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ORIGINAL PAPER

Age-Related Changes in Ang II Receptor Localization and Expression in the Developing Auditory Pathway

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Abstract

We studied Ang II receptor localization in different nuclei of the auditory system, by means of binding autoradiography, during brain development. The inferior colliculus (IC), a large midbrain structure which serves as an obligatory synaptic station in both the ascending and descending auditory pathways, exhibited high Ang II AT_2 binding at all ages (P0, P8, P15, P30), being maximal at P15. These observations were confirmed by *in situ* hybridization and immunofluorescence at P15, demonstrating that AT_2 receptor mRNA localized at the same area recognized by AT_2 antibodies and anti β III–tubulin suggesting the neuronal nature of the reactive cells. Ang II AT_1 receptors were absent at early developmental ages (P0) in all nuclei of the auditory system and a low level was observed in the IC at the age P8. AT_2 receptors were present at ventral cochlear nucleus and superior olivary complex, being higher at P15 and P8, respectively. We also explored the effect of prenatal administration of Ang II or PD123319 (AT_2 antagonist) on binding of Ang II receptors at P0, P8, P15. Both treatments increased significantly the level of AT_2 receptors at P0 and P8 in the IC. Although total binding in the whole IC from P15 animals showed no difference between treatments, the central nucleus of the IC exhibited higher binding. Our results supports a correlation between the timing of the higher expression of Ang II AT_2 receptors in different nuclei, the onset of audition and the establishment of neuronal circuits of the auditory pathway.

Keywords AT_2 receptors · Inferior colliculus · Superior olivary complex · Cochlear nucleus · Brain development

Introduction

It is now accepted that central renin-angiotensin system (RAS) is not only involved in fluid homeostasis and blood pressure control, but it can also modulate brain areas involved in cognition, motor control, and sensory integration [1]. In a previous study we reported Ang II receptor localization in the brainstem and cerebellum at P15, a critical age for movement acquisition [2]. In this study we identified AT_2 binding in several brainstem nuclei related to either sensory or motor control activity. At the stage P15, we reported the presence of Ang II receptors, with predominance of AT_2 receptors, localized in the inferior colliculus (IC) [2], which was also observed earlier during development (P0 and P8) [3].

The IC is a large midbrain nucleus that integrates auditory information from brainstem nuclei and cortical regions and serves as the primary source of auditory projections to the thalamus [4, 5]. The IC has its origin from the tectum at early embryonic stage (E12) [6, 7].

Among the brainstem nuclei involved in the auditory pathway are the cochlear nuclear (CN) complex, which includes the ventral (VCN) and dorsal (DCN) nuclei and the superior olivary complex (SOC). The SOC comprises three nuclei, the lateral superior olive (LSO), medial superior olivary complex (MSO), and the medial nucleus of the trapezoid body (MNTB). The complex neuroanatomical connections of the IC and the numerous neurotransmitters involved, suggest a central role of the IC at integrating external signals and its responses [4, 5]. It is well-recognized that, in rodents, the onset of hearing arises at postnatal day

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12 (P12), while maturation of the connections continues for several days [8, 9].

Recent studies [10] provided experimental evidences in relation with the developmental mechanisms involved in the formation and refinement of the auditory system, confirming previous ones [11, 12]. It has been well-established that the CN complex, the primary relay station of the central auditory system originates from distinct regions of the rhombomere (r2–r5) neuroepithelium during embryonary stages (E13–E18). Similarly, the SOC arises mainly from the r5 [10].

Nuyt et al. performed ontogenic studies regarding AT_2 and AT_1 Ang II receptor localization [13, 14] by in situ hybridization in the developing brain (E19 to P28). In relation to the auditory system, the authors identified AT_2 mRNA in the cochlear nuclei and the ventricular nuclei at P7, and the SOC at E19, E21. These authors only refer AT_2 mRNA localization in the IC at the stage P7 [13]. In adult rat, low mRNA expression for Ang II AT_2 receptors in the IC and AT_1 receptors in the SOC was reported [15]. It is well known that AT_2 receptor subtype diminishes its expression level with age, and thus several data support a potential role for these receptors in brain development [2, 3, 16].

Since the onset of audition (P12) [8, 9] correlates with the maximum expression level of Ang II AT_2 receptors in several brainstem nuclei, we decided to explore a possible correlation between the pattern of AT_2 receptors expression level and its localization with maturation of the auditory system. To the best of our knowledge, no previous studies have been focused on the potential role of Ang II receptors in the auditory pathways. Previous data reported Ang II receptors during development of the visual pathway [17, 18] which involves the Superior Colliculus (SC). Michells et al. [17] suggested that Ang II receptors in the SC might be regulated by retinal input. Coudé et al. [18] reported that administration of Ang II in the SC yielded a strong reduction in the amplitude of visual evoked potentials with participation of both receptor subtypes. Recently, Quiñones et al. [19] demonstrated participation of Ang II AT_1 receptors in fear conditioning of the auditory system.

In the rat, organogenesis takes place during the third gestational week and maturation of several tissues, pursues during the first postnatal weeks. Such is the case of the cerebellum, and also of the auditory system. Thus, we applied a model to evaluate the effect of overstimulation or blockade of Ang II receptors during organ development [3, 20], by performing different treatments during the third gestational week (G13–G21). Based on the observation of the loss of the Purkinje cell layer in animals treated with the AT_2 antagonist PD123319, we proposed a role for AT_2 receptors in neuronal migration [3, 21]. Recently, Guimond et al. [22] reviewed the potential role of Ang II AT_2 receptors in neuronal differentiation and neuronal migration and its role in development.

Anatomical and physiological experiments demonstrate that the IC is involved in a great diversity of functional roles in the auditory system integrating acoustic stimuli from the ears and playing an important role in sound localization and aversive behavior [19, 23]. In the rat, the IC is divided according to morphologically different cell types into three subregions with unique connections: the central nucleus (CIC), dorsal cortex (DCIC) and external cortex (ECIC). The ECIC is found around the CIC laterally and ventrally, which has also been called the lateral cortex [5, 24]. Fredrich et al. [25] analyzed the interconnection between the different nuclei which integrates the rat auditory pathway by means of immunofluorescence and retrograde staining. Both inhibitory signals (glycine, GABA) as well as excitatory signals were identified.

The present study was designed to understand the potential role of Ang II receptors during development. We performed a study of Ang II receptor localization and expression in the rat developing inferior colliculus and auditory pathway and studied the effect of overstimulation or inhibition of Ang II receptors during pregnancy to evaluate potential changes in receptor subtype distribution and expression.

Materials and Methods

Animals

All animal experiments were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health (2011, 8th. Ed.). All efforts were made to minimize the number of animals used and their suffering.

Pregnant Wistar rats weighing 230–250 g were kept in a dark–light cycle (12:12 h), maintained at 22 ± 1 °C and fed with standard rodent food and water *ad libitum*. Daily vaginal smears were taken and day 0 of pregnancy was assessed by a sperm- positive vaginal smear. Pups ($n = 5–6$) were sacrificed at postnatal day 0 (P0), P8, P15 or P30. The whole brains were immediately removed and snap frozen in isopentane at − 30 °C and stored at − 80 °C until use.

Animal Treatment During Pregnancy

On the 13th day of pregnancy (G13), osmotic mini-pumps (Alzet model 2001; Palo Alto, CA) were implanted subcutaneously between the scapulae bones, as described [3, 20]. Alzet osmotic mini-pumps were filled with sterile saline vehicle (control animals), Ang II (Sigma Chemical Co, St. Louis, MO) or the AT_2 antagonist PD123319 (1-(4-dimethylamino)-3-methylphenyl)-methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1-himidazo [4,5c]pyridine-6-carboxylic acid)ditrifluoroacetate (RBI-Sigma, Natick, MA

01760, USA). Treatments lasted for 1 week (G13–G21) and the doses used for the different treatments, 1.0 mg/kg/day, corresponded to 41 µg/kg/h [3, 20]. We performed four independent treatments of pregnant mothers, 5–6 pups from each treatment group were sacrificed at P0, P8 or P15 and whole brains were immediately removed, snap frozen and stored at − 80 °C until use.

Autoradiography of Ang II Receptors

Binding by autoradiography was performed as described previously [2, 3, 20]. Briefly, rats (n = 5–6 per age) were sacrificed by decapitation, the brains carefully dissected, snap-frozen and stored at − 80 °C until use. Consecutive coronal sections of midbrain and hindbrain from rats at different ages were obtained at the selected level [26, 27]. Coronal sections (16 µm) were cut with a cryostat at − 20 °C (Microm, Zeiss Inc.), thaw-mounted onto gelatin-coated glass slides, and desiccated at 4 °C overnight. Sections were preincubated in 10 mM sodium phosphate buffer (PBS) pH 7.4, containing 120 mM NaCl, 5 mM disodium EDTA, 0.005% bacitracin (Sigma, St. Louis, CO), and 0.2% proteinase free bovine serum albumin (BSA) (Sigma) and incubated in fresh buffer with 0.2 nM [^{125}I] Ang II (DuPont-NEN, sp. act. 2200 Ci/mmol), a concentration below the Kd value, for 2 h. Non-specific binding was determined with an excess of Ang II (10^{-6} M). After incubation, slides were rinsed, in fresh ice-cold 50 mM Tris buffer, pH 7.6, followed by ice-cold water, and dried under a stream of cool air. For Ang II receptor subtype identification, consecutive sections were incubated with 0.2 nM [^{125}I] Ang II, in the presence of 10^{-6} M of AT_1 antagonist Losartan (DuPont-Merck Pharmaceutical Co., Wilmington, DE) to define AT_2 receptors, or 10^{-6} M PD123319, to define AT_1 subtype. The dried labeled sections were apposed to Kodak BioMax MR film in X-ray cassettes. Films were developed with D19 Kodak developer (4 min, 4 °C), after 15–20 days exposure. Autoradiographic images were semi-quantified by densitometry with Scion software for Windows. For statistical comparisons, values for the different age groups were obtained within the same film. Results were expressed as optical density units from a 256 grey scale.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry and immunofluorescence staining were performed as described previously [16]. Briefly, coronal sections were obtained (14–16 µm) with a cryostat and sections were incubated with goat anti-AT_2 antibody (1:50 dilution, sc-7420, Santa Cruz Biotechnology) for 48 h at 4 °C. Following blocking of the endogenous peroxidase and washings, sections were incubated with biotinylated-secondary anti-goat antibody (1:200, Goat ExtrAvidin Peroxidase kit, Sigma, MO). As negative control, the primary antibodies were omitted and no specific labeling was observed.

For Immunofluorescence staining, coronal sections (12 µm) were incubated overnight at 4 °C with primary antibody, rabbit anti-AT2 (1:100 dilution, sc-9040, Santa Cruz Biotechnology) or anti βIII-tubulin (1:100 dilution, Millipore). Sections were washed with PBS and incubated with secondary antibody, Alexa fluor 488 (1:200 dilution, Molecular Probes, Invitrogen) for 2 h at room temperature (RT) in the dark. Images of immunostained sections were acquired with an epifluorescence microscope (Nikon Eclipse 50i). The resolution, brightness, and contrast of the images were optimized using the Adobe Photoshop CS software (Adobe Systems Inc., San Jose, CA, USA) but not otherwise manipulated. Specificity of the rabbit anti-AT2 antibodies was previously assayed [16].

Histological Analysis

Parallel sections to those used for autoradiography, immunohistochemistry and immunofluorescence staining were stained with hematoxylin and eosin (H&E) to enable anatomical identification of binding sites. Brain nuclei were defined with reference to a stereotaxic atlas of the developing rat nervous system for P0 animals [26] or adult animals [27].

In situ Hybridization

In situ hybridization was performed by using Digoxigenin (DIG)-labeled antisense and sense (control) riboprobes. Riboprobes were prepared from a fragment of AT_2 receptor (586 bp), subcloned in pGEM-T-easy vector, by *in vitro* transcription using T7 or SP6 RNA polymerases and the DIG-dUTP labeling kit (Roche Diagnostics). Coronal sections (10 µm) were fixed in 4% paraformaldehyde/PBS, rehydrated in PBS, treated with 10 mg/ml proteinase K (5 min, RT) and post fixed with freshly prepared 4% paraformaldehyde (5 min), followed by acetylation with 0.25% acetic anhydride in 0.9% triethanolamine. After that, sections were incubated in hybridization buffer (5× saline-sodium citrate (SSC) buffer, 50% formamide, yeast RNA 50 µg/ml, heparin 50 µg/ml, 1% SDS, 1 mg/ml of DIG-labeled riboprobe), at 70 °C in humified chamber, overnight. Washes were carried out in 50% formamide/5× SSC at 65 °C, followed by washes in 50% formamide/2× SSC. Sections were blocked with 2% Blocking Roche reagent in TBST, incubated with alkaline phosphatase-coupled anti-digoxigenin antibody (dilution 1:2500, 2 h, RT). Labeled sections were exposed to BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium, Promega) in the dark and color developed within 2–3 days, rinsed in PBS, mounted in Mowiol (Sigma–Aldrich).

Statistical Analysis

Statistical differences among groups were obtained with the *One-way Analysis of Variance* (ANOVA) followed by Tukey–Kramer's Multiple Comparisons test (GraphPad Prism 5). Data are expressed as mean ± SEM (standard error of the mean) and statistical significance was accepted with probability value of $P < 0.05$.

Results

Ang II Receptors Localization and Expression in the IC During Development

Binding Autoradiography

Coronal sections from the midbrain rat at the stages P0, P8, P15 and P30 containing the IC were obtained in a cryostat, level P0- 69 for P0 animals and level equivalent to Bregma − 8.64 for other ages. Adjacent sections were used for binding autoradiography with [^{125}I] Ang II (0.2 nM) and receptor subtype evaluation. The IC is a bilateral prominent structure at early developmental stages (Fig. 1a). Binding was quantified by using Scion program (Fig. 1b). Both AT_1 and AT_2 receptors are present in the IC, although binding to AT_1 receptors was considerably lower and very little variation was observed between the stages studied (Fig. 1b). AT_2 receptor subtype predominates at all stages in the IC, with maximal expression level at P15 (Fig. 1a, c). Although AT_2 receptors remain in the adult animals, receptor density in adults was considerable lower than binding density at P15 (Fig. 1c). The central area of the IC (CIC) exhibited higher AT_2 binding than other areas, at stages P8 to P30.

Immunolabeling of AT_2 Receptors in the IC

In order to confirm the presence of AT_2 receptors in the IC, we performed immunohistochemistry and immunofluorescence staining of coronal sections from P15 animals at the − 9.30 level. Immunohistochemical and immunofluorescence staining (Supplementary Fig. S1) with anti-AT_2 antibody did confirm the presence of AT_2 receptors at the IC. In order to establish the nature of the cells recognized by AT_2 antibody, we performed immunolabeling of consecutive sections with anti-βIII tubulin, a neuronal marker. Both AT_2 and β-III tubulin antibodies recognized cells located at the same areas, thus suggesting the neuronal nature of AT_2 labeled cells (Supplementary Fig. S1).

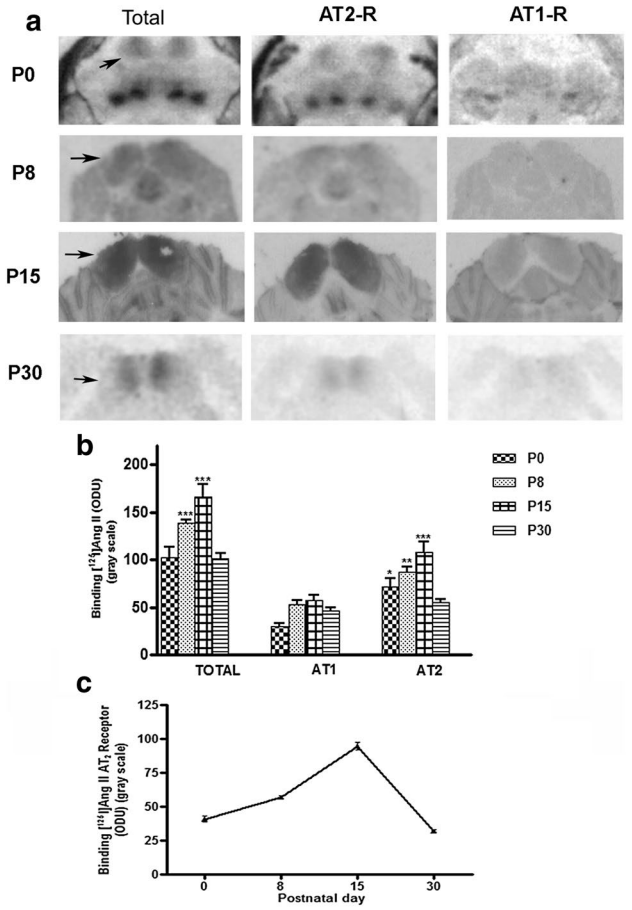

Fig. 1 Localization of Ang II receptors in the inferior colliculus (IC) from rats at different developmental stages: P0, P8, P15 and P30. **a** Autoradiographic localization of Ang II receptors to midbrain at the level equivalent to Bregma − 8.64. Consecutive coronal sections were incubated with [^{125}I] Ang II (0.2 nM) in the absence (Total) or in the presence of Losartan (AT_2 receptors, 10^{-6} M) or PD123319 (AT_1 receptors, 10^{-6} M). Arrow points to the IC. **b** Quantitative data are mean ± SEM of total specific binding, AT_1 subtype (AT1-R) and AT_2 subtype (AT2-R) (n = 5–6 animals per age). For Total binding, ***p < 0.001, P8 and P15 vs. P0 and P30; for AT2 receptors **p < 0.01, P8 vs. P0, P15 and P30 and ***p < 0.001, P15 vs. P0 and P30. **c**. Variation of AT_2 receptor binding during ICs development

In situ Hybridization

To verify the presence of mRNA in the IC, we performed in situ hybridization with either *antisense* or *sense* digoxigenin labeled riboprobes in coronal sections containing the ICs (Supplementary Fig. S2) from P15 animals. The *antisense* riboprobe recognized the same area identified by binding autoradiography and immunolabeling. In this way we confirmed that both protein and mRNAs were present in the ICs of P15 rats, mainly localized at the CIC.

Ang II Receptors in the Auditory System

The previous results provide evidence of an increase in binding to Ang II AT_2 receptors with development, peaking at the stage P15 in the IC, a central station in the auditory pathway. The maximal expression of Ang II AT_2 receptors correlates with the onset of audition, which in the rat is at P12 [8, 9]. Thus, we decided to evaluate the presence of Ang II receptors in the different nuclei involved in the auditory pathway during development, at the midbrain and brainstem levels. Figure 2 shows detailed structures obtained under light microscope of binding at the stages P8 (Fig. 2a) and P15 (Fig. 2b), including the IC, VCN and SOC nuclei. No binding was observed at the VCN and the SOC in newborn brains. Table 1 summarizes the relative level of Ang II receptors during development at these nuclei. As shown in Fig. 1, AT_2 receptors were present at all ages in IC, being maximal at P15. Meanwhile at the VCN AT_2 binding was observed at ages P8 and P15 and low AT_1 binding at the different ages. Binding of Ang II receptors in the SOC was detected at P8 and P15, exhibiting the highest AT_2 binding at P8, while low AT_1 binding was observed at P15. It is well known that in adulthood the SOC exhibits binding of the AT_1 subtype [28, 29]. To the best of our knowledge, the presence of AT_2 receptors in developing brain was first reported in the SOC by Nuyt et al. [13] at E19 and E21. In the adult, with only the exception of the SOC, most of the nuclei related to auditory pathways exhibit a predominance of Ang II AT_2 receptors. Although the presence of Ang II receptors has been previously described at some of the studied areas, this is a first study where a possible correlation with the maturation of the auditory system is performed. At the stage P0, most of the structures, with exception of the IC are not developed or did not show Ang II binding.

Prenatal Treatment with Ang II or PD123319

To evaluate the potential role of Ang II receptors during early developmental stages, we performed prenatal administration of Ang II or PD123319 (G13–G21) for a week

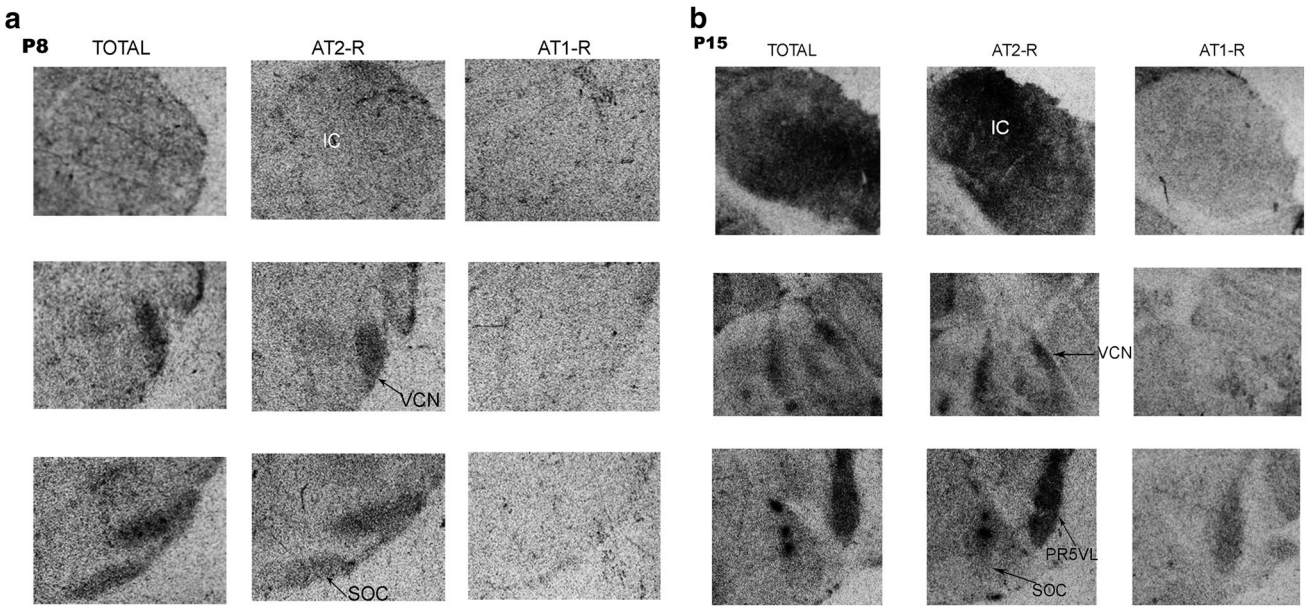

Fig. 2 Autoradiographic localization of Ang II receptors in the developing auditory system. Consecutive coronal sections were incubated with [^{125}I] Ang II (0.2 nM) in the absence (Total) or in the presence of Ang II (NS, 10^{-6} M), Losartan (AT_2 receptors, 10^{-6} M) or PD123319 (AT_1 receptors, 10^{-6} M) and processed as indicated under Methods. Images from the film were captured under light microscope, magnification 100×. **a** Midbrain sections of P8 animals, level equivalent to −9.30. **b** Midbrain sections of P15 animals, level −9.30. *IC* inferior colliculus complex, *VCN* ventral cochlear nucleus, *SOC* superior olivary complex, *Pr5VL* principal sensory trigeminal nucleus

Table 1 Localization and relative level of Ang II receptors at the auditive pathway during development

	AT_2 receptor			*AT_1 receptor*		
	P0	*P8*	*P15*	*P0*	*P8*	*P15*
Inferior colliculus (IC)	+	++	+++		+	+
Ventral cochlear nuclei (VCN)		++	++		+	+
Superior olivary complex (SOC)		++	+			+

before birth. In previous studies we demonstrated that both Ang II or PD123319 were able to pass by the fetoplacental barrier and did affect cerebellar development as well as Ang II receptors expression and localization [3]. Since the hindbrain and midbrain structures involved in the auditory pathway (CN, SOC and the IC), has their origin early during developmental stages [7, 10] we assume that the treatments should affect Ang II receptor expression or localization in the postnatal period.

Receptor localization was assayed by binding autoradiography with [^{125}I] Ang II in coronal sections from P0, P8 and P15 brains from treated animals. Figure 3 shows receptor localization following treatments at the IC for different ages: P0 (Fig. 3b), P8 (Fig. 3c), and P15 (Fig. 3d). For comparison with the histological level (P0–69) the atlas image for newborn brain was included (Fig. 3a). Quantification of Ang II receptors at the IC (total binding, AT_2 and AT_1 receptors) at the different stages is shown at Fig. S3.

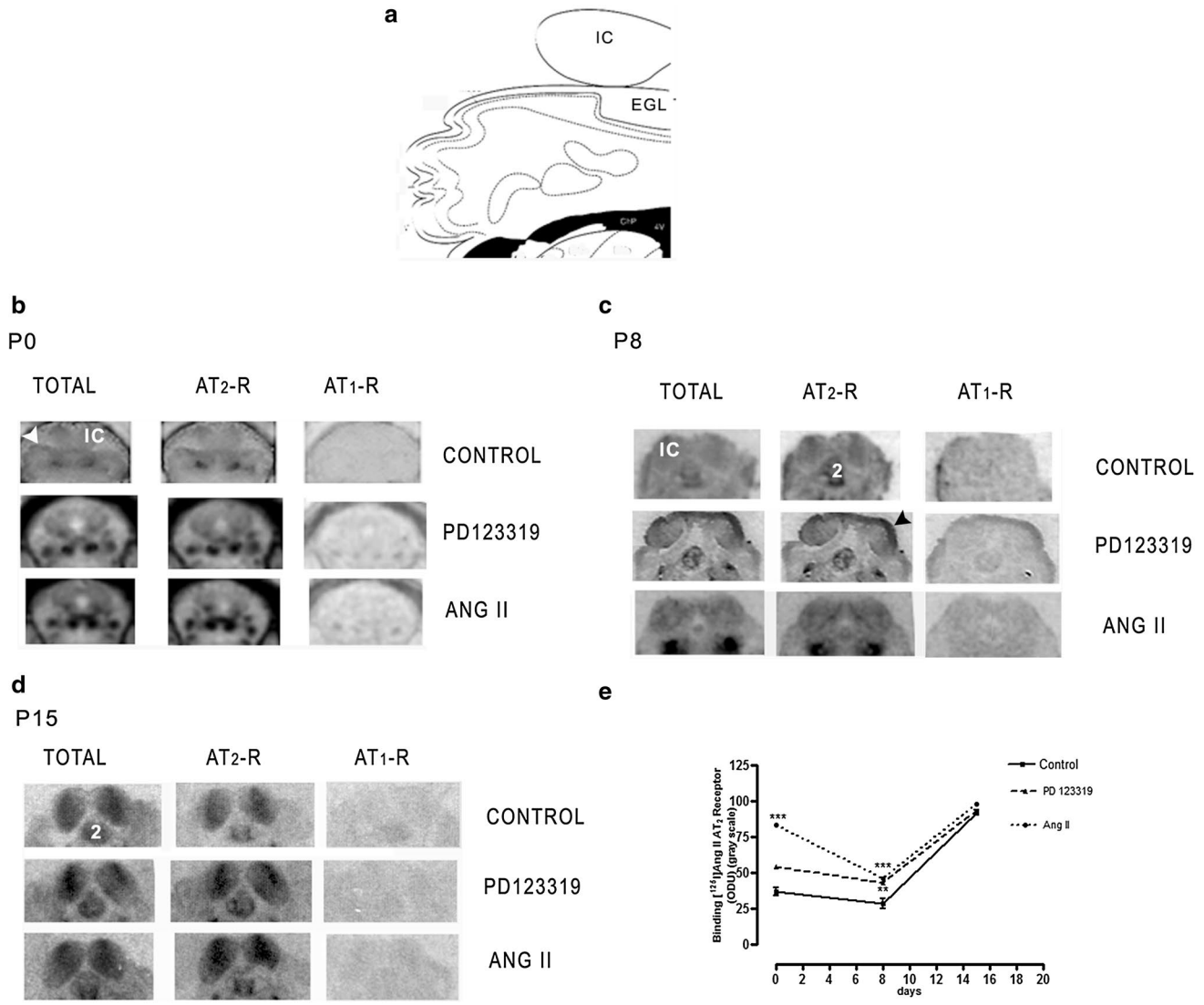

Fig. 3 Autoradiographic localization of Ang II receptors at the midbrain level of animals treated during late pregnancy. Consecutive coronal sections from pups P0 (level P069), P8 and P15 (Bregma −9.30) born from mothers treated during late pregnancy (G13–G21) with saline (control) Ang II or PD123319 were obtained as described. Sections were incubated with [^{125}I] Ang II (0.2 nM) in the absence (Total) or in the presence of Ang II (NS, 10^{-6} M), Losartan (AT_2 receptors, 10^{-6} M) or PD123319 (AT_1 receptors, 10^{-6} M). Images for NS (non specific binding) were not included, but considered for quantification. **a** Schematic coronal sections from rat P0 at level P0–69 modified from Paxinos et al. [26] showing the relative position of the IC with reference to the Cerebellum. **b–d** Autoradiographic localization of Ang II receptors at the level of the inferior colliculus complex, stages P0, P8 and P15. *IC* inferior colliculus complex, 2: cerebellar area, white arrowhead: cerebellum, black arrowhead: Superior colliculus. **e** Variation of binding to AT_2 receptors at the IC with development in control and treated animals. Data are mean ± SEM from 4 independent treatments of pregnant mothers, (20–24 animals from each treatment). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. control

Treatment with Ang II increased significantly the level of AT_2 receptors at birth (P0) and P8, while at P15 receptor binding was comparable to that of control animals (see Fig. 3e). Blockade of Ang II AT_2 receptors with PD123319 during the last gestation week also induces an increase in the level of Ang II AT_2 receptors at P0 and P8 (Fig. 3e). In both cases, animals had the capacity to recover after 2 weeks without treatment. On the other hand, either overstimulation with Ang II or blockade of AT_2 receptors do not modify the level of AT_1 receptor, which remains low at all stages.

Although binding in the whole IC is comparable at the stage P15 in control and treated animals (Fig. 3e), it appears highly localized and increased in the central nucleus (CIC) of the IC (Fig. 3d). Figure 4 shows the IC at higher magnification in P15 and binding quantification in the CIC. Thus, we observed increased expression of AT_2 receptors in the CIC of P15 animals treated either with PD123319 or Ang II (Fig. 4).

Discussion

The predominance of Ang II AT_2 receptors at early developmental stages supports a potential role for these receptors in development, recognized by different authors [2, 3, 13–17, 20–22, 28, 29]. In previous papers, we showed Ang II AT_2 receptors localization in either motor or sensorial areas of the brainstem in P15 animals [2] and P0 and P8 animals following treatment with Ang II or its antagonists [3]. We described high binding in the IC, a major station in the auditory pathway in P15 animals [2]. This observation prompted us to evaluate the presence of Ang II receptors at different nuclei of the developing auditory system.

Development of the auditory system starts during embryonic life and continues postnatally. The IC, a midbrain nucleus, has its origin from the tectum at early embryonic stage (E12) [6, 7], while the CN and the SOC originates from the rhombomere (r2–r5) neuroepithelium [10]. It is

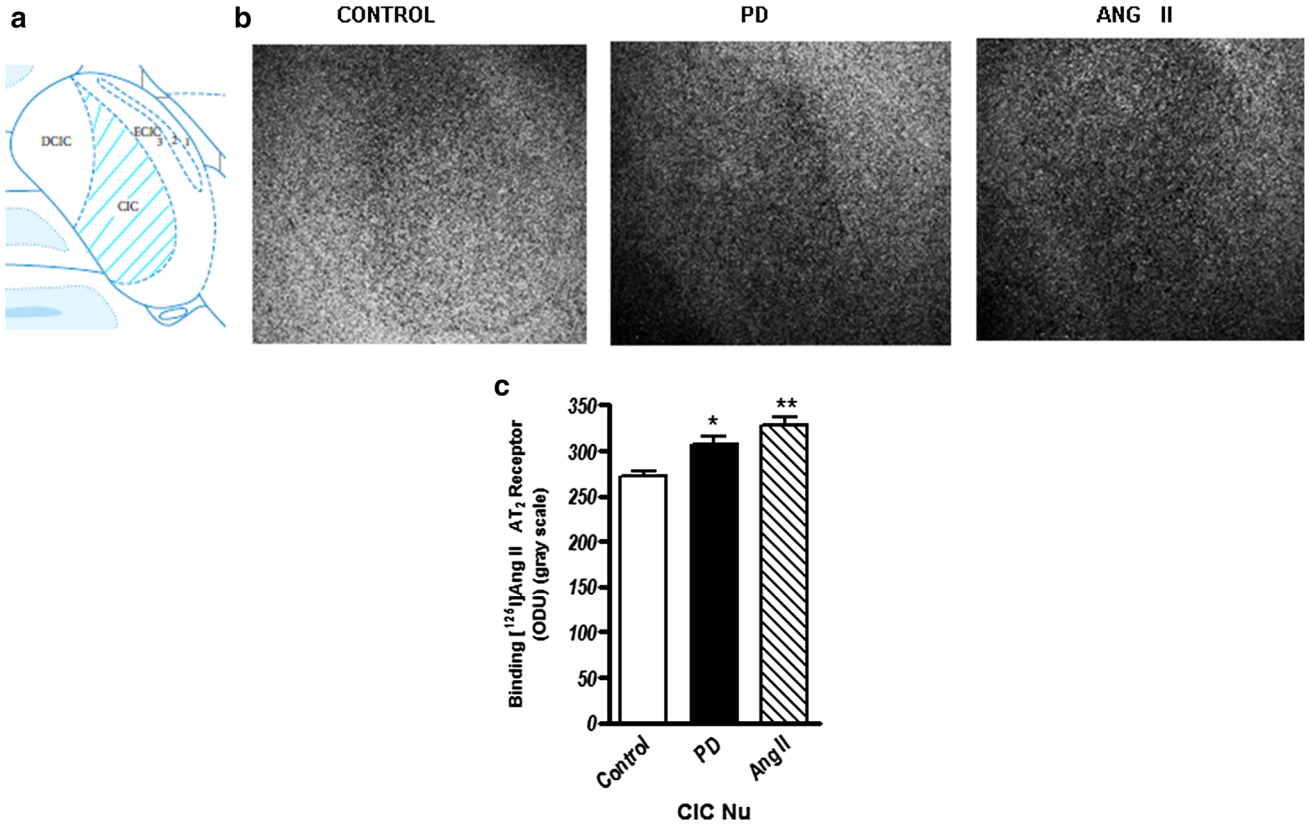

Fig. 4 AT_2 binding densities at the CIC in P15 animals treated with PD123319 or Ang II during pregnancy. Consecutive coronal sections were incubated with [^{125}I] Ang II (0.2 nM) in the absence (Total) or in the presence of Ang II (NS, 10^{-6} M), Losartan (AT_2 receptors, 10^{-6} M) or PD123319 (AT_1 receptors, 10^{-6} M) and processed as indicated under Methods. Images for NS (non specific binding) were not included, but considered for quantification. **a** Schematic sections from brain at Bregma −9.30, showing the CIC (shadow area)(modified from [27]). **b** Autoradiographic localization of AT_2 receptors at the level of the CIC in control and treated animals with PD123319 or Ang II during pregnancy. Images from the film were captured under light microscope, magnification 100×. **c** Quantification of binding to AT_2 receptors in the CIC, P15 rat brain: Data are mean ± SEM from 4 independent treatments of pregnant mothers, (5–6 animals per treatment group). $^{**}p < 0.01$, $^{*}p < 0.05$ vs. control

well-established that the onset of hearing takes place at P12 [8, 9] while maturation of the connections continues for several days [30, 31]. In the present paper we evaluated receptor localization and expression at different ages (P0, P8, P15 and P30) for different brainstem nuclei (CN, SOC) and the IC, a critical midbrain station for the auditory pathway.

By using binding autoradiography (semi-quantitative), we demonstrated a predominance of Ang II AT_2 receptors in the IC, at all stages P0 to P30, being maximal at the stage P15. The presence of Ang II AT_2 receptors in the IC was corroborated by immunofluorescence and its mRNA by in situ hybridization. Thus, both protein and mRNAs were present at the same nucleus, being higher at the CIC. Cells immunostained by Ang II AT_2 antibodies localized at the same areas labeled by βIII−tubulin antibody, a marker of neuronal cells. In this way, we showed a differential expression of AT_2 receptors in the IC, encompassing the onset of the audition (P12). The IC is a major station in the auditory pathway which coordinates the interaction between brainstem nuclei and the auditory cortex. The presence of AT_2 receptors in the IC was reported by Nuyt et al. [13], only at the stage P7 by in situ hybridization.

Different nuclei which participate in the auditory pathway have been reported as expressing Ang II receptors [13, 14]. However, no correlation has been established between the binding to Ang II receptors or mRNA expression level and the maturation of the auditory pathway. By using binding autoradiography we demonstrated increasing level of Ang II AT_2 receptors during development of the IC, VCN, and the SOC. Table 1 reports the presence or absence and relative level of Ang II receptor subtypes at the different nuclei during development. Although in the adult, the SOC has been identified as expressing mainly Ang II AT_1 receptors, we observed AT_2 receptor subtype at the stages P8 and P15 in the SOC. This is the first report of the presence of Ang II AT_2 receptors in the SOC at early postnatal stages, since Nuyt et al. [13] only reported the presence of mRNA during embryonic stages (E19, E21). Thus, we are reporting for the first time a time correlation between the onset of audition and the development of brain nuclei involved in the auditory system.

Although the size of the IC remains rather constant during postnatal development [32], synaptogenesis continues postnatally. While neurons originate during embryonic stages, the final cytoarchitecture of the brain requires neuronal migration, synaptogenesis and circuit refinement. In the rat, synaptogenesis and circuits refinement occur postnatally [11]. In the present study, while similar size was observed at the different postnatal stages, the expression level of Ang II AT_2 receptors encompasses the maturation of the auditory system. These observations suggest a possible participation of AT_2 receptors in the development of the auditory system.

In order to evaluate the potential role of Ang II receptors during development of the auditory pathway, we performed overstimulation (with Ang II) or blockade (PD123319) of AT_2 receptors during the late gestational period (G13–G21). Previous data reported an increase of Ang II AT_2 receptors following overstimulation of the receptors with Ang II in a cell line [33]. To our surprise, both treatments increased the expression level of AT_2 receptors in the IC in P0 and P8 pups and in the CIC in P15 animals. In cerebellum, we observed the loss of the Purkinje cell layer in animals treated with the AT_2 antagonist PD123319 and suggested a role for AT_2 receptors in neuronal migration [16], in agreements with observations from other authors [22].

Several authors identified the presence of neurotransmitters in the CIC, identifying glutamatergic predominance in this area, together with a minor GABA-ergic and glycinergic prevalence [4, 34]. In the IC and auditory system Ca^{2+}-binding proteins co-localizing with amino acid neurotransmitters have been described [35]. The CIC has been identified as the active area which establishes neuronal communication with the brainstem nuclei as well as the cortical auditory system [10]. Sturm et al. [31] by using laser-scanning photostimulation characterized the intrinsic connectivity in the CIC, supporting that refinement of afferent projections to the CIC takes place postnatally, in agreement with other authors [11, 30]. We observed that, although in P15 animals, the level of AT_2 receptors in the whole IC is comparable in control and treated animals, treatment with either Ang II or PD123319 increased the level of AT_2 receptors in the CIC.

In summary, the coincidence in time and space of the higher expression of Ang II AT_2 receptors in the different nuclei which integrate the auditory system, particularly in the CIC, with the onset of audition and maturation of the auditory pathway do suggest the possible participation of Ang II AT_2 receptors in this process.

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