THE ROLE OF ESTROGEN AND DEHYDROEPIANDROSTERONE IN SYNAPTIC REMODELING

Ph.D. Thesis

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Publications

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Introduction

Gonadal steroids and synaptic plasticity

Almost 100 years ago, Ramon y Cajal (1911) suggested that neurons are capable of making morphological changes in response to their environment. At that time Cajal's idea was not accepted, neuronal plasticity was only considered as a behavior- and adaptation-induced change in the transmission strength of existing synapses, without any morphological implications. Since then accumulating experimental evidence have indicated that morphological neuroplasticity is taking place in the brain, which contributes to the functional adaptation to the changing environmental conditions. It has become clear that several factors are able to influence neuroplastic mechanisms and among these, gonadal steroids represent a group of endogenous compounds that can powerfully regulate cellular and morphological changes in the brain.

Gonadal steroids exert both organizational and activational effects on steroid responsive tissues in the central nervous system (CNS). Organizational effects are those which are permanent and occur during the fetal-neonatal period, when estrogens and aromatizable androgens play an important role in modulating neuronal development and neuronal circuit formation. As a result, several areas of the central nervous system become sexually differentiated. Activational effects are transitory and fluctuate considerably as the hormonal milieu changes. A characteristic feature of activational effects is that they are acting not only during development, but also in adult life, more or less affecting almost every aspect of brain physiology.

Hormone-induced changes in the synaptic connectivity of the hypothalamic arcuate nucleus

Most of the data concerning synaptic remodeling in developing and adult neuroendocrine brain areas have been obtained from the arcuate nucleus (ARC). The ARC is located at the base of the hypothalamus, surrounding by the ventral part of the third ventricle, in close apposition to the endocrine median eminence and the pituitary gland and is intimately connected to both by neuronal and neurohemal connections. The arcuate nucleus is sexually dimorphic: the number of axo-somatic synapses is higher in females than in males.

In adult female rats the nucleus exhibits a natural phasic synaptic remodeling, which could be linked to the fluctuation in hormone levels during the estrus cycle. The number of axo-somatic synapses on arcuate neurons decreased between the morning and afternoon of proestrus following estradiol peak and remained low during estrus morning, and then rose again to the metestrus/diestrus level.

More detailed analysis in the ARC has confirmed that estradiol plays a fundamental role in the induction of synaptic remodeling. Studies in ovariectomized rats have shown that the administration of a single dose of 17β-estradiol induces a reversible decline in the number of arcuate axo-somatic synapses. The effect is specific, because not all types of synapses are affected. Quantitative postembedding immunocytochemical analysis has indicated that the majority of axo-somatic synaptic terminals on arcuate neurons of ovariectomized rats are GABA-immunoreactive (GABA-IR). In the case of synapses on dendritic shafts this proportion was about 50 %, while the spine synapses received only non-GABAergic innervation. The administration of a single dose of 17β-estradiol resulted in a significant decrease in the number of GABA-IR axo-somatic synapses and had no effect on the number of axo-dendritic synapses. In addition, there was a significant increase in the volume density of excitatory spine synapses.

The fluctuation in the number of axo-somatic contacts may certainly involve some sort of displacement of synapses from the perikarya, rather than a degenerative loss of axons/synapses. By studying the cellular and molecular background of hormone-induced synaptic remodeling, it has become clear that astrocytes may also contribute to synapse formation. The surface density of glial fibrillary acidic protein (GFAP) immunoreactive cell somata and processes, the number of astroglial profiles in the arcuate neuropil and the lenght of perikaryal membrane covered by glial processes are increased in the afternoon of proestrus and in the morning of estrus compared to other phases of the estrus cycle or to ovariectomized rats. This phenomenon is also observable in ovariectomized females one day after injection of a single-dose of 17β-estradiol.

Role of local estradiol synthesis in synaptic changes

Considering the molecular mechanisms of hormone-induced synaptoplastic changes, the local synthesis of estradiol in the brain seems to be also important. These steroids accumulate in the brain independently of the supply by peripheral endocrine

glands and can be synthesized de novo in the nervous system from steroid precursors. Among neurosteroids dehydroepiandrosterone (DHEA) and pregnenolone were the firsts found in the rat brain. DHEA has diverse effects in the brain: and in some cases these correlate with the physiological role of this steroid as a precursor for bioactive androgens and estrogens.

Early researches have focused on the hippocampal formation, because in both men and women as well as in laboratory animals, falling levels of androgens and estrogens have been implicated as a contributory factor to the decline in cognitive function and to the appear of neurodegenerative disorders that occur late in life. The apical dentritic spine density on CA1 pyramidal neurons decreases between the late afternoon of proestrus and late afternoon of estrus phases in the CA1 subfield of the hippocampus. In addition administration of 17β-estradiol in OVX rats increased CA1 spine density, but in castrate males the same treatment was without effect. Recent experimental data have indicated that not only 17β-estradiol, but also DHEA is able to induce a rise in CA1 spine density in OVX females, as well as in orchidectomized (ORCH) males. In females, letrozole, a selective nonsteroidal aromatase inhibitor, completely blocks the effects of DHEA on CA1 spine synapse number. In males, by contrast, aromatization does not appear to play a significant role: the synaptic effects of DHEA are completely unaffected by letrozole administration. These data suggest that in females the DHEA-induced increase in CA1 spine synapse density is mediated via intracerebral estrogen biosynthesis.

Aims of the work

The main aims of our studies were the following:

1. To test whether the synaptic remodeling of arcuate neurons that occurs naturally as the consequence of physiological, short term variation in the level of gonadal hormones during the ovarian cycle, is specific for certain types of synapses.

2. To determine whether DHEA induces synaptic remodeling in the arcuate nucleus and, if so, whether these effects are mediated directly by the neurosteroid or by 17β-estradiol synthesized from DHEA.

3. To determine the specificity of estrogen action on synaptic remodeling at the level of postsynaptic cells, we studied the changes in synaptic connectivity of the dopaminergic subpopulation (TIDA neurons) of the arcuate nucleus.

Materials and methods

Animals and surgical procedures

All animals were raised and maintained on a 12h light/dark cycle in standard laboratory conditions, with tap water and regular rat chow available *ad libitum*. During handling of animals, the authors conformed to the guidelines of our institutional ethical committee for the use of laboratory animals following the UE legislation (86/609/EEC), and appropriate measures were taken to minimize the number of animals used and cause minimal stress to them.

1. To study the fluctuation of synapse density during the estrus cycle three-month-old normally cycling Wistar female albino rats were used. Daily vaginal smears were taken for over 2 weeks, and only those animals with regular cycles were used. They were killed between 10 and 12 h of proestrus, estrus, metestrus and diestrus days and between 18 and 20 h on proestrus day. Each group contained 5 animals.

2. To examine the effect of DHEA as a precursor of bioactive androgens and estrogens adult female CFY rats were used. They were ovariectomized and one month later (n=5) received DHEA (Fluka), given in the form of a single subcutan injection. Control animals (n=5) were OVX for the same length of time and then treated with vehicle alone. An additional group of rats received the same DHEA treatment after injection of the aromatase inhibitor letrozole.

3. To analyse the hormonal treatment specificity at the level of postsynaptic cells in the experiments, female and male CFY albino rats were used. Two-month-old animals were gonadectomized and one month later, they were s.c. injected either with a single dose of 17β–estradiol (Sigma Chemical Co., St. Louis) dissolved in sesame oil or with a single injection of the oil vehicle alone. Each group contained 6 animals.

Preembedding immunostainig

Rats were transcardially perfused with fixative and the brains were removed and postfixed for an additional 3 hours. Then, 50 μm thick coronal Vibratome sections were made from mid arcuate nucleus region for light microscopic tyrosine hydroxilase (TH) immunostaining performed according to the routine PAP procedure.

Sections were first immersed in 20% normal goat serum (NGS) for 30 min and then incubated in rabbit polyclonal anti-TH antibody (Chemicon), at 1:1000 dilution for overnight in room temperature. The following day, sections were incubated for 2 hr in goat-anti-rabbit IgG (GAR) and then in peroxidase-antiperoxidase complex (PAP), diluted 1:1000 at room temperature. Peroxidase was reacted with diaminobenzidine and hydrogen peroxide.

Postembedding immunostaining

For electron microscopy, sections were osmicated (1% OsO_4 in PB) for 30 min, dehydrated in ethanol and embedded in araldite. After embedding sections were trimmed and ribbons of serial ultrathin sections were collected on Formvar coated single slot gold grids. Postembedding immunostaining for GABA was carried out using a modification of the method of Somogyi and Hodgson (1985).

Morphometry

The outline of the arcuate nucleus was drawn in one of every two serial sections on a paper using a Leitz microscope equipped with a camera lucida (Leica Microsystems, Wetzlar, Germany). The area occupied by the arcuate nucleus in each section was measured for each drawing with the aid of a microprocessor system and an image analyzing program, and the volume of the nucleus was calculated according to the Cavalieri principle.

The number of axo-somatic, axo-dendritic and spine synapses per unit of volume was estimated on electron micrographs from rats of different experimental conditions in double blind fashion, using the unbiased disector method of Sterio (1984). Each experimental group was composed of five rats and counting was performed on three blocks/animal. In each block we counted 20–24 disectors, providing 60–72 disectors per animal. The synapses were counted in the consecutive “look up” and “reference” sections and in order to increase sampling, the procedure was repeated in such a way that the “reference” and “look up” sections were reversed. We considered a structure as a synapse if the bouton and the postsynaptic membrane were in direct contact and at least three synaptic vesicles were present in the presynaptic bouton.

The number of synapses per unit volume was calculated according to the formula

$$Nv=\sum Q / Vdis$$

where $\sum Q$ represented the number of synapses present in the "reference" section which disappeared in the "look-up" section. Vdis is the disector volume (Sterio, 1984). Section thickness was determined by using the Small's (1968) minimal fold method.

Statistical analysis

The data from the same animals were pooled since no variations were detected through the three blocks in any groups of rats. Volume measurements were analyzed by analysis of variance (ANOVA). For synapse counts, a Kruskal-Wallis one-way nonparametric analysis of variance test was selected for multiple statistical comparisons. The Mann-Whitney *U* test was used to determine significant differences between two independent groups. A level of confidence of $P<0.05$ was adopted for statistical significance.

Results

Fluctuation of synapse density in the arcuate nucleus during the estrus cycle

Volume measurements show that there are no significant changes in the total volume of the arcuate nucleus during the estrus cycle.

Electron microscopic analysis performed after postembedding GABA immunostaining revealed GABA-immunopositive nerve endings forming synaptic contacts on both neuronal somata and dendritic shafts of arcuate neurons. According to stereological measurements, about two thirds of axo-somatic synapses were GABAergic on proestrus morning. About 50 %, of the synapses on dendritic shafts were labeled with GABA, while the synapses on dendritic spines received only non-GABAergic innervation.

The synaptic inputs to arcuate neurons showed variations during the estrus cycle. The number of GABAergic axo-somatic synapses decreased significantly from proestrus morning to proestrus afternoon and remained low on the day of estrus, then rose again in metestrus and remained at the same levels in diestrus and in the morning of proestrus. We have observed that the number of GABAergic and non-GABAergic synapses on dendritic shafts did not alter during the estrus cycle. In contrast, the density of synapses on dendritic spines showed a highly significant increase on proestrus afternoon, this value remained high on estrus day and returned to the base level on the next two days. Apart from the changes observed in synapse density we did not find any fine structural alterations, signs of possible degeneration of terminals in either days of the estrus cycle.

DHEA-induced axo-somatic synaptic changes in the arcuate nucleus

Like estrogen, DHEA treatment also resulted in a significant decrease in the number of GABAergic axo-somatic terminals, while the non-GABAergic population was unaffected. When the animals were pretreated with letrozole, a selective nonsteroidal aromatase inhibitor, this remodeling was not observed.

Estradiol-induced synaptic remodeling of tyrosine hydroxilase immunopositive neurons in the arcuate nucleus

In agreement with literature data we found a sexually dimorphic pattern of tyrosine hydroxylase immunoreactive (TH-IR) neurons, the males having more labeled cells in the ventrolateral region. Most of these ventrolateral neurons are non-dopaminergic monoenzymatic neurons, expressing individual complementary enzymes of the DA synthetic pathway: only tyrosine hydroxylase or aromatic L-amino acid decarboxylase. For that reason we performed the morphometric measurements in the dorsomedial part (between 2.8-3.5 mm caudal from bregma) where the two genders do not differ in respect of the number of dopaminergic neurons.

In electron microscopic pictures the DAB reaction reveals the TH-IR neurons; the postembedding immunostaining is highly specific and clearly labels the GABAergic terminals.

Morphometric analysis shows that there is a sexual dimorphism concerning the total number of axo-somatic synapses, arcuate neurons receive more synaptic terminals in females than in males.

In spite of this difference, however, the pattern of synaptic connectivity i.e. the ratio of GABAergic and non-GABAergic synapses is similar in both sexes. TH-IR neurons had significantly more GABAergic than non-GABAergic axo-somatic synapses ($P< 0.05$) with a ratio of about 2:1, while the non-dopaminergic arcuate neurons received about equal numbers of these two types of synapses.

The effect of 17β-estradiol is specific in the sense that not all arcuate neurons are affected by the structural synaptic remodeling. In females the significant decrease in numerical density of GABAergic synapses 24 hours after the hormonal treatment was observed only in the TH-IR group when compared to OVX animals. The changes in the number of axo-somatic synapses on the non-labeled arcuate neurons are not significant. In orchidectomized (ORCH) males, however, the synaptic connectivity of TH-IR neurons is not affected, while in the non-labeled population an increase of GABAergic terminals can be observed.

Discussion

Synaptic changes in the arcuate nucleus during the estrus cycle

The arcuate nucleus is a relevant neuroendocrine control center, involved in the regulation of reproduction, growth, energy balance and food intake. Synaptic changes within the nucleus during the estrus cycle may have an important share in coordinating these functions under variable environmental states. Previous quantitative postembedding immunocytochemical analyses at electron microscopic level have shown that the majority of axo-somatic synaptic terminals on arcuate neurons of ovariectomized rats are GABA-immunoreactive. Our present findings indicate that this is also the case in ovary-intact cycling females also, but only for the metestrus and diestrus phases and for the morning of proestrus. We have detected that the number of GABAergic axo-somatic synapses on arcuate neurons decreased between the morning and afternoon of proestrus and remained low during estrus morning, and then rose again in metestrus and diestrus. The number of non-GABAergic axo-somatic synapses does not change significantly.

As in previous studies, we have not observed signs of axonal or synaptic degeneration in the arcuate nucleus during the estrus cycle. We suggest that the changes in the number of axo-somatic synapses are associated to modifications in the astroglial coverage of arcuate neuronal somata and therefore astrocytes may play an active role in the synaptic displacement. Indeed, astroglial coverage of neuronal somata is increased in the afternoon of proestrus and the morning of estrus. Different interactions of astroglial processes with neuronal somata and neuronal dendrites may explain that the changes in GABAergic synapses during the estrus cycle are not observed in dendrites. However, the synaptic changes appear to present selectivity for specific synapses, since non-GABAergic synapses are not affected.

In contrast to the decrease in the number of axo-somatic synapses, the number of synapses on dendritic spines, most probably excitatory glutamatergic inputs, showed a marked increase from the morning to the afternoon of proestrus. The changes in the number of excitatory synaptic inputs on dendritic spines and inhibitory synaptic contacts on neuronal somata may play important role in the regulation of arcuate neuronal excitability.

Effects of DHEA in axo-somatic synaptic remodeling in the arcuate nucleus

Accumulating experimental data indicate that neurosteroids are also involved in neuroplastic changes. We have observed that similarly to estrogen, DHEA treatment also induces a significant decrease in the numerical density of GABAergic axo-somatic synapses in the arcuate nucleus, while the non-GABAerg synapses remain unaffected. The adult rat produces very little adrenal DHEA, so the present data reflect the effect of DHEA injection against a low endogenous background of this steroid.

Theoretically, DHEA could affect synaptic changes in OVX female rats via one or a combination of several potential mechanisms: 1) via androgen agonist activity of either DHEA itself or one of its metabolites; 2) through aromatization of DHEA to estrogen; or 3) through conversion of DHEA to estrogenic C-19 metabolites via pathways independent of aromatization.

In females the GABAergic axo-somatic synaptic density after short term DHEA treatment is dependent on aromatization. The aromatase inhibitor letrozole completely abolished the effect of DHEA on axo-somatic synapse number.

Role of estradiol in synaptic plasticity of tyrosine hydroxilase containing arcuate neurons

Estradiol induced synaptic remodeling is not confined to the presynaptic elements, it seems to be also specific at the level of postsynaptic cells, as well. Previous studies had reported that GABAergic nerve terminals make extensive synaptic contacts with cells containing tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis in the arcuate nucleus. This anatomical organization makes possible that GABAergic neurons directly influence the activity of these tubero-infundibular dopaminergic (TIDA) neurons and the release of dopamine from TIDA axon terminals. We selected these dopaminergic neurons and studied the changes in their synaptic connectivity.

The data from the 17β-estradiol treated animals clearly show that similarly to females the hormone is able to induce synaptic remodeling also in males, and in both sexes the GABAergic synapses are involved in the plastic changes. The hormonally-triggered synaptic plasticity, however, is sexually dimorphic in the sense that in females the TH immunoreactive neurons, while in males the non-labeled ones are affected. In ovariectomized females the number of GABAergic axo-somatic synapses

in TIDA neurons is significantly lower 24 h after estradiol treatment. This observation is in agreement with our earlier results obtained by using systemic application of the tracer Fluorogold and confirms that the retrograde labeling identifies TIDA neurons.

The physiological relevance of these observations can be understood by considering the role of TIDA neurons in the regulation of prolactin secretion. It is generally accepted that this process is paced by a light-entrained circadian rhythm, and modulated by a very complex and sensitive balance of stimulatory and inhibitory inputs. Experimental data show that exogenous estrogen stimulates the secretion of PRL in OVX rats; the effect is complex, the hormone may influence the PRL homeostasis at several anatomical sites, which include the hypothalamus and pituitary.

In our interpretation estrogen plays a complex role in the process: i.) as an initial effect it increases the prolactin secretion and ii.) it induces morphological synaptic remodeling in the arcuate nucleus, which, with a time delay, results in an increased activity of TIDA neurons and inhibits PRL secretion.

The observation that estradiol-induced remodeling of GABAergic synapses occurs not only in females but also in males suggest that this hormone has a central role in the structural synaptic plasticity of the arcuate nucleus.

Our present results give additional information to the effect of estradiol on the DA system of the arcuate nucleus. The data clearly show that the hormone induced synaptic remodeling, which was described earlier, is specific in the sense that it affects mainly the tuberoinfundibular dopaminergic system.

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