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Ontogeny of Angiotensin Type 2 and Type 1 Receptor Expression in Mice

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Abstract

In the current experiment, we determined AT2R and AT1R protein expression by Western blot analysis in developing normal mice. The results indicate that, (1) in all detected brain regions and in the spinal cord, adult mice exhibited significantly higher AT2R expression and lower AT1R expression in total protein extracts compared to fetuses and neonates; (2) other major organs, including heart, lung, liver, and kidney, exhibited the same expression pattern as the brain and spinal cord; (3) reciprocal changes in AT2R and AT1R expression were found in the total protein extracts from the brainstems of mice from one day prenatal to six weeks of age. There was a negative correlation between AT2R and AT1R protein expression; (4) in both membrane and cytosolic fractions from the brainstem, adult mice exhibited higher AT2R and lower AT1R expression than did fetuses and neonates; (5) in the brainstem, there were no significant differences in AT2R and AT1R mRNA levels among fetal, neonatal, and adult mice. The above results reconfirmed our previous finding in rats that adult animals have higher AT2R and lower AT1R expression compared to fetuses and neonates. These data imply an involvement of AT1R in fetal development and of AT2R in adult function.

Keywords

Angiotensin receptors; development; protein expression; mRNA expression

1. Introduction

The Renin-Angiotensin System (RAS) plays multiple roles in sympathetic regulation, cardiovascular control, water and electrolyte balance, and hormone secretion[1;2]. Angiotensin type 1 receptors (AT1R) and Angiotensin type 2 receptors (AT2R) are two main effectors of this system, through which Angiotensin II exerts the majority of its functions. A better understanding of AT1R and AT2R expression profiles during growth and maturation is essential to estimate the significance of this system. It has long been assumed that AT2R is abundant only during fetal life. Immediately after birth, the expression of this receptor decreases thus making the AT1R the dominant receptor subtype[3;4]. Available evidence for this notion however, is derived from studies utilizing autoradiography[5;6], competition binding assay[7;8], and *in situ* hybridization[9;10]. These techniques detect an affinity of ligand-receptor or mRNA but do not directly evaluate receptor protein expression. Employing Western blot analysis, we recently demonstrated that, in the brainstem, liver, and kidney, adult rats exhibit a significantly higher AT2R and lower AT1R protein expression when compared to fetuses and neonates[11]. To our knowledge, this is the first report of

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developmental changes of these two receptors based on protein expression. More importantly, our data contradict the currently prevailing concept based on other techniques. In the current study, we evaluated developmental changes in AT2R and AT1R expression in various tissues and organs of mice to extend our previous findings in rats.

2. Methods

2.1. Animals

A total of 73 male c57BL/6 mice, including fetuses (~ 3 days before birth), neonates (~ 3 days after birth), juvenile (1 – 6 weeks), and adults (10 – 14 weeks) were used in this study. The individual fetuses were taken from different pregnant female mice, and individual neonates were taken from different litters. The sex of the fetuses and neonates was identified by the sex determining region Y (SRY) expression employing RT-PCR. The primers used are given in Table 1. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and were carried out under the guidelines of the American Physiological Society and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2. Preparation of Tissues

All mice were euthanized by CO_2. The cerebral cortex, hypothalamus, cerebellum, brainstem, spinal cord, heart, lung, liver, and kidney were removed and immediately frozen on dry ice, and stored at −80 °C. The samples were stored for a maximum of 2 weeks. The whole heart and whole right kidney from each fetus and neonate were used due to their small size, but only partial tissues from other organs were used. All tissues were taken from the same regions of each organ. For example, the brainstem samples were taken from a rostral coronal section 0.5 – 2.0 mm of the obex. Kidney samples of adult mice were taken as a 0.5 – 1.0 mm horizontal section from the middle of the right kidney, which included the renal cortex, medulla, calyx, and vessels. The hepatic anterior lobe and the lower portion of left lung were taken for analysis.

2.3. Western Blot Analysis of Total Protein Extract

The tissues were homogenized in RIPA buffer (50 mM Tris/HCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS) and total protein was extracted from the homogenates. The concentration of protein extracted was measured using a protein assay kit (Pierce; Rockford, IL) and adjusted with equal volumes of 2X 4% SDS sample buffer. The samples were boiled for 5 min followed by loading on a 10% SDS-PAGE gel (30 μg protein/per well) for electrophoresis using a Bio-Rad mini gel apparatus at 40 mA/-gel for 45 min. The fractionized protein on the gel was transferred onto a PVDF membrane (Millipore) and electrophoresed at 300 mA for 90 min. The membrane was first probed with AT2R primary antibodies (Rabbit polyclonal to Angiotensin II Type 2 Receptor, ab78747, Abcam, 1:1000 dilution) and secondary antibody (goat anti-rabbit IgG-HRP, Santa Cruz, 1:5000), and then treated with enhanced chemi-luminescence substrate (Pierce; Rockford, IL) for 5 min at room temperature. The bands on the membrane were visualized and analyzed using UVP BioImaging Systems. After obtaining the AT2R blot density, the membrane was treated with Restore Western Blot Stipping Buffer (Thermo Scientific) to remove the AT2R signal, followed by probing with AT1R primary antibodies (AT1R rabbit polyclonal IgG, sc-1173, Santa Cruz Biotechnology Inc, 1:500) and finally with GAPDH primary antibodies (GAPDH mouse monoclonal IgG, sc-32233, Santa Cruz Biotechnology Inc, 1:1000) using the same process as the AT2R antibodies to obtain the AT1R and GAPDH blot densities. The final reported data are the normalized AT2R and AT1R band density divided by the GAPDH density.

2.4. Western Blot Analysis of Membrane and Cytosolic Protein Extracts

The membrane and cytosolic protein fractions from mouse brainstems were extracted using ProteoExtract Tansmenbrane Protein Extraction Kit (Novagen Madison, WI, USA), according to the procedures recommended by the manufacturer. Briefly, brainstems were homogenized in glass homogenizers in buffer with protease inhibitor cocktail. The homogenate was then centrifuged at 1000 × g at 4 °C for 5 min. The supernatant contained the cytosolic protein fraction. The pellet was resuspended in buffer with protease inhibitor cocktail, and centrifuged at 16,000 × g at 4 °C for 15 min. This supernatant was enriched with integral membrane proteins. The membrane and cytosolic fractions were then stored at −80°C until analyzed. The Western blot procedures used to determine AT2R and AT1R protein expression in membrane and cytosol were the same as that described above for total protein extract.

2.5. Validation of AT2R and AT1R antibodies

In order to characterize the specificity of antibodies used in this experiment, we carried out a positive control for both receptor antibodies. The primary procedures for this test were the same as for western blotting described in section 2.3. We replaced the tissue with rat adrenal medulla (a positive control for AT2R antibody) or rat paraventricular nucleus (PVN) (a positive control for AT1R antibody). In addition, we carried out an antibody preabsorption assay using these positive tissues with blocking peptide treated antibodies. The antibodies were preabsorbed according to the description by Shekhar A et al[12]. Briefly, a 1:1000 dilution of the AT2R antibody or 1:500 dilution of AT1R antibody was incubated overnight at room temperature in 1 ml of 0.1 M PBS with 0.1% Tween 20 alone or with 5×, 10×, or 20× concentrations of AT2R antibody blocking peptide (ab90842, Abcam) or AT1R antibody blocking peptide (sc-1173P, Santa Cruz Biotechnology). On the following day, the treated antibodies were subjected to western blotting as described above.

2.6. Real-Time RT PCR

Total RNA was extracted from brainstems with TRIZOL Reagent (Invitrogen), which was then reverse transcribed into double-stranded cDNA using iScript cDNA Synthesis Kit (BIO-RAD). Templates (50 ng cDNA) were subjected in triplicate to Real-time PCR using a thermocycler (PTC-200 Peltier Thermal Cycler with CHROMO 4 Continuous Fluorescence Detector, BIO-RAD). SsoFast EvaGreen Supermix (BIO-RAD) and AT2R, AT1R, GAPDH specific primers were used for relative quantitation. Target genes were normalized to GAPDH levels, expressed as $2(-\Delta C_t)$, where ΔC_t is $\{C_t[\text{target}]-C_t[\text{GAPDH}]\}$. The primers were designed using free online software at https://www.genscript.com/ssl-bin/app/primer and synthesized in the Eppley Institute Molecular Biology Core Laboratory on the campus of the University of Nebraska Medical Center. Please see Table 1 for details of gene-specific primers.

2.7. Statistical Analyses

All data are described as the mean ± SE. A one way ANOVA was used followed by the Student-Newman-Keuls *post hoc* analysis where appropriate. Pearson Correlation was performed to assess the relationship between the changes of AT1R and AT2R protein expression in developing mice. Statistical analysis was done with the aid of SigmaStat software. A P value < 0.05 was considered statistically significant.

3. Results

3.1. AT2R and AT1R protein expression in various brain regions and spinal cord

We measured AT2R and AT1R total protein expression in extracts from cerebral cortex, hypothalamus, cerebellum, brainstem, and spinal cord of fetal, neonatal, and adult mice. In all detected brain regions and in the spinal cord, adult mice exhibited a significantly higher AT2R and significantly lower AT1R protein expression than did fetuses and neonates (Figure 1). However there were no significant differences between fetal and neonatal mice.

AT2R and AT1R protein expression in other organs

To determine if the above expression pattern also existed in non-neural tissues, we measured AT2R and AT1R expression in total protein extract from heart, lung, liver, and kidney (Figure 2). Heart, liver, and kidney exhibited the same expression profile as did neural tissue. Even though lung tissue of adult mice had higher AT2R expression than that of fetal and neonatal mice, there was no significant difference in AT1R expression among the three groups.

3.2. Correlation of AT2R and AT1R protein expressions

To analyze the correlation of AT2R and AT1R expression during development, we measured their expression levels in whole cell protein extract from brainstem samples of eight groups of mice over 6 weeks of age. As can be seen in panel A of Figure 3, AT2R expression gradually increased, whereas AT1R expression gradually decreased, from fetal to 6 weeks of age. Panel B shows the mean data of blot density for developing changes of AT1R and AT2R. Acute alterations of both AT2R and AT1R expression occur between 1 day and 2 weeks of post-natal life. There were no significant differences in their expression before 1 day or after 2 weeks. Panel C of figure 3 shows the relationship between AT2R and AT1R expression from which we can clearly see a strong, negative correlation over the range of developmental ages studied (correlation coefficient: $r = -0.92$, $P = 0.001$, $n = 5$/group).

3.3. AT2R and AT1R expressions in membrane and cytosol

As two G protein coupled receptors, the mature AT2R and AT1R are integrated into the cell membrane, and therefore it is of interest to determine their expression in the membrane and cytosol. These data are shown in Figure 4. Both membrane (panel A) and cytosol (panel B) protein extracts from brainstems exhibit higher AT2R and lower AT1R protein expression in adult mice compared to fetus and neonates; similar to that observed for total protein. Panel A of Figure 4 shows a very weak GAPDH blot (cytosol marker) and a very strong Na^+/K^+ ATP blot (membrane marker). This suggests a high quality membrane extract. We noticed that the Na^+/K^+ ATP expression was visibly higher in adult mice compared to fetus and neonate. Therefore we could not utilize this membrane marker as an internal control for AT2R and AT1R expression. In contrast, there was no difference in GAPDH expression among these three groups of mice. We therefore used GAPDH blot density to normalize the AT2R and AT1R expression in membrane, which resulted in a higher ratio of AT2R or AT1R to the GAPDH than usual (Figure 4A).

3.4. Characterization of the AT1R and AT2R antibodies

To confirm the specificity of antibodies, we employed rat adrenal medulla as an AT2R positive tissue and PVN as an AT1R positive tissue. Blots 1 and 5 of Figure 5 show a single strong band at 50~ kDa for AT2R immunostaining in adrenal medulla protein extract and a predominant band at ~ 45 kDa for AT1R immunostaining in PVN protein extract. When the antibodies were preincubated with three different concentrations of blocking peptides, the

immunostaining bands gradiant weakened at 5 × and 10 × concentration of peptides and completely disappeared at 20 ×, These are shown in blots 2–4 (AT2R blots) and 6–8 (AT1R blots). These data demonstrate that the AT2R antibody and AT1R antibody used in this experiment were specific for the AT2R and AT1R antigens.

3.5. AT2R and AT1R mRNA expression

Figure 6 shows the AT2R and AT1R mRNA expression in the brainstems from fetal, neonatal, and adult mice using real-time RT-PCR. There were no significant differences in AT1R mRNA expression levels among these three groups of mice. The AT2R mRNA expression appears somewhat higher in fetuses than that in neonates and adults, but the difference did not reach statistical significance.

Discussion

We have examined developmental changes of AT2R and AT1R protein expression in various neural and non-neural tissues of normal mice. Neural tissue included cerebral cortex, hypothalamus, cerebellum, brainstem, and spinal cord. Non-neural tissue included heart, lung, liver, and kidney. Our major findings are that, in all tissues, adult mice exhibited a significantly higher AT2R, and a significantly lower AT1R protein expression than did fetal and neonatal mice. These results confirmed our previous findings in rats[11] and extend this observation to additional organs and tissues. This developing expression pattern was also demonstrated in the cell membrane and cytosolic components. In addition, we documented a negative relationship in the developing changes of protein expression between these two receptors from fetus to 6 weeks of age. However, we did not find significant differences in AT2R and AT1R mRNA levels among fetal, neonatal, and adult mice.

The current findings of lower AT2R protein expression in the fetal mice, together with our previous data from fetal rats, further calls into question the well-accepted concept concerning the maturation of this receptor. Even though the AT2R has been demonstrated to be dramatically higher in the fetus using autoradiography and thus has been considered to be involved in development for around thirty years[3;4], the evidence linking this receptor to growth or maturation has been absent. In contrast, the AT2R has been well documented to mediate antiproliferation[1;3;13]. Moreover, AT2R deficient mice do not exhibit any obvious developmental abnormalities[14;15], directly contradicting the hypothesized role of this receptor in growth or development. On the other hand, data reported from functional experiments in fetal animals are consistent with our finding of a lower AT2R expression and AT1R as the predominant receptor subtype in the fetus. In the ovine fetus, intracerebroventricular injection of Ang II elevated plasma vasopressin[16], up-regulated hypothalamic c-fos expression[17;18], and evoked a pressor response[19;20], which were completely abolished by the AT1R antagonist losartan, but not by the AT2R antagonist PD 123319[21;22]. Moreover, a functional AT1R has also been demonstrated to be present in the fetal cardiovascular system [23;24]. In the ovine fetus, intravenous injection of Ang II elicited a significant increase in arterial blood pressure, which was abolished by the AT1R antagonist losartan[25;26]. These results strongly suggest that in the fetus the AT1R but not the AT2R is the predominant Ang II receptor subtype[27], and therefore the AT1R may play a more important role during fetal life than was previously expected, especially in the regulation of cardiovascular system and sympathetic outflow[28].

Another intriguing finding of the present study is the age-related down regulation of AT1R expression, with the highest level in the fetus and the lowest in adult tissue. These results are consistent with previous reports from other laboratories that the RAS is activated more during the early stages of development[29]. In the neonatal period in humans, Miyawaki et al.[30] reported higher plasma Ang II at day 0 than that at day 7. In children aged between 1

week and 13 years, Fiselier et al.[31] found an age-related decrease in plasma Ang II concentration. In the adult, Broughton et al.[32] documented lower Ang II level compared to children. An age-related decrease in plasma Ang II also could be found in sheep[33] and rabbits[34]. In addition to Ang II, the plasma concentration of other major components of the RAS, including renin, Ang I, angiotensin-converting enzyme (ACE), and aldosterone all inversely correlate with age[31]. The higher AT1R expression in the fetus observed in the current study implies a potential involvement of this receptor in development. In fact, accumulating evidence demonstrates a critical role of AT1R but not AT2R in development, especially in renal genesis[35;36]. Blockade of AT1R in newborn rats induced an arrest in nephrovascular maturation and renal growth[37;38], whereas blockade of AT2R did not alter renal morphology[38]. Mice with targeted deletion of $AT1_aR/AT1_bR$ exhibit renal histological abnormalities[39]. However, AT2R-deficient mice have histologically normal kidneys[15].

Finally, the data from the current study demonstrated an inverse expression pattern between AT1R and AT2R during development. This phenomenon leads us to postulate that there is a potential cross-talk between these two receptors that may reciprocally regulate the expression of each. An inverse expression pattern has been reported in some pathological conditions. In a rat model of cold-induced hypertension, the hypothalamus and brainstem exhibit higher AT1R and lower AT2R mRNA and binding compared with control rats[40]. In the rostral ventrolateral medulla we have previously found higher AT1R and lower AT2R protein in rats with chronic heart failure compared to sham rats[41]. In the renal cortex[42] and cerebral microvessels[43] of spontaneously hypertensive rats higher AT1R and lower AT2R protein expression has been demonstrated compared to normotensive control rats. This inverse expression pattern between AT1R and AT2R is also gender, sex hormone, and cytokine relevant. In the kidney, male rats have higher AT1R but lower AT2R mRNA than do female rats[44]. In female rats following ovariectomy, estrogen replacement significantly down-regulated AT1R and up-regulated AT2R expression in the adrenal glands[45] and kidneys[44]. Interleukin-6 treatment[46], nicotine exposure[47], and chronic hypoxia[48] have also been demonstrated to evoke an inverse expression pattern of AT1R and AT2R. In the current study, we found a highly negative correlation between AT1R and AT2R protein expression from fetus to six weeks of age. To our knowledge, this is the first report to quantify this inverse relationship between these two receptors.

The exact mechanism(s) underlying these developmental changes in AT1R and AT2R expression are unknown. Increasing evidence however, supports the hypothesis that the reciprocal regulation between these two receptors may play some role in this respect. Indeed, the AT2R exhibits a profound inhibitory influence on AT1R expression. Interestingly, this negative regulation seems to have “tonic” characteristics because higher AT1R expression could be observed when AT2R signaling was blocked genetically or pharmacologically. In some organs and tissues, including the hypothalamus, adrenal gland, kidney, liver, spleen, lung, and aorta, AT2R gene-deficient mice exhibit significantly higher AT1R expression and mRNA compared with wild-type mice[49–52]. Pharmacological inhibition of AT2R by PD123319 in adult rats has been reported to enhance AT1R expression in the brain[53]. The AT1R, on the other hand, exhibits an inhibitory influence on AT2R expression. It has been demonstrated that AT1R blockade up-regulated AT2R expression in the brain[43;54;55], muscular gastric layer[56], and adipose tissue[57]. Stimulation of the AT1R has been reported to down regulate AT2R expression in PC12 cells through the Gq and ERK1/2 phosphorylation pathways[58]. Similar data has been provided from endothelial cells[59].

The cause for the differences between our findings utilizing western blot analysis and the previous findings employing receptor binding assays is not clear. One possibility is that the results from western blotting represent the total receptor protein, while binding assays only

elucidate receptor proteins capable of binding to its ligand. In this case, the conclusion from binding assays seems more reasonable because it indicates functional protein. However, functional experiments do not support this postulation. As we indicated above, in the ovine fetus both central and peripheral administration of Ang II elicited a significant increase in blood pressure[26;60] which was completely abolished by the AT1R blocker losartan, but not by the AT2R blocker PD123319[21;25]. These findings directly contradict the data derived from binding assays that the AT2R is the predominant Angiotensin II receptor subtype in fetal life[3;4]. Moreover in the membrane extracts, which supposedly includes the most of mature receptor protein, we demonstrated a lower AT2R and higher AT1R expression in fetal mice compared to the adult by western blotting (Figure 4). There are several limitations of the binding assay as applied to Angiotensin receptors. Even though the iodinated Ang II and its analogues are supposed to have equal affinities for AT1R and AT2R when used in receptor binding assays, several studies demonstrate that this is not always true. For example, Rowe et al.[61] found that ^{125}I[Sar^{1}Ile^{8}]Ang II had a four-fold higher affinity for the AT1R in rat brain nuclei compared to the AT2R. In contrast, in ovine tissues, ^{125}I[Sar^{1}Ile^{8}]Ang II has been demonstrated to have a four-fold greater affinity for AT2R than AT1R[62]. However, in another study, ^{125}I[Sar^{1}Ile^{8}]Ang II labeled both subtypes equally in the rat adrenal and brain whereas ^{125}I[Ang II] did not[63]. These differences in radioligand affinity for Angiotensin receptors may result in significant shifts in dissociation constants and half-maximal displacements of [Sar^{1}Ile^{8}] Ang II (Sar IC_{50}s, derived from competition assays) from tissues with different proportions of AT1R and AT2R[62]. While these differences may appear small with respect to the affinities of AT1R/AT2R antagonists (e.g. losartan and PD123319), they can result in the derivation of inaccurate proportions of receptor subtypes when using submaximal concentrations of the radioligand[62].

For western blots, on the other hand, we recognize that caution is warranted with regard to the specificity of antibodies we used, especially because our data are opposite to the prevailing notion. The AT1R antibody in this experiment was purchased from Santa Cruz Biotechnology (sc-1173), which was used and validated by other laboratories[64–66]. The AT2R antibody was purchased from Abcam (ab78747), which was also applied and confirmed by another laboratory recently[67;68]. To confirm the validity and specificity of these two antibodies within our own laboratory, we carried out positive controls and antibody preadsorption experiments. Using rat PVN tissue as the positive control for AT1R antibody, we obtained a single strong band at ~45 kDa (blot 1, Figure 5), which is consistent with previous estimations of the AT1R molecular weight[12;69]. Employing rat adrenal medulla as the positive control for AT2R antibody, we obtained a distinct band at 50~ kDa (blot 5, Figure 5). This molecular weight is consistent with Abcam datasheet (http://www.abcam.com/Angiotensin-II-Type-2-Receptor-antibody-ab78747.pdf), and published data by other groups using AT2R antibody from Cell Signaling Technology[70] or from Santa Cruz Biotechnology[71], and our previous results by AT2R antibody from Santa Cruz Biotechnology (sc-9040)[11], which is another rigorously validated, well-characterized AT2R antibody[72]. The AT1R has a calculated mass of 40,889 Daltons as deduced from its 359 amino acid sequence[73;74] and the molecular mass of AT2R is predicated to be 41,346 Daltons based on its 363 amino acid sequence[75;76]. However, the major specific bands of AT1R and AT2R obtained from western blot were larger than the sizes predicted by their amino acid sequences due to the glycosylation of receptor proteins[69]. This phenomenon is much more obvious for the AT2R[77;78]. Moreover, our data also show that both AT1R and AT2R western blot signals were eliminated by preadsorption of the antibodies with blocking peptides (blots 2–4 for AT2R and blots 6–8 for AT1R, Figure 5), further demonstrating the specificity of the antibodies we used. Overall, these data support our assertion that the primary antibodies used in the present experiment were detecting the AT1R and AT2R antigens.

In summary, the current study showed an age-related increase in AT2R protein expression in the developing mouse from fetus to six weeks of age, with the lowest level in the fetus and the highest level in the adult. This finding is diametrically opposite to the currently held notion concerning AT2R expression during development. In contrast to AT2R, an age-related decrease in AT1R protein expression was observed. More interestingly, the developmental expression changes between AT2R and AT1R protein appear to be closely and negatively related. Combing these data with the previous report describing AT1R and AT2R expression patterns in developing rats[11] and in rats with chronic heart failure[41] and hypertension[40;42;43], we propose the AT1R and AT2R expression profile shown in figure 7. We believe that at all ages the AT1R always acts as the predominant subtype receptor. The final output of RAS function, namely Ang II signaling, is therefore always dominated by the AT1R. Thus administration of Ang II invariably evokes hypertension in all intact animals, including in the fetus[19;20;25;26]. This hypothesis, however, raises the question: what is the real role of the AT2R? Based on previous reports and the results from the present experiment, we further suggest that the AT2R might act as a “brake” on AT1R signaling (i.e. as a buffer) via directly contradicting AT1R function and/or indirectly down regulating AT1R expression.

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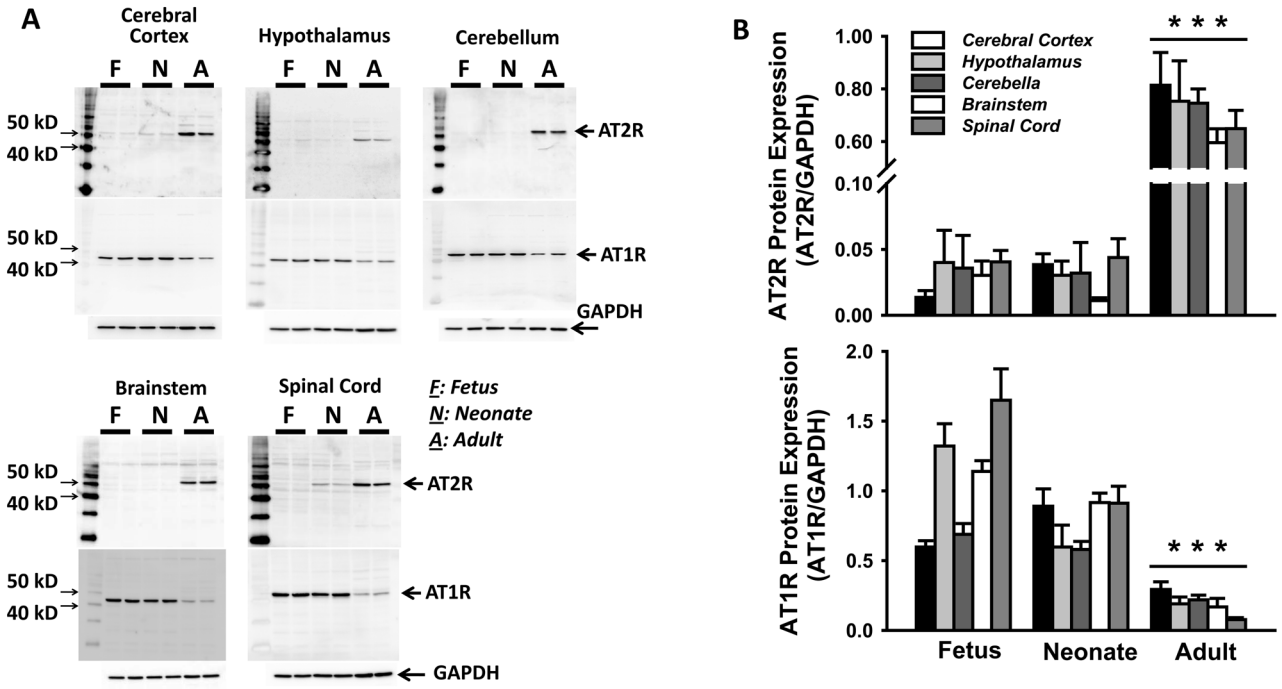

Figure 1.

AT2R and AT1R protein expression from total protein extracts of various brain regions and spinal cord of fetal, neonatal, and adult mice. ***$P < 0.001$ *vs* counterpart brain regions or spinal cord from fetus and neonate; n = 4/group.

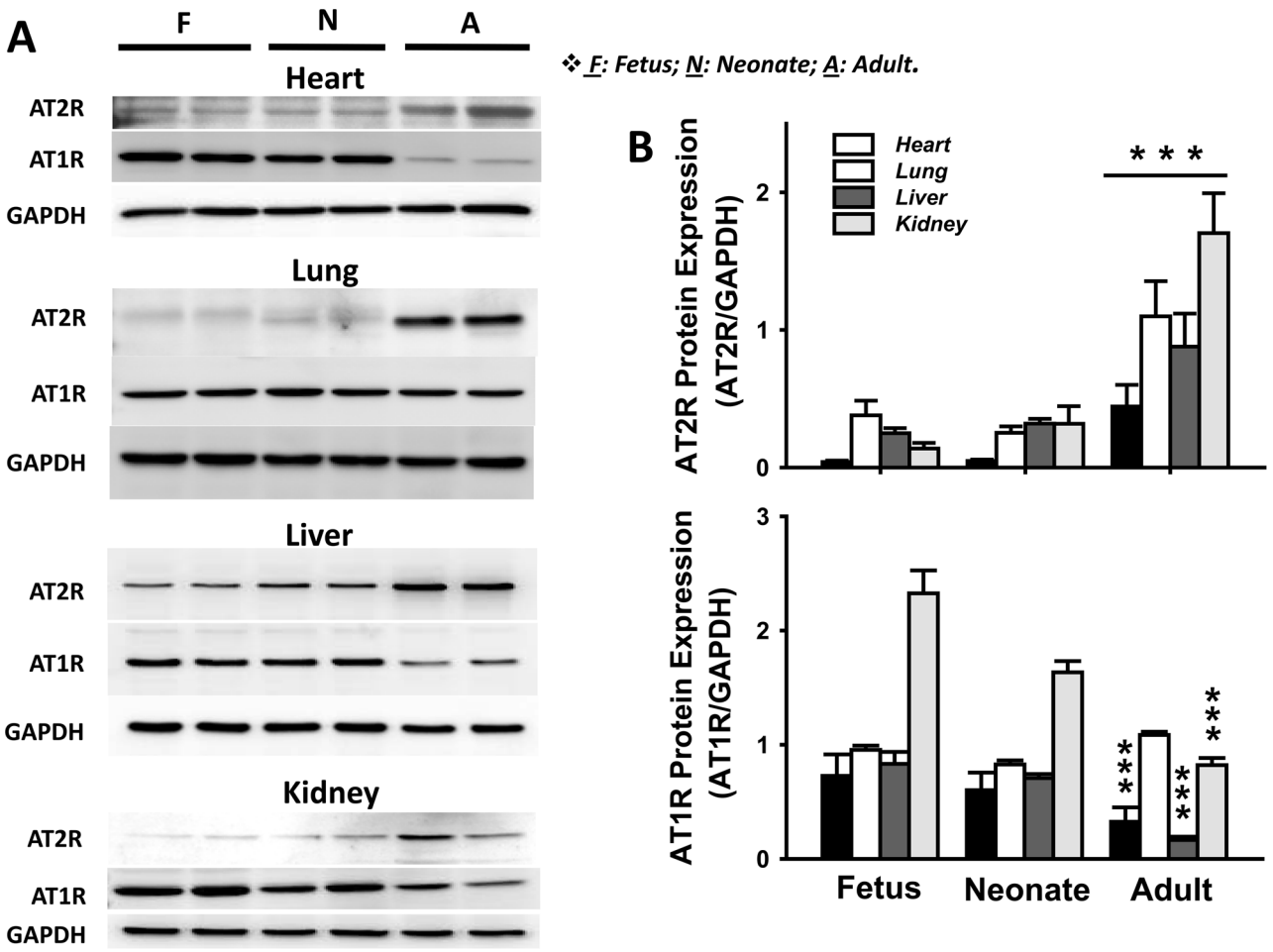

Figure 2.

AT2R and AT1R expression from total protein extracts from heart, lung, liver, and kidney of fetal, neonatal, and adult mice. ***$P < 0.001$ *vs* counterpart organs from fetus and neonate; n = 4/group.

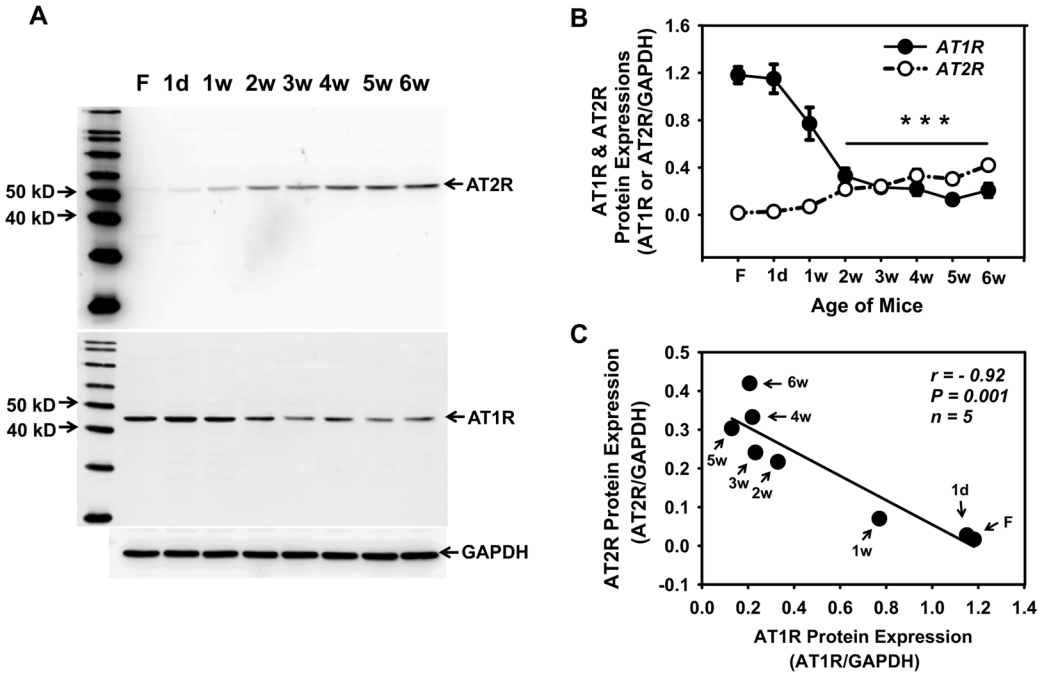

Figure 3.

Consecutive AT2R and AT1R expressions from total protein extracts of brainstem from fetus to 6 week old mice. Panel A: A representative Western blot shows AT2R and AT1R expression. Panel B: Line plot showing the developing changes of AT2R and AT1R from fetus to 6 week old mice. ***$P < 0.001$ *vs* fetus, 1 day, and 1 week mice; n = 5/group. Panel C: Correlation for the developing change between AT2R and AT1R.

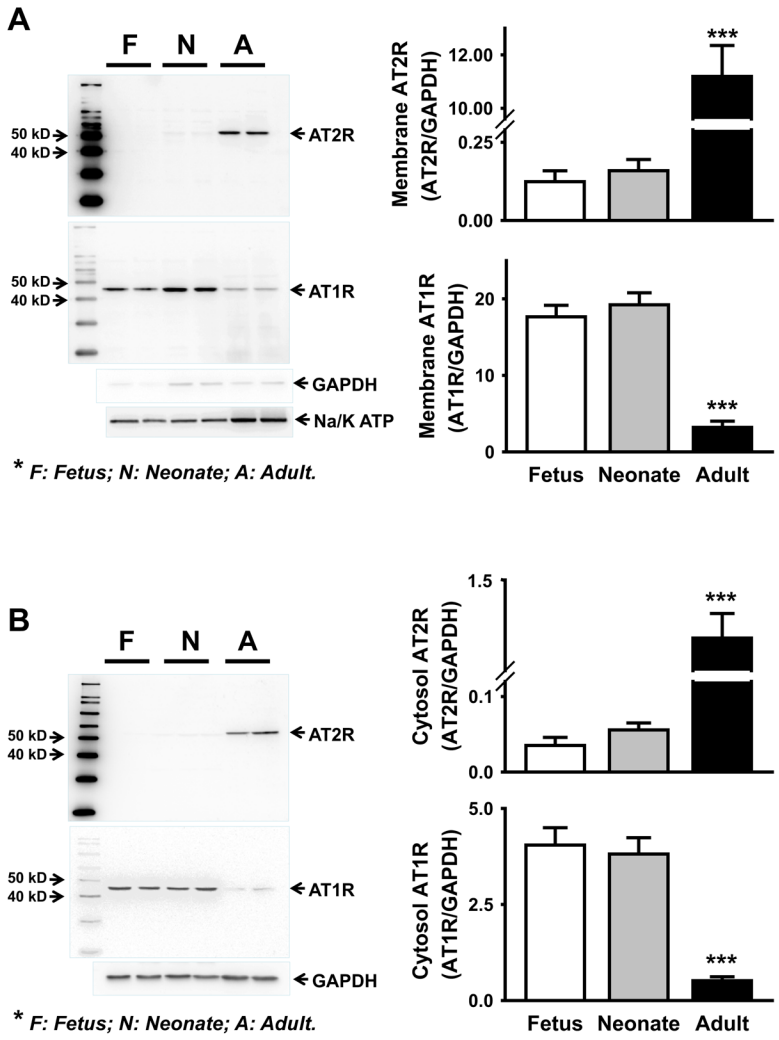

Figure 4.

AT2R and AT1R expression in membrane (A) and cytosolic (B) protein extracts from brainstem of fetus, neonate, and adult. ***$P < 0.001$ *vs* counterpart in fetus and neonate; n = 4/group.

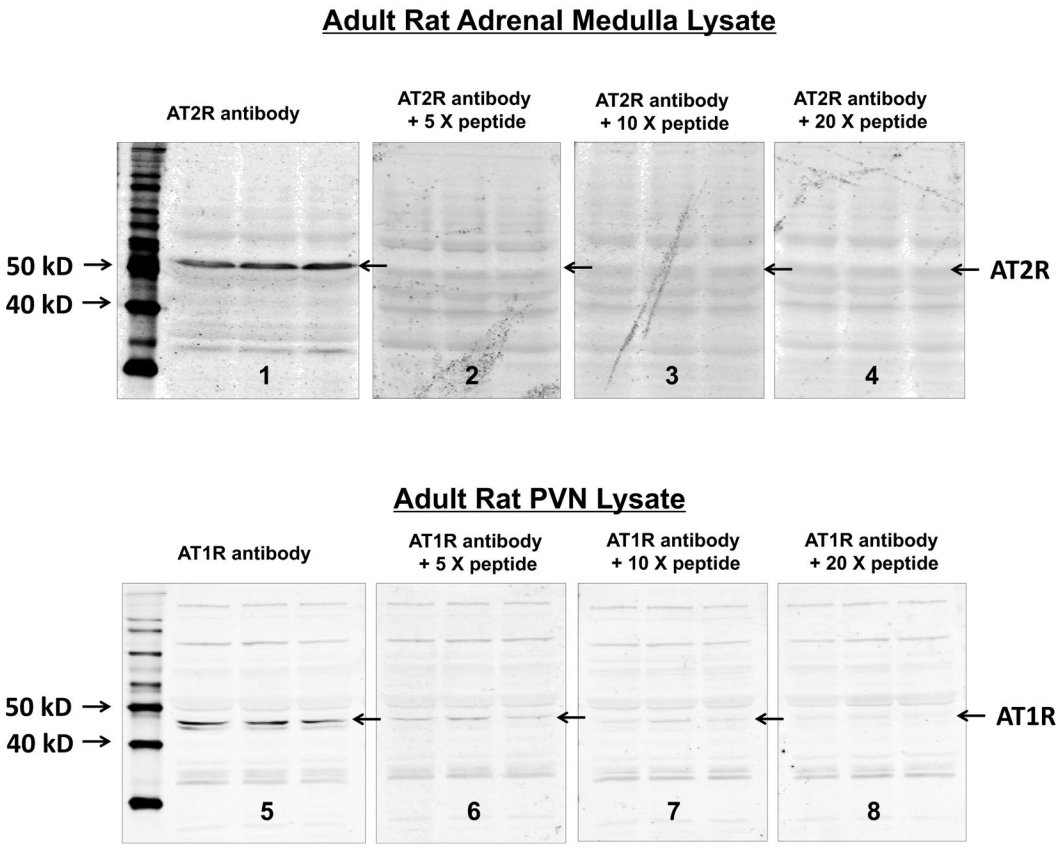

Figure 5.

AT2R and AT1R positive tissue control test and antibody preabsorption test. The upper panels show the AT2R immunostaining bands from rat adrenal medulla extract with intact AT2R antibody (blot 1, positive control test) or preabsorbed AT2R antibodies (blots 2–4, preabsorbtion test). The lower panels show the AT1R immunostaining bands from rat PVN extract with intact AT1R antibody (blot 5, positive control test) or preabsorbed AT1R antibodies (blots 6–8, preabsorption test).

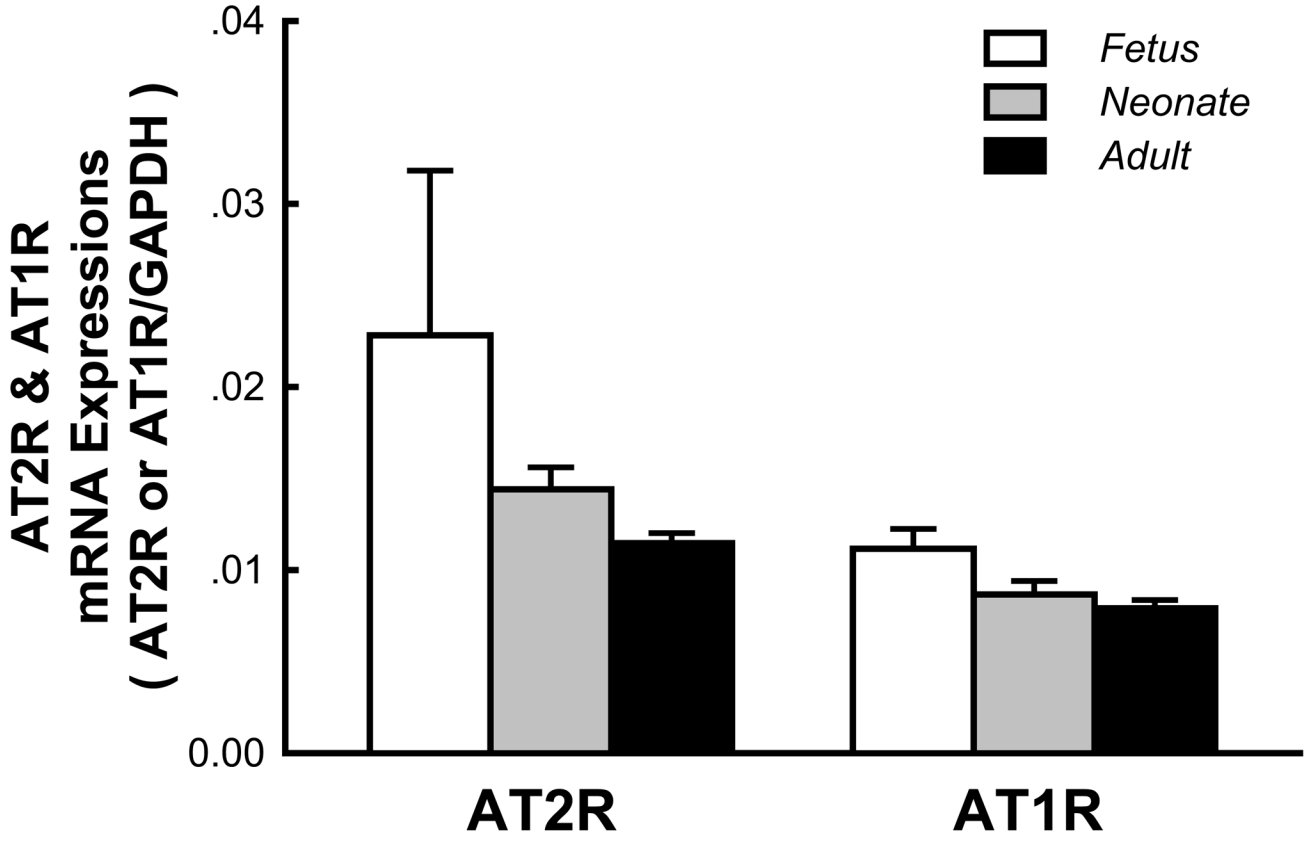

Figure 6.
AT2R and AT1R mRNA expression in the brainstem of fetal, neonatal, and adult mice. n = 6/group.

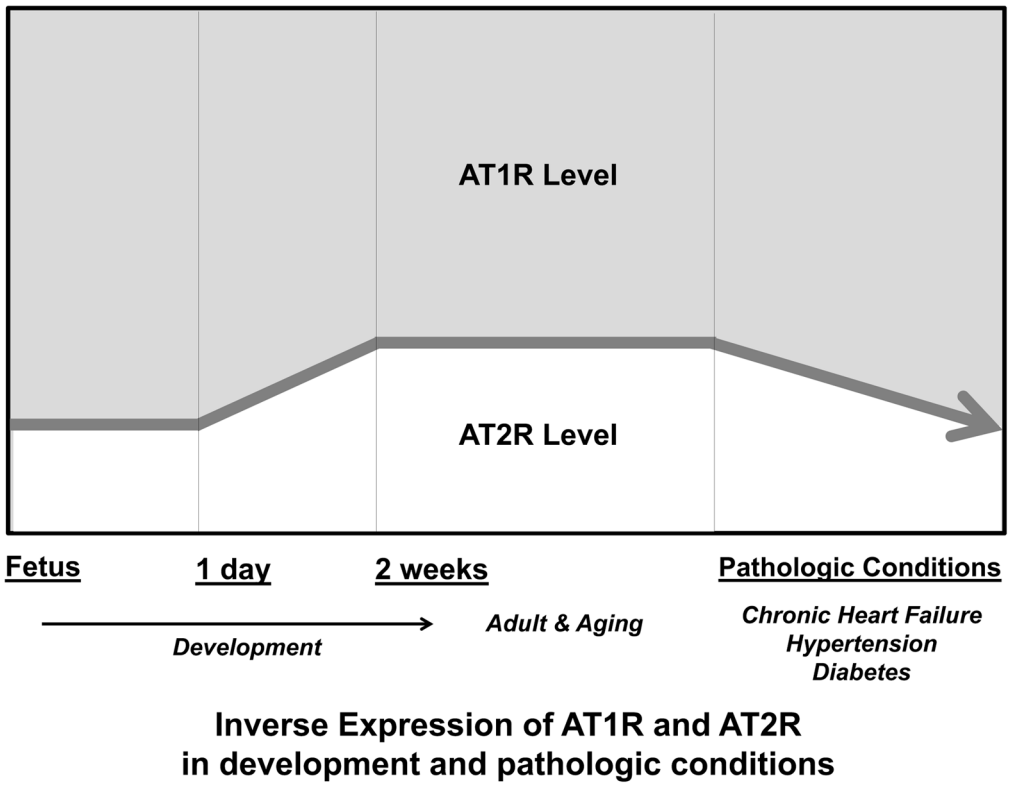

Figure 7.
Proposed AT2R and AT1R expression profile during development and under pathological conditions.

Table 1

Gene-specific primers for real-time PCR.

Name of genes (Accession No.)	Forward primers	Reverse primers	Amplicon Size (nt*)
Mouse AT2R (NM_007429)	GAAGCTCCGCAGTGTGTTTA	TGGCTAGGCTGATTACATGC	147
Mouse AT1R (NM_177322)	GTGTTCCTGCTCACGTGTCT	GATGATGCAGGTGACTTTGG	108
Mouse GAPDH (NM_001001303)	ACAACTTTGGCATTGTGGAA	GATGCAGGGATGATGTTCTG	133
Mouse Sry (NM_011564)	CTCATCGGAGGGCTAAAGTG	AAGCTTTGCTGGTTTTTGGA	166

* nt, nucleotide numbers.