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Gonadal Hormone Regulation of Neuronal–Glial Interactions in the Developing Neuroendocrine Hypothalamus

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Recent evidence indicates that, in addition to their well known effects on neurons, gonadal steroids may exert part of their neural effects through astroglia. In adult female rats astroglia participate in the phasic remodelling of synapses that takes place during the estrous cycle in the arcuate nucleus of the hypothalamus under the influence of estradiol. Astroglia also appear to be involved in the genesis of sex differences in synaptic connectivity. Gonadal steroids influence hypothalamic astroglia differentiation *in vitro* and *in vivo*. In monolayer mixed neuronal–glial cultures from fetal rat hypothalami, estradiol induces a progressive differentiation of astrocytes from a flattened epithelioid morphology to bipolar, radial and stellate shapes. This effect of estradiol on astroglia is dependent on the expression of specific molecules on the neuronal surface, such as the polysialic acid-rich form of the neural cell adhesion molecule. In the rat arcuate nucleus *in situ*, perinatal androgen influences astroglia gene expression and differentiation, resulting in a sex difference in astroglia organization by postnatal day 20. By this day, the amount of neuronal surface covered by astroglial processes is higher in males than in females. This difference in the coverage of neuronal surface by astroglia may be directly related to the reduced number of synaptic contacts that is established on the soma of male neurons compared to females.

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INTRODUCTION

One of the main difficulties in the study of cellular mechanisms involved in the sexual differentiation of the central nervous system is the extreme complexity of cellular interactions in neural tissue. Effects of gonadal steroids, exerted on neurons that express the appropriate hormone receptors, may be transmitted transsynaptically, both retrogradely and anterogradely, to steroid-insensitive neurons. Furthermore, non-neuronal components of the nervous system, such as ependymal cells, astroglia, oligodendroglia, microglia and brain blood vessels may be affected, directly or indirectly, by gonadal hormones as well. For instance, *in vitro* studies have demonstrated the presence of progesterone, estrogen and androgen receptors in glial cells [1, 2]. Furthermore, immunoreactivity for estrogen receptor has been detected in hypothalamic astroglia *in vivo* [3]. On the other hand, sexual dimorphisms in astroglia have been reported in several brain areas [4–8] and gonadal hormones have been shown to affect astroglia cell shape, immunoreactivity and gene expression *in vivo* and *in vitro* [2, 9–18]. Since neurons and glia maintain direct cell–cell interactions, effects on one cellular type may be transmitted to the other through cell adhesion molecules and/or by the secretion of soluble factors. Therefore, effects of gonadal steroids on glia may result in the modification of neuronal function, while glial cells, in turn, may be affected by hormonally-driven changes in neuronal activity.

Here we will review current evidence for the participation of astroglia in the sexual differentiation of the hypothalamus. We will first examine how estradiol promotes neuro–glial remodeling in the hypothalamic arcuate nucleus of adult rats. This hormonal effect, linked to synaptic plastic changes, provides some instructive insights into how hormonally modulated neuro–glial interactions may be involved in the formation of synaptic connectivity. Then, we will discuss

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recent data that suggest that specific interactions between neurons and astroglia participate in the sexual differentiation of synaptic connectivity in the hypothalamus.

GONADAL HORMONES MODULATE NEURO-GLIAL INTERACTIONS

The arcuate nucleus of the rat hypothalamus has been extensively explored in the past years as a model for the study of cellular mechanisms related to the effects of gonadal steroids on synaptic connectivity. This nucleus is a key center for the control of pituitary secretions and contains numerous neuronal elements that express receptors for gonadal steroids. Matsumoto and Arai, in a series of pioneering studies [19–21], defined the role of androgens and estrogens on the modulation of synaptogenesis in the arcuate nucleus and demonstrated that the genesis of sexually dimorphic synaptic contacts on arcuate neurons is dependent on perinatal androgen. More recent experiments indicate that, in addition to this organizational effect of androgens, gonadal steroids have activational effects on arcuate synapses in adult animals as well. In adult female rats, the number of synaptic terminals in contact with the soma of arcuate neurons fluctuates during the estrous cycle [22]. The number of axo-somatic synapses shows a prominent decrease from the morning to the afternoon of proestrus, remains at low levels during the morning of estrus and recovers to diestrus levels by the afternoon of estrus. These changes reflect a transitory disconnection of synapses and do not involve any degeneration or sprouting of synaptic terminals. Since neither the length of remaining synaptic plates nor the neuronal soma volume are modified, the amount of neuronal soma surface covered by presynaptic terminals is significantly decreased in the afternoon of proestrus and in the morning of estrus, compared to other phases of the estrous cycle.

In addition to classical synaptic interactions, arcuate neuronal somas, as is true for many other neurons, have abundant contacts with glial cell processes. The decrease in the synaptic coverage of arcuate neuronal somas in the afternoon of proestrus and in the morning of estrus is compensated by an increase in the amount of neuronal membrane surface covered by glial processes. During these stages of the estrous cycle, a multiple layer of glial processes is interposed between arcuate somas and the disconnected pre-synaptic terminals, preventing the formation of synaptic contacts [13]. This glial reaction may be a response that is in part directed to fill the space left by the disconnected presynaptic terminals. However, in homology to the role played by glial cells on the elimination of axo-somatic synapses from the surface of axotomized motoneurons [23–26], it has been proposed that the ensheathing of arcuate neurons by glial processes may have an active role in the transient disconnection of synaptic terminals [13, 22]. Also in the hypothalamus, glial cells appear to be involved in the modulation of synaptic plasticity in the paraventricular and supraoptic nucleus of adult rats [27] and in arcuate and LHRH neurons of adult monkeys [28, 29].

Administration of estradiol to adult ovariectomized rats results in the induction of coordinated synaptic and glial changes in the arcuate nucleus, with similar characteristics to those observed during the estrous cycle [30]. This suggests that estrous-cycle associated axo-somatic and glial plasticity is driven by fluctuations of the levels of this hormone in plasma. The effect of estradiol on arcuate axo-somatic synapses is blocked by the simultaneous administration of progesterone [30], a treatment that also results in the abolishment of estrogen effects on glial cells [13]. Furthermore, estradiol predominantly affects the γ-amino-butyric acid (GABA)-immunoreactive synapses [31]. Therefore, glial cells, by stripping inhibitory GABAergic synapses from the surface of arcuate neurons, may actively contribute to the estrogen-dependent increase in arcuate neuronal firing that accompanies LH release [32, 33].

Further immunocytochemical studies, by using antibodies for the specific astrocytic cytoskeletal marker glial fibrillary acidic protein (GFAP), indicate that the glial cells involved in the synaptic plastic changes in the arcuate nucleus are astroglia [13]. Both the surface density of GFAP immunoreactive profiles and the number of astroglial cell processes in the neuropil of the arcuate nucleus are increased in the afternoon of proestrus and in the morning of estrus compared to other phases of the estrous cycle. Furthermore, these parameters show a rapid and reversible increase in ovariectomized rats after the injection of 17β-estradiol, with a maximal effect by 24 h after the administration of the hormone. All these changes were accompanied by the interposition of glial processes between synaptic terminals and arcuate neurons [13].

In summary, these findings indicate that astroglia participate in the activational effects of estradiol on the structural reorganization of arcuate synapses in adult rats. Since plastic reorganization events in the adult brain may share common cellular mechanisms with developmental processes, the question is raised as to whether astroglia are also involved in the genesis of sex differences in synaptic connectivity.

GONADAL HORMONES INFLUENCE THE DIFFERENTIATION OF HYPOTHALAMIC ASTROGLIA

The effect of gonadal steroids on glial cell differentiation has been tested *in vitro*. In primary glial cultures from newborn rat forebrains, cell growth is inhibited by progesterone and stimulated by estradiol while both hormones increase the synthesis of GFAP by the cultured astrocytes [2]. In monolayer mixed

neuronal–glial cultures from E15–E16 rat hypothalami, estradiol induces a progressive differentiation of GFAP-immunoreactive astrocytes from a flattened epithelioid morphology to bipolar, radial and stellate shapes. This effect is dose-dependent, reversible and specific, since the potent estradiol antagonist tamoxifen blocks the effect of estradiol [12, 18]. The transformation of epithelioid astrocytes into cells with a bipolar shape occurs as early as 30 min after the addition of estradiol to the cultures [18]. This short term hormonal effect may be mediated by the reorganization of glial cytoskeleton. However, the development of elongated radial forms and stellate astrocytes requires longer hormonal exposure [18] and may be dependent on the synthesis of cytoskeletal proteins, since there is increased GFAP synthesis in cultures chronically exposed to estradiol.

HORMONAL EFFECTS ON ASTROGLIA DIFFERENTIATION DEPEND ON SPECIFIC NEURONAL-GLIAL INTERACTIONS

An important feature of the effects of estradiol on hypothalamic astroglia differentiation *in vitro* is that a direct contact between neurons and glial cells is necessary for the manifestation of these effects [18]. As indicated above, estradiol affects astroglial differentiation in mixed neuronal–glial cultures. However, in pure glial cultures, when neurons are absent, the differentiation of astrocytes is neither modified by estradiol nor by medium conditioned by estradiol-treated mixed cultures. Astroglia recover their sensitivity to estradiol after addition of neurons to the glial cultures. Moreover, the hormonal effect is dependent on pre-existing direct contact between living neurons and glia and appears to involve specific hypothalamic neuro–glia interactions, since it is not observed in co-cultures of hypothalamic glia with cerebellar neurons [18].

A role for specific neuro–glial interactions in the transmission of hormonal effects to astroglia is also supported by recent findings that indicate that the effect of estradiol on hypothalamic astroglia differentiation *in vitro* is dependent on the expression of the polysialic acid (PSA)-rich embryonic form of the neural-cell adhesion molecule (N-CAM). In embryonic brain, N-CAM contains more than 30% PSA. In most brain regions PSA–N-CAM is gradually replaced during the perinatal and early postnatal periods by N-CAM isoforms containing less PSA. However, PSA–N-CAM expression persists in the adult rat in several brain areas that show neuro–glial plasticity, such as the hypothalamo–neurohypophysial system, the arcuate nucleus and the median eminence [34]. In primary rat hypothalamic cultures, immunoreactive PSA–N-CAM is found on neuronal membranes while no immunostaining is detected on astroglia. Enzymatic removal of PSA from neuronal membranes prevents the effect of estradiol on astroglia, while it does not affect morphological changes induced by molecules that act directly on astroglial cells, such as bFGF (Garcia-Segura *et al.*, unpublished). Thus, expression of PSA–N-CAM on the neuronal surface is necessary for the manifestation of the estradiol-induced morphological changes in astroglia. PSA–N-CAM may modulate adhesion between neuronal and glial membranes and may also act on membrane receptors and activate intracellular signaling in astroglia resulting in cytoskeletal reorganization and cell shape changes.

In regards to the role played by neuronal membranes in the transmission of hormonal effects to astroglia, it is important to reconsider the results of freeze-fracture studies of arcuate neurons showing the existence of structural sex differences in neuronal membranes (see ref. [35] for a recent review). Arcuate neuronal membranes from male and female rats differ in the content of intramembrane protein particles (IMPs). Membranes from females have more IMPs of small diameter (<10 nm) than males, while neuronal membranes from males have a modest excess of large IMPs (>10 nm). Small IMPs in arcuate neuronal membranes bind concanavalin A and may, therefore, represent glycoproteins involved in cellular recognition. The sexually dimorphic membrane phenotype is dependent on perinatal androgen and appears to be generated by hormonal modifications in endocytotic activity of arcuate neurons. Sex differences in the organization of neuronal plasma membrane in the rat arcuate nucleus are generated before birth [35]. Thus, membrane differences precede by several days the onset of sex differences in astroglia and synapses (see below). The significance of sex differences in arcuate neuronal membranes may be appreciated when one considers the results of the experiments discussed above, showing that estradiol induced differentiation of hypothalamic astroglia is dependent on interactions of glial cells with neuronal surfaces.

ANDROGEN EFFECT ON ASTROGLIA DEVELOPMENT IS LINKED TO SEXUAL DIFFERENTIATION OF SYNAPTIC CONNECTIVITY

Gonadal steroids also affect astroglia differentiation in hypothalamic development *in vivo*. In the rat arcuate nucleus, the surface density of GFAP immunoreactive profiles and the number of astroglial cell processes per unit volume of neuropil, increase progressively with postnatal age (Fig. 1). By postnatal day 20, males show a higher level of GFAP immunoreactive profiles than females (Fig. 1) and a higher number of astroglial cell processes in the neuropil (Fig. 2). These sex differences are dependent on perinatal androgen. Administration of testosterone to newborn females increases the surface density of GFAP immunoreactive profiles to male levels by postnatal day 20 (Fig. 1) and also results in the abolishment of sex differences in number of astroglial

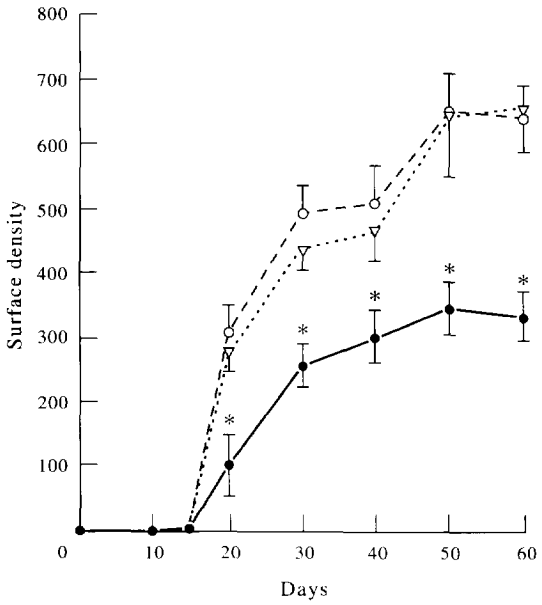

Fig. 1. Surface density of cell bodies and cell processes immunoreactive for GFAP in the arcuate nucleus of the rat hypothalamus represented versus postnatal age (days). Surface density of GFAP immunoreactive material was determined as described in [13]. Continuous line, intact females. Dotted line, intact males. Dashed line, females injected with 1.25 mg of testosterone on the day of birth. Data are mean ± SEM from 6 rats. Asterisks indicate values significantly different from those of intact males and androgenized females ($P < 0.01$).

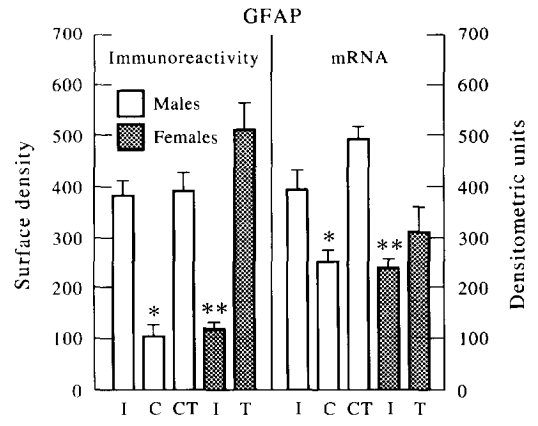

Fig. 3. Surface density of GFAP-immunoreactive cell bodies and cell processes and GFAP mRNA signal levels in the arcuate nucleus of 3-month-old-rats. Surface density of GFAP-immunoreactive material was determined using a morphometric grid as described in [13]. Messenger RNA signal levels were determined by densitometry of arcuate cryostat sections after *in situ* hybridization histochemistry. I, intact animals. C, neonatal castration. T, animals injected with 250 μg testosterone on the day of birth. Data are mean ± SEM from 6 rats.

cell processes (Fig. 2). The effect of testosterone on astroglia differentiation may be consecutive to its conversion to estradiol in the hypothalamus, where high levels of aromatase enzyme have been found [36].

The effect of perinatal androgens on arcuate astroglia has been further tested by measuring GFAP mRNA levels by *in situ* hybridization (Fig. 3). GFAP mRNA levels are sexually dimorphic in the arcuate nucleus of postpubertal rats, with females having lower levels than

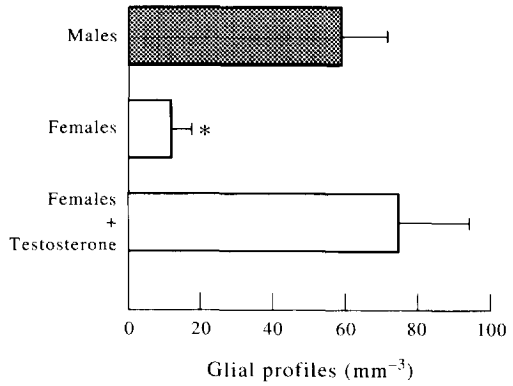

Fig. 2. Number of astroglial processes in the neuropil of the arcuate nucleus of 20-day-old rats. Data from intact males, intact females and females injected with 1.25 mg of testosterone on the day of birth are represented. Astroglial processes were identified and counted as described in [13]. Data are mean ± SEM from 6 rats. Asterisks indicate values significantly different ($P < 0.01$) from those of intact males and androgenized females.

males. Androgenization of females increases GFAP mRNA to male levels. In contrast, castration of newborn males reduces GFAP mRNA to female levels while testosterone administration to castrated males increases GFAP mRNA to intact male levels. GFAP mRNA changes are accompanied by differences in GFAP immunoreactivity (Fig. 3). Therefore, perinatal androgen influences astroglial gene expression, astroglia differentiation and growth of astroglial cell processes in the arcuate nucleus. However, the number of GFAP immunoreactive cells is unchanged in the arcuate nucleus after manipulation of the early postnatal levels of testosterone, suggesting that the hormone does not affect astroglia proliferation. Application of 5′-bromodeoxyuridine (BrdU) immunocytochemistry for the analysis of cell proliferation further supports this conclusion. Newborn male and female rats were injected with BrdU, an analogue of thymidine that is incorporated into the DNA during the S-phase of the cell cycle. The injection of BrdU was repeated at postnatal days 2 and 3. Cells that incorporated BrdU were detected by immunocytochemistry in histological sections from the arcuate nucleus of 20-day-old rats. Astroglia proliferation was assessed by counting the number of cells that were immunoreactive for both BrdU and GFAP. No significant sex difference in the number of immunoreactive cells was detected. Furthermore, the injection of testosterone to newborn females did not affect the number of labelled cells (Fig. 4). Therefore, perinatal testosterone, while affecting astroglia morphology and GFAP expression, does not appear to alter astroglia proliferation in the developing arcuate nucleus.

The effect of androgen on astroglia differentiation has several possible functional consequences.

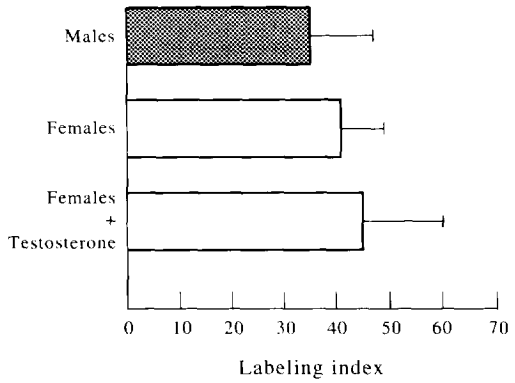

Fig. 4. Percentage of GFAP immunoreactive cells immunostained with BrdU (labeling index) in the arcuate nucleus of 20-day-old rats. BrdU was injected to intact males, intact females and androgenized females as described in text. Data are mean ± SEM from 6 rats.

Astrocytes play an active role in the maintenance and modulation of synaptic transmission [37, 38] and influence neuronal activity by regulating the glucose supply, extracellular ion concentrations, cerebral blood flow, neurotransmitter levels, as well as by releasing neuroactive substances [39]. Tanycytes, a specialized astroglia cell type present in the arcuate nucleus and median eminence, may be involved in the regulation of LHRH release [15, 40]. These cells are a source of trophic factors that modulate neuroendocrine events, such as transforming growth factor-α (TGF-α) and insulin-like growth factor I (IGF-I) [15, 41]. In addition, astroglia participate in the metabolism of gonadal hormones [42–45] and in the synthesis of neurosteroids [46, 47].

During development, astroglia guide migrating neurons and growing axons and modulate neuronal differentiation [48–50]. For instance, pathfinding by growing LHRH axons may be aided by channels provided by tanycytes [40, 51]. Astroglia are also involved in the regulation of synaptogenesis. The number of axo-somatic synapses on arcuate neurons increases progressively during postnatal development to reach a plateau by day 20. Male and female rats have a similar number of axo-somatic synapses until postnatal day 10. Then, the rate of synapse formation increases in females, reaching, by day 20, a significantly higher number of axo-somatic synapses compared to males. The difference appears to be dependent on perinatal androgens, since the androgenization of females results in the development of a pattern of synaptic connectivity similar to males [35]. Androgens may modulate the amount of membrane available on arcuate neurons for the establishment of synaptic contacts by increasing the number of astroglial processes wrapped around the neuronal surface. Actually, by postnatal day 20 the amount of cellular surface of arcuate neuronal somas covered by glial processes is higher in males than in females. Furthermore, androgenization of females increases the proportion of neuronal surface covered by glial processes to male levels [35]. It should be noted that there is a coincidence in the time of apparition of sex differences in both axo-somatic synapses and astroglial parameters: both events take place by postnatal day 20. This suggests that synaptic and glial changes may be interdependent and that androgens may in part modulate the number of axo-somatic synapses on arcuate neurons by affecting astroglia differentiation.

CONCLUSION

The studies reviewed in this paper suggest that astroglia are involved in the genesis of structural and functional sex differences in the hypothalamus. *In vitro* studies have shown that the effects of gonadal steroids on astroglia differentiation are transmitted through molecules located on the neuronal surface. During the differentiation of the arcuate nucleus *in situ*, testosterone, either directly or after aromatization to estradiol, affects the organization of neuronal plasma membrane. The resulting sexual dimorphism of neuronal membranes may be the source of the observed sex differences in neuro–glial interactions and these, in turn, may generate differences in synaptic inputs between males and females and may result in sex differences in neuronal function. Therefore, organizational effects of gonadal steroids during hypothalamic differentiation depend, at least in part, on specific neuro–glia interactions.

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