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Development of fetal brain renin–angiotensin system and hypertension programmed in fetal origins

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Abstract

Since the concept of fetal origins of adult diseases was introduced in 1980s, the development of the renin–angiotensin system (RAS) in normal and abnormal patterns has attracted attention. Recent studies have shown the importance of the fetal RAS in both prenatal and postnatal development. This review focuses on the functional development of the fetal brain RAS, and ontogeny of local brain RAS components *in utero*. The central RAS plays an important role in the control of fetal cardiovascular responses, body fluid balance, and neuroendocrine regulation. Recent progress has been made in demonstrating that altered fetal RAS development as a consequence of environmental insults may impact on “programming” of hypertension later in life. Given that the central RAS is of equal importance to the peripheral RAS in cardiovascular regulation, studies on the fetal brain RAS development in normal and abnormal patterns could shed light on “programming” mechanisms of adult cardiovascular diseases in fetal origins.

Keywords

Renin-angiotensin system; Fetal brain; Development

1. Introduction

Since the concept of fetal origins of adult health and disease was first introduced in the late 1980s (Barker and Osmond, 1986), the development of the renin–angiotensin system (RAS) in normal and abnormal patterns before birth has attracted significant attention. The RAS has been studied extensively in adults (Fitzsimons, 1998; Paul et al., 2006), but to a much less degree in fetuses. Recent progress in perinatal studies has been made in demonstration of the importance of fetal RAS in both prenatal and postnatal development.

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In the classic definition, the substrate of RAS, angiotensinogen, a glycoprotein, is synthesized and released from the liver, and is cleaved in the circulation by renin secreted from the juxtaglomerular apparatus to generate decapeptide angiotensin (Ang) I. Angiotensin converting enzyme (ACE), a membrane-bound metalloprotease, which is in high density on the surface of pulmonary vascular endothelium, converts Ang I to the octapeptide Ang II. Ang II, the most active peptide of the RAS, binds to Ang II type 1 (AT1R) and type 2 (AT2R) receptors for its physiological or pathophysiological functions (Fitzsimons, 1998; Paul et al., 2006).

Several decades ago, the first evidence of central effects of Ang II was shown with the demonstration that circulating Ang II increased blood pressure *via* a central nervous system (CNS) (Bickerton and Buckley, 1961). The existence of an isolated brain RAS was proposed by the discovery of the renin-like activity in the brain (Fischer-Ferraro et al., 1971; Ganten et al., 1971). Since these initial discoveries, most intrinsic components of the RAS, including angiotensinogen, angiotensins, and converting enzymes, have been well demonstrated in the brain (Saavedra, 1992). More recent developments of molecular biological methods, including transgenic technology and use of infusion gene REN-eGFP (renin-enhanced green fluorescent protein) (Lavoie et al., 2004), have been used to demonstrate the existence of the brain RAS independent of the peripheral RAS.

Studies over the last decades have shown that the central RAS plays an important role in the control of fetal cardiovascular responses, body fluid balance, and neuroendocrine regulation. This review aims at the development of the central RAS during fetal period and examines the ontogeny of the local RAS components in the developing brain *in utero* as well as their functional development before birth. This review also pays attention to alterations of the development of the fetal brain RAS by environmental factors, and its impact on *in utero* “programming” of hypertension in later life. Given that the central RAS and its receptors in the brain are of equal importance to the peripheral RAS in the control of blood pressure, we presumed that studies on the development of the fetal brain RAS in normal and abnormal patterns should shed light on “programming” mechanisms for adult cardiovascular diseases in fetal origins.

2. Components of the fetal brain RAS during development

2.1. Angiotensinogen

Angiotensinogen has been widely studied in both adult and fetus. In adult brain, the angiotensinogen sequence is identical to that in the liver (Campbell et al., 1984) and both angiotensinogen mRNA and protein have been detected (Campbell et al., 1984; Deschepper et al., 1986; Ohkubo et al., 1986; Imboden et al., 1987; Lynch et al., 1987; Thomas and Sernia, 1988). In the fetal brain, angiotensinogen immunoreactivity has been found to be present on day 19 of gestation in rats (Sood et al., 1987a,b). Further studies showed the existence of angiotensinogen in choroid plexus and ependymal cells lining the 3rd ventricle on 18th day of gestation in the rat fetus (Mungall et al., 1995).

Transgenic mice containing the human angiotensinogen (HAGT) gene were utilized to determine the developmental regulation of HAGT expression (Yang and Sigmund, 1998). HAGT expression in rodents was first detected at embryonic day 8.5 and was abundant after day 9.5. Northern blot analysis showed moderate levels of HAGT mRNA in the fetal brain from gestation day (GD) 16.5 onward. In situ hybridization performed on tissue sections revealed that HAGT mRNA became widely distributed in the fetal brain at GD 13.5 (Yang and Sigmund, 1998). Moreover, rat brain angiotensinogen mRNA was shown on day 15 of gestation, and its levels were about 10-fold less than those of days 17–19 of gestation (Lee et al., 1987; Kalinyak et al., 1991; Yang and Sigmund, 1998). From GD 15 to GD 20,

angiotensinogen mRNA was more abundant in the brain than in the liver in rat fetuses. Soon after birth, the level in the brain increased to a concentration of 3-fold above fetal levels whereas that in the liver increased 30-fold within 12 h after birth (Kalinyak et al., 1991). Thus, the temporal difference of brain angiotensinogen mRNA and protein, and the ontogenetic and transitional difference between central and peripheral angiotensinogen mRNA levels suggest intrinsic functions of the brain angiotensinogen during fetal life, which is likely to contribute to the differentiation and/or proliferation in the CNS.

Immunocytochemical localization of brain angiotensinogen in the choroid plexus and ependymal cells lining the third ventricle has been observed on GD 18 in rats. This initial angiotensinogen expression was followed by a rapid progression of staining appearing in astrocytes in the paraventricular nucleus, medial preoptic area, ventromedial and arcuate hypothalamic nuclei (Sernia et al., 1997). In general, neuroglial staining was higher in regions proximal to the cerebral ventricles and cerebral aqueduct. In the rat brain, angiotensinogen is expressed by GD 16–18 (Sernia et al., 1997). From the cell density and intensity of staining, a rapid increase in angiotensinogen occurs between GD 20 and postnatal day 0, followed by further and smaller increases postnatally. The timing of this appearance and development of brain angiotensinogen supports the conception for establishment of a central RAS by late gestation. Its predominance in the fetal hypothalamic nuclei and in the thalamic, cerebellar, and cortical neurons suggests major roles in brain maturation, in prenatal fluid and electrolyte balance, and in sensorimotor development (Mungall et al., 1995).

Antisense deoxyoligonucleotides to angiotensinogen mRNA inhibit *in vitro* growth of neuroblastoma cells, indicating a significant role for angiotensinogen in gene expression. While it is accepted that astrocytes express angiotensinogen, neuronal angiotensinogen also has been demonstrated by immunohistochemistry (Sood et al., 1987a,b; Thomas and Sernia, 1988; Sood et al., 1990; Mungall et al., 1995). Brain angiotensinogen expression is regulated in part by glucocorticoids (Sernia et al., 1997).

2.2. Renin

Renin and its mRNA have been investigated in the adult brain (Hirose et al., 1980; Speck et al., 1981; Schelling et al., 1982; Dzau et al., 1986; Hermann et al., 1987; Saavedra, 1992; Vila-Porcile and Corvol, 1998; Paul et al., 2006). However, information on renin in the fetal brain is limited. Given that other major components of the RAS such as angiotensinogen, AT1R and AT2R were found in the rat fetal brain before day 19 of gestation (see details below), it is likely that the ontogeny of the fetal brain renin also may appear at an earlier developmental age. Sood and co-workers identified the renin activity accompanied with angiotensinogen, ACE, and Ang II in the fetal rat brain on day 19 of gestation using immunochemical methods (Sood et al., 1987a,b, 1989, 1990), albeit the brain renin isoform differs from that in the peripheral circulation (Lavoie et al., 2004).

2.3. Angiotensin converting enzyme (ACE)

ACE immunoreactivity was shown in the human fetal brain (Strittmatter et al., 1986; Schutz et al., 1996), and both ACE mRNA and protein have been detected in the choroid plexus and other brain regions (Mendelsohn et al., 1984; Strittmatter et al., 1984; Chai et al., 1987; Whiting et al., 1991; Rogerson et al., 1995; Baltatu et al., 1998) in fetal rats and rabbits. In the rat fetal brain on day 19 of gestation, ACE was detected in the choroid plexus, subfornical organ, and posterior pituitary, but not in extrapyramidal structures and the anterior pituitary (Strittmatter et al., 1986; Tsutsumi et al., 1993). After birth, ACE was identified in the caudate-putamen (Strittmatter et al., 1986). Our recent functional studies have shown that exogenous Ang I applied into the lateral ventricle of ovine fetuses at 70–90% gestation can induce Ang II-like physiological actions, such as increased blood pressure and release of the neuropeptide

vasopressin (Xu, in preparation), suggesting the existence and functions of ACE in the fetal brain.

2.4. Other enzymes for forming Ang II

Several studies have demonstrated that enzymes including tonin, chymase, and cathepsin D also may be involved in the formation of Ang II in the brain in adults (Dzau et al., 1982; Klickstein et al., 1982; Baltatu et al., 1997; Sumitani et al., 1997). However, the ontogeny and functions of these enzymes related to the central RAS during the early developmental period in the fetal brain have not been reported, and thus present an intriguing area for future investigation.

2.5. Angiotensins (angiotensin peptides)

In adults, Ang I, Ang II, Ang III, and Ang-(1–7) have been demonstrated in the brain in rats, rabbits, and primates (Ferrario et al., 1991). In rats, amino acid sequences of these brain angiotensins are the same as those in plasma (Ganten et al., 1983). Notably, Ang II and Ang-(1–7) are the most widely studied in the brain of adult rats and dogs (Phillips and Stenstrom, 1985; Schiavone et al., 1990; Ferrario et al., 1991; Krob et al., 1998). In rat fetuses at GD 20, Ang II was formed and released from primary cultures in brain cells such as neurons (Weyhenmeyer et al., 1980; Gadbut et al., 1991). During the past decade, a series of studies, including ours, have demonstrated physiological functions of Ang II in the ovine fetal brain, at both near-term and pre-term (El-Haddad et al., 2000, 2001, 2002, 2005; Shi et al., 2004a,c; Xu et al., 2003, 2004, 2005). These studies strongly support the hypothesis that the fetal local RAS in the brain is relatively mature and plays an important role in physiological functions, including the central regulation of cardiovascular and neuroendocrine responses. However, angiotensin peptides used in these experiments were exogenous, and future studies must establish the existence of endogenous Ang II, and its functional ontogeny in the brain during fetal neural development.

2.6. Angiotensin II receptor subtypes

2.6.1. Concept of developmental AT1R and AT2R—In adult, Ang II receptors in the brain have been extensively studied, and the central RAS and AT1R play a key role in the regulation of cardiovascular responses, sodium and water balance, and elaboration of pituitary gland hormones. The AT2R also is expressed during fetal development (Saavedra, 1992; Fitzsimons, 1998; Paul et al., 2006). Tsutsumi et al. showed that both central AT1R and AT2R were located in the brain at GD 18 in fetal rats by quantitative autoradiographic analysis (Tsutsumi et al., 1991a,b). In the fetal rat brain, from GD 11 before birth to postnatal day 28, Nuyt and colleagues used radiolabeled cRNA probes for *in situ* hybridization to determine the development of the two AT1R subtypes (AT1aR and AT1bR) and AT2R mRNA (Nuyt et al., 1999, 2001). These investigators showed that the first appearance of AT1aR mRNA was at GD 19 in many brain regions, and that of AT2R mRNA appeared at GD 13 in the differentiating lateral hypothalamus, and GD 15 in other brain areas (Nuyt et al., 1999, 2001). Primary cultures of astrocytes obtained from various brain regions of GD 17 and postnatal day 1 rats express Ang II binding sites belonging to the AT1R subtype (Bottari et al., 1992). In the rat anterior pituitary at GD 19, AT1R also was prominent (Tsutsumi et al., 1993). These data are of interest because the AT1R subtype has been suggested to be predominant in adults, while AT2R predominates in fetuses. However, this concept should be treated cautiously. The idea of predominant AT1R or AT2R before or after birth can be correct only in general. For certain cells, tissue, or organs, that view may not always be true as AT1R may be predominant in astrocytes of the fetal brain and other tissues (Bottari et al., 1992; Tsutsumi et al., 1991a,b; Xu et al., 2004). Fig. 1 shows a comparison of AT1R and AT2R between the fetal and adult brain (Fig. 1).

2.6.2. Ontogeny and location of AT₁R and AT₂R in the fetal CNS—*In situ*

hybridization has demonstrated the ontogenic development of AT₂R mRNA in the fetal and neonatal rat brain. For example, brain AT₂R mRNA is detected at GD 13 in the lateral hypothalamic neuroepithelium, at GD 15 in the subthalamic and hypoglossus nuclei, at GD 17 in the motor facial nucleus, pedunculopontine nucleus, cerebellum, and the inferior olivary complex, at GD 19 in the thalamus, interstitial nucleus of Cajal, bed nucleus of the supraoptic decussation, nuclei of the lateral lemniscus, locus coeruleus, and supragenual nucleus, and at GD 21 in the lateral septal and medial amygdaloid nuclei, medial geniculate body, and the superior colliculus (Table 1).

In rat brain nuclei at GD 19, AT₁R is located in the subfornical organ, paraventricular nucleus, nucleus of the solitary tract, choroid plexus, and anterior pituitary, and is sensitive to incubation with GTP gamma S (GTP γ S). The sensitivity of AT₂R to GTP γ S was heterogeneous. In the ventral thalamic, rostral hypoglossal, medial geniculate nuclei, and in the locus coeruleus, binding to AT₂R was sensitive to GTP γ S, and these areas belong to the AT_{2a}R subgroup. Conversely, in the inferior olive, medial cerebellar nucleus, and caudal part of the hypoglossal nucleus, areas belonging to the AT_{2b}R subgroup, binding was insensitive to GTP γ S. AT₂ receptors also were present in cerebral arteries (Tsutsumi et al., 1993). Table 1 summarizes the localization of the AT₁R and AT₂R in the fetal rat brain.

2.6.3. Ang-(1–7), Ang IV, and the fetal brain—

The angiotensin protein-coupled oncogene, Mas, may be the intrinsic binding site for Ang-(1–7) (Jackson et al., 1988; Kostenis et al., 2005; Santos et al., 2005). This is important because Ang-(1–7) has been investigated for its functions in fluid-electrolytic homeostasis in the ovine fetus (Moritz et al., 2001). In the adult CNS, Mas is colocalized with arginine vasopressin (AVP) in the supraoptic nuclei (SON) and paraventricular nuclei (PVN), and active in the posterior pituitary for its functions in diuresis and natriuresis balance (Felix et al., 1991; Krob et al., 1998), and may be relevant to amygdale and hippocampus functions (Walther et al., 1998; Von Bohlen und Hahlbach et al., 2000).

As compared to AT₁R and AT₂R, AT₄R, a receptor subtype for angiotensin IV (Ang IV), has been studied mainly in the adult brain. Ang IV has been suggested to play a role in adult learning, memory, and neural plasticity (Wright and Harding, 1997, 2004); however, its existence, distribution, and relevant functions in the fetus are unknown. Several lines of evidence point to an important contribution by the brain RAS to non-classic physiologies mediated by the recently discovered AngIV and AT₄R system. This system appears to interact with brain matrix metalloproteinases to modify extracellular matrix molecules, thus permitting synaptic remodeling critical to the neural plasticity presumed to underlie memory consolidation, reconsolidation, and retrieval (Wright and Harding, 2004). Evidence also supports an inhibitory role by Ang II activation on the AT₁R subtype, and a facilitory role by Ang IV activation of the AT₄R subtype, on neuronal firing rate, long-term potentiation, and associative and spatial learning. The discovery of the AT₄R subtype, and its facilitory influence upon learning and memory, suggests an important role for the brain RAS in normal cognitive processing and perhaps in the treatment of dysfunctional memory disease states in adults (Wright and Harding, 2004). Nonetheless, the anatomical and physiological ontogeny and functions of Ang IV and AT₄R in the fetal brain are still unclear.

3. Functional development of the fetal brain RAS

3.1. Development of the brain RAS and cardiovascular regulation

3.1.1. History of studying brain functions in fetal cardiovascular responses—

Several decades ago, in an attempt to determine whether the CNS plays a role in the blood pressure in the fetus, Macdonald and co-workers decapitated pig fetuses at 40–43 days of

gestation, and measured those decapitated fetuses and intact fetuses (as control) between 35 and 112 days of gestation (term 114 days) (Macdonald et al., 1983). In late gestation, the blood pressure of the decapitated fetuses was significantly lower than that of intact fetuses. These observations demonstrate that blood pressure regulation in the pig varies with gestational age, and the changes occurring in late gestation require central systems within the brain. Among those systems, the brain RAS has attracted great attention in fetal cardiovascular regulation (Xu et al., 2003). Although decapitation might cause other responses, this was an early study on the central control of fetal blood pressure *in utero*.

Ang II receptors are predominant in the fetal hypothalamic nuclei, thalamic, cerebellar, and cortical neurons. The timing of appearance of these receptors supports the establishment of a renin–angiotensin system by late gestation (Mungall et al., 1995). Dysfunction of the fetal hypothalamus rich in Ang II receptors can impact on fetal tissue maturation and hence have profound consequences in postnatal life (Chen et al., 2005).

3.1.2. Central Ang II and fetal blood pressure—During the past decade, a series of studies, including ours, demonstrated that intracerebroventricular (icv) Ang II application in both near-term (~130 GD) or pre-term (~100 GD) ovine fetuses can produce an increase of systolic, diastolic, and mean arterial pressure, in association with a significant decrease of heart rate, indicating in the fetus a central role of Ang II in the regulation of blood pressure and heart rate (Shi et al., 2004a,c; Xu et al., 2003, 2004). We have found in our recent studies that icv application of Ang I also can increase blood pressure in the ovine fetus at near-term *in utero* (manuscript in preparation). This suggests that local angiotensin converting enzyme in the fetal brain was functional in converting Ang I into Ang II in cardiovascular regulation. These studies have demonstrated that the local RAS in the fetal brain is functional, and its activation is critical in regulation of fetal blood pressure. In determining the brain pathways in response to central Ang II stimulation in the fetal brain, we employed c-fos mapping approach. These experiments demonstrated intense c-fos expression in the fetal SON and PVN, the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), median preoptic nucleus (MnPO) in the forebrain, and in the area postrema (AP), tractus solitarius nuclei (NTS), and lateral parabrachial nucleus (LPBN) in the hindbrain (Shi et al., 2004a,c; Xu et al., 2003, 2005). In addition, double labeling showed that icv Ang II activated neurons marked by expression of c-fos in the fetal hypothalamus contained AVP. Vasopressin receptors are subdivided into at least two types, V1 and V2 subtypes. Using V1-, but not V2-receptor antagonist, could partially inhibit the central Ang II-increased fetal blood pressure (Shi et al., 2004a), suggesting that icv Ang II-increased fetal blood pressure partially depends on the neuroendocrine mechanism, via hypothalamic release of AVP.

To investigate the importance of the hypothalamic-pituitary system on the ontogenic changes in RAS activity, Chen et al. used the technique of hypothalamic-pituitary disconnection (HPD) in near-term fetus. HPD did not alter renal renin or prorenin content. However, in the kidney and lung, HPD fetuses had increased AT1R, but not AT2R, mRNA and protein expression. These studies demonstrated that blockade of the substances naturally occurring in the fetal hypothalamus was associated with tissue-specific alterations in expression of AT1R and AT2R in the fetus (Chen et al., 2005).

3.1.3. Fetal brain Ang II receptors and cardiovascular regulation—Studies by ourselves (Hum et al., 2004; Xu et al., 2004) and others (Millan et al., 1991; Tsutsumi et al., 1993; Shanmugam et al., 1994; Wright and Harding, 1995; Nuyt et al., 2001) have shown AT1R protein and mRNA expression in the central RAS pathways, including the PVN, SON, and MnPO. Intense expression of AT1R in those brain areas suggests that an increase of blood pressure associated with icv Ang II-induced cellular activation labeled by c-fos expression in the fetal central network (Xu et al., 2004) was based on AT1R. This finding was further

supported later by using AT1R and AT2R antagonists in the fetal brain. Icv administrated losartan, an AT1R antagonist, but not PD123,319, an AT2R blocker, could suppress central Ang II-increased fetal blood pressure (Shi et al., 2005). This indicates that fetal brain AT1R plays an important role in physiological or pathophysiological responses to the actions of central Ang II. Of note, a high dose of icv losartan itself also increased fetal arterial pressure, which differed from observations in adult animals with similar treatments (Shi et al., 2004b). Although we do not have data to explain the difference, abundant fetal brain AT2R could be one of the causes for different phenomenon observed after central application of the Ang II receptor antagonist.

Disruption of the mouse AT2R gene also resulted in a significant increase in blood pressure and increased sensitivity to the pressor action of Ang II. Thus AT2R mediates a depressor effect, and antagonizes the AT1R-mediated pressor responses by Ang II (Ichiki et al., 1995). In general, both AT1R and AT2R are widely located in the fetal sheep brain, at least at late gestation. This differs from the local RAS in the adult brain. Although a number of studies have investigated the function of central RAS-mediated fetal cardiovascular regulation, many questions remain. For example, why does icv losartan behave differently at a high dose in the fetal brain from that of the adult? Is the cellular activation marked by c-fos in the fetal brain RAS pathways a direct or indirect action of icv Ang II? How is development of the fetal brain RAS regulated? Answers to these questions may lead to further understanding of the development of action sites and central pathways in the fetal brain in response to Ang II.

3.2. Body fluid homeostasis

3.2.1. Fetal swallowing—With fetal swallowing, amniotic fluid was ingested spontaneously (Wislocki, 1921). The swallowed fluid is an admixture of amniotic fluid, lung liquid, and perhaps salivary secretions. Net amniotic fluid volume results from a balance of fetal fluid production, mainly via urine and lung liquid, and that of absorption, via swallowing and perhaps intramembranous flow through the fetal amniotic membranes (Gilbert and Brace, 1989, 1990). Several investigators have investigated fetal swallowing in the sheep. In 1973, Bradley and Mistretta first placed an electromagnetic flow probe in the near-term ovine fetal esophagus, and recorded swallowed volume of 16–44 ml/kg daily (Bradley and Mistretta, 1973). Swallowing activity occurred primarily in 2–7 bouts of activity, suggesting a potential association of swallowing with fetal neurobehavior (Harding et al., 1984). Thereafter, swallowing was measured by electromyogram activity of laryngeal adductor muscles establishing the relationship of swallowing to fetal breathing movements and low voltage high frequency (REM-like pattern) electrocorticogram (ECoG) activity. During the last third of gestation, swallowing bouts were always accompanied by fetal breathing movements. Bouts occurred at intervals of 2.3 h, predominantly during the periods of low-voltage ECoG activity and active eye movements (Harding et al., 1984). Later, investigators used electromyogram wires placed on the fetal thyrohyoid muscle and the nuchal and thoracic esophagus, with an ultrasonic flow probe placed around the fetal thoracic esophagus, to measure electromyogram activity and esophageal fluid flow. Analysis of the electromyogram waveforms detects individual muscle contractions and subsequently defines a “swallow” as a timed sequence of waveform progression from the thyrohyoid to the thoracic esophagus. Bouts of swallowing are defined as three or more successive swallows occurring at rates greater than three per minute. The volume swallowed is measured by the integral of the flow velocity waveform from the ultrasound flow meter (Sherman et al., 1990; Ross and Nijland, 1998). Spontaneous ovine fetal swallowing activity is associated with fetal behavioral state monitored continuously for 24 h. Therefore, factors that impact on fetal neurobehavior may influence swallowing activity, and thereby fetal body fluid homeostasis. Development of this technology has allowed researchers the opportunity to study fetal swallowing behavior and “thirst” responses *in utero* (Ross et al., 1994).

3.2.2. Body fluids and the fetal brain RAS—The diverse localization of the local RAS throughout the brain has suggested a variety of potential functions. The strong stimulation and tonic function of central RAS, especially, central Ang II, has been studied intensively in adults (Fitzsimons, 1998). The brain RAS-mediated behavioral and endocrine responses are important in the regulation of body fluid balance, including water and salt intake, renal excretion and reabsorption (the relationship between the fetal brain RAS and endocrine is discussed below).

Although a number of studies have revealed the roles of the central RAS in regulation of body fluids in adults, the functional development of the fetal brain RAS in body fluid balance has been studied recently. Intracerebroventricular Ang II injection in the chronically catheterized sheep fetuses-induced vigorous swallowing associated with low voltage ECoG high frequency in late gestation (Ross et al., 1994; Xu et al., 2001; El-Haddad et al., 2000, 2001, 2002, 2005). In addition, icv Ang II-induced fetal swallowing activity could be blocked by an angiotensin receptor antagonist (El-Haddad et al., 2001, 2005). These physiological experiments provide evidence that the fetal brain RAS is active at late gestation and appear to play an important role in the control of fetal dipsogenic responses during development.

Further studies have demonstrated the functional development of the central areas in response to Ang II. Shi et al showed that the nuclei activated by icv Ang II in the brain of ovine fetuses include the SFO, MnPO, OVLT, SON, and PVN in the forebrain, and the NTS and LPBN in the hindbrain (Shi et al., 2004a,c; Xu et al., 2003, 2004, 2005). The data suggest that all those areas in the fetal brain have been developed for actions of central Ang II. Based on the evidence that icv Ang II-stimulated fetal swallowing activity was associated with activation of the brain nuclei, including the SFO, OVLT, AP, and LPBN (Xu et al., 2001), two conclusions can be considered. First, the brain Ang II is important to fetal behavioral development in body fluid regulation. Second, the central pathways related to the fetal brain RAS are relatively intact in response to Ang II stimulation. This is important for the brain RAS pathways in cooperation with the peripheral RAS. Under normal physiological conditions, Ang II cannot cross the blood–brain–barrier (BBB). The BBB is relatively impermeable to low-molecular-weight amino acids, even at 60% of gestation (~75 GD) in sheep (Stonestreet et al., 1996). However, several central regions, including the SFO, OVLT, and AP, lack a BBB, thus Ang II in the peripheral circulation can act on the brain via those central “windows”. In a recent study in the sheep, we demonstrated that intravenous Ang II can induce cellular activity marked with intensive c-fos expression in the SFO, OVLT, MnPO, SON, and PVN (Shi et al., 2003, 2004c). The peripheral Ang II acts on the fetal hypothalamus indirectly via the circumventricular organs (CVOs, such as SFO, OVLT in the forebrain) which lack the BBB, but are rich of Ang II receptors. These results are of interest because they indicate that pathways from the SFO, OVLT, and MnPO to the fetal hypothalamus have been established, and are functional during late gestation (Fig. 2). Importantly, this finding also provides a novel approach of using the existing method (c-fos mapping with *in vivo* fetal model) to study functional development of fetal brain pathways *in utero*. Understanding this aspect of developmental physiology also is important to understanding abnormalities which may occur. Alteration during development of the fetal brain RAS may result in problems that impact not only on prenatal but also on postnatal health and wellbeing.

3.3. Neuroendocrine axis

3.3.1. Vasopressin and oxytocin—In adults, accumulating evidence has demonstrated the magnocellular neuroendocrine cells of the hypothalamus are critical in maintenance of body fluid homeostasis by releasing AVP and oxytocin (OT) in response to a variety of stimuli, including central administration of Ang II (Mahon et al., 1995; Lauand et al., 2007). Recent studies have demonstrated icv Ang II can elicit a significant increase of plasma AVP in both

near-term (~135 GD) and pre-term (~95 GD) ovine fetuses (Ross et al., 1994; Shi et al., 2004a; Xu et al., 2005) without altered fetal plasma osmolality, sodium, and hematocrit levels. In addition, the data from pre-term ovine fetuses showed that the increase of fetal plasma AVP is Ang II dose-dependent. The fetal plasma OT also was increased in response to icv Ang II during the last third of gestation. These results suggest that the hypothalamic-neurohypophyseal system is functional at least at 70–90% of gestation, and Ang II plays an important role in fetal neuroendocrine functions. Moreover, as mentioned above, increased plasma AVP contributes, in part, to the central Ang II-induced pressor responses (Shi et al., 2004a). Nonetheless, the role of fetal OT released by central Ang-II remains to be clarified, although it is likely to be involved in body fluid homeostasis.

Further studies using double labeling have shown many central Ang II-activated AVP-containing neurons in the fetal hypothalamus with marked c-fos expression (Shi et al., 2004a; Xu et al., 2003, 2004, 2005). Angiotensin receptor antagonist experiments also have demonstrated that losartan, but not PD123319, inhibited an increase of plasma AVP concentrations in response to icv Ang II (Shi et al., 2006). These data thus suggest that in the developing fetus, the brain AT1R mechanism is critical in the central Ang II-related neuroendocrine responses. Previous studies on central Ang II-released AVP in the fetus mainly focused on fetal cardiovascular and body fluid regulation. It is well known that AVP and OT also have other neurophysiological functions either in the local areas of the brain or released into the peripheral circulation. These remain unknown. Further studies are needed in this regard.

3.3.2. Hypothalamic-pituitary-adrenal (HPA) axis—In the adult, in addition to Ang II-mediated AVP and OT responses, Ang II in the brain also stimulated adrenocorticotrophic hormone (ACTH) secretion (Ganong, 1993). Ganong has demonstrated that circulating Ang II can act on the CVOs to increase corticotropin-releasing hormone secretion (Ganong, 2000), indicating that Ang II plays an important role in HPA axis regulation.

During fetal development, progressive maturation of the HPA axis is associated with an increase of plasma ACTH and cortisol; the peak was at the onset of birth in sheep (Currie and Brooks, 1992). The fetal HPA axis responds to maternal stress during late gestational period (Ohkawa et al., 1991). By employing immunohistochemical techniques, Bishop and King found corticotropin-releasing factor (CRF) at rodent GD 10 in scattered puncta that appear to approximate cell bodies throughout the cerebellar plate (Bishop and King, 1999). The localization of type 1 corticotropin releasing factor receptor (CRF-R1) also has been demonstrated in the embryonic mouse brain (King et al., 2003).

Keiger et al demonstrated in the developing ovine brain that CRF mRNA levels are differentially regulated by cortisol in a region-specific manner. Fetal plasma cortisol levels were chronically elevated at 70% of gestation (100 days) to physiological levels found at 90% of gestation, when glucocorticoid-induced maturational changes are known to occur in the HPA axis. Cortisol treatment increase CRF mRNA levels 3.5-fold in the medulla oblongata of fetuses, indicating that cortisol up-regulates CRF gene expression at 70% of gestation in the fetal ovine brainstem (Keiger et al., 1995).

Keller-Wood et al. identified the changes in genomic expression of critical components of the HPA axis in the second half of gestation in fetal sheep. These workers isolated CRF mRNA from the pituitary, hypothalamus, hippocampus, and brain stem in fetal sheep at 80, 100, 120, 130, and 145 days gestation. They showed that both glucocorticoid receptors and mineralocorticoid receptors were highly expressed in the pituitary and hippocampus; and hypothalamic CRF expression was increased at the end of gestation compared with younger ages. Overall, these results demonstrate the expression of both glucocorticoid receptors and

mineralocorticoid receptors in the fetal brain as being important for the control of the HPA axis (Keller-Wood et al., 2006). Feedback control of glucocorticoid production also is established *in utero* (Reichardt and Schütz, 1996).

Notably, both the brain RAS and HPA develop during late gestation, and present certain functions that have been established in previous studies *in utero* (Fig. 3). However, still lacking is direct evidence for a link between the fetal RAS and HPA as has been demonstrated in the adult. Thus, future studies for that possible association in either neurophysiology or pathology in fetal models are required.

3.3.3. Other hormones—Interactions between central angiotensin and other hormone systems have been demonstrated in studies of mature animals. Intraventricular injection of Ang II has been shown a suppressive action on the release of prolactin (PRL), growth hormone (GH), and thyroid-stimulating hormone (TSH) (Franci et al., 1997). To determine the role of endogenously released Ang II in hypothalamic-pituitary hormone release, specific antiserum directed against Ang II microinjected into the third ventricular of the conscious, ovariectomized rats, resulted in elevation of PRL, GH, and TSH (Franci et al., 1997). This suggests that the Ang II cells in the brain have a physiological significance in suppressing release of those hypothalamic neurohormones.

In vitro studies also demonstrated that Ang II can cause the release of PRL from the pituitary glands of adults and neonates (Kacsóh et al., 1993; Aléssio et al., 1994; Eckert et al., 2003). Ang II can cause a rapid and short-lasting stimulation on PRL release from the organ-cultured tilapia pituitary (Eckert et al., 2003).

In anterior pituitary cell aggregates, cultured in the presence of the glucocorticoid dexamethasone (DEX), Ang II showed a dual effect on growth hormone by stimulating GH release in aggregates from 2-week-old rats, whereas its effect was inhibitory in cultures from adult rats. These data suggest that the glucocorticoid-dependent stimulus-effect coupling of Ang II on GH release is involved in both stimulatory and inhibitory components, and that the stimulatory component predominates during immature life, while the inhibitory one predominates during adult life (Robberecht and Denef, 1990). In addition to the effect of Ang II on GH, GH itself also up-regulates, directly and not via insulin-like growth factor 1 (IGF-I), angiotensin receptors of the AT1a subtype in astrocytes by a transcriptional mechanism (Wyse and Sernia, 1997).

Because of the link between the fetal RAS and the HPA mentioned above, we know that RAS is quite mature in the fetal brain at late gestation and that other hormone systems such as PRL, GH, and TSH are relatively intact at the same pregnant period, and we also know there is an active interaction between the brain RAS and other hormones in adults. However, we do not know if a functional link between the fetal brain RAS and other hormone systems has been established before birth. This deserves further study.

3.4. Roles of the RAS in development

A growing body of evidence suggests that angiotensin may have a functional role in growth and differentiation. AT2R promotes vascular differentiation and contributes to vasculogenesis (Yamada et al., 1999), nephron tubular development (Wolf, 2002), and neural differentiation (Li et al., 2007). Alterations in AT2R signaling may change the delicate balance between growth stimulation and inhibition, leading to alterations in development (Wolf, 2002). It has been proposed that during fetal development the RAS is up-regulated. On the other hand, inhibition of the RAS by ACE inhibitors or other blockers may produce specific development abnormalities in tissue and organs (Wolf, 2002).

Using *in vitro* receptor autoradiography, Cook et al. identified specific 125I-Sar1, Ile8 Ang II binding in several brain areas in fetal rats, and observed significant differences in the concentration of binding sites, at different ages in several brain nuclei. With the knowledge that RAS components appear early in development, and have an association with cellular growth, it is not unlikely that an irregularity occurring during neurogenesis could contribute to abnormalities of developmental as well as subsequent diseases such as hypertension (Cook et al., 1993). In fetal tissues, AT2 receptors are abundantly and widely expressed, but in the adult, they are present only in restricted tissues such as in the atretic ovary. Although the function of AT2R is not as well defined as that of AT1R, its abundance in the fetal brain and wound tissue suggests a role in growth and development. Mukoyama et al. (1993) cloned a cDNA encoding a unique 363-amino acid protein with pharmacological specificity, tissue distribution, and developmental pattern of the AT2R. It shares 34% homology with the AT1R, including a seven-transmembrane domain topology, suggesting that this receptor may belong to a unique class of seven-transmembrane receptors that include somatostatin SSTR1, dopamine D3, and frizzled protein Fz. All members of this class exhibit fetal, developmental, and neuronal-specific expression. A conserved motif in the third intracellular loop, distinguishing this class from “classical” G protein-coupled receptors, may mediate novel intracellular effects (Mukoyama et al., 1993). The putative negative regulatory region located between the positions –453 and –225 may play an important role in the transcriptional control of AT2R expression, along with the cell growth. In confluent cells, enhanced interferon regulatory factor 1 expression antagonizes the interferon regulatory factor 2 effect, and increases the AT2R expression. Thus, it was proposed that these transcriptional factors influence cell growth in part by regulating AT2R expression (Horiuchim et al., 1995; Mogi et al., 2007). AT2 receptors appear to act as modulators of complex biological programs involved in embryonic development, cell differentiation, tissue protection and regeneration, as well as in programmed cell death (Unger, 1999).

Li et al. demonstrated that AT2R signalling-induced neural differentiation via an increase in MMS2, one of the ubiquitin-conjugating enzyme variants. The AT2R, MMS2, Src homology 2 domain-containing protein-tyrosine phosphatase 1 (SHP-1) and newly cloned AT2R-interacting protein (ATIP) were expressed highly in fetal rat neurons, declining after birth (Li et al., 2007). A study in PC-12W cells showed that the AT2R not only inhibited growth factor-induced proliferation, and enhanced nerve growth factor (NGF)-mediated growth arrest, but also induced morphological differentiation in cells of neuronal origin. These data support the hypothesis that the AT2R promotes differentiation in neuronal cells (Meffert et al., 1996). Ang II-induced MMS2 expression was enhanced with the AT1R blocker valsartan but inhibited by the AT2R blocker PD123319 (Li et al., 2007). The increase in AT2R-induced MMS2 mRNA expression was enhanced by overexpression of ATIP. Following AT2R stimulation, ATIP and SHP-1 were translocated into the nucleus after formation of their complex. Furthermore, increased MMS2 expression may mediate the inhibitor of DNA binding 1 proteolysis, and promote DNA repair. These results provide new insight into the contribution of AT2R stimulation to neural differentiation, via transactivation of MMS2 expression involving the association of ATIP and SHP-1 (Li et al., 2007).

Antisense to angiotensinogen mRNA inhibited *in vitro* growth of neuroblastoma cells, indicating a significant role for angiotensinogen in mitogenic functions (Sernia et al., 1997). Together, the RAS plays an important role in brain development, and AT2R has attracted greater attention in cellular differentiation. In spite of that, the role of AT2R in neural development is unclear. Hopefully, over the next a few years we should gain new knowledge and answers to questions on angiotensin functions in development. Given the rapid progress in research on fetal angiotensin, this may serve as a model for the action of peptides on cellular functions in general.

3.5. Others

In addition to its classical role in the regulation of blood pressure and body-fluid homeostasis, angiotensin in the brain has more subtle functions involving complex mechanisms such as learning and memory. The profound effects on behavior produced by angiotensin are of broad interest to neuroscientists. For example, researchers have found that most of the behavioral effects by Ang II described so far are linked with AT1R. The neuronal effect of Ang II in the inferior olivary nucleus, blocked specifically by AT2R antagonists, suggests an involvement in motor control (Mosimann et al., 1996). Disruption of the AT2R gene attenuated exploratory behavior and lowered body temperature, suggesting that AT2R regulates brain functions in the control of the behavior (Ichiki et al., 1995). How, and to which extent, the local RAS and its receptors in the fetal brain influence development of learning, memory, and other CNS functions deserves new studies.

4. Fetal origins of hypertension and abnormal development of the brain RAS

4.1. Nutrition and hypertension

Inadequate nutrition compromises fetal development and poses long-term health risks for the offspring. Protein restriction in food during pregnancy increases rat offspring blood pressure by 20–30 mmHg (Sahajpal and Ashton, 2005). An early study suggested that this was associated with a reduction in nephron number and increased glomerular sensitivity to Ang II *in vivo* (Woods et al., 2001). More recent studies have proposed a link between impaired nephrogenesis, decreased activity of the RAS, and the onset of hypertension in rats exposed *in utero* to a maternal low-protein diet (Langley and Jackson, 1994; Gilbert et al., 2007). Increased AT1 receptor expression, which may be a consequence of the direct effect of protein restriction, could contribute to the elevated blood pressure of this model (Sahajpal and Ashton, 2003). In offspring rats with history of maternal protein restriction during pregnancy, by 4 weeks after birth, AT1 receptor expression had increased (62%) and AT2 expression was decreased (35%). Renal renin activity, tissue Ang II, and plasma aldosterone concentrations did not differ. Increased AT1 receptor expression in kidneys is consistent with greater haemodynamic sensitivity to Ang II *in vivo* (Sahajpal and Ashton, 2003). This may result in an inappropriate reduction in glomerular filtration rate, salt and water retention, and an increase in blood pressure.

Substantial epidemiologic and experimental data now support the concept that the “fetal origins” is important in determining subsequent risk for the development of cardiovascular and metabolic diseases (Barker and Osmond, 1986; Barker, 1995; Huxleym et al., 2000; Godfrey and Barker, 2001). Intrauterine programming of hypertension is associated with evidence of increased RAS activity. Pladys and co-workers investigated whether arterial baroreflex and blood pressure variability are altered by *in utero* programming of hypertension secondary to isocaloric protein deprivation and whether activation of the RAS plays a role in this alteration. Pregnant rats were fed low-protein (9%) diet during gestation. Mean arterial blood pressure (MABP) and blood pressure variability were significantly greater in the adult offspring of the 9% protein-fed mothers. Arterial baroreflex generated by i.v. infusion of phenylephrine and nitroprusside was significantly shifted toward higher pressure; i.v. ACE inhibitor normalized MABP and shifted the arterial baroreflex curve of the 9% offspring toward lower pressure. Intracerebroventricular injection ACE inhibitor and AT1R antagonist significantly reduced MABP of the offspring. The brain AT1R expression in the subfornical organ and the vascular organ of the lamina terminalis was increased in the offspring (Pladys et al., 2004). These data demonstrate a major tonic role of the brain RAS in hypertension associated with antenatal nutrient deprivation.

4.2. Glucocorticoid and programming hypertension related to the fetal RAS

In rats, maternal treatment with the glucocorticoid corticosterone resulted in a nephron deficit in association of development of hypertension in the offspring (Singh et al., 2007). Maternal corticosterone treatment caused a nephron deficit of 21% in male and 19% in female offspring. Mean arterial pressures were elevated significantly in offspring of both sexes. Real-time PCR revealed that maternal corticosterone increased fetal expression of AT1R and AT2R in the peripheral tissue. Changes in the RAS may be contributing to the phenotypes in development of hypertension.

Any factor which disrupts fetal development *in utero* may induce maladaptive changes that contribute to the risk of diseases. Such factors include exposure of the mother to hypoxia, malnutrition, or glucocorticoid at critical gestational periods. Although Jobe et al. reported that pre-term fetal betamethasone administration did not alter neonatal pulmonary systems, renal functions were changed by a 20-min period of mild hypoxia in the offspring (Jobe et al., 1996). It was proposed that permanent changes in gene expression and functions of the brain and kidney could be crucial in the development of adult-onset hypertension as a result of prenatal exposure to inadequate factors such as cortisol (Dodic et al., 2002c) (environmental insults like hypoxia, stress, and malnutrition also could be inadequate factors to fetuses during pregnancy). Studies in sheep demonstrated alterations in the expression of hypothalamic angiotensinogen and AT1R in the medulla oblongata (Wintour et al., 2003). The brain is one of organs most vulnerable in early development, particularly in an extremely primitive state of development.

In sheep, mean arterial pressure was significantly higher in the adult offspring whose mothers had been treated with cortisol during pregnancy. Prenatal cortisol treatment led to up-regulation of angiotensinogen and AT1R mRNA in the hippocampus in fetuses at 130 GD. The first evidence that relatively short prenatal exposure to cortisol can program high blood pressure in the adult female and male offspring of sheep was reported by Dodic et al. (2002b). Their findings suggest that altered gene expression in the hippocampus could have a significant effect on hippocampal development, and on postnatal behavior (Dodic et al., 2002b). Further studies showed when pregnant ewes and their fetuses were exposed to dexamethasone for 2 days early in pregnancy (days 26–28), some aspects of the local RAS (particularly in the kidney and brain) could have participated in the “programming” event (Dodic et al., 2001). The levels of mRNA for AT1R and AT2R, as well as angiotensinogen, are increased in the kidney of dexamethasone-treated fetuses in late gestation in sheep. Similar increases in AT1R mRNA in the medulla oblongata of the fetal brain and large increases of mRNA for angiotensinogen occur in the hypothalamus. These findings suggest that both kidney and brain regions are affected by events that also “program” high blood pressure in the offspring of animals in which the intra-uterine environment has been perturbed at certain stage (Dodic et al., 2001). In sheep, dexamethasone administered between 26 and 28 days of gestation resulted in an increased expression of angiotensinogen in the fetal hypothalamus. In addition, the medulla showed higher expression of the AT1R. Brief prenatal exposure of the pregnant ewe to dexamethasone leads to hypertension in adult offspring of both sexes. Importantly, the mechanisms leading to programming of hypertension may be linked with the brain angiotensin system (Dodic et al., 2002a).

In addition to malnutrition and glucocorticoids, many other environmental factors also may cause programming of hypertension *in utero*. For example, recently we demonstrated that the offspring rats with prenatal history of exposure to nicotine during pregnancy showed a higher blood pressure to Ang II simulation in association with alterations of the Ang II receptor levels in the heart (Xiao et al., 2008). Such associations need to be tested in greater detail.

5. Conclusions and future directions

Since the hypothesis of the fetal origins of adult diseases about 20 years ago, physiology has become active in the study of hypertension and other adult diseases in fetal origins. *In utero* programming of hypertension via alteration of the RAS before birth has attracted great attention. Accumulated evidence strongly supports the hypothesis that the RAS plays an important role in contributing to “program” cardiovascular diseases *in utero*. Notably, most previous studies in this field have focused on the peripheral RAS and its roles in prenatal imprinting. Considering that the local RAS in the brain and its central receptor systems are as important as the peripheral RAS in the regulation of blood pressure, neuroendocrine, and body fluid homeostasis, more studies on the fetal brain RAS and its roles in “programming” are expected. The challenge is for us to understand in depth the mechanisms by which antenatal stress can alter normal neurophysiology and abnormal pathophysiology of the fetal brain RAS.

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Abbreviations

ACE	angiotensin converting enzyme
ACTH	adrenocorticotrophic hormone
Ang	angiotensin
AP	area postrema
AT1R	angiotensin II receptor subtype 1
AT2R	angiotensin II receptor subtype 2
ATIP	AT2R-interacting protein
AVP	arginine vasopressin
BBB	blood–brain-barrier
CNS	central nervous system
CRF	corticotropin-releasing factor
CRF-R1	corticotropin releasing factor receptor
CVOs	circumventricular organs
DEX	dexamethasone
ECoG	electrocorticogram
GD	gestation day
GH	growth hormone
GTP γ S	GTP gamma S
HAGT	human angiotensinogen
HPA	hypothalamic-pituitary-adrenal axis
HPD	hypothalamic-pituitary disconnection

Icv	intracerebroventricular
IGF-I	insulin-like growth factor 1
LPBN	lateral parabrachial nucleus
MABP	mean arterial blood pressure
MnPO	median preoptic nucleus
NGF	nerve growth factor
NTS	tractus solitarius nuclei
OT	oxytocin
OVLT	organum vasculosum of the lamina terminals
PRL	prolactin
PVN	paraventricular nuclei
RAS	renin–angiotensin system
REN-eGFP	renin-enhanced green fluorescent protein
SFO	subfornical organ
SHP-1	Src homology 2 domain-containing protein-tyrosine phosphatase 1
SON	supraoptic nuclei
TSH	thyroid-stimulating hormone

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Fig. 1.

AT1R and AT2R in the adult (B) and fetal (A) brain. C: indicates AT1R proteing density from preterm to term in the ovine fetal brain. AP, area postrema; AT1R, Ang II type 1 receptor; AT2R, Ang II type 2 receptor; LPBN, lateral parabrachial nucleus; MnPO, median preoptic nucleus; NTS, nuclei of tractus solitarius; OVLT, organum vasculosum of the lamina terminals; PVN, paraventricular nuclei; SFO, subfornical organ; SON, supraoptic nuclei.

Fig. 2.

The RAS pathways in the fetal forebrain. The SFO and OVLT lack of the BBB, but rich of AT1R. Intravenous Ang II cannot pass the BBB, this peptide acts on the SFO and OVLT, not only induced c-fos in the SFO and OVLT, but also in the SON and PVN. This shows functional projections between the CVO and the PVN/SON have been established *in utero* at the gestational age tested. Ang II, angiotensin II; BBB, blood–brain barrier; CVO, circumventricular organ; MnPO, median preoptic nucleus; OVLT, organum vasculosum of the lamina terminalis; PVN, paraventricular nuclei; RAS, renin–angiotensin system; SFO, subfornical organ; SON, supraoptic nuclei.

Fig. 3. Comparison of physiological functions of Ang II in the fetal and adult hypothalamus. ACTH, adrenocorticotrophic hormone; Ang II, angiotensin II; AVP, arginine vasopressin; CRF, corticotropin-releasing factor; OT, oxytocin; PRL, prolactin; TSH, thyroid-stimulating hormone.

Table 1

Genes of angiotensin receptors (AT1R and AT2R) in rat fetal brain.

Brain region	AT1 mRNA	AT2 mRNA
Telencephalon		
Anterior olfactory nucleus	√(21)	
Cingulate cortex	√(21)	
Piriform cortex	√(21)	
Septohippocampal nucleus	√(21)	
Lateral septal nucleus		√(21)
Medial amygdaloid nucleus		√(21)
Diencephalon		
Median preoptic nucleus	√√(19)	
Paraventricular nucleus	√√(19)	
Thalamus		√√(19)
Medial geniculate body		√(21)
Subthalamic nucleus		√(15)
Bed nucleus of the supraoptic decussations		√(19)
Differentiating lateral hypothalamic area		√√√(13)
Mesencephalon		
Periaqueductal gray matter (raphedorsal)	√√(19)	
Red nucleus	√√(21)	√√(15)
Interstitial nucleus of Cajal		√(17)
Superior colliculus		√(21)
Pedunculopontine nucleus		√(17)
Pons		
Nucleus of the lateral lemniscus		√(19)
Locus coeruleus		√(19)
Supragenual nucleus		√√(15)
Motor nucleus of the trigeminal nerve		√√(21)
Principal sensory nucleus of the trigeminal nerve		√√(17)
Superior olivary complex		√√(19)
Cerebellum		√√(17)
Medulla oblongata		
Nucleus raphe pallidus	√√(19)	
Motor facial nucleus	√√(19)	√√(17)
Nucleus of the solitary tract	√(19)	
Ambiguous nucleus	√(19)	
Differentiating reticular formation		√√√(15)
Gigantocellular reticular nucleus		√√(17)
Medullary reticular nucleus		√(19)
Lateral reticular nucleus		√√(17)
Spinal nucleus of the trigeminal nerve		√(21)
Inferior olivary complex		√(17)

Brain region	AT1 mRNA	AT2 mRNA
Parasolitary nucleus		√(19)
Motor hypoglossal nucleus		√(15)
External cuneate nucleus		√(21)
Circumventricular organs		
Vascular organ of the lamina terminalis	√√(19)	
Subfornical organ	√√(21)	

("√, √√, √√√" indicates immunoreactive intensity from mild, moderate, to the highest, respectively. 13 and 21: gestation days 13 and 21).