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Ovary-Dependent Degeneration in the Hypothalamic Arcuate Nucleus*

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ABSTRACT. The effects of estradiol valerate and constant light exposure on the histological appearance of the arcuate nucleus were assessed in female rats. Both of these treatments caused significant increases in the numbers of reactive microglial cells and astrocytic granules in the nucleus. Ovariectomy before either treatment prevented the glial reaction, indicating that the experimental manipulations triggered the secretion of an ovarian product which appears to be selectively toxic to the arcuate nucleus. The fact that monthly injections of estradiol valerate in male rats produced the same profile of degeneration in the arcuate nucleus suggests that the neuropathological agent may itself be estradiol. Ovariectomy also significantly reduced arcuate microglial reactivity associated with normal aging, which suggests that cyclic surges of endogenous estradiol may be capable of gradually producing an arcuate lesion. This phenomenon may account for the hypothalamic reproductive failure associated with normal aging in the rat. (*Endocrinology* **107:** 274, 1980)

TREATMENT with estradiol valerate (EV) has been shown to produce pathological changes in the hypothalamic arcuate nucleus of the rat. Female rats given multiple (1) or single (2) injections of 2 mg EV exhibited a gradually progressive multifocal degeneration of axons, axon terminals, and dendrites in the arcuate nucleus. These degenerative foci contained active phagocytic microglial cells and reactive astrocytes, both of which were easily identifiable at the light microscopic level (2, 3). Animals given a single injection of 2 mg EV developed, in addition to the lesion, an anovulatory syndrome characterized by small multicystic ovaries and persistent vaginal estrus as well as high normal plasma LH values, low normal FSH values, and moderately elevated plasma estradiol (E_2) levels (20–30 pg/ml) by 6 weeks after injection. These alterations in endocrine and reproductive functions are reminiscent of those that characterize the anovulatory condition after surgical anterior hypothalamic deafferentation (4–6). This suggests the possibility that degenerating synaptic structures in the EV-treated rats may represent a selective disruption of connections between the medial preoptic area (MPOA) and the medial basal hypothalamus (MBH), resulting in impaired transmission of LH-releasing stimuli emanating from the MPOA. The observation that EV-lesioned animals exhibit a reduced LH surge after electrochemical stimulation of the MPOA (3) supports this hypothesis.

Although the morphological and functional changes described above are clearly triggered by the administration of estradiol, the exact role of the steroid in this system is unknown. It is not clear whether the initial dose of EV produces an irreversible effect on the hypothalamus which gradually becomes manifest or whether constant exposure to only moderately elevated plasma E_2 levels (2) is required to produce the lesion. If minor elevations in plasma E_2 result in hypothalamic damage, other persistent estrous models should show similar hypothalamic changes.

We have previously pointed out that the arcuate nucleus of the young, normally cycling female rat shows some glial reactivity which increases with age and is particularly prominent in aged anovulatory females. Consequently, it has been suggested that the EV injection greatly accelerates what appears to be a normal age-related process (2). Although the age-associated persistent estrous female presents a neurohistological and reproductive endocrine picture similar to that of the EV-treated model, it is not known if the age-related changes are steroid dependent.

The present study was undertaken to elucidate these points and better define the endocrinological and physiological conditions that result in neuropathological events in the arcuate nucleus. In the present study,

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astrocytic and microglial reactivities are used as histological indices of the severity of the degenerative process within the arcuate nucleus. These glial markers enable us to evaluate quantitatively the effectiveness of various treatments (*e.g.* constant light exposure, gonadectomy, *etc.*) in accelerating or, conversely, inhibiting the arcuate reaction.

Materials and Methods

Histology

Tissue preparation. The brains of all animals used in this study were fixed by intracardial perfusion of a solution containing 1% glutaraldehyde and 1% formaldehyde buffered to pH 7.4 with 0.12 M phosphate. Brains were removed and the hypothalami were dissected out. The hypothalamic blocks were postfixed, dehydrated, infiltrated, and embedded according to a standard procedure described in detail elsewhere (7). Four to six coronal sections (1 μm thick) were cut per animal at mid levels of the nucleus, anterior to the formation of deep tuberoinfundibular sulci. The lateral recesses were sharply defined on either side of the third ventricle, and two small clusters of pars tuberalis appeared on either side of the median eminence at the base of the brain, nestled within shallow tuberoinfundibular sulci. This level corresponds to level P_3 in the cytoarchetectonic atlas of Szentagothai *et al.* (8). Each section included two fields of arcuate nucleus, one on either side of the third ventricle. Sections were stained with toluidine blue.

Two of the four to six sections per animal were selected at random for quantitative analysis of arcuate glial activity. Thus, for each animal, four fields of the arcuate nucleus were evaluated, since each section contained two arcuate fields.

Histological parameters. The number of reactive microglial cells and astrocytic granules was determined in each field (four per animal) of the arcuate nucleus. Microglial cells were identified as small fusiform or ellipsoidal cells exhibiting heterochromatic nuclei, and a darkly staining cytoplasm containing dense granules. Since the number of cytoplasmic inclusion granules within microglial cells reflects phagocytic activity (9–11) and arcuate microglial cells in young normal animals rarely contained more than four such inclusion granules, a microglial cell was designated as reactive and counted as such if it contained five or more inclusion granules.

The total number of reactive microglial cells per animal (the sum of four fields) was then taken as a quantitative index of arcuate microglial reactivity for that animal.

Astrocytes are identified as small pale cells with euchromatic ellipsoidal nuclei. These cells radiate numerous cytoplasmic extensions and processes which course through the neuropil, often for considerable distances. These processes comprise the bulk of the cytoplasmic volume of the astrocyte. Arcuate astrocytes and their processes characteristically contain dense granular inclusions, and these increase under pathological conditions (1–3). Since most astrocytic granules appear in pale astrocytic processes dissociated from the parent cell (due to the thinness of the section), it was decided that the number of astrocytic granules per field was a more reliable index of astrocytic activity than the number of astrocytic perikarya containing a designated number of granules. Thus, for each animal, the number of astrocytic granules in the four arcuate fields was totaled to give a single index of astrocytic reactivity per animal.

Light micrographs depicting astrocytic granules and reactive microglial cells appear in a previous publication (3).

All data were analyzed by the Mann-Whitney test. In the case of the EV dose-response histograms for the male, a log-linear regression analysis was also performed.

Treatment of animals

All animals used in this study were Virgin cyclic Wistar rats, weighing 130–170 g, obtained from Canadian Breeding Farms and Laboratories. All animals were given food and water *ad libitum* and kept on a regular 12-h light, 12-h dark cycle, except for the groups exposed to constant light (see below).

Normal cyclic controls (group I). Animals were obtained, and vaginal cyclicity was established and followed for 2 weeks. The animals were then kept for a period of 4 months, during which time vaginal cyclicity was monitored by means of daily smears. At the end of this 4-month period, all animals that had exhibited regular 4-day cycles were killed by intracardial perfusion, as described above. All animals were killed at the same time regardless of the stage of the cycle that each exhibited on the day of perfusion.

EV-treated rats (group II). After exhibiting 2 weeks of normal cycles, animals received a single sc injection of 2 mg EV in 0.2 ml vehicle (sesame oil). Vaginal smears were taken daily, and by 4 weeks after injection, most animals exhibited persistent estrus (group IIa). The remaining animals (about 25%) continued to cycle throughout the duration of the experiment (group IIb). All animals were perfused 4 months after EV injection.

EV-treated ovariectomized rats (group III). Animals, identical to those in group II, were ovariectomized 1 week before EV treatment. They were perfused 4 months after EV injection.

Constant light-exposed rats (group IV). Animals in which normal vaginal cyclicity had been observed for 2 weeks were then quartered in a constantly lit room for 4 months before perfusion. Vaginal smears were taken daily. All animals began to exhibit persistent estrus after about 1 month of constant light exposure.

Ovariectomized constant light-exposed rats (group V). These animals were treated in an identical manner to those of group IV, except that they were ovariectomized 2 days before being quartered in the constantly lit room.

Ovariectomized rats (group VI). Animals were ovariectomized after 2 weeks of normal vaginal cycles and kept in the same quarters and under the same conditions as the normal controls (group I) for 4 months before perfusion.

Males. Normal, young, sexually mature male Wistar rats were obtained and housed under conditions identical to those described for the control females. Untreated animals served as controls and were kept for 4 months before perfusion. Three groups of rats each received, respectively, 2, 0.2, and 0.02 mg EV in 0.2 ml sesame oil sc per month for 4 months before perfusion. Animals were always injected on the same day of each month.

Results

Effects of constant light (group IV) and EV treatment (group IIa) on the arcuate nucleus in intact females

Figure 1 shows the response of arcuate glia to these two manipulations. EV injection and constant light exposure significantly elevated both microglial and astrocytic reactivities above the control level ($P < 0.05$). Furthermore, both of these treatments produced similar increases in glial activity, *i.e.* constant light is just as effective if not more so in generating the arcuate neuropathological process.

Effects of constant light and EV treatment on the arcuate nucleus in ovariectomized females (groups III and V)

Figure 1 shows that the arcuate glial reactions to EV treatment and constant light exposure are significantly reduced in animals ovariectomized before treatment ($P < 0.05$). Ovariectomy caused a significant and similar reduction in microglial reactivity in both EV-treated and constant light-exposed animals ($P < 0.05$). The reduction in astrocytic reactivity produced by ovariectomy was the same in both groups. The numbers of reactive microglial cells and astrocytic granules in the ovariectomized EV-treated and ovariectomized constant light animals were within the control range (group I; $P > 0.05$).

Differences between the astrocytic and microglial responses

Significantly elevated microglial reactivity appears to be a concomitant feature of persistent estrus, as can be seen in Fig. 1. EV-treated females that did not go into persistent estrus (group IIb) exhibited the same numbers of reactive microglia as normally cycling controls (group I). In contrast, the behavior of the astrocytes exhibited greater variability. EV-treated animals that did not go into persistent estrus exhibited significantly fewer astrocytic granules than EV-treated animals that went into persistent estrus ($P < 0.05$), albeit significantly more than normally cycling controls ($P < 0.05$).

Effect of ovariectomy

Since the ovaries are indispensable for the development of the EV- and constant light-accelerated neuropathological reactions in the arcuate nucleus, the question arises as to whether they contribute to the pathological changes in this nucleus associated with normal aging.

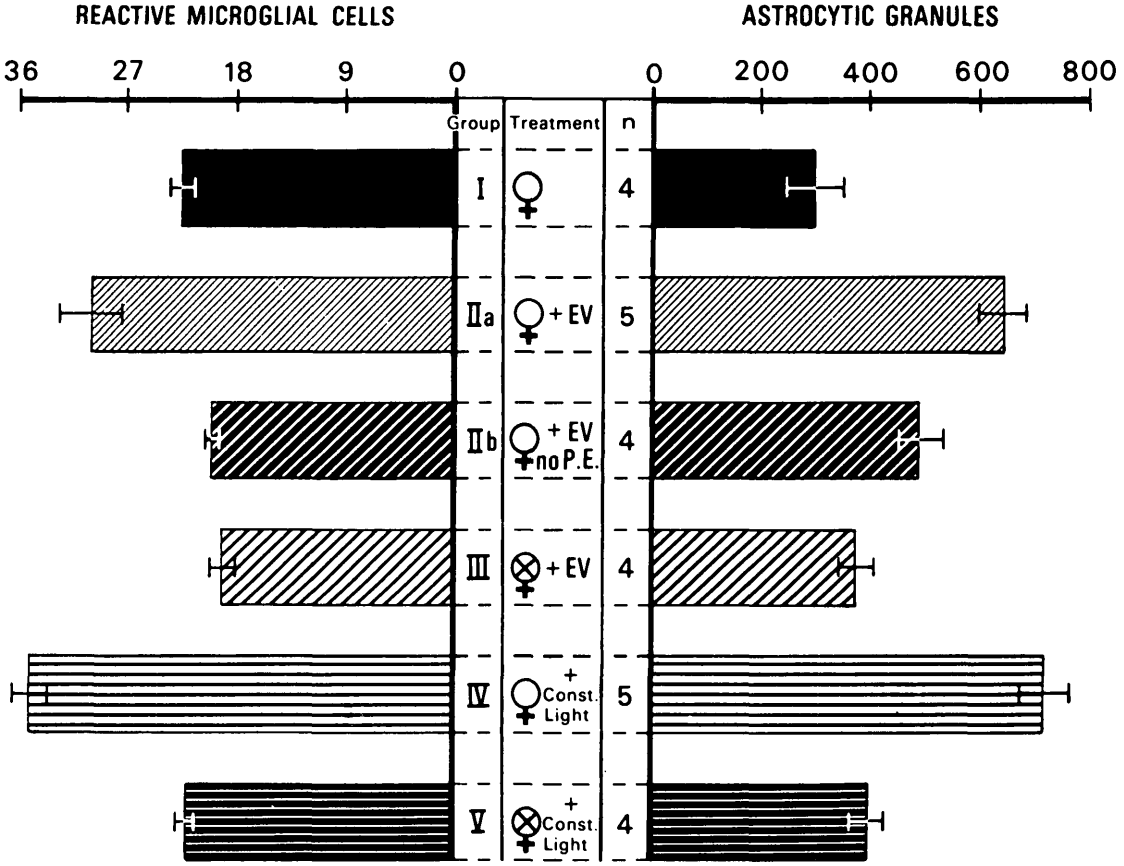

FIG. 1. Glial response to various treatments. The *left section* (REACTIVE MICROGLIAL CELLS) indicates the mean number and SE of reactive microglial cells per animal for each experimental group. The *right section* (ASTROCYTIC GRANULES) indicates the mean number and SE astrocytic granules per animal for each experimental group (determined from the same four sections per animal). The group number refers to specific experimental categories designated by the same numbers in *Materials and Methods*. The treatment is a figurative summary of each of these categories. n, Number of animals in each group.

Figure 2 shows the effects of ovariectomy on the development of reactive gliosis in otherwise normal female rats. Ovariectomy significantly reduced microglial activity compared to that in normal controls of the same age. Astrocytic activity, however, appeared to be unaffected by ovariectomy within the time range examined ($P > 0.05$).

EV-dose response relationship in the male

Monthly injections of EV were given to male rats for a period of 4 months in order to determine whether males were susceptible to the EV-induced arcuate lesion. Figure 3 shows that injections of 0.02 mg EV/month produced a significant glial response in the arcuate nucleus ($P < 0.05$).

The correlation coefficient for the astrocytic responses over the dose range tested was 0.67, indicating a significant dose-response relationship ($P < 0.05$). The correlation coefficient for the microglial response over the same dose range was not significant ($P > 0.05$). Maximal numbers of reactive microglia were obtained with 0.02 mg/month, whereas the administration of 0.2 mg/month was required in order to reach a plateau of astrocytic responsiveness.

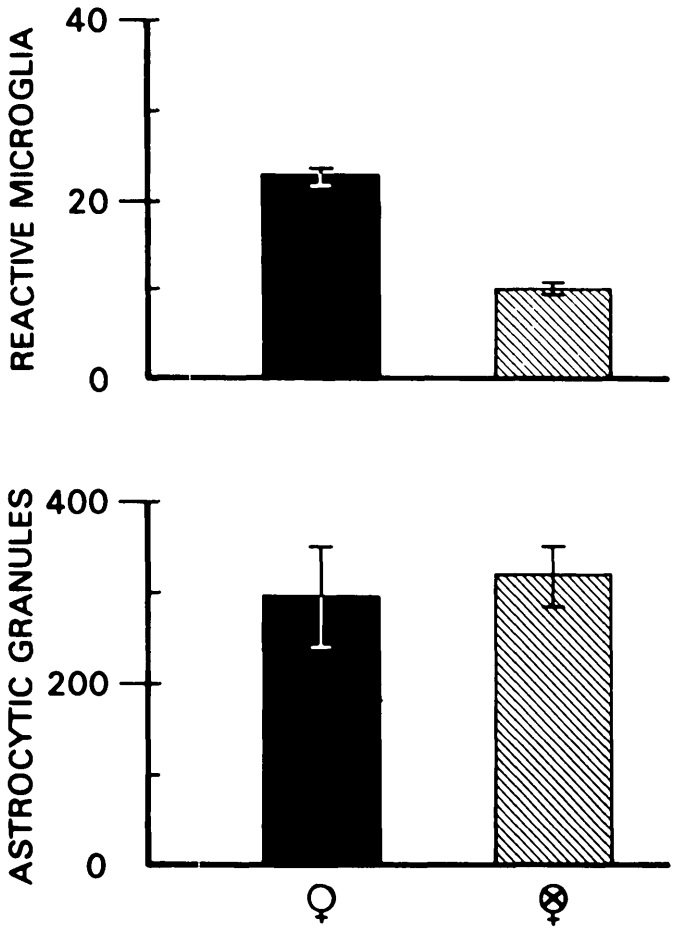

FIG. 2. Effect of ovariectomy on arcuate glial activity. The mean number and SE of reactive microglial cells and astrocytic granules per animal is shown (as in Fig. 1). ■, Normal intact controls (group I). ▨, Ovariectomized rats (group VI). There were four animals per group.

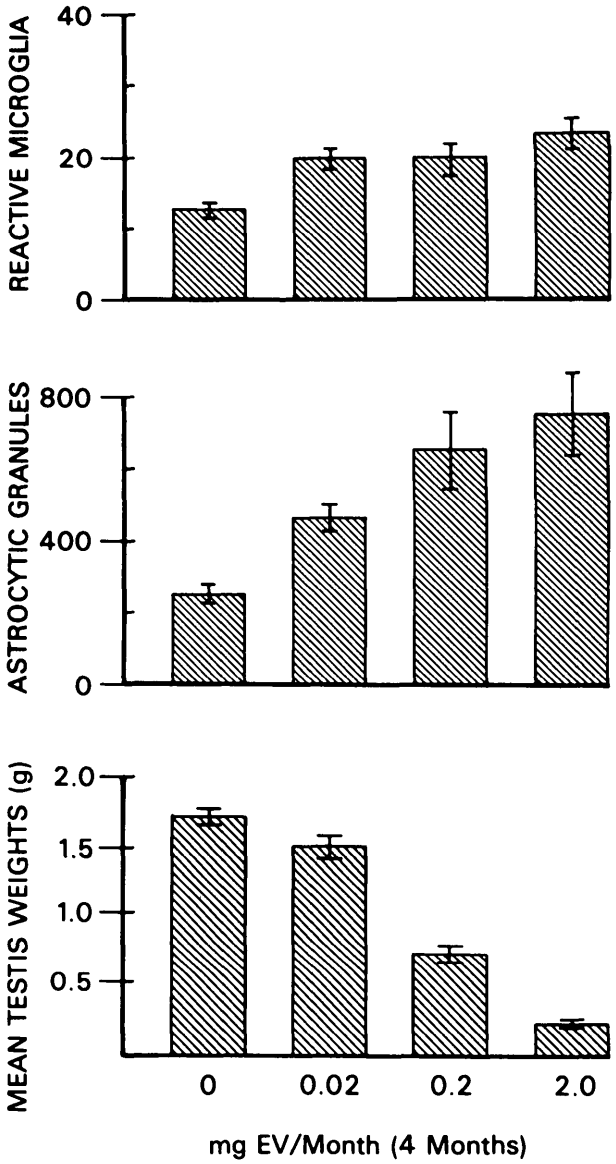

FIG. 3. Dose-response relationship in the male. The *top two histograms* show the microglial and astrocytic responses to different monthly doses of EV in the male. The *bottom histogram* shows the effect of these doses on the mean testis weight. n = 4 for the control (zero dose); n = 3 for all other dose categories.

Figure 3 also shows the effect of different doses of EV per month on mean testis weight. Although the maximum astrocytic response was produced at 0.2 mg EV/month, 2.0 mg/month produced yet a further reduction in the mean testis weight. The correlation coefficient for the testis weight dose-response histogram was 0.97, which was highly significant ($P < 0.05$).

Discussion

Glial indices

In the present study, microglial and astrocytic activities have been used as an index of a neuropathological

process in the arcuate nucleus. These glial parameters are widely used in neuropathology as dependable, albeit indirect, indications of neuronal damage. Microglial activity is a particularly reliable parameter, since the granules contained within these cells represent phagocytized debris. The applicability of the microglial marker to the EV lesion is further substantiated by electron microscopic observations of degenerating axons, axon terminals, and dendrites in the immediate vicinity of and often partially sequestered by engorged microglia (1, 2). Consequently, glial appearance, particularly microglial activity, undoubtedly reflects neuronal damage in this model as it does in other systems.

The significance of the rather unusual astrocytic reaction is somewhat more ambiguous. There is evidence that the astrocytic granules may contain endogenous peroxidase (12–14), the role of which is unclear in this system. Furthermore, there is no direct morphological association between astrocytic activity and neuronal debris as there is in the case of the microglial cells. It is possible that astrocytes may respond directly to estradiol. This could explain the different responses of astrocytes and microglial cells in the EV-treated females that did not exhibit persistent estrus. Although the single injection of estradiol may have produced insufficient neuronal damage to trigger a microglial response (and persistent estrous), it may have exerted a direct and irreversible effect on the astrocytes. Supporting this possibility is the observation that male and gonadectomized female rats receiving a single injection of EV showed greater astrocytic activity after 4 months than did noninjected controls (Brawer, J. R., H. Schipper, and F. Naftolin, unpublished results). Microglial activity in both of these groups was within the control range. The failure of some females given EV to exhibit persistent estrus has been reported previously (2).

Changes in granule-containing glia of the hypophysiotropic hypothalamus during sexual maturation have been reported in the rat (15). Goldgefter (16) observed a decrease in glial granule clusters in ovariectomized rats in response to estradiol treatment [in contrast to the findings of Kroon and Goosens (17)]. It should be emphasized, however, that Goldgefter examined the response of glia to estradiol 6–16 h after the administration of the steroid, as opposed to our observations made months after injection. Furthermore, in none of these studies was it clear which glial cell type exhibited granules. Finally, aside from Brawer and Sonnenschein (1), no one has correlated alterations in glial granule content with neuronal degeneration.

Functional significance

The functional significance of the EV-induced arcuate lesion was assessed in a previous study (3). It was found that the LH surge after electrochemical stimulation of the MPOA was significantly reduced in EV-treated rats with histologically identifiable glial reactions in the arcuate nucleus. Pituitary responsiveness tested with a LHRH analog was also somewhat reduced, but this decline by itself was insufficient to account for the change observed after MPOA stimulation. This suggests that the histologically identifiable lesion in EV-treated animals reflects, at least in part, a disconnection between the MPOA and the MBH, resulting in acyclicity characterized by features similar to those produced by surgical anterior deafferentation of the MBH (4–6).

The possibility exists, however, that the reduced LH surge after preoptic stimulation in EV-lesioned animals may be the result of an indirect effect of the altered steroid milieu.

It was noted previously that the arcuate lesion and the persistent estrus syndrome became evident weeks after an injection of 2 mg EV. At this time, plasma E_2 levels were only moderately elevated (2). This indicates that either the pathological process once initiated by the EV proceeds independently of any other influence or that the pathological process develops as a result of constant exposure to relatively low concentrations of plasma E_2. In the present study, constant light exposure, a condition which produces stable plasma E_2 concentrations of only about 10 pg/ml (18), was as effective in producing the arcuate lesion as was the EV injection. Furthermore, ovariectomy before either treatment significantly reduced or eliminated the arcuate reaction. These findings indicate that the arcuate nucleus of the adult female rat is exquisitely sensitive to an ovarian product which is neurotoxic to specific target neurons. That this ovarian product may be E_2 is suggested by the EV dose-response curve in the male (Fig. 3). It is also noteworthy that the region of degeneration is particularly rich in E_2-binding sites (19, 20). It may be, therefore, that specific connections subserving sexual cyclicity are susceptible to E_2 in any concentration in the adult female rat.

The fact remains, however, that neither the results presented here nor those of previous studies (1–3) constitute direct evidence that E_2 is the primary pathogen.

Aging

The sensitivity of the arcuate nucleus to neurotoxic effects of an ovarian product (possibly E_2) in the adult female rat may help to clarify the process of age-related anovulation. It has long been known that early age-associated reproductive failure in female rats is primarily a hypothalamic event. Most female rats exhibit persistent estrus at an age at which the pituitaries and ovaries are fully capable of responding to their characteristic hormonal stimuli (21–23). Furthermore, sexually senescent rats in persistent estrus will cycle in response to injections of norepinephrine (24).

It has been previously suggested that this hypotha-

lamic aging phenomenon may be reflected in the age-associated increase in reactive gliosis in the arcuate nucleus (2). The role of an ovarian product in the development of age-associated anovulation has been demonstrated by Aschheim (25). He noted that if young cyclic rats are ovariectomized and allowed to age for 24 months, cyclicity will resume after implantations of ovarian grafts from senescent rats. Normally, the majority of intact rats exhibit senile persistent estrus by 24 months, suggesting that the chronic absence of an ovarian product (presumably estrogen) in Aschheim's model delayed hypothalamic aging, thereby permitting cyclicity of the ovarian graft.

In the present study, reactive gliosis of the arcuate nucleus is invariably associated with anovulation and is also ovary dependent. The arcuate nuclei of old anovulatory animals are histologically indistinguishable from those of animals in persistent estrus as a result of EV injection or constant light exposure (Brawer, J. R., H. Schipper, and F. Naftolin, unpublished results). Furthermore, in animals that were ovariectomized for 4 months, there were significantly fewer reactive microglial cells than in normally cycling controls of the same age (Fig. 2). It seems, therefore, that both functional and histological manifestations of hypothalamic aging are, at least in part, a function of regular exposure to ovarian E_2. It may be that each preovulatory surge of E_2 destroys a fraction of the neuronal connections responsible for cyclicity. After a critical number of such exposures, there may not be sufficient afferent connections left to sustain cyclicity, and persistent estrus ensues.

It would appear that estrogen may exert direct control over the hypophysiotropic circuitry throughout life. In the neonatal animal, E_2 appears to influence sexual differentiation through a synaptogenic effect (26, 27). Likewise, the onset of puberty may be correlated with an E_2 facilitation of synapse formation in the MBH (27). In the adult animal, however, E_2 appears to exert a dystrophic effect, resulting in degeneration of specific neuronal elements involved in the regulation of the cyclic drive.

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