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NEUROPLASTIC CHANGES IN THE CENTRAL NERVOUS SYSTEM: THE ROLE OF GONADAL HORMONES

PhD Thesis

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INTRODUCTION

During recent decades, it has become a generally accepted view that structural neuronal plasticity is remarkably involved in the functional adaptation of the central nervous system. Thus, cellular morphology in the brain is in continuous transition throughout the life span, as a response to environmental stimuli. The effects of the environment on neuroplasticity are mediated by, to some extent, the changing levels of circulating gonadal steroid hormones. Today, it is clear that the function of gonadal steroids in the brain extends beyond simply regulating reproductive and/or neuroendocrine events. In addition, or even more importantly, gonadal steroids participate in the shaping of the developing brain, while their actions during adult life are implicated in higher brain functions such as cognition, mood and memory. A large body of evidence indicates that gonadal steroid-induced functional changes are accompanied by alterations in neuron and synapse numbers, as well as in dendritic and synaptic morphology. These structural modifications are believed to serve as a morphological basis for changes in behavior and cellular activity. Due to their growing functional and clinical significance, the specificity, timeframe, as well as the molecular and cellular mechanisms of hormone-induced neuroplasticity have become the focus of many studies.

Accumulating evidence indicates that gonadal steroids exert both *organizational* and *activational* effects on different parts of the central nervous system. The organizational effects are permanent and are acting during development, mainly in the fetal-neonatal period when estrogens and aromatizable androgens modulate neuronal development and the formation of neuronal circuits. As a result, several areas of the central nervous system become sexually differentiated. The activational effects are transitory and fluctuate considerably as the hormonal milieu changes. A characteristic feature of activational effects is that they occur not only during development, but also in adult life, more or less affecting almost every aspect of brain physiology. Despite years of research, we have just begun to gain insight into the mechanisms underlying gonadal steroid-induced neuroplasticity.

Hormonally-induced synaptic changes in hypothalamic nuclei

Considering the fact that gonadal hormones have been found to induce plastic changes, it was logical to expect that these changes occur mainly in brain areas involved in the neuronal control of reproductive or neuroendocrine events. This is the reason why the early research, looking for signs of structural plasticity, has concentrated on the hypothalamus. Most of the data concerning synaptic remodeling in developing and adult neuroendocrine brain areas have been obtained from the arcuate nucleus.

The first experiments have clearly indicated that the neuronal plasticity in this nucleus is significantly enhanced by estrogen. The synapses exhibited a phasic

remodeling, which could be linked to the fluctuation in hormone levels during the estrous cycle. Further studies have revealed that the effect of hormone is specific, because not all types of synapses are affected. Quantitative postembedding immunocytochemical analysis at the electron microscopic level has indicated that the majority of axosomatic synaptic terminals on arcuate neurons of ovariectomized rats are GABA-immunoreactive. The administration of a single dose of 17β-estradiol resulted in a significant decrease in the number of these GABA-IR synapses. In contrast, estradiol administration had no significant effect on the number of immunonegative axosomatic synapses. These data clearly show that physiological levels of estradiol induce a remodeling of the GABAergic axosomatic inhibitory input to arcuate neurons. More recently, we have shown that estradiol treatment does not elicit changes in the number of axodendritic arcuate synapses, although there is a significant increase in the volume density of excitatory spine synapses.

The role of estrogen receptors in the synaptic remodeling

Although it has been known that estrogen exerts a profound influence on synaptic plasticity in the central nervous system, a definitive demonstration of the role of the estrogen receptors in this process has remained elusive. To address this question we have studied the anteroventral periventricular nucleus (AvPv) which plays a particularly important role in the neural control of gonadotropin secretion. It contains high densities of neurons that express receptors for ovarian steroid hormones and appears to provide direct projections to gonadotropin releasing hormone neurons in the hypothalamus. Moreover, it receives inputs from a variety of distinct sensory systems known to influence secretion of luteinizing hormone from the anterior pituitary. It has also been shown that both ERα and ERβ are expressed in this nucleus, thus, the AvPv appears a good model to get information on this aspect of neuronal plasticity.

To understand the cellular mechanism of this complex phenomenon, it is important to know if the presence or absence of estrogen receptors (ERα and ERβ) in neurons have any influence on the hormone-induced synaptic remodeling. We performed unbiased stereological measurements in the AvPv and with the combination of preembedding ER and postembedding GABA immunostaining we could demonstrate differences in the synaptic connectivity and in the synaptic remodeling in neurons expressing different ERs.

Synaptic changes in the tubero-infundibular dopaminergic neurons

The fact that the hormonal treatment affects only GABAergic synapses clearly indicate some sort of specificity and raises the question whether there is a kind of specificity at the level of postsynaptic cells, as well. We attempted to identify a population of cells within the nucleus that responds to endocrine changes differently from surrounding populations. Previous studies had reported that GABAergic nerve terminals make extensive synaptic contact 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 (DA) 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 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 population within the arcuate nucleus and quantify GABAergic and non-GABAergic axo-somatic synapses in ovariectomized and ovariectomized + 17 ß-estradiol treated female; in orchidectomized and orchidectomized + 17 ß-estradiol treated male adult rats.

Sexually dimorphic expression of c-Fos in the accessory olfactory bulb

The molecular mechanisms of estrogen induced plasticity are not known, but it is believed that it could also act via intracellular signaling mechanisms. In this respect, estrogen has also been implicated in the regulation of brain c-Fos. This proto-oncogene belongs to the family of immediate early genes and can act as a transcription activator through various signal transduction pathways. C-Fos may also be involved in neuronal plasticity, and it has recently been shown that the number of c-Fos immunoreactive cells decreases with age in certain areas of the brain and spinal cord. Apart from being centrally involved in gene regulation, the exact mechanism and the physiological roles of c-Fos have not yet been elucidated. There is even less information on basal levels of c-Fos in the accessory olfactory bulb of the rat.

The AOB is a good model for studying hormonally regulated synaptic plasticity *in vivo* for at least two reasons. One is that it is a sexually dimorphic structure – it is bigger in males than females. In rodents it is under endocrine regulation and it plays a part in the detection of pheromones. Another reason that the olfactory system is suitable for plasticity studies is that the primary sensory neurons undergo a constant turnover, even during adulthood. We hypothesized that since (a) the differing hormonal milieu between male and female rats is associated with morphological and physiological differences in the AOB, and (b) c-Fos plays an important role in co-ordinating neuronal gene activity, then the number of AOB cells expressing c-Fos in response to estrogen treatment may change. Furthermore, the amount of c-Fos expressed by cells may also be altered because both synaptic plasticity and gene activity are age related functions. To test our hypotheses, we investigated c-Fos expression as a function of sex, age, and 17ß-estradiol treatment in the AOB of rats.

AIMS

The main aims of our studies were the following:

1. To determine the importance of ERα and ERβ estrogen receptors in the 17ß-estradiol-induced synaptic remodeling.

2. To investigate the role of the postsynaptic neuron in the specificity of estrogen action and to characterize the changes in synaptic connectivity of the dopaminergic subpopulation (TIDA neurons) of the arcuate nucleus.

3. To study the effect of age and 17ß-estradiol on the expression of c-Fos in the accessory olfactory bulb, a highly plastic brain region responsible for the regulation of sexually dimorphic behavior.

MATERIALS AND METHODS

Animals and surgical procedures

In the experiments CFY and Wistar albino rats were used. The animals were raised and maintained in standard laboratory conditions, with tap water and regular rat chow available *ad libitum* on a 12h:12h dark-light cycle. Two-month-old rats were anesthetized with Nembutal and were ovariectomized or orchidectomized. One month later, the rats received subcutaneous injections of either a single dose (45-100μg/kg body wt.) of 17ß-estradiol dissolved in sesame oil or vehicle.

Estrogen receptor immunostaining

The animals were anesthetized with nembutal and perfused through the left cardiac ventricle with 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, the brains were placed at 4^{o}C in glutaraldehyde-free fixative for an additional 3 hours. After washing in TBS, 50 μm thick coronal Vibratome sections were made for immunostaining using ERα (Santa Cruz Biotechnology) and ERβ (Zymed Laboratories Inc.) antibodies. The immunostaining was performed according to the avidin-biotin (ABC) procedure using tetramethylbenzidine (TMB) as the chromogen. Sections were analyzed by light microscopy and processed for electron microscopy, they were osmicated (1% OsO_4 in 0,1 M PB) for 30 min, dehydrated in ethanol and flat embedded in araldite between liquid release-coated slides and coverslips.

TH immunostaining

Using the routine fixation and sectioning protocol (see above) 50 μm thick coronal Vibratome sections were cut through the middle of the arcuate nucleus (Bregma -2.30 - -3.60) and processed for tyrosine hydroxylase immunostaining

performed according to the routine peroxidase-antiperoxidase (PAP) procedure using 3,3'-diaminobenzidine (DAB) as the chromogen. Sections were analyzed by light microscopy before embedding.

c-Fos immunostaining

Free-floating sections were first treated with a 0.3% H_2O_2 solution in methanol for 30 minutes followed by polyclonal rabbit anti-rat c-Fos (Santa Cruz Biotechnology, CA) antiserum at 1:20.000 dilution. Sections were then immersed in secondary antisera containing anti-rabbit IgG conjugated with biotin (1:500). The reaction was visualized by the avidin-biotin complex method using the Vectastain™ kit, developed with DAB and intensified with nickel chloride.

Postembedding immunogold GABA staining

Serial ultrathin sections were collected on Formvar-coated single slot gold grids. Postembedding immunostaining for GABA was carried out using a modification of the immunogold method of Somogyi and Hodgson. Briefly, 80-90 nm sections, collected on formvar-coated nickel slot grids were first etched in 1% periodic acid (5 min) and 1% Na metaperiodate (5 min). Sections were then incubated in a rabbit serum against GABA (gift of P. Somogyi) and the immunoreactivity was revealed with immunoglobulin conjugated colloidal gold (15 nm, diluted 1:20). All sections were contrasted with uranyl acetate and Reynold's lead citrate and examined in a Zeiss EM 902 electron microscope.

Quantitative analysis

Light microscopy

The slides were examined using a Zeiss-Jenalumar light microscope and counting was performed using the optical disector technique with the aid of a Sony digital video camera mounted on the microscope. All c-Fos immunoreactive nuclei were counted and the mean number/volume–were used for further analysis. Statistical differences were determined using the two-tailed Student's *t* test with an alpha value of 0.05.

Electron microscopy

The numerical density of axo-somatic synapses was determined by the unbiased disector method by Sterio (1984). Section thickness was determined by using the Small's minimal fold method.

There were six rats in each experimental group and three tissue blocks/animal were counted. The data from the same animals were pooled since no variations were detected in the three blocks from any group of rats. The Mann-Whitney test was used to determine the significance of differences between the mean values of data groups. A level of confidence of $P < 0.05$ was adopted for statistical significance.

RESULTS

Axo-somatic synaptic plasticity upon estrogen receptor expressing neurons in the anteroventral periventricular nucleus

In agreement with literature data we found that the AvPv contains high number of neurons that express estrogen receptor. The distribution of the two types is different: ERα-immunoreactive neurons are found through the whole nucleus, the ERβ-positive cells are localized in the medial-most portion of the nucleus. For EM studies we used sections from the rostral part of AvPv; in this plane the ERβ-IR neurons are localized in a well defined layer within 50μm from the ependymal lining and more laterally only ERα expressing neurons can be found. Two areas were selected for morphometric analysis of synaptic density. According to Orikasa et al. (2002) there is a high co-localization of the two ERs (~ 83%), therefore ERβ immunoreactive cells from area close to the ventricle represent a subpopulation expressing both ER receptors, while labeled neurons from the more lateral area can be defined as pure ERα expressing ones.

In electron microscopic pictures the nuclear TMB reaction product (crystalline spicules) reveals the estrogen receptor immunoreactive neurons; the postembedding immunostaining is highly specific and clearly labels the GABAergic terminals. Morphometric analysis show that in ovariectomized animals the axo-somatic innervation pattern of AvPv neurons is not the same in different subpopulations of the nucleus. In the ER-labeled neurons the number of GABAergic axo-somatic synapses is higher than the non-GABAergic ones. In contrast to this proportion, in the non-labeled neuron population the majority of the axo-somatic synapses is non-GABAergic.

The three subpopulations of AvPv neurons react differently to the estradiol treatment. In case of non-labeled neurons and neurons with ERα immunoreactivity there is a small decrease in number of both the GABAergic and non-GABAergic axo-somatic synapses. In neurons co-expressing ERα and ERβ, the density of non-GABAergic axo-somatic synapses remains stable, while we could observe a significant increase in number of GABA positive terminals.

17β-estradiol-induced synaptic remodeling on the TIDA neurons of the arcuate nucleus

There is a well-defined sexually dimorphic pattern of TH-IR neurons in the arcuate nucleus; males had more labeled cells in the ventrolateral region. This is the reason why we performed the morphometric measurements in the dorsomedial region of the arcuate nucleus (between 2,3-3,6 mm caudal from bregma) where the two sexes do not differ with respect to the number of dopaminergic neurons.

The electron micrographs revealed clear DAB reaction product indicative of tyrosine hydroxylase immunoreactive neurons; the postembedding immunogold staining was highly specific and clearly labeled the GABAergic terminals. We have found a sex difference in the total number of axo-somatic synapses; arcuate neurons receive more synaptic terminals in females than in males.

Although the total number of axo-somatic synapses was higher over all in females, the pattern of synaptic connectivity that is the ratio of GABAergic to non-GABAergic synapses was similar in the gonadectomized males and females. 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.

According to our data not all arcuate neurons were affected by the structural synaptic remodeling. In the ovariectomized females, 17ß-estradiol significantly reduced the numerical density of GABAergic synapses onto TH-IR neurons alone In contrast, in orchidectomized males the synaptic connectivity of TH-IR neurons was not affected by 17ß-estradiol, whereas 17ß-estradiol increased the density of GABAergic terminals onto non-dopaminergic neurons.

Age dependent and sexually dimorphic changes of c-Fos expression in the accessory olfactory bulb

C-Fos immunoreactivity was localized to the cell nuclei and no immunoreactivity was observed in any of the negative controls. The mitral and granular cell layers of the accessory olfactory bulb showed strong immunoreactivity, but only a few labeled cells were found in the glomerular layer. We found no difference in c-Fos immunoreactivity between mitral and granular cell layers in the AOB, and the numerical data had comparable variances. Furthermore, in all the cases of observed difference (e.g. effect of age or estradiol treatment) the changes were always comparable and with similar significance for the two different layers. For this reason we provide numerical data only for the mitral cell layer.

Estrogen treatment resulted in a temporary increase in c-Fos expression both in the mitral and granular cell layers. The number of immunoreactive cells was higher 1h after injection compared to ovariectomized controls. However, a significant increase in cell counts actually attributed to 17β-estradiol treatment was noted only 4h post-injection, and this was statistically significant compared to control animals. Immunopositive cell counts observed either 1h or 24h post-injection did not differ depending on whether oil or oil plus estrogen injection was administered. It is known that c-Fos can be rapidly, but transiently induced by stress. In light of this, compared to the ovariectomized control group, the observed increase in immunoreactive cells 1h after injection indicates that the effects in c-Fos expression are presumably, in part due to handling effects. 24 hours after the injection the number of labeled cells was comparable with the control value.

Studying the age-dependent changes, in juvenile animals we found high number of c-Fos immunopositive cells with similar values in both sexes. Much stronger immunoreactivity was observed in females of reproductive age, which was substantially higher compared to the immunoreactivity observed in adult male counterparts. There were very few or no immunoreactive cells noted in old rats, and no statistically significant differences in cell counts were observed between sexes.

DISCUSSION

Our results clearly demonstrates that 17ß-estradiol induces remodeling of axo-somatic synapses in the AvPv in a specific way, because the effect of the hormone differs in different subpopulations of neurons within the nucleus. By using double immunolabeling we evidence that the presence of estrogen receptor beta is decisive in this process. We found a significant increase in the number of GABAergic axo-somatic synapses in ovariectomized female rats treated with 17ß-estradiol; this change in synapse number was observed only in neurons which express estrogen receptor beta.

These data confirm earlier observations by Langub et al. (1994) who reported similar increase in axo-somatic synapses of estrogen receptor immunoreactive neurons. With the combination of preembedding estrogen receptor (ERα and ERß) and postembedding GABA staining we made this observation more specific, because we could demonstrate a well-defined selectivity of estrogen effect at both pre- and postsynaptic levels. Our data show that the hormonal treatment affects only GABAergic synapses which clearly indicate some sort of specificity, but at the same time we have found differences at the level of postsynaptic cells, as well. The synaptic connectivity of neurons expressing no estrogen receptors or only the ERα type does not change after 17ß-estradiol treatment, while there is a hormone-induced increase in the number of synapses in the subpopulation having both ERα and ERß receptors.

It is interesting to note that the number of synapses changes in opposite directions in two different brain regions; the hormone induces a decrease in the number of axo-somatic synapses in the arcuate nucleus while in the anteroventral periventricular nucleus a significant increase can be observed. This suggests that gonadal hormones are facilitating synaptic plasticity in different brain areas, but are not promoting a specific direction to the plastic changes.

In experiments studying the dopaminergic (TIDA system) of the arcuate nucleus we have found that the synaptic connectivity of TH-positive neurons are also specifically affected by the hormone. In this study, we used double immunolabeling to localize and separately analyze the synapses on neurons of the TIDA system and demonstrate that the response to the hormonal treatment is different in the dopaminergic vs. non-dopaminergic neurons.

According to our data, 17ß-estradiol induces synaptic remodeling not only in females, but 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, inhibitory synapses onto the TH immunoreactive neurons, whereas in males, inhibitory synapses onto non-dopaminergic neurons are affected by the hormone. In ovariectomized females 17ß-estradiol induced synaptic remodeling in the TIDA neurons: the number of GABAergic axo-somatic synapses was significantly lower 24 h after the hormone treatment. The non-labeled arcuate neurons were not affected by 17ß-estradiol, since the synaptic connectivity of those neurons remained 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. We found that the synaptic connectivity of these "hypophysiotropic neurons" was different from the other neurons that did not take up the tracer, and that the neurons projecting to the median eminence underwent synaptic remodeling in response to 17ß estradiol. The present data confirm, by inference, that the retrograde labeling by Fluorogold identifies TIDA neurons.

The specificity of the estrogen effect on the dopaminergic neurons shown in the present studies supports the notion that the hormonally induced synaptic remodeling has functional consequences. The decrease in the number of GABAergic inhibitory inputs onto TH-IR neurons in females that was observed after 24 hours of estradiol treatment may result in an increase in their activity, i.e. elevated DA release. The estradiol-induced synaptic changes can be paralleled by the physiological observations too, they correspond well to the termination phase of the prolactin surge. We interpret these data to mean that estrogen plays a complex role in the process: in that estradiol i.) initially increases prolactin secretion and ii.) over time, induces morphological synaptic remodeling in the arcuate nucleus, that eventually results in increased activity of TIDA neurons and inhibits PRL secretion.

A possible approach to study the effect of estrogen on the intracellular signaling mechanisms is to follow the expression pattern of c-Fos. The results of our study indicate for the first time that basal c-Fos expression in the accessory olfactory bulb is sexually dimorphic and decreases with age. Estrogen treatment increases the number of cells expressing c-Fos in the AOB.

Because c-Fos is known to be involved in central gene co-ordination, the large number of immunoreactive cells observed in juvenile rats may reflect a higher growth rate and differentiation. We found that the number of c-Fos labeled cells in adult males is lower compared to adult females. Importantly, this is in contrast to the juveniles where we observed no sex-related differences in the number of labeled cells. This observation may be explained by considering the changing hormonal milieu of animals during various stages of life. As the cyclic influence of estrogen and progesterone on the cascade of cellular signaling has

been documented, we have concluded that increased c-Fos expression in the adult females observed in our study could be due to higher levels of circulating estrogen.

The AOB is a sexually dimorphic region perceptive to a variety of sex related queues and plays an important role in sexual behaviour. Altogether, our results suggest that in the AOB there is a population of estrogen-receptive neurons in which c-Fos is involved in the cascade of cellular events. Furthermore, it is probable that this regulation of neuronal activation in the AOB occurs through ER-ß and not the alpha subtype, since only the ER-ß is present in this region.

LIST OF PUBLICATIONS

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[*]E.Cs. and A.K. contributed equally to this article.

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