THE ROLE OF ESTROGEN AND DEHYDROEPIANDROSTERONE IN SYNAPTIC REMODELING

Ph.D. Thesis

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Abbreviations

ARC	-	arcuate nucleus
AVPv	-	anteroventral periventricular nucleus
CA1	-	cornu ammonis field 1
CNS	-	central nervous system
DA	-	dopamine
DAB	-	diaminobenzidine
DHEA	-	dehydroepiandrosterone
ER	-	estrogen receptor
ER-IR	-	estrogen receptor immunoreactive
GABA	-	γ-aminobutyric acid
GABA-IR	-	γ-aminobutyric acid immunoreactive
GFAP	-	glial fibrillary acidic protein
i.e.	-	id est
i.p.	-	intraperitoneal
IR	-	immunoreactive
ORCH	-	orchidectomized
OVX	-	ovariectomized
PSA-NCAM	-	polysialylated form of neural cell adhesion molecule
s.c.	-	subcutan
SNB	-	spinal nucleus of bulbocavernosus
PRL	-	prolactin
TIDA	-	tubero-infundibular dopaminergic
TH	-	tyrosine hydroxilase
VMN	-	ventromedial nucleus

Contents

1. Introduction

1.1. 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 and until as late as the middle of the last century (Hebb, 1949), 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 has indicated that morphological neuroplasticity is taking place in the brain, which contributes to the functional adaptation to the changing conditions of the external and internal environment. Although the magnitude and distribution of these neuroplastic alterations are more prominent in developing animals, the adult brain also retains a remarkable capacity for structural and functional modifications. 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.

It is now well accepted that gonadal steroids exert both organizational and activational effects on steroid responsive tissues in the central nervous system (Arnold and Breedlove, 1985). 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 (CNS) become sexually differentiated (MacLusky and Naftolin, 1981; Arnold and Gorski, 1984; Arai et al., 1986; Matsumoto and Arai, 1980, 1981a, 1983, 1986). The structural sexual dimorphisms exist at many morphological levels including neuron numbers (Breedlove and Arnold, 1983; Chowen et al., 1992, 1993), dendritic length (Greenough et al., 1977), neuronal membrane organization (Garcia-Segura et al., 1985), synaptic formation (Matsumoto and Arai, 1980, 1981a), neuronal connectivity (Sakuma and Pfaff, 1981) and neuropeptide receptor numbers (Hammer, 1984).

The first detailed description of sexual dimorphism in the vertebrate brain has been reported by Nottebohm and Arnold (1976), who demonstrated that the three vocal control areas of songbirds are significantly larger in males than in females. In

the preoptic region, similar structural differences were found in other species, including the medial preoptic nucleus of quail (Panzica et al., 2001) and rat (Gorski et al., 1978), the sexually dimorphic nucleus of the preoptic area in rat (Gorski et al., 1980) and human (Hofman and Swaab, 1989), as well as the anterior hypothalamus–preoptic area in lizard (Crews et al., 1990).

The influence of gonadal hormones has also been shown on the spinal nucleus of the bulbocavernosus (SNB) in rats, where the number of neurons is higher in males than in females. In this case, the involvement of gonadal steroid hormones is supported by the finding that prenatal treatment with testosterone propionate (TP) was able to masculinize the number of SNB neurons in female rats (Breedlove and Arnold, 1983).

Regarding organizational/developmental effects on synaptic patterns in the ventromedial nucleus (VMN), sexual dimorphism has been found in the synaptic organization of the ventrolateral subdivision. The numerical densities of dendritic spine and shaft synapses in adult male rats were higher than in females. A dimorphic pattern in the numerical density of spine synapses occurs as early as postnatal day 5 and is present throughout postnatal life. Treatment of neonatal female rats with testosterone increased the numerical density of axo-dendritic synapses, inducing a pattern similar to adult males. On the other hand, administration of tamoxifen, a selective estrogen receptor modulator, to newborn male rats significantly reduced the numerical density of spine synapses to levels comparable to those of normal female rats (Pozzo and Aoki, 1991).

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. Gonadal steroid hormones earlier were thought to alter behaviour by changing the activity of existing neuronal circuits. This is the reason why early studies focused on hormone-induced neurochemical changes such as synthesis of neurotransmitters and neuropeptides, or alterations in receptor numbers (Cardinali and Vacas, 1978; Romano et al., 1989). However, accumulating experimental data indicate that responses to gonadal steroids in the adult brain include well-defined morphological changes, as well. They modulate nuclear volume (Commins and Yahr, 1984), dendritic morphology (DeVoogd and Nottebohm, 1981), innervational patterns (DeVries et al., 1984) and number of synaptic input (Garcia-

Segura et al., 1986; Olmos et al., 1989; Matsumoto, 1991; Naftolin et al., 1993; Parducz et al., 1993; Perez et al., 1993). For example, by using confocal microscopy, Calzio and Flanagan-Cato (2000) have shown that in ovariectomized (OVX) rats estrogen treatment increased dendritic spine density by 48 % in the ventrolateral VMN but had no effect on spine density in the dorsal part of nucleus. In the anteroventral periventricular nucleus (AVPv) of cycling females ultrastructural analysis revealed that between proestrus and estrus there was a 39 % increase in axo-somatic synapses upon AVPv neurons. In the subsequent 24 h to metestrus, the number of axo-somatic synapses decreased by 22 %, while ovariectomy resulted in more axo-somatic synapses in the nucleus. Furthermore, estrogen receptor immunoreactive (ER-IR) neurons received significantly more synaptic input at proestrus and estrus than non-ER-IR neurons (Langub et al., 1994).

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

Due to years of research, a general view has emerged indicating that gonadal steroids affecting nearly all structural parts of the synaptic formation, influence synaptic remodeling in specific steroid sensitive areas of the central nervous system of the adult mammal under both physiological and experimental conditions. In the brain synaptic changes were expected to occur mainly in areas involved in the neuronal control of reproductive or neuroendocrine events. Early research efforts concentrated on the hypothalamus, one of the main autonomic centers in the brain (Matsumoto and Arai, 1980, 1986; Güldner, 1982; Clough and Rodriguez-Sierra, 1983; Garcia-Segura et al., 1986). However, it should be noted that in addition to those areas involved in reproduction or other endocrine function, synaptic connectivity also appears to be under hormonal influence in cognitive areas of the brain, such as the cerebral cortex (Medosch and Diamond, 1982; Juraska, 1991) and the hippocampal formation (Meyer et al., 1978; Wooley at al., 1990; Gould et al., 1990; Juraska, 1991; Parducz and Garcia-Segura, 1993).

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, it exhibits different astrocyte morphology (Mong et al., 2002) and the number of axo-somatic synapses is higher in females than in males by 20 days of age. This gender difference is maintained throughout adult life. The anatomical organization, its afferent and efferent connections clearly indicate that this structure plays an important neuroendocrine role (Figure 1.). Earlier studies showed that it is involved in the mediation of luteinizing hormone, prolactin and growth-hormone secretion (Chronwall, 1985), and has decisive interactions in the regulation of the estrus cycle and sexual behavior. The nucleus contains over fifteen identified neurotransmitters such as acetylcholine (ACh), dopamine (DA), γ-aminobutyric acid (GABA), growth hormone-releasing hormone (GHRH) and somatostatin. The large number of these different transmitters provides several possibilities for sensitive regulation within the nucleus. One of the possible ways of such regulation is the hormonally induced synaptic remodeling, which occurs in different brain areas and has intensively been studied in the arcuate.

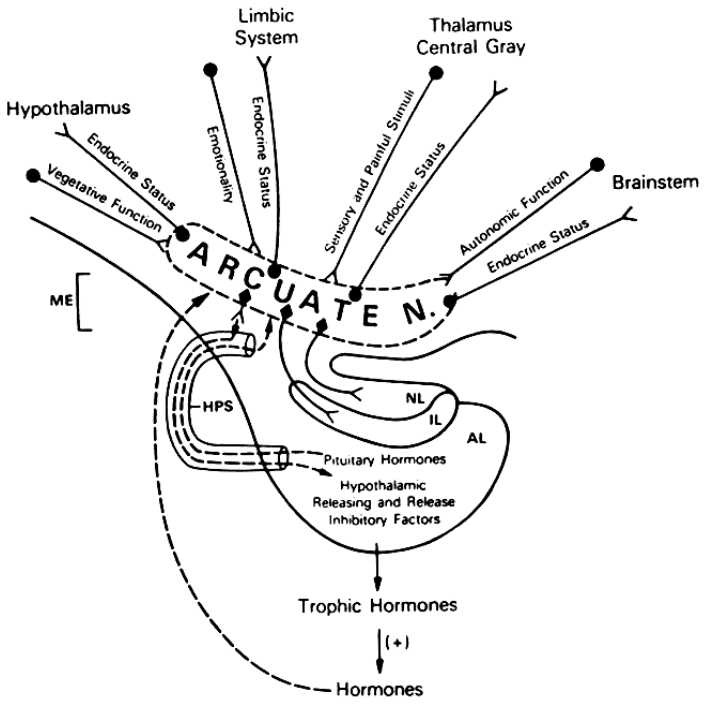


Figure 1. An illustration about the general organization of the arcuate nucleus. The nucleus plays a key role in integrating emotionality, vegetative, homeostatic, autonomic, endocrine functions and sensory information (Chronwall, 1985). ME, median eminence; NL, neural lobe; IL, intermediate lobe; AL, anterior lobe; HPS, hypothalamic portal system.

Arai and Matsumoto (1978) have provided evidence on the effect of neonatal estrogen treatment on synapse formation during postnatal development in female rats. In isolated ARC three weeks of estradiol benzoate treatment restored the deafferentation-induced loss of axo-dendritic synapses (Matsumoto and Arai, 1979). These pioneering experiments have clearly indicated that the neuronal plasticity in the ARC is significantly enhanced by estrogen (Matsumoto and Arai, 1981b). 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 (Olmos et al., 1989).

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, resulting in plasma levels of the hormone similar to those detected during proestrus, induces a reversible decline in the number of arcuate axo-somatic synapses (Perez et al., 1993). Furthermore, studying the estradiol-induced synaptic remodeling revealed that the effect is specific, because not all types of synapses are affected (Parducz et al., 1993). GABAergic axons are abundant in the ARC and form axo-somatic synapses on estrogen-sensitive cells (Leranth et al., 1985). Both GABA levels and GABA receptors are modulated by gonadal steroids (Apud et al., 1985; Nicoletti et al., 1985) and a large population of GABA neurons within the nucleus expresses estradiol and/or progesterone receptors (Leranth et al., 1985; Flugge et al., 1986). Quantitative postembedding immunocytochemical analysis at the electron microscopic level 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 immunonegative axo-somatic synapses on these neurons. In contrast, estradiol administration did not induce changes in the number of axo-dendritic arcuate synapses, but there was a significant increase in the volume density of excitatory spine synapses (Parducz et al., 2002). The effect of 17β-estradiol is not confined to the presynaptic elements, it seems to be also specific at the

level of postsynaptic cells, as well. By using systematic application of the retrograde tracer Fluorogold it has been established that the “hypophysiotropic neurons” that project to the median eminence receive less axo-somatic inputs than non-labeled neurons. A 17β-estradiol-induced synaptic remodeling takes place in this subpopulation of ARC neurons as evidenced by a decrease in the numerical density of axo-somatic synapses (Parducz et al., 2003).

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 significantly contribute to synapse formation. Several authors have demonstrated that there is a negative correlation between the level of glial ensheathing and the number of synaptic contacts on hypothalamic neurons in a variety of species (Montagnese et al., 1988; Theodosis and Poulain, 1992; Perera and Plant 1997; Hatton, 2002). It suggests that glial cell activity might represent a mechanism that underlies some aspects of synaptic plasticity. Estradiol-induced synaptic changes on ARC neurons of adult rats are accompanied by prominent morphological modification of arcuate glial cells. The surface density of glial fibrillary acidic protein (GFAP), a specific astrocytic marker, 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 (Garcia-Segura et al., 1994b). This phenomenon is also observable in ovariectomized females one day after injection of a single-dose of 17β-estradiol, when glial processes cover the majority of the surface of arcuate neuronal somata (Garcia Segura et al., 1994). Alteration of these cell membrane interactions is partly mediated by the highly polysialylated isoform of neural cell adhesion molecule (PSA-NCAM). Arcuate neurons express high levels of PSA-NCAM not only during development, but also in adult life. The presence of large quantities of the PSA moiety on NCAM is a necessary prerequisite for estrogen-induced synaptic remodeling in the adult female arcuate nucleus, because the 17β-estradiol-induced reduction in the number of GABAergic axo-somatic synapses did not occur after infusion of anti-PSA antibodies or *in vivo* enzymatic removal of PSA from NCAM (Hoyk et al., 2001).

It is also well documented that the structure of the arcuate neuronal membrane exhibits reversible changes in adult female rats, which closely follows the fluctuation of plasma estradiol levels during the estrus cycle (Garcia-Segura et al., 1988). The density of the intramembrane particles in ovariectomized animals was decreased following 17β-estradiol injection and this was associated with an increased number of exo/endocytotic images (coated pits). Since these organelles are thought to play a role in trafficking molecules to and from the membrane, it may be concluded that estradiol increases the turnover of membrane components (Parducz et al., 1996).

Although it has become generally accepted that the gonadal hormone-induced synaptic remodeling plays an important role in various activities of the CNS, we have only very limited data on the time-course of structural synaptic changes. Earlier studies have focused on the phenomenon itself and the effects have been reported following long-term hormone treatments. The fact that synaptic changes seem to be linked to the hormonal variations during the estrus cycle has indicated that the morphological alterations may occur in hours rather than days, but no direct experimental evidence has been published so far supporting this hypothesis. Electrophysiological recordings, however, have clearly shown that such synaptic remodeling may be induced in less than an hour, as far as synaptic alterations are associated with changes in cellular activity. Kis et al. (1999) have recorded a significant increase in the spontaneous activity of ARC neurons, 25 min after the i.p. administration of 17β-estradiol. The authors used an *in situ* preparation, i.e. all of the synaptic connections to the cells remained intact, allowing the investigators to follow the development of the hormonal effect on the same units. The observed onset of action at around 25 min following the estradiol injection makes the involvement of direct membrane effects unlikely. Instead, the authors proposed that the enhanced firing might be the result of the diminishing inhibitory synaptic input to arcuate neurons (Kis et al., 1999).

1.3. 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. The concept of neurosteroids has emerged from an observation made by Baulieu and his coworkers (Baulieu, 1981). 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 (Figure 2.).

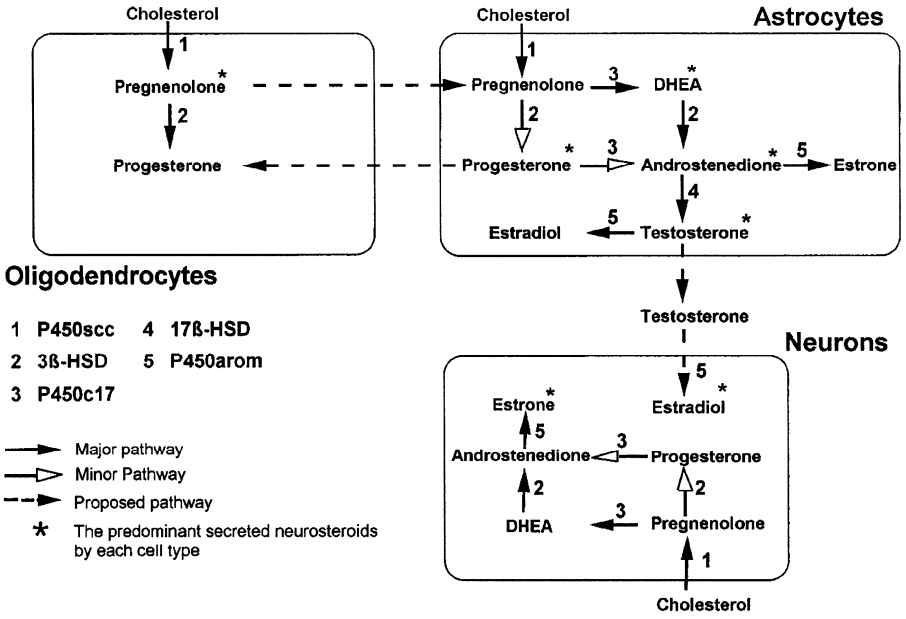


Figure 2. A schematic view of the neurosteroidogenic pathway in oligodendrocytes, astrocytes and neurons in the rat brain. Closed arrows, major steroidogenic pathway; open arrows, minor steroidogenic pathway; dotted arrows, proposed steroidogenic pathway. *, the predominantly produced neurosteroids by each cell type (Zwain and Yen, 1999).

Among neurosteroids dehydroepiandrosterone and pregnenolone were the firsts found in the rat brain (Corpechot et al; 1983). DHEA has diverse effects in the brain: interacts with γ-aminobutyric acid type A ($GABA_A$), N-methyl-D-aspartate (NMDA) and sigma 1 receptor (Majewska, 1992; Bergeron et al., 1996; Maurice et al., 2001) and has neuroprotective properties (Bologa et al., 1987; Kimonides et al., 1998; Bastianetto et al., 1999; Cardounel et al., 1999; Li et al., 2001; Tomas-Camardiel et al., 2002). Furthermore, DHEA down-regulates glucocorticoid receptors, regulates protein kinase C (PKC) signaling and microglia reactivity (Cardounel et al., 1999; Racchi et al., 2001; Wang et al., 2001). In some cases, the effects of DHEA in the nervous system correlate with the physiological role of this steroid as a precursor for bioactive androgens and estrogens (Labrie et al., 2003), i.e. it can be converted to testosterone and consecutively by aromatization to estradiol (Azcoitia et al., 2001;

Veiga et al., 2003). Conversion of testosterone to estradiol in the rat brain is catalyzed by cytochrome P450 aromatase. It is expressed exclusively by neurons under normal conditions in the central nervous system (Naftolin, 1994; Lephart, 1996; Balthazart and Ball, 1998). Aromatase containing neurons are mainly localized in the lateral septal region, the bed nucleus of the stria terminalis, the amygdala, several hypothalamic nuclei, the medial preoptic area, the nucleus accumbens and several regions of the cortex, especially in the piriform lobe (Jakab et al., 1993, 1994; Sanghera et al., 1991).

Early researches have focused on the hippocampal formation, a region of the brain vital for processing of mnemonic information, 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. During the female reproductive cycle, physiological levels of gonadal steroids greatly influence the density of pyramidal cell dendritic spines and spine synapses in the CA1 subfield of the hippocampus. The apical dentritic spine density on CA1 pyramidal neurons decreases between the late afternoon of proestrus and late afternoon of estrus phases of the cycle. Spine density then appears to cycle back to proestrus high values over a period of several days (Wooley et al., 1990; Wooley and McEwen, 1992). In addition administration of estradiol in OVX rats increased CA1 spine density (Gould et al., 1990; Leranth et al., 2000), but in castrate males the same treatment was without effect (Leranth et al., 2003). Recent experimental data have indicated that not only 17β-estradiol, but also the neuroactive dehydroepiandrosterone is able to induce synaptic changes in the CA1 subfield of the hippocampus. DHEA 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 (Hajszan et al., 2004) and almost completely blocks the effect of testosterone (Leranth et al., 2004) 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 (MacLusky et al., 2004). These data suggest that in females the DHEA-induced increase in CA1 spine synapse density is mediated via intracerebral estrogen biosynthesis.

These observations, combined with the well documented decline in circulating DHEA level that occurs during aging, suggest that this steroid may contribute to age-

related neurodegenerative processes. Because DHEA itself has only weak hormonal activity, it appears potentially capable of providing replacement therapy targeted to the organs that contain the enzyme systems necessary for conversion of DHEA to biologically more active steroids, without the side effects associated with systemic androgen or estrogen replacement (Yen et al., 1995; Labrie at al., 2003).

1.4. 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 study 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.

2. Materials and methods

2.1. Animals and surgical procedures

All animals were raised and maintained on a 12 h 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 under Nembutal anesthesia. One month later, rats (n=5) received DHEA (Fluka), given in the form of a single subcutan injection (4 mg/kg in 20 % β-cyclodextrin). 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 (1 mg/kg in 200 µl 2.5 % carboxymethylcellulose). The letrozole injection preceded the DHEA by 1 hour to allow time for complete aromatase blockade before administration of the androgen.

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 under Nembutal anesthesia and one month later, they were s.c. injected either with a single dose (100 µg/100 g body wt.) 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.

2.2. Preembedding immunostainig

The animals were perfused through the left cardiac ventricle, first with 50 ml of 0.9 % NaCl then with 250 ml of 1 % glutaraldehyde and 1 % paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, the brains were removed and placed at 4 ^{0}C in glutaraldehyde-free fixative for an additional 3 hours. After washing in TBS, 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 (Sternberger, 1979). Sections were first immersed in 20 % normal goat serum (NGS) for 30 min to diminish non-specific staining and then incubated in rabbit polyclonal anti-TH antibody (Chemicon), at 1:1000 dilution in TBS buffer for overnight in room temperature. Following three 10 min washes in TBS buffer, sections were incubated for 2 hr in goat-anti-rabbit IgG (GAR) at 1:1000 dilution, then washed 3 times again, and incubated for 2 hr in peroxidase-antiperoxidase complex (PAP), diluted 1:1000 at room temperature. Peroxidase was reacted with diaminobenzidine and hydrogen peroxide.

2.3. Postembedding immunostaining

For electron microscopy, sections were osmicated (1 % OsO_4 in PB) for 30 min, dehydrated in ethanol and embedded in araldite either between liquid release-coated (Electron Microscopic Sciences, Fort Washingtin PA) slides and coverslips or capsules. 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), described in detail in Parducz et al. (1993).

2.4. 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 (Uylings et al., 1986).

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.

2.5. Statistical analysis

The data from the same animals were pooled since no variations were detected through the three blocks in any groups of rats. The *N* for statistical analysis was the number of animals. Volume measurements were analyzed by analysis of variance (ANOVA). For synapse counts, because the *F* test demonstrated a significant nonhomogeneous variance between groups, 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.

3. Results

3.1. 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 (0.69 ± 0.04; 0.70 ± 0.01; 0.69 ± 0.01; 0.70 ± 0.02 and 0.68 ± 0.02 mm^3 in the morning of proestrus, the afternoon of proestrus, the morning of estrus, the morning of metestrus and the morning of diestrus, respectively. $P = 0.97$).

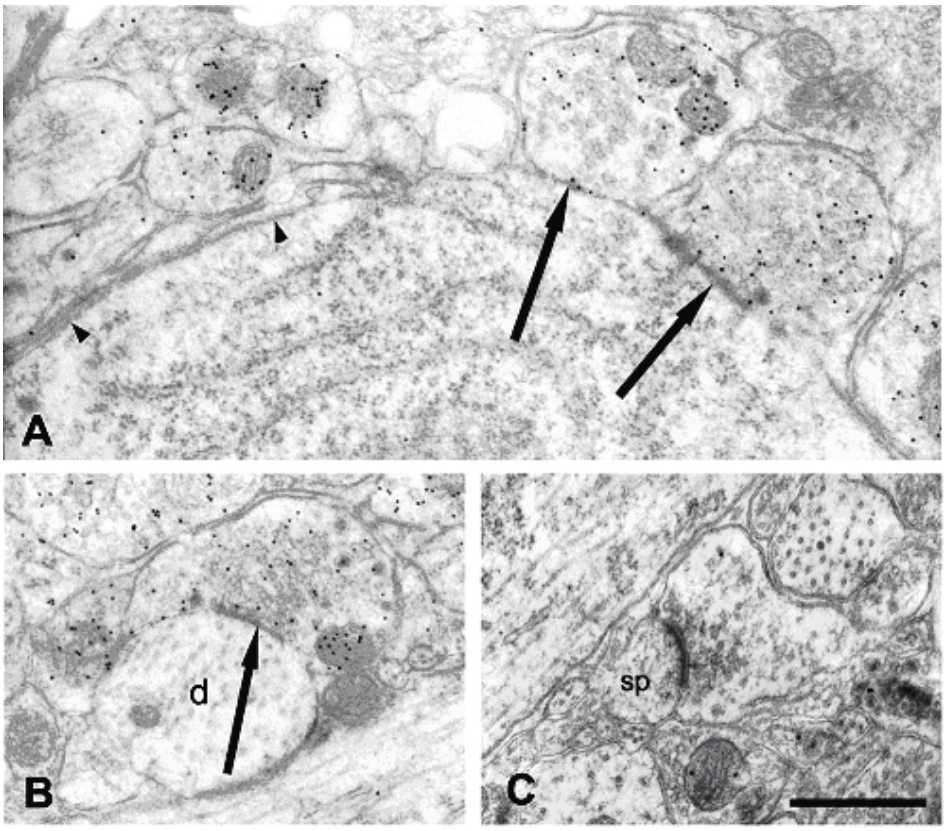
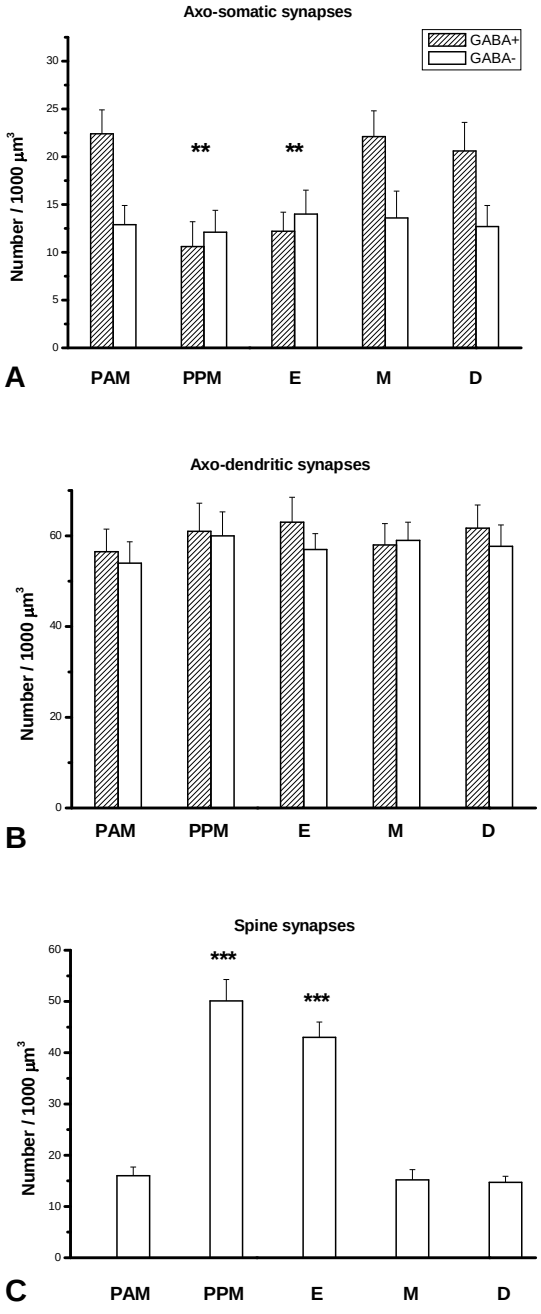


Figure 3. Axo-somatic (A), axo-dendritic (B) and dendritic spine (C) synapses from the arcuate nucleus of female rats killed on metestrus day. Bar: 1 μm

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 (Figure 3.).



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 (Figure 4.A). We have observed that the number of GABAergic and non-GABAergic synapses on dendritic shafts did not alter during the estrus cycle (Figure 4.B). 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 phases (Figure 4.C). Apart from the changes observed in synapse density we did not find any fine structural alterations, signs of possible degeneration of terminals in either phases of the estrus cycle.

Figure 4**.** Cyclic changes in the numerical density of axo-somatic (A), axo-dendritic (B) and dendritic spine synapses (C) in the arcuate nucleus. Each experimental group was composed of five rats and 60 disectors per animals were counted. PAM, proestrus morning; PPM, proestrus afternoon; E, estrus; M, metestrus; D, diestrus. The data are expressed as mean ± S.D. ** $P < 0.01$, *** $P < 0.001$ vs. PAM values.

3.2. 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 (Figure 5.).

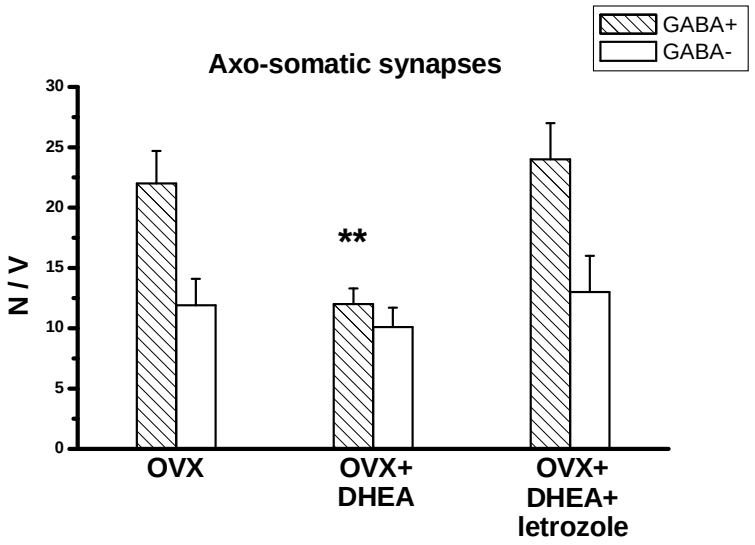


Figure 5. DHEA-induced changes in the numerical density of GABAergic and non-GABAergic axo-somatic synapses of arcuate nucleus. Data are presented as mean ± S.D. ** Significantly different from the density of GABAergic cells in all other treatment groups (Scheffe test, $P < 0.01$).

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

In agreement with literature data (Cheung et al., 1997) 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 (Ershov et al., 2005). For that reason we performed the morphometric measurements in the dorsomedial part of the nucleus (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 (Figure 6.).

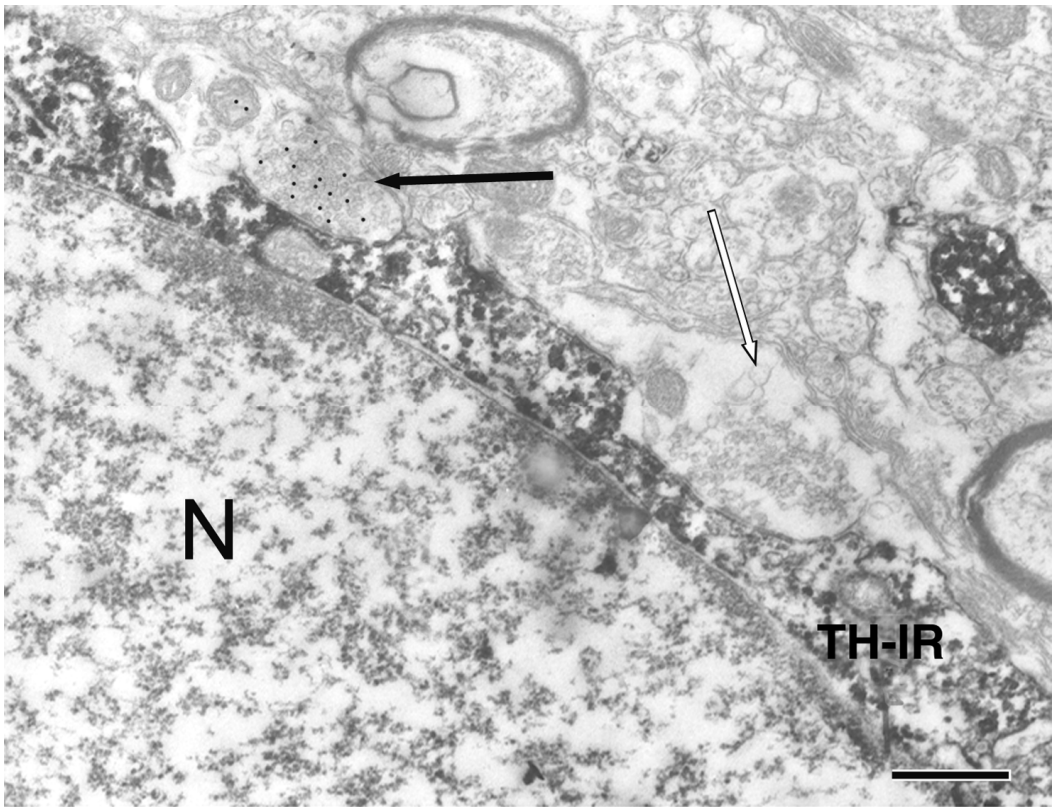


Figure 6. GABAergic (black arrow) and non-GABAergic (white arrow) nerve terminals synapsing on TH-IR neuron in the arcuate nucleus of orchidectomized male rat. N, nucleus; TH-IR, tyrosine hyroxylase immunorective neuron; bar: 0.5 μm

Morphometric analysis shows (Table 1.) 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.

Table 1. Number of synapses in the arcuate nucleus of female and male rats

	Female	Male
TH-IR neurons	31.9 ± 3.3	22.4 ± 2.1**
Non-labeled neurons	24.2 ± 2.9	12.8 ± 1.9**

The number of axo-somatic synapses in female and male animals is statistically different. ** $P < 0.01$

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 (Figure 7.A OVX and Figure 8.A ORCH), while the non-dopaminergic arcuate neurons received about equal numbers of these two types of synapses (Figure 7.B OVX and Figure 8.B ORCH).

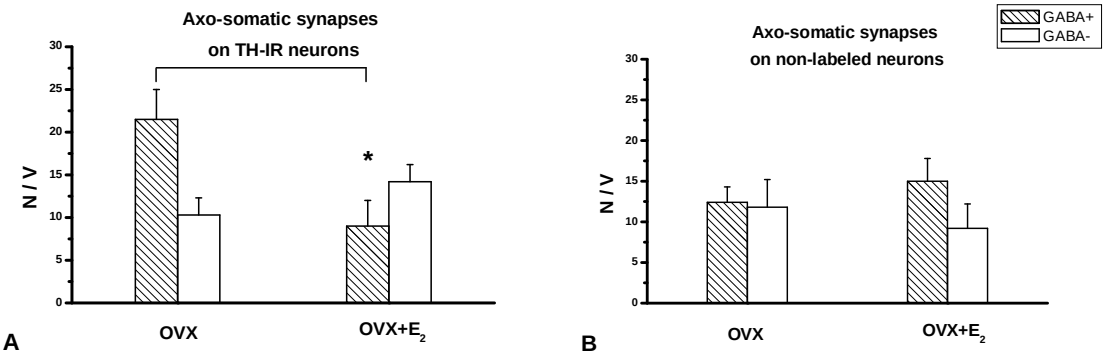


Figure 7. The effect of 17β-estradiol on the number of GABAergic and non-GABAergic axo-somatic synapses on TH-IR (A) and non-labeled (B) arcuate neurons of ovariectomized rats. The number of synapses was counted by the Sterio method and the data are expressed as number of synaptic contacts per unit volume of perikaryon (1000 μm^3). There is a significant decrease of GABAergic terminals synapsing on TH-IR neurons. * $P < 0.05$.

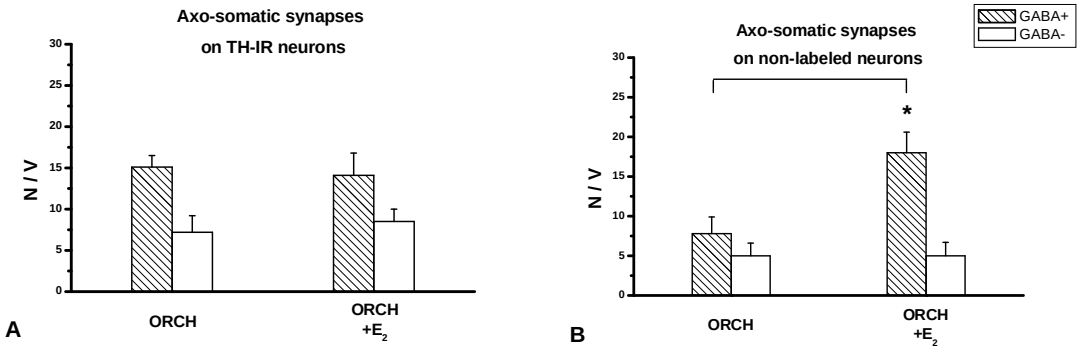


Figure 8. Numerical density of axo-somatic synapses in the male arcuate nucleus on tyrosine hydroxylase (TH+) immunopositive (A) and non-labeled (TH-) neurons (B). 17β-estradiol treatment results in an increase of GABAergic axo-somatic synapses on non-labeled neurons. * $P < 0.05$.

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 (Figure 5.). 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 (Figure 8.).

4. Discussion

4.1 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 (Chronwall, 1985). 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 (Parducz et al., 1993). 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. Using unbiased morphometric analyses we have also confirmed previous observations on fluctuation in the number of axo-somatic synapses on arcuate neurons during the estrus cycle (Olmos et al., 1989). In agreement with these studies, we have detected that the number of 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. Using postembedding immunocytochemisty, we have found that only GABAergic axo-somatic synapses fluctuated during the estrus cycle, the number of non-GABAergic axo-somatic synapses did not change significantly. The number of GABAergic and non-GABAergic synaptic inputs on dendritic shafts did not show significant changes. The fluctuation in the number of synapses during the estrus cycle follows the oscillation of estradiol levels in plazma (Naftolin et al., 1972). In addition estradiol treatment also promotes the reduction in the number of GABAergic axo-somatic synapses (Garcia-Segura et al., 1994a; Parducz et al., 2002) and does not affect synapses on dendritic shafts in the arcuate nucleus (Parducz et al., 2002).

To exclude the possibility that alterations of the hormone level result in volumetric changes, morphometric measurements were performed and in agreement with previous data (Garcia-Segura et al., 1994b; Leal et al., 1998) we observed no significant variations in the volume of the arcuate nucleus between the different

phases of the estrus cycle. The differences detected in the Nv parameter were not due to differences in the total volume of the nucleus.

As in previous studies (Olmos et al., 1989), 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 (Garcia-Segura et al., 1994a). Indeed, astroglial coverage of neuronal somata is increased in the afternoon of proestrus and the morning of estrus and glial processes are interposed between the pre- and postsynaptic membranes (Garcia-Segura et al., 1994a,b). There is strong evidence that cell membrane interactions are mediated by the embryonic highly polysialylated form of neural cell adhesion molecule (Hoyk et al., 2001). 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 this regard, it is interesting to note that GABA may affect astroglia morphology in the arcuate nucleus (Mong et al., 2002). In addition, there is a significant population of GABAergic synapses that remains connected to arcuate somata in the afternoon of proestrus and in the morning of estrus. Therefore, an unspecific synaptic displacement by glial processes, even if it was restricted to perikarya, cannot completely explain the selectivity in the synaptic changes. It is therefore possible that different synaptic contacts present a different degree of stability and resistance to glial displacement. Alternatively, changes in glial processes may follow the displacement of GABAergic synapses and may not play an active role in the synaptic changes. In both cases the GABAergic synaptic inputs that fluctuate during the estrus cycle may represent a specific subpopulation of GABAergic synapses of low stability.

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. Spine synapses remained increased in the morning of estrus phase and decreased in metestrus and diestrus. Similar fluctuations during the estrus cycle have been detected in other cell types, such as in CA1 pyramidal neurons (Woolley et al., 1990). In both arcuate neurons and hippocampal CA1 cells, estradiol treatment also promotes an

increase in the number of spine synapses (Wooly and McEwen 1992; Parducz et al., 2002).

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. The sum of opposite changes in the number of axo-somatic GABAergic inhibitory synapses and in the number of excitatory synapses on dendritic spines might result in modifications in neuronal excitability during the estrus cycle. Electrophysiological recordings have shown that these fluctuations are associated to an increased neuronal firing frequency in a subpopulation of arcuate neurons (Kis et al., 1999; Parducz et al., 2002).

Our present findings suggest that physiological changes of estradiol level during the estrus cycle may affect axo-somatic GABAergic synapses and the non-GABAergic (excitatory) synapses on dendritic spine in the arcuate nucleus. This does not exclude that other factors may also be involved in the regulation of synapses during the estrus cycle. Previous semiquantitative studies showed that progesterone alone did not affect the number of axo-somatic synaptic profiles, but when administered with estradiol, blocked the effects of estradiol on synapses (Perez et al., 1993). However, it cannot be exluded that rising levels of progesterone may be involved in the recovery in synapse numbers in metestrus phase.

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

Accumulating experimental data indicate that neurosteroids are also involved in neuroplastic changes (Hajszan et al., 2004; Leranth et al., 2004; MacLusky et al., 2004). 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 (Punjabi et al., 1983), 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 (which include testosterone and dihydrotestosterone); 2) through aromatization of DHEA to estrogen; or 3)

through conversion of DHEA to estrogenic C-19 metabolites via pathways independent of aromatization. DHEA is extensively converted to 5-androstene-3β,17β-diol (androstenediol) by 17β-hydroxysteroid dehydrogenase. Androstenediol has estrogen agonist activity (Ho and Levin, 1986; Littlefield et al., 1990). Similarly, dihydrotestosterone is converted to 5α-androstan-3α,17β-diol (3αA-diol) as well as its 3β-isomer (3βA-diol). Both 3αA-diol and 3βA-diol have weak estrogenic activity (Ho and Levin, 1986), whereas 3αA-diol has also been implicated in androgen regulation of GABA-benzodiazepine receptor function (Frye, 2001).

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. GABAergic and non-GABAergic synapse density observed in the arcuate nucleus of letrozole and DHEA-treated animals was statistically indistinguishable from those in OVX rats.

Previous work has demonstrated expression of aromatase in the arcuate neurons (Roselli et al., 1985; Sanghera et al., 1991). However, aromatase activity is considerably higher in other areas of the brain, including suprachiasmatic nucleus, amygdaloid complex and preoptic area (Roselli et al., 1985). This raises the possibility that DHEA-induced enhancement of GABAergic axo-somatic synapse density in the arcuate nucleus could be mediated indirectly via action on aromatase-rich regions.

4.3. 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 (van den Pol, 1986; Decavel and van den Pol, 1992). 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. Dopamine is transported via the portal system of the median eminence to the anterior pituitary

gland where it binds to receptors on the lactotroph cells and following a series of postreceptor events, results in inhibition of prolactin (PRL) secretion. We selected these dopaminergic neurons and studied the changes in their synaptic connectivity. With the combination of preembedding TH immunostaining and the postembedding immunogold method we could identify two different neuron populations within the nucleus and quantify GABAergic and non-GABAergic axo-somatic synapses in ovariectomized and ovariectomized + 17β-estradiol-treated female; in orchidectomized and orchidectomized + 17β-estradiol-treated male adult rats.

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 17β-estradiol induces synaptic remodeling in TIDA neurons: the number of GABAergic axo-somatic synapses is significantly lower 24 h after the hormone treatment. The non-labeled arcuate neurons are not affected by 17β-estradiol, since their synaptic connectivity remains unchanged. This observation is in agreement with our earlier results obtained by using systemic application of the tracer Fluorogold to label the subpopulation of arcuate neurons that project to the median eminence (Parducz et al., 2003). We found that the synaptic connectivity of these “hypophysiotropic neurons” is different from the other, non-labeled population, and they respond to 17β-estradiol treatment by synaptic remodeling. The present data confirm that the retrograde labeling by Fluorogold 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 (Freeman et al., 2000). Experimental data show that exogenous estrogen stimulates the secretion of PRL in OVX rats (Chen and Meites, 1970; Caligaris et al., 1974). The effect is complex, the hormone may influence the PRL homeostasis at several anatomical sites, that include the hypothalamus and pituitary (Freeman et al., 2000).

The specificity of the estrogen effect on the dopaminergic neurons shown in the present paper supports the notion that the hormonally induced synaptic remodeling has functional consequences. The decrease in the number of GABAergic inhibitory inputs on TH-IR neurons in females observed 24 hours after estradiol treatment may result in an increase in their activity, i.e. elevated DA release. This hypothesis is supported by literature data, which clearly show that the basal TIDA neuronal activity is decreased by ovariectomy, and is restored by estrogen (DeMaria et al., 1998; Freeman et al., 2000). Estradiol-induced synaptic changes can be paralleled by the physiological observations too, they correspond well to termination phase of the prolactin surge (DeMaria et al., 2000).

In our interpretation estrogen plays a complex role in the process: 1) as an initial effect it increases the prolactin secretion (Chen and Meites, 1970; Caligaris et al., 1974) and 2) 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. There is certain specificity in the hormone action, because in females the TIDA neurons, while in males the non-labeled neuron population is affected by the change. In orchidectomized males we found a significant increase of GABAergic axo-somatic synapses in the non-labeled neurons. It is known that the arcuate dopaminergic system in male animals is target of estrogen: in orchidectomized animals the hormone increases the basal activity of TIDA neurons (Shieh and Pan, 1996), but more detailed studies are needed to get the mechanism of this action right.

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 TH immunoreactive neurons, i.e. the tuberoinfundibular dopaminergic system.

5. Summary

It is known that gonadal steroids induce synaptic plasticity in several areas of the developing and adult nervous system. Most of the data concerning synaptic remodeling have been obtained from the hypothalamic arcuate nucleus. The nucleus integrates different hormonal and neural signals to control neuroendocrine events, feeding, energy balance and reproduction. Previous studies have shown that in adult female rats the arcuate nucleus undergoes a 17β-estradiol-triggered phasic synaptic remodeling as a function of changing hormone levels, resulting in a decrease in the number of inhibitory synaptic inputs, an increase in the number of excitatory synapses and an enhancement of the frequency of neuronal firing (Perez et al., 1993; Parducz et al., 2002; Kis et al., 1999).

Our findings indicate that there is a significant decrease in the number of GABAergic axo-somatic synapses on proestrus afternoon and estrus day compared to other phases of the estrus cycle. This decrease in GABAergic synapses is accompanied by an increase in the number of dendritic spine synapses. The synaptic density appears to cycle back to proestrus morning values on metestrus day. In contrast, the number of synapses on dendritic shafts does not change during the cycle. These results indicate that a rapid and selective synaptic turnover of arcuate synapses occurs in physiological circumstances.

Recent experimental data indicate that neurosteroids are also important in synaptic remodeling (Hajszan et al., 2004; Leranth et al., 2004; MacLusky et al., 2004). We have observed that in females 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-GABAergic synapses remain unaffected. Because DHEA and its metabolites have multiple effects in the CNS, more detailed studies are needed to determine the exact mechanism of their actions.

The specificity of the hormonal effect is not confined to the presynaptic element, it is observable at the level of postsynaptic cells, as well (Parducz et al., 2003). We selected the tyrosine hydroxylase immunoreactive neurons in the arcuate and studied the changes in their synaptic connectivity. Our study shows that the effect of 17β-estradiol is sex and cell specific in the sense that not all arcuate neurons are affected by the structural synaptic remodeling. In ovariectomized females hormonal

treatment results in a significant decrease in the numerical density of GABAergic axo-somatic contacts synapsing on TH-IR neurons, while in orchidectomized and 17β-estradiol-treated males the non-labeled neurons receive higher number of inhibitory terminals. Our data indicate that the hormonally induced plastic changes in synaptic connectivity of TH-IR neurons may serve as the morphological basis for the cyclic regulation of the anterior pituitary.

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8. Appendix