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Synapse formation in response to estrogen in the medial amygdala developing in the eye

(intraocular transplantation/sexual differentiation/dendritic synapses)

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ABSTRACT **Medial amygdaloid tissue, taken from female rats immediately after birth, was transplanted into the anterior chamber of the eye in adult ovariectomized host rats in order to elucidate the influence of estrogen on synapse formation without contribution of neural afferents. After injections of estradiol benzoate or oil vehicle to the hosts for 20 successive days, the grafts were processed for semiquantitative electron microscopic study to examine synaptic density in the neuropil. The number of synapses on dendritic shafts vs. dendritic spines was not significantly different in the control group. In contrast, in the grafts exposed to estrogen, shaft synapses occurred more frequently than spine synapses. Synaptic density on shafts was significantly greater in these grafts than that in the controls, although the density on spines did not differ between the two groups. These data show that estrogen affects the medial amygdaloid neurons themselves and specifically facilitates the formation of dendritic shaft synapses *in oculo*. Our previous report raises the possibility that the specific increase of shaft synapses induced by sex steroids is involved in the process of sexual differentiation of neuronal networks from the inherently feminine pattern to the masculine pattern in the medial amygdala. Therefore, the present findings may provide evidence that sexual differentiation triggered by sex steroids is accomplished by intrinsic factors in the neurons of the medial amygdala.**

Recent studies indicate that sex steroid hormones modulate and promote neuronal maturation and neurite growth (1–4) in certain brain regions that belong to a sex steroid-concentrating neuronal system (5). Furthermore, synaptogenesis can be facilitated by estrogenic action in the hypothalamic arcuate (6) and medial amygdaloid nuclei of early postnatal rats (7). The presence of a sex difference in synaptic organization and its dependence on the neonatal hormone environment also has been reported in these nuclei (8–10) and the preoptic area (11).

Our data have shown (7, 9) that the medial amygdala is one of the representative regions where sexual differentiation in synaptic pattern is brought about in males by the organizational action of sex steroids; otherwise, the feminine pattern of neuronal circuitry develops (7, 9). However, it is unclear whether extrinsic or intrinsic influences are of major importance in permitting the emergence of sexually dimorphic connections in this region, which includes both the medial amygdaloid neurons and some of the afferent fiber systems (12–16) containing receptors for sex steroids (5).

Here we used the intraocular transplantation of medial amygdaloid tissue of the newborn rat to analyze the possible influence of estrogen on the development of the synaptic pattern when isolated from afferent fiber systems.

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MATERIALS AND METHODS

Medial amygdaloid tissue taken from female pups of Sprague–Dawley rats (Japan Charles River Inc.; Atsugi, Japan) immediately after parturition was transplanted homologously into the anterior chamber of the eye of adult host females (age 8–10 wk). As described by Olson and Malmfors (17), the eyes of anesthetized hosts ovariectomized 2–5 days earlier were treated with atropine, and a small incision was made through the cornea. A tissue fragment consisting mainly of the medial amygdaloid nucleus dissected out from the brain of a decapitated pup was put into the anterior chamber of the eye through the corneal slit by means of a fine pipette under a dissection microscope. The hosts were injected subcutaneously with oil vehicle or 2 μg of estradiol benzoate (Teikoku Hormone Mfg.) dissolved in 0.1 ml of sesame oil for 20 successive days from the day of grafting.

On the day after the last injection, the anesthetized hosts were perfused with 1% glutaraldehyde/1% paraformaldehyde/0.1 M phosphate buffer solution, and the grafts were removed from the anterior chamber of the host's eye, postfixed with 2% (vol/vol) osmium tetroxide in the phosphate buffer, and dehydrated and embedded as described by Palay and Chan-Palay (18). Eleven grafts derived from the hosts injected with oil and 13 grafts from those given estrogen were processed for semiquantitative electron microscopic study. For evaluation of the density of dendritic synapses, the procedures described in our previous papers (7, 9) were modified. Synapses on dendritic shafts (shaft synapses) and synapses on dendritic spines (spine synapses) were differentially counted on 120 electron micrographs taken randomly from a single ultrathin section of the middle part of each graft. Photographs (×10,000, enlarged to ×21,000 for counting) taken of the neuropil were coded so that the experimental conditions would be unknown. The number of synapses in each graft was converted into the density per field of 5,000 μm^2. Statistical analysis was made by Student's t test.

In order to examine the sympathetic catecholaminergic innervation into the grafts from the host's iris, a number of hosts were intraperitoneally given 5-hydroxydopamine (20 mg/kg of body weight; Sigma) four times over a period of 48 hr (19) from the day of the last injection of estrogen or oil. Five hr after the last dose, the hosts were perfused under anesthesia, and four grafts in each group were processed for electron microscopic observation. In addition, four or three grafts in estrogenized or control groups, respectively, were dissected out with their attached irises from hosts that had received intraperitoneal injection of nialamide, a monoamine oxidase inhibitor (250 mg/kg of body weight; Sigma), 5 hr earlier to increase catecholamine content in sympathetic fibers and their terminals. They were processed for the Falck–Hillårp method (20) and examined by fluorescence microscopy.

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RESULTS

Twenty-one days after transplantation, the grafts were oval or flattened in shape and continuous with the host's iris, being well vascularized. The ultrastructural features of the neuronal and glial cell bodies and the intertwined neuropil consisting of dendritic, axonal, and glial processes (Fig. 1) were apparently identical to those of tissues *in situ*. Numerous synaptic contacts were found on the dendritic shafts and spines and neuronal somata. These observations indicate that the medial amygdala of neonatal rats can successfully survive and grow *in oculo*.

The number of dendritic synapses in a field of 5,000 μm^2 ranged from 452 to 1,105 in 11 grafts obtained from the hosts given oil and from 549 to 1,065 in 13 grafts derived from the hosts treated with estrogen. The size of the tissue was inconstant at the time of grafting. This might be one of the critical factors in variability of synaptic density among grafts. However, it evidently increased when compared with that in the medial amygdaloid nucleus of 1-day-old females, and the mean number was almost comparable to that of normal adult females reported earlier (7).

The present work focused on the distribution of the dendritic synapses. As shown in Fig. 2*A*, the number of synapses was not different from those on the dendritic shafts and on the spines in any graft in the control group. Interestingly, however, the number of shaft synapses was larger than that of spine synapses in 12 out of 13 grafts exposed to estrogen through the hosts (Fig. 2*B*). The mean synaptic density on the shafts in these 13 grafts (mean ± SEM, 456 ± 25) was significantly greater than that in the 11 grafts in the control group (348 ± 31, $P < 0.01$), whereas, in the mean synaptic density on the spines, there was no significant difference between the estrogenized (308 ± 22) and control group (384 ± 34). Therefore, we suggest that treatment with estrogen through the hosts promoted synaptogenesis on the dendritic shafts in the medial amygdaloid grafts. However, the formation of spine synapses does not seem to be influenced by estrogen. The mean shaft/spine ratio of postsynaptic elements in the estrogenized grafts (1.5 ± 0.09) was significantly larger than that of the oil-treated grafts (0.9 ± 0.03, $P < 0.001$).

Because the grafts became well fused with the host's iris, rich in sympathetic fibers, it was possible that these fibers grew into the grafts and made synaptic contacts with neuronal elements in the grafts. However, only a few synapses (ranging from one to seven per 5,000 μm^2) containing granular vesicles labeled with 5-hydroxydopamine, which were regarded as catecholaminergic boutons (19), were found in the neuropil in the middle part of the grafts. The incidence of catecholaminergic synapses did not change even after the direct application of 5-hydroxydopamine into the anterior chamber of the hosts' eyes in our preliminary study. Furthermore, the medial amygdaloid grafts were devoid of fluorescent fibers except for a few fibers arising from the catecholaminergic plexus of the iris in the basal part of the grafts, even after nialamide treatment. From the electron and fluorescent microscopic observations, there seemed to be no significant contribution of iridic catecholaminergic fibers to the synapse formation in the grafts in either estrogenized or control groups.

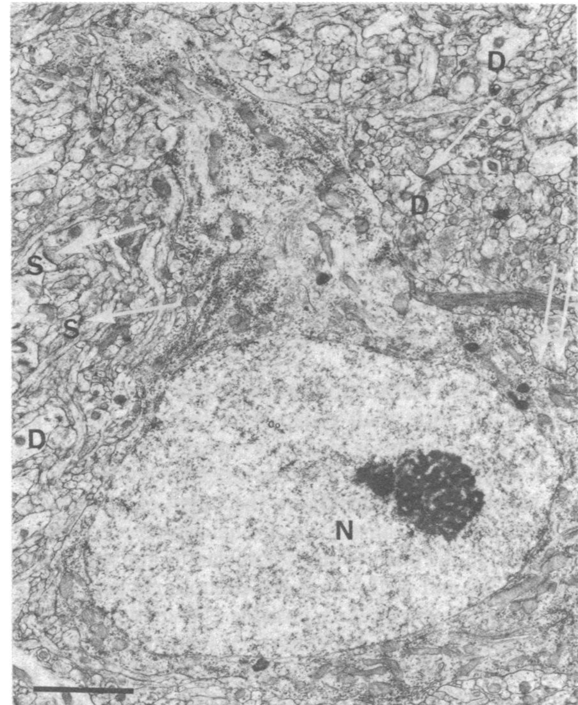

FIG. 1. Electron micrograph of an intraocular medial amygdaloid graft of the control group. Somatic synapse (white double arrows), dendritic shaft synapse (white arrow) on dendritic shafts (D), and dendritic spine synapses (white arrow) on dendritic spines (S) are seen in intertwined neuropil. N: Neuronal nucleus. (Bar = 2 μm.)

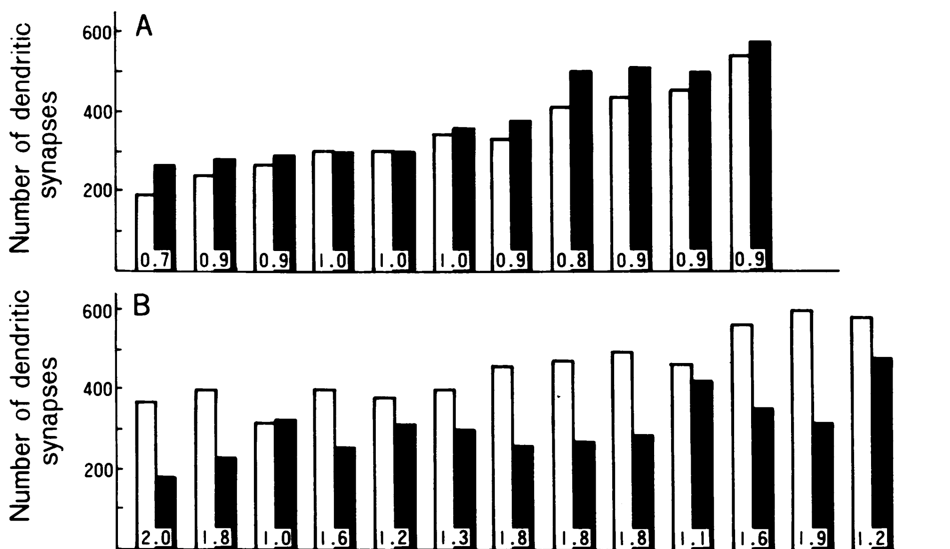

FIG. 2. Number of dendritic synapses per field of 5,000 μm^2 in the neuropil of the middle part of the intraocular medial amygdaloid grafts obtained from the host injected with oil vehicle (*A*) or 2 μg of estradiol benzoate (*B*) for 20 successive days. The values of dendritic shaft synapses (□) and of dendritic spine synapses (■) are shown in each graft in the order of increasing number. The number at the bottom of the pair of bars indicates the shaft/spine ratio of postsynaptic elements.

DISCUSSION

The present results indicate that intraocular grafts of the medial amygdala develop and have complex neuronal connections similar to those of the medial amygdaloid nucleus *in situ* (7). Because the incidence of synapses labeled with 5-hydroxydopamine was quite low, the participation of iridic catecholaminergic fibers does not seem to be significant in quantifying synaptic density. According to the report by Fallon *et al.* (21), the medial amygdala is inherently innervated by few catecholaminergic fibers. The situation may be different from the cerebral cortex, which receives a considerable adrenergic input *in situ* (22); in this case, iridic sympathetic fibers innervate intraocular cortical grafts (23).

In the present study, estrogen facilitates specifically the formation of dendritic shaft synapses in the developing medial amygdala *in oculo*. This is in good accordance with our previous study *in situ* (7). It is likely that the medial amygdaloid neurons responsive to sex steroids (5) are the target of estrogen in the ectopic condition because the neuronal estrogen receptors can be detected in the embryonic hypothalamus grafted in the choroidal pia (24) and in the neonatal preoptic/hypothalamic tissues cultured *in vitro* (2). A possible explanation for the predominance of shaft synaptogenesis in response to the organizational action of estrogen in the medial amygdala both *in situ* and in the ectopic site is that the synaptic distribution is governed largely by intrinsic factors that cause the organotypical development of the medial amygdaloid neurons. According to a Golgi study on dendritic morphology in intraocular hippocampal grafts (25) and autoradiographic analysis of the time schedule of neurogenesis in the embryonic cerebral cortex transplanted to tectal or cortical regions (26), organotypical programs similar to those observed during normal development operate in ectopic neural tissues. The present report provides electron microscopic evidence for organotypical development of synaptic organization in the ectopic medial amygdala. If this is the case, it may imply the presence of a considerable number of intranuclear connections within the medial amygdaloid nucleus.

We have reported that the synaptic organization in the medial amygdaloid nucleus was sexually dimorphic, and the dimorphism was attributed to a specific and permanent increase of shaft synapses in neonatal male rats in response to sex steroids (9). In the present study, treatment with estrogen through the host animals caused neuronal networks to mimic the masculine medial amygdala in the graft tissues from newborn females. Therefore, these data suggest that sexual differentiation triggered by sex steroids is achieved by intrinsic factors in the neurons of the medial amygdala. Because the extraamygdaloid afferents (13, 14, 16, 27) and intraamygdaloid connections arising from the basomedial or posterior cortical nuclei (12, 15) could be eliminated by the intraocular transplantation in the present experiments, the postsynaptic elements on the shafts seemed to be a possible candidate on which the effect of estrogen is manifested. Recently it has been reported that α-bungarotoxin-binding capacity in the posterior medial amygdala of the mouse is sexually dimorphic and permanently modified by neonatal sex steroid manipulation (28). It is of interest to consider the possible correlation between sexual differentiation of the synaptic organization and neonatal activation of postsynaptic elements by sex steroids. However, the possibility cannot be excluded that the presynaptic elements of the connections in the medial amygdaloid neurons also are involved in the process of estrogen-induced synaptogenesis. There is evidence suggesting that estrogen facilitates axonal growth in the preoptic/hypothalamic tissues *in vitro* (2). It is not clear whether glial components might contribute to the organizational responses of these neural substrates to estrogen.

Finally, findings on sexual differences caused by the organizational actions of sex steroids have been accumulating not only in the amygdala but also in the preoptic area (11, 29), hypothalamic arcuate nucleus (8, 10), and spinal cord (30). The fact that all of the sexually dimorphic regions belong to a sex steroid-concentrating neuronal system (5, 30) reinforces the possibility that sex steroids directly exert an influence on each of these regions and elicit the organotypical dimorphism.

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