Role of the hypothalamic opioid system in estradiol-induced polycystic ovarian syndrome.

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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September, 1992

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ISBN 0-315-87850-9

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ABSTRACT

Long-term exposure to physiological levels of estradiol induced by a single IM injection of estradiol valerate has previously been shown to cause a selective lesion in the hypothalamic arcuate nucleus and to induce a cascade of hypothalamic-pituitary deficits which ultimately result in polycystic ovaries and acyclicity. Several lines of evidence, including a selective increase in ^{3}H-naloxone binding in the anterior hypothalamus of EV-treated animals, had previously suggested that the hypothalamic opioid system may be involved. To test this possibility, the intrahypothalamic distribution of mu, delta and kappa opioid receptor types was examined by *in vitro* radioautography using the opioid ligands ^{125}I-FK-33 824, ^{125}I-DTLET and ^{125}I-DPDYN, respectively as selective markers. The density and distribution of these receptors in the hypothalamus of normal rats was then compared to that of rats injected with estradiol valerate in order to verify the location of the previously described increase in ^{3}H-naloxone binding and to identify the specific opioid receptor type involved. Analysis of opioid receptor changes following long-term exposure to estradiol revealed that mu opioid binding densities were significantly increased in the medial preoptic area of EV-treated animals. Delta and kappa opioid binding densities were unchanged in the medial preoptic area although a slight decrease in delta sites was observed in the suprachiasmatic nucleus. Hypothalamic ß-endorphin concentrations were concomitantly decreased in EV-treated animals, suggesting that observed increases in mu opioid binding were due to a compensatory up-regulation of receptors secondary to loss of ß-endorphin input from the arcuate nucleus. To confirm this interpretation, mu opioid

receptor binding was measured in the MPOA of animals treated with monosodium glutamate, which destroys the arcuate nucleus. Results indicated that mu opioid receptor binding densities were inversely proportional to hypothalamic ß-endorphin concentrations in the same animals supporting the existence of a causal relationship between chronic reductions in hypothalamic ß-endorphin concentrations and mu opioid receptor upregulation in the medial preoptic area.

To further document the decreased concentrations of ß-endorphin in the hypothalamus of EV-treated animals, light microscopic immunocytochemistry for ß-endorphin was performed in colchicine treated control and EV-injected rats. ß-endorphin neurons were digitized and counted across the entire rostrocaudal axis of the mediobasal hypothalamus. Additionally, adjacent sections were labeled with antibodies against tyrosine-hydroxylase, neurotensin and somatostatin, in order to see whether other arcuate neuronal populations were affected by the EV treatment. Eight weeks following EV treatment, a 60% decrease in the total number of ß-endorphin-immunoreactive neurons was detected in the arcuate nucleus, while neuron numbers for nearby neuronal populations were unchanged. These results were confirmed in biochemical experiments demonstrating reduced hypothalamic ß-endorphin concentrations in the absence of changes in neuropeptide-Y and met-enkephalin in EV-treated rats as compared to controls. 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 as compared to controls. Furthermore, the estimated number of neurons lost (~3500) corresponded precisely with the total number of ß-endorphin

neurons lost (~3600) as estimated using quantitative immunocytochemistry. Together, these findings strongly suggest that ß-endorphin neurons are selectively destroyed following long-term exposure to estradiol. In order to determine the involvement of free radicals in this cytotoxic reaction, EV-injected rats were co-treated with the potent antioxidant, vitamin E, and hypothalamic beta-endorphin concentrations measured by radioimmunoassay. Our results demonstrated that EV-treated animals co-treated with vitamin E displayed hypothalamic ß-endorphin concentrations similar to controls. In addition, these animals maintained regular estrous cycles and displayed normal ovarian morphology. These findings suggest that estradiol-induced neurotoxicity of ß-endorphin neurons involves the production of free radicals and further supports the notion that the loss of these neurons is important to the induction of chronic anovulation and polycystic ovaries resulting after EV treatment.

RÉSUMÉ

L'administration à des rats femelles d'une seule injection IM de valérate d'estradiol (VE) entraîne une exposition prolongée à des taux physiologiques d'estradiol. Il a déjà été démontré que ce traitement cause une lésion sélective au niveau du noyau arqué de l'hypothalamus et engendre une cascade de déficits de l'axe hypothalamo-hypophyso-ovarien menant à la formation d'ovaires polykystiques et à l'arrêt de la cyclicité. Plusieurs données, dont une augmentation de liaison spécifique du naloxone-^{3}H dans l'hypothalamus antérieur de rattes traitées au VE, avaient déjà impliqué le système opioide hypothalamique dans l'étiopathologie de ce modèle. Pour tester cette hypothèse, la distribution des récepteurs mu, delta et kappa respectivement marqués par les ligands sélectifs, ^{125}I-FK 33-824, ^{125}I-DTLET and ^{125}I-DPDYN a été examinée par radioautographie *in vitro* dans l'hypothalamus de rattes normales. La densité et la distribution de ces récepteurs furent ensuite comparées à celles observées chez des rattes injectées au VE, afin de localiser le site de l'augmentation de la liaison spécifique du naloxone-^{3}H et d'identifier le type de récepteur impliqué. Les résultats mirent en évidence une augmentation sélective des récepteurs mu dans l'aire préoptique médiane (APM) des rattes traitées aux estrogènes et une réduction moins importante mais néanmoins significative des récepteurs delta dans le noyau suprachiasmatique. Des dosages radioimmunologiques chez les mêmes animaux démontrèrent une baisse concomitante de la concentration hypothalamique de beta-endorphine suggérant que l'augmentation de la liaison mu puissent refléter une régulation compensatrice de ce type de récepteurs due à une perte d'afférentes beta-endorphiniques dans l'APM. Pour

vérifier cette interprétation, l'effet de la destruction du noyau arqué par le monosodium glutamate (MSG) sur la liaison mu dans l'APM fut examiné dans une série d'expériences complémentaires. Les résultats démontrèrent que la densité des récepteurs mu était inversement proportionnelle aux concentrations intrahypothalamiques de beta-endorphine, et donc qu'il pouvait y avoir relation causale entre les réductions chroniques de concentration beta-endorphine et l'augmentation des récepteurs mu dans l'APM.

Afin d'identifier l'origine de la diminution des concentrations hypothalamiques de beta-endorphine observée chez les animaux injectés au VE, les neurones à beta-endorphine du noyau arqué furent identifiés par immunohistochimie chez des animaux contrôles et traités au VE. Les neurones à la tyrosine-hydroxylase, neurotensine et somatostatine ont été identifiés en parallèle afin de déterminer si ces populations étaient elles aussi affectées par le traitement au VE. Huit semaines après le début du traitement, le nombre total de neurones immunoreactifs à la beta-endorphine dans le noyau arqué était réduit de 60%, alors que le nombre de neurones des populations voisines demeurait inchangé. Ces résultats furent confirmées par des expériences radiolimmunologiques démontrant une diminution de la concentration hypothalamique de beta-endorphine en l'absence de changement des concentrations de met-enképhaline ou de neuropeptide-Y chez les animaux traités au VE. Le décompte du nombre total de cellules du noyau arqué, effectué à l'aide d'une nouvelle méthode stéréologique, devait en outre révéler une diminution significative de ces neurones total de neurone chez les animaux injectés aux VE par rapport aux animaux contrôles. Le nombre total

de neurones disparus (~3500) s'est avéré correspondre précisément au nombre total de neurones beta-endorphine-immunoréactifs affectés par le traitement au VE. Nos résultats suggèrent donc que les neurones beta-endorphinergiques du noyau arqué sont sélectivement détruits après traitement par le valérate d'estradiol. Afin d'apprécier le rôle des radicaux libres dans cette perte cellulaire, des rattes traitées au VE ont aussi été traitées à la vitamine E, un puissant antioxydant, et les niveaux de beta-endorphine hypothalamiques mesurés par radioimmunoassai. Nos résultat démontrent que les concentrations hypothalamiques de beta-endorphine sont les mêmes chez les animaux traités au VE et à la vitamine E que chez les contrôles. De plus, ces animaux démontrent des cycles estrus réguliers et une morphologie ovarienne normale. Ces résultats suggèrent que la neurotoxicité des neurones beta-endorphinergiques par l'estradiol implique la production de radicaux libres et sont en faveur du concept que la perte de ces neurones entraîne l'anovulation chronique et la formation d'ovaires polykystiques chez les animaux traités au VE.

ACKNOWLEDGEMENTS

It is with feelings of immense gratitude and respect that I acknowledge the generous support of my co-supervisors, Drs. James Brawer and Alain Beaudet as I could not have benefited from more competent supervisors.

My interest in this field of research was originally motivated by Dr. Brawer's teachings. Through his courses, he transmitted the excitement of past scientific discoveries and invited one to the challenge of scientific research. I have learned a great deal from his manner of assessing and answering scientific questions and sincerely thank him for the knowledge, support and guidance provided to me during my undergraduate research project and throughout my graduate studies.

The accomplishments derived from this thesis are largely due to the organized and well-structured supervision I received from Dr. Beaudet. His laboratory provides one with all that is necessary to grow and develop ones practical and analytical skills. I sincerely thank him for his conscientious supervision, his support, and encouragement.

The research performed over the past four years would have been much more difficult and much less enjoyable without the help of many persons. I especially thank Kathy Leonard who was always available to lend a hand and who has saved me many hours of labour by making the day to day workings of the lab so functional and efficient. My

gratitude is extended to Dalia-Piccioni Chen without whom I could not have completed all phases of the animal work. Her expert assistance has been invaluable.

Upon joining the lab, I benefitted from the work and excellent teaching of Filoteo Pasquini, Catherine Jomary and Dominique Marcel who taught me the iodination procedures and provided me with the binding protocols fo the delta, kappa and mu ligands utilized in this study. I am grateful for the support of Dr. John Bergeron and for discussions and collaborations with Dr. Hyman Schipper.

Finally, I wish to acknowledge my friends and co-workers, Marie-Pierre Faure, Colin Holmes, John Woulfe, Louise Lafortune, Chris Mascott, Francoise Villemain, Farzin Fharadi-Jou, Ivana Gritti, Frank McCarthy and from other laboratories, Victor Viau, Wayne Rowe, Sean Marett, Dominique Richard and Stéphane Oliet for making this work environment so stimulating.

I am grateful to the Medical Research Council of Canada for granting me a studentship from 1989-1992.

PUBLICATIONS

Journals:

Desjardins, G.C. and J. Brawer (1989) Development and maintenance of a polycystic condition in ovaries autotransplanted under the kidney capsule of the rat. Anat. Rec. 225:118-123.

Desjardins, G.C., Brawer, J. and A. Beaudet (1990) Distribution of mu, delta and kappa opioid receptors in the hypothalamus of normal rats. Brain Res. 536:114-123.

Desjardins, G.C., A. Beaudet, J. Brawer (1990) Alteration in opioid parameters in the hypothalamus of rats with estradiol valerate-induced polycystic ovarian disease. Endocrinology 127:2969-2976.

Desjardins, G.C., J. Brawer and A. Beaudet (1992) Monosodium-glutamate induced reductions in hypothalamic ß-endorphin content result in mu opioid receptor upregulation in the medial preoptic area. (Neuroendocrinology, in press).

Desjardins, G.C., J. Brawer and A. Beaudet (1992) Estradiol is selectively neurotoxic to hypothalamic ß-endorphin neurons. (Endocrinology, in press)

Desjardins, G.C., A. Beaudet, H. Schipper and J. Brawer (1992) Vitamin E treatment protects against estradiol-mediated ß-endorphin loss. (Endocrinology, in press)

Liu, Zhao, **Desjardins, G.C.** and M. Avoli (1992) Pilocarpine treatment-induced seizures and hippocampal cell loss. (in preparation)

Abstracts:

Desjardins, C., Brawer, J. and A. Beaudet (1989) Comparative distribution of mu, delta and kappa opioid receptors in the hypothalamus of normal and estrogenized female rats. International Narcotics Research Conference, P-147, Ste-Adèle, Quebec, July 9-14.

Desjardins, C., Brawer, J. and A. Beaudet (1989) Distribution comparative des recepteurs opioides mu, delta et kappa dans l'hypothalamus du rat normale et du rat traites aux estrogenes. XIX^{me} Coloque International de Neuroendocrinologie Expérimentale, Rouen, France, Sept. 12-15, 1989.

Desjardins, C., Brawer, J. and A, Beaudet (1991) Monosodium-induced reductions in hypothalamic ß-endorphin content result in mu opioid receptor upregulation in the medial preoptic area of the rat. IBRO, Montreal, Quebec.

Desjardins, C., Brawer, J. and A, Beaudet (1991) Selective ß-endorphin cell loss and mu opioid receptor up-regulation following long-term exposure to estradiol. Society for Neuroscience, New Orleans.

Desjardins, C., A. Beaudet, H. Schipper and J. Brawer (1992) Vitamin E protects against estradiol-mediated ß-endorphin loss. Endocrine Society, San Antonio, Texas. June 23-27.

Faure, M.-P., **Desjardins, C.**, A. Beaudet (1992) Insulin-like growth factor receptor (IGF1r): Morphological evidence for preferential action on cholinergic neurons. Growth Factors, Washington D.C., June 1-6.

CLAIM FOR ORIGINAL WORK

The results presented in this thesis represent an original contribution to the knowledge of the long-term action of estradiol on the hypothalamus and more particularly, on its effects on the hypothalamic ß-endorphin system. The work derived from this thesis has been presented orally and in poster form at various meetings including the Narcotics Research Conference, 1988, the Societé Française de Neuroendocrinologie Expérimentale in 1989, the meetings of The Society for Neuroscience, 1991 and 1992 as well as the Endocrine Society Meeting of 1992.

Chapter 1 presents an overview of the research literature pertaining to the involvement of opioids in the control of reproduction and particularly, highlights evidence demonstrating the influence of estradiol on ß-endorphin control of gonadotropin secretion.

Chapter 2 describes the intra-hypothalamic distribution of mu, delta and kappa opioid receptors in normal rats. The distribution is somewhat different from that described previously and contributes original findings such as the novel demonstration of delta and kappa opioid receptors in the suprachiasmatic nucleus.

Chapter 3 provides a quantitative analysis of mu, delta and kappa opioid binding densities in the hypothalamus of normal, EV-injected and E_2-implanted rats. These are the first demonstrations that in EV-treated rats, mu opioid receptors are chronically up-regulated and hypothalamic ß-endorphin concentrations are reduced as compared to controls.

Chapter 4 utilizes a well characterized model of arcuate nucleus cell destruction

to test and confirm the novel hypothesis that the chronic mu opioid receptor up-regulation in the medial preoptic area may be a direct consequence of the loss of ß-endorphin neurons in the arcuate nucleus following EV treatment.

Chapter 5 demonstrates using quantitative immunocytochemistry, radioimmunoassay and unbiased stereological measurements that 60% of ß-endorphin neurons ~~are~~ appear to be destroyed following EV injection whereas other neuronal populations such as tyrosine hydroxylase, neurotensin-, somatostatin-, neuropeptide-Y- and met-enkephalin-containing neurons are spared.

Chapter 6 presents findings indicating that the mechanism of estradiol neurotoxicity involves the production of free radicals.

Chapter 7 contains a brief overview of what is known concerning steroid induced neuron cell death and concludes on the significance of ß-endorphin cell loss to general mechanisms of hypothalamic aging.

MANUSCRIPTS AND AUTHORSHIP:

The following is an excerpt from the *Guidelines Concerning Thesis Preparation*, which we are required to include in the introductory sections of any thesis to which it applies (so that external readers will be informed of Faculty regulations).

"Candidates have the option, subject to approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory.

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To my family,

past, present and future

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CHAPTER 1: INTRODUCTION

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8.0 Objectives of the present study

1.0 Hypothalamus

The hypothalamus functions as the central regulator of many essential homeostatic systems including the energetic, adaptive and reproductive systems [Dillman, 1976]. Via its afferent connections, the hypothalamus receives information from limbic structures such as the hippocampus and amygdala, visceral and somatic brainstem nuclei, the thalamus and the retina, in addition to receiving direct information from the cerebrospinal fluid [Bleier, 1985]. Within the hypothalamus, this extremely diversified input is transduced and efferent signals are conveyed through projections to the parasympathetic and sympathetic nervous systems, reciprocal connections with limbic structures, or via the hypothalamic regulation of the secretion of pituitary hormones [Bleier, 1985]. The unique connections of the hypothalamus provide a teleological explanation, if not a complete understanding of its role as the central pacemaker of all diurnal, circadian and menstrual rhythms in mammals.

2.0 Hypothalamic-pituitary-ovarian axis

The hypothalamic-pituitary-ovarian axis is often described as a closed-loop endocrine system where the final endproduct, estradiol, exerts negative and positive feedback effects at all levels of the axis to influence the secretion of hormones regulating its own production. Recently, it has become obvious that other systems such as those regulating energy balance, stress responses or immune function [Bateman, 1990] share signalling molecules which interact at multiple levels with the hypothalamic-pituitary-ovarian axis. For example, undernourishment will result in the complete arrest of cyclicity in many mammalian species [Finch, 1990] including man, as is observed in such disorders as

anorexia nervosa. Likewise, psychogenic stress [Lee, 1985] and infection [Ghareeb, 1981] have been shown to temporarily compromise the reproductive system. The cellular mechanisms underlying these phenomena are not understood despite the tremendous knowledge gained on the regulation of the secretion of hypothalamic, pituitary and ovarian hormones.

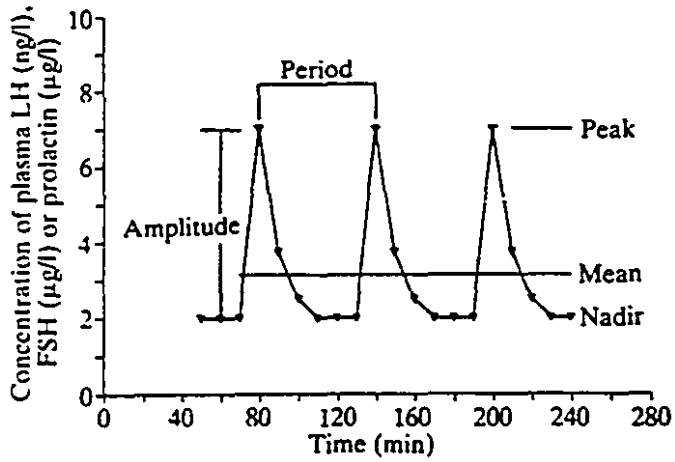
2.1 The estrous cycle

In female rodents, as in other mammals, the reproductive stages representing oocyte maturation, ovulation and preparation of the uterus for implantation of the embryo, succeed each other in a highly regulated and cyclic fashion. Collectively, these stages comprise the estrous cycle which in rats has a duration of approximately 4-5 days. Original observations of the estrous cycle were based upon the distinct alterations in sexual behavior observed to occur in female rats at regularly repeating intervals. It was shown, for example, that the frequency of lordosis responses (i.e. adoption of mating posture) of mated female rats was selectively increased for a 12 hour period every 4-5 days [Young, 1961]. It was later ascertained that this estrous behavior occurred coincident with clearly discernable changes in uterine weight, vaginal cytology and ovarian histology [Adler, 1981]. The assessment of vaginal cytology, which consists in swabbing the vaginal lumen and examining the cells obtained under a microscope, remains a simple and reliable index of estrous cyclicity today and forms the basis for the division of the estrous cycle into four main stages. Diestrus I and diestrus II are characterized by a vaginal smear displaying few, small rounded cells and moderately elevated uterine weights. The growth and development of ovarian follicles are the main

features of this period. Primordial follicles consisting of an ovum surrounded by a single layer of granulosa cells have been shown to develop increasing layers of granulosa cells which induce a thecal layer to differentiate from the surrounding stromal tissue and envelop the growing follicle. and have been described during diestrus [Adler, 1981]. The proestrus stage witnesses an exponential growth in ovulatory follicles, increased uterine weight and the occurrence of unique large, nucleated cells in the vaginal smear. On the afternoon of proestrus, ovulatory follicles rupture and release the ovum into the uterine tubes. Estrous is then characterized by a progressive decline in uterine weight and the appearance of large numbers of irregularly shaped, squamous, cornified cells in the vaginal smear. Ovaries at this time, display the remnants of ovulatory follicles, the corpus lutea, ovarian follicles as well as large numbers of follicles undergoing atresia or in various stages of development [Midgley, 1979; Adler, 1981] .

2.1.1 Characteristic changes in LH and FSH

The regulation of the estrous cycle has been shown to be exerted by luteinizing hormone (LH) and follicle stimulating hormone (FSH), both released from pituitary gonadotropes in pulsatile fashion. In early studies, technical limitations prevented the detection of pulses of LH and FSH release, however, since then the importance of the pulsatile pattern of release of these and other hormones has been appreciated. A schematic diagram of the parameters of a pulsatile mode of secretion is illustrated below as a guide for future discussions.



Pulsatile hormone patterns have been characterized as follows: mean hormone levels refer to the average hormone concentration measured in all samples over a given sampling period. Mean pulse peak and nadir are the average of the peak and nadir values of all pulses observed during the sampling period. Mean pulse amplitude is the average difference between the peak and the preceding nadir values of all pulses detected during the sampling period. Finally, the mean pulse period has been obtained by dividing the total time of sampling by the total number of pulses observed [from Grosser, 1986].

The pulsatile pattern of LH and FSH has been shown to vary throughout the estrous cycle according to the steroidal milieu [Kalra, 1974; Gallo, 1981; Fox, 1985]. A transient rise in FSH during the early hours of diestrus, initiates the recruitment of new ovarian follicles and promotes the division of granulosa cells (reviewed in Adler, 1981). FSH receptors located on granulosa cells mediate an increase in cyclic AMP production which in turn induces the expression of the aromatase enzyme responsible for converting androgens to estrogens. Simultaneously, LH, acting exclusively on thecal cells, promotes the production large amounts of androgens. These diffuse across the basement

membrane separating thecal and granulosa cells and are converted by the aromatase enzyme to estrogens [Midgley, 1979; Adler, 1981]. The pulsatile pattern of LH release at diestrus I, is characterized by pulse frequencies of approximately 1 pulse/hour and amplitudes of 50 pg/ml [Gallo, 1981; Fox, 1985]. During diestrus II, it has been suggested that the secretion of progesterone and peptides such as inhibin from growing follicles exert negative feedback effects at the pituitary level to decrease the synthesis [Mercer, 1989; Gharib, 1990] and secretion of FSH and to a lesser degree, of LH [Rivier, 1986; Culler, 1989]. Thus, during diestrus II up to the afternoon of proestrus, FSH secretion is reduced and LH pulse amplitude decreases steadily resulting in reduced mean secretion of LH despite conserved LH pulse frequency [1 pulse/hour; Gallo, 1981; Fox, 1985]. At the ovarian level, the combined action of FSH and the estradiol microenvironment in dominant follicles induces the expression of LH receptors on granulosa cells, a necessary step in the induction of ovulation. In the other follicles not stimulated by large amounts of local E_2 production, the appearance of LH receptors does not occur and follicles are either growth arrested or stimulated to undergo atresia. On the afternoon of proestrus, high levels of estradiol [attaining values greater than 40 pg/ml; Kalra, 1974] secreted by the dominant ovarian follicles exert positive feedback effects on the hypothalamus and pituitary gland which ultimately result in a massive surge of LH secretion [Kalra, 1974; Blake, 1976]. This surge in LH has been shown to be the result of an increase in both frequency (up to 3 pulses/hour) and amplitude of LH pulses [Gallo, 1980; Fox, 1985]. Mean LH values during the LH surge range between 800-2600 ng/ml. The LH surge then induces specific changes in the granulosa

and thecal cells of ovarian follicles resulting in ovulation [Adler, 1981]. Estradiol concentrations subsequently decline to approximately 10 pg/ml [Kalra, 1974]. During the estrous phase, the corpus luteum secretes large amounts of progesterone which exerts multiple effects at the pituitary and hypothalamic levels among which are a decrease in the frequency of LH release [Mahesh, 1985]. The estrus period displays the lowest frequencies of LH release seen during the entire cycle (1 pulse/2 hours) and relatively low peak amplitudes (app. 30 ng/ml) of release [Fox, 1985; Gallo, 1981]. After the demise of the corpus luteum, steroid secretion declines, permitting another transient increase in FSH which initiates the recruitment of new follicles and the completion of another cycle.

2.2 Regulation by LHRH

The secretion of LH and FSH has been shown to be under the direct control of luteinizing-hormone-releasing hormone (LHRH), released in a pulsatile fashion from tuberoinfundibular hypothalamic neurons. Parallel measurements of LHRH and LH release using push pull cannulae in the rat have shown that each peak of LH is preceded and/or coincides with a peak in LHRH release [Levine, 1982]. In animals treated with LHRH antibodies or in which endogenous LHRH secretion was inhibited by hypothalamic lesions, marked reductions in mean LH and FSH concentrations were observed [Knobil, 1974]. Moreover, normal levels of FSH and LH could be restored by exogenous LHRH administration [Knobil, 1974], indicating that LHRH is both necessary and sufficient for the release of LH and FSH. Certain lines of evidence suggest that FSH may be under the control of another as of yet undiscovered

hypothalamic factor [Culler, 1986]. LHRH has been shown to stimulate the synthesis of both specific mRNA subunits of LH and FSH [Gharib, 1990]. Furthermore, the relative stimulation of LH and FSH subunit MRNA expression was shown to be highly dependent on the frequency and amplitude of LHRH stimulation [Gharib, 1986; Gharib, 1987]. Indeed, it was shown early on that the pituitary gland was acutely sensitive to the frequency of LHRH discharge [Knobil, 1974]. In gonadectomized, hypothalamus-lesioned animal receiving exogenous LHRH pulses, LH release was reduced when LHRH pulse frequency was increased to 2 pulses per hour or reduced to 1 pulse per 3 hours from the normal 1 pulse per hour, indicating that pituitary responsiveness to LHRH was maximal at the physiological frequency of LHRH release. Interestingly, although LH release was always reduced when LHRH pulse frequency was altered, FSH secretion was increased at slower frequencies, suggesting that alterations in LHRH pulse parameters could differentially regulate the release of FSH and LH as has now been observed in the case of LH and FSH synthesis. Since LHRH is so difficultly measured in rodents, the pattern of LH release has usually been interpreted as reflecting similar changes in the pattern of LHRH release. However, this view is not entirely supported by evidence derived from studies of the estrous cycle or during the post-ovariectomy rise in LH secretion (see section 2.2).

The precise regulation of LH and FSH release by LHRH over the course of the estrous cycle remains controversial, primarily due to the difficulty in accurately measuring minute changes in LHRH release in rodents. Using a specific radioimmunoassay for LHRH, Kalra et al. (1986) have described attenuated levels of

LHRH release from the mediobasal hypothalamus (MBH) *in vitro* during the estrous phase and progressive increases in the episodic release of LHRH during the period of diestrus, paralleling changes in LH release described during these periods [Fox, 1985]. Similar results were obtained by Parnet et al., 1990 when LHRH concentrations were measured in median eminence extracts [Parnet, 1990]. However, these findings were not confirmed when measurements of LHRH efflux from the MBH or pituitary were assessed using push pull canula *in vivo* [Ramirez, 1987; Park, 1989; Levine, 1982]. In these cases, LHRH levels were described as stable for the entire period from estrus to diestrus with the frequency of LHRH release remaining at approximately 1 pulse every 50 min, despite the fact that LH release was markedly varied during this period [Ramirez, 1987]. In contrast, most studies are in agreement in demonstrating increased release of LHRH at the time of the LH surge [Kalra, 1986; Levine, 1982; Parnet, 1990].

It is uncertain whether the pulsatile mode of release of LHRH is derived from intrinsic excitation within these neurons or from the pulsatile release of stimulatory and inhibitory afferents within the hypothalamus. The former possibility is supported by the recent demonstration that immortalized LHRH neurons in culture are capable of pulsatile LHRH release [Martinez de la Escalera, 1992]. Various other hormonal and neural inputs would then play permissive or suppressive roles on the synthesis, storage and release of LHRH and modify various parameters of LHRH secretion such as nadir, pulse frequency, duration or amplitude.

2.3 Regulation by estradiol

It is well established that estradiol exerts negative feedback effects on the synthesis, storage and release of LH and FSH [Schally, 1972; Knobil, 1974; Karsch, 1987; Gharib, 1990]. Ovariectomy, which removes the inhibitory influence of estradiol, has been consistently shown to result in increases in serum LH and FSH [Yamamoto, 1970; Sherwood, 1980; Wise, 1981]. The pulsatile pattern of LH release in gonadectomized rats is characterized by high amplitude, short duration (20-30 minutes) LH pulses on a high nadir [Weick, 1978; Clayton, 1981; Leipheimer, 1983]. Ovariectomy was also shown to result in increases in the pituitary mRNA content of the α and ß subunit of LH and FSH [Gharib, 1986; Gharib, 1987; Papavasiliou, 1986] due to an increased transcription of these genes [Shupnik, 1988] which in most cases was reversed by estradiol replacement [Gharib, 1986; Gharib, 1987; Papavasiliou, 1986]. Conversely, potent stimulatory feedback effects of estradiol on LH and FSH secretion have been described [Schally, 1972; Knobil, 1974; Karsch, 1987]. These are most clearly illustrated during the LH surge of the normal estrous cycle. Whether these estradiol-induced changes in the synthesis and secretion of LH and FSH are mediated via alterations in the synthesis and secretion of LHRH or are the result of direct actions on the pituitary remains controversial. As both pituitary gonadotropes [Simanov, 1977] and hypothalamic neurons have been shown to possess estradiol receptors [Pfaff, 1968], it is likely that estradiol's effects are mediated at multiple loci within the hypothalamic pituitary axis. Early evidence obtained from hypothalamic-lesioned monkeys replaced with hourly infusions of LHRH demonstrated that LH secretion was suppressed after estradiol administration, suggesting a pituitary site of action [Knobil, 1974]. In

addition, rats whose pituitary glands were removed from the action of hypothalamic LHRH by transplantation to the kidney capsule also exhibited suppression of LH release following estradiol administration [Strobl, 1988]. Evidence derived from *in vitro* studies of isolated rat pituitary cells indicated that estradiol both suppressed LH responsiveness to LHRH [Frawley, 1984; Emons, 1984] and/or increased basal LH release and responsiveness to LHRH [Drouin, 1976; Drouin, 1981; Kamel, 1987]. Stimulatory effects of estradiol have been observed during the estrous cycle, where it was noted that as estradiol levels rise, the pituitary responsiveness to LHRH increased [Kalra, 1973]. It was also shown in vivo that the preovulatory LH surge was preceded by a rise in LH ß mRNA levels [Shupnik, 1989; Zmeili, 1986] suggesting that the pituitary gland was directly, positively regulated by estradiol. The opposing effects of estradiol on pituitary sensitivity to LHRH *in vitro* have been shown in ewes to be entirely dependent on the duration of incubation since exposure to estradiol for up to 12 hours resulted in an enhancement of pituitary sensitivity to LHRH whereas incubations greater than 20 hours suppressed LH sensitivity to LHRH [Clarke, 1988; Laws, 1990].

Although LHRH neurons have been shown to be devoid of estradiol receptors [Shivers, 1983], several lines of evidence suggest that estradiol may modulate the synthesis, storage and release of LHRH via alterations in the actions of neuromodulatory inputs to LHRH neurons [Kalra, 1983a; Kalra, 1983b] or via direct membrane mediated effects [Dufy, 1982; Kelly, 1982]. Recent evidence suggests that estradiol exerts facilitatory actions on LHRH secretion from the hypothalamus [Kalra, 1989] and not inhibitory effects as was suggested by earlier work. Studies in ovariectomized rodents

treated with estradiol to induce an LH surge have shown that LHRH concentrations are increased in the median eminence at the time of the surge, suggesting that estradiol exerts positive feedback effects on LHRH at this time [Wise, 1981; Fink, 1982]. In addition, 2-4 weeks following ovariectomy, it was shown that LHRH concentrations in the median eminence were reduced [Kalra, 1981b; Fink, 1982], findings which were originally interpreted to suggest increased secretion of LHRH. A few studies reported increased LHRH levels in the pituitary stalk plasma after ovariectomy [Fink, 1982; Sherwood, 1980] however doubts have been cast on the validity of the experimental design of these studies [Kalra, 1989]. Indeed, these results were not reproduced when LHRH levels were measured directly from the ME or in extracted plasma. Studies have shown that LHRH release is diminished at this time of maximal LH secretion [Kalra, 1989; Leadem, 1984]. Furthermore, a series of studies suggested that E_2 replacement in long-term ovariectomized rats resulted in a increase in LHRH release [Dlunzen, 1986; Leadem, 1984; Ramirez, 1987], supporting the view that estradiol is stimulatory to LHRH release.

The regulation of LHRH synthesis by estradiol has nonetheless remained equivocal. Estradiol administration to ovariectomized rats has been shown to stimulate [Pfaff, 1989; Rothfeld, 1987; Roberts, 1989; Park, 1990] or inhibit LHRH mRNA content in the hypothalamus [Wray, 1989; Zoeller, 1988; Rothfeld, 1987; Toranzo, 1989] as determined by both Northern blotting and *in situ* hybridization. Likewise, ovariectomy was shown to have diverse effects on LHRH mRNA content in the medial preoptic area of the hypothalamus [Roberts, 1989; Toranzo, 1989; Kelly, 1989].

3.0 Neural circuitry involved in the regulation of gonadotropin secretion

3.1 LHRH neurons

3.1.1 Distribution

In rodents, LHRH immunoreactive nerve cell bodies have been predominantly localized to the preoptic-anterior hypothalamic regions, although a few neurons have been shown to extend throughout the entire hypothalamus ranging from the diagonal band of Broca, dorsal septal nuclei, periventricular, anterior and lateral preoptic area, rostrally, to the retrochiasmatic zone and cell poor region between the arcuate and ventromedial nucleus, caudally [for reviews, see Setalo, 1976; Sternberger, 1978; Witkin, 1982; Krey, 1983; Shivers, 1983; Liposits, 1984 as well as Hoffman, 1990; Silverman, 1987; Merchentaler, 1984]. Scattered LHRH immunoreactive cells have also been found in the organum vasculosum of the stria terminalis, subfornical organ and the olfactory bulb. This distribution of immunoreactive LHRH neurons has been found to conform to that of neurons immunoreactive for LHRH mRNA as determined by double studies using immunocytochemistry and *in situ* hybridization [Ronnekliev, 1989; Shivers, 1986].

The total number of LHRH neurons has been estimated in the rat at less than 1000 neurons for the entire hypothalamus [Shivers, 1983; Silverman, 1987]. These neurons are usually fusiform in shape with a process extending at each pole and are typically small [10μm] neurons oriented in a dorsoventral direction [Ibata, 1979; Rethelyi, 1981; Bennett-Clarke, 1982; King, 1982; Shivers, 1983; Witkin, 1982]. At the electron microscopic level, LHRH neurons were found to possess an invaginated nuclear

membrane and a large nucleolus. These were often replete with flattened saccules of endoplasmic reticulum and contained a prominent Golgi apparatus. LHRH immunoreactivity could be localized within these neurons in peripheral granules of approximately 70-95 nm located mainly in the cell periphery.

3.1.2 Projections of LHRH neurons

LHRH neurons have been shown to send extensive projections to the median eminence where a concentration of terminals has been localized in the lateral wings of external zone of the median eminence [Kordon, 1974; Ibata, 1979; Shivers, 1983; Liposits, 1984]. Upon ultrastructural examination, LHRH terminals were observed to contact the basement membranes surrounding pericapillary spaces of the portal plexus of the median eminence [Ibata, 1977] in accordance with light microscopic results. In addition, LHRH axons were also shown to contact tanycytes processes in the ME [Rethelyi, 1981] and to project to the ventricular border of the third ventricle but not to cross the ependyma. The origin of LHRH neurons projecting to the median eminence was a point of controversy for many years. The results derived from various studies where ME LHRH levels were measured following selective destruction of hypothalamic nuclei were often contradictory probably due to differences in the extent of damage caused to surrounding hypothalamic tissue. Nevertheless, a majority of studies support the idea of a relative enrichment of ME-projecting-LHRH neurons in the medial preoptic area [Samson, 1979]. LHRH ME levels have been shown to be reduced following bilateral destruction of the medial preoptic area [MPOA; Samson, 1979] but not following destruction of the arcuate nucleus of the mediobasal hypothalamus induced

by neonatal monosodium glutamate treatment [Nemeroff, 1977; Badger, 1982]. These findings are corroborated by two recent studies utilizing combined retrograde tracing of wheat germ agglutinin (WGA) injected directly into the median eminence (ME) and immunocytochemistry for LHRH containing neurons [Merchenthaler, 1989; Silverman, 1987]. ME projecting LHRH neurons were found to be widely distributed throughout the hypothalamus but a concentration of double labeled WGA and LHRH neurons was detected in the septal-anterior hypothalamic regions. The proportion of tuberoinfundibular LHRH neurons to total LHRH cells was estimated at 50% in untreated [Silverman, 1987] and 70% in colchicine treated animals [Merchentaler, 1989]. Interestingly, it has been shown that immediately preceding the preovulatory surge in LH secretion, 35-50% of all LHRH neurons expressed the immediate early gene c-fos [Hoffman, 1990], a marker of neuronal stimulation; thus, suggesting that a large proportion of all tuberoinfundibular LHRH neurons are activated in concert during the LH surge to produce a synchronous and massive release of LHRH into the pituitary portal vasculature.

It has been shown at both light and electron microscopic levels that LHRH neurons also project to the organum vasculosum of the lamina terminalis, the olfactory bulb, islands of Calleja and the piriform cortex. In addition, LHRH neurons have been shown to project to medial and cortical amygdaloid nuclei via the stria terminalis or amygdalo-fugal pathway [Clayton, 1979; Barry, 1979; Jennes, 1982; Silverman, 1976; Sternberger, 1978]. Some of the fibers in the latter projection system continue to the hippocampus where they generally terminate in the stratum radiatum or molecular layer.

LHRH fibers arising from the preoptic area have been shown to course with the ventricle along the borders of the thalamus and terminate in the midbrain central grey [Liposits, 1984], the interpeduncular nucleus [Silverman, 1976; Witkin, 1982] and the superior colliculi [Silverman, 1976; Liposits, 1984; Chen, 1989; Leranth, 1988].

Collectively, these results suggest that LHRH may act as both a neurohumoral factor controlling the secretion of pituitary hormones and as a neurotransmitter/modulator affecting information transmission in other systems.

3.1.3 Afferents

The medial preoptic nucleus of the hypothalamus, which has been shown to contain a majority of LHRH neurons, receives diverse inputs from hypothalamic, limbic and mesencephalic nuclei. The chemical identity of terminals synapsing on LHRH neurons has been investigated using a variety of double labeling strategies at the electron microscopic level. GABAergic immunoreactive terminals have been observed forming symmetrical synapses on LHRH reactive dendrites and perikaria in the medial preoptic nucleus [Leranth, 1985] and could arise locally from GABAergic neurons located in this region [Hokfelt, 1984] or from other hypothalamic nuclei such as the arcuate nucleus [Hokfelt, 1984]. Also, tyrosine-hydroxylase positive terminals have been shown to impinge on LHRH neurons and to form small undifferentiated synapses on their perikaria and dendrites [Leranth, 1986; Chen, 1989]. These fibers have been postulated to arise mainly from the incertohypothalamic dopamine neurons located within the hypothalamus [Hokfelt, 1984]. Serotonergic axons labeled by high resolution radioautography have also been shown to terminate on LHRH neurons labeled

immunocytochemically in the preoptic area [Kiss, 1985]. These afferents are likely to arise from the demonstrated projections of the dorsal and median raphe nuclei to the medial preoptic area [Hokfelt, 1984]. Furthermore, LHRH neurons in the medial preoptic area were shown to receive direct synaptic input from ß-endorphin/ACTH containing neurons [Leranth, 1986; Chen, 1989]. By combining a biotinylated antirabbit IgG, revealed using avidin biotin HRP complex and an IgG revealed with tetramethyl benzidine which forms a blue crystalline product [Chen, 1989], the proportion of ß-endorphin positive terminals impinging on LHRH cells was estimated at only 10% of total number of terminals labeled in this area. These are likely to arise from ß-endorphin neurons located in the arcuate nucleus [Bloom, 1978; Gee, 1979]. In a double electron microscopic study by Chen, 1988, noradrenaline containing axon terminals were shown to contact LHRH cell somata and dendrites with a proportion greater than that previously shown for ß-endorphin [Chen, 1988]. Projections from the locus ceruleus, the commisural portion of the solitary nucleus (A2 group) and the A1 region are likely to account for the noradrenergic innervation of LHRH neurons in this region [Hokfelt, 1984]. Finally, LHRH-immunoreactive synaptic terminals from axon collaterals comprise another 10% of all terminals impinging on LHRH immunopositive dendrite and perikaria, providing anatomical evidence for LHRH autoregulation [Leranth, 1985; Witkin, 1985; Chen, 1989 although see Pelletier, 1984].

4.1 Overview of hypothalamic opioid system

4.1 Discovery

Reports of high affinity stereospecific binding sites for opioid drugs first appeared in 1973 [Pert and Snyder, 1973; Simon, 1972; Terenius, 1973] and isolation of met-enkephalin, leu-enkephalin and ß-endorphin rapidly followed [Hughes and Kosterlitz, 1975; Golstein, 1975]. Multiple opioid receptor types were first postulated in order to explain the distinct behavioral syndromes observed after injection of various opioid compounds into the chronic spinal dog [Martin et al., 1976; Gilbert and Martin, 1976]. These investigators proposed the existence of three types of opioid receptors; μ, selective for morphine-like compounds, δ, selective for enkephalin-like compounds and σ, selective for ethylketocyclazocine-like compounds. To date, there have been reports of more than six different opioid receptor types (including mu, delta, kappa, epsilon, lambda and zeta)[Goldstein, 1984; Akil, 1984; Schultz, 1981; Garzon, 1984; Grevel, 1985] as well as several accounts of multiple opioid receptor subtypes (μ_1, μ_2, $kappa_1$, $kappa_2$, $kappa_3$) [Zukin, 1988], however only mu, delta and kappa opioid binding sites have been investigated in parallel behavioral and pharmacological studies. It is not known whether mu, delta and kappa opioid receptors represent different proteins, or different post-translational or conformational alteration of the same amino acid sequence as none of these have been cloned.

The endogenous ligands for these opioid receptors are derived from three distinct precursor molecules, pro-opiomelanocortin [POMC], proenkephalin and prodynorphin which are cleaved in a tissue specific manner during processing through the secretory pathway to yield various biologically active molecules. Among the most abundantly produced in the brain are ß-endorphin, met-enkephalin and dynorphin, each derived

from pro-opiomelanocortin, proenkephalin and prodynorphin, respectively. The affinity of these peptides for each receptor type varies, but in general, ß-endorphin has highest affinity for mu, met-enkephalin for delta and dynorphin for kappa receptor types [Mansour, 1986; Paterson, 1984]. Competition studies nonetheless suggest that under conditions of high opioid release or in areas of particular enrichment in given opioid receptor types, met-enkephalin may bind to both delta and mu opioid receptors and ß-endorphin to both mu and delta [Paterson, 1984].

4.2 Opioid peptide distribution

In the central nervous system, it has been well established that virtually all POMC mRNA containing neurons are located in the mediobasal hypothalamus extending from the retrochiasmatic area anteriorly to the mammilary bodies caudally, mostly within the arcuate nucleus but also in the latter most periarcuate region [Gee, 1983]. These perikaria are immunoreactive to all POMC peptide products including ß-endorphin, α-melanocyte stimulating hormone, adrenocorticotrophic hormone [Bloom, 1978; Jacobowitz, 1978; Watson, 1978; Khachaturian, 1985]. A small cluster of ß-endorphin neurons also exists in the nucleus tractus solitarius in the caudal medulla [Khachaturian, 1983]. It has been shown that this group extends relatively short local projections to various nuclei in the brain stem [Pilcher, 1986]. In contrast, the arcuate ß-endorphin neurons have been shown to extend long projections throughout the telencephalon, effectively innervating all structures with the exception of the cortex, striatum, hippocampus, cerebellum and olfactory bulb [Khachaturian, 1986]. In the diencephalon, ß-endorphin containing fibers project forward via the medial preoptic area where many

ß-endorphin terminals have been shown to synapse with elements in this nucleus [Leranth, 1986; Chen, 1989]. Other fibers continue rostrally toward the bed nucleus of the stria terminalis and the amygdala [Mezey, 1985].

Neurons containing met-enkephalin are much more numerous and widespread than those containing ß-endorphin [Finley, 1981; Khatchaturian, 1985]. The proenkephalin gene contains six copies of met-enkephalin and one for leu-enkephalin explaining why met-enkephalin is relatively more abundant. In the rat brainstem, met-enkephalin immunoreactive neurons have been localized by immunocytochemistry in the spinal dorsal grey, spinal trigeminal, lateral reticular, dorsal tegmental, reticularis gigantocellularis and paragigantocellularis and interpeduncular nuclei as well as in the dorsal raphe and periaqueductal grey [Finley, 1981; Khatchaturian, 1985]. In the telencephalon, met-enkephalin neurons exist in the cerebral cortex, olfactory tubercle, amygdala, hippocampus, striatum, septum and bed nucleus of the stria terminalis. In the diencephalon, met-enkephalin neurons have been localized by immunocytochemistry in the medial preoptic area, ventromedial nucleus, periventricular and arcuate nuclei, with more numerous cell bodies occurring in the paraventricular and supraoptic nuclei. However, using *in situ* hybridization, proenkephalin-containing neurons were more abundant in the ventrolateral division of the ventromedial nucleus and no positive cells were detected in the arcuate or periventricular nuclei [Harlan, 1987]. In general, met-enkephalin neurons are small and send only short projections which along with their widespread distribution make them likely candidates for interneurons participating in local neural circuitry.

Dynorphin producing neurons may also give rise to α or ß-neoendorphin and leu-enkephalin, but not to met-enkephalin. Their cell bodies have been localized in the cortex, striatum, amygdala, hippocampus, periaqueductal grey, parabrachial and spinal trigeminal nuclei, nucleus tractus solitarius, lateral reticular nucleus and the dorsal horn of the spinal cord. In the hypothalamus, dynorphin perikaria have been localized predominantly in the preoptic area, lateral hypothalamic area, supraoptic and paraventricular nuclei in addition to the arcuate nucleus [Khachaturian, 1982; Vincent, 1982; Watson, 1981]. Hypothalamic dynorphin containing neurons in the arcuate nucleus have been shown to project to the median eminence [Meister, 1989] whereas those in the magnocellular cell groups give rise to a rich fiber plexus in the preoptic area [Khachaturian, 1985; McGinty, 1983].

4.3 Opioid receptor distribution

The presence of mu, delta and kappa opioid receptors in the hypothalamus has been ascertained using radioreceptor assays [Chang, 1979; Wolozin, 1982] and *in vitro* radioautography [Atweh, 1977; Duka, 1981; Goodman, 1980; Herkenham, 1982; Lynch, 1985; Mansour, 1988; Mansour, 1986, McLean, 1986; Pearson, 1980; Quirion, 1983; Tempel, 1987; Wamsley, 1983; Wolozin, 1982] however, the relative hypothalamic concentrations of each subtype and their intrahypothalamic distribution remain controversial. A majority of studies have reported a low or negligible concentration of mu opioid binding sites in the hypothalamus relative to other brain regions [Herkenham, 1982; Lynch, 1985; Mansour, 1986, McLean, 1986; Tempel, 1987; Wamsley, 1983]. Others have reported that the hypothalamus is enriched in mu

opioid receptors relative to delta and kappa opioid binding [Chang, 1979; Wolozin, 1982; Duka, 1981; Goodman, 1980; Pearson, 1980]. Mu opioid receptors have been described as being highly concentrated in the medial preoptic nucleus, the ventromedial nucleus, the suprachiasmatic nucleus and the mammillary bodies [McLean, 1986]. However, high levels of mu opioid binding were not detected in these regions in other studies [Mansour, 1988]. Instead, elevated mu opioid binding was demonstrated in the dorsomedial and lateral hypothalamic areas.

The concentration of delta opioid binding sites in the hypothalamus has been consistently described as low to moderate relative to mu and kappa opioid receptor types [Duka, 1981; Goodman, 1980; Lynch, 1985; Mansour, 1986; Tempel, 1987; Wolozin, 1982]. The distribution of these binding sites has been described as low or absent in the entire preoptic region of the hypothalamus, the magnocellular nuclei, the arcuate nucleus while a moderate degree of delta opioid binding has been described in the ventromedial nucleus [Mansour, 1986, Goodman, 1980].

Kappa opioid binding sites have been described as high relative to mu and delta opioid binding in the hypothalamus [Lynch, 1985; Mansour, 1987; Morris, 1986; Sharif, 1989; Tempel, 1987] however, another study reported a relative paucity of kappa opioid binding in the rat hypothalamus [Jomary, 1988]. In the studies describing high concentrations of kappa opioid binding, the ventromedial and dorsomedial nuclei and suprachiasmatic were most densely labeled whereas the arcuate nucleus, supraoptic nucleus, paraventricular nucleus and ME were moderately labeled [Mansour, 1986; Pearson, 1980; Tempel, 1987]. Most of these *in vitro* radioautographic studies have

examined the distribution of opioid receptors throughout the entire central nervous system and hence were not aimed at specifically clarifying the intrahypothalamic distribution of these receptor types. A detailed analysis of the intrahypothalamic and intranuclear distribution of mu, delta and kappa opioid receptors has not been performed. Also the chemical identity of hypothalamic elements possessing opioid receptors has not been determined, hence it is not known whether LHRH neurons located in the medial preoptic area possess opioid receptors. Current radioautographic data would suggest that predominantly kappa and then mu opioid receptors in this region could mediate the actions of opioids on LHRH. Pharmacological [Gopalan, 1989] and anatomical data [Leranth, 1986; Chen, 1989] indicate that mu opioid receptors in the medial preoptic area may be located on presynaptic noradrenergic terminals impinging on LHRH neurons and/or LHRH neurons themselves.

4.4 Projections of the arcuate opioid-containing neurons to the medial preoptic nucleus

Electrophysiological studies by Renaud et al, 1977 originally demonstrated that 11.5% of neurons in the arcuate nucleus could be orthodromically stimulated via the MPO. Moreover, that significant numbers of arcuate neurons project to the MPO was confirmed by microlesion studies demonstrating a significant amount of synaptic degeneration in the medial preoptic nucleus following lesions of the medial arcuate nucleus [Zaborsky, 1979]. Although the arcuate-medial preoptic projection undoubtedly contains more than one neurotransmitter population, double retrograde and immunocytochemical studies have shown that 20% of POMC expressing neurons are

retrogradely labeled after injection of the fluorescent dye fast blue, into the medial preoptic area [Wilcox, 1986]. Mezey et al, (1985) also demonstrated using immunocytochemistry that ACTH/ß-endorphin immunoreactive axons originating from the arcuate nucleus form a dense network in the MPO. In addition, arcuate neurons expressing leu-enkephalin have been shown to project to the medial preoptic area [Finley, 1981; Yamano, 1988] and, as mentioned previously dynorphin neurons located in magnocellular nuclei also project to this region [Khachaturian, 1985; McGinty, 1983]. LHRH neuronal cell bodies located in the medial preoptic area are in close proximity to ß-endorphin, met-enkephalin and dynorphin containing cells bodies or their projections and have been shown to receive direct synapses from ß-endorphin terminals [Leranth, 1985; Chen, 1989].

5.0 Opioid regulation of gonadotropin release

Even before the discovery of the endogenous opioid peptides, the effects of opioids on LH secretion had already been anticipated by a study demonstrating that treatment with morphine, an exogenous opioid analogue, inhibited ovulation in rats [Barraclough, 1955]. It is now generally accepted that this inhibitory action of opioids occurs via pronounced suppression of LH release (for reviews see Kalra, 1983a, Kalra, 1983b]. Numerous studies are in agreement in demonstrating the pronounced inhibitory effects of morphine, endogenous opioid peptides or their analogues on the release of LH from intact and gonadectomized rats either under normal conditions [Bruni, 1977; Ching, 1983; Cicero, 1980; Cicero, 1979; Johnson, 1982; Kalra, 1980a; Kinoshita, 1980; Koves, 1980, Pang, 1977; for review see Kalra, 1983b] or during the preovulatory or

gonadal steroid induced LH surge [Ching, 1983; Kalra, 1980]. Thus, intracerebroventricular injections of ß-endorphin were shown to significantly inhibit LH release in both male [Kalra, 1984; Cox, 1982; Kinoshita, 1980; Wood, 1980] and female rats [Kalra, 1983] to a degree greater than that exerted by other opioids [Cox, 1982; Wood, 1982] and even that induced by estradiol [Kalra, 1981b; Kalra, 1982; Leipheimer, 1983]. Nonetheless, a variety of experimental paradigms have consistently shown that all three main opioid types i.e. ß-endorphin, met-enkephalin and dynorphin exert inhibitory influences on LH release [Kalra, 1983b, Kalra 1984; Mallory, 1990; Leadem, 1987]. In contrast, leu-enkephalin, which has preferential affinity for delta opioid receptors [Cox, 1982; Wood, 1982] has been found to stimulate LH release in ovariectomized rats [Leadem, 1983]. Met-enkephalin has also been reported as having stimulatory effects on LH release [Motta, 1982] however, this finding was not reproduced in other studies [Leadem, 1987].

The effects opioids on LH release have been purported to be centrally mediated since *in vitro* studies using hemipituitary preparations have indicated that opiate antagonist, naloxone, exerted no effect on basal LH release [Bruni, 1977; Cicero, 1979; Gabriel, 1983]. Furthermore, the anterior pituitary has been described as containing a paucity of mu, delta and kappa opioid binding sites [Robsen, 1983; but see Rotten, 1986], suggesting that opioids do not act directly on gonadotropes to inhibit the synthesis and or storage of LH. Most of the evidence for opioid modulation of LH release suggests that opioids act on LH indirectly, via alterations in LHRH secretion [Drouva, 1981; Ching, 1983; Adler, 1984; Kalra, 1987a; Leadem, 1985] or changes in pituitary

sensitivity to LHRH [Blank, 1986; Ferin, 1984; Sarkar, 1985].

5.1 Mechanism of action of opioids on LH release

Endogenous opioids peptides have been shown to inhibit the release of LHRH from hypothalamic explants [Wilkes, 1980] and have been shown to diminish the accumulation and efflux of LHRH from ME nerve terminals [Kalra, 1981b; Drouva, 1981; Ching, 1983, Adler, 1984; Kalra, 1987a; Leadem, 1985]. *in vitro* studies have also shown that morphine or met-enkephalin treatment [Rotsztejn, 1978] had no effect on the unstimulated release of LHRH from isolated ME *in vitro*, but inhibited dopamine induced release of LHRH, suggesting that opioids may have direct modulatory actions on LHRH terminals in the median eminence [Rotszejn, 1978]. The demonstrated synapses between ß-endorphin containing terminals and LHRH neuronal cell bodies in the medial preoptic area also suggest that ß-endorphin may act directly on neuronal perikaria and dendrites to affect the synthesis, storage and/or release of LHRH.

Another mechanism of opioid suppression of LH release may be by diminishing LH sensitivity to LHRH. Thus ß-endorphin released in the pituitary portal vasculature was shown to attenuate LHRH induced LH release [Blank, 1986; Ferin, 1984; Sarkar, 1985].

5.3 Intrahypothalamic site of action of opioids on LH release

The precise hypothalamic site of action of opioids in the inhibition of LH has been extensively investigated. When ß-endorphin was infused intracerebrally at various brain sites, it was shown that infusions near the ventromedial nucleus, anterior hypothalamic

area, septal complex and diagonal band of Broca failed to significantly suppress LH release whereas infusions near the medial preoptic area and arcuate nucleus-ME complex inhibited LH release [Weisner, 1983]. Similar sites of opioid action were identified in studies where naloxone, the opioid receptor antagonist was implanted in different hypothalamic nuclei [Kalra, 1981c]. In this case, LH release was significantly stimulated following implantation in the medial preoptic area and arcuate nucleus but not in other hypothalamic nuclei. In male rats, subcutaneous administration of the long acting opioid analogue, FK 33-824 also suppressed LH release suggesting that peripherally secreted opioids may act on sites outside the blood brain barrier to inhibit LH release [Wilkinson, 1984]. Morphine infusion in the raphe nucleus or in the amygdaloid region was also shown to suppress LH [Johnson, 1982; Lakoski, 1982].

5.3.1 Involvement of opioid receptor types

In general, endogenous opioids or their analogues acting on the mu opioid receptor have been shown to exert the most potent effects on LH release (Pfeiffer, 1987). Intracerebroventricular injections of selective mu, delta and kappa opioid analogues have shown that mu opioid agonist induce pronounced suppression of LH release at 10 fold lesser concentrations than those of delta opioid agonists and at these concentrations kappa ligands were without effect at [Leadem, 1987]. Recently, in a study where mu, delta and kappa opioid analogues were infused directly into the medial preoptic area [Mallory, 1990], opioid analogues acting at mu opioid receptors were found to be more potent than those acting on delta receptors. The activation of kappa opioid receptors in this region had no effect on LH release [Mallory, 1990]. Thus, in general, the

effects of various endogenous opioids on LH release correspond well with the demonstrated prevalence of mu opioid receptors in the medial preoptic area.

5.3.2 Alterations in LH pulse parameters

It has been shown that a bolus injection of ß-endorphin causes a marked suppression of LH pulse amplitude while leaving other parameters of LH release unchanged [Kalra, 1982]. In contrast, the continuous slow infusion of ß-endorphin caused an almost complete cessation of the entire LH pulsatile pattern effectively reducing it to a tonic mode of release. Mallory and Gallo have shown that at a given concentration, a mu opioid analogue, injected in the medial preoptic area caused a significant suppression of LH pulse amplitude but not LH pulse frequency, nadir, peak or duration [Mallory, 1990]. At this same dose, delta opioid agonist were without effect. At higher doses however, mu opioid agonists were shown to significantly reduce both LH pulse amplitude and frequency and delta opioid agonists (at these higher concentrations) began to suppress LH pulse amplitude. These findings emphasize the fact that minute alterations in the secretion of ß-endorphin may cause significant variation in LH release parameters.

5.3.3 Endogenous opioid peptide participation in the inhibitory effects of gonadal steroids on LH release

Naloxone, the classical opioid receptor antagonist, induces increases in LH release when administered to intact rats suggesting that LH is under tonic opioid inhibition [Bruni, 1977, Ching, 1983, Cicero, 1979; Cicero, 1980; Gabriel, 1983; Kalra, 1981a]. It was originally observed that the stimulatory effects of naloxone on LH release varied

according to the steroidal milieu. Following gonadectomy, naloxone was unable to cause significant increases in the release of LH [Bhanot, 1983; Bhanot, 1984]. These and other studies led to the belief that endogenous opioid peptides, particularly ß-endorphin, mediated the estradiol negative feedback actions on LHRH and LH. However, this interpretation was later shown to be incompatible with the time course of effects occurring after gonadectomy and with events known to occur during the estrous cycle. For example, it was shown that naloxone stimulation of LH release could be discerned for up to 2 weeks following gonadectomy, a time where circulating estradiol levels are extremely low [Kalra, 1983a]. Moreover, steroid replacement blunted this effect [Masotto, 1990]. During the estrous cycle, tonic opioid inhibition of LH is halted only on the afternoon of proestrus [Kalra, 1983a; Kalra, 1986]. Aside from this brief period, it appears that no changes in tonic opioid inhibition occur throughout the despite dramatic changes estradiol concentrations [Kalra, 1974; Gallo, 1981].

5.4 Opioid-neurotransmitter interactions in the regulation of LH secretion

Ample evidence suggests that noradrenaline (NA) stimulates the release of LH from intact or ovariectomized rats [reviewed by Barraclough, 1982]. The site of permissive action of NA on LH release has been confirmed to be in the POA/AH [Jarry, 1990] and it has been shown that the this effect is mediated via α 1 receptor subtype of noradrenaline receptors [Weick, 1978]. It is now well established that the stimulatory effects of naloxone on LH release are largely mediated by a dishinbition of NA stimulation of LH release [Van Vugt, 1981; Kalra, 1986]. It was shown early in studies

that the stimulation of LH release by naloxone in E_2,P primed ovariectomized rats could be blocked by DDC (dethyldithiocarbamate) a NA synthesis inhibitor and that these effects could be reversed by clonidine, a NA receptor agonist [Simpkins, 1980]. Other studies using a variety of NA synthesis inhibitors and α NA receptor antagonists also demonstrated that naloxone induced LH release was mediated by NA activation [Van Vugt, 1981].

It has been suggested that the inhibitory effects of dopamine (DA) on LHRH are mediated by DA-stimulation of ß-endorphin release since small doses of naloxone prevented the reductions in LHRH release from isolated MBH-ME previously observed following DA administrations [Rasmussen, 1991]. Anatomic evidence supports the idea of close interactions between the dopamine and ß-endorphin neurons in the arcuate nucleus. Reciprocal synaptic connections between these two populations have been described [Leranth, 1986]. Also, evidence that DA can stimulate ß-endorphin release [Rasmussen, 1987] and POMC gene expression has been presented [Allen, 1988]. However, others have shown that DA inhibits ß-endorphin release from the hypothalamus [Vermes, 1985]. These contradictory results have been interpreted to suggest that different subpopulations of DA neurons may differentially activate ß-endorphin and LHRH neurons and thus may either inhibit or stimulate LHRH release [Rasmussen, 1991]. The specific and independent actions of dopamine on LHRH release and ß-endorphin remain unclear. However, these reveal that the response to dopaminergic activity in the MBH is dependent on the status of neighbouring neuronal populations such as ß-endorphin.

6.0 Estradiol action on the opioid system

6.1 Effects on ß-endorphin content and release

It has been shown that 3 weeks of E_2 treatment decreased hypothalamic ß-endorphin levels in ovariectomized rats [Wardlaw, 1982; Forman, 1986] relative to untreated controls, although no changes in hypothalamic ß-endorphin concentration were detected 3 weeks after ovariectomy [Wardlaw, 1982]. Conversely, hypothalamic ß-endorphin concentrations were found to be elevated in animals ovariectomized for 3 weeks and in accordance with previous findings estradiol replacement for 2 days reduced ß-endorphin concentrations to control levels [Le, 1990]. Interestingly, ß-endorphin levels in peripheral plasma were increased relative to ovariectomized animals in both intact and estradiol-replaced rats suggesting that estradiol may exert differential effects on hypothalamic and pituitary ß-endorphin secretion or on the synthesis and release of ß-endorphin from a given population. Indeed, evidence obtained from studies of perifused mediobasal hypothalamus demonstrated that estradiol stimulated the release of ß-endorphin in a dose-dependent fashion [Nakano, 1991]. The regulatory influences of estradiol on ß-endorphin have also been investigated in the context of fluctuating concentrations of both hormones during the estrous cycle. Using micropunches of hypothalamic slabs, Barden, demonstrated that ß-endorphin-immunoreactive levels in the median eminence and suprachiasmatic nucleus were at their highest during the afternoon of proestrus whereas ß-endorphin-ir concentrations in the arcuate nucleus were at their lowest at this time [Barden, 1981]. Although reduced ß-endorphin concentrations in the arcuate nucleus during the afternoon of proestrus were confirmed

in a later study, the median eminence was shown to contain moderate concentrations of ß-endorphin at this time. In the medial preoptic nucleus, ß-endorphin concentrations were lowest during proestrus, rose abruptly during estrus and then gradually declined during diestrus I and II. These results fit in well with the suppressed pattern of LH release observed during estrus, and with the relative lack of ß-endorphin suppression of LH release during the proestrus surge [Kalra, 1984; Van Vugt, 1981].

6.2 Effects on POMC mRNA expression

Using the RNA dot blot method for quantification of mRNA levels, Wilcox and Roberts (1985) demonstrated that implantation with E_2-containing capsules for 3 days caused a 40% decrease in POMC mRNA relative to controls. These findings were reproduced using *in situ* hybridization which further revealed that POMC mRNA levels were depressed at all levels of the arcuate nucleus except the most caudal one [Tong, 1990]. Later, it was shown in males that the effects of steroids on POMC mRNA expression were biphasic. An initial stimulation of POMC mRNA was observed for up to 6 hrs, whereupon a pronounced inhibition of POMC mRNA synthesis occurred [Wilcox, 1985].

6.3 Effects on opioid receptor levels

In addition to exerting pronounced changes in ß-endorphin release, varying E_2 levels have been shown to alter hypothalamic opioid receptor binding. During the estrous cycle, it was shown in both rats and hamsters that ^{3}H-naloxone binding in the medial preoptic area was lowest at proestrus and highest during estrus [Hammer, 1985; Ostrowski, 1987]. In a detailed study substantiating these findings, Jacobsen and Kalra

described significant reductions in ^{3}H-naloxone binding in the MBH and POA of EB-primed, P injected rats on the afternoon of proestrus as compared to the morning [Jacobsen, 1988]. ^{3}H-naloxone binding was not altered in sham-treated animals nor was it in other brain regions such as the cortex. Similar findings were observed in ovariectomized rats replaced with levels of estradiol designed to mimic those occurring normally during the estrus cycle [Hammer, 1985]. In another study by Casulari, 1988 mu opioid receptors measured on hypothalamic plasma membrane were decreased in the afternoon of EB-treated compared to controls. These studies parallel those of Jacobsen and Kalra, 1989 who demonstrated increase dihydromorphine (DHM) binding from the morning of proestrous to 12:00 followed by pronounced decreases in DHM during the afternoon of the LH surge. In these studies, the changes in ^{3}H-naloxone binding were shown to be due to changes in Bmax and not in the affinity of receptors. Together, these studies suggest a physiological role for E_2 (and P) in decreasing opioid binding during the LH surge.

7.0 Estradiol-valerate induced neuroendocrine cascade

7.1 Site of irreversible estradiol-induced damage

A variety of treatments which result in chronic exposure to E_2 have been shown to give rise to persistent vaginal cornification (chronic estrus), polycystic ovaries and acyclicity, including exposure to constant light [Cambell, 1980]; implantation with E_2 [Brawer, 1983; Schipper, 1981], feeding with E_2 [Kohama, 1989], injection with estradiol valerate [Brawer, 1983; Brawer, 1986; Garcia-Segura, 1988], and aging [Ascheim, 1976]. The mechanism whereby E_2 induces acyclicity and polycycstic

ovaries is not thought to involve permanent alterations in the ovarian synthetic apparatus or ovarian responsiveness since under certain condition such as cessation of constant light exposure [Cambell, 1980], hemiovariectomy [Hemmings, 1983; Farookhi, 1985] or stimulation with LHRH [Hemmings, 1983], the ovaries revert to a normal histological appearance and function. Thus, estradiol exposure does not permanently alter ovarian biosynthetic machinery and polycystic ovaries are believed to be the response of normal ovaries to an abnormal LH signal. Fundamental changes in pituitary function also do not seem to be causal to the development of estradiol-induced polycystic ovaries and acyclicity. Although changes in pituitary receptor levels [Carriere, 1988] and responsiveness to LHRH have been described [Carriere, 1989; Simard, 1987], these are all expected to result from a reduced hypothalamic LHRH signal. Several lines of evidence suggest that the action of estradiol in producing cystic anovulatory ovaries occurs at the level of the hypothalamus.

7.2 Morphological changes in the hypothalamic arcuate nucleus

In many of these models of chronic E_2 exposure including EV injection, administration via chronic release capsules, and aging, a neurodegenrative lesion occurs in the arcuate nucleus concomitant with the loss of ovarian cyclicity [Brawer, 1975; Brawer, 1978; Brawer, 1983; Schipper, 1981]. The lesion was first described in 1975 in the arcuate nucleus of female rats induced by estradiol valerate, a long acting 17 ß-estradiol derivative [Brawer, 1975]. This cytopathologic lesion was characterized at the light microscopic level by isolated foci of dark astrocytic granules located throughout the arcuate nucleus but not in any other brain region [Brawer, 1978]. At the

ultrastructural level, all the features of a classic neurodegenerative lesion were described including an invasion of microglia, an increase in number and size of reactive astrocytes, the appearance of collapsed myelin figures as well as degenerating axon terminals and dendrites. The latter were often observed in close proximity to dark cytoplasmic inclusion within astrocytic processes [Brawer, 1981]. The proliferation both in size and number of these unique astrocytic granules has been utilized as an index of degenerative activity in the hypothalamus [Schipper, 1981; Schipper, 1989]. Others have shown that the number of synapses per surface of dendritic shafts in the arcuate nucleus was significantly reduced after 8 and 16 weeks but returned to control levels at 32 weeks [Garcia-Segura, 1988], suggesting that a considerable amount of synaptic reorganization occurs at these periods. Additionally, several signs of neuronal degeneration such as significant increases in myelinic axons, dendrites and synaptic terminals were observed 4 weeks following EV treatment. However, the expression of these degenerative structures was transient such that by 8 or 16 weeks, no significant differences between controls were detected [Garcia-Segura, 1988]. These results are not in complete accord with the results of Schipper et al., 1981, demonstrating progressive increases in the number of astrocytic granules and microglial infiltrates from 24-52 weeks following EV treatment, however, since the indices of degeneration measured in these two studies are different, it may be that the collapsed myelin figures and astrocytic granules represent different aspects of the neurodegenerative process and are therefore not temporally correlated. Also, the larger dose of EV administered by Garcia-Segura et al., may have resulted in a different pattern of estradiol exposure.

Despite these well characterized morphological alterations in the arcuate nucleus, the chemical identity of the neurons undergoing degeneration has not been identified. In an attempt to identify these, immunocytochemical staining for tyrosine hydroxylase was performed in the arcuate nucleus of control and EV-treated rats at the electron microscopic level [Piotte, 1985]. No differences in neuron number nor in fine structural appearance of these neurons was observed between control and EV-treated groups indicating that dopamine containing neurons of the arcuate nucleus were not the primary targets of the estradiol-induced lesion [Piotte, 1985]. It has also been shown that the integrity of the LHRH neurons is not affected by a variety of manipulations which result in constant estrus including chronic estradiol exposure. Leranth et al., 1986 and King, 1980 have demonstrated that the number of LHRH immunoreactive terminals in the median eminence and the ultrastructural appearance of LHRH neurons in the medial preoptic area remains unchanged following induction of chronic estrus and acyclicity [Leranth, 1986; King, 1980]. These findings support the hypothesis that the EV-treatment causes a deafferentation of the LHRH neurons resulting in irregular release of LHRH [Brawer, 1981; Brawer, 1986], and do not conform with the idea that these neurons are directly compromised by estradiol.

7.3 Steroid specificity of arcuate lesion

The neurotoxic effects of estradiol on the hypothalamic arcuate nucleus have been shown to be steroid specific [Brawer, 1983]. Animals implanted with E_2 containing pellets showed marked increases in the mean number of astrocytic granules or microglial cells counted in the arcuate nucleus whereas in animals implanted with either

DHT alone or E_2 and DHT, these parameters were unchanged with respect to vehicle implanted controls. The protection from E_2 effects by DHT was suggested to be either direct or via the peripheral conversion of DHT to 5 α-andorstan-3 α, 17ß-diol since this metabolite was measured at two fold higher concentrations than DHT in the plasma of animals implanted with DHT [Brawer, 1983]. In animals implanted with T alone, no significant change in astrocytic granules was observed but microglial responses were increased in EV-treated rats probably due to central conversion of T to E_2 [Naftolin, 1988].

The neurodegeneration induced by chronic E_2 exposure is also observed in aged rats and mice [Schipper, 1981]. Astrocytic and microglial indices became gradually elevated from 6 months to 14 months of age. The degree of astrocytic reaction seen in the 52 week old untreated rat was similar to that seen 8 weeks after chronic estradiol exposure in ovariectomized rats [Brawer, 1983]. Moreover, the age associated degenerative process was entirely prevented by early ovariectomy (i.e. at 2 months; Schipper, 1981), suggesting that long-term exposure to physiological levels of estradiol during the course of normal aging could induce a hypothalamic lesion similar to that seen after experimentally induced chronic estrus and acyclicity.

7.4 Physiological changes in LHRH and LH

The hypothalamic lesion induced by E_2 is observed concomitant with marked changes in the pituitary content and secretion of LH and FSH. For example, the pituitary content of LH, whose synthesis is dependent on LHRH stimulation [Liu, 1976], was found to be decreased in animals with EV induced polycystic ovarian disease [Schulster,

1984; Simard, 1987]. In contrast, that of FSH was not altered in these same animals. Moreover, the post-castration rise in LH was attenuated in EV-treated animals as compared to controls [Simard, 1987] and LH release in response to repeated LHRH stimulations was reduced as compared to controls [Hemmings, 1983]. The pituitary content of LHRH receptors, which is often used as an index of LHRH stimulation [Clayton, 1981], was decreased in EV-treated animals [Carriere, 1989] and ovariectomy induced a significant increase in LHRH receptors in the pituitary gland of normal but not EV-treated animals [Carriere, 1988]. These results are consistent with a reduced content and secretion of LH in response to LHRH stimulation [Simard, 1987] and strongly suggest that LHRH secretion is reduced following EV treatment.

7.4.1 Changes in pulsatile pattern of release of LH

When the pulsatile pattern of secretion of LH was measured eight weeks following a single EV injection, using indwelling atrial catheters it was shown that LH pattern of release exhibited very low amplitude pulses (133 pg ml; peak 242 pg ml at 8 weeks) of 20-30 min duration with a frequency of approximately one pulse per hour [Grosser, 1987; McCarthy, 1990]. Mean LH levels in EV-treated animals were reduced to approximately 145 pg/ml [Grosser, 1987]. The development of this suppressed pattern of LH secretion from 0 to 8 weeks was characterized by a progressive decline in LH pulse frequency, amplitude and peak height such that at 8 weeks LH pulse amplitudes were reduced to approximately 130% of nadir values [Grosser, 1987; McCarthy, 1990]. This pattern of LH release did not resemble that of controls in estrus nor did it any other normal stage of the estrous cycle [Grosser, 1987; Gallo, 1981; Fox, 1985].

Animals in estrus exhibited pulse amplitudes greater than six-fold those observed in EV-treated animals and pulse periods were almost twice as long as animals in estrus but similar to that described for animals in diestrus [Grosser, 1987; Gallo, 1981]. The dramatic reductions in LH pulse amplitude in the absence of significant changes in other pulsatile parameters suggests that LH release in the EV-treated animal is chronically inhibited. It is therefore likely that the disregulation in LHRH release described above results in a chronically inhibited pattern of LH secretion which then induces the ovaries to become acyclic and polycystic [McCarthy, 1990; Brawer, 1986].

7.5 Comparison of EV-treated to E_2-implanted model of induced persistent estrus

In contrast to the effects on LH secretion induced by EV injection, the effects of chronic E_2 implantation for 8 weeks reveal a bimodal pattern of LH release consisting of relatively frequent (43 min) low but variable amplitude pulses (0.22 ± 0.165 ng ml), interrupted by large, broad based episodes of LH release lasting approximately 2 hours (10 ng/ml). These differences in the pulsatile pattern of LH release in the EV and E_2 implanted models are reflected in differential ovarian responses. For the first 14 days following EV injection, mean paired ovarian weights were found to be similar to those of controls. Between the second and third weeks after treatment, ovarian weight dropped significantly to lower than 50% control values [Brawer, 1986; Wilkinson, 1983]. In contrast, E_2 implanted animals exhibited enlarged ovaries at 8 weeks post-implantation [Brawer, 1989]. However, in both models, a dramatic reduction in healthy secondary ovarian follicles and a complete absence of corpora lutea was observed at 8 weeks post treatment. The histological appearance of the EV treated ovary has been

described as macrocystic because of the characteristic large cysts occupying an impoverished stroma. E_2-implanted ovaries display a microcystic condition with numerous small cysts embedded within a hypertrophied and luteinized stroma. These differences in histology are likely to reflect differences in the LH signalling pattern (as described above) as well as differences within the local steroid milieu. In this respect, it has been postulated that the hypertrophied ovary of the E_2 implanted animals reflects increased androgen conversion. Additionally, increased androgen levels do not occur in the EV treated animal [Hemmings, 1983]. Increased androgen (DHEA, T or androstenedione) production by the E_2-implanted ovary would also account for the slower progression of the hypothalamic lesion seen in this model relative to animals treated with EV [Brawer, 1983; Brawer, 1978] since DHT and α-diol have been shown to antagonize the neurotoxic effects of E_2 on the hypothalamus [Brawer, 1983].

7.6 Involvement of the hypothalamic opioid system in the EV-induced polycystic ovarian condition

The strongest evidence for the involvement of opioids in the generation of EV-induced acyclic condition comes from studies indicating that prolonged treatment (7 days) with the long-acting opioid antagonist naltrexone reversed the cystic appearance of ovaries and restored normal estrus cycles in EV-treated animals [Carriere, 1989]. In this study, it was also demonstrated that the content of pituitary LHRH receptors, normally reduced in the EV-treated animal, was also restored to normal levels following naltrexone treatment, suggesting that the disregulation in LHRH secretion involved the endogenous opioid peptides. Indeed, EV-treated were shown to display selective

increases in ^{3}H-naloxone binding in the hypothalamus but not the amygdala as compared to controls [Wilkinson, 1983; Wilkinson, 1985]. ß-adrenergic binding (as assessed by 3H-dihydroalprenolol binding) was unchanged in the hypothalamus, amygdala and cortex although the affinity of the ß-adrenergic receptor was reduced in the hypothalamus of EV-treated animals [Wilkinson, 1983]. Later, it was demonstrated that these selective increases in hypothalamic opioid binding reciprocated the conditions and features of the EV-induced hypothalamic lesion. Also, increases in ^{3}H-naloxone binding were steroid specific in that they did not occur in animals treated with either DHT or testosterone, but did in animals treated with E_2 or estradiol and T. Thus, increased opioid binding did not occur in EV-treated animals ovariectomized at the time of treatment. These findings strongly suggested that the hypothalamus of EV-treated animals was under chronic opioid inhibition and that the presence of increased opioid binding in the EV-treated animals was dependent on the presence of a demonstrable hypothalamic lesion.

7.0 Objectives of the present study

Although the short-term regulatory influences of estradiol on the hypothalamic-pituitary-ovarian axis have been extensively investigated, the effects of long-term estradiol exposure have not. Given that all female mammals are chronically exposed to estrogens throughout life, and that cumulated estradiol exposure has been implicated in the arrest of cyclicity, studies on the chronic effects of estradiol are clearly necessary for a complete understanding of the action of these steroids on the hypothalamic pituitary ovarian axis. The overall objective of the current thesis was to characterize the neural substrates underlying estradiol's actions on the hypothalamus using a well characterized model of estradiol induced acyclicity, the EV-treated rat.

Several lines of evidence had previously suggested that the hypothalamic opioid system played an important role in the etiology of the EV-induced syndrome. EV-treated animals exhibited a suppressed pattern of plasma LH characterized by a diminution in pulse amplitude in the absence of significant changes in other pulse parameters relative to estrus controls [Grosser, 1987; McCarthy, 1990]. Indeed, these changes in the pulsatile pattern of LH release resemble those which have been described following exogenous administration of mu opioid agonist in the medial preoptic area of the hypothalamus [Mallory, 1990; Leadem, 1986]. In addition, EV-treated animals injected with the opioid antagonist, naltrexone, exhibit a complete reversal of the polycystic ovarian syndrome including a resumption of ovarian cyclicity and the disappearance of cystic ovarian follicles [Carriere, 1990]. Finally, EV-treated rats display selective increases in the binding of ^{3}H-naloxone to hypothalamic homogenates

as compared to normally cycling controls Wilkinson, 1985; Wilkinson, 1985].

In Chapter 2, the normal intrahypothalamic distribution of mu, delta and kappa opioid binding sites was elucidated using *in vitro* radioautography with selective mu (^{125}I-FK 33 824), delta (^{125}I-DTLET) and kappa (^{125}I-DPDYN) opioid ligands. As the location of the previously demonstrated increase in ^{3}H-naloxone binding and the opioid receptor type involved were unknown, Chapter 3 examined the comparative distribution of these three opioid receptor types in controls and in rats chronically exposed to estradiol by either implantation with estradiol containing capsules or by a single injection of estradiol valerate. Chapter 4 examined the relationship between alterations in opioid binding and hypothalamic opioid peptide concentrations using *in vitro* radioautography of mu opioid binding sites in a well characterized model of arcuate nucleus destruction, the neonatally monosodium glutamate-treated rat.

The changes in opioid binding believed to be involved in the maintenance of the anovulatory condition induced by EV treatment were previously shown to be dependent on the presence of a hypothalamic lesion. Although this estradiol-induced lesion had been described at the light and electron microscopic levels, the chemical identity of the affected neurons had not been identified. For this purpose, in Chapter 5, several neuronal populations of the arcuate nucleus including ß-endorphin, tyrosine hydroxylase, somatostatin and neurotensin were examined by quantitative immunocytochemistry and ß-endorphin, met-enkephalin and neuropeptide-Y were measured by radioimmunoassay in control and EV-treated. Finally, the mechanism of estradiol-induced neurotoxicity was investigated in Chapter 6, by treating EV-injected animals with vitamin E, a potent

inhibitor of lipid peroxidation.

CHAPTER 2

Distribution of mu, delta and kappa opioid receptors in the hypothalamus of the rat

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16 text pages; 6 plates

Key words: opioid receptors; radioautography; hypothalamus; rat

Published in: Brain Research, 536 (1990) 114-123

ABSTRACT

The radioautographic distribution of mu, delta and kappa opioid binding sites was examined by *in vitro* radioautography in the rat hypothalamus using the highly selective ligands ^{125}I-FK 33 824, ^{125}I-azidoDTLET and ^{125}I-DPDYN, respectively. Levels of mu opioid binding sites varied considerably amongst hypothalamic nuclei. Mu opioid labeling was dense in the medial preoptic area, medial preoptic nucleus, suprachiasmatic nucleus and ventromedial nucleus, whilst the supraoptic nucleus, paraventricular nucleus, arcuate nucleus and dorsomedial nucleus were devoid of labeling. Delta opioid labeling was sparse throughout most of the hypothalamus, however, moderate binding densities were detected in the suprachiasmatic and ventromedial nucleus. Kappa opioid labeling was also scant throughout the hypothalamus with the exception of the suprachiasmatic nucleus which was very densely labeled. Our results indicate that the three opioid receptors types are differentially distributed within the hypothalamus, although a significant overlap exists. In general, the distribution of hypothalamic opioid receptors correlates well with that of opioid-containing terminal fibers and may represent the anatomical substrate for opioid involvement in the hypothalamic regulation of autonomic, behavioral and neuroendocrine functions.

Opioid peptides have been shown to play a major role in the hypothalamic regulation of a variety of autonomic, behavioral, and neuroendocrine functions [37]. For example, intra-hypothalamic administration of opioid analogues has been shown to decrease blood pressure [29], stimulate eating [16] and modulate the release of a number of hypothalamic hypophysiotropic factors [26]. The effects of exogenously administered opioids are highly dependent on the receptor selectivity and site of administration of the drugs utilized, illustrating the importance of a precise knowledge of the distribution of opioid receptor types within the hypothalamus for a better understanding of their mode of action.

All three main types of opioid receptors, namely mu, delta and kappa opioid receptors, have been shown to be present in mammalian hypothalamus using radioreceptor assays [8,54] or radioautographic techniques [1,9,15,20,21,31,32,34,35,40,41,49,51,54]. Radioautographic studies have revealed striking differences in the regional distribution of these three different receptor types in rat brain. There are, however, discrepancies in the literature concerning the relative proportion of each of these receptor types in the hypothalamus. For example, some investigators have claimed the hypothalamus to be particularly rich in mu opioid receptors [1,8,9,15,40], while others have reported very few or "negligible" amounts of mu opioid binding sites in this brain region [20,21,32,34,35,41,49,51,54]. The density of delta opioid binding sites has usually been described as being low [15,31,34,35,49,54] to moderate [9] throughout the rat hypothalamus. The density of kappa opioid binding sites was found in early studies to be higher than that of mu and delta opioid binding sites [31,32,49,54], but more recent studies using selective ligands suggest that kappa opioid receptors might actually be sparser than originally reported, at least

when defined in terms of affinity for dynorphin-like peptides [24].

In the present study, we have reevaluated the inter-hypothalamic distribution of mu, delta and kappa opioid binding sites labeled *in vitro* with the highly selective ligands ^{125}I-FK 33 824 [37], ^{125}I-azidoDTLET [6] and ^{125}I-DPDYN [11,12], respectively. Our results indicate that the three types of opioid receptors are differentially distributed in the rat hypothalamus and that their distribution differs, in some instances, from that described previously.

MATERIALS AND METHODS

Animals

Adult cycling female Wistar rats (n=7; 14 weeks) were purchased from Charles River Canada Inc. Only animals exhibiting at least two consecutive estrus cycles were used in this study. All rats were sacrificed by decapitation and their brains were rapidly removed , frozen by immersion in isopentane (-50°C) for 15 seconds and stored at -80°C.

Radioligands

Mu opioid receptors were labeled with the synthetic met-enkephalin analog FK 33-824, [D-Ala^2,N-Me-Phe^4,Met-(O)^5-ol]-Enkephalin (FK). The ligand was iodinated using the lactoperoxidase method and monoiodinated ^{125}I-FK was purified by gel filtration (specific activity:2000-2300 Ci/mmole). ^{125}I-FK was previously shown to bind to rat brain sections with a k_d of 1.2 nM [17,37] and to have good selectivity for mu receptors [37] (K_iDTLET/K_iFK= 15.9).

Delta opioid receptors were labeled with the selective photoaffinity probe azidoDTLET, [D-Thr^2]-Leu-Enkephalin-Thr, (graciously provided by B.P. Roques, Université René Descartes, Paris) . The compound was iodinated using the chloramine T method [22] and monoiodinated ^{125}I-azidoDTLET isolated using reverse-phase high performance liquid chromatography (HPLC) on a μBondapak C_{18} column with 0.25 M triethylamine formate, pH 3, (50%), and acetonitrile (50%) as the eluent (specific activity: 900-1000 Ci/mmole). ^{125}I-azidoDTLET was found to bind to rat brain sections with a K_d of 2.1 nM (Pasquini, F., Jomary, C., Roques, B.P. and Beaudet, A.,

unpublished) and to have good selectivity for delta receptors [6] (K_IDAGO/K_IDTLET= 9.8).

Kappa opioid receptors were labeled with the synthetic dynorphin analog DPDYN, [D-Pro[10]]-Dynorphin 1-11], (graciously provided by J. E. Gairin, CNRS, Toulouse). The compound was iodinated using the chloramine T method [22] and monoiodinated ^{125}I-DPDYN was isolated using reverse-phase HPLC on a μBondapak C_{18} column with 0.25 M triethylammonium phosphate, pH 3.5, (85%), and acetonitrile (15%) as the eluent (specific activity:1000-1200 Ci/mmole). ^{125}I-DPDYN was previously shown to bind to rat brain sections with a k_d of 0.3 nM and to have good selectivity for kappa receptors (K_IDSLET/K_IDPDYN= 140, K_IDAGO/K_IDPDYN= 48) [12,13].

Tissue Preparation

The brains were cut on a cryostat at -17°C, and serial sections (20 μm) were collected across the hypothalamus from the decussation of the anterior commissure rostrally, to the mammillary bodies, caudally. The sections were thaw-mounted on microscope slides that had been previously dipped in a gelatin chrome alum solution containing polyethyleneimine (.001% v/v). Two sections were placed on each slide and each consecutive slide was placed in separate slide boxes to be processed as follows: 1) incubation with ^{125}I-FK (μ) 2) incubation with ^{125}I-DTLET (δ) 3) incubation with ^{125}I-DPDYN (k) 4) staining with cresyl violet 5) co-incubation with non-radioactive naloxone (μ), levorphanol (δ) or DPDYN (k) for determination of non-specific binding.

In Vitro Radioautography

For determination of mu opioid binding, sections were incubated for 45 minutes at

room temp. in 0.05 M TRIS-HCL, pH 7.4 containing 1 nM ^{125}I-FK with and without 1 μM naloxone. For delta opioid binding, the sections were incubated with 1 nM ^{125}I-DTLET in the presence or absence of 1 μm levorphanol. After incubation, all sections were rinsed in 6 consecutive baths of ice-cold buffer and briefly dipped in distilled water. For determination of kappa opioid binding, sections were incubated for 45 minutes at room temp. with 0.3 nM ^{125}I-DPDYN in 0.05 M TRIS-HCL, pH 7.4 containing 10 μM bestatin with and without unlabeled DPDYN and rinsed for four hours in ice-cold TRIS-HCL containing 2% ovalbumin. All sections were then radioautographed by apposition to tritium-sensitive film (Amersham). After 3, 6 and 8 days of exposure for ^{125}I-FK, ^{125}I-DTLET, and ^{125}I-DPDYN respectively, the films were developed in Kodak GBX at 13°C for 5 min and fixed in Kodak rapid fixer.

For each of the three ligands, a series of sections were processed using liquid emulsion coating techniques. These sections were labeled as above except that 0.25 M sucrose was added to the incubation medium and buffer to maintain isosmolarity. After incubation with either ^{125}I-FK or ^{125}I-azidoDTLET, sections were post-fixed in a 3.5% glutaraldehyde solution on ice for 30 min. After incubation with ^{125}I-DPDYN, sections were post-fixed in a 2% paraformaldehyde solution on ice for 30 min. These fixation protocols were previously shown to allow for regionally proportional retention of a major fraction of the bound radioactivity [17,25]. All sections were subsequently dipped in distilled water, dehydrated in ethanols, cleared in xylene and rehydrated in an inverse series of ethanols. Once dry, these were coated by dipping in a 1:1 solution of NTB-2 emulsion, developed four weeks later in Dektol for 1.5 min. at 17°C, and stained with

cresyl violet.

Quantitation

Film radioautographs were quantitated using a computer-assisted microdensitometry program (Bioquant). Cresyl violet-stained sections were first used to outline anatomical boundaries and hypothalamic areas of interest on the computer screen. Radioautographic images were then aligned to anatomical boundaries and light transmittance was measured within the outlined hypothalamic nuclei. Quantitative measurements of binding site density were then obtained by converting light-transmittance values to nCi/mg tissue wet weight using ^{125}I-microscales (20 μm; Amersham) as standards [3,42].

RESULTS

When viewed in whole brain sections taken across the hypothalamus, selectively labeled mu, delta and kappa opioid binding sites showed distinct distributional patterns. Mu opioid binding sites were prominent within the internal granular layer of the frontoparietal cortex, neostriatal striosomes, the bed nucleus of the stria terminalis, and the amygdala, whereas delta opioid binding sites predominated in supra- and infragranular layers of the cortex, the entire caudate putamen and the amygdala (Fig. 1a, b). Kappa opioid binding sites showed overall lower binding densities than mu and delta sites and were mainly enriched in the superficial and deep layers of the cortex, the globus pallidus and the endopiriform nucleus (Fig. 1c).

Distribution of hypothalamic mu opioid binding sites

The density of mu opioid receptor labeling varied considerably across the different hypothalamic areas (Fig. 2, a-f). In most instances, the labeling remained confined within well-defined anatomical boundaries, as delineated in adjacent Nissl-stained sections, however, in the rostral hypothalamus, it occasionally exceeded nuclear limits (Fig. 2, a-f). At the level of the decussation of the anterior commissure, moderate to dense ^{125}I-FK binding was observed in the medial preoptic area (MPA) and medial preoptic nucleus (Fig. 2a, d). The median preoptic (MnPO) and anteroventral preoptic (AVPO) nuclei showed moderate binding densities whereas the lateral preoptic area (LPO) and striohypothalamic nucleus (SHy) were only weakly labeled. Labeling within the medial preoptic nucleus was contiguous dorsally with that of the bed nucleus of stria terminalis (BNST), forming a characteristic "V"-shaped pattern. More caudally, high

labeling densities were observed in the suprachiasmatic nucleus (SCh) as well as in the anterior hypothalamic area (AHA) and in the bed nucleus of the stria terminalis (BNST) (Fig. 2b,e). Low to moderate binding densities overlayed the lateral hypothalamic area (LH), whereas the periventricular nucleus (Pe), supraoptic nucleus (SON) and lateroanterior nucleus (LA) were conspicuously devoid of labeling (Fig. 2b, e). Within the suprachiasmatic nucleus, mu opioid binding sites were found by high resolution radioautography to be mainly concentrated within the ventrolateral segment of the nucleus and particularly along its ventral border (Fig. 3a, a'). At the level of the arcuate nucleus (Arc), low to moderate labeling was noted in the posterior pole of the anterior hypothalamic nucleus (AHP), the ventromedial nucleus (VMH), and the ventralmost portion of the lateral hypothalamic area (LH; Fig. 2c, f). The ventromedial nucleus was relatively densely labeled in its dorsomedial division, but only sparsely in its ventrolateral division (Fig. 2c, f). By contrast, the diffuse portion of the dorsomedial nucleus (not shown) and lateral hypothalamic area were less intensely reactive. The arcuate nucleus and underlying median eminence (ME) were virtually devoid of label (Fig. 2c, f). Rostral to the mammillary bodies, moderate binding densities were detected throughout the medial hypothalamus, excluding the arcuate nucleus-median eminence complex (Arc,ME; Fig. 4a). Only low binding densities were apparent in the lateral hypothalamic area (LH) (Fig. 4a). Finally, in the caudalmost portion of the hypothalamus, very high levels of mu opioid binding were detected in the ventral segment of the premammillary nucleus (not shown), the supramammillary nucleus (SuM) and the medial portion of the medial mammillary nucleus (MMn; Fig.

4b). Moderate binding densities were noted in the posterior hypothalamus (PH) as well as within the dorsal part of the premammillary nucleus (PMD; Fig. 4b).

Distribution of hypothalamic delta opioid binding sites

The distribution of ^{125}I-azidoDTLET-labeled delta opioid binding sites was much less extensive than that of mu opioid sites. With the exception of the suprachiasmatic (SCh) and ventromedial nuclei (VMH), all areas of the hypothalamus exhibited weak and diffuse labeling (Fig. 2g-i). Within the suprachiasmatic nucleus, ^{125}I-azidoDTLET binding was moderate and mainly confined within the ventrolateral segment of the nucleus (Fig. 2b, h). Light microscopic examination of liquid emulsion coated sections revealed no apparent enrichment along the ventral border of the nucleus, as observed with the mu agonist (Fig. 3b, b'). More dorsally, weak radiolabeling was apparent in the medial preoptic nucleus, tapering out dorsally towards the bed nucleus of the stria terminalis (BNST) as for mu opioid binding (Fig. 2h). Further caudally, moderate delta opioid binding was detected in the posterior portion of the anterior hypothalamic nucleus (AHP), the ventral portion of the lateral hypothalamic area (LH) and the ventromedial division of the ventromedial nucleus (VMH), again according to a pattern reminiscent of that of mu sites (Fig. 2c, i).

Distribution of hypothalamic kappa opioid binding sites

As can be seen in Fig 2 (j-l), the suprachiasmatic nucleus (SCh) was the only hypothalamic nucleus to exhibit high densities of ^{125}I-DPDYN binding sites. In liquid emulsion processed material, these were found to be concentrated in the ventral half of the nucleus rostrally, and in the inferolateral division, caudally (Fig. 3c,c'). In both

areas, the label extended further dorsally than either mu or delta receptor binding. Within the remainder of the hypothalamus, low densities of ^{125}I-DPDYN binding sites were apparent at the level of the medial preoptic area (MPA) rostrally (Fig. 2j), and at the level of the ventromedial nucleus (VMH), arcuate nucleus (Arc) and median eminence (ME) caudally (Fig. 2l).

Quantitation of mu, delta and kappa opioid binding sites confirmed the qualitative distribution described above (Fig. 5). Prominent with all three ligands was the relative enrichment of the suprachiasmatic and ventromedial hypothalamic nuclei. Also apparent from Fig. 5 was the selective enrichment of the preoptic area in mu, as opposed to delta or kappa sites.

DISCUSSION

The present results confirm that the rat hypothalamus contains measurable amounts of mu, delta and kappa opioid receptors. Mu opioid binding sites were detected throughout the hypothalamus and found to be concentrated most highly in the medial preoptic area and suprachiasmatic nucleus. Considerably lower densities of delta and kappa opioid binding sites were labeled throughout the hypothalamus except in the ventromedial and suprachiasmatic nuclei.

The mu opioid ligand utilized in the present study, ^{125}I-FK, has been shown to bind with high affinity (k_d=1.2 nm) and high selectivity to mu opioid receptors [37]. Our findings of relatively high levels of mu opioid binding in rat hypothalamus conform to the biochemical data of Chang et al. [8] and Pearson et al. [40], and the radioautographic data of Goodman et al. [15]. Other studies have reported low or negligible mu opioid binding in this region [32,35,49]. These apparent discrepancies could be due to sex differences [18,38] or to variations in the endocrine status of the animals [18,19]. They could also reflect differential affinities of hypothalamic mu opioid binding sites for the ligands used for their visualization. Under the present experimental conditions, the density of hypothalamic mu opioid binding sites was still relatively low as compared to regions such as the thalamus or neostriatum. It did compare, however, with binding densities found in layers 1 and 4 of the cerebral cortex (see Fig. 1a) and can therefore hardly be considered "negligible". The intrahypothalamic distribution of mu opioid receptors reported in the present study conformed for the most part with the previous radioautographic observations of McLean et al. [35] but differed from earlier data by

Mansour et al. [32], in that only low levels of mu opioid binding were detected in the dorsomedial nucleus and the lateral hypothalamic area in our material.

The delta opioid ligand utilized in the present study, ^{125}I-azidoDTLET, has been shown to bind with high affinity (K_d=2.1nm) and high selectivity to the delta opioid receptor [6]. This ligand revealed only low densities of opioid binding sites in the rostral hypothalamus and somewhat higher levels in the ventromedial nucleus in accordance with earlier reports on the distribution of delta opioid receptors in rat brain [15,32,34,35,49]. It also provided for the first demonstration of relatively high concentrations of delta opioid binding sites in the suprachiasmatic nucleus.

The kappa opioid ligand utilized in this study is an iodinated synthetic analogue of dynorphin A-(1-11), [D-Pro^{10}]dynorphin A-(1-11) or ^{125}I-DPDYN, which was shown to bind with high affinity (K_a= 0.3 nM) and good selectivity to kappa opioid binding sites [12,13]. Except for the suprachiasmatic nucleus which was densely labeled, ^{125}I-DPDYN labeling was low and diffusely distributed throughout the rat hypothalamus. These results are in agreement with earlier radioautographic observations [24] as well as with biochemical data suggesting that in the rat forebrain, only 9% of all opioid receptors are of the kappa variety [33]. However, they markedly differ from the results of studies based on the use of non-selective opioid ligands in the presence of saturating concentrations of mu and delta selective drugs, which describe within most hypothalamic structures higher levels of kappa labeling densities than detected in the present study [31,32,34,49,54]. This discrepancy could be due to the recognition by ^{125}I-DPDYN of only a subset of kappa binding sites. There is indeed pharmacological evidence for the existence of

multiple kappa receptors in the mammalian central nervous system [36] . The pharmacological and distributional characteristics of 125-DPDYN binding to guinea pig brain [24] suggest that it most likely recognizes dynorphin/U-69593-sensitive K_1 sites. However, it may not bind with the same affinity to benzomorphan-sensitive k_2 sites, or at least to the fraction of these that has been reported to display moderate affinity to dynorphin.

Although mu, delta and kappa opioid receptor types were distributed in unique patterns, there was a significant degree of overlap between them. For example, all three ligands labeled the bed nucleus of the stria terminalis and displayed a "V"-shaped labeling pattern in the region of the medial preoptic nucleus. The three ligands were also densely concentrated within the suprachiasmatic nucleus, and this labeling was confined for the most part to the ventral segment of the nucleus. Additionally, mu and delta opioid labeling overlayed the ventromedial nucleus and posterior aspect of the anterior hypothalamic nucleus, radiating toward the central nucleus of the amygdala in what presumably corresponds to the ventral amygdalofugal pathway [56]. The significant overlap in the hypothalamic distributions of mu, delta and kappa opioid binding sites is consistent with the hypothesis that these different opioid receptor types may correspond in part to interconvertible forms of a single receptor [7].

Areas where dense mu opioid labeling was detected, such as the bed nucleus of the stria terminalis, the medial preoptic area, the ventromedial nucleus and the amygdala are extensively interconnected [30,44,56]. This observation suggests that mu opioid binding sites may be associated with neuronal systems interlinking these different nuclear

groups. For instance, mu opioid binding sites detected in the medial preoptic area and bed nucleus of the stria terminalis may reside on luteinizing hormone releasing hormone (LHRH) neurons which have been shown to bridge these two regions in the rat [2,4,46]. Similarly, mu, and to a lesser extent delta opioid binding sites detected in the amygdala and the ventromedial nucleus may be associated with neurons known to interconnect these two regions [30,44,56]. Interestingly, the ventromedial nucleus and the amygdala have been linked to a number of behaviours such as satiation, dependence and female reproductive behaviours [28] in which endogenous opioids play a prominent role [37].

Although most opioid peptides have been shown to bind to more than one opioid receptor type, competition studies on rat brain sections or homogenates have shown that derivatives of proopiomelanocortin, pro-enkephalin and pro-dynorphin bind with highest affinity to mu, delta and kappa opioid receptors, respectively [34,39]. Many of the distributional features reported here for opioid receptors in rat hypothalamus conform with these pharmacological observations in that areas known to be rich in ß-endorphin, leu-enkephalin and dynorphin axon terminals exhibited the highest mu, delta and kappa labeling densities, respectively. For instance, the medial preoptic nucleus, which displayed the highest mu opioid binding densities, is also the region where the highest ß-endorphin fibre immunoreactivity has been detected in the hypothalamus [5,23,36,43,53]. Similarly, the ventromedial nucleus which exhibited relatively high delta binding densities, is the region of the hypothalamus where amongst the highest densities of leu-enkephalin immunoreactive fibres have been reported [10]. Finally, the high density of kappa opioid binding sites measured in the suprachiasmatic nucleus conformed with the

high concentration of dynorphin immunoreactive fibres detected in this nucleus [55]. In contrast, there was little correspondence between the distribution of mu, delta and kappa opioid binding sites and that of nerve cell bodies immunoreactive for ß-endorphin, leu-enkephalin and dynorphin, respectively. Thus, the arcuate nucleus which contains virtually all ß-endorphin immunoreactive nerve cell bodies in the hypothalamus [14,36,53] was virtually devoid of labeling with all three opioid ligands, suggesting that ß-endorphin-containing neurons express few, if any opioid receptors. Similarly, only marginal amounts of delta opioid binding were detected in regions of the hypothalamus known to contain high concentrations of leu-enkephalin immunoreactive cell bodies such as the paraventricular and supraoptic nuclei [45,48]. Finally, areas where dynorphin-containing cell bodies have been found, ie. the arcuate nucleus, paraventricular nucleus, supraoptic nucleus and lateral hypothalamic area, [27,50,52] were devoid of kappa opioid labeling. These results suggest that neither delta nor kappa opioid receptors are synthesized by enkephalin or dynorphin-containing cells.

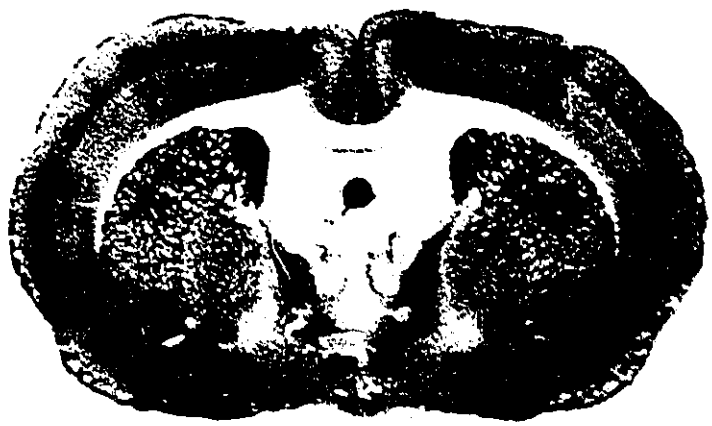
This study provides the first comprehensive distribution of mu, delta and kappa opioid binding sites in the hypothalamus of normal female rats. The three opioid receptor types are differentially distributed throughout the hypothalamus, yet display some degree of overlap. The distribution of opioid receptors correlates well with that of opioid peptide-containing axon terminals, and provides the anatomical substrate for opioid regulation of a variety of autonomic and neuroendocrine functions.

ACKNOWLEDGEMENTS

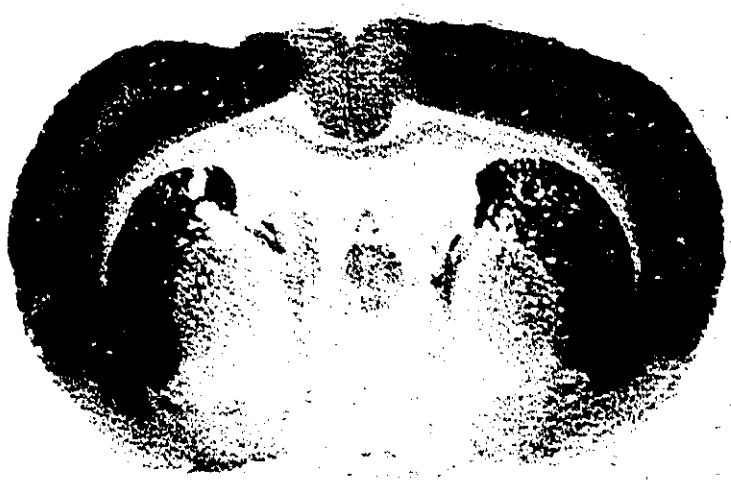
The authors would like to express their gratitude to Dr. C. Jomary, F. Pasquini and D. Marcel for their help with the iodination of the peptides. Additionally, the excellent photographic workmanship of Mr. C. Hodge and the assistance of K. Leonard are gratefully acknowledged. This work was supported by grant MT-7366 and a Scientist award to A.B. from the Medical Research Council of Canada.

Figure 1.

Selective labeling of mu, delta and kappa opioid binding sites in rat brain. Sections were incubated with ^{125}I-FK 33 824, ^{125}I-DTLET and ^{125}I-DPDYN to label mu (a), delta (b) and kappa (c) opioid receptors, respectively. Note the relative density of hypothalamic labeling with respect to other brain structures.



(a)

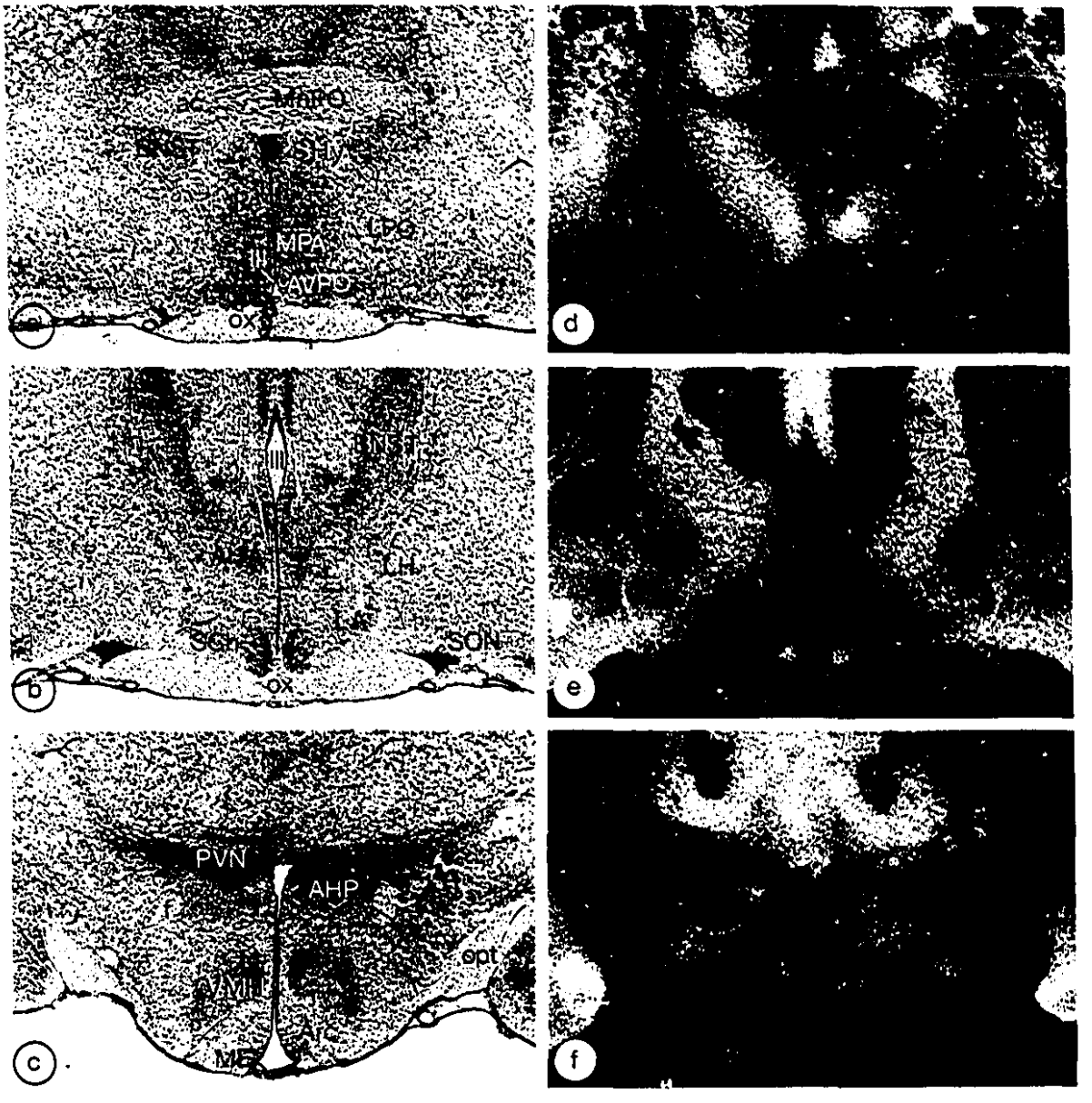


(b)

(c)

Figure 2.

Distribution of mu, delta and kappa opioid binding sites in rat hypothalamus. Cresyl violet stained sections taken at the level of the decussation of the anterior commissure (ac;a), the suprachiasmatic nucleus (SCh;b) and the ventromedial nucleus (VMH;c) are used as anatomical references. Film radioautographs from sections incubated with the mu opioid ligand, ^{125}I-FK 33 824 (d-f), the delta opioid ligand, ^{125}I-azidoDTLET (g-i) and the kappa opioid ligand ^{125}I-DPDYN (j-l) are illustrated in darkfield. Nomenclature as per Paxinos and Watson, 1986. III: 3rd ventricle, AHA: anterior hypothalamic area, anterior, AHP: anterior hypothalamic nucleus, posterior, BNST: bed nucleus stria terminalis, f: fornix, LA: lateroanterior hypothalamic nucleus, LH: lateral hypothalamic area, LPO: lateral preoptic area, ME: median eminence, MPA: medial preoptic area, opt: optic tract, ox: optic chiasm, PVN: paraventricular nucleus, Pe: periventricular hypothalamic nucleus, SCh: suprachiasmatic nucleus, SHy: striohypothalamic nucleus, SON: supraoptic nucleus. Scale bar: 1 mm.


MnPO
SHy
MPA
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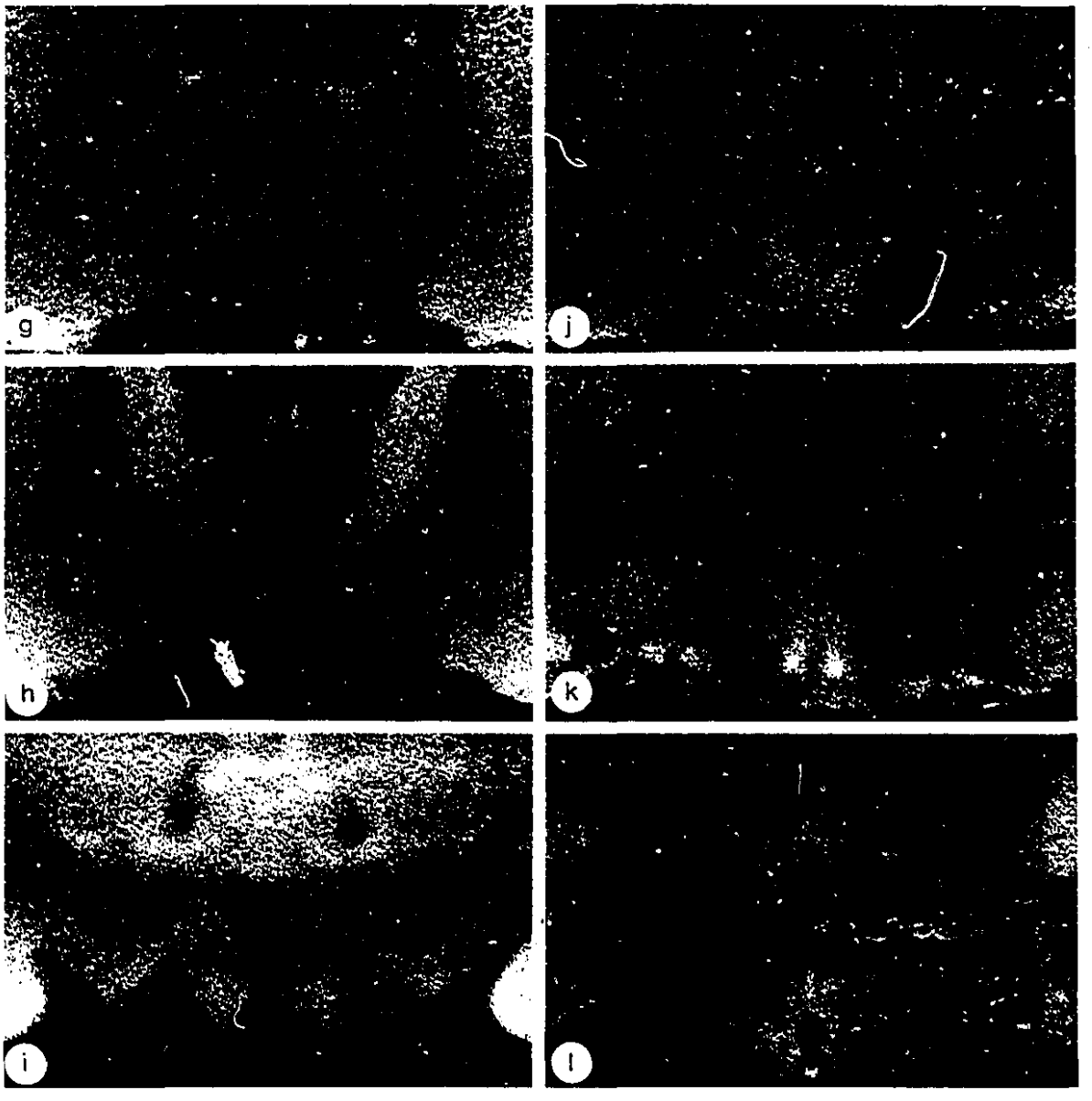
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Figure 3.

High resolution radioautographic distribution of mu, delta and kappa opioid binding sites within the suprachiasmatic nucleus. Bright (a-c) and darkfield (a'-c') micrographs of emulsion-coated sections incubated with ^{125}I-FK 33 824 (mu), ^{125}I-azidoDTLET (delta) or ^{125}I-DPDYN (kappa). Note that mu opioid labeling (a') is mainly confined to the ventrolateral segment of the suprachiasmatic nucleus and along its ventral border. Delta opioid labeling (b') is also mainly confined to the ventrolateral segment of the nucleus, but shows no obvious enrichment along the ventral border. Kappa opioid labeling (c') is restricted to the ventral half of the nucleus, rostrally, and to the inferolateral segment, caudally. In both areas, the labeling extends further dorsally than either mu or delta receptor binding. Scale bar: 200 μm.

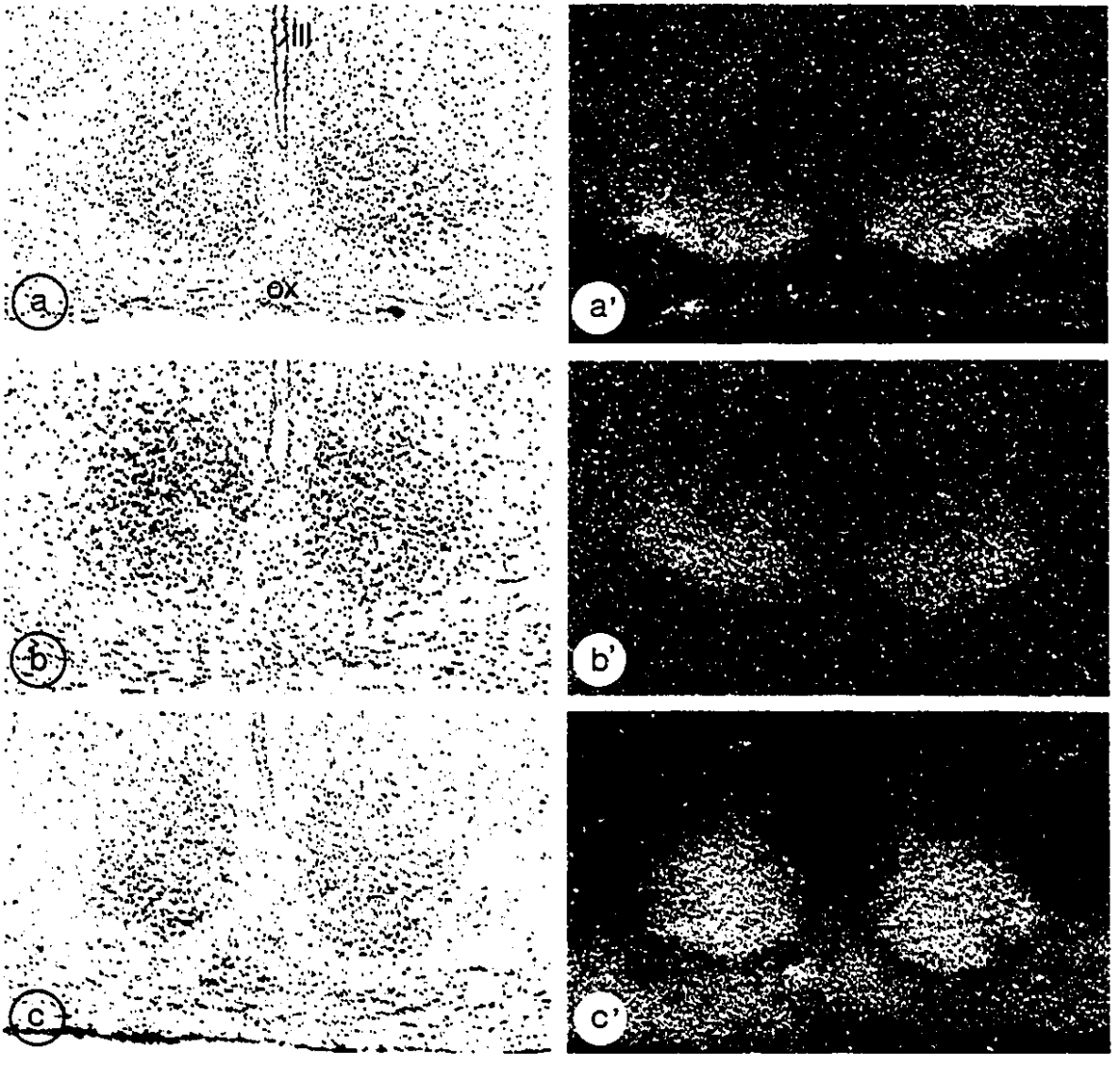
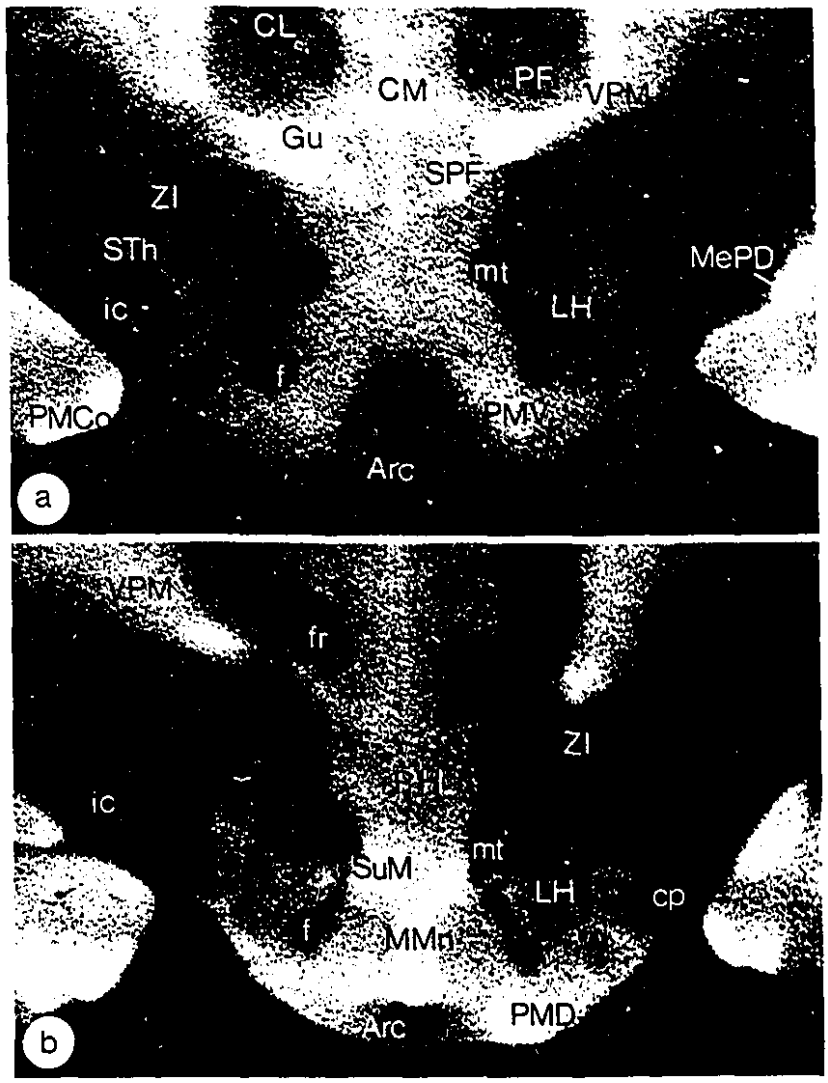

III
ox
a
a'
b
b'
c
c'

Figure 4.

Distribution of mu opioid labeling in the caudal hypothalamus. Darkfield radioautographs taken at the level of the infundibular stem (a) of the mammillary bodies (b). III: 3rd ventricle, Arc: arcuate nucleus, CL: centrolateral thalamic nucleus, CM: centromedial thalamic nuclei, cp: cerebral peduncle, f: fornix, Gu: gustatory thalamic nucleus, ic: internal capsule, LH: lateral hypothalamic area, MePD: medial amygdaloid nucleus, posterodorsal, mfb: medial forebrain bundle, MMn: medial mammillary nucleus, median, PF: parafascicular thalamic nucleus, PMCo: posteromedial cortical amygdaloid nucleus, PMD: premammilary nucleus, dorsal, PMV: premammillary nucleus, ventral, SPF: subparafascicular thalamic nucleus, STh: subthalamic nucleus, VPM: ventroposteromedial thalamic nucleus, ZI: zona incerta. Scale bar: 1 mm.


CL
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PMD
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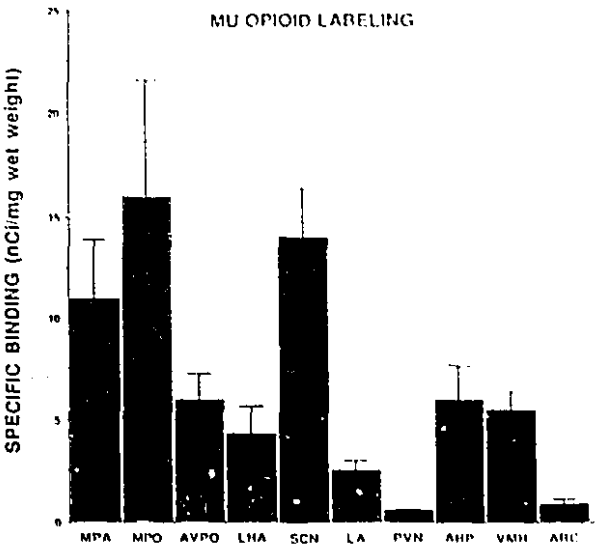
Figure 5.

Quantitative distribution of mu, delta and kappa opioid labeling densities within various hypothalamic nuclei. Light transmittance values measured using a computer-assisted microdensitometry system and converted to nCi/mg tissue wet weight using ^{125}I-microscales (20μm; Amersham) as standards. Results represent the mean $\pm$ S.E.M. of measurements taken in 6 adjacent sections/ animal (n=7). Densities for different opioid receptor types cannot be directly compared since only one concentration of ligand was tested in the present study and Scatchard analyses were not performed.AHP: anterior hypothalamic nucleus, posterior, AVPO: anteroventral preoptic nucleus, ARC: arcuate nucleus, LA: lateroanterior nucleus, LH: lateral hypothalamic area, MPA: medial preoptic area, MPO: medial preoptic nucleus, PVN: paraventricular nucleus, SCN: suprachiasmatic nucleus, VMH: ventromedial hypothalamic nucleus.


MU OPIOID LABELING
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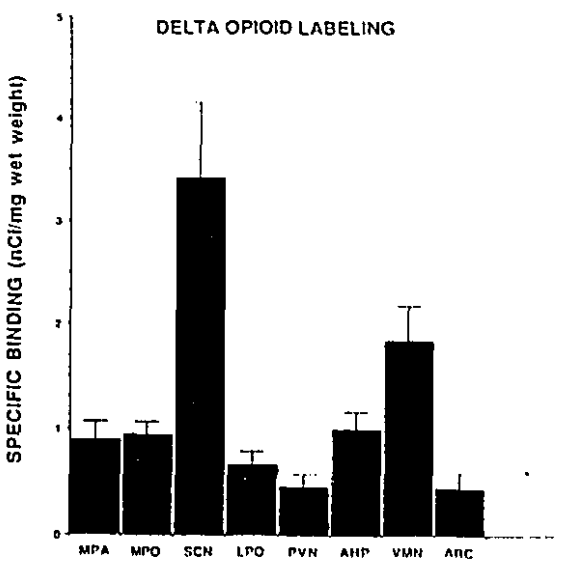
MPO
AVPO
LHA
SCN
LA
PVN
AHP
VMH
ARC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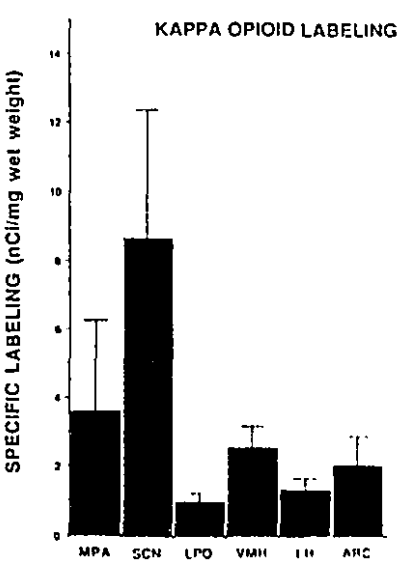
SPECIFIC BINDING (nCi/mg wet weight)
MPA
MPO
SCN
LPO
PVN
AHP
VMN
ARC


KAPPA OPIOID LABELING
SPECIFIC LABELING (nCi/mg wet weight)
MPA
SCN
LPO
VMH
ARC

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Connecting text# 1

Significant increases in ^{3}H-naloxone binding have been consistently observed in hypothalamic homogenates of EV-treated animals (Wilkinson, 1983; Wilkinson, 1985) relative to controls. However, the location of this increase remains to be determined. Since ^{3}H-naloxone is a non-selective opioid antagonist, it is also not known which type(s) of opioid receptor(s) account for the increases in opioid binding. In order to answer these questions, the intrahypothalamic distribution of the three main opioid receptor types were quantitated and compared to that of EV-treated animals as described below. Additionnally, it had previously been noted that the increases in ^{3}H-naloxone binding in EV-treated animals occurred only under conditions where a hypothalamic lesion was shown to occur, suggesting that the increase in opioid binding was linked to arcuate neuron damage. As an additional control for the effects of long-term estradiol exposure in the absence of a demonstrable hypothalamic lesion, EV-treated rats were compared to animals implanted with E_2 for 8 weeks, a time where these rats do not display a hypothalamic lesion.

CHAPTER 3

Alterations in opioid parameters in the hypothalamus of rats with estradiol-induced polycystic ovarian disease

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Abbreviated title: estradiol effects on opioid system

17 text pages; 5 plates

Key words: opioid receptors; ß-endorphin; hypothalamus; estradiol; polycystic ovarian disease

Published in: Endocrinology 127, (1990) 2969-2976

ABSTRACT

The distribution and density of selectively labeled mu, delta and kappa opioid binding sites were examined by *in vitro* radioautography in the hypothalamus of normal, estradiol valerate (EV)-injected and estradiol (E_2)-implanted female rats. Hypothalamic ß-endorphin concentration was also examined by radioimmunoassay in these three groups of animals. Quantitative analysis of film radioautographs demonstrated a selective increase in mu opioid binding in the medial preoptic area of EV-treated, but not of E_2-implanted rats. However, both these estrogenized groups exhibited a reduction in the density of delta opioid binding in the suprachiasmatic nucleus. Statistically significant changes between either estrogenized groups were not observed for kappa opioid binding. Results on the hypothalamic concentration of ß-endorphin indicated a marked reduction in EV-injected animals with respect to controls. In contrast, the E_2-implanted animals exhibited ß-endorphin concentrations similar to controls. The present results confirm the increase in opioid receptor binding previously reported in the hypothalamus of EV-treated rats and further demonstrate that this increase is confined to the medial preoptic area and exclusively concerns mu opioid receptors. The concomitant reduction in ß-endorphin levels observed in the same group of animals suggests that the observed increase in mu opioid binding could reflect a chronic up-regulation of the receptor in response to compromised ß-endorphin input. Given the restriction of this effect to the site of origin of LHRH neurons and the demonstrated inhibitory role of opioids on LHRH release, it is tempting to postulate that such up-regulation could lead to the suppression of the plasma LH pattern that

characterizes polycystic ovarian disease in the EV-treated rat.

INTRODUCTION

Chronic anovulation and polycystic ovaries (PCO) occur in the rat as a consequence of a single large intramuscular injection of estradiol valerate (EV) [10,28,53]. Evidence from our laboratory indicates that the estradiol treatment produces a permanent defect at the hypothalamic level, resulting in an aberrant pattern of luteinizing hormone releasing hormone (LHRH) release [56]. This pattern in turn results in a unique anomalous plasma LH pattern to which the ovaries respond by becoming polycystic [14,25,37].

Although the relationship between the aberrant pattern of LH release and the cystic ovarian condition has been fairly well established [14,25,37], substantially less is known about the EV-induced hypothalamic defect that gives rise to it. Several lines of evidence from our laboratory, however, suggest that the primary hypothalamic impairment involves endogenous opioid neurons. The attenuated pulsatile plasma LH pattern associated with PCO, for example, indicates chronic suppression of the hypothalamo-pituitary gonadal axis [25,37]. Opioids are potent inhibitors of LH release under a variety of conditions [5,32,47]. We have also shown that opioid binding in the anterior hypothalamus-preoptic area (as assessed with tritiated naloxone) is elevated in EV-induced PCO, suggesting the possibility of hypothalamic supersensitivity to opioids [59,60]. Finally, we have shown that treatment of animals with EV-induced PCO with naltrexone significantly elevates the number of pituitary LHRH receptors and restores normal ovarian morphology [12].

The present study was undertaken in order to elucidate the role of the hypothalamic

opioid system in EV-induced PCO. In order to determine the receptor selectivity and precise localization of the elevation in opioid binding previously reported after EV-treatment [59,60], we have employed *in vitro* radioautography using specific ligands for mu, delta and kappa receptors [17]. Since ß-endorphin has been specifically implicated in the suppression of gonadotropin secretion [32], we have also measured the concentration of ß-endorphin in the hypothalamus of animals with EV-induced PCO. Animals exposed to chronic estrogenization by means of subcutaneously implanted estradiol (E_2)-containing silastic capsules also exhibit a type of polycystic ovarian condition [38]. This treatment has also been shown to result in elevated naloxone binding, albeit less intense than that observed after in EV-injection [60]; consequently, we have also examined the same opioid parameters in E_2-implanted rats. Our results indicate that the EV treatment, but not E_2 implants produce a highly specific elevation of mu receptors in a restricted region of the medial preoptic area concurrent with a reduction in ß-endorphin concentration.

MATERIALS AND METHODS

Animals

Five-week old female Wistar rats (175-200g), purchased from Charles River Canada Inc. (St-Constant, Quebec), were maintained under conditions of controlled light (lights on between 0700h - 2100h) and temperature (22°C). Animals had free access to pelleted rat food and water. Only animals exhibiting at least two consecutive estrus cycles as assessed by daily examination of vaginal smears were used in this study. Fourteen animals were given a single IM injection of 2mg estradiol valerate (EV). This treatment has been shown to result in a permanent polycystic ovarian condition accompanied by a characteristic abnormal plasma gonadotropin pattern by 4 weeks after treatment [7,10]. A second group of 12 animals were each implanted subcutaneously with a single silastic capsule (o.d. 3.18 mm, i.d. 1.98 mm, length 2 mm) containing crystalline estradiol (E_2). This treatment maintains a constant serum concentration of 30-50 pg/ml E_2 [8,38]. A third group of 19 animals were normal cycling controls, age-matched to the two experimental groups. Eight weeks following the EV injections or implantation of the chronic release capsules, all animals were killed by decapitation (9-10 a.m.). Control animals were at different stages in their estrus cycle at the time of sacrifice. Twenty-one animals (i.e. 7 controls, 7 EV-injected and 7 E_2-implanted) were used for radiautographic studies, in which case their brains were rapidly removed, frozen by immersion in isopentane (-50°C), and stored at -80°C until sectioned. The remaining 24 animals were used for the determination of ß-endorphin concentration and release.

Radioligands

FK 33-824, [D-Ala2,N-Me-Phe^4,Met-(O)^5-ol]-Enkephalin, (Sandoz) was used to label mu opioid receptors. The peptide was iodinated using the lactoperoxidase method and monoiodinated ^{125}I-FK 33-824 was isolated by gel filtration (specific activity: 2000 Ci/mmole). The K_d of ^{125}I-FK 33-824 binding to rat brain sections was previously estimated at 0.8 nM [26,45] and its selectivity index at 15.9 for K_IDTLET/K_IFK 33-824 [45].

AzidoDTLET, [D-Thr^2]-Leu-Enkephalin-Thr, graciously provided by B.P. Roques, was used to label delta opioid receptors. The compound was iodinated by means of the chloramine T method [29] and monoiodinated ^{125}I-azidoDTLET was isolated using reverse-phase high performance liquid chromatography on a μBondapak C_{18} column with 0.25 triethylammonium formate, pH 3, (50%) and acetonitrile (50%) as the eluent (specific activities: 1000 Ci/mmole). The K_d of ^{125}I-azido DTLET binding to rat brain membrane preparations was estimated at 15 nM [6] and its selectivity index at 9.8 for K_IDAGO/K_IDTLET [6].

DPDYN, [D-Pro^{10}]-Dynorphin 1-11, graciously provided by J. E. Gairin, was used to label kappa opioid receptors. As above, the peptide was iodinated using the chloramine T method [29], and monoiodinated ^{125}I-DPDYN was isolated by reverse-phase HPLC on a uBondapak C_{18} column using 0.25 M triethylammonium phosphate, pH 3.5, (85%), and acetonitrile (15%) as the eluent (specific activities: 1200 Ci/mmole). The k_d of ^{125}I-DPDYN binding to membrane preparations was estimated at 0.3nM [21,22] and its selectivity index at 140 and 48 for K_IDSLET/K_IDPDYN and K_IDAGO/K_IDPDYN, respectively [21,22].

Tissue Preparation

The hypothalami of the 21 animals (ear marked for radioautographic studies) were serially sectioned on a cryostat from the decussation of the anterior commissure, rostrally, to the mammillary bodies, caudally. Sections (20um-thick) were thaw-mounted on microscope slides subbed with a 10% gelatin/ 1% chrome alum solution containing 0.001% v/v polyethyleneimine solution. Two sections were placed on each slide and each consecutive slide was placed in one of five slide boxes to be processed as follows: 1) incubation with ^{125}I-FK 33-824 (μ) 2) incubation with ^{125}I-azidoDTLET (δ) 3) incubation with ^{125}I-DPDYN (k) 4) incubation with non-radioactive naloxone (μ), levorphanol (δ) or cold DPDYN (k) for determination of non-specific binding. 5) staining with cresyl violet.

In Vitro Radioautography

For determinations of mu opioid binding, frozen sections from the hypothalami of normal, estradiol-implanted and estradiol-injected animals were incubated simultaneously for 45 minutes at room temp. in 0.05 M TRIS-HCL (pH 7.4) containing 1 nM ^{125}I-FK 33-824 with or without 1 uM naloxone. Delta opioid binding was determined in sections from the same three series by incubation in the same buffer containing 1 nM ^{125}I-azidoDTLET in the presence or absence of 1 uM levorphanol. Incubation with ^{125}I-azidoDTLET was performed in the dark to prevent covalent linkage of the ligand to the tissue. For determinations of kappa opioid binding, sections were incubated for 45 min at room temp. in 0.05 M TRIS-HCL (pH 7.4) containing 10 μM bestatin and 0.3 nM ^{125}I-DPDYN with or without unlabeled DPDYN. After incubation, sections labeled with

^{125}I-FK 33-824 or ^{125}I-azidoDTLET were rinsed for 2 mins in 6 consecutive baths of ice-cold TRIS-HCl buffer. Sections incubated with ^{125}I-DPDYN were rinsed for four hours in ice-cold TRIS-HCL containing 2% albumin. All sections were air-dried at room temp and radioautographed by apposition to ^{3}H-sensitive film (Amersham). After 3,6 and 8 days of exposure for ^{125}I-FK 33-824, ^{125}I-azidoDTLET, and ^{125}I-DPDYN respectively, the films were developed in Kodak GBX at 13°C for 5 min and fixed in Kodak rapid fixer.

Quantitation

Film radioautographs were quantitated using a computer-assisted microdensitometry system (Bioquant). The adjacent cresyl violet stained section, were used to outline the anatomical boundaries of each hypothalamic nuclei of interest in every animal. Radioautographic images were then superimposed over corresponding drawings and light transmittance was measured within the outlined hypothalamic nuclei. Quantitative measurements of binding site density were then obtained by converting light-transmittance values to nCi/mg tissue wet weight using ^{125}I-microscales (Amersham; 20μm) as standards [4,52].

Determination of ß-endorphin concentrations

Twenty-four rats (12 control, 7 EV-injected and 5 E_2-implanted) were decapitated and their hypothalami were quickly removed and homogenized in 1 ml acetic acid (2N) using a Polytron. The tissue was separated from the medium by centrifugation and the supernatant utilized for the determination of ß-endorphin concentration using a commercially available radioimmunoassay (RIA) to human ß-endorphin (1-31;

Peninsula). The % cross reactivity with methionine-enkephalin was 0% and with gamma-endorphin was >0.001%.

Statistical analysis

Statistical analysis of all data was performed using a one-way analysis of variance followed by t-test and only p values of less than 0.05 were considered significant.

RESULTS

Effects of EV-treatment on mu, delta and kappa opioid binding

The topographic distribution of ^{125}I-FK 33-824-labeled sites was similar in the hypothalami from control, E_2-implanted and EV-injected animals and conformed to that previously described for mu opioid receptors [17,23,40]. Briefly, the medial preoptic and suprachiasmatic nuclei exhibited dense mu opioid labeling, the ventromedial and anterior hypothalamic nuclei were moderately labeled, whereas the arcuate, dorsomedial and magnocellular nuclei displayed very sparse labeling. Computer-assisted measurements of ^{125}I-FK 33-824 binding densities in different hypothalamic nuclei revealed a trend toward increased concentrations of mu opioid labeling in most hypothalamic nuclei of EV-treated animals compared to normal controls. A significant increase in mu opioid binding, however, was only observed in the medial preoptic nucleus (MPO) and the anteroventral preoptic nucleus (AVPO) (Figs. 1a,b; 2). There were no significant differences in ^{125}I-FK 33-824 binding between E_2-implanted animals and normal controls (Fig. 2).

The pattern of ^{125}I-azidoDTLET binding was similar in the hypothalami of control, EV-injected and E_2-treated animals and resembled that described previously for delta opioid receptors [17,23,36,40]. Moderate delta opioid labeling was detected in the ventromedial, anterior hypothalamic and suprachiasmatic nucleus. Densitometric measurement of ^{125}I-azidoDTLET binding within different hypothalamic nuclei revealed a significant decrease in delta opioid labeling in the suprachiasmatic nucleus (SCN) of both EV- and E_2-treated groups as compared to normal controls (Figs. 3a,b; 4). No

alteration in delta opioid labeling was detected in the medial preoptic, anteroventral preoptic or any other nuclei from either of these two experimental groups (Fig. 4).

The topographic distribution of ^{125}I-DPDYN-labeled binding sites was similar in normal, EV-injected and E_2-treated groups and resembled that described previously for kappa opioid receptors [17,31] . The suprachiasmatic nucleus was the only region of dense kappa opioid labeling although the medial preoptic and arcuate nuclei contained low to moderate labeling densities. Densitometric measurement of kappa binding densities revealed a trend toward decreased labeling in the SCN of EV-injected and E_2-treated animals as compared to controls, however this change was not statistically significant (Fig. 5).

Effects of EV treatment on concentrations of hypothalamic concentrations of ß-endorphin

Hypothalamic ß-endorphin concentrations as measured by RIA were significantly lower in EV-treated animals (82.7$\pm$7.0 pg ß-endorphin/mg tissue; n=7) than in normal controls (148.9$\pm$19.0; $p<0.05$; n=12). In contrast, the mean hypothalamic ß-endorphin levels in the E_2-treated rats (161.0$\pm$16.9; n=5) was similar to that of normal controls.

DISCUSSION

It has been previously demonstrated that opioid binding sites, as determined by ^{3}H-naloxone binding, are elevated in hypothalamic homogenates from EV-treated rats [59,60]. Furthermore, this increase was shown to be the result of an increase in the density of opioid binding sites rather than of an alteration in the affinity of the sites. The present results indicate that this increase in binding is attributable to a selective elevation in mu opioid receptors and that it is predominantly localized to the medial preoptic nucleus (MPO) and the anteroventral preoptic nucleus (AVPO) of EV-treated animals. Given that these two nuclei correspond to regions where LHRH cell bodies are located [34,54,55], our observations support the hypothesis that the hypothalamic defect underlying the suppressed LH pattern in EV-treated animals [56,37] is due to hypersensitivity of LHRH neurons to the inhibitory action of endogenous opioids [60]. Whether this effect occurs through mu opioid binding sites located directly on LHRH neurons remains to be determined but the recent demonstration of synaptic contacts between ß-endorphin-immunoreactive axons and LHRH neurons in the rat MPO [13,35] makes this a likely possibility. Another possibility is that the opioid receptors affected are located presynaptically on catecholaminergic elements impinging upon LHRH cells. There is indeed evidence that the presynaptic stimulatory action of noradrenaline on LHRH release may be under opioid control [19]. Mu-selective agonists have been shown to decrease the release of noradrenaline from MPO slices [20] and to decrease the *in vivo* turnover rate of noradrenaline in the MPO [24], resulting in both cases in a marked reduction in LH release.

The specific elevation in mu opioid binding in the MPO and AVPO was found to coincide with a reduction in hypothalamic levels of ß-endorphin. Furthermore, preliminary (unpublished) data from our laboratory suggest that both basal and veratridine evoked release of ß-endorphin are attenuated in hypothalamic slices from EV-treated animals. Taken together, these results may be taken as an indication that the EV treatment compromises the ß-endorphin neuronal network. This functional defect may well reflect the neuronal degeneration observed in the arcuate nucleus of EV-treated animals [9,11].

Three lines of evidence suggest to us, that the decrease in hypothalamic ß-endorphin and the concomitant elevation in mu opioid binding in the MPO may be causally linked. First, ß-endorphin neurons have been shown to project extensively to the MPO [41,61] and, as mentioned above, ß-endorphin terminals have been observed making direct synaptic connections on LHRH-immunoreactive elements in this nucleus [13,35]. Second, there is evidence that ß-endorphin interacts primarily with mu opioid receptors [51]. Third, opioid target neurons have been shown to compensate for a reduced opioid input by expressing an increased number of receptors [2,44]. The increased mu opioid receptor binding observed in the present study could therefore result from diminished ß-endorphin release in the MPO. The ensuing hypersensitivity of the target neurons, presumably LHRH neurons or noradrenergic axons, would render them chronically susceptible to inhibition, either by residual ß-endorphin or by other endogenous opioids.

In contrast to EV-treated rats, the animals chronically estrogenized with E_2-containing implants did not differ from normal controls in the density of mu opioid

binding in the MPO and AVPO. They also compared to normal controls with regards to hypothalamic ß-endorphin concentration. These data suggest that E_2-implanted animals are not subjected to the same type of hypothalamic insult as are EV-treated rats. The E_2-implanted animal does exhibit pathological changes in the arcuate nucleus similar to those seen in the EV-treated rat, albeit at much longer time intervals [8]. It is therefore possible that the arcuate damage in this group of animals is brought about by different mechanisms or concerns other neuronal populations than in EV-treated rats.

The major differences reported herein for the two estrogenized models are not unexpected. The E_2-implanted rats exhibit a polycystic ovarian condition very different from that seen in EV-treated rats. The ovaries are much larger and the stroma is greatly hypertrophied. The cysts are numerous, small and surrounded by a grossly hypertrophied theca [38]. This is in stark contrast to the small atrophied ovaries containing a few very large cysts with modest theca seen in the EV-treated rats [7,37]. As would be predicted on the basis of ovarian morphology, the plasma gonadotropin patterns in the E_2-implanted model differ considerably from those in the EV-treated rats [38]. In contrast to the suppressed LH pattern seen in the latter, E_2-implanted rats exhibit large LH episodes of relatively long duration [38]. The present study supports the involvement of different etiological factors in the two disorders and suggests that a major difference between the two is the presence in EV-treated but not E_2-implanted rats of chronic opioid inhibition of LHRH secretion.

The decrease in delta binding in the SCN of both EV-injected and E_2-implanted rats was an unexpected finding. Since this change was observed in both experimental

models, it would appear that the mechanism underlying it is fundamentally different from that underlying the increase in mu opioid receptors in the MPO in EV-injected rats. In particular, this mechanism is unlikely to be the result of a local increase in the concentration and/or release of endogenous ß-endorphin, in the light of the present biochemical results. Furthermore, ß-endorphin cells do not appear to project extensively to the SCN [49,57], and the ligands with the highest affinity for delta receptors are derived from pro-enkephalin A [15,51]. The decrease in delta opioid binding observed in the SCN more likely represents a physiological response to the constant, maintained plasma concentrations of estradiol characteristic of both estrogenized models [37,53]. It is now well established that central opioid binding may be regulated by gonadal steroids [27,50]. For instance, the density of opioid binding sites has been shown to vary during the estrus cycle, being highest at diestrous and lowest at proestrus [27,50]. This mechanism may be part of a more general process of receptor sensitivity regulation through which gonadal steroids may affect hypothalamic function [39]. The suprachiasmatic nucleus could even represent a target of choice in this regard given that both neurotensin [46] and α-bungarotoxin [42] binding were also found to be down-regulated by gonadal steroids in this area.

The functional significance of the decrease in delta opioid binding within the SCN is, as of yet, unclear. Indirect evidence suggests that the suppressed LH pattern typifying EV-induced PCO may be subject to diurnal variation. There are, in the EV-induced polycystic ovary, unique large follicular structures (type III large follicular structures) that are very different from the majority of degenerate cystic, precystic and

atretic follicles [7,18]. In all likelyhood, it is the eventual atresia of these large follicular structures that produces the precystic and subsequent cystic follicles [7,38]. It would seem that for such an advanced state of folliculogenesis to occur, large gonadotropin pulses must occur. Since we have only examined gonadotropin patterns during the daytime [25,37], it may be that large gonadotropin episodes occur at night. This is, in fact, what occurs in the human cystic ovarian condition secondary to hypothalamic amenorrhea [33]. If such is the case, the changes in delta opioid binding in the SCN could be of considerable significance since this nucleus is a major regulator of diurnal rhythmicity [30,43,48].

In conclusion, the present study supports the view that cystic ovarian disease encompasses a variety of expressions, each with it's unique underlying pathogenic mechanism [37]. EV-induced polycystic ovarian disease may result from a chronic up-regulation of mu opioid receptors on and/or in the vicinity of LHRH neurons rendering them chronically susceptible to opioid inhibition. In contrast, the E_2-implanted model of PCO is not associated with specific alterations in mu opioid receptors or in hypothalamic concentration of ß-endorphin but rather seems to reflect a physiological adaptation of the hypothalamus and/or pituitary to chronic circulating estradiol levels. As previously indicated, the EV-induced PCO resembles in many respects the multifollicular ovarian condition seen in women with hypothalamic amenorrhea [1]. It is of considerable interest that the hypothalamic opioid system seems to play a key role in this type of cystic ovarian disease [33,58]. In contrast, the E_2-implanted PCO exhibits some similarity to the Stein-Leventhal disease [1,38], a condition which does not appear

to be associated with enhanced hypothalamic opioid tone [3,16].

Figure 1.

Radioautographic distribution of ^{125}I-FK 33-824-labeled mu opioid binding sites in the medial preoptic area of normal (a) and EV-injected (b) rats. Note the striking increase in the density of mu opioid labeling in the medial preoptic nucleus (MPO) of EV-treated rats. Dark field. III: third ventricle, ac: anterior commissure, LH: lateral hypothalamic area. Nomenclature as per Paxinos and Watson (1986). Scale bar: 1 mm.

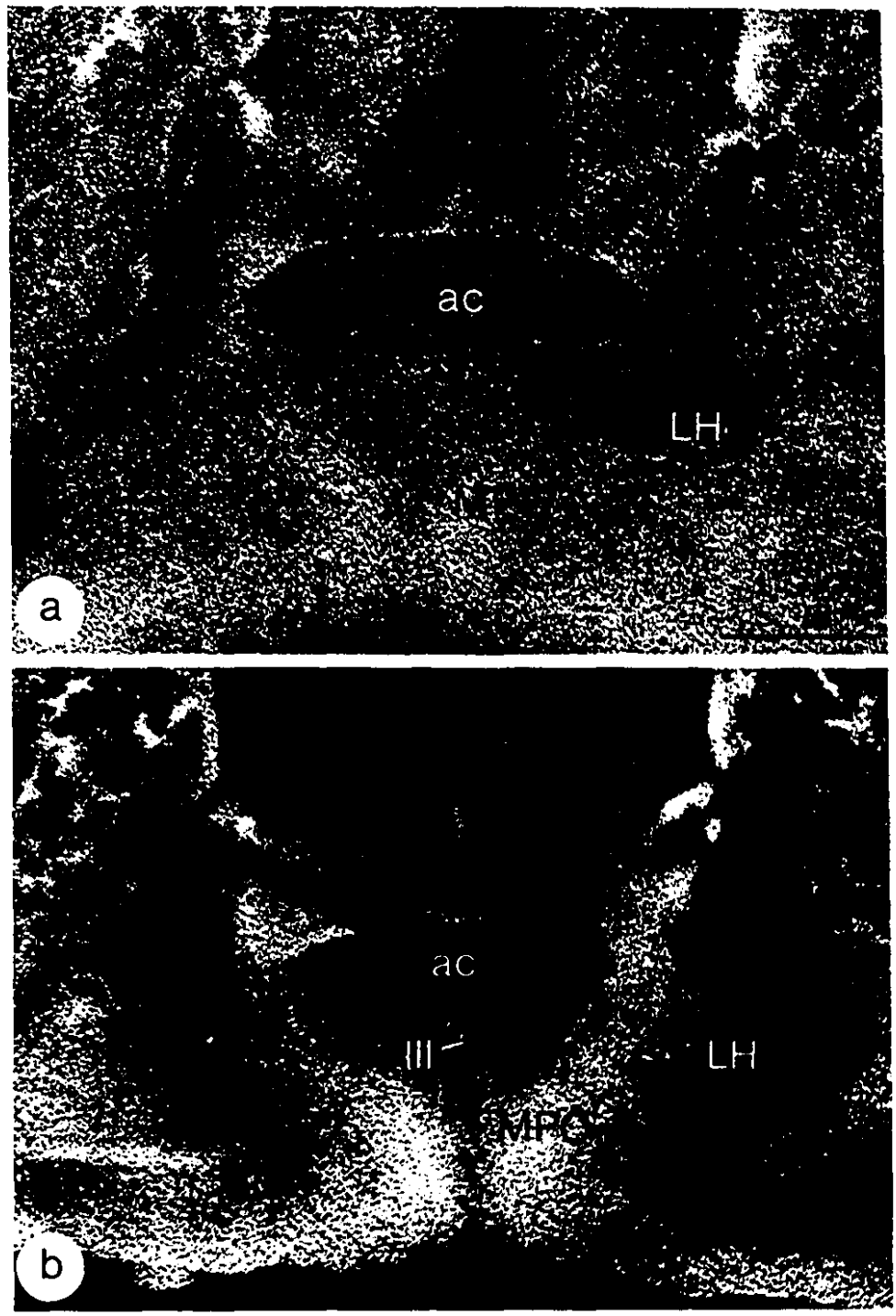
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Figure 2.

Quantitative distribution of mu opioid labeling densities in hypothalamic nuclei of normal, EV-injected and E_2-implanted rats. Light transmittance was measured using a computer-assisted microdensitometry system (Bioquant) and values were converted to nCi/mg tissue wet weight using ^{125}I-microscales as standards (Amersham; 20μm). Light transmittance was measured in at least 6 sections/ hypothalamic region/ animal. Bars represent the mean $\pm$ S.E.M. (n=7). Data from the three groups of animals were compared using one way ANOVA and p values of less than 0.05 were considered significant (*). AHP: anterior hypothalamic nucleus, posterior, AVPO: anteroventral preoptic nucleus, ARC: arcuate nucleus, LA: lateroanterior nucleus, LHA: lateral hypothalamic area, MPA: medial preoptic area, MPO: medial preoptic nucleus, PVN: paraventricular nucleus, SCN: suprachiasmatic nucleus, VMH: ventromedial hypothalamic nucleus.

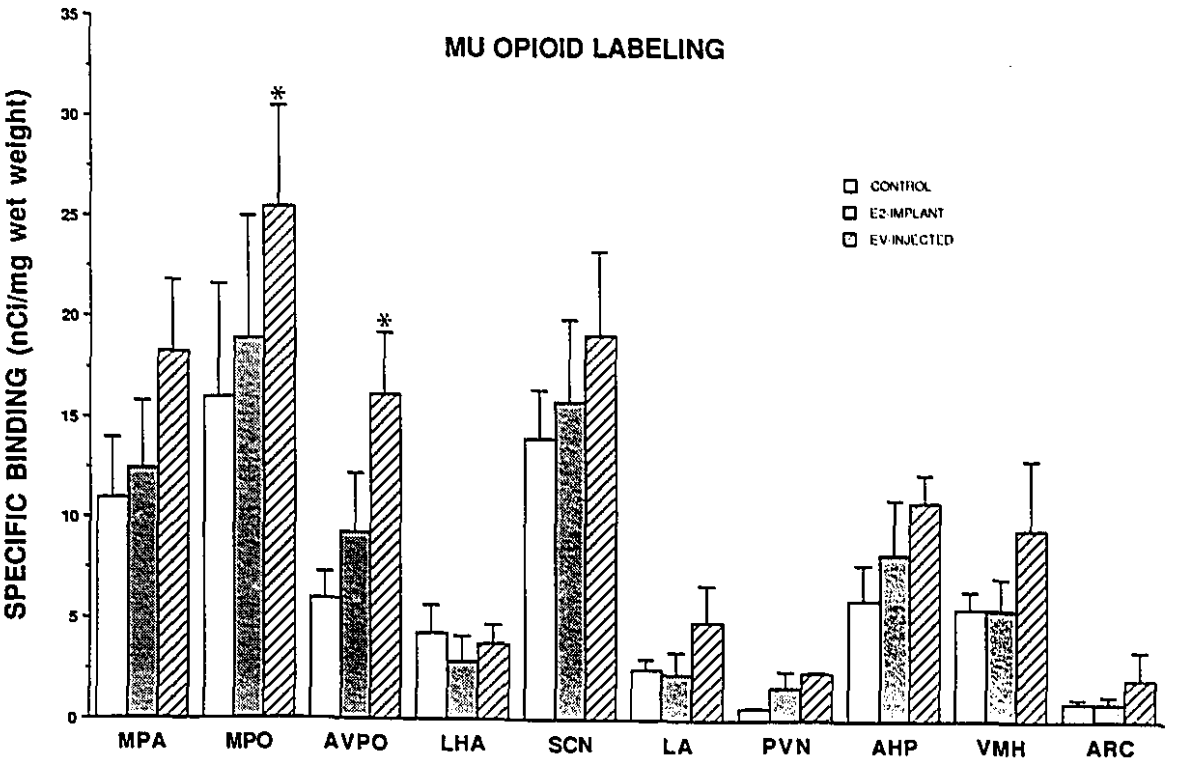

MU OPIOID LABELING
SPECIFIC BINDING (nCi/mg wet weight)
35
30
25
20
15
10
5
0
CONTROL
E2-IMPLANT
EV-INJECTED
*
*
MPA
MPO
AVPO
LHA
SCN
LA
PVN
AHP
VMH
ARC

Figure 3.

Radioautographic distribution of ^{125}I-azidoDTLET-labeled delta opioid binding sites in the suprachiasmatic nucleus (SCh) of normal (a) and E_2-implanted (b) rats. Note the decrease in the density of delta opioid labeling in the suprachiasmatic nucleus (SCh) of E_2-implanted rats. III: third ventricle, BNST: bed nucleus of the stria terminalis, LH: lateral hypothalamic area, ox: optic chiasm. Scale bar: 1 mm.

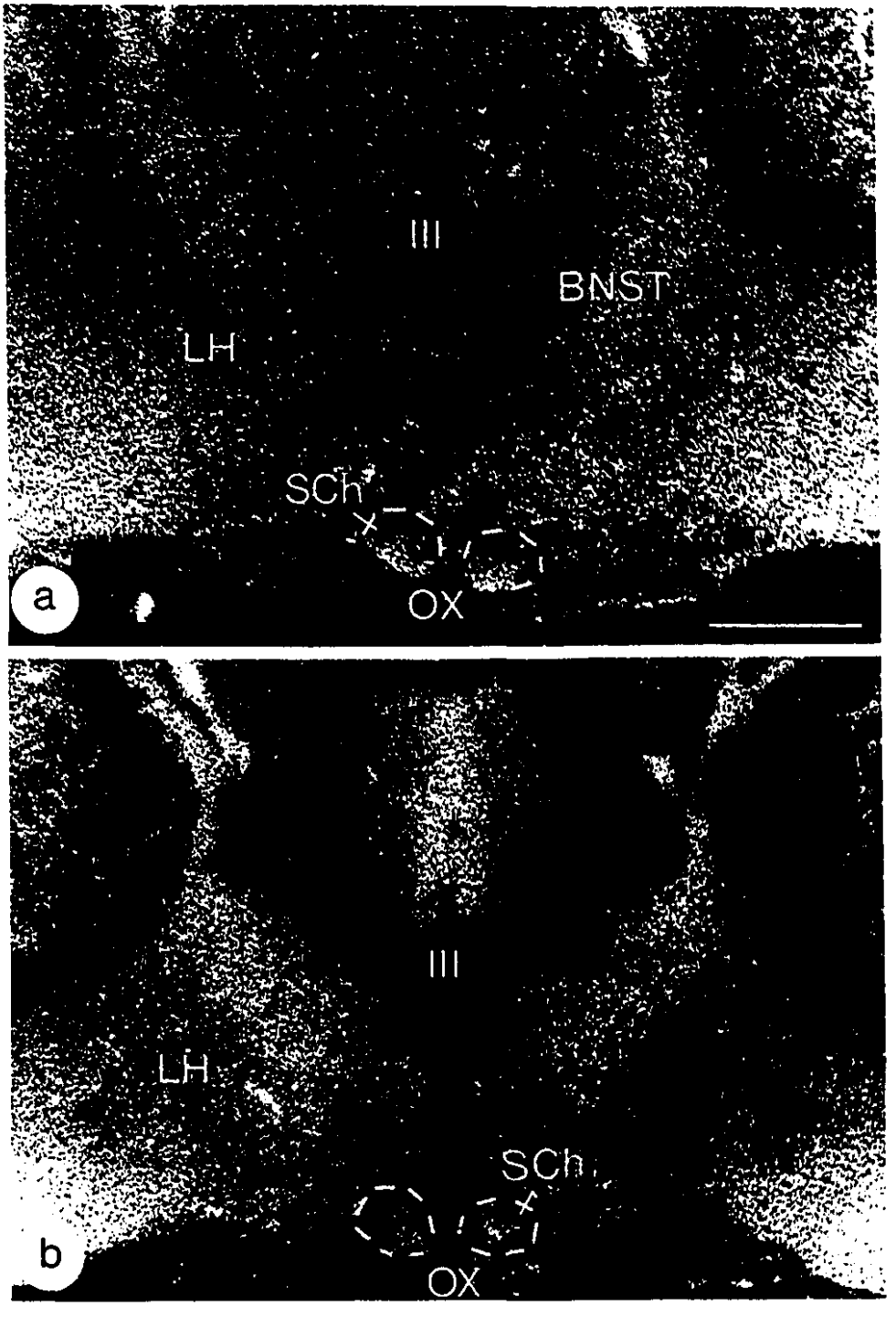

III
BNST
LH
SCh
OX
a
III
LH
SCh
OX
b

Figure 4.

Quantitative distribution of delta opioid labeling densities in hypothalamic nuclei of normal, EV-injected and E_2-implanted animals. Quantitative measurements of opioid labeling were obtained as for mu opioid labeling. Bars represent the mean $\pm$ S.E.M. (n=6). Abbreviations as in Fig. 2.

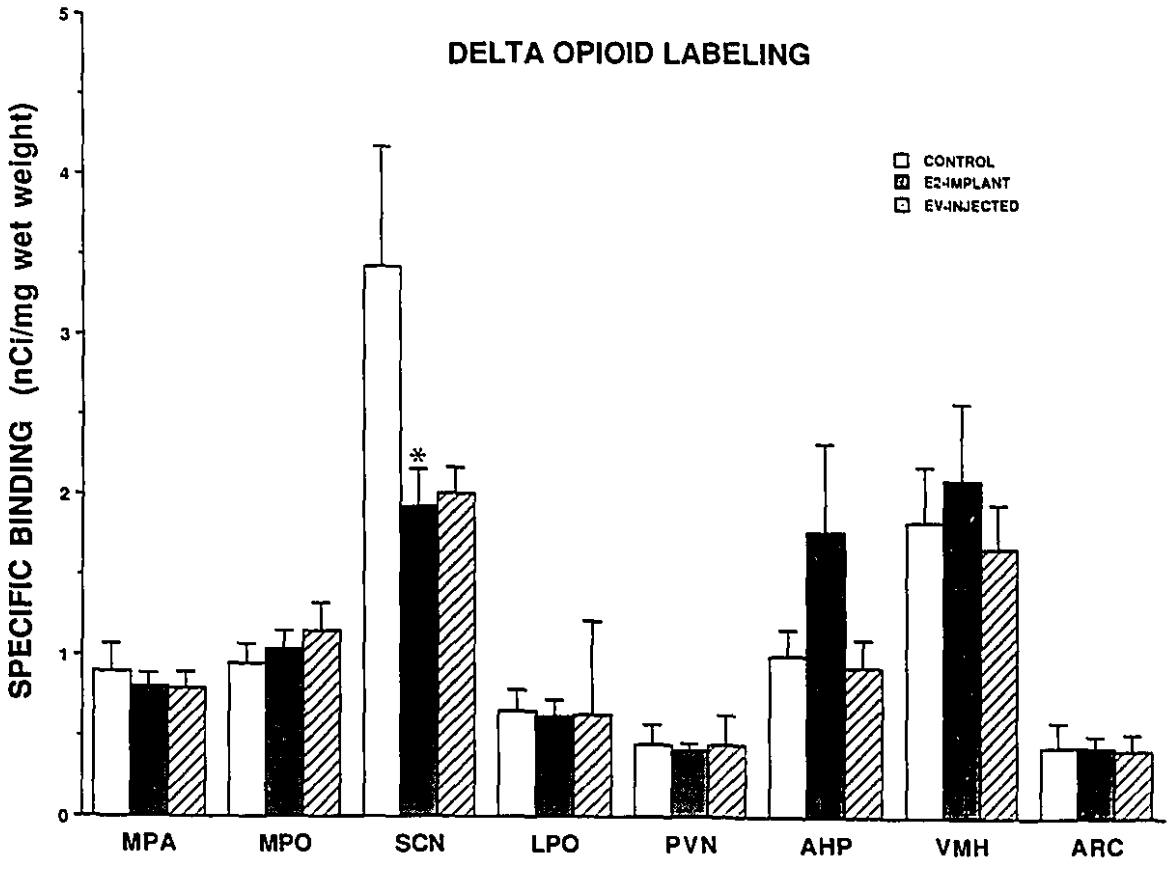
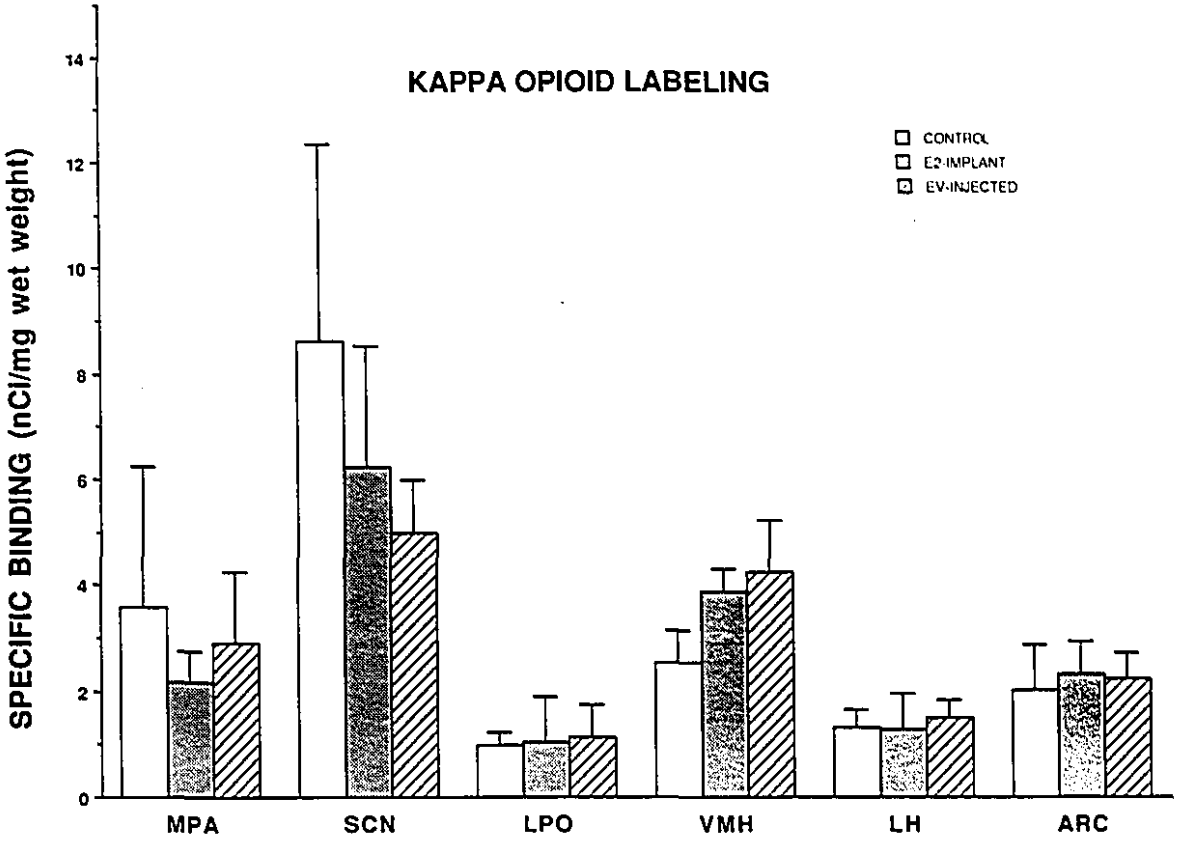

DELTA OPIOID LABELING
SPECIFIC BINDING (nCi/mg wet weight)
CONTROL
E2-IMPLANT
EV-INJECTED
*
0
1
2
3
4
5
MPA
MPO
SCN
LPO
PVN
AHP
VMH
ARC

Figure 5.

Quantitative distribution of kappa opioid labeling densities in hypothalamic nuclei of normal, EV-injected and E_2-implanted rats. Bars represent mean $\pm$ S.E.M. (n=6).


KAPPA OPIOID LABELING
SPECIFIC BINDING (nCi/mg wet weight)
0
2
4
6
8
10
12
14
CONTROL
E2-IMPLANT
EV-INJECTED
MPA
SCN
LPO
VMH
LH
ARC

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the assistance of D. Piccioni-Chen and K. Leonard. We also thank P.A. Lapchak for his tutoring. This work was supported by grant MT-7366 and a Scientist Award to A.B. from the Medical Research Council of Canada.

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Connecting text #2

The precise reason for the increased mu opioid binding in the MPOA is unknown. To test the hypothesis that decreases in hypothalamic ß-endorphin concentrations result in increased mu opioid receptor binding in the medial preoptic area, hypothalamic beta-endorphin concentrations were measured in monosodium glutamate-treated rats and compared to mu opioid binding densities in the MPOA of the same animals.

CHAPTER 4

Monosodium glutamate-induced reductions in hypothalamic ß-endorphin content result in mu opioid receptor upregulation in the medial preoptic area

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Short title: MSG-induced mu opioid receptor upregulation

14 text pages; 3 plates

Key words: ß-endorphin; opioids; receptors; hypothalamus; radioautography; arcuate nucleus

In press: Neuroendocrinology, 1992

ABSTRACT

Estradiol valerate (EV) treatment in the rat induces a lesion of the hypothalamic arcuate nucleus, resulting in significant decreases in hypothalamic ß-endorphin. In addition, the EV treatment causes a selective increase in mu opioid binding in the medial preoptic area (MPOA). Since ß-endorphin neurons located in the arcuate nucleus project extensively to the MPOA, we have hypothesized that the EV-induced loss of these afferents induces a compensatory up-regulation of mu opioid receptors in opioid target neurons. In order to test this hypothesis, we have utilized MSG-treated animals as a model of ß-endorphin cell loss and hence of ß-endorphin deafferentation of the MPOA. Neonatal MSG treatment has been shown to result in the destruction of 80-90% of arcuate neurons accompanied by pronounced decreases in ß-endorphin concentrations in both the arcuate nucleus and MPOA. Mu opioid binding sites were radioautographically labeled in sections from the MPOA of sham and MSG injected animals using the met-enkephalin analogue ^{125}I-FK 33-824, and quantitated by computer-assisted densitometry. The remainder of the hypothalamus of these same animals was utilized for the determination of ß-endorphin concentration. The hypothalami of rats treated with MSG exhibited 62% ($p<0.01$) less ß-endorphin than saline injected controls. In addition, mean mu opioid binding densities in the MPOA were 24% ($p<0.05$) above controls in the MSG treated group. Linear regression analysis of hypothalamic ß-endorphin concentrations and mu opioid binding densities within the same animals yielded an inverse proportional relationship with a coefficient of correlation of -0.85 and a goodness of fit of 0.7. These results substantiate the

hypothesis that EV-induced destruction of ß-endorphin neurons in the arcuate nucleus may result in chronic mu opioid receptor upregulation in the MPOA and further suggest that ß-endorphin may regulate mu opioid receptor density in this area.

INTRODUCTION

Anovulatory acyclicity and polycystic ovaries occur in the rat 4-8 weeks following a single subcutaneous injection of estradiol valerate (EV) [1-3]. Evidence from our laboratory indicates that the EV treatment produces an intractable impairment in the hypothalamic pattern of LHRH delivery [3,4], which in turn results in selective defects in pituitary LH production, storage and release [3]. This cascade of hypothalamic and pituitary defects ultimately translates into a unique attenuated plasma pattern of LH, to which the ovaries respond by becoming polycystic [5-6].

Our investigation into the nature of the primary hypothalamic impairment responsible for this cascade of events has revealed significant alterations in opioid function. We have shown that ^{3}H-naloxone binding in the anterior hypothalamus is elevated in EV-treated rats [7]. This is due to a selective increase in mu opioid receptors within the region of the medial preoptic area (MPOA) most densely populated with LHRH neurons [8], suggesting that the LHRH neuronal system may become abnormally sensitive to the inhibitory action of endogenous opioids [9]. The resultant opioid inhibition could then account for the suppressed, low-amplitude plasma LH pattern characterizing EV-induced polycystic ovarian condition (PCO) [6]. In accordance with this hypothesis, treatment with the opioid antagonist, naltrexone, was found to increase the pituitary content of LHRH receptors and to restore cyclicity and normal ovarian morphology in EV-treated rats [4].

Early anatomical studies of the hypothalamus of EV-treated rats had disclosed the presence of a multifocal lesion of the arcuate nucleus consisting of neuronal

degeneration and reactive gliosis [1,10]. More recently, we also found that the concentration of ß-endorphin was significantly reduced in the hypothalamus of EV-treated animals [8]. It has already been well established that hypothalamic ß-endorphin neurons are concentrated in the arcuate nucleus [11,12] and that this population of cells projects extensively to the MPOA [13]. It was, therefore, tempting to speculate that the observed decrease in hypothalamic ß-endorphin concentrations observed after EV treatment was due to the loss or impairment of ß-endorphin cells in the arcuate nucleus and that in turn, the ß-endorphin denervation of the MPOA was responsible for the increase in mu opioid binding detected in that area [8]. The validity of this hypothesis is predicated on the notion that opioid target neurons in the MPOA respond to a reduced ß-endorphin input by upregulating mu receptors. This possibility appears likely given that mu opioid upregulatory responses to diminished ligand availability have been demonstrated in other brain regions [14-18]. Nevertheless, it remains to be determined whether such a mechanism applies to the MPOA, especially since in the EV model, the loss of ß-endorphin neurons and upregulation of mu opioid receptors may be caused independently by unrelated actions of estradiol.

In order to determine whether the upregulation of mu opioid receptors observed in the MPOA of EV-treated rats could indeed be a response to partial ß-endorphin deafferentation, we resorted to a model of arcuate neuron loss other than EV-treatment, the monosodium glutamate (MSG)-treated animal. Neonatal treatment with MSG has been shown to produce a fairly selective and complete lesion of the arcuate nucleus [19,20] accompanied by pronounced decreases in ß-endorphin in both the arcuate

nucleus and the MPOA [21,22]. We have therefore measured mu opioid binding densities by film radioautography in adult rats in which the arcuate ß-endorphin system had been compromised by means of neonatal treatment with MSG, and correlated the results with hypothalamic ß-endorphin concentrations measured by RIA in the same animals. Our data indicate that the MSG-induced arcuate lesion, resulting in significant reduction in hypothalamic ß-endorphin, does indeed evoke an increase in mu opioid binding in the MPOA. These results substantiate the hypothesis that destruction of arcuate ß-endorphin neurons is responsible for the chronic mu opioid receptor upregulation observed in the MPOA of animals treated with EV.

MATERIALS AND METHODS

Animals

Three pregnant Wistar rats were obtained from Charles River Canada Inc. (St-Constant, Quebec) and maintained in a controlled environment with free access to water and pelleted rat food. Female pups each received subcutaneous injections of MSG (n=10; 4 mg/g body weight; Sigma Chemical Co., St-Louis, Miss.) dissolved in 0.9% NaCl [19-22] or equivalent volumes of isosmolar NaCl (10%; n=8) on days 1,3,5,7 and 9 after birth. At 8 weeks of age, animals were decapitated (09:00-10:00 h) and their brains rapidly frozen in isopentane (-54°C) for 15 seconds and stored at -80°C until used.

Brains from both control (n=8) and MSG-treated rats (n=8) were cut into 20 μm sections on a cryostat from the anterior commissure, rostrally, to the suprachiasmatic nucleus, caudally, and thaw-mounted onto gelatin-coated slides. Alternate slides were used for *in vitro* autoradiaugraphic determination of mu opioid binding densities, whereas the remaining slides were utilized for the determination of non-specific binding and cresyl violet staining. The remainder of each hypothalamus was dissected from the brain and utilized for the determination of ß-endorphin concentration.

Radioautography

Mu opioid receptors were labeled *in vitro* using the met-enkephalin analogue ^{125}I-FK 33-824 which has been shown to bind with high affinity (Kd= 1.2nM) and selectivity to mu opioid receptors (K_iDTLET/K_iFK=16)[23]. The ligand was iodinated using the lactoperoxidase method and purified by gel filtration (specific activity ≈1700

Ci/mmole). Alternate sections from MSG-treated and sham-treated animals were labeled in parallel by incubation for 45 min at room temp. in 0.05 M TRIS-HCL, Ph 7.4 containing 1 Nm ^{125}I-FK 33-824. Remaining sections from both groups were incubated in the presence of 1 μM naloxone for determination of non-specific binding. After labeling, the sections were rinsed in six consecutive baths of ice-cold buffer, dipped in distilled water, and radioautographed by apposition to tritium-sensitive Hyperfilm (Amersham). Films were developed after 3 days of exposure in Kodak GBX at 13°C and binding densities quantitated using a computer-assisted densitometry program (Biocom). To this aim, a minimum of 15 sections taken across the entire extent of the medial preoptic and anteroventral preoptic nuclei (i.e. MPOA), as well as through the central portion of the anterior commissure were quantitated per animal. Within each section, the regions of interest were outlined on a high resolution computer screen and light transmittance values automatically averaged and recorded within them. These values were then automatically converted to absolute values (Nci/mg wet weight) by interpolation from an ^{125}I-microscales standard (20μm;Amersham). Non-specific binding was neglected in the calculation of specific binding densities since these slides produced virtually no image on the radioautographic film.

Radioimmunoassay

For the determination of ß-endorphin concentrations, the hypothalami of all animals were dissected from the remainder of the brain, weighted and homogenized in 1 ml of 2 N acetic acid using a Polytron. The homogenate was then centrifuged and ß-endorphin levels were measured in the supernatant using a RIA to human ß-endorphin

(RIK 8616 Peninsula Labs., Inc., Belmont Calif.). The antibody provided in this assay exhibits a 92% cross-reactivity with rat ß-endorphin, and 0% cross-reactivities with met-enkephalin and gamma-endorphin. Cross-reactivity with acetylated ß-endorphin (1-27) and with acetylated ß-endorphin (1-26) is 50% and 30%, respectively. The sensitivity of the assay was 10 pg/tube. The intraassay variability was $<10\%$ and the interassay variability was $<16\%$.

Statistics

Mean values for ß-endorphin concentrations and mu opioid labeling densities in control and MSG-treated rats were compared using the Student's T-test. Each of these values, obtained within the same MSG-treated rat, was then compared by linear regression analysis.

RESULTS

MSG-treated rats exhibited many of the features previously described by others after MSG-induced damage of the arcuate nucleus including a loss of coat sleekness, stunted growth, and obesity (33% greater increase in body weight than shams) [20,26,27]. Upon histological examination of cresyl violet-stained sections of the hypothalamus, the arcuate region appeared profoundly modified (Fig.1). Most of the arcuate nucleus was destroyed with only the dorsomedial portion remaining albeit dislocated ventrally.

The radioautographic distribution of specifically labeled mu opioid binding sites within the rostral hypothalamic area was comparable to that described previously [8,28] and topographically similar between control and MSG-treated groups. Mu opioid labeling was dense within the MPOA whilst the septohypothalamic nucleus (Shy) and lateral preoptic area (LPO) were moderately labeled (Fig. 2). The optic chiasm and anterior commissure were virtually devoid of labeling. Within the MPOA of MSG-treated rats, mean specific mu opioid binding densities were found to be significantly higher than in controls (Fig.2; Table 1). In contrast, there was no significant difference between the two groups in the densities of labeling measured in the anterior commissure (Fig. 2).

Measurements of hypothalamic ß-endorphin concentrations revealed a three-fold reduction in MSG-treated animals as compared to controls (Table 1).

When mean mu opioid binding densities for individual animals in the MSG-treated group were directly correlated with hypothalamic ß-endorphin concentrations in the same animals, an inverse proportional relationship was apparent. Regression analysis

yielded a regression coefficient of -0.85 and a goodness of fit of 0.7 (Fig. 3).

DISCUSSION

In conformity with previous reports, neonatal treatment with MSG was found here to result in the histological disappearance of most of the arcuate nucleus as well as in a marked reduction in the hypothalamic content of immunoreactive ß-endorphin [20,22,26]. The MSG lesion thus clearly eliminated a significant portion of the ß-endorphin cells that project afferents to the MPOA [13]. Although neonatal MSG treatment had previously been shown to increase opioid binding in the midbrain of the mouse [29], the present study is the first to demonstrate increased opioid binding in the MPOA. Furthermore, the strong inverse correlation between hypothalamic ß-endorphin concentrations and mu opioid binding suggests that this elevation in binding is a consequence of ß-endorphin deafferentation of opioid target neurons.

The concept that mu receptors may be regulated by the availability of endogenous opioids is supported by a large literature. Chronic *in vivo* or *in vitro* administration of opioid antagonists such as naloxone and naltrexone has been shown to result in mu opioid receptor upregulation within whole homogenates [14,16,18,30-32] or selective limbic regions [15,17,33] of the rat brain. In addition, it was shown that treatment with these antagonists increased the total number of mu opioid receptors (B_{max}) as opposed to changing their affinity (K_d)[14,16-18,29-31]. Increases in mu opioid receptor binding were found to be two-fold greater in microsomes than in synaptic membrane fractions [14] indicating that part of the newly expressed opioid binding sites may be intracellular.

The present results, therefore, support the hypothesis that the increase in mu opioid

binding observed in the MPOA of animals treated with EV represents an upregulation of mu opioid receptors secondary to the drop in hypothalamic ß-endorphin measured in these animals [8]. Based on the postulated association of mu opioid binding sites with LHRH-containing neurons within the MPOA [34], such an upregulation may be hypothesized to engender a hypersensitivity of the LHRH system to the inhibitory action of either residual ß-endorphin or other endogenous opioids. Indeed, recent evidence has confirmed the involvement of mu receptors in the LHRH-rich MPOA-anterior hypothalamic area in the suppression of plasma LH. Application of a mu opioid agonist to the MPOA-anterior hypothalamic area significantly suppressed plasma LH in ovariectomized rats [35]. Furthermore, this effect was shown to result from a reduction in the plasma LH pulse amplitude, as opposed to frequency, nadir, or duration. Only at high doses of the agonist was mean LH pulse frequency also reduced [35]. We have shown that the diminution in the mean plasma LH concentration in the EV-treated rat [3,37] is likewise due to a reduction in the pulse amplitude in the absence of alterations in any other pulse parameter [6], thereby strengthening the idea that upregulation of mu opioid receptors is functionally linked with decreases in plasma LH release.

In the present study, the reduction in hypothalamic ß-endorphin engendered by MSG treatment (−62%) was greater than that produced by EV (−44%) [8]. Furthermore, the disruption in the reproductive function in the MSG exposed rats, although variable [24,38] is less severe than in EV-treated animals [2,3]. Indeed, it has been reported that plasma and pituitary LH concentrations, as well as LH responses to LHRH are, if

anything, elevated in MSG-treated females [39] rather than diminished as they are in the EV-treated rat [3]. This has not, however, been confirmed by others [24]. In any event, there are clearly significant differences between the MSG- and EV- lesioned models. The MSG lesion is induced during the neonatal period, an interval during which the hypothalamus retains considerable plasticity. In the course of development, the opioid deficit resulting from the MSG-induced arcuate lesion, may be compensated for by one or more of the numerous regulatory inputs into the LHRH system. Furthermore, the effect of EV treatment on the arcuate nucleus appears to be far more selective than that of MSG. The EV induced lesion consists of well delineated foci of degeneration within an otherwise normal appearing neuropil. Moreover, we have shown that the EV-lesion spares the dopaminergic arcuate neurons [40], and we have preliminary evidence that other systems are likewise unaffected (unpublished). In contrast, treatment of neonates with MSG results in the loss of 80-90% of all neurons in the arcuate nucleus [19,26]. Since there is a large variety of transmitter/peptide-specific neuronal populations in the arcuate nucleus that contribute to the regulation of LHRH neuronal activity, the loss of inhibitory and excitatory influences on LHRH may balance each other leaving no net effect.

In conclusion, the present results demonstrate that the diminution in ß-endorphin resulting from the destruction of arcuate neurons by MSG is accompanied by a compensatory increase in mu opioid binding within the MPOA. It is proposed that the increase in mu opioid binding observed in the MPOA of animals injected with EV is engendered by the same mechanism and is thus a direct consequence of the reduction

of hypothalamic ß-endorphin measured in these animals.

ACKNOWLEDGEMENTS

The authors thank Kathy Leonard and Dalia Piccioni-Chen for their expert technical assistance. This work was supported by MRC grant # MT-7366 and by an MRC studentship to C.D.

Figure 1.

Sections from the hypothalamus of (a) sham-injected and (b) MSG-treated animals showing the massive cytotoxic effect of MSG treatment on arcuate neurons (arrows). Abbreviations: III, third ventricle; Arc, arcuate nucleus; ME, median eminence; VMH, ventromedial nucleus. Scale bar: 200 μm.

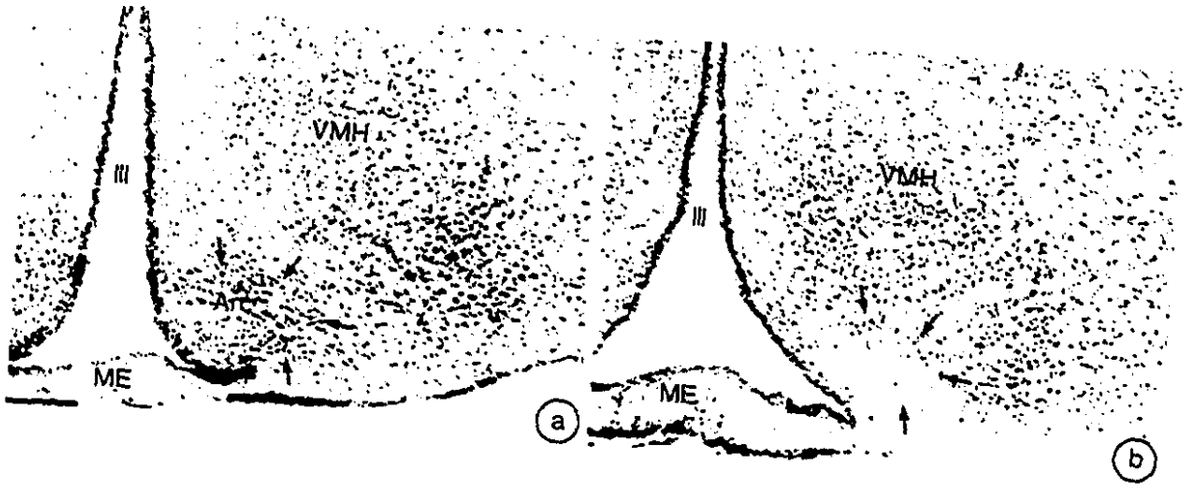

VMH
III
ARC
ME
a
VMH
III
ME
b

Figure 2.

Cresyl violet-stained section (a) and mu opioid binding in the MPOA of (b) control and (c) MSG-treated rats. Fresh frozen sections were labeled with ^{125}I-FK 33 824, apposed to ^{3}H-sensitive film and photographed in dark field. Note the dense concentration of mu opioid binding in the MPOA and the increase in labeling density in the MSG-treated group. Abbreviations: III, third ventricle; ac, anterior commissure; LPO, lateral preoptic area; MPOA, medial preoptic area; oc, optic chiasm; Shy, septohypothalamic nucleus. Scale bar: 500 μm.

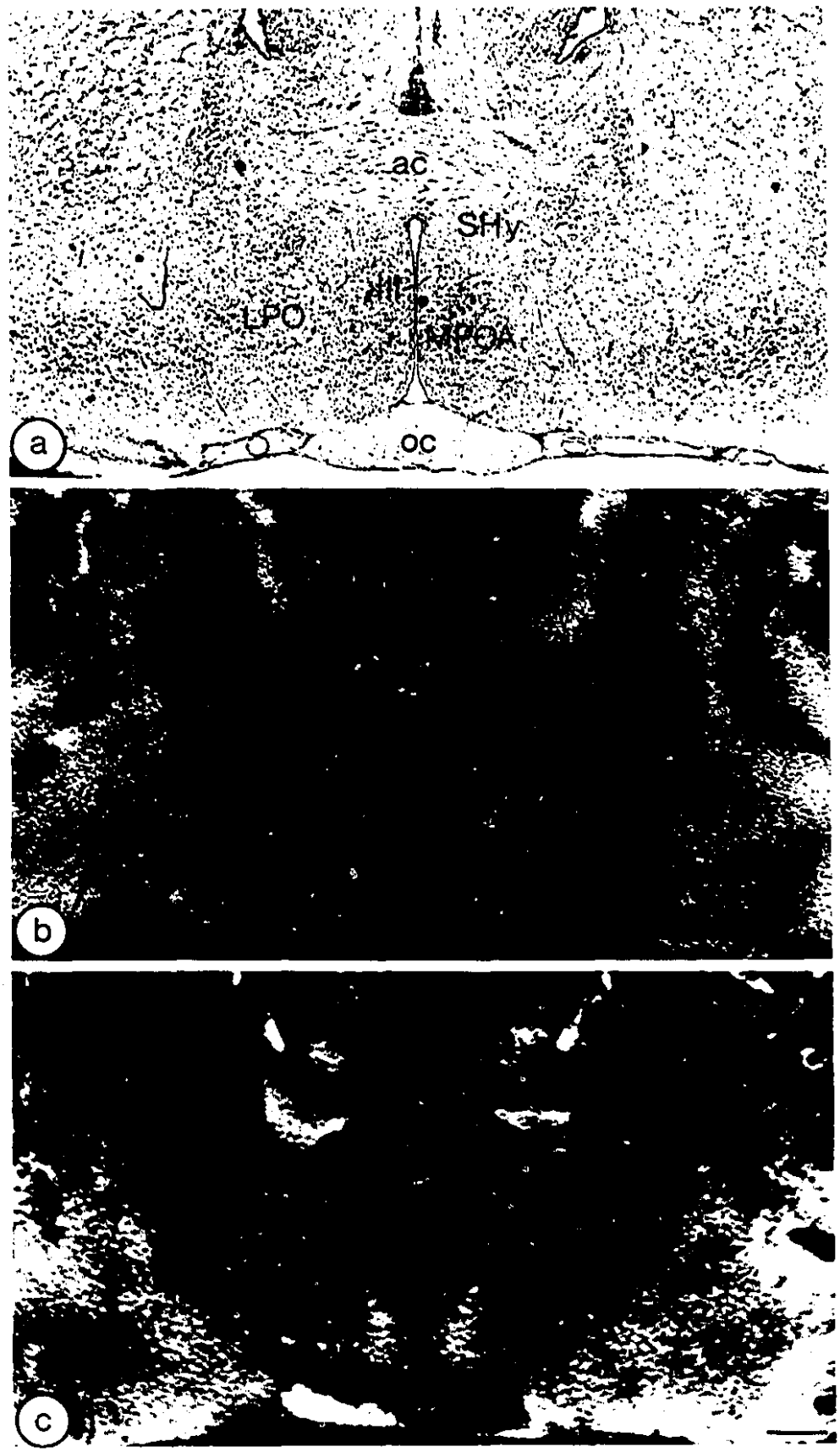

ac
SHy
LPO
MPOA
a
oc
b
c

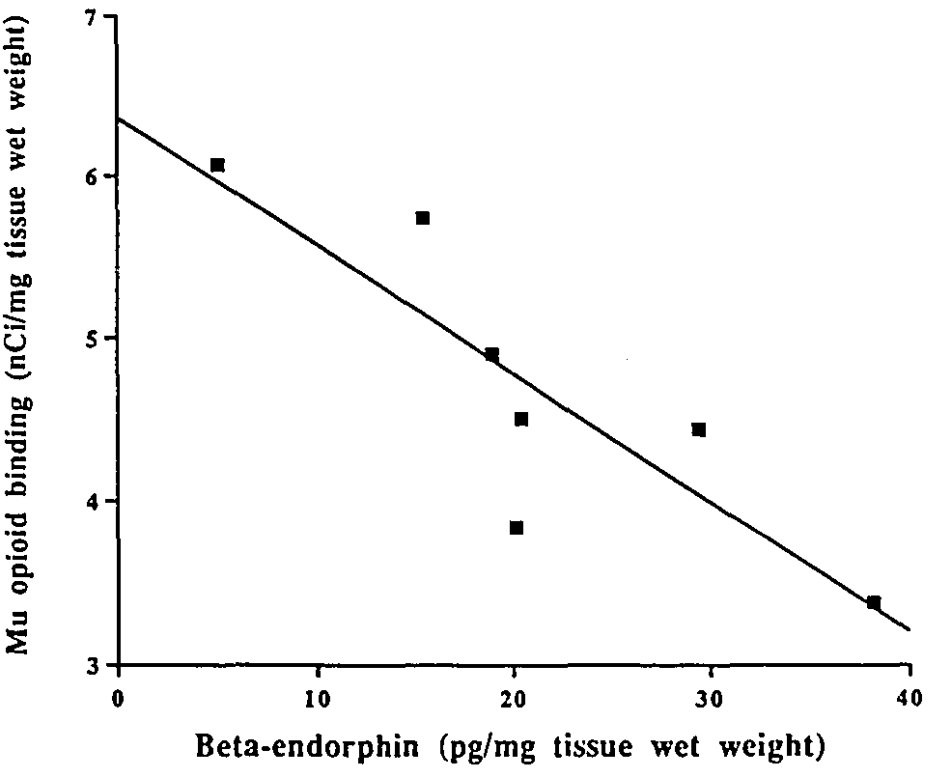
Table 1. Mean hypothalamic beta-endorphin levels and mu opioid binding in the MPOA of sham and MSG-treated rats.

	Beta-endorphin levels (pg/mg wet weight)	Mu opioid binding in MPOA (nCi/mg wet weight)
sham	55.1 ± 7.1	3.7 ± 0.2
MSG-treated	21.0 ± 4.0** (↓62%)	4.6 ± 0.3* (↑24%)

*p<0.05, **p<0.01, n=7,8

Figure 3.

Linear regression of hypothalamic ß-endorphin concentration as measured by RIA (pg/mg tissue wet weight) versus mean MPOA mu opioid binding per animal as determined by film radioautographic densitometry (Nci/mg tissue wet weight). The regression coefficient was -0.85 and goodness of fit 0.7.


Mu opioid binding (nCi/mg tissue wet weight)
7
6
5
4
3
0
10
20
30
40
Beta-endorphin (pg/mg tissue wet weight)

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Connecting text #3

Given that the chemical identity of the hypothalamic neurons affected by estradiol toxicity had never been identified, it was of considerable interest to determine whether the reduction in ß-endorphin concentration observed in EV-treated animals was due to an actual cell loss or to decreased synthesis of ß-endorphin. After preliminary experiments revealed drastic reductions in the number of ß-endorphin immunoreactive neurons following EV treatment, a series of experiments examining other neurotransmitter/peptides as well as stereological measurements of total neurons number were performed in order to determine whether other neuronal populations were likewise affected by EV treatment.

CHAPTER 5

Estradiol is selectively neurotoxic to hypothalamic ß-endorphin neurons.

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Abbreviated title: E_2-mediated toxicity of ß-endorphin neurons

18 text pages; 7 plates

In press, Endocrinology, 1992

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 following EV treatment, a 60% decrease in the total number of beta-endorphin-immunoreactive neurons was detected in the arcuate nucleus. In contrast, neurotensin-, somatostatin- and tyrosine-hydroxylase -immunoreactive neuron numbers were unchanged, suggesting that the effects of estradiol were selective for beta-endorphin neurons. Further evidence for the selectivity of estradiol's actions was provided by radioimmunoassays indicating decreases in hypothalamic beta-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 as compared to controls. The estimated number of neurons lost (~3500) corresponded precisely with the total number of beta-endorphin neurons lost (~3600) as estimated using quantitative immunocytochemistry. These results confirm the selectivity of estradiol's effects towards the beta-endorphin cell population and demonstrate that the observed decrease in beta-endorphin immunoreactivity reflects actual cell loss. Evidence indicates that the selective neurotoxic effect of estradiol on hypothalamic beta-endorphin neurons contributes to reproductive senescence, suggesting that steroids may participate in the disruption of the biological functions that they normally facilitate.

INTRODUCTION

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 prior to 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 physiologic 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 pathologic 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, 8, 9). Indeed, the contribution of estradiol to hypothalamic aging has been appreciated for at least 25 years (10). As of 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 beta-endorphin neurons in the arcuate nucleus of the rat. This specific beta-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 beta-endorphin in the central nervous system.

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, Que.) were housed in groups of three 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 eight weeks of age, 17 animals were anaesthetized with ether and injected IM with 2mg estradiol valerate (EV) dissolved in sesame oil (Delestrogen, Squibb). This treatment has been shown to result initially in a supraphysiologic 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 three groups to be processed for immunocytochemistry, radioimmunoassay, 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 (0.2cc/100g body weight) and injected ICV with 60ug of colchicine dissolved in 30ul of a 0.9% saline solution. Forty-eight hours later, animals were reanesthetized and sacrificed by perfusion with 450 ml of 4% paraformaldehyde solution in 0.12M Sorensen buffer. After removing the brain, these were cryoprotected in a

30% sucrose solution for 24 h at 4°C. These brains were then rapidly frozen by immersion in isopentane (-55°C) for 20 sec and stored at -80°C. Brains were serially sectioned (30μm) from the posterior chiasmatic area rostrally, to the mammillary bodies, caudally. Beta-endorphin immunopositive neurons were counted in the first 3 adjacent sections out of seven (i.e. 90μm of each 210μm 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 beta-endorphin antibody, a rabbit anti-human beta-endorphin (Immunocorps, Montreal), was tested by incubating the diluted antiserum overnight with 100μg/ml of rat beta-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% NGS. All other antisera including tyrosine hydroxylase (Eugene Tech), neurotensin and somatostatin (Incstar) were similarly raised in rabbits and tested by these respective companies. Free floating sections were collected in Sorensen's buffer 0.12M 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 the inactivation of endogenous peroxidases, then rinsed 3 X 8 min in Tris saline and incubated for 30 min in 1% normal goat serum. After 2 X 8 min rinses in Tris saline, sections were incubated overnight at room temperature with primary antisera at a concentration of 1:2000 for beta-endorphin and 1:750 for TH, NT and SRIF. The following day,

sections were rinsed 3 X 8 min in Tris saline containing 0.2% BSA and 1% NGS and then incubated for 30 min with a goat anti-rabbit IgG (Sternberger-Meyer, Immunocytochemicals, Inc.). Following 2 X 8 min rinses in the same buffer, sections were incubated for 30 min with rabbit peroxidase anti-peroxidase PAP (Sternberger-Meyer ICN), rinsed 2 X 8 min in Tris saline and reacted with Tris water containing 0.05g of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 3mg H_20_2 for six minutes. The sections were mounted on gelatin coated slides, dehydrated and defatted in a graded series of alcohols, cleared in xylene, and coverslipped with Permount.

Radioimmunoassays

Hypothalami were dissected from the brains using the optic chiasm and mammillary bodies as rostrocaudal borders, and the lateral recess of the hypothalamus as lateral borders. These were homogenized in 1ml HCL 1N for 30s using a Polytron. Samples were centrifuged for 30s at 10 000g and the supernatant utilized for the determination of beta-endorphin concentration. The beta-endorphin antibody in the radioimmunoassay (RIK 8626; Peninsula) is an rabbit-anti-human beta-endorphin which displays 80% cross-reactivities with rat beta-endorphin. It does not cross-react with met-endorphin, gamma-endorphin or beta-lipotropin. The NPY antibody (RIK-7172; Peninsula) shows cross-reactivities with peptide YY, vasoactive intestinal polypeptide and avian pancreatic polypeptide of .003%, .001%, and .007%, respectively. The met-enkephalin antibody (#18100 Incstar) displays 2.8% cross-reactivity with leu-enkephalin and $<$.003% cross-reactivities with alpha- and beta-endorphin.

Morphometry and quantitation of immunoreactive cell number

Morphometric analysis was performed using a computer-assisted image analysis system (Biocom). For all beta-endorphin immunostained sections, the location, number, surface area, long diameter and form factor (defined as 4π X area $\div$ 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 3 adjacent 30 μm sections was plotted on a rostrocaudal axis. Estimates of the total numbers of beta-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., 1983 (11).

In the case of neurotensin-, somatostatin-, and tyrosine hydroxylase-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 30μm section out of every seven sections was plotted in a rostrocaudal 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 utilized. Fresh (unfixed) brains were snap frozen in isopentane (-54°C) and serially sectioned at 30μm on a cryostat (-17°C). Sections from control and EV-treated rats were stained in parallel for cresyl-violet. Neuron density (Nv) was determined using the optical dissector method described by West et al., 1990 (12). In one out of each ten 30μm section, the arcuate and periarcuate area (including the arcuate nucleus and ventrolateral periarcuate region) was outlined at low power on an image analysis system which calculates the area outlined automatically (Biocom). At high power, a window of appropriate dimensions (A=400μ^2) was generated and the number of neurons or glia (Q) coming into focus within the thickness of the section (t) were 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 x A. Estimates of total neuron numbers were obtained by multiplying the neuron density with the volume of the arcuate nucleus estimated using the principle of Cavalieri. 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" ($V = t \times \Sigma\, a_i$). 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 the Student's t-test, as were those from the radioimmunoassays. The precision of the stereological estimates of total neuron numbers were analyzed as described in West et al., 1990 (12) using a modified analysis of variance. Based on the formula, $OCV^2=CV^2 + OCE^2$ for adjacent sampling levels, where OCV = the observed relative variance at one sampling level (i.e. SD/mean), CV = the true relative variation i.e. unknown and OCE = the computable variation of the stereological estimate (CE see p.18 West et al., 1990), the true biological variance (CV), i.e. that contributed by the estradiol exposure, accounted on average for more than 85% of the observed variability among groups.

RESULTS

In control animals, an abundance of beta-endorphin immunoreactive neurons were observed distributed along the ventrolateral aspect of the mediobasal hypothalamus within the arcuate and periarcuate region as previously described (Fig.1a) (13-16). In sharp contrast, EV-treated animals exhibited a considerably smaller number of beta-endorphin immunoreactive cells (Fig. 1b). The few immunopositive neurons detected were small, shrivelled and often exhibited tortuous and swollen dendrites, indicative of neuronal degeneration (Fig. 1b). However, a small subset of the spared beta-endorphin cells located in the lateral periarcuate region, was comprised of larger neurons that appeared not to be damaged by the EV treatment.

Computer-assisted quantitation of beta-endorphin immunopositive neurons throughout the entire rostrocaudal 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 rostrocaudal level) (Fig. 2). The total number of beta-endorphin neurons estimated in controls was 5523 ± 820 (mean$\pm$S.E.M) whereas in EV treated animals this value was 1992 ± 480 ($p<0.01$) indicating a net immunoreactive cell loss of approximately 3500 beta-endorphin neurons. Reductions in the number of beta-endorphin immunopositive neurons were most pronounced in the caudal 2/3 of the arcuate nucleus (Fig. 2) and in its most medial aspect.

Morphometric analysis of beta-endorphin neurons are summarized in Table 1. EV-treated animals displayed significant reductions in mean neuron form factor as compared to controls. Although a slight trend towards 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 beta-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).

Long-term exposure to estradiol did not produce any significant change in the mean number of neurotensin-, somatostatin- or tyrosine hydroxylase-immunoreactive neurons counted throughout the arcuate nucleus (Fig. 3). However, both somatostatin and tyrosine hydroxylase neuronal populations stained more intensely and exhibited a trend toward increased numbers following EV treatment (Fig. 3 and 4). At level -2.82 μm from bregma, TH-immunoreactive neuron numbers were significantly increased relative to controls ($p<0.05$). Morphometric analysis of tyrosine-hydroxylase-immunoreactive neurons indicated that following 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 ($0.528\pm.002$ vs. $0.544\pm.002$; $p<0.05$) as compared to controls.

As seen in table 2, beta-endorphin concentrations as determined by radioimmunoassay were reduced by more than 60% in the EV-treated group as compared to untreated controls. In contrast, hypothalamic concentrations of met-enkephalin (met-EK) and neuropeptide Y (NPY) were unchanged in the EV-treated group with respect to controls (Table 2).

Unbiased stereological measurements of mean arcuate volume, neuronal 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 mean number of neurons per mm^3) was reduced in EV-treated animals as compared to controls although this did not reach statistical significance. Total neuron number estimations revealed a loss of 3500 neurons throughout the arcuate region following 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 beta-endorphin neurons within the arcuate nucleus, while sparing other coextensive neuronal populations.

The mean number of beta-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 beta-endorphin immunoreactive cells parallels the overall 60% reduction in beta-endorphin concentration in hypothalamic homogenates. In contrast, none of the other neurotransmitter/peptide populations examined in the hypothalamus were found to be affected by EV treatment. Concentrations of NPY and met-ENK, 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 beta-endorphin, neurotensin-, somatostatin- and tyrosine hydroxylase-immunoreactive neuronal populations were not decreased following EV treatment. Both tyrosine hydroxylase and somatostatin 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 rostrocaudal level, there was a significant increase in mean cell

surface area and neuronal form factor of TH-immunoreactive cells in the EV-treated group as compared to 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 beta-endorphin. Indeed, beta-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 somatostatin neurons are similarly disinhibited by the loss of beta-endorphin. In any event, the absence of reductions in all neuropeptide populations examined concomitant with pronounced decreases in beta-endorphin concentrations, strongly suggest that estradiol's deleterious effects are selectively targeted to beta-endorphin neurons.

Beta-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 beta-endorphin neurons are in the process of degeneration and that the decreases in beta-endorphin immunoreactive neuron number and hypothalamic concentrations of beta-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 by West et al. (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). Following 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 beta-endorphin immunopositive neurons following 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 as compared to those located in the arcuate nucleus proper and thus are unlikely to affect the comparison of total arcuate neuron numbers with total beta-endorphin neurons lost. Taken together, these data indicate that estradiol is selectively neurotoxic to arcuate beta-endorphin neurons.

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

The mechanism underlying the pathogenic effect of estradiol on hypothalamic beta-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 which are highly sensitive to circulating estradiol levels (1, 6). These astrocytes, identified in both rodent and human brains (22), may transform catechol estrogens (2-, 4-hydroxyestrogen), generated spontaneously in the

brain from circulating estradiol (23), to o-semiquinone free radicals (24). These free radicals would, in turn, cause the 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 beta-endorphin loss (26). The unique vulnerability of beta-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 beta-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 beta-endorphin neurons observed in EV-treated animals results in a marked increase in mu 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 beta-endorphin evoked a compensatory upregulation of mu opioid receptors on target neurons/terminals in the MPOA rendering them supersensitive to the inhibitory action of either residual beta-endorphin or other endogenous opioids (i.e. met-enkephalin). This interpretation is supported by the observation of mu opioid binding site upregulation 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, reinitiated 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 beta-endorphin concentrations (8, 30, 31, 32, 33, 34), diminution in POMC MRNA (35, 36), and loss of beta-endorphin neurons (37) have all been associated with aging in female rodents. However, despite these decreases in beta-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 of noradrenaline and to 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 re-establishes normal cyclicity in senescent rats for extended periods of time, however, suggesting that they affect events which are 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 pituitary-adrenal response to stress (44). Similarly, the chronic estradiol-induced destruction of hypothalamic beta-endorphin neurons demonstrated in the present study results in the 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.

Figure 1. Immunocytochemically labeled beta-endorphin neurons in the periarcuate region of a) control and b) EV-treated rats. Beta-endorphin-immunoreactive neurons were drastically reduced in the EV-treated group even though darkly stained cells were still apparent (arrows). Abbreviations: III: third ventricle, ME: median eminence. Scale bar: 250μm.

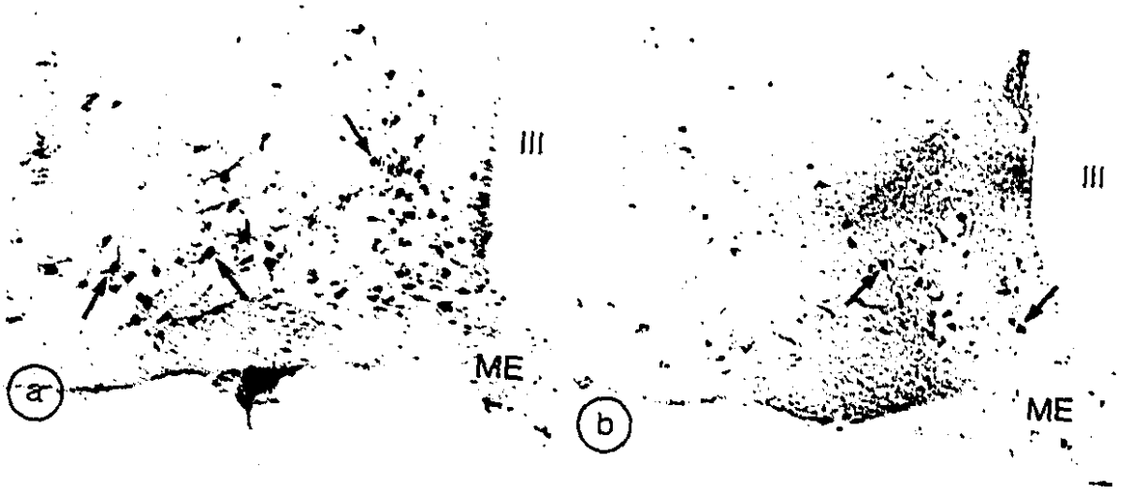
III
ME
a
b
III
ME

Figure 2. Rostrocaudal distribution of mean beta-endorphin-immunoreactive neurons in control and EV-treated rats. Mean beta-endorphin neurons were plotted on a rostrocaudal axis by comparison of section level to the rat brain atlas of Paxinos and Watson, 1986. EV-treated animals displayed significant reductions in beta-endorphin neurons at every level of the arcuate nucleus with an average 60% drop in mean beta-endorphin neurons ($p<0.05$ to $p<0.001$).

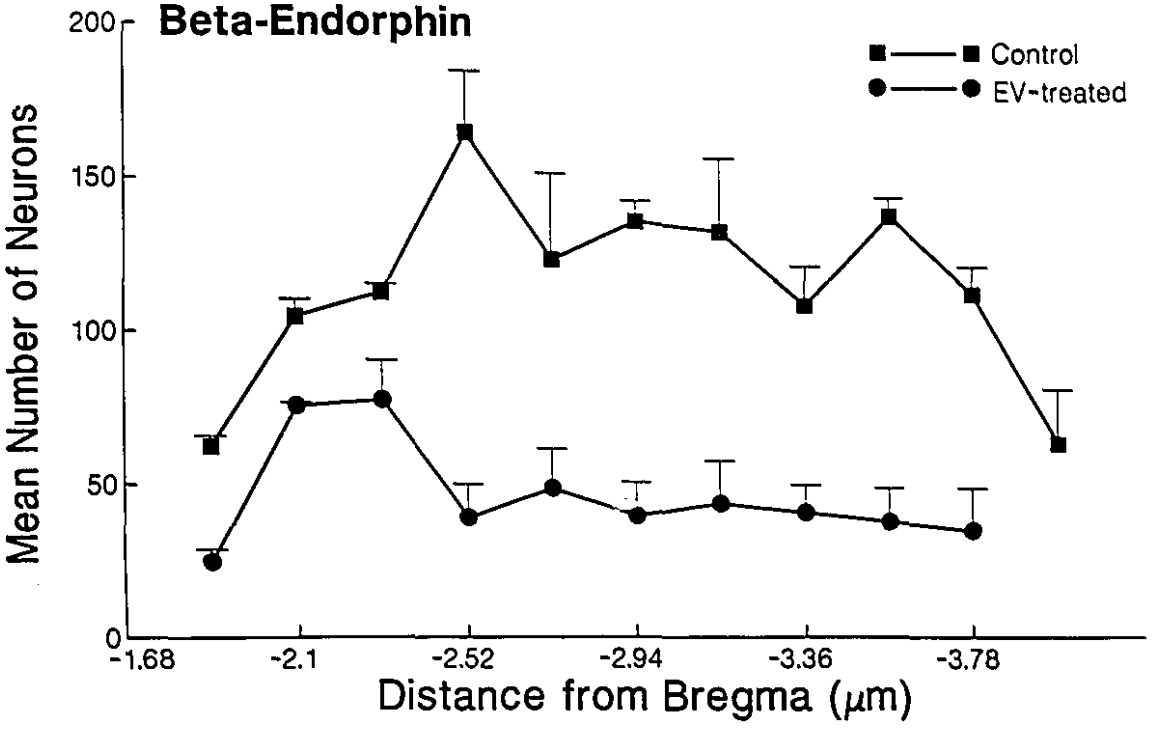
Beta-Endorphin
Control
EV-treated
Mean Number of Neurons
200
150
100
50
0
-1.68
-2.1
-2.52
-2.94
-3.36
-3.78
Distance from Bregma (μm)

Figure 3. Rostrocaudal distribution of mean neurotensin (NT), somatostatin (SRIF) and tyrosine hydroxylase (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 towards 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 (* indicates $p < 0.05$).

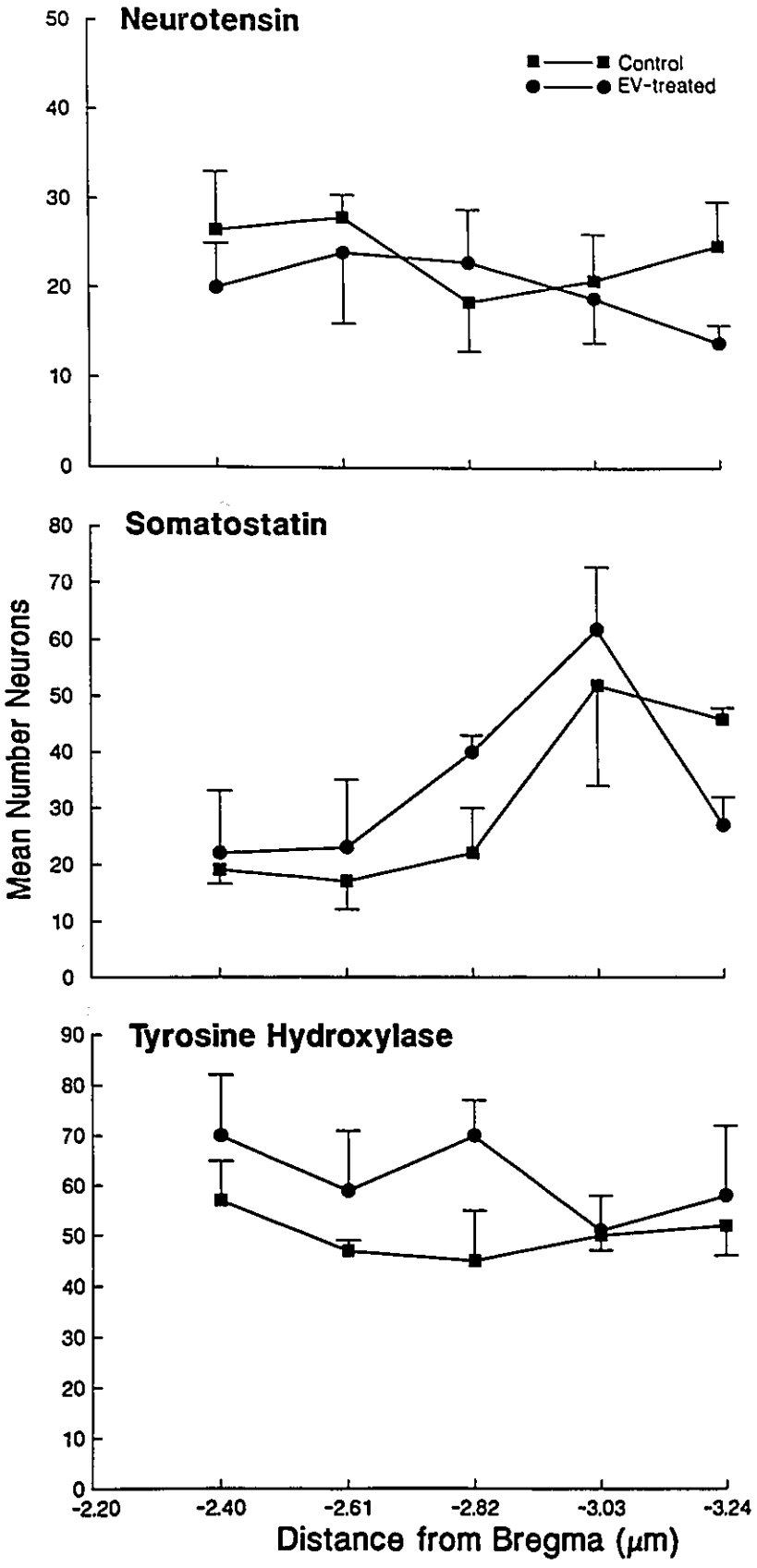
Neurotensin
Control
EV-treated
50
40
30
20
10
0
Somatostatin
80
70
60
50
40
30
20
10
0
Mean Number Neurons
Tyrosine Hydroxylase
90
80
70
60
50
40
30
20
10
0
-2.20
-2.40
-2.61
-2.82
-3.03
-3.24
Distance from Bregma (μm)

Figure 4. Immunocytochemically labeled neurotensin (top), somatostatin (middle) and tyrosine hydroxylase (bottom) immunoreactive neurons in control (a, c and e) and EV-treated (b, d and f) rats. Neither neurotensin, somatostatin nor tyrosine hydroxylase immunoreactive staining was reduced following EV-treatment. In fact, both somatostatin and tyrosine hydroxylase neurons appeared somewhat more darkly stained in the EV-treated group as compared to controls. Scale bar: 350μm.

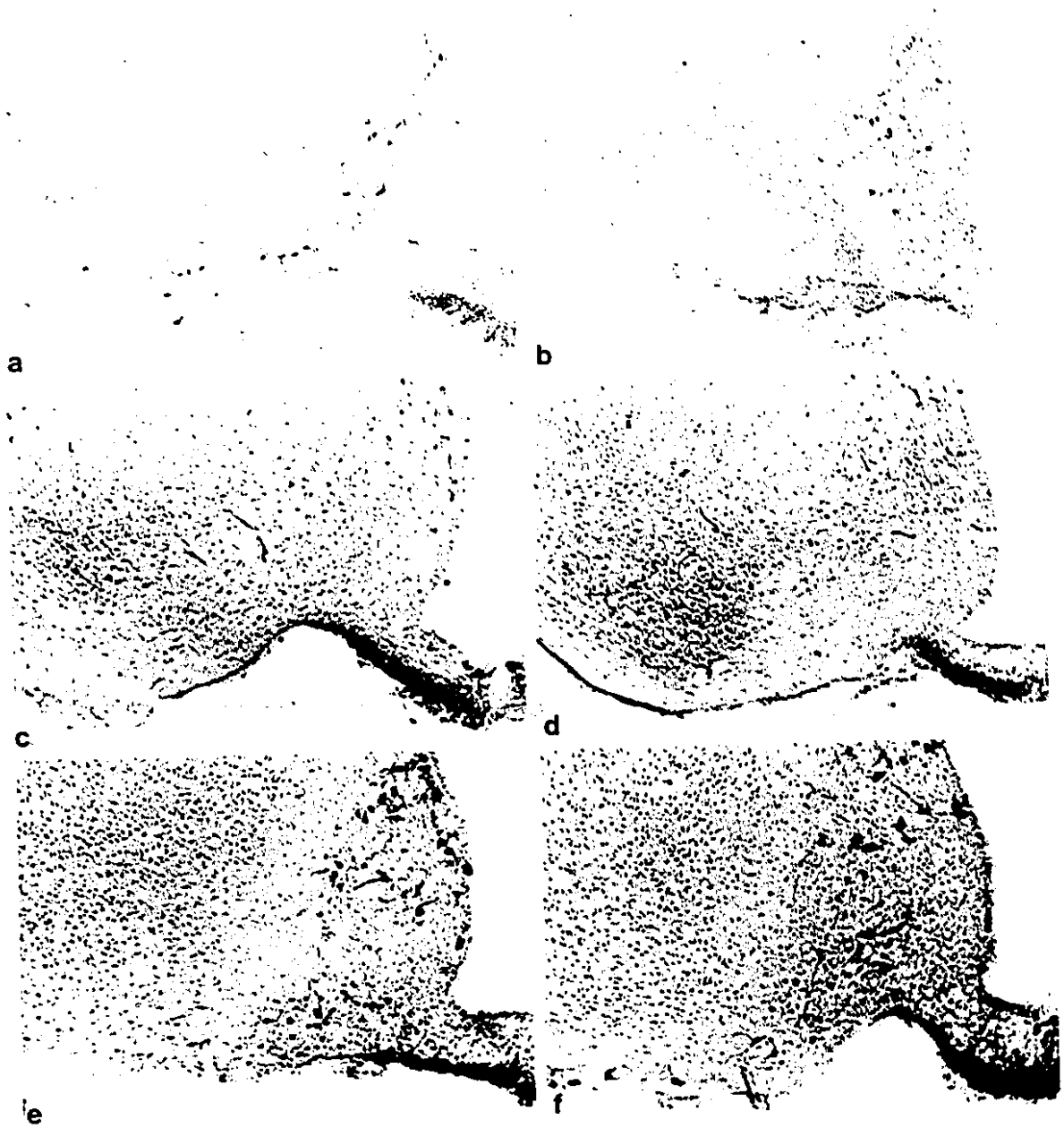
a
b
c
d
e
f

Table 1. Effect of EV treatment on morphometric parameters of arcuate beta-endorphin neurons.

	Control	EV-treated
Surface area (μm^2)	84.6±2	80.9±1
Long diameter (μm)	16.9±1	15.7±1
Form factor	0.52±0	0.45±0**

Values are expressed as mean±S.E.M. for n=5. ** indicates significantly different from controls; $p<0.001$.

Table 2. Effect of EV treatment on radioimmunoassayable beta-endorphin, met-enkephalin and neuropeptide Y in rat hypothalami.

	Beta-endorphin (pg/mg)	Met-enkephalin (pg/mg)	Neuropeptide-Y (pg/mg)
control	112±8.9	2892±287	354±23
EV-treated	46±8.2**	3178±275	324±16

Values are expressed as mean±S.E.M. pg/mg wet weight for n=7. ** indicates significantly different from controls $p<0.01$.

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

	Neurons			Glia	
	Arcuate volume (mm^3)	Nv (n/mm^3)	N	Gv (g/mm^3)	G
Controls	0.136 (0.05)	1.8371 $X10^5$	**23 820** (0.07)	0.7592 $X10^5$	**10 672** (0.07)
			CV .123		CV .077
			BV .101		BV .030
EV-treated	0.123 (0.04)	1.6371 $X10^5$	**20 249** (0.07)	0.7466 $X10^5$	**9 140** (0.09)
			CV .148		CV .090
			BV .130		BV .050

Cresyl-violet stained sections from control and EV-treated rats were utilized 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., 1988 [Gundersen, 1988]. At high power, a window of appropriate dimensions ($A=400\mu^2$) 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 which 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 amongst groups was then compared to the variance contributed by the sampling procedure in order to ascertain what proportion of change was due to true biological variance.

In both control and EV-treated groups the biological variance accounted for more than 85% of the observed variance indicating that the values were highly accurate.

Abbreviations: Nv=neurons per volume; N=neurons; Gv=glia per volume; G=glia; CV=coefficient of variation; BV=biological variation. Coefficients of error are shown in parentheses.

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Connecting text 4

The mechanism of action of estradiol to induce the cell death of ß-endorphin neurons in the arcuate nucleus is unknown. However, experiments from the laboratory of Dr. H. Schipper have suggested that the conversion of catechol estrogens, generated spontaneously in the brain to free radicals may occur by catalysis in peroxidase positive granules located in unique astrocytes in the arcuate nucleus. These granules have been shown to proliferate in response to estradiol exposure and have been seen in close proximity to degenrating neuronal elements at the electron microscopic level.

Connecting text #4

The demonstration of selective beta-endorphin cell death following long-term estradiol exposure has not been previously described, hence virtually nothing is known regarding its underlying mechanism(s). Work from the laboratory of Dr. Hyman Schipper has nonetheless suggested that free radicals generated in unique peroxidase-positive astrocytes may be involved in the neurotoxic effects of long-term estradiol exposure [Schipper, 1991]. The following study examines this question by treating EV injected animals with the potent antioxidant, vitamin E.

CHAPTER 6

Vitamin E treatment protects against estradiol-induced ß-endorphin loss.

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8 text pages; 3 plates

Key words: neurotoxicity; alpha-tocopherol; lipid peroxidation; cyclicity; rats

In press, Endocrinology, Rapid Communication, 1992.

ABSTRACT

Estradiol valerate (EV) treatment has been shown to result in the destruction of 60% of ß-endorphin neurons in the hypothalamic arcuate nucleus. The mechanism of estradiol neurotoxicity has been postulated to involve the production of lipid damaging free radicals from nearby peroxidase positive astrocytes. In this study, we have examined whether treatment with a powerful antioxidant, Vitamin E, might prevent the diminution in ß-endorphin concentrations previously shown to be associated with cell loss. Our results demonstrate that Vitamin E was indeed able to protect ß-endorphin neurons from the neurotoxic action of estradiol. Furthermore, by doing so, vitamin E also prevented the appearance of persistent estrus and ovarian pathology normally associated with EV treatment.

INTRODUCTION

Estradiol valerate (EV) treatment, which results in tonic ovarian secretion of physiological levels of estradiol, results in a cascade of hypothalamic-pituitary-ovarian deficits which ultimately result in acyclicity and polycystic ovaries (2, 4). Several studies have shown that in rodents, such chronic (8 weeks) exposure to estradiol causes a neurotoxic lesion in the hypothalamic arcuate nucleus characterized by degenerating axons and dendrites and a proliferation of peroxidase positive astrocytic granules (3, 18). We have recently shown that this lesion is due to the selective degeneration of ß-endorphin neurons (8). Using quantitative immunocytochemistry, radioimmunoassay and unbiased stereological methods, 60% reduction in the total number of ß-endorphin neurons in the arcuate nucleus was detected following EV treatment. Furthermore, neighbouring neuronal populations such as met-enkephalin-, neuropeptide-Y-, somatostatin-, neurotensin- and tyrosine hydroxylase-containing neurons were spared from the neurotoxic effects of estradiol (8). The loss of ß-endorphin neurons was shown to engender a compensatory mu opioid receptor up-regulation in the medial preoptic area (MPOA) (6, 7) which may be responsible for the supersensitivity of elements in this region, including LHRH neurons, to the inhibitory actions of endogenous opioids (5). Indeed, several indices of LHRH stimulation, such as pituitary LHRH receptor numbers, are reduced in the EV-treated rat (21). Furthermore, patterns of plasma LH release are reduced, and the features of this pattern, decreased amplitude of secretion with no change in frequency, nadir or duration (16), are consistent with the activation of mu opioid receptors in the MPOA (12, 14). The development of the

suppressed pattern of LH release coincides with the formation of polycystic ovaries and the appearance of persistent estrus (2). These features of the EV-treated animal are reversed by treatment with the opiate antagonist, naltrexone, consistent with the idea of a supersensitivity to opioids engendered by the loss of ß-endorphin neurons.

The mechanism whereby estradiol induces the degeneration of hypothalamic ß-endorphin neurons is not understood. We have previously postulated that the production of lipid-damaging free radicals from nearby peroxidase positive astrocytes may be involved [Schipper, 1989]. Brain tissues are able to convert estradiol both spontaneously and enzymatically to catechol estrogen (2- or 4-hydroxyestradiol) (13). These, in turn, have been shown to be readily transformed to 0-semiquinone free radicals by peroxidase positive astrocytes in culture (19). These astrocytes detected in both rodent and human brains (20), contain peroxidase positive granules which proliferate following EV treatment and have often been located in close proximity to degenerating elements (3, 18). Additionally, in peripheral tissues, estradiol has been shown to stimulate the production of delta-aminolevulinic acid synthetase, the rate limiting enzyme in heme biosynthesis (9, 17). Heme and other peroxidases are catalysts in the formation of free radicals (13). Thus, estradiol may serve as both substrate and stimulator of the production of lipid damaging free radicals. In order to test the hypothesis that ß-endorphin neurons are destroyed following EV treatment by free radical mediated lipid peroxidation, animals injected with EV were concomitantly treated with vitamin E (alpha-tocopherol), a potent antioxidant. The effects of dietary alpha-tocopherol on lipid peroxidation have been extensively studied (11). It has been

shown to accumulate in tissues in a concentration dependent manner and to inhibit both iron-induced and spontaneous lipid peroxidation in brain and peripheral tissues .

Our results demonstrate that long-term treatment with alpha-tocopherol prevents the EV-induced loss of ß-endorphin immunoreactivity associated with the disappearance of these neurons. Treatment with Vitamin E alone also protected ß-endorphin neurons from age-associated declines in hypothalamic ß-endorphin content. These results suggest that the mechanism whereby estradiol induces cell loss involves the production of free radicals. Furthermore, animals co-treated with EV and alpha-tocopherol displayed regular cycling patterns and healthy ovaries containing numerous corpora lutea, therefore indicating that the protection of ß-endorphin neurons by alpha-tocopherol also prevented acyclicity and ovarian pathology.

MATERIALS AND METHODS

Adult female Wistar rats (Charles River, Canada) were housed under conditions of controlled light (12L:12D) and temperature (22°C). Animals were divided into four groups (n=9) and treated as follows: the first group was subjected to a single 2mg IM injection of estradiol-valerate (EV; Delestrogen, Squibb) which is known to result in the destruction of ß-endorphin neurons (11). The second group was injected with EV and immediately treated with 150 IU alpha-tocopherol (alpha-tocopherol; Sigma) by daily ingestion through the diet (powdered purina rat chow). The third group was treated with alpha-tocopherol only, and group four were normally cycling controls. Eight weeks following the initiation of treatment, animals were sacrificed (between 0900 and 1100) and their brains rapidly removed and frozen in isopentane 50°C for 15 sec. Vaginal cytology was monitored for two weeks prior to sacrifice. Ovaries were removed and stored in Bouin's for histological processing of paraffin sections in hematoxylin and eosin.

Radioimmunoassays

Hypothalami were dissected from the brains and homogenized in 1ml HCL 1N for 30s using a Polytron. Samples were centrifuged for 30s at 10 000g and the supernatant utilized for the determination of ß-endorphin concentration. The ß-endorphin antibody in the radioimmunoassay (RIK 8626; Peninsula) is an rabbit-anti-human ß-endorphin which displays 80% cross-reactivities with rat ß-endorphin. It does not crossreact with met-endorphin, gamma endorphin or ß-lipotropin. The NPY antibody (RIK-7172; Peninsula) shows cross reactivities with peptide YY, vasoactive intestinal polypeptide

and avian pancreatic polypeptide of .003%, .001%, and .007%, respectively. The met-enkephalin antibody (#18100 Incstar) displays 2.8% cross reactivity with leu-enkephalin and <.003% cross reactivities with alpha- and ß-endorphin.

Statistics

The means for each group of animals in the long-term Vitamin E experiment (controls, EV-treated, Vitamin E and EV-treated and Vitamin E treatment alone) were compared by one way analysis of variance followed by Student's t-test. The unpaired student's t-test was used for comparing controls to short-term Vitamin E treated rats.

RESULTS AND DISCUSSION

Animals treated with a single injection of EV displayed significant reductions in hypothalamic ß-endorphin concentrations as compared to controls (fig. 1; $p<0.001$), in agreement with what we have previously shown to occur as a result of cell loss. In contrast, EV-injected animals fed diets supplemented with alpha-tocopherol displayed ß-endorphin concentrations similar to controls, suggesting that alpha-tocopherol treatment prevented estradiol-induced loss of ß-endorphin immunoreactivity (fig. 1). Animals treated with alpha-tocopherol only, exhibited significantly higher levels of ß-endorphin than did untreated controls (fig. 1; $p<0.01$), implying that the well documented senescent decline in hypothalamic ß-endorphin content (1, 8) is also prevented by alpha-tocopherol treatment. This maintenance of ß-endorphin levels by Vitamin E in the EV-treated and maturing rat may be interpreted to suggest either that alpha-tocopherol prevented estradiol-induced loss of ß-endorphin neurons, or alternatively, that it increased the expression of ß-endorphin in viable neurons. To distinguish between these two possibilities, rats were treated (as before) for 3 days with alpha-tocopherol in order to determine whether this short term treatment would result in increases in ß-endorphin concentrations. If the maintenance of ß-endorphin concentrations by alpha-tocopherol were due to an increase in ß-endorphin expression, 3 days of alpha-tocopherol treatment should be sufficient to induce a stimulation of ß-endorphin synthesis. If, on the other hand, alpha-tocopherol were protecting ß-endorphin neurons from lipid peroxidation due to its antioxidant properties, such short term treatment would not be expected to yield any differences in ß-endorphin

concentrations since only a negligible degree of lipid peroxidation is expected to occur during this time frame, and such short term treatment has previously been shown to be ineffective in preventing free radical induced degeneration of neurons (10). Our results demonstrate that short-term treatment with alpha-tocopherol failed to alter ß-endorphin concentrations with respect to controls (87.68+13 vs. 88.33+18; n=4; t-value 0.292), indicating that vitamin E did not increase the expression of ß-endorphin. Additionally, in order to ascertain whether Vitamin E might have non-specific actions on other neuronal populations in the arcuate nucleus, we also measured hypothalamic concentrations of met-enkephalin and neuropeptide-Y in the long-term Vitamin E treated rats. Met-enkephalin and neuropeptide-Y concentrations were unaffected by Vitamin E treatment (Table 1), supporting the idea that alpha-tocopherol acts as an antioxidant, and does not non-selectively change the expression of neuropeptides. Taken together, these results suggest that Vitamin E treatment protects ß-endorphin neurons from both estradiol and age-associated lipid peroxidation.

In addition, we monitored vaginal cyclicity and ovarian morphology in the aforementioned groups of long-term Vitamin E treated rats. As previously shown, animals treated with EV developed polycystic ovaries devoid of corpora lutea and were in persistent estrus for the two week period preceding sacrifice (Figure 2b). In contrast, 6 out of 9 of the EV-treated animals fed diets containing Vitamin E, displayed healthy ovaries with abundant corpora lutea (Figure 2a, c). These same animals displayed regular cycling patterns. Animals treated with Vitamin E only were indistinguishable from controls. The results suggest that alpha-tocopherol treatment also prevents the

EV-induced appearance of persistent estrus and ovarian pathology.

The prevention of estradiol degenerative effects by alpha-tocopherol treatment suggests that the mechanism whereby estradiol induces neurotoxicity involves the production of free radicals. These are likely to be O-semiquinone free radicals generated by peroxidase positive granules in nearby astrocytes. The astrocytes themselves may be protected from the lipid peroxidating effects of free radicals by their elevated glutathione oxidase content. A differential distribution of antioxidant enzymes within arcuate neuronal populations could account for the selective vulnerability of ß-endorphin neurons to the neurotoxic effects of estradiol. The fact that alpha-tocopherol also mediated the preservation of regular cycling and normal ovarian morphology after EV treatment further implies that protection of ß-endorphin neurons from lipid peroxidation prevents persistent estrus and ovarian pathology.

Figure 1. Mean hypothalamic beta-endorphin concentrations in homogenates from control, EV-treated, vitamin E and EV-cotreated as well as vitamin E-treated groups as determined by RIA. Beta-endorphin concentrations in the EV-treated group were significantly reduced with respect to controls ($p<0.01$; t-test; $n=9$) whereas animals treated with vitamin E alone displayed significant increases ($p<0.05$; t-test; $n=9$).

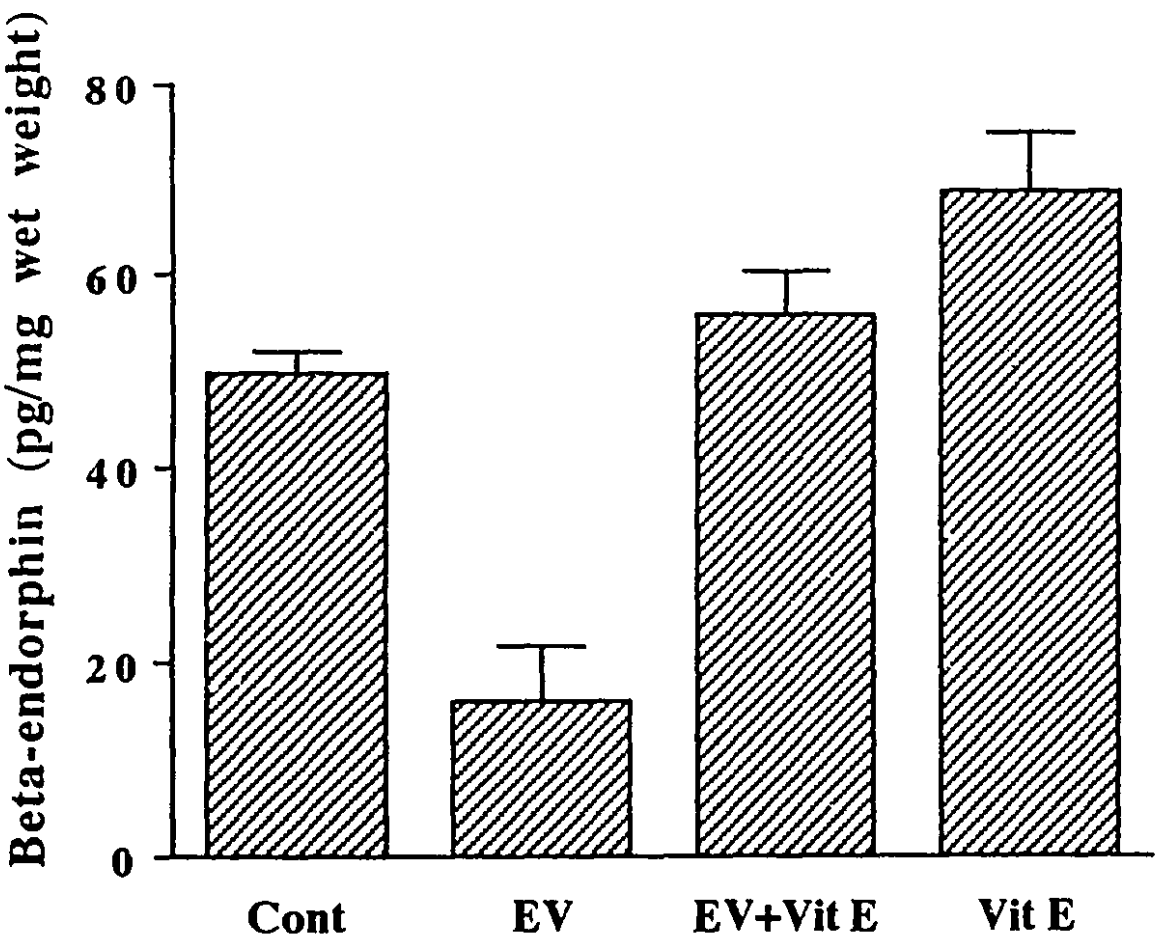
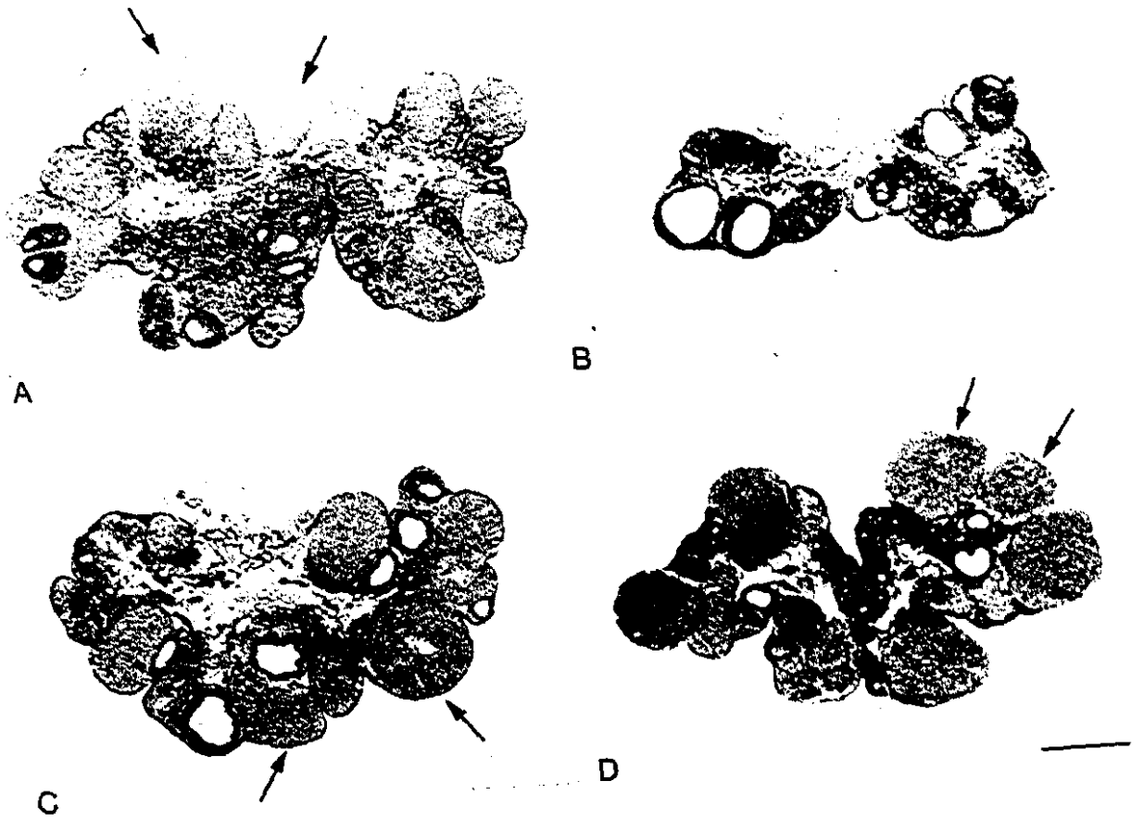

Beta-endorphin (pg/mg wet weight)
80
60
40
20
0
Cont
EV
EV+Vit E
Vit E

Table 1. Effects of long-term Vitamin E treatment on hypothalamic met-enkephalin and neuropeptide-Y concentrations.

Values are expressed as the mean ± S.E.M. for n=9; *indicates significantly different from age-matched controls ($p<0.05$).

	Met-enkephalin (pg/mg)	Neuropeptide-Y (pg/mg)
control	4494±411	212±10
EV-treated	5284±496	269±19*
EV+Vitamin E	4089±302	221±13
Vitamin E	4579±385	227±14

Figure 2. Representative hematoxylin and eosin stained ovarian sections derived from a) controls, b) EV-treated, c) vitamin E and EV co-treated and d) vitamin E treated groups. Arrows indicate corpora lutea which were numerous in all groups except the EV-treated animals.


A
B
C
D

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CHAPTER 7

GENERAL DISCUSSION

One of the main findings of this thesis is the demonstration that chronic estradiol exposure results in the destruction of greater than 60% of ß-endorphin neurons in the arcuate nucleus of the hypothalamus while leaving other nearby neuronal populations intact. It is thus likely that the degenerated dendrites and axons previously described at the electron microscopic level following EV treatment [Brawer, 1975; Brawer, 1978; Brawer, 1980] correspond to ß-endorphin neurons. Although the mechanism responsible for the selective vulnerability of ß-endorphin neurons to estradiol neurotoxicity remains unknown, work from the laboratory of Dr. Hyman Schipper has demonstrated that a unique population of Gomori-positive astrocytes may be involved [Schipper, 1990; Schipper, 1991]. Indeed, anatomical studies of the arcuate nucleus following long-term estradiol treatment described, in addition to dendrites and axons in various stages of degeneration, increased numbers of microglial cells and an pronounced increase in the number and size of peroxidase positive granules located in astrocytes [Brawer, 1978, Brawer, 1980; Schipper, 1990]. Peroxidase positive astrocytic granules have been shown to be rich in ferrous iron [McLaren, 1992] that behaves as a non-enzymatic peroxidase activity [Schipper, 1990], a feature consistent with the presence of a metalloporphyrin such as heme [Schipper, 1990]. Such heme peroxidases catalyze the oxidation of catechols such as catechol estrogens (2-OH-E_2 or 4-OH-E_2) generated both enzymatically and spontaneously in the brain [MacLusky, 1981] to reactive O-semiquinone free radicals [Liehr, 1990]. Indeed, it has been shown

using electron spin resonance that astrocytic cultures enriched with peroxidase-positive granules were able to generate E_2-semiqinones upon addition of catechol estrogens [Schipper, 1991]. E_2-semiquinones are unstable and have been shown to participate in the redox cycling between catechol estrogens and E_2-quinones [Liehr, 1990], reactions which can generate large amounts of free radicals such as superoxide ($O_2^{\cdot}$) and the hydroxyl anion ($OH^{\cdot}$) [Liehr, 1990]. The results presented here on vitamin E mediated protection of ß-endorphin neurons following EV injection support the idea that free radicals are produced under these conditions and that they participate in lipid peroxidative damage to ß-endorphin neurons. The participation of these unique peroxidase positive astrocytes in the generation of free radicals could account for the selectivity of estradiol's effects on the arcuate nucleus, since these astrocytes have been shown to be concentrated in the arcuate area [Schipper, 1990]. Glial cells may themselves be protected from the damaging effects of free radicals [Schipper, 1991] by their demonstrated enrichment in suphydryl content [Srebro, 1970]. However, the reasons for the selective vulnerability of the ß-endorphin population remain unclear.

Evidence from other models of neurodegenerative disease suggest that a relative enrichment or deficiency in the antioxidant enzymes of the central nervous system, such as superoxide dismutase (SOD), catalase or glutathione may predispose neurons to free radical induced damage. For example, it was shown using *in situ* hybridization, that the mRNA for superoxide dismutase was preferentially localized in vulnerable cortical neurons of Alzheimer's patients [Delacourte, 1988]. Additionnally, *post mortem* examination of the brains of Parkinsonian patients revealed increases in SOD mRNA

concentrations in the substantia nigra relative to controls [Ceballos, 1990]. Superoxide dismutase (SOD) converts the superoxide anion to hydrogen peroxide, a compound which itself may react with lipid membranes and induce peroxidation. Thus, depending on the redox milieu, so-called protective enzymes may either predispose or protect neurons from peroxidative damage. It can not be excluded, however, that increased in SOD mRNA expression is the result of a compensatory mechanism in vulnerable neurons confronted with increase lipid peroxidation.

Several other factors may account for the selective vulnerability of ß-endorphin neurons to chronic estradiol exposure. Certain receptor and channel systems have been shown to be involved in excitotoxic mediated neuronal cell death [Choi, 1988].
It is possible that the unique properties of ß-endorphin neurons, such as en enrichment in these potentially deleterious systems, accounts for their selective susceptibility to cell death. For example, NMDA receptors, which transduce the effects of glutamate and mediate long-term potentiation, have been found in high concentrations in the hippocampus and among other regions, the arcuate nucleus [Collindridge, 1987]. A relative enrichment in these receptors might, upon stimulation, cause excessive calcium entry in ß-endorphin neurons and result in cytoskeletal breakdown and DNA dissolution [Choi, 1988], while neighbouring neurons not endowed with such receptors would remain relatively protected. Additionnally, P-channels, which have been shown to mediate calcium entry in a variety excitotoxic conditions [Uchitel, 1992] have also been shown to be differentially distributed in central nervous system. Subsets of neurons

have been shown to be enriched with P-channels in the CA1 region of the hippocampus, the lateral habenular nucleus, the lateral substantia nigra, the inferior olive and the arcuate nucleus [Hillman, 1991]. A preferential localization of these receptors on ß-endorphin neurons would also account for their relative susceptibility to cell damage. Such a situation would nevertheless entail increased ß-endorphin susceptibility to cell death in response to a variety of toxic stimuli, including but not only, chronic estradiol exposure. That this may indeed be the case is supported by the recently described gold-thio-glucose lesion of the hypothalamus, which has been shown to selectively destroy neurons in the most lateral regions of the arcuate and periarcuate, the precise location of large ß-endorphin neurons [Bergen, 1992]. The idea that ß-endorphin neurons may more sensitive to destruction than other arcuate neuronal populations merits further research using double labeling strategies such as *in situ* hybridization for POMC mRNA with other detection methods for either protective enzymes or potentially deleterious receptor systems.

Another contribution of the present thesis concerns the elucidation of a functional link between the loss of ß-endorphin neurons in the arcuate nucleus and the development of polycystic ovaries and anovulation. The selective loss of beta-endorphin neurons following EV treatment has been shown to result in a compensatory up-regulation of mu opioid receptors in the MPOA. Increased mu opioid receptors in the region of LHRH neurons may mediate a chronic inhibition of LHRH release thus accounting for the suppressed pattern of LH release which engenders acyclicity and polycystic ovaries in these animals. Indeed, it has previously been shown that mu opioid agonists infused

into the MPOA result in a suppressed pattern of LH release [Mallory, 1990; Leadem, 1989] identical to that described in EV-treated animals [i.e reduced pulse amplitude in the absence of other changes) and that this reduced secretion was restored by treatment with naltrexone, an opioid receptor antagonist [Carriere, 1990]. The question arises as to the nature and origin of the endogenous ligand acting upon the upregulated mu receptors in the MPO to account for this inhibition. Two possibilities exist. Residual beta-endorphin normally secreted in this region or met-enkephalin secreted from nearby neurons could equally be responsible for LH inhibition under these conditions. The fact that long-term estradiol treatment has been shown to increase methionine-enkephalin immunoreactivity in the MPOA [Yuri, 1992] strengthens the latter possibility. Nevertheless, the current observation of reduced ß-endorphin neuron numbers concomitant with anatomical and functional correlates of opioid hypersensitivity appear somewhat paradoxical. For example, it is not known how mu opioid receptors, presumably up-regulated due to reduced ligand availability, can remain upregulated if acted upon by residual ß-endorphin or met-enkephalin. It may be that mu opioid receptors are incapable of down-regulation, since these have often been shown to up-regulate in response to diminished ligand availability [Moudy, 1985; Paden, 1987; Barg, 1989; Morris, 1989; Tempel, 1985; Tempel, 1984], while reports of opioid receptor down-regulation following chronic stimulation have been scarce. Also, there are other examples of denervation-induced chronic up-regulation of receptors, concomitant with functional supersensitivity.* The denervated muscle displays an up-regulation of acetylcholine receptors and spasticity, despite the initial loss of acetylcholine innervation

NB:
* Tardive dyskinesia (chronic dopamine blockade-induced dopamine receptor supersensitivity) may be a valid analogue to the [illegible]

[Kandel, 1991].

This cascade of events, the loss of beta-endorphin neurons, compensatory up-regulation of mu opioid receptors in the MPOA and inhibition of LH secretion, nonetheless represents an examination of estradiol's effects at a relatively late end point (i.e. 8 weeks after treatment). It is likely that factors other than the loss of ß-endorphin neurons interfere with the regulation of estrous cyclicity at earlier time points. Indeed, impairments in reproductive function due to the EV injection occur long before the degeneration of large numbers of neurons. It is known that animals treated with EV display irregular vaginal smears immediately after treatment and that a majority of animals display chronic estrus by approximately 2 weeks [Schulster, 1984]. However, a demonstrable hypothalamic lesion is apparent at only 3 months following E_2-implantation [Brawer, 1983], suggesting that the disturbances in cyclicity observed much earlier are not the result of beta-endorphin cell loss or the cascade of events described above. Indeed, Kalra has shown that 17 days of estradiol exposure results in disturbances in opioid regulation which are not reversed by naltrexone [Fuentes, 1992]. This finding is consistent with the opioid up-regulation due to cell loss representing a late endpoint to the multiple actions exerted by estradiol.

The loss of ß-endorphin neurons following chronic estradiol exposure due to EV injection bears several implications with regards to the regulation of the reproductive cycle. In female rats, mice and sheep, cumulative exposure to E_2 over life has been shown to participate in the arrest of cyclicity [Mobbs, 1984; Finch, 1984]. The original studies of Ascheim in rats had clearly shown that young animals heterografted with the

ovaries from old acyclic animals resumed cyclicity upon transplantation whereas the ovaries of young animals implanted into old rats ceased cycling [Ascheim, 1976]. Furthermore, these studies demonstrated that early ovariectomy resulted in the preservation of the ability to support estrous cycles in old rats implanted with young ovaries, whereas several markers of reproductive aging, including loss of ovarian cycles, inhibition of E_2-induced LH surge, and reduced post-ovariectomy rise in LH were accelerated by chronic E_2 exposure in young rats and mice [Mobbs, 1984; Finch, 1984; Simard, 1987]. The EV-induced model of chronic persistent estrus and acyclicity featured here has been considered a model of accelerated reproductive aging [Brawer, 1983; Schipper, 1981; Finch, 1984]. It is thus possible that the loss of ß-endorphin neurons described herein represents a physiologically appropriate cell death which contributes to age-associated arrest of cyclicity.

Other steroids have been shown to cause selective cell death. The most well characterized example is that of glucocorticoid induced hippocampal cell loss [Sapolsky, 1986], however prologued vasopressin exposure has also been shown to result in specific renal impairments [Miller, 1984]. An analogy may be drawn between the neurotoxic effects of estradiol and those of glucocorticoids. Chronic exposure to high physiological levels of glucocorticoids has been shown to induce the selective degeneration of CA1 hippocampal neurons in rodent and primate species including man [Sapolsky, 1986]. The loss of these neurons is said to cause a diminution in the negative feedback regulation of glucocorticoid secretion thus inducing a vicious circle of hippocampal neuron loss and excessive glucocorticoid secretion. In accordance with

what has been shown for estradiol, chronic exposure to circulating glucocorticoids throughout life was also shown to result in the loss of hippocampal neurons in aged rats and treatment with glucocorticoids was shown to accelerate various parameters of aging in the hypothalamic-pituitary-adrenal axis [Sapolsky, 1986]. Together, these results suggest that cumulative steroid exposure contributes to the demise of the endocrine systems which they normally facilitate. It follows from this idea that steroids play a role in inducing senescent changes in organisms. This notion has been previously postulated [Finch, 1990; Mobbs, 1992] and is supported by evidence from phylogenic studies [Finch, 1990]. It is also interesting to note that the general aging theory of Dillman [Dillman, 1976], which proposes increasingly elevated hypothalamic thresholds to the negative feedback effects of steroids, conforms to the general effects chronic estradiol and glucocorticoid exposure described above.

The mechanism underlying glucocorticoid-induced neurotoxicity has not been ascertained. *In vitro* studies using primary hippocampal cultures have revealed that although high physiologic levels of glucocorticoids alone did not kill hippocampal neurons [Sapolsky, 1992], the treatment predisposed these cells to a variety of other insults such as mild ischemia, which otherwise would not have been lethal. The catabolic actions of glucocorticoids has been purported to place hippocampal neurons on a so-called "metabolic cliff" from which they are highly susceptible to damage and can only be rescued by glucose or hexose. Our findings on the mechanism of estradiol neurotoxicity suggest that in vivo, lipid peroxidation may be partially responsible for ß-endorphin cell loss. However, since a multiplicity of factors cause free radical

production, including ischemia and metabolic insufficiency, it remains possible that mechanisms of estradiol neurotoxicity are complementary or similar to those described in culture for glucocorticoid neurotoxicity. Because of the widespread action of steroids during ontogeny and throughout the organism, steroids such as glucocorticoids and estradiol, are ideally suited to regulate cell differentiation and growth, as well as cell death throughout life. Glucocorticoids have been shown under certain conditions, to be inhibitory to cell growth. During reactive synaptogenesis in experimentally induced lesion models, treatment with glucocorticoids abolished a series of molecular changes such as increased tubulin synthesis associated with reactive synaptogenesis [Poirier, 1992]. Alternatively, glucocorticoids have been shown to be necessary for neuronal survival. Long-term adrenalectomy caused the loss of dendate gyrus neurons, thus illustrating how the same hormone may promote the survival and death of different neuronal populations within the same structure [Sloviter, 1989; Sapolsky, 1991]. Similarly, a variety of studies have illustrated the trophic effects of E_2 on hypothalamic neurons during critical periods of development and the effect this has on the development of sexual dimorphisms [Hutchison, 1991]. Interestingly, it has been shown that the trophic effects of estradiol are not restricted to the neonatal period and that morphological alterations of neurons by estradiol is a daily phenomenon [Frankfurt, 1991]. For example, acute treatment with estradiol benzoate results in increased dendritic spine density in the ventromedial nucleus of the hypothalamus, but not in the dendate gyrus or other regions of the hippocampus. Increased spine density is also observed in intact animals at proestrus, when E_2 levels are elevated, indicating that these

structural changes occur regularly throughout the 4 day estrous cycle. It is clear from these studies that particular subpopulations of neurons are susceptible to steroid-induced structural alterations during adult life.

Additionnally, molecular biological evidence suggest the involvement of steroids in regulating physiological cell death. The concentration of GR varies markedly throughout the cell cycle [Cidlowski, 1979]. What is particular about this variation is that the direction of change of GR concentration is opposite that of virtually every other cellular protein [Murray, 1991]. In fact, only cell cycle regulating proteins such as the cyclins have been shown to vary in this fashion [Murray, 1991], suggesting that glucocorticoids acting via glucocorticoid receptors can regulate cell cycle stage. Interestingly, it has recently been shown that estrogens may also interact with the cyclins [Thomas, 1992]. Thus, steroids, particularly glucocorticoids and estradiol appear as prime candidates for the regulation and dysregulation of physiological processes involving neuronal cell death and survival throughout life.

CHAPTER 9: SUMMARY

The results contained within this thesis may be summarized as follows:

1) Mu, delta and kappa opioid receptors are present within the rat hypothalamus and are differentially distributed. Highest concentrations of mu opioid receptors are located in the medial preoptic area whereas delta opioid receptors are most highly concentrated in the ventromedial nucleus. Kappa opioid receptors are highest in the suprachiasmatic nucleus although significant concentrations of mu and delta opioid receptors were also detected in this nucleus.

2) EV treatment results in significant increases in mu opioid receptors in the anteroventral preoptic nucleus of the hypothalamus, in the absence of any changes in other nuclei. Delta opioid receptors in the suprachiasmatic nucleus are decreased following chronic estradiol exposure whereas kappa opioid receptors are unchanged. Concomitantly, hypothalamic ß-endorphin concentrations are reduced in the EV-treated group but not in the estradiol-implanted rats as compared to controls.

3) Destruction of the arcuate nucleus by neonatal treatment with monosodium glutamate (MSG) induces increases in mu opioid receptors in the medial preoptic area. MSG-induced reductions in ß-endorphin concentrations were inversely proportional to the degree of opioid binding measured in the medial preoptic area.

4) EV treatment results 8 weeks later in significant reductions in ß-endorphin concentrations and in ß-endorphin-immunoreactive neuron numbers. Neuropeptide-Y and met-enkephalin concentrations are unaltered following EV treatment nor are tyrosine-hydroxylase, somatostatin or neurotensin neuron numbers. The total number of neurons

in the arcuate nucleus is reduced after EV injection. The total number of neurons lost corresponds closely with the estimated number of ß-endorphin neurons lost. Together, these results suggest that EV treatment results in the selective destruction of more than 60% of ß-endorphin neurons in the arcuate nucleus of the hypothalamus.

5) The reductions in ß-endorphin concentrations seen in EV treated rats are prevented by daily administration of vitamin E. Furthermore, vitamin E prevents the appearance of constant estrus and polycystic ovaries normally associated with the EV treatment.

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