To elucidate the steroid-synthesis in the mammalian brain (i.e., neurosteroid), we immunohistochemically studied various kinds of steroidogenic cytochrome P450 enzymes in the rat brain. The primary antibodies used in this study were rabbit polyclonal antibodies to cholesterol side-chain cleavage (SCC), C21-hydroxylase (C21), 11β-hydroxylase (11β), 17α-hydroxylase/C17-20 lyase (17α) and aromatase (AROM). The immunoreactivities for the microsome enzymes, C21 and 17α were located in the neuronal cell-bodies and proximal parts of their fibers, while mitochondria enzymes, SCC and 11β were located in the cell-bodies and their fibers and terminals. These immunoreactivities were distributed in the limbic structures of prosencephalon including hippocampus and amygdaloid complex, the hypothalamus including preoptic area, the cerebellar cortex, and some of other regions. All the sets of these enzymes did not always coexist in the identical cells, however, in the hypothalamus and cerebellum these enzymes are thought to work one after another in adjacent cells, forming the “steroidogenic cellular circuits”. These findings strongly suggest that the steroid-synthesis occurs in the neurons; and the neurosteroids exist in the mammalian brain.

Key words: Neurosteroid, Cytochrome P450, Steroidogenesis, Brain, Immunohistochemistry

I. Introduction

The steroid hormone (trans-trans-trans type steroid) is biosynthesized as shown in Fig. 1 by the catalytic reaction of following steroidogenic cytochrome P450 enzymes: cholesterol side chain cleavage (SCC; cholesterol to pregnenolone); 3β-hydroxysteroid dehydrogenase (3β; pregnenolone to progesterone); 17α-hydroxylase/C17-20 lyase (17α; pregnenolone or progesterone to androgens through 17α-hydroxy-pregnenolone or -progesterone); C21 hydroxylase (C21; progesterone or 17α-hydroxy-progesterone to 11-deoxy-corticoesterone or -cortisol); 11β-hydroxylase (11β; 11-deoxy-corticoesterone or -cortisol to corticosterone or cortisol); and aromatase (AROM; androgens to estrogens). The enzymes, SCC and 11β are present on the mitochondria membrane; while the others are on the microsome membrane. In the rats, 17α is found in reproductive organs, but not in the adrenal glands. In the ovarian follicle, AROM exists in granulosal cells different from SCC-, 3β- and 17α-containing thecal cells.

The autoradiographic studies on “steroid-concentrating neurons” [19, 26] had suggested the possible biosynthesis of steroids in the brain. After 1987, this hypothesis became elucidated by two serial-researches. One consists of our consecutive studies [6, 27, 30, 32-34] on the endogenous digitalis-like substance (EDLS). The EDLS, which causes natriuresis by the result of inhibition of sodium-pump (Na⁺, K⁺-ATPase), is cis-trans-cis type steroid, and is secreted in the hypothalamus. The other consists of histochemical studies on steroidogenic cytochrome P450 enzymes by Le Goascogne, Robel, Baulieu and their coworkers in France [2, 3, 8, 12, 15]. In their articles, the steroid synthesized in the nervous system is designated as “neurosteroid”. However they only reported SCC in the white matter and some neurons [15] and glial cells [8]; 3β and its mRNA in glial cells [2, 3, 12]; and absence of 17α in the brain [16]. Thereafter the distribution of AROM in the brain was histochemically or biochemically demon-
Fig. 1. Schematic drawings of steroid-synthesizing steps. The steroidogenic cytochrome P450 enzymes are abbreviated as SCC (cholesterol side chain cleavage), 17α (17α-hydroxylase/C17-20 lyase), C21 (C21 hydroxylase), 11β (11β-hydroxylase), and AROM (aromatase).

III. Results

The immunoreactivity of all the antibodies appeared as a dark brown color in the cytoplasm of their positive cells. In the control study, strong immunoreactivity for SCC and 17α was observed in the thecal cells of ovarian follicles (Fig. 2); and AROM was in the granulosal cells; while C21 and 11β were not detected in any regions of the ovary. The immunoreactivity of SCC, C21 and 11β was found only in the endocrine cells of the adrenal cortex, while 17α- and AROM-reactivities were not detected anywhere in this gland. In the brain, this immunoreactivity was mainly found in the neurons. It was widely distributed as shown in the Table 1. All this positive immunoreactivity disappeared when the primary antibodies were preabsorbed with immunogen proteins.

In and around the ventral pallidum, C21- and AROM-containing multipolar neuronal cell-bodies were scattered (Fig. 3A). The distribution area of immunopositive neurons extended to the bed nucleus of stria terminalis and the islands of Calleja. In the bed nucleus of stria terminalis, C21- and AROM-containing cell-bodies were localized; C21-, 11β- and AROM-fibers were also
neurosteroid

Table 1. Distribution of steroidogenic cytochrome P450 enzymes in rat brain

<table>
<thead>
<tr>
<th></th>
<th>SCC</th>
<th>C21</th>
<th>11β</th>
<th>17α</th>
<th>AROM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islands of Calleja</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Ventral pallidum</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
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<tr>
<td>Diagonal band</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Bed nucleus of stria terminals</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
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<tr>
<td>Lateral septal nucleus</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
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<tr>
<td>Hippocampal formation</td>
<td>O</td>
<td>O</td>
<td>O</td>
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<tr>
<td>Central amygdaloid nucleus</td>
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<tr>
<td>Medial amygdaloid nucleus</td>
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<tr>
<td>Basolateral amygdaloid nucleus</td>
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<tr>
<td>Medial preoptic area</td>
<td>O</td>
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<tr>
<td>Lateral preoptic area</td>
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<tr>
<td>Hypothalamic periventricular area</td>
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<td>Paraventricular nucleus</td>
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<tr>
<td>Supraoptic nucleus</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
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<tr>
<td>External layer of median eminence</td>
<td>*</td>
<td>*</td>
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<td>*</td>
</tr>
<tr>
<td>Medial habenular nucleus</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
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<tr>
<td>Nucleus of posterior commissure</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
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<tr>
<td>Cerebellar cortex</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
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<tr>
<td>Dorsal motor nucleus of vagus</td>
<td>O</td>
<td>O</td>
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<td>O</td>
</tr>
<tr>
<td>Cranial motor nuclei</td>
<td>O</td>
<td>O</td>
<td>O</td>
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</tr>
</tbody>
</table>

O : cell bodies  * : nerve fibers and terminals  □ : both of them

In the hippocampal formation, C21-containing cell-bodies were located (Fig. 3D). These positive cell-bodies were multiformic in shape and scattered in the stratum radiatum of CA1-CA3. A small number of multiformic cell-bodies were also found in the stratum oriens of CA1 and CA2; and those of bipolar type were seen in the polymorphic area just facing the granular layer of the dentate gyrus. The proximal parts of their nerve fibers were scattered in all the hippocampal areas especially in the granular layer of dentate gyrus. Using the anti-11β, an accumulation of fine dots of immunoreactivity with a diffuse pattern was detected in the stratum oriens of CA2-CA3, and granular and polymorphic layers of dentate gyrus. The central, medial and basolateral nuclei of amygdala contained cell-bodies that are immunopositive to anti-C21 and anti-AROM (Fig. 3E, F). Weakly stained fibers of AROM were also distributed in these nuclei (Fig. 3E, F).

The cell-bodies containing SCC, C21 and AROM were also observed in the medial preoptic area. In addition to this area, AROM-neurons were also found in lateral preoptic area. In the hypothalamic periventricular area, immunopositive cell-bodies of SCC, 11β and 17α were situated (Fig. 4A). These neurons were small to medium-sized, and oval-shaped or multiform. The magnocellular neurons of paraventricular nucleus (Fig. 4B) and supraoptic nucleus (Fig. 4C) also showed immunopositive for C21 and 11β, and SCC and 11β, respectively. Only proximal parts of their fibers were found in these areas, while SCC- and 11β-containing fibers and terminals were detected in the external layer of the median eminence (Fig. 4D). In other regions, 11β-immunoreactive fibers, and AROM-neurons were distributed in medial habenular nucleus, and nucleus of posterior commissure, respectively.

In the cerebellar cortex, 11β-immunoreactivity was seen in the molecular and granular layers showing strong staining of Bergmann glial cells (Fig. 5A); 17α-reactivity was recognized in the cell-bodies and dendrites of Purkinje cells (Fig. 5B); and AROM-reactivity was found in the Golgi cells of granular layer (Fig. 5C).

The dorsal motor nucleus of vagus contained SCC-, C21- and AROM-immunoreactive neurons (Fig. 5D). In the somatic and special visceral motor nuclei of trigeminal, facial, glossopharyngeal and vagus (nucleus ambiguus), accessory and hypoglossal (Fig. 5D) nerves, dense accumulation of strongly stained AROM-containing fibers were diffusely present among the motoneurons’ neuropil areas.

IV. Discussion

As mentioned by Robel and Baulieu, the term neurosteroid applies to those steroids that are both synthesized in the nervous system, either de novo from

Fig. 2. Photomicrograph of section of rat ovarian follicle after immunostaining with anti-cytochrome P450 cholesterol side chain cleavage antibody. Scale bar=200 μm.
cholesterol or from steroid hormone precursors and that accumulate in the nervous system to levels at least in part independent of steroidogenic gland secretion rates. The EDLS, cis-trans-cis type steroid that had been considered to be found only in plants, was biochemically, physiologically and histologically proved to be a neurosteroid in mammals by our consecutive studies [6, 27, 30, 32-34]. In contrast to EDLS, we have not been able to obtain precise...
The distribution of the steroidogenic cytochrome enzymes in the brain was immunohistochemically studied in the present study. The antibodies used in this study are thought to be specific, because they had been biochemically characterized [7, 11, 13, 25], and succeeded in the immunohistochemical absorption tests and control staining using steroidogenic organs (ovary and adrenal gland). In the present results, however, all the sets of steroidogenic enzymes did not always coexist in identical cells. Therefore the possibility of the existence of iso-enzymes might be considered. Since the steroids themselves are able to pass through the cell-membrane or blood-brain-barrier easily, neurosteroids are thought to be synthesized from not only cholesterol but also steroid hormone intermediate-precursor that is transported from other regions. In the hypothalamus and cerebellum, steroidogenic enzymes existed adjacently. These findings suggest that different types of neurons share the steroidogenic steps in the "steroidogenic cellular circuits", as well as the case of sex-steroids in ovarian follicles.

The enzyme SCC in the brain has already been demonstrated immunohistochemically [8, 15]. In these reports, SCC is localized in the white matter, scarce clusters of neuronal cell-bodies in the cerebral cortex, and cultured glial cells. In another immunohistochemical study, it was described that 17\(\alpha\) was not detectable in the brain of either the rat or guinea-pig [16]. The distribution of C21 and 11\(\beta\) in the brain has not yet been investigated. Therefore, our present study first demonstrates the precise distribution of these enzymes in the mammalian brain. Our study also reveals that most immunoreactivities are localized in the neurons, and that microsome enzymes, C21 and 17\(\alpha\) are restrictively observed in the cell-body and proximal parts of fibers, while mitochondria enzymes, SCC and 11\(\beta\) are found in not only cell-bodies but also in nerve fibers.
In the hippocampus, the existence of glucocorticoid receptor [1, 5, 9, 35], glucocorticoid effects [17], GABA-A receptor (hypothetical membrane-type steroid-receptor) [10], and 11β-hydroxysteroid dehydrogenase (glucocorticoid metabolism) [14] were reported. These previous studies support the distribution of C21 and 11β in the CA1-CA3 of hippocampus and dentate gyrus, which is obtained in this study.

The "steroidogenic cellular circuit" is formed in the hypothalamic periventricular region and cerebellar cortex. The steroid-metabolism (or synthesis in part) and sites of steroid-action have been considered in these areas. This study also shows neurosteroid enzyme in the Bergmann glial cells, a kind of astrocyte in the cerebellum. This type of glial cell also contains D-amino acid oxidase [4], heme oxygenase-I [17] and GABA-A receptor [10], besides the glutamate receptor. Neurosteroid is thought to not only regulate target gene expression (e.g., heme oxygenase-I) but also effect the membrane receptor (e.g., GABA-A receptor) in the cerebellar neurons and glial cells including Bergmann glial cells.

The enzyme AROM in the brain was investigated biochemically [20-22] and immunohistochemically [23, 24, 28, 29]. Our result is fundamentally the same as their previous reports. However, dense accumulation of AROM-immunoreactive nerve fibers in the somatic and special visceral motor nuclei, has not been reported previously. This observation of the present study is supported by the hypothesis that the motoneurons are maintained by sex-steroids [18]. This also indicates the probable cause in of sex difference in the frequency of motor neuron disease. The brain AROM has been thought to convert from androgens of steroidogenic gland-origin to neuro-estrogens. Considering all the present evidence, various kinds of steroid hormones including estrogens are synthesized in the brain from cholesterol or steroid hormone precursors by the catalytic reaction of steroidogenic cytochrome enzymes. These neurosteroids affect mammalian nervous system as neuromodulator and/or neurotrophic factor.
V. References


