRNA is expressed in the limbic cortex, neocortex, olfactory bulb, hippocampus, amygdala, preoptic-hypothalamic neuroendocrine circuits, ventral tegmental area, substantia nigra, in motor nuclei of cranial nerves, in brainstem nuclei controlling autonomic function, and pituitary gland (14-15, 52). NPR-C mRNA is expressed in many rat brain regions, including the frontal and retrosplenial granular cortices, medial preoptic nucleus, ventral cochlear nucleus, choroid plexus, deep layers of the neocortex and limbic cortex, posterior cortical amygdala, ventral subiculum, amygdalohippocampal area, and dentate gyrus in the rat brain [13]. In monkey brain, in situ hybridization analysis has demonstrated that NPR-C mRNA is localized to neurons in cerebral cortex and cerebellum [53], suggesting a species difference in cerebellar NPR-C expression. Moreover, in the forebrain, NPR-C shows a lack of overlap with the distribution of NPR-A and NPR-B mRNAs [13, 15].

Although several studies showed the expression of NPR mRNAs in the CNS [17-18, 54-55], a few studies demonstrated their protein expression in the CNS [20-21, 52], NPR-A protein was observed in several regions, including the oculomotor nucleus, red nucleus, locus coeruleus, parabrachial nucleus, the principal trigeminal sensory nucleus, dorsal motor nucleus of the vagus, the hypoglossal nucleus, the gracile nucleus, the cuneate nucleus, the nucleus ambiguus, the reticular formation, the lateral reticular nucleus, and the inferior olivary nucleus. In rat, NPR-B immunoreactivity were found to be localized in the ventral tegmental area, substantia nigra, caudate-putamen, nucleus accumbens, frontal cortex, hippocampus, cortex, cerebellum [52]. NPR-C protein was observed in several regions of the brainstem, including the periaqueductal gray, red nucleus, locus coeruleus, dorsal tegmental nucleus, nucleus of the trapezoid body, ventrolateral pons including A5, cranial motor nuclei, dorsal motor nucleus of the vagus, nucleus ambiguus, and

ventrolateral medulla including A1 and inferior olivary nucleus [21].

The distribution of natriuretic peptide receptor immunoreactivities in the brainstem showed a close anatomical relationship among them. The presence of both guanylyl cyclase receptors (NPR-A and NPR-B) in the same regions suggests the action of ANP, BNP and CNP in these regions, which indicate similar functions of natriuretic peptide hormones in the same regions. On the other hand, the presence of NPR-B and NPR-C in the same regions may suggest a role of NPR-C in limiting the CNP in these areas for local action, or a synergetic role of NPR-B and NPR-C in modulating CNP functions.

In the retina, NPR-A and NPR-B are also localized to neuronal elements, including bipolar cells and amacrine cells, in addition to the two plexiform layers and Müller glial cells [55, 56]. Double labeling experiments in rat retina have revealed expression of NPR-A and NPR-B in dopaminergic amacrine cells, whereas cholinergic amacrince cells express NPR-B [56], suggesting that natriuretic peptides have different regulatory systems in dopaminergic and cholinergic amacrine cells in rat retina.

Several studies demonstrated that NPRs are localized to glial cells in several regions of the brain [58-62]. It was reported that NPR-A and NPR-B are found only in non-neuronal elements in both mixed cultures of the CNS and brain slices. Also, incubation of astrocytes in culture with ANP increases intracellular cGMP levels [58]. NPR-A is predominantly expressed in SHR (spontaneously hypertensive rat) and WKY (Wistar-Kyoto, normotensive) rat astrocyte glial cultures [63]. NPR-B mRNA has been demonstrated in astrocyte cultures [64]. However, in situ hybridization study on rat brain didn't detect NPR-B glial signal [14]. Cultured astrocytes also express abundant NPR-C [63]. In bullfrog retinal Müller glial cells, receptor of ANP is also functionally expressed [66]. Taken together, these findings suggest that natriuretic peptides have functions in glial cells.
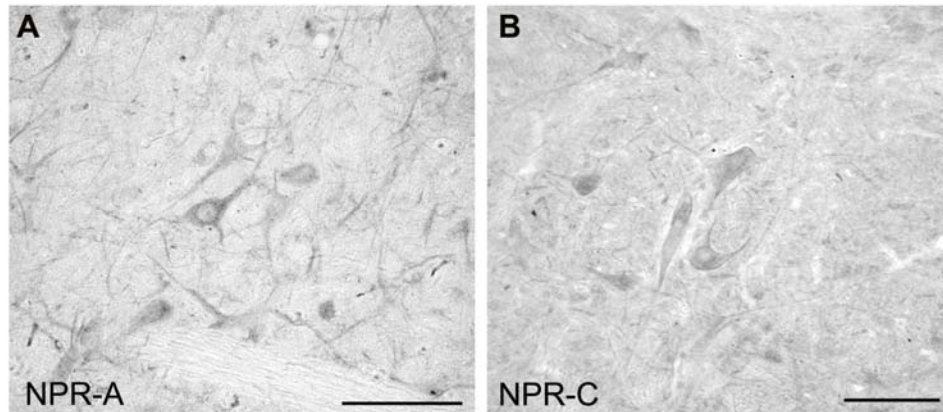
Figure 4. Examples of the localization of NPR-A and NPR-C immunoreactivities in the neurons of the brainstem. Bar = 50 µm.

Possible function of NPRs in the CNS

The important role of the ANP/NPR-A system in the physiological regulation of arterial blood pressure and volume has been emphasized in various genetic mouse models. Targeted deletion of the peptide (ANP$^{-/-}$) or its receptor (GC-A$^{-/-}$), leads to severe, chronic arterial hypertension, cardiac hypertrophy, and sudden death [67-69]. In contrast, overexpression of ANP or NPR-A elicits a "dose-dependent" fall in arterial blood pressure [71]. ANP and NPR-A transcripts were found to be up-regulated for prolonged periods in rat cerebral cortex following acute cortical spreading depression [70]. It is of interest

that NPR-C appears to mediate the mitogenic action of natriuretic peptides in Schwann cells [16, 29, 72]. Mice lacking the NPR-C gene Npr3 show reduced blood pressure and skeletal deformities associated with a considerable increase in bone turnover [73]. In addition, the elimination of NPR-C by antisense has also been reported to attenuate inhibitory effects of CNP on evoked neurotransmitter efflux in PC12 cells [18]. The anti-proliferative actions of ANP on astrocytes are reported to be mediated by NPR-C [54]. In rat hypothalamus, CNP inhibits the calcium current in magnocellular neurosecretory cells through its effect on NPR-C [57]. Natriuretic peptides inhibit

the stimulated proliferation of astrocytes via actions at NPR-C [54], and enhance the survival of PC12 and embryonic basal forebrain cells [74].

	NPR-A	NPR-B	NPR-C
Telencephalon			
- Main olfactory bulb	+++	++	-
- Anterior olfactory nucleus	-	++	-
- Amygdala	-	+++	++
- Cortex	+	+++	+++
- Basal forebrain	-	+	-
- Basal ganglia	-	++	-
- Hippocampus	+	++	+
Diencephalon			
- Hypothalamus	+	+++	+
- Thalamus	-	++	-
Circumventricular organs			
- Area postrema	++	++	-
- median eminence	-	+++	-
- subfornical organ	+++	+++	-
Cerebellum	+	+	+
Brainstem			
A) Midbrain			
- Periaqueductal gray	-	+++	++
- Oculomotor nucleus	++	++	++
- Trochlear nucleus	-	+++	++
- Red nucleus	+++	+	+++
- Dorsal raphe nucleus	-	+	+
- Substantia nigra	-	+	-
B) Pons			
- Pontine nucleus	+	+++	+++
- A5 region	-	+	++
- Facial nucleus	-	++	+++
- Abducens nucleus	-	+++	+
- Parabrachial nucleus	++	+++	+
- Vestibular nucleus	-	+	++
- Trigeminal motor nucleus	+	+++	+++
- Locus coeruleus	+	-	+++
- Cochlear nucleus	-	+++	++
C) Medulla			
- Spinal trigeminal nucleus	+	+++	-
- Hypoglossal nucleus	+++	+++	+++
- Dorsal motor nucleus of vagus	+++	++	++
- Nucleus ambiguous	+++	++	++
- Ventrolateral medulla	-	++	+++
- Solitary nucleus	-	+	-
- Cuneate nucleus	+++	++	-
- Lateral reticular nucleus	++	++	+++
- Inferior olive	+++	+++	++

Table 1. Distribution of NPRs in the brain.

In the monkey brainstem, NPR-A immunoreactivity overlaps with cholinergic neurons in the parabrachial nucleus, the dorsal motor nucleus of the vagus, the hypoglossal nucleus, and the nucleus ambiguus [20]. Also, a large proportion of cholinergic neurons in the cranial motor nuclei and dorsal tegmental nucleus express NPR-C [21]. Furthermore, NPR-B has been detected in brainstem motor nuclei [14], suggesting its presence in cholinergic neurons. These data suggest a possible function of all natriuretic peptides in cholinergic neurons.

Previous reports have shown the role of central cholinergic pathways in the secretion of hormones involved in fluid and electrolyte balance [75]. For example, cholinergic stimulation of the anteroventral third ventricle induces an increase in plasma ANP as well as a marked elevation of peptide content in the medial basal hypothalamus, neuro and adenohypophysis [75]. Furthermore, cholinergic neurons in the dorsal motor nucleus of the vagus are preganglionic parasympathetic neurons that send axons to visceral organs such as the heart and kidney. Also, some of the cholinergic neurons in the nucleus ambiguus project to the heart, and thereby play an important role in cardiac regulation of arterial blood pressure [76, 77]. Microinjection of ANP into the nucleus ambiguus causes a marked bradycardia [78]. These findings, along with the previous results showing the presence of NPRs in cholinergic neurons in the dorsal motor nucleus of the vagus and nucleus ambiguus [20-21], suggest the involvement of natriuretic peptides in cardiovascular control through activation of cholinergic neurons expressing natriuretic peptide receptors.

A previous study has also demonstrated that natriuretic peptides and their receptors are expressed in the eye [56, 79-80], and recently all natriuretic peptide hormones and NPR-B were detected in cholinergic retinal amacrine cells [56, 81]. These observations suggest that natriuretic peptides also play a role in regulating eye functions through cholinergic neurons in the brainstem.

NPRs were detected in regions contain catecholaminergic neurons. NPR-A was

seen in the locus coeruleus which did not overlap with TH-positive neurons [20]. However, NPR-C immunoreactivity was observed in several catecholaminergic cell groups, including the A6, A5, A1, C3 and C1 cell groups. Furthermore, it has been reported that some of these nuclei contain NPR-B mRNA [14]. Since NPR-B is a specific receptor for CNP, these data suggest that NPR-C may bind CNP, but not ANP or BNP, in catecholaminergic neurons of these regions.

The interactions of natriuretic peptides with catecholaminergic neurons have been well documented in previous studies. Locus coeruleus neurons have a presser function. For example, the selective stimulation of noradrenergic cell bodies in the locus coeruleus elicits decreases in blood pressure, renal sympathetic activity and heart rate [82-84]. Furthermore, the ventrolateral medulla contains catecholaminergic neurons which are known to be important in cardiovascular control. Natriuretic peptides induce significant increases in TH mRNA through a cGMP-dependent phosphodiesterase pathway and protein kinase G-dependent mechanisms, which in turn results in stimulation of catecholamine synthesis in PC12 cells [85]. Furthermore, in spontaneously hypertensive rats, local microperfusion of ANP or an NPR-C agonist into the anterior hypothalamus reduces the release of noradrenalin from nerve terminals, an effect accompanied by an increase in arterial blood pressure [86]. All these results suggest that natriuretic peptides regulate cardiovascular function, partly by modulating catecholaminergic neurons.

In addition to the established roles for natriuretic peptides in regulating neuroendocrine and cardiovascular functions, and fluid-electrolyte balance [5], NPs and NPRs were found in regions not related to the mentioned functions. A previous study showed that CNP inhibits dopamine release by stimulation of NPR-B receptors and the increase in intracellular GMP concentration [87]. In addition, CNP can regulate cocaine-induced dopamine release and expression of immediate early genes in brain neurons [52]. Interestingly, CNP has been found to improve learning and consolidation of

learning in a passive avoidance paradigm and dopamine has been shown to be one of the mediating neurotransmitters in the effect of CNP on learning [88]. Recent reports have shown that only the natriuretic peptides binding to NPR-C inhibit adrenergic neurotransmitter efflux [17-18]. Moreover, natriuretic peptides and their receptors, NPR-A and NPR-B, have been found in dopaminergic retinal amacrine cells [56, 81]. Taken together, these results suggest that CNP may regulate the activity of catecholaminergic neurons in several regions of the rat brainstem through binding to NPR-C. The functional significance of NPRs in several regions in the brain remains to be elucidated.

Perspectives

Genetic models and pharamcological studies have shown that the NPs play important roles in the regulation of cardiovascular homeostasis, skeletal growth. In addition, the NP signaling system may be important for cardiomyocyte development as shown by their expression at early stages of heart development and in ES cell-derived cardiomycoytes, and their expression after transplantation is important to maintain cardiac functions. On the other hand, NPRs are widely distributed throughout the brain in regions related to cardiovascular control and in other regions related to other functions, suggesting the involvement of NP system in broad range of functions in the brain. A better understanding of the functions and regulation of NP system may provide an opportunity to clarify their roles during development and in the CNS.

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