Endocrine (1995) 3, 827-831

Estrogen modulation of two subpopulations of β-endorphin neurons in ovariectomized guinea pigs distinguished by peripherally injected fluorogold

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β-endorphin released by neurons in the arcuate nucleus affects the output of several neuroendocrine systems and estrogen levels modulate the production and secretion of β-endorphin. We used intraperitoneal injection of fluorogold to retrogradely label the cell bodies of neurons that project outside the blood–brain–barrier in conjunction with immunohistochemistry for β-endorphin to dual label the subpopulation of β-endorphin neurons that project to the median eminence or other sites of access to the peripheral circulation. We found that some identified β-endorphin neurons in the arcuate nucleus of ovariectomized guinea pigs sequestered fluorogold. Approximately 7% of β-endorphin-containing cells co-localized with fluorogold. The effect of estrogen on the number of identified β-endorphin cells was examined. A single estradiol benzoate injection to ovariectomized guinea pigs 24 h prior to sacrifice dramatically decreased the total number of β-endorphin cells identified in the rostral, medial and the caudal portions of the arcuate nucleus. Also, a significantly smaller percentage of fluorogold-filled cells was found to contain β-endorphin immunoreactivity in the estrogen-treated group. These data suggest that a subpopulation of β-endorphin neurons has access to the peripheral circulation and may alter the output of neurosecretory terminals at the level of the median eminence. Furthermore, estrogen affects this subpopulation and the general population of β-endorphin neurons in the arcuate nucleus in a similar manner.

Keywords: Fluorogold; β-endorphin; guinea pig; arcuate; estrogen

Introduction

The neurosecretion of β-endorphin influences a number of neural systems including most, if not all, neuroendocrine systems (Millan & Herz, 1985). In the forebrain of the mammal, the cell bodies of the neurons that produce β-endorphin and its precursor pro-opiomelanocortin (POMC) appear to be limited to the area in and around the arcuate nucleus (ARC) of the hypothalamus (Bloom *et al.*, 1978; Khachaturian *et al.*, 1984; Thornton *et al.*, 1994). These neurons project to many areas of the forebrain. It is unknown if β-endorphin cells in guinea pigs project to the median eminence (ME) and act either as neuroendocrine neurons by releasing β-endorphin into the perivascular spaces of the external layer of the ME or to modify output by other neuroendocrine terminals. Anatomical evidence in other species is equivocal as to whether ARC β-endorphin neurons act as neuroendocrine cells or on other neuroendocrine terminals. Electron microscopic evaluation of rat median eminence has found evidence for β-endorphin projections to the internal layer of the ME (Hisano *et al.*, 1982) but not for β-endorphin terminals in the external layer (Ibata *et al.*, 1985).

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Received 30 May 1995; accepted 23 August 1995

There is considerable evidence suggesting that the synthesis as well as the secretion of β-endorphin from ARC neurons is affected by changes in estrogen levels. For example, the administration of estrogen to ovariectomized rats and ewes alters POMC mRNA levels in the ARC (Wilcox & Roberts, 1985; Tong *et al.*, 1990; Rosie *et al.*, 1992; Treiser & Wardlaw, 1992; Weiland *et al.*, 1992; Whisnant *et al.*, 1992; Broad *et al.*, 1993; Petersen *et al.*, 1993). Levels of β-endorphin in the portal blood, thought to represent neurosecretion by ARC β-endorphin neurons, also co-vary with estrogen level. β-endorphin was lower in ovariectomized monkeys and rats than in intact animals (Wehrenberg *et al.*, 1982; Sarkar & Yen, 1985). The amount of β-endorphin present in the cell body is determined by the interaction of the rates of synthesis and processing of POMC and the transport and release of β-endorphin. We have shown previously that the number of immunocytochemically-identified, β-endorphin-containing neurons in the ARC of ovariectomized guinea pigs is sensitive to steroid conditions (Thornton *et al.*, 1994).

We thought that it would be informative to examine whether a subpopulation of β-endorphin neurons has access to the peripheral circulation. Further, we asked if the response of this subpopulation of β-endorphin neurons to estrogen replacement was similar to that of the general population of β-endorphin neurons. To do this, we used peripheral injection of fluorogold (FG) to label neurons projecting outside the blood–brain–barrier (Merchenthaler, 1990) in ovariectomized oil- and estrogen-treated guinea pigs and then performed immunocytochemistry to identify β-endorphin-containing cells.

Results

When injected peripherally into ovariectomized guinea pigs, fluorogold fluorescence in the hypothalamus was readily apparent in the paraventricular, supraoptic and arcuate nuclei (Figure 1A). The concentrations of fluorescent particles were often relatively larger and more dense in the paraventricular and supraoptic nuclei than those seen in the arcuate nucleus. On occasion, long 'fibers' were observed that appeared to emanate from the third ventricle and course toward the median eminence (Figure 1A). Confirming a previous report in guinea pigs using peripheral injection of Granular Blue as the retrograde tracer (Poulain *et al.*, 1990), we found concentrations of FG in the retrochiasmatic area of the mediobasal hypothalamus underneath the third ventricle and in all areas of the ARC. Sections from a control animal that received only the vehicle in which the fluorogold was dissolved had no areas of concentrated autofluorescence, only a uniformly low background level.

When fluorogold-filled cells were examined in the ARC, a relatively small proportion were found to be coincident with β-endorphin immunoreactivity in both treatment groups (Figure 1B and C). Of 949 fluorogold-containing profiles examined throughout the arcuate nuclei of the 15 animals, 51 (5.4%) were found to be colocalized with β-endorphin. The estrogen-treated group of animals had a significantly smaller

percentage of fluorogold-containing cells that also were identified as β-endorphin-containing than did the oil-treated group (3.4 ± 0.8% *vs* 7.4 ± 2.1%; $P < 0.05$; Figure 2A).

When a random sample of β-endorphin-containing cells was examined it was found that a number of them also contained FG. Of 922 β-endorphin cells examined from the 15 animals, 50 also contained fluorogold. The percent of β-endorphin cells that also contained fluorgold from ovariectomized EB-treated animals ($n = 7$) tended to be smaller (4.0 ± 1.5%) than the percent of double-labeled cells in the ovariectomized oil-treated group (6.7 ± 1.7%; $n = 8$) but the differences between the two groups did not reach statistical significance ($P < 0.1$; Figure 2B).

The number of β-endorphin-containing cells in the mediobasal hypothalamus was found to vary depending on whether estrogen was administered (Figure 3). There were 38–56% fewer identifiable β-endorphin cells in the four

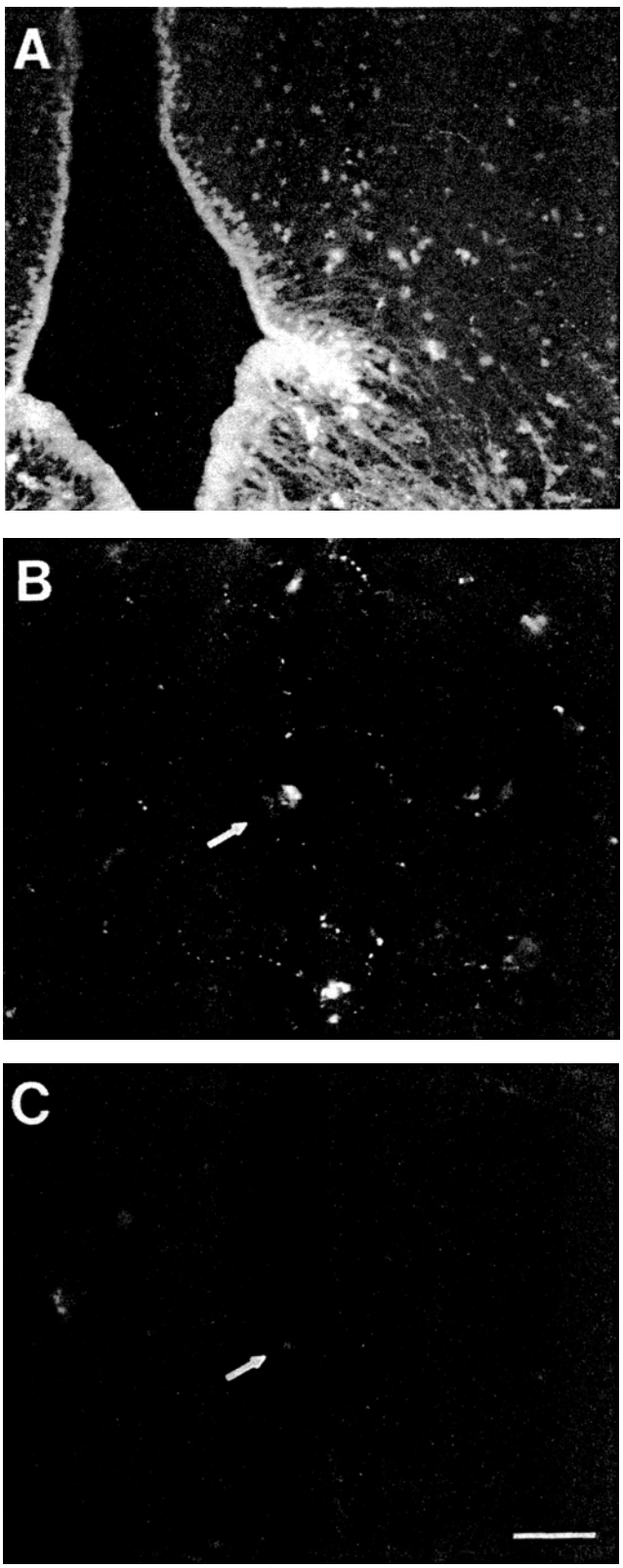

Figure 1 The appearance of fluorogold fluorescence in the caudal arcuate nucleus of an oil-treated, ovariectomized guinea pig (A) and an example of a cell stained for β-endorphin (B) that also was retrogradely labeled with fluorogold (C) from the medial arcuate nucleus of an oil-treated, ovariectomized guinea pig. Arrows point to the same cell in B and C. Bar indicates 50 μm in A and 20 μm in B and C

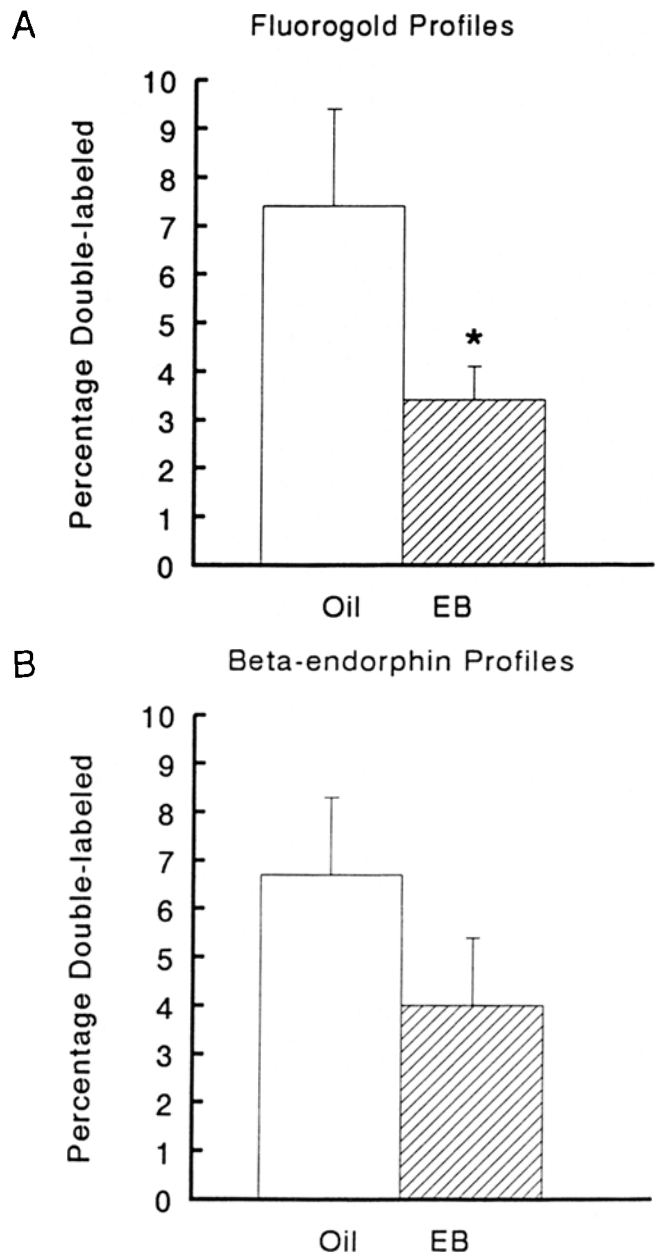

Figure 2 The percentage of cells labeled with both fluorogold and β-endorphin. Tissue was from ovariectomized guinea pigs injected with EB (shaded bars) or with vehicle (open bars) 24 h previously. (A) Fluorogold accumulations that were co-localized with β-endorphin immunoreactivity were a small percentage of the sample of fluorogold-filled profiles examined. The arcuate nucleus of estrogen-treated animals ($n = 7$) had fewer double-labeled cells than in control animals ($n = 8$). (B) β-endorphin-labeled cells that also contained fluorogold were a small percentage of the sample of β-endorphin cells examined. The same sections were analysed as in (A). Data are presented as mean ± SEM. $^*P < 0.05$

rostrocaudal areas of the mediobasal hypothalamus in tissue from EB-treated animals than there were from oil-treated control tissue. Analysis of variance showed that the main effect for steroid was significant ($P<0.001$) but the main effect for area was not. Planned comparison analyses comparing oil *vs* EB cell counts at each of the rostrocaudal areas found that the rostral, medial and caudal areas of the arcuate nucleus but not the retrochiasmatic area had significantly fewer β-endorphin cells in sections from EB-treated animals than sections from oil-treated animals (see Figure 3). The direction and relative magnitude of these results were confirmed when additional sections from the medial ARC of the 15 animals were run in three different assays and counted by another observer (data not shown).

Discussion

In the present study, peripheral injection of fluorogold was found to label cells of the paraventricular, supraoptic and arcuate nuclei. Magnocellular neurons in the paraventricular and supraoptic nuclei project outside the blood–brain–barrier to the posterior pituitary and have been reported to sequester peripherally injected tracers such as fluorogold in several species (Merchenthaler, 1990; Poulain *et al.*, 1990; Silverman *et al.*, 1990; McShane *et al.*, 1994). The arcuate nucleus of the guinea pig was reported to contain cells that sequester peripherally injected Granular Blue and some of these cells were found to co-localize with progesterone receptors (Poulain *et al.*, 1990). Other cell types in the arcuate nucleus that have access to substances in the peripheral blood have not been identified previously in guinea pigs. To our knowledge, β-endorphin neurons have not been examined for accumulation of fluorogold or similar tracers after peripheral injection in any species prior to this study. Our results show that a subpopulation of β-endorphin neurons does sequester fluorogold after peripheral injection. This suggests that β-endorphin release by these neurons could reach the portal vasculature. Furthermore, these data also suggest that β-endorphin might act by a presynaptic action on the terminals of neurosecretory neurons ending near the fenestrated capillaries of the median eminence. These results provide an anatomical basis for physiological studies that have inferred that β-endorphin in the portal blood could have been released from neurons in the ARC (Wehrenberg *et al.*, 1982; Sarkar and Yen, 1985) as well as studies that have found opioids and/or opioid antagonists to affect neurosecretory systems at the level of the median eminence (Forman *et al.*, 1983; Panerai *et al.*, 1983; Baige *et al.*, 1992). Thus, these results provide another piece of evidence supporting the hypothesis that the ME is a site of action for ARC β-endorphin neurons.

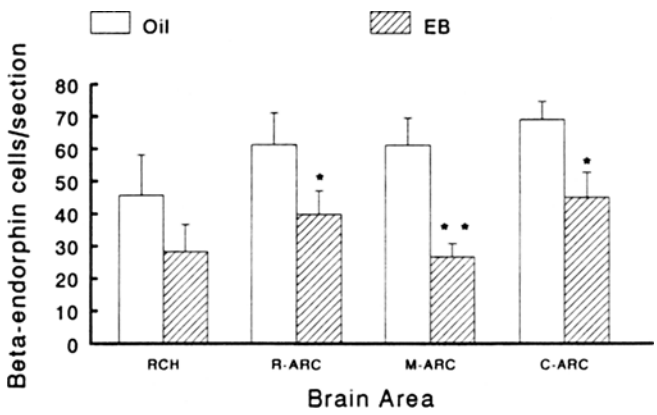

Figure 3 Effects of estrogen on the number of β-endorphin cells in four areas of the mediobasal hypothalamus. Data are from eight oil-treated animals and 7 EB-treated animals and are presented as the mean ± SEM. $*P<0.05$, EB *vs* Oil; $**P<0.01$, EB *vs* Oil

It is possible that some cells projecting to the internal layer of the ME or to the ventral aspect of the ARC could have been exposed to low levels of fluorogold since peripheral injection of tracers has been reported to penetrate into parts of the ARC (Balin *et al.*, 1986). In these studies we did note that the median eminence and the ventral-most portion of the ARC sometimes had a higher level of fluorescence than the dorsal aspect of the ARC which might indicate some diffusion of the FG since it was not seen in the non-FG treated animal. Nevertheless, the observation that fluorogold can reach terminals of some β-endorphin neurons indicates that a pathway exists by which blood-borne molecules can act on β-endorphin cells and we propose that β-endorphin secreted by these terminals could follow this pathway to reach the portal blood and its environs.

The data presented here indicate that the number of neurons in ovariectomized guinea pigs which contain sufficient β-endorphin to be detected is dramatically reduced 24 h after estrogen is reintroduced to the animal. This decline in cell number is evident throughout the rostrocaudal extent of the arcuate nucleus. We interpret our data to indicate that many of the neurons which had detectable levels of β-endorphin in their cell body under ovariectomized conditions had a significant decrease in their level of this neuropeptide 24 h after EB administration.

There are several physiological mechanisms that might underlie a decreased level of neuropeptide in β-endorphin neurons. One family of mechanisms is that the estrogen treatment caused less β-endorphin to be produced due to decreases in transcription, translation or various processing steps. There appear to be no data available in the guinea pig on these processes. Inferring from other species is problematic. The data in the rat, perhaps the best studied model, have indicated that POMC mRNA levels, for example, are altered by numerous interacting factors such that the direction of change is not easily predicted at a given point in time. Age, time after castration, endocrine condition and the time of day at sacrifice all appear to influence levels of POMC mRNA (e.g. Wilcox & Roberts, 1985; Wise *et al.*, 1990; Bohler *et al.*, 1991; Kerrigan *et al.*, 1991; Weiland *et al.*, 1992; Petersen *et al.*, 1993; Scarbrough *et al.*, 1994).

Another mechanism that could lead to a decreased amount of β-endorphin in hypothalamic neurons is an increased level of secretory activity resulting in a larger amount of β-endorphin transported from the soma than is replenished. Indeed, decreased β-endorphin levels have been observed in the face of increased POMC mRNA levels in the same experimental animals (Markowitz *et al.*, 1992; Bronstein *et al.*, 1993) presumably due to increased output of β-endorphin. Recent studies suggest that estrogen may stimulate β-endorphin neurons. For example, β-endorphin release can be increased by estrogen treatment of perifused mediobasal hypothalamic tissue (Nakano *et al.*, 1991). Furthermore, estrogen can disinhibit β-endorphin neurons in guinea pig hypothalamic slices by causing them to be less sensitive to opioid- and GABA-mediated inhibition (Kelly *et al.*, 1992; Lagrange *et al.*, 1994). β-endorphin cell bodies in guinea pigs may be highly susceptible to depletion of their neuropeptide under stimulatory conditions. Using a staining method identical to that used in the present study we have observed dramatic decreases in the number of β-endorphin cells following a single 1 h stimulation with medium containing 30 mM potassium in an *in vitro* slice preparation as compared to unstimulated controls (Loose, unpublished results).

This study and one other has examined the effect of estrogen on β-endorphin cell counts in guinea pigs. The previous study found that 24 h after an EB injection there were significantly more β-endorphin cells as compared to ovariectomized controls (Thornton *et al.*, 1994). Based on the results of this previous study we had expected also to see higher cell counts in the EB-treated animals. We have no definitive

explanation for the divergent results of these two studies. Differences of potential importance include use of different strains of guinea-pig (Topeka *vs* Hartley), different treatment protocols (e.g. 1 EB injection *vs* 2 EB injections), different times after ovariectomy (8 *vs* 18 days), different primary antibodies, and different immunohistochemical techniques. In any case, it appears that the guinea pig is similar to the rat in that the amount of β-endorphin in the hypothalamus can be dramatically altered and is quite sensitive to experimental conditions. Further studies will be needed to determine which factors are contributing to the differences observed.

A relatively small percentage of identified β-endorphin-containing cells were found to sequester FG in both estrogen-treated and oil-treated ovariectomized guinea pigs. Sequestration of FG requires both access to areas outside the blood–brain–barrier and sufficient secretory activity to take up detectable amounts of FG. Indeed, when fluorogold was injected just prior to a steroid-induced LH surge in mice, a period of presumptive hypersecretion by LHRH neurons, a greater percentage of LHRH neurons had detectable levels of FG than when fluorogold was injected into ovariectomized mice (Silverman *et al.*, 1990). Thus, our estimate of 7% co-localization should be considered a lower end estimate as there is no reason to suspect that the release rate of β-endorphin from ARC neurons is maximal in the ovariectomized condition. Rather, the evidence from other species is that low estrogen conditions are correlated with lower β-endorphin output into the median eminence than under high estrogen conditions (Wehrenberg *et al.*, 1982; Sarkar and Yen, 1985).

We found that a significantly smaller percentage of FG-filled cells stained for β-endorphin in the estrogen-treated condition than in the oil-treated condition. These data imply either that the number of β-endorphin cells that were detected and that have access to the peripheral circulation decreased or that the number of non-β-endorphin cells sequestering FG increased with respect to the number of β-endorphin cells. The trend toward a decrease in the percent of β-endorphin neurons sequestering FG coupled with the large decrease in detected β-endorphin cells in the EB-treated guinea pigs as compared to the control animals supports the prior interpretation. Thus, we suggest that some of the β-endorphin neurons that could act as neuroendocrine cells are affected by estrogen in a manner similar to the non-neuroendocrine population of β-endorphin cells.

In conclusion, a subpopulation of β-endorphin neurons has been identified that has access to the peripheral circulation. Thus, these neurons may act either as neuroendocrine neurons themselves or by modulating the output by other neurons terminating at the median eminence. In addition, we have found that 24 h after estrogen is administered to ovariectomized guinea pigs, a dramatic decrease in the number of β-endorphin cells throughout the rostrocaudal extent of the arcuate nucleus can be detected. Estrogen may increase the secretory activity of some β-endorphin neurons, including a potential neuroendocrine subpopulation, leading to a decrease in the stored levels of this neuropeptide in their somata.

Materials and methods

Animals and animal treatments

Young adult Hartley guinea pigs (Hilltop Lab Animals Inc., Scottdale, PA) were maintained on a light schedule of 14 h of light beginning at 0600. Guinea pig chow and water were available at all times. At least 2 weeks after arrival, 16 females (492 ± 42 g) were ovariectomized using the inhalant anesthetic methoxyflurane (Pitman-Moore Inc, Mundelein, IL). Eleven days later at 10 a.m. the females were injected subcutaneously with 25 μg estradiol benzoate (EB) dissolved in 0.25 ml corn oil (EB-treated group) or 0.25 ml of the vehicle alone (control group). Twenty-four hours later the guinea pigs were injected intraperitoneally with 20 mg/kg fluorogold (Fluorochrome Inc., Englewood, CO) dissolved in 0.9% saline at a concentration of 20 mg/ml. Preliminary tests in guinea pigs examined the effects of four doses of FG and two survival times. Injection of 3 mg/kg FG results in barely detectable filling of cell bodies, whereas a 9 mg/kg dose resulted in much improved levels of fluorescence. Both 15 mg/kg and 20 mg/kg FG doses resulted in slightly brighter fluorescence than 9 mg/kg. Survival periods of 6 and 12 days after FG injections resulted in similar patterns of FG fluorescence. Five days after fluorogold injection the animals received a second injection of EB or vehicle at 10 a.m. and 24 h after this injection the animals received an overdose of pentobarbital (65 mg/kg) and were killed by decapitation. To summarize, the EB-treated animals were injected twice with 25 μg EB (11 and 17 days after ovariectomy) and fluorogold was administered 24 h after the first EB injection and they were killed 24 h after the second EB injection. Control animals were treated identically except they received the oil vehicle instead of EB.

Tissue preparation and immunocytochemistry

The procedures for obtaining tissue slices, fixation and immunocytochemistry for β-endorphin neurons were similar to those previously described (Ronnekleiv *et al.*, 1990). In brief, the brain was rapidly removed (<2 min) and placed into an ice-cold, oxygenated (95% O_2, 5% CO_2) modified Krebs-Ringer buffer. The buffer contained NaCl, 124 mM; KCl, 5 mM; NaH_2PO_4, 1.25 mM; $MgSO_4$, 2 mM; $CaCl_2$, 2 mM; $NaHCO_3$, 26 mM; dextrose, 10 mM; HEPES, 3 mM; and HEPES-Na, 2 mM; and was approximately pH 7.3 at 4°C. While immersed, the brain was cut using a razor blade into a block which contained primarily the hypothalamus and preoptic area. The rostral pole of this block was glued with cyanoacrylate to a chilled stainless steel chuck, the dorsal surface was supported by an agar strip and this assemblage was immersed in ice-cold buffer for slicing on a vibrating microtome (World Precision Instruments, Sarasota, FL). Neurons in slices prepared in this manner remain electrophysiologically active for many hours (Loose & Kelly, 1990; Loose & Jacobs, 1992).

A 2 mm thick coronal section was cut that included most of the mediobasal hypothalamus excluding the mammillary bodies. Immediately after slicing, the sections were fixed by immersion for 4 h in cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4; PB) then were placed into 30% sucrose in PB. The next morning the slices were rapidly frozen into embedding medium using dry ice and stored at −80°C until cut on a cryostat into 20 μm thick sections. Sections were thaw-mounted onto poly-L-lysine coated slides and stored at −20°C until processed for immunocytochemistry. β-endorphin neurons were identified using the R-13 primary antibody (Weber *et al.*, 1982) at a 1:1000 dilution. This antiserum was reported to bind equally well to β-lipotropin, β-endorphin (1-31) and -(1-27) but not to β-endorphin (1-9) or to Met-enkephalin. Thus, this antibody would be expected to recognize a number of POMC-derived peptides as well as β-endorphin. For brevity, we will refer to identified cell profiles as β-endorphin-containing cells. Visualization of the β-endorphin-containing cells was accomplished using Texas Red as follows. Slides were washed for 20 min in PB on a shaker platform, edge blotted and 1–2 drops of primary antibody were added so that the entire section was covered. The slides were placed on damp paper in airtight boxes at 4°C for approximately 40 h. Slides were washed for 30 min in PB and edge blotted then 1–2 drops of the secondary antibody (donkey anti-rabbit gamma globulin conjugated to Texas Red; 1:50; Jackson Immunoresearch Labs, Westgrove, PA) were applied. The primary and secondary antibodies were diluted in tris(hydroxymethyl)aminomethane (0.5%) buffer containing 0.12% Na_2HPO_4, 0.048% KH_2PO_4, 0.7% NaCl, 0.5% Triton X-100 and 0.7% lambda carrageenan,

type 4. Following a 2 h incubation in the humid boxes at room temperature the slides were again washed for 30 min in PB. Coverslips were applied using a glycerol-PB medium (29:1) containing 5% n-propyl gallate and were sealed with collodion.

Specificity of the Texas Red fluorescence for β-endorphin was evaluated in two ways. Tissue was processed as above except (1) the primary antibody was omitted or (2) 30 μg/ml rat β-endorphin was added to the primary antibody shortly prior to incubation. Autofluorescence in the absence of fluorogold was also evaluated by processing tissue from an animal that did not receive fluorogold. Sections in which the primary antibody had been deleted or co-incubated with β-endorphin exhibited no staining of cell bodies or fibers. Tissue from an animal not exposed to fluorogold showed very little autofluorescence under our conditions following processing for immunocytochemistry whether or not the β-endorphin primary antibody was included. Sections were viewed using a Leitz Ortholux microscope and the N2 cube for Texas Red and the D2 cube for fluorogold. Photomicrographs were taken with technical pan film (Eastman Kodak Company, Rochester, NY). Tissue from one animal in the EB-treated group had no FG in any hypothalamic nuclei and the data from this animal were excluded from the study.

Sections were analysed at 400 μm intervals from the retrochiasmatic area to the premammillary area of the hypothalamus (five or six per animal). Three cell counts were performed on each section by a single investigator who was blind to the treatment or animal from which the sections were obtained. All identifiable β-endorphin cells were counted at 400 × magnification. Because fluorogold-filled cells often were lightly filled and the perimeter of the cell was not discernible a cell count of all fluorogold-filled cells was not practical. Therefore, two sampling procedures were employed. A total of 10 to 15 clearly distinguishable concentrations of fluorogold particles were identified per section at 625 × using the D2 cube and each concentration of grains was then evaluated for β-endorphin immunoreactivity by switching to the N2 cube. Also, 10 to 15 distinct β-endorphin-positive cells were selected randomly per section and subsequently examined for the presence of fluorogold particles. Since the number of double-labeled cells per section was small, the percent of cells double-labeled per animal was determined by combining the counts from all of the sections analysed. In order to test for steroid effects that might be limited to anatomical subareas of the β-endorphin population, each section was assigned prior to image analysis to one of four subareas of the mediobasal hypothalamus along the rostro-caudal dimension, the retrochiasmatic area, the rostral arcuate, the medial arcuate and the caudal arcuate. When two sections from the same animal were assigned to the same subarea, the mean of the β-endorphin cells counted was used as a single data point. The retrochiasmatic area was distinguished by the presence of β-endorphin cells under the third ventricle and no infundibular sulcus, the rostral arcuate by the slight formation of the infundibular sulci, the medial arcuate by the full development of the median eminence and the caudal arcuate by the separation of the infundibular stalk from the median eminence.

The *t*-Test for independent samples was used to evaluate the effects of steroid on the percent of cells that were double labeled and a two way repeated measure analysis of variance (steroid condition by anatomical area) was used to examine the number of β-endorphin cells in the section. Four planned comparison analyses were performed, each comparing oil *vs* EB cell counts in one of the four anatomical areas evaluated (Keppel, 1982). Any difference with a P value less than 0.05 was considered a significant difference.

Acknowledgements

This work was supported in part by NIH NS29970, the Howard Hughes Foundation and a Research and Development Grant from Oberlin College to M.D.L.

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