Immunohistochemical Detection of Angiotensin II Receptors in Mouse Cerebellum

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Abstract

Angiotensin II (AT) receptors, including AT receptor type 1 (AT1R) and type 2 (AT2R), are expressed in the rodent central nervous system, but their distributions and activation states are still unclear. In this study, we have performed immunohistochemical analyses of AT receptors in mouse cerebellum using our "in vivo cryotechnique" (IVCT). We used antibodies against amino-terminal domains of AT receptors, which are considered to undergo conformational changes upon the binding of AT. Immunoreactivity of AT1R was detected in mouse cerebellum and was highest in the outer tissue areas of molecular layers using IVCT. Surprisingly, the AT1R immunoreactivity in the cerebellar cortex was remarkably reduced following 5 and 10 min of hypoxia. The correlation of localization with GFAP and also hypoxia-induced decrease of its immunoreactivity were similarly observed by immunostaining of AT2R in the cerebellar specimens. These findings demonstrated that IVCT is useful to reveal dynamically changing immunoreactivities usually affected by receptor-ligand binding as well as hypoxia and also suggested that functional activities of AT receptors are time-dependently modulated under hypoxia in the central nervous system in comparison with the adrenal gland.

Keywords

In vivo cryotechnique • Angiotensin II receptors • Cerebellum • Bergmann glia • Hypoxia

31.1 Introduction

Angiotensin II receptors, type 1 (AT1R) and type 2 (AT2R), belong to the superfamily of seven-transmembrane G protein-coupled receptors [9] and mediate the physiological functions of angiotensin II (AT), an octapeptide hormone regulating cardiovascular homeostasis, in various organs of animals [4]. Some previous studies also demonstrated that AT receptors were often expressed in neurons and glia of the central nervous system (CNS) and play some functional roles in

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S. Ohno et al. (eds.), *In Vivo Cryotechnique in Biomedical Research and Application for Bioimaging of Living Animal Organs*, DOI 10.1007/978-4-431-55723-4_31

hemodynamic control, differentiation, neuronal plasticity, and cell survival [1, 5, 8, 18, 20]. Although both AT1R and AT2R are expressed in animal cerebellum [2, 3, 6, 11, 19], their distributions and activation states under physiological and pathological conditions have not been well understood.

In the last one and a half decades, our "in vivo cryotechnique" (IVCT) has become well known as a powerful tool to retain native ultrastructures in brain tissue sections, such as extracellular spaces in mouse cerebellar cortex [16, 17]. In addition, using IVCT followed by freeze-substitution (FS) fixation, unstable signal molecular components in animal organs were instantly captured in situ and could be immunohistochemically visualized without technical artifacts due to anoxia and ischemia [22]. Therefore, the functional activities of AT receptors could be examined using IVCT along with specific antibodies, whose immunoreactivities usually depend on the dynamic binding of ligands and agonists to the receptors [10]. G protein-coupled receptors, including AT1R and AT2R, undergo rapid conformational changes of extracellular amino-terminal regions upon the binding of AT ligands and agonists [7]. Therefore, some immunoreactivity changes of specific antibodies against the amino-terminal regions of such receptors could reflect alterations of ligand-binding activities and rapid adaptation for the intracellular metabolism and microenvironment.

In the present study, we used the IVCT and also antibodies against amino-terminal regions of AT receptors and performed immunohistochemical analyses of mouse cerebellum. We also examined their immunoreactivity changes under normal or hypoxic conditions using IVCT. Our present results demonstrated that immunoreactivities of dynamically changing AT1R and AT2R were clearly detected using IVCT and were closely related to Bergmann glia and some astrocytes immunopositive for glial fibrillary acidic protein (GFAP). In addition, the AT1R immunoreactivity in the mouse cerebellum was diminished under hypoxic conditions for 5 min. The hypoxia-induced immunostaining change of AT2R mostly resembled that of AT1R.

31.2 Different Preparation Methods for Mouse Cerebellum and Adrenal Gland

For the control group, IVCT was performed with an in vivo cryoapparatus (IV-II; EIKO Engineering, Hitachinaka, Ibaraki, Japan) on living mouse cerebellum under normal respiration conditions (Fig. 31.1b), as reported previously [15]. For the experimental hypoxia groups, IVCT was performed at 1, 5, 10, or 15 min after opening the thoracic cavity [21]. To expose the cerebellum of anesthetized mice, a part of the cranial bone was carefully removed with a dental electric drill [16]. IVCT was performed by directly cutting the cerebellum with a cryoknife precooled in liquid nitrogen (−196 °C) (Fig. 31.1b) [13] and then immediately pouring isopentane-propane (IP) cryogen (−193 °C) over it under the in vivo cryoapparatus (Fig. 31.1b).

31.3 Immunolocalization Comparison of AT1R with Different Preparation in Cerebellum of Mouse

The immunolocalizations of AT1R examined using QF-FS were mostly similar to those prepared using IVCT (Fig. 31.2a–f), and immunoreaction products appeared to be clustered or linear dot patterns in the mouse cerebellar cor-

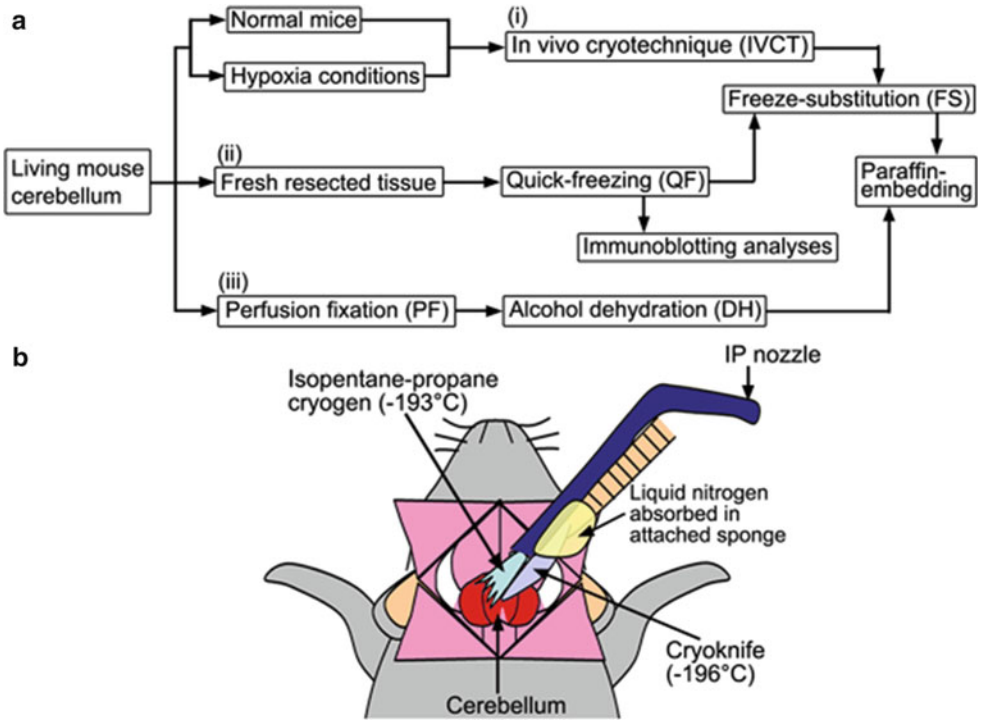

Fig. 31.1 (**a**) A flow chart of the three different preparation procedures, namely, in vivo cryotechnique with freeze-substitution (i), quick-freezing of fresh resected tissues followed by freeze-substitution (ii), and conventional perfusion fixation with alcohol dehydration (iii) for mouse organs, such as cerebellum or adrenal glands, which are finally embedded in paraffin wax. (**b**) A schematic drawing of the in vivo cryotechnique for an anesthetized mouse cerebellum under an in vivo cryoapparatus. It is frozen in vivo with a cryoknife and simultaneously has isopentane-propane (*IP*) cryogen (−193 °C) poured on it

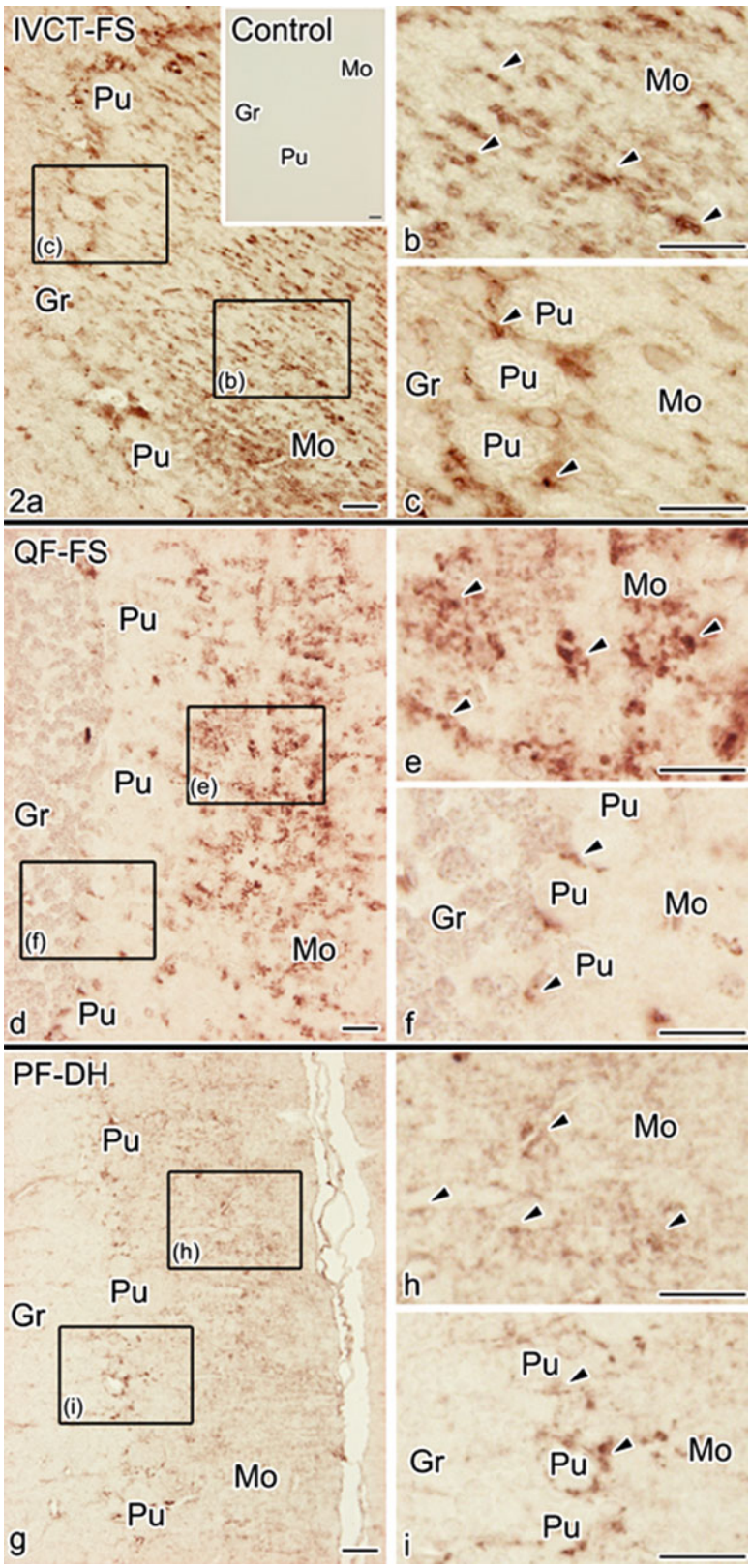

Fig. 31.2 Immunostaining of AT1R with the three different preparation methods, namely, IVCT-FS (**a**–**c**), QF-FS (**d**–**f**), and PF-DH (**g**–**i**). Immunoreaction products in the specimens prepared using IVCT-FS are more clearly detected in molecular layers, Purkinje cell layers, and granular layers than those prepared using QF-FS and PF-DH. At higher magnification, immunoreaction products, recognized as dot patterns, are less clear in the molecular layers using QF-FS and PF-DH (**b**, **e**, **h**, *arrowheads*) and also less clear in Purkinje cell layers with QF-FS and PF-DH (**c**, **f**, **i**, *arrowheads*). Bars, 20 μm

tex. However, compared with the AT1R immunoreactivity detected using IVCT (Fig. 31.2a–c), it was more weakly detected in all three layers using QF-FS (Fig. 31.2d–f). Using the conventional PF-DH (Fig. 31.2g–i), the AT1R immunoreactivity was less obviously detected in the three layers.

Our previous studies demonstrated that both QF-FS and PF-DH easily caused technical artifacts of morphology because of hypoxia/ischemia inevitably induced by the conventional chemical fixation [17]. Therefore, the decreased AT1R immunoreactivity following both QF-FS and PF-DH preparations indicated that the immunostaining of AT1R was inevitably altered by hypoxic conditions.

31.4 Immunoreactivity of AT1R at Several Hypoxia Time

To confirm this possibility, the immunoreactivity of AT1R was examined in mouse cerebellum prepared using IVCT under different intervals of hypoxia. IVCT was similarly performed at three time points under hypoxia after opening the thoracic cavity, as shown in Fig. 31.1b. Following 1 min of hypoxia, the immunoreactivity of AT1R was slightly decreased, but still clearly detected in all three layers of cerebellar cortex (Fig. 31.3a, b). However, after 5 and 10 min of hypoxia, it was remarkably reduced and undetectable in the three layers (Fig. 31.3e, f, i, j). By contrast, the immunoreactivities of both calbindin (Fig. 31.3c, g, k) and GFAP (Fig. 31.3d, h, l) were mostly unchanged under such hypoxic conditions.

31.5 Immunoreactivity of AT2R at Several Hypoxia Time

Next, considering the hypoxic conditions, the immunoreactivity of AT2R in the cerebellar cortex was still detected at 1 min of hypoxia (Fig. 31.4a), which was similar to that of AT1R (Fig. 31.3b), but it was remarkably reduced at 5 or 10 min of hypoxia (Fig. 31.4b, c).

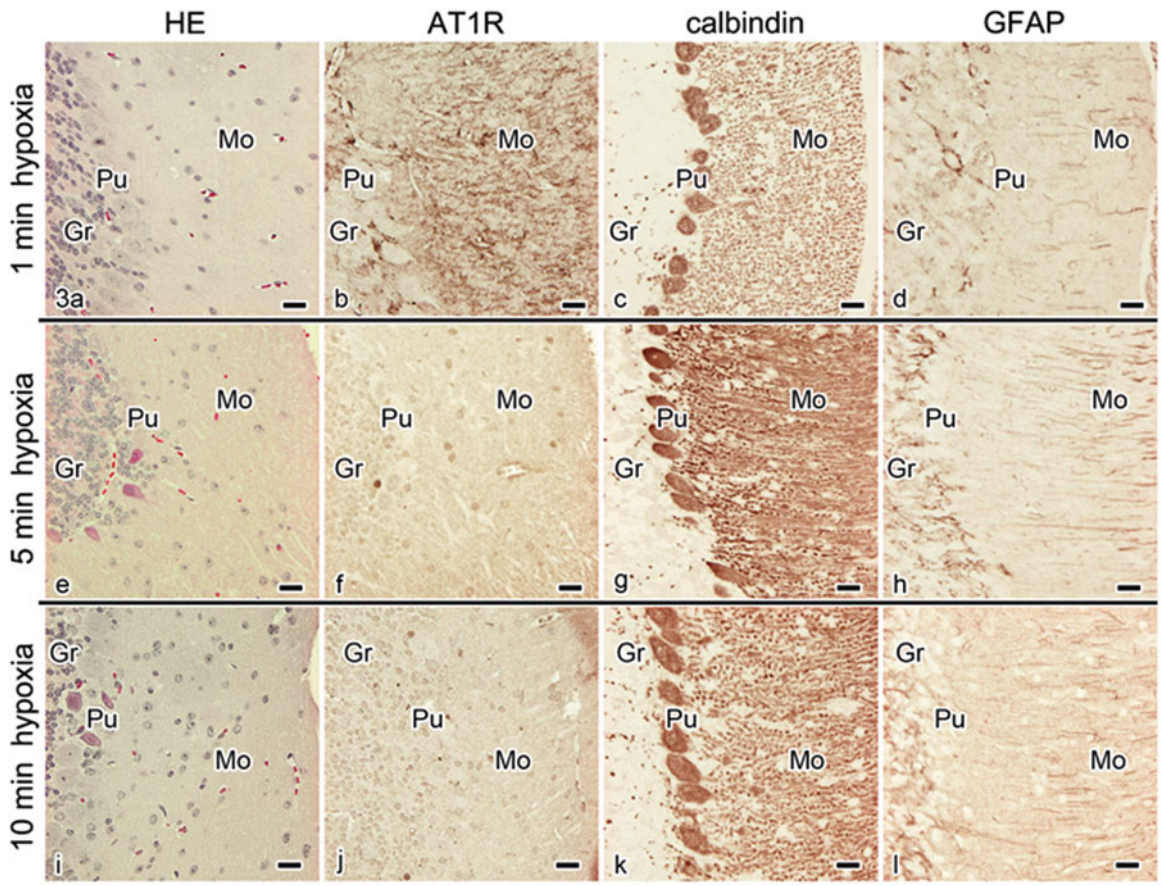

Fig. 31.3 Light microscopic images of HE staining (**a**, **e**, **i**) and immunostaining of AT1R (**b**, **f**, **j**), calbindin (**c**, **g**, **k**), and GFAP (**d**, **h**, **l**) under 1 (**a**–**d**), 5 (**e**–**h**) and 10 (**i**–**l**) min hypoxia in serial sections of the mouse cerebellum, as prepared using IVCT-FS. At 1 min after hypoxia, AT1R immunoreactivity is still detected, whereas its intensity is slightly decreased in comparison with those of normal mice, as shown in Figs. 31.2d and 31.3a. At 5 (**f**) and 10 (**j**) min of hypoxia, AT1R immunoreactivity is remarkably reduced in all three layers, namely, molecular layer (*Mo*), Purkinje cell layer (*Pu*), and granular layer (*Gr*). Moreover, as seen by HE staining (**e**, **i**), some Purkinje cells show more eosinophilic cytoplasm at 5 and 10 min after hypoxia. By contrast, immunostaining of calbindin or GFAP does not change following hypoxia. Bars, 20 μm

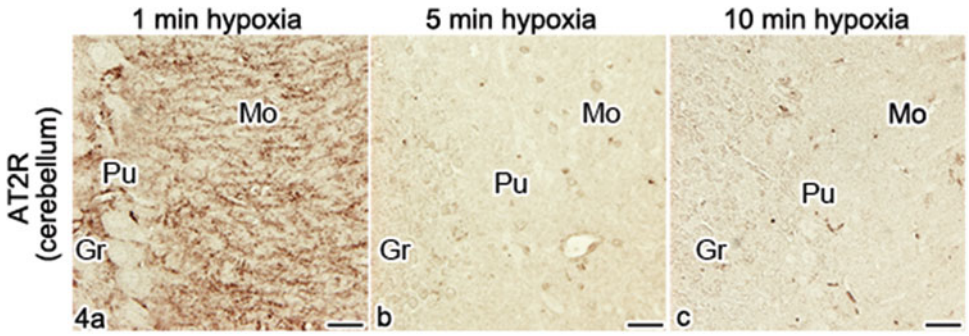

Fig. 31.4 Immunostaining of AT2R under hypoxic conditions in mouse cerebellum, as prepared using IVCT-FS. (**a**)–(**c**) In the cerebellum, at 1 min after hypoxia (**a**), immunoreaction products are still detected as dot patterns, but their immunostaining intensities are slightly decreased in comparison with those of normal mice. After 5 and 10 min of hypoxia (**b**, **c**), they are remarkably reduced in all three molecular (*Mo*), Purkinje cell (*Pu*), and granular layers (*Gr*). Bars, 20 μm

31.6 Concluding Remarks

With IVCT-FS, several technical problems in clarifying the native tissue morphology and the precise immunolocalizations of signal molecules or receptors were avoidable, including tissue shrinkage and the translocation of soluble proteins caused by chemical fixation and alcohol dehydration [17, 21, 22]. The meshwork-like structures induced by the tiny ice crystal formation probably facilitate effective penetration of antibodies during immunohistochemical steps, resulting in better formation of antigen-antibody complexes [14]. Moreover, freeze-substitution (FS) fixation has an additional benefit of avoiding antigen-retrieval treatments. Therefore, we were able to obtain clearer immunolocalizations of AT1R and AT2R in the mouse cerebellum. In the present study, both AT1R and AT2R were immunolocalized in the molecular, Purkinje cell, and granular layers of mouse cerebellum. Our findings are consistent with previous reports, showing that AT receptors were located in the cerebellar cortex [2, 11]. It is also interesting that the immunoreactivity of AT receptors became rarely detectable in the mouse cerebellum following hypoxia. The antibodies used in this study are raised against the extracellular N-terminal regions of the AT receptors [10], which undergo conformational changes upon receptor activation [12]. One possibility for the reduced immunoreactivity is that molecular structures of the AT receptors, AT1R and AT2R, bound to ligands can be effectively retained using IVCT, and it could capture dynamic detachment of AT ligands from the AT receptors and conformational alteration of the receptors from activated to nonactivated states, as reported previously for rhodopsin [22]. The present figures were already published in our paper, Histochem Cell Biol (2013) 140:477–490, and cited with their permissions.

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