0013-7227/92/1321-0086$03.00/0
Endocrinology

Vol. 132, No. 1
Printed in U.S.A.

Estradiol Is Selectively Neurotoxic to Hypothalamic β-Endorphin Neurons

G. CLARISSA DESJARDINS, JAMES R. BRAWER, AND ALAIN BEAUDET

Departments of Anatomy and Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada H3A 2B4

ABSTRACT

The neurotoxic effects of estradiol on hypothalamic arcuate neurons were examined in a model of chronic estrogenization induced by means of a single injection of estradiol valerate (EV). Eight weeks after EV treatment, a 60% decrease in the total number of β-endorphin-immunoreactive neurons was detected in the arcuate nucleus. In contrast, the numbers of neurotensin-, somatostatin-, and tyrosine hydroxylase-immunoreactive neurons were unchanged, suggesting that the effects of estradiol were selective for β-endorphin neurons. Further evidence for the selectivity of estradiol's actions was provided by RIAs indicating decreases in hypothalamic β-endorphin concentrations, but not in Met-enkephalin or neuropeptide-Y concentrations. Cell counts performed in Nissl-stained material using unbiased stereological methods revealed a reduction in the total number of neurons in the EV-treated group compared to that in the controls. The estimated number of neurons lost (~3500) corresponded precisely with the total number of β-endorphin neurons lost (~3600), as estimated using quantitative immunocytochemistry. These results confirm the selectivity of estradiol's effect on the β-endorphin cell population and demonstrate that the observed decrease in β-endorphin immunoreactivity reflects actual cell loss. The evidence indicates that the selective neurotoxic effect of estradiol on hypothalamic β-endorphin neurons contributes to reproductive senescence, suggesting that steroids may participate in disruption of the biological functions that they normally facilitate. (*Endocrinology* **132:** 86–93, 1993)

A SINGLE injection of estradiol valerate (EV) given to a normally cycling female rat initiates a progressive multifocal lesion throughout the hypothalamic arcuate nucleus (1). In addition to degenerating neuronal elements, the lesion foci within the arcuate nucleus contain reactive microglial cells as well as an unusual variety of reactive astrocytes, characterized by numerous peroxidase-positive dense inclusions (2, 3, 4). If the ovaries are removed before EV injection, this hypothalamic lesion does not occur, suggesting that the pathology results from exposure to endogenous gonadal steroids recorded after the original EV insult to the neuroendocrine axis (2). Indeed, we have shown that chronic exposure to physiological concentrations of estradiol, but not androgens (3) or progestins (5), generates the arcuate pathology in young gonadectomized male and female rats (3, 6). Furthermore, comparable lesions have been shown to occur spontaneously in female rats and mice in response to physiological levels of estradiol during the course of normal aging (4).

The pathological action of estradiol on the hypothalamus and its role in hypothalamic aging have been extensively investigated in a variety of estrogenized and aging rodent models (3, 7–9). Indeed, the contribution of estradiol to hypothalamic aging has been appreciated for at least 25 yr (10). As yet, however, the specific neuronal circuitry affected by the toxic action of estradiol is unknown. We report here, using quantitative neuroanatomical and stereological techniques, that chronic exposure to estradiol results in the selective destruction of 60% of β-endorphin neurons in the arcuate nucleus of the rat. This specific β-endorphin cell loss might underlie many of the neuroendocrine changes that occur during reproductive senescence and may serve as an excellent model with which to examine the role of β-endorphin in the central nervous system.

Received June 26, 1992.
Address all correspondence and requests for reprints to: G. C. Desjardins, Neurobiology Group, Montreal Neurological Institute, 3801 University Street, Montreal, Quebec, Canada H3A 2B4.

Materials and Methods

Animals

Animals in this study were treated according to the practices and procedures approved by McGill University's Policy on the Handling and Treatment of Laboratory Animals. Thirty-four Wistar female rats (Charles River Canada, St. Constant, Quebec, Canada) were housed in groups of 3 and maintained in a controlled environment, with free access to pelleted rat chow and water. After acclimatization, vaginal cyclicity was monitored daily by examination of vaginal smears. Only animals exhibiting normal estrous cycles were used in these experiments. At 8 weeks of age, 17 animals were anesthetized with ether and injected im with 2 mg EV dissolved in sesame oil (Delestrogen, Squibb, Princeton, NJ). This treatment has been shown to result initially in a supraphysiological serum concentration of estradiol, which, after 2 weeks, stabilizes at 20–30 pg/ml (1, 3). The remaining 17 animals served as age-matched controls. Eight weeks after the injection of EV, experimental and control animals were each divided into 3 groups to be processed for immunocytochemistry, RIA, and stereological estimates of total neuron numbers.

Preparation of tissue for immunocytochemistry

EV-injected (n = 5) and control rats (n = 5) were anesthetized with sodium pentobarbital (0.2 cc/100 g BW) and injected intracerebroventricularly with 60 μg colchicine dissolved in 30 μl 0.9% saline solution. Forty-eight hours later, animals were reanesthetized and killed by perfusion with 450 ml 4% paraformaldehyde solution in 0.12 M Sorensen buffer (NaH_2PO_4, Na_2HPO_4). After removal, brains were cryoprotected in a 30% sucrose solution for 24 h at 4 C, then rapidly frozen by immersion in isopentane (−55 C) for 20 sec and stored at −80 C. Brains were serially sectioned (30 μm) from the posterior chiasmatic area

rostrally to the mammillary bodies caudally. β-Endorphin-immunopositive neurons were counted in the first three adjacent sections of seven (*i.e.* 90 μm of each 210 μm sectioned). The remaining sections were processed for neurotensin (NT), somatostatin (SRIF), tyrosine hydroxylase (TH), and specificity controls.

Immunocytochemistry

All antisera used in this study were purchased from and characterized by commercial suppliers. The β-endorphin antibody, a rabbit antihuman β-endorphin (Immunocorps, Montreal, Quebec, Canada), was tested by incubating the diluted antiserum overnight with 100 μg/ml rat β-endorphin-(1–31) (Sigma). This preadsorption of the antibody resulted in a complete absence of immunostaining, as did replacement of the primary antibody with 1% normal goat serum (NGS). All other antisera, including TH (Eugene Tech, Ramsey, NJ) and NT and SRIF (Incstar, Stillwater, MN), were similarly raised in rabbits and tested by these respective companies. Free-floating sections were collected in Sorensen's buffer (0.12 M; pH 7.6). The sections were first incubated for 30 min in Tris-saline containing 0.1% sodium azide and 0.3% hydrogen peroxide for inactivation of endogenous peroxidases, then rinsed three times for 8 min each time in Tris-saline and incubated for 30 min in 1% NGS. After two 8-min rinses in Tris-saline, sections were incubated overnight at room temperature with primary antiserum at a concentration of 1:2000 for β-endorphin and 1:750 for TH, NT, and SRIF. The following day, sections were rinsed three times for 8 min each time in Tris-saline containing 0.2% BSA and 1% NGS, then incubated for 30 min with a goat antirabbit immunoglobulin G (Sternberger-Meyer Immunocytochemicals, Inc., Laval, Quebec, Canada). After two 8-min rinses in the same buffer, sections were incubated for 30 min with rabbit peroxidase-antiperoxidase (Sternberger-Meyer ICN), rinsed twice for 8 min each time in Tris-saline, and reacted with Tris-water containing 0.05 g 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and 3 mg H_2O_2 for 6 min. The sections were mounted on gelatin-coated slides, dehydrated and defatted in a graded series of alcohols, cleared in xylene, and coverslipped with Permount.

RIAs

Hypothalami were dissected from the brains using the optic chiasm and mammillary bodies as rostro-caudal borders, and the lateral recess of the hypothalamus as the lateral border. These were homogenized in 1 ml 1 N HCl for 30 sec using a Polytron (Brinkmann Instruments, Westbury, NY). Samples were centrifuged for 30 sec at 10,000 × *g*, and the supernatant was used for determination of the β-endorphin concentration. The β-endorphin antibody in the RIA (RIK 8626, Peninsula, Belmont, CA) is an rabbit antihuman β-endorphin that displays 80% cross-reactivity with rat β-endorphin. It does not cross-react with Met-enkephalin, γ-endorphin, or β-lipotropin. The neuropeptide-Y (NPY) antibody (RIK-7172, Peninsula) shows cross-reactivities with peptide-YY, vasoactive intestinal polypeptide, and avian pancreatic polypeptide of 0.003%, 0.001%, and 0.007%, respectively. The Met-enkephalin antibody (no. 18100, Incstar) displays 2.8% cross-reactivity with Leu-enkephalin and less than 0.003% cross-reactivities with α- and β-endorphin.

Morphometry and quantitation of immunoreactive cell number

Morphometric analysis was performed using a computer-assisted image analysis system (Biocom, Les Ulis, France). For all β-endorphin-immunostained sections, the location, number, surface area, long diameter, and form factor (defined as $4\pi \times \text{area} \div \text{perimeter}^2$) of immunoreactive neurons were recorded and stored automatically, using a polygonal transformation function that scans the digitized image of the section and delineates structures within a defined grey level interval. The parameters determined for this automatic polygonal transformation (*i.e.* grey level, step, luminosity, and contrast) were kept constant for the entirety of the analysis and between control and EV-treated animals. The mean number of neurons counted (automatically) in three adjacent 30-μm sections was plotted on a rostro-caudal axis. Estimates of the total numbers of β-endorphin-immunopositive neurons for each animal were obtained by interpolation of neuron numbers between measured points and by summing the measured and interpolated values, as described by Shivers *et al.* (11).

In the case of NT-, SRIF-, and TH-immunostained sections, immunopositive neurons were identified by the observer and counted automatically on the image analysis system. The mean number of neurons counted in one 30-μm section of every seven sections was plotted on a rostro-caudal axis. In addition, a subpopulation of TH-immunopositive neurons chosen at random in control and EV-treated animals was outlined manually so as to automatically obtain data on TH neuron surface area, long diameter, and form factor.

Estimate of the total number of neurons in the arcuate nucleus

For estimates of total arcuate neuron numbers, 5 EV-injected and 5 control rats were used. Fresh (unfixed) brains were snap-frozen in isopentane (−54 C) and serially sectioned at 30 μm on a cryostat (−17 C). Sections from control and EV-treated rats were stained in parallel with cresyl violet. Neuron density (Nv) was determined using the optical dissector method described by West and Gundersen (12). In 1 of each 10 30-μm sections, the arcuate and periarcuate areas (including the arcuate nucleus and ventrolateral periarcuate region) were outlined at low power on an image analysis system that calculates the area outlined automatically (Biocom). At high power, a window of appropriate dimensions ($A = 400\ \mu m^2$) was generated, and the number of neurons or glia (Q) coming into focus within the thickness of the section (t) was counted. Once these measurements had been repeated over the sampled surface using a systematic sampling procedure, an unbiased estimation of neuron density was obtained using the formula $Nv = Q/t \times A$. Estimates of total neuron numbers were obtained by multiplying Nv with the volume of the arcuate nucleus, estimated using the principle of Cavalieri (12). According to this principle, an unbiased estimate of volume V may be obtained from "the sum of the areas (a_i) of the individual profiles of the object on a set of n systematically positioned parallel sections through the object that are separated by a known constant, t ($V = t \times \sum a_i$). The procedure for systematic sampling was devised in a pilot study, such that the total error contributed by each sampling level [*i.e.* the level of individual animals, sections, field of vision (window), and number of neurons counted within each field] to the estimation of total neuron numbers was less than 7%, *i.e.* to what can reasonably be expected to be derived from true biological variation (12).

Statistics

Results derived from the quantitation of immunoreactive cells in control and EV-treated animals were compared by Student's t test, as were those from the RIAs. The precision of the stereological estimates of total neuron numbers was analyzed as described in West and Gundersen (12), using a modified analysis of variance. Based on the formula, $OCV^2 = CV^2 + OCE^2$ for adjacent sampling levels, where OCV is the observed relative variance at one sampling level (*i.e.* SD/mean), CV is the true relative variation (*i.e.* unknown), and OCE is the computable variation of the stereological estimate (CE; see p. 18 of Ref. 12). The true biological variance (CV), *i.e.* that contributed by the estradiol exposure, accounted, on the average, for more than 85% of the observed variability among groups.

Results

In control animals, an abundance of β-endorphin-immunoreactive neurons was observed distributed along the ventrolateral aspect of the mediobasal hypothalamus within the arcuate and periarcuate regions, as previously described (Fig. 1a) (13–16). In sharp contrast, EV-treated animals exhibited a considerably smaller number of β-endorphin-immunoreactive cells (Fig. 1b). The few immunopositive neurons detected were small and shrivelled, and often exhibited tortuous and swollen dendrites, indicative of neuronal degen-

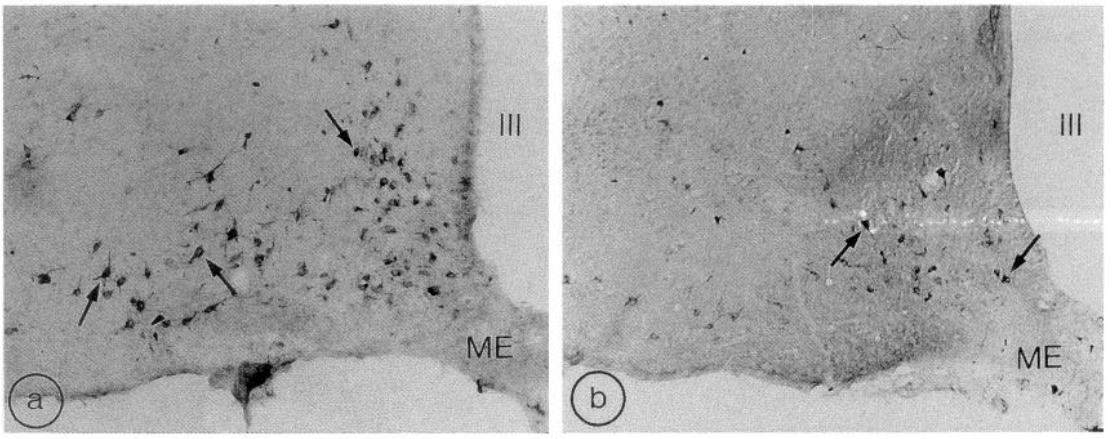

FIG. 1. Immunocytochemically labeled β-endorphin neurons in the arcuate and periarcuate region of control (a) and EV-treated (b) rats. β-Endorphin-immunoreactive neurons were drastically reduced in the EV-treated group even though darkly stained cells were still apparent (*arrows*). III, Third ventricle; ME, median eminence. Magnification, ×12.5.

eration (Fig. 1b). However, a small subset of the spared β-endorphin cells located in the lateral periarcuate region was comprised of larger neurons that appeared not to be damaged by EV treatment.

Computer-assisted quantitation of β-endorphin-immunopositive neurons throughout the entire rostro-caudal extent of the arcuate nucleus revealed an average 60% reduction in EV-treated animals compared to controls ($P < 0.001$ to $P < 0.05$ at each rostro-caudal level; Fig. 2). The total number of β-endorphin neurons estimated in controls was 5523 ± 820 (mean ± SEM), whereas in EV-treated animals, this value was 1992 ± 480 ($P < 0.01$), indicating a net immunoreactive cell loss of approximately 3500 β-endorphin neurons. Reductions in the number of β-endorphin-immunopositive neurons were most pronounced in the caudal two thirds of the arcuate nucleus (Fig. 2) and in its most medial aspect.

Morphometric analysis of β-endorphin neurons is summarized in Table 1. EV-treated animals displayed significant reductions in mean neuron form factor compared to controls. Although a slight trend toward reduced mean surface area and long diameter was also detected in the EV-treated group, this value did not reach statistical significance. Analysis of the frequency distribution profiles of β-endorphin cell surface area and long diameter in control and EV-treated groups indicated a relative sparing of larger neurons in the latter group (data not shown).

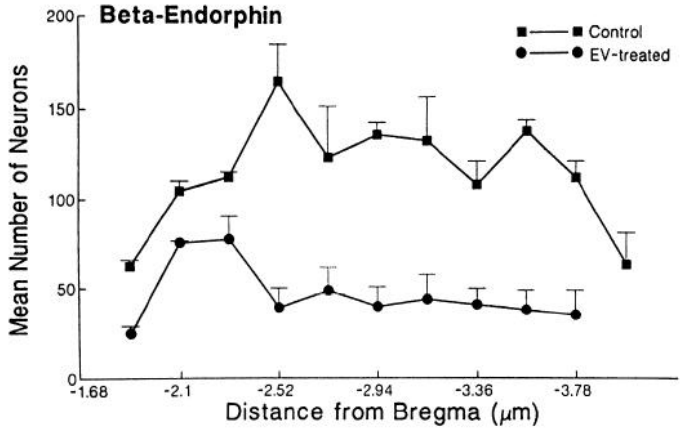

FIG. 2. Rostro-caudal distribution of mean β-endorphin-immunoreactive neurons in control and EV-treated rats. Mean β-endorphin neurons were plotted on a rostro-caudal axis by comparison of section level to the rat brain atlas of Paxinos and Watson (45). EV-treated animals displayed significant reductions in β-endorphin neurons at every level of the arcuate nucleus, with an average 60% drop in mean β-endorphin neurons ($P < 0.05$ to $P < 0.001$).

Long term exposure to estradiol did not produce any significant change in the mean number of NT-, SRIF-, or TH-immunoreactive neurons counted throughout the arcuate nucleus (Fig. 3). However, both SRIF and TH neuronal populations stained more intensely and exhibited a trend toward increased numbers after EV treatment (Figs. 3 and 4). At level −2.82 μm from the bregma, TH-immunoreactive neuron numbers were significantly increased relative to control values ($P < 0.05$). Morphometric analysis of TH-immunoreactive neurons indicated that after EV treatment, these neurons exhibited significant increases in cell surface area (76.2 ± 1 *vs.* 89.8 ± 1; $P < 0.001$; n = 4) as well as in neuron form factor (0.528 ± 0.002 *vs.* 0.544 ± 0.002; $P < 0.05$) compared to controls.

As shown in Table 2, β-endorphin concentrations, as determined by RIAs, were reduced by more than 60% in the EV-treated group compared to those in untreated controls. In contrast, hypothalamic concentrations of Met-enkephalin and NPY were unchanged in the EV-treated group with respect to controls (Table 2).

Unbiased stereological measurements of mean arcuate vol-

TABLE 1. Effect of EV treatment on morphometric parameters of arcuate β-endorphin neurons

	Control	EV-treated
Surface area (μm²)	84.6 ± 2	80.9 ± 1
Long diameter (μm)	16.9 ± 1	15.7 ± 1
Form factor	0.52 ± 0	0.45 ± 0[a]

Values are expressed as the mean ± SEM (n = 5).
[a] Significantly different from controls, $P < 0.001$.

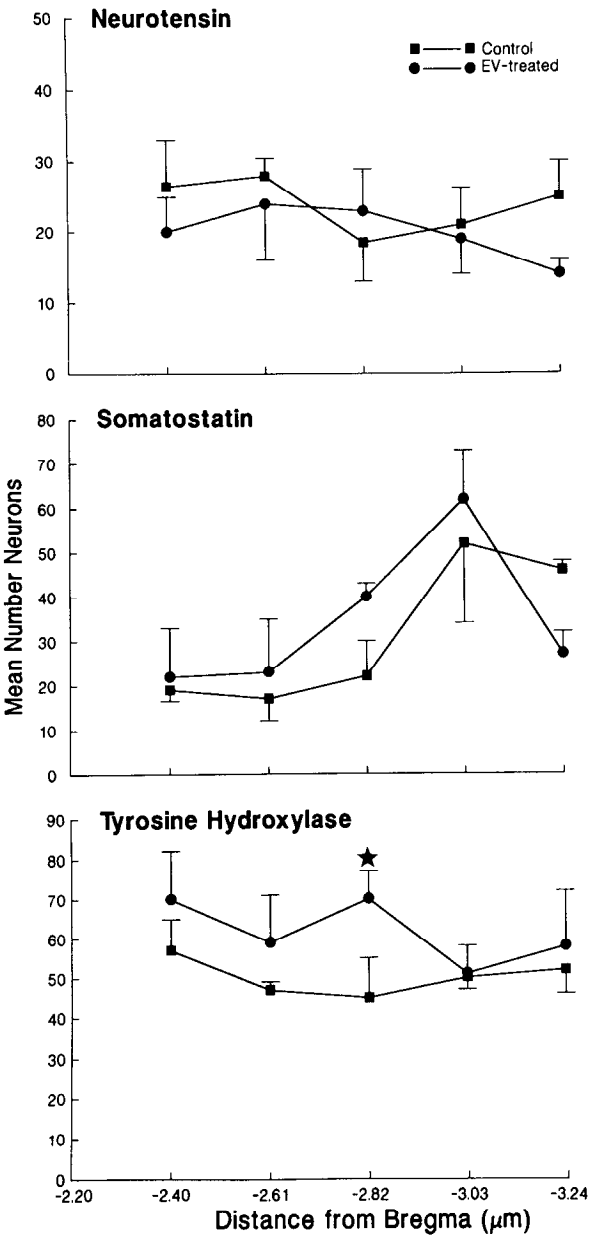

FIG. 3. Rostro-caudal distribution of mean NT-, SRIF-, and TH-immunoreactive neurons in control and EV-treated rats. Neither SRIF, NT, nor TH neuronal populations displayed significant reductions after EV treatment. SRIF- and TH-immunoreactive neurons exhibited a trend toward increased numbers of neurons in the EV-treated animals, although this did not reach significance. Only at one level of the arcuate nucleus of EV-treated rats were TH-immunoreactive neurons more numerous than in controls (*, $P < 0.05$).

ume, neuron density, and total number of neurons are summarized in Table 3. EV-treated animals exhibited slightly smaller volumes of the arcuate/periarcuate region than did untreated controls. Neuronal density (expressed as the mean number of neurons per mm^3) was reduced in EV-treated animals compared to controls, although this did not reach statistical significance. Total neuron number estimations revealed a loss of 3500 neurons throughout the arcuate region after EV treatment. EV treatment did not significantly affect glial cell density or total glial cell numbers at this time point.

Discussion

Long term exposure to physiological levels of estradiol has been shown to induce a permanent lesion in the hypothalamic arcuate nucleus of rats and mice (1–4). The present findings demonstrate that exposure to high physiological/low pharmacological concentrations of estradiol initiated by EV treatment results in the selective destruction of β-endorphin neurons within the arcuate nucleus, while sparing other coextensive neuronal populations.

The mean number of β-endorphin-immunoreactive neurons estimated in the arcuate nucleus of controls was 5523 ± 820 compared to 1992 ± 483 in EV-treated animals, indicating an average loss of approximately two thirds of all immunoreactive neurons, or 3500 neurons. This decline in the population of β-endorphin-immunoreactive cells parallels the overall 60% reduction in β-endorphin concentration in hypothalamic homogenates. In contrast, none of the other neurotransmitter/peptide populations examined in the hypothalamus was found to be affected by EV treatment. Concentrations of NPY and Met-enkephalin, two peptides involved in the control of reproductive function, were similar in hypothalamic homogenates from control and EV-treated rats. Also, in sections adjacent to those labeled for β-endorphin, NT-, SRIF-, and TH-immunoreactive neuronal populations were not decreased after EV treatment. Both TH and SRIF neurons were, if anything, more numerous and more intensely labeled than in controls. Although these changes only reached statistical significance in the case of TH neurons at one rostro-caudal level, there was a significant increase in mean cell surface area and neuronal form factor of TH-immunoreactive cells in the EV-treated group compared to those in controls, suggesting that EV treatment exerted stimulatory effects on TH immunoreactivity. These changes may be the result of a disinhibition of dopamine neurons secondary to the loss of β-endorphin. Indeed, β-endorphin has been shown to inhibit the synthesis (17, 18), turnover (18, 19), and release (18) of dopamine from the hypothalamus. Further studies are needed to determine whether SRIF neurons are similarly disinhibited by the loss of β-endorphin. In any event, the absence of reductions in all neuropeptide populations examined concomitant with pronounced decreases in β-endorphin concentrations strongly suggests that estradiol's deleterious effects are selectively targeted to β-endorphin neurons.

β-Endorphin-immunoreactive neurons in the EV-treated group were more spiny and more tortuous than those in control animals, as evidenced by their significant reductions in form factor. This finding supports the view that β-endorphin neurons are in the process of degeneration and that the decreases in β-endorphin-immunoreactive neuron number and hypothalamic concentrations of β-endorphin observed after EV treatment reflect actual cell loss and not merely reductions in peptide expression. To confirm this interpretation, arcuate neuronal density and total neuron numbers were estimated using the optical dissector method described

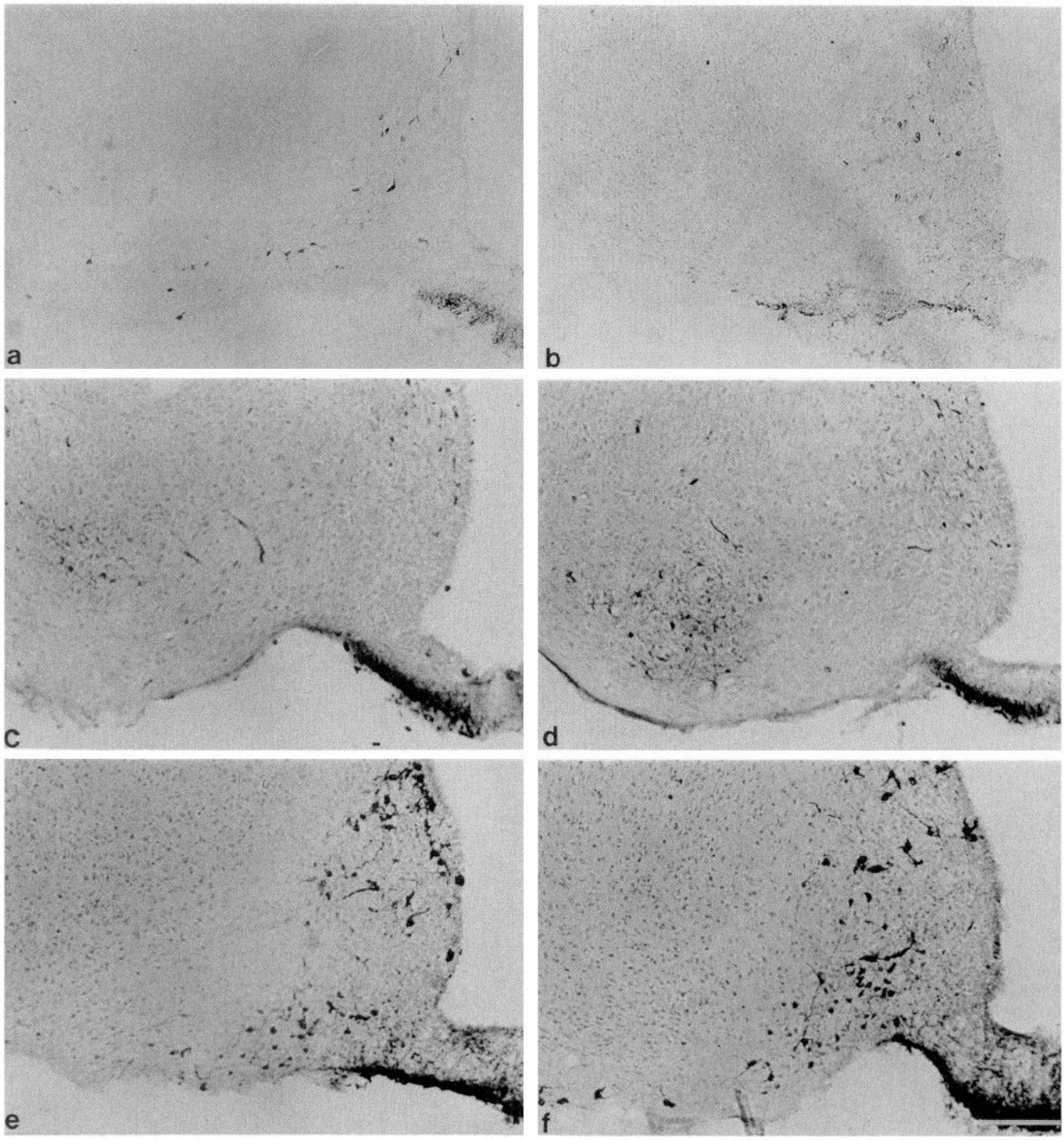

FIG. 4. Immunocytochemically labeled NT (*top*)-, SRIF (*middle*)-, and TH (*bottom*)-immunoreactive neurons in control (a, c, and e) and EV-treated (b, d, and f) rats. Neither NT-, SRIF-, nor TH-immunoreactive staining was reduced after EV treatment. In fact, both SRIF and TH neurons appeared somewhat more darkly stained in the EV-treated group than in controls. *Scale bar*, 350 μm.

by West and Gundersen (12). In control animals, mean neuron number (coefficient of error) was 23,829 (0.07), in conformity with what has previously been reported on the basis of Golgi staining (20) or classical stereology (21). After EV treatment, the mean number of neurons was reduced to 20,249 (0.07), paralleling what has been reported in aged rats (20). Furthermore, the absolute mean decrease in the number of neurons in the arcuate nucleus of EV-treated animals was equivalent to the mean loss of β-endorphin-immunopositive neurons after EV treatment (*i.e.* 3500 neurons). Although some of the laterally located neurons may have fallen beyond the area circumscribed for our volumetric calculations, these appear to be relatively spared by the EV treatment compared to those located in the arcuate nucleus proper and, thus, are unlikely to affect the comparison of total arcuate neuron numbers with total β-endorphin neu-

TABLE 2. Effect of EV treatment on radioimmunoassayable β-endorphin, Met-enkephalin, and NPY in rat hypothalami

	β-Endorphin (pg/mg)	Met-Enkephalin (pg/mg)	NPY (pg/mg)
Control	112 ± 8.9	2892 ± 287	354 ± 23
EV-treated	46 ± 8.2[a]	3178 ± 275	324 ± 16

Values are expressed as the mean ± SEM (picograms per mg wet wt; n = 7).

[a] Significantly different from controls, $P < 0.01$.

rons lost. Taken together, these data indicate that estradiol is selectively neurotoxic to arcuate β-endorphin neurons.

The reductions in neuron form factor observed in the β-endorphin population remaining after EV treatment suggests that at longer time intervals (*i.e.* >8 weeks), a greater number of β-endorphin neurons are likely to degenerate; this conforms to previous evidence demonstrating that the EV-induced lesion is progressive (2, 3). Since virtually all β-endorphin projections in the central nervous system originate in the arcuate nucleus (13, 14), EV-treated animals may serve as an excellent model to examine the effects of selective β-endorphin depletion.

The mechanism underlying the pathogenic effect of estradiol on hypothalamic β-endorphin cells is unknown. A unique feature of the arcuate nucleus which may account for its susceptibility to estradiol neurotoxicity is the presence of unusual peroxidase-positive astrocytes that are highly sensitive to circulating estradiol levels (1, 6). These astrocytes, identified in both rodent and human brains (22), may transform catechol estrogens (2- and 4-hydroxyestrogen), generated spontaneously in the brain from circulating estradiol (23), to *o*-semiquinone free radicals (24). These free radicals would, in turn, cause lipid peroxidation of neuronal membranes and eventually lead to cell death (25). In support of an etiological role for free radicals in the present model, we have shown that treatment with vitamin E, a potent antioxidant, prevents EV-induced β-endorphin loss (26). The unique vulnerability of β-endorphin neurons could result from a variety of factors, including a selective insufficiency in free radical-scavenging enzymes or a unique proximity to free radical-generating peroxidase-positive astrocytes (3).

Selective estradiol-induced destruction of the arcuate β-endorphin system provides a conceptual framework uniting diverse observations on the neuroendocrine consequences of long term estradiol exposure. We have shown that the loss of hypothalamic β-endorphin neurons observed in EV-treated animals results in a marked increase in μ-opioid binding in the medial preoptic area (MPOA) (27, 28), a region rich in LHRH neurons (11). This suggests that the estradiol-induced loss of β-endorphin evokes a compensatory up-regulation of μ-opioid receptors on target neurons/terminals in the MPOA, rendering them supersensitive to the inhibitory action of either residual β-endorphin or other endogenous opioids (*i.e.* Met-enkephalin). This interpretation is supported by the observation of μ-opioid-binding site up-regulation in animals in which the arcuate nucleus had been destroyed by neonatal monosodium glutamate treatment (28). The subsequent opioid supersensitivity could account for the suppressed pattern of LH release and acyclicity observed in EV-treated rats. Indeed, treatment with the opioid antagonist naltrexone reinitiates cyclicity and normal ovarian morphology in these animals (29). The supersensitivity of the neuroendocrine axis to the inhibitory action of endogenous opioids in the EV-treated rat may parallel events occurring in the aging female rodent. Decrements in hypothalamic β-endorphin concentrations (8, 30–34), diminution in POMC mRNA (35, 36), and loss of β-endorphin neurons (37) have all been associated with aging in female rodents. However, despite these decreases in β-endorphin parameters, the aging hypothalamus has been shown to be supersensitive to the inhibitory action of endogenous opioids (38). Indeed, LH secretion, which is inhibited by opioids, declines with age (36, 39).

In aged animals, this suppressed pattern of LH release may be temporarily reversed by a variety of treatments, including opioid antagonists (38), dopamine receptor antagonists, and adrenergic agonists (40, 41). These and other observations have prompted the suggestions that age-induced suppression of LH is a result of decreased stimulation (from facilitatory systems such as noradrenaline) or, conversely, that it is due to increased inhibition due to increased dopaminergic and/or serotonergic tone. The infusion of opioids in the MPOA has been shown to reduce the turnover

TABLE 3. Unbiased stereological estimates of total neuron and glial cell numbers in the arcuate nucleus of control and EV-treated rats

	Arcuate vol (mm^3)	Neurons		Glia	
		Nv (n/mm^3)	N	Gv (g/mm^3)	G
Controls	0.136 (0.05)	1.8371×10^5	23,820 (0.07) CV 0.123 BV 0.101	0.7592×10^5	10,672 (0.07) CV 0.077 BV 0.030
EV-treated	0.123 (0.04)	1.6371×10^5	20,249 (0.07) CV 0.148 BV 0.130	0.7466×10^5	9,140 (0.09) CV 0.090 BV 0.050

Cresyl violet-stained sections from control and EV-treated rats were used for the estimation of total neuron numbers within the arcuate nucleus. Briefly, neuron density (Nv) was determined using the optical dissector method described by Gundersen *et al.* (0). At high power, a window of appropriate dimensions (A = 400 μm^2) was generated on the computer screen, and the number of neurons (or glia) coming into focus within the thickness of the section (t) were counted (Q). These measurements were repeated over the sampled surface of the arcuate using a systematic sampling procedure that resulted in less than 7% coefficient of error. An unbiased estimation of neuron (or glial) density was obtained using the formula Nv = Q/TXA. Estimates of total neuron numbers were obtained by multiplying the average neuron density with the total volume of the arcuate nucleus for each animal. The total observed variance among groups was then compared to the variance contributed by the sampling procedure to ascertain what proportion of change was due to true biological variance (BV). In both control and EV-treated groups, the biological variance accounted for more than 85% of the observed variance. CV, Coefficient of variation; N, neurons; Gv, glia per volume; G, glia.

of noradrenaline and increase the turnover of dopamine and serotonin (42, 43). Thus, a single feature shared by all of these treatments is that they temporarily override the effects of opioid supersensitivity and the subsequent diminution in LH secretion. None of these treatments reestablishes normal cyclicity in senescent rats for extended periods of time, however, suggesting that they affect events secondary to the putative irreversible estradiol-induced hypothalamic damage.

The present results contribute to the novel concept that in addition to their well established physiological roles, steroids act as selective neurotoxins. The well characterized neurotoxic effects of chronic glucocorticoid exposure on hippocampal neurons has been shown to decrease the negative feedback regulation of the pituitary-adrenal response to stress (44). Similarly, the chronic estradiol-induced destruction of hypothalamic β-endorphin neurons demonstrated in the present study results in functional disconnection of the neuroendocrine loops regulating reproductive cyclicity (29). Steroids, thus, appear to participate in the disruption of the biological functions that they normally facilitate.

References

1. **Brawer JR, Naftolin F, Martin J, Sonnenshein C** 1978 Effects of a single injection of estradiol valerate on the hypothalamic arcuate nucleus and on reproductive function in the female rat. Endocrinology 103:501–512
2. **Brawer JR, Schipper H, Naftolin F** 1980 Ovary-dependent changes in the hypothalamic arcuate nucleus. Endocrinology 107:274–279
3. **Brawer JR, Schipper H, Robaire B** 1983 Effects of long-term androgen and estradiol exposure on the hypothalamus. Endocrinology 112:194–199
4. **Schipper HM, Brawer JR, Nelson JF, Felicio LS, Finch CE** 1981 Role of the gonads in histologic aging of the hypothalamic arcuate nucleus. Biol Reprod 25:413–419
5. **Schipper HM, Piotte M, Brawer J** 1990 Effects of progestins on the age-related accumulation of astrocytic granules in the hypothalamic arcuate nucleus. Brain Res 527:176–179
6. **Schipper HM, Lechan RM, Reichlin S** 1990 Glial peroxidase activity in the hypothalamic arcuate nucleus: effects of estradiol-valerate induced persistent estrus. Brain Res 507:200–207
7. **Kohama SG, Anderson CP, Osterburg HH, May PC, Finch CE** 1989 Oral adminsitration of estradiol to young C57BL/6J mice induces age-like neuroendocrine dysfunctions in the regulation of estrous cycles. Biol Reprod 41:227–232
8. **Forman LJ, Marquis DE, Stevens R** 1985 The effect of chronic estrogen treatment on immunoreactive beta-endorphin levels in intact female rats. Proc Soc Exp Biol Med 179:365–372
9. **Brawer JR, Munoz M, Farookhi R** 1986 Development of the polycystic ovarian condition (PCO) in the estradiol valerate-treated rat. Biol Reprod 35:647–655
10. **Ascheim P** 1964 Resultats fournis par la greffe heretrochrone des ovaires dans l'etude de la regulation hypothalamo-hypophyso-ovarienne de la ratte senile. Gerontologia 10:65–75
11. **Shivers BD, Harlan RE, Morrell JI, Pfaff DW** 1983 Immunocytochemical localization of luteinizing hormone-releasing hormone in male and female rat brains. Neuroendocrinology 36:1–12
12. **West MJ, Gundersen HJG** 1990 Unbiased stereological estimation of the number of neurons in the human hippocampus. J Comp Neurol 296:1–22
13. **Bloom F, Battenberg E, Rossier J, Ling N, Guillemin R** 1978 Neurons containing beta-endorphin in rat brain exist seperately from those containing enkephalin: immunocytochemical studies. Proc Natl Acad Sci USA 75:1591–1595
14. **Watson SJ, Richard CW, Barchas JD** 1978 Adrenocorticotropin in rat brain:immunocytochemical localization in cells and axons. Science 200:1180–1182
15. **O'Donohue TL, Miller RL, Jacobowitz DM** 1979 Identification, characterization and stereotaxic mapping of intraneuronal alpha-melanocyte stimulating hormone-like immunoreactive peptides in discrete regions of the rat brain. Brain Res 176:101–123
16. **Mezey E, Kiss JZ, Mueller GP, Eskay R, O'Donohue TL, Palkovitz M** 1985 Distribution of pro-opiomelanocortin derived peptides, adrenocorticotrope hormone, alpha-melanocyte-stimulating hormone and beta-endorphin in the rat hypothalamus. Brain Res 328:341–347
17. **Alper RH, Demarest KT, Moore KE** 1980 Morphine differentially alters synthesis and turnover of dopamine in central neuronal systems. J Neural Transm 48:157–165
18. **Reymond MJ, Porter JC** 1983 A role for morphine on the release of dopamine into hypothalamic pituitary blood and on synthesis of dopamine in tuberoinfundibular neurons. Brain Res 262:253–258
19. **Van Loon GR, Ho D, Kim C** 1980 Beta-endorphin induces decreases in hypothalamic dopamine turnover. Endocrinology 106:76–80
20. **Szentagothai J, Flerko B, Mess B, Halasz B** 1968 Hypothalamic Control of Anterior Pituitary, an Experimental-Morphological Study. Akademiai Kiado, Budapest, pp 40–68
21. **Hsu HK, Peng MT** 1978 Hypothalamic neuron number of old female rats. Gerontology 24:434–440
22. **Schipper HM** 1991 Gomori-positive astrocytes: biological properties and implications for neurologic and neuroendocrine disorders. Glia 4:365–377
23. **Liehr JG, Roy D** 1990 Free radical generation by redox cycling of estrogens. Free Radical Biol Med 8:415–423
24. **Schipper HM, Kotake Y, Janzen E** 1991 Catechol oxidation by peroxidase-positive astrocytes in primary culture: an electron spin resonance study. J Neurosci 11:2170–2176
25. **Tappel AL** 1973 Lipid peroxidation damage to cell components. Fed Proc 32:1870–1874
26. **Desjardins CG, Beaudet A, Schipper HM, Brawer J**, Vitamin E protects against estradiol-induced hypothalamic beta-endorphin loss. 74th Annual Meeting of The Endocrine Society, San Antonio, TX, 1992 (Abstract 1523)
27. **Desjardins GC, Beaudet A, Brawer JR** 1990 Alterations in opioid parameters in the hypothalamus of rats with estradiol-induced polycystic ovarian disease. Endocrinology 127:2969–2976
28. **Desjardins GC, Brawer J, Beaudet A**, Monosodium-induced reductions in hypothalamic beta-endorphin content result in mu opioid receptor upregulation in the medial preoptic area. Annual Meeting of the Third International Brain Research Organization (IBRO), Montreal, Quebec, Canada, 1992, p 106 (Abstract P60.23)
29. **Carriere PD, Farookhi R, Brawer JR** 1989 The role of aberrant hypothalamic opiatergic function in generating polycystic ovaries in the rat. Can J Physiol Pharmacol 67:896–901
30. **Barden N, Dupont A, Labrie F, Merand Y, Rouleau D, Vauchy H, Roissier JR** 1981 Age-dependent changes in the beta-endorphin content of discrete rat brain nuclei. Brain Res 208:209–212
31. **Forman LJ, Sonntag WE, Van Vugt DA, Meites J** 1981 Immunoreactive β-endorphin in the plasma pituitary and hypothalamus of young and old male rats. Neurobiol Aging 2:281–284
32. **Dorsa DM, Smith ER, Davidson JM** 1984 Immunoreactive beta-endorphin and LHRH levels in the brains of aged male rats with impaired sex behavior. Neurobiol Aging 5:115–120
33. **Rogers J, Shoemaker WJ, Morgan DG, Finch CE** 1985 Senescent changes in tissue weight and immunoreactive beta-endorphin, enkephalin, and vasopressin in eight regions of C57BL/6J mouse brain and pituitary. Neurobiol Aging 6:1–9
34. **Wardlaw SL, Thoron L, Frantz AG** 1982 Effects of sex steroids on brain beta-endorphin. Brain Res 245:327–331
35. **Nelson JF, Bender M, Schachtner BS** 1988 Age-related changes in proopiomelanocortin meessenger ribonucleic acid levels in hypothalamus and pituitary of female C57BL/6J mice. Endocrinology 123:340–344
36. **Wise PM, Scarbrough K, Larson GH, Lloyd JM, Weiland NG,**

Chiu S 1991 Neuroendocrine influences on aging of the female reproductive system. Front Neuroendocrinol 12:323–356

37. **Miller MM, Joshi D, Billiar RB, Nelson JF** 1991 Loss during aging of beta-endorphin neurons in the hypothalamus of female C57BL/6J mice. Neurobiol Aging 12:239–244
38. **Field EA, Kuhn CM** 1989 Opiate antagonist treatment reinstates estrous cycles in middle-aged persistent estrous rats. Biol Reprod 40:714–719
39. **Finch CE, Felicio LS, Mobbs CV, Nelson JF** 1984 Ovarian and steroidal influences on neuroendocrine aging processes in female rodents. Endoc Rev 5:467–497
40. **Clemens JA, Amenomori Y, Jenkins T, Meites J** 1969 Effects of hypothalamic stimulation, hormones and drugs on ovarian function in old female rats. Proc Soc Exp Biol Med 132:561–563
41. **Meites J** 1988 Neuroendocrine basis of aging in the rat. In: Everitt AV, Walton JR (eds) Regulation of Neuroendocrine Aging. Karger, Basel, pp 24:37–50
42. **Gopalan C, Gilamore DP, Brown CH, Wilson A** 1989 Effects of opiates on biogenic amine turnover in specific hypothalamic areas on the afternoon of pro-estrus in the rat-I. Catecholamines. Biogen Amines 6:597–606
43. **Gopalan C, Gilamore DP, Brown CH, Leigh A** 1989 Effects of opiates on biogenic amine turnover in specific hypothalamic areas on the afternoon of pro-estrus in the rat-II. Serotonin. Biogen Amines 6:607–614
44. **Sapolsky RM, Krey LC, McEwen BS** 1986 The neuroendocrinology of stress and aging: the glucocorticoid cascade. Endocr Rev 7:284–301
45. **Paxinos G, Watson C** 1986 The Rat Brain in Stereotaxic Coordinates. Academic Press, Sydney