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Melanin concentrating hormone and estrogen receptor-α are coexstensive but not coexpressed in cells of male rat hypothalamus

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Abstract

In male rats, estradiol (E_2) exerts marked anorectic effects. One mechanism proposed for this effect is an E_2-mediated down-regulation of the orexigenic neuropeptide melanin concentrating hormone (MCH). Previous anatomical work has shown that both MCH and estrogen receptor α (ERα) are found in quantity in the lateral hypothalamic area (LHA), a structure long associated with appetite and ingestive behavior. It has been hypothesized that the most direct manner by which E_2 could affect MCH expression and feeding would be via classical nuclear ERα located in MCH neurons. To evaluate this notion, we performed double-label immunohistochemistry for MCH and ERα in male rat hypothalamus. We report here that MCH neurons do not contain ERα, suggesting that the primary locus for estrogenic control of feeding is not the MCH neurons themselves. Rather, we show substantial overlap in the anatomical distribution of both cell types, raising the possibility that E_2 influences MCH signaling indirectly via adjacent ERα-containing cells.
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Since its discovery in neurons of the mammalian hypothalamus [3,26], melanin concentrating hormone (MCH) has been studied as a key regulator of energy balance [16]. Central administration of MCH promotes feeding [5,17,18], while genetic ablation of the *Mch* gene produces a lean phenotype often characterized by hypophagia [1,21]. It has also been demonstrated that the *Mch* gene is up-regulated as a compensatory response to energy restriction [17]. According to one model for the hypothalamic control of food-intake and energy balance, MCH-containing neurons near the lateral hypothalamic area (LHA) are "second order" neurons, once removed from the site of adiposity peptide (i.e. leptin) signaling in the arcuate nucleus [8,20,27]. Anatomical data suggest that a condition of negative energy balance is relayed to MCH cells via orexigenic neuropeptide Y (NPY)- and agouti-related protein (AgRP)-containing neurons, while the opposite condition is signaled by neurons containing the anorectic peptides proopiomelanocortin (POMC) and α-melanocyte stimulating hormone (α-MSH) [4,6,7]. MCH-containing neurons presumably integrate this information from the arcuate, organizing feeding behavior accordingly via their projections to brain structures like the nucleus accumbens [9].

While increased MCH activity appears to bias animals towards attaining a state of positive energy balance, estradiol (E_2) has the opposite effect, producing weight loss and aphagia when given at supraphysiologic doses [2,14]. Additionally, genetic ablation of estrogen receptor α (ERα) produces an obese phenotype [10]. The presence of ERα in LHA, near the main population of MCH-containing neurons, and in arcuate nucleus has prompted investigations of whether this peptide mediates the aphagic effects of E_2 [13,15], presumably by impairing MCH signaling. In male rats, *Mch* expression decreases markedly with E_2 administration [15], and it has been suggested that this change may be responsible for E_2's anorexic effects. The most straightforward manner in which E_2 could affect *Mch* expression would be via action at nuclear estrogen receptors located in MCH neurons. Alternatively, the presence of estrogen receptors in neighboring cells of the LHA or arcuate would indicate an indirect, afferent-driven mechanism of *Mch* regulation. To determine the anatomical relationship between ERα and MCH in the hypothalamus, we performed double-label immunohistochemistry for these substances and mapped their distribution in male rats.

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In this experiment, 40 μm paraformaldehyde-fixed, frozen sections from hypothalami of four adult (∼350 g) male Long-Evans rats (Harlan, Indianapolis, IN, kept and used in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*), were first labeled for ERα (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) with goat anti-rabbit secondary antibody (1:600, Vector, Burlingame, CA) and visualized using nickel-intensified 3′3-diaminobenzidine (DAB) and an avidin–biotin peroxidase kit (Elite Kit, Vector), according to procedures described previously [19]. Because there is little evidence for the expression of ERβ near the MCH neuronal field [11], labeling for that receptor subtype was not performed. After ERα labeling was complete and tissue was reblocked with normal goat serum, a second round of labeling was conducted for MCH (1:2500, Phoenix Pharmaceuticals, Belmont, CA, secondary antibody as above) using only DAB as the chromogen. Serial double immunolabeling revealed a pattern of opaque, black, nuclear ERα label and translucent brown labeling in cytosol for MCH. In no case were antigens found outside their expected cellular compartments (e.g. black label in cytosol or brown label in nucleus).

Control sections from each animal were incubated in absence of primary antibodies for MCH, ERα, or both, and in all cases labeling for that antigen was absent. Major landmarks and labeled cells from each of six consistent rostrocaudal levels of hypothalamus were drawn using a microscope and camera lucida. These drawings were digitally scanned and transposed using Adobe Illustrator software to corresponding levels of a reference atlas [23].

Anatomical distribution of each antigen was consistent with previously published research reports using single-label immunohistochemistry or *in situ* hybridization [3,22,24] (Figs. 1A and 2). Both MCH- and ERα-labeled cells were sparse at the level of the paraventricular nucleus (PVN). Most MCH-labeled neurons there appeared dorsally in zona incerta (ZI), above the fornix and stretching medially to the third ventricle. ERα-labeled cells appeared in the ventrolateral aspect of the ventromedial hypothalamic nucleus (VMH) and arcuate nucleus, and ascended along the ventricle through the periventricular area, eventually mingling with MCH-labeled neurons in PVN. More caudally, as each cell type became more numerous, this general pattern continued, but ERα-labeled cells also occurred alongside MCH neurons in the ZI and LHA. Interestingly, in no case were MCH neurons seen to express ERα (Fig. 1B). It is also notable that MCH neurons avoided the dorsomedial hypothalamic nucleus (DMH) and VMH, often appearing between the two structures as the population stretched medially to near the third ventricle (Fig. 2). Together, these observations present a heterogeneous picture of MCH and ERα distribution in hypothalamus where there are areas of overlap (LHA, PVN, ZI) as well as partial exclusion (ERα but not MCH in VMH).

The absence of nuclear ERα in MCH neurons may help to elucidate the mechanism of estradiol-induced anorexia. Previously, it was assumed that E_2 must act directly at estrogen receptors in MCH neurons to reduce expression of that peptide, thereby inducing aphagia [15]. Indeed, this model is parsimonious and has intuitive appeal. Our data suggest a more complex model,

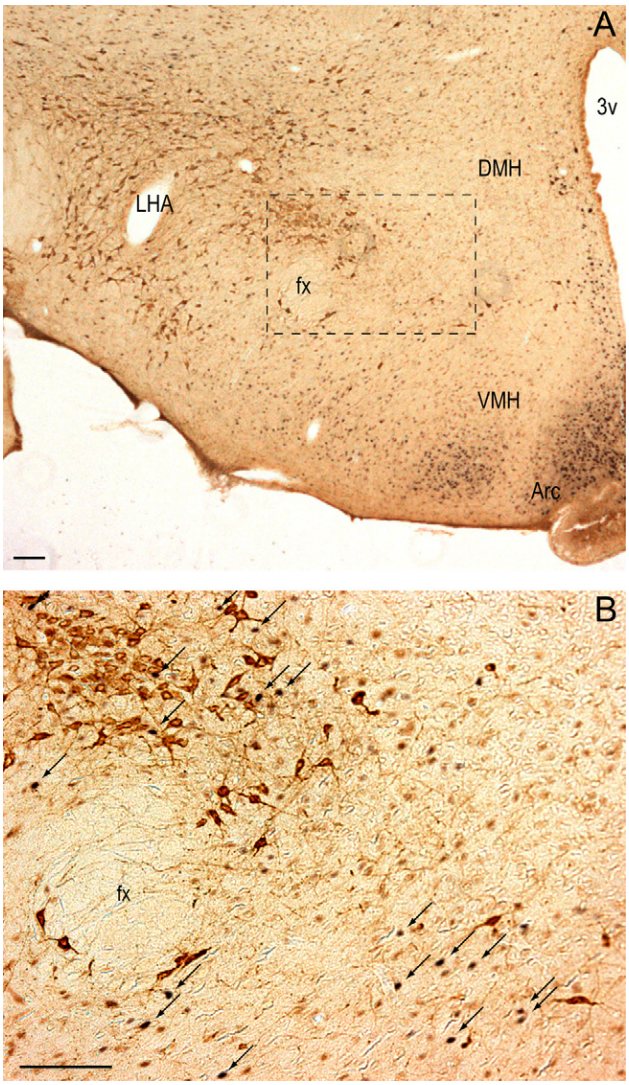

Fig. 1. (A) Micrograph of MCH and ERα immunopositive cells in hypothalamus 3.25 mm caudal to bregma. ERα-labeled nuclei are most apparent in arcuate nucleus and VMH. MCH-labeled neurons are most apparent in LHA. Dashed box indicates area of magnification. Scale bar = 100 μm. (B) Higher magnification detail of ERα-positive nuclei (arrows) and MCH-positive neurons (brown cytosolic label). Scale bar = 100 μm. Arc, arcuate nucleus; DMH, dorsomedial hypothalamic nucleus; fx, fornix; LHA, lateral hypothalamic area; 3v, third ventricle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

where E_2 may still act locally in the hypothalamus, particularly in those structures where ERα- and MCH-containing cells are found to mingle. Ultimately, regulation of MCH expression and appetite by ERα and its ligand E_2 appears to be indirect and not within MCH neurons themselves. Given their close proximity, it seems possible that local afferents from ERα-containing cells synapse on MCH neurons to affect their electrical properties and transcriptional activity. Additional anatomical work will be required to confirm this synaptic arrangement and to identify transmitters used to signal between ERα-expressing cells and neighboring MCH neurons. One might anticipate, however, that ERα-containing cells would convey a net-inhibitory signal on both domains of MCH neuronal function.

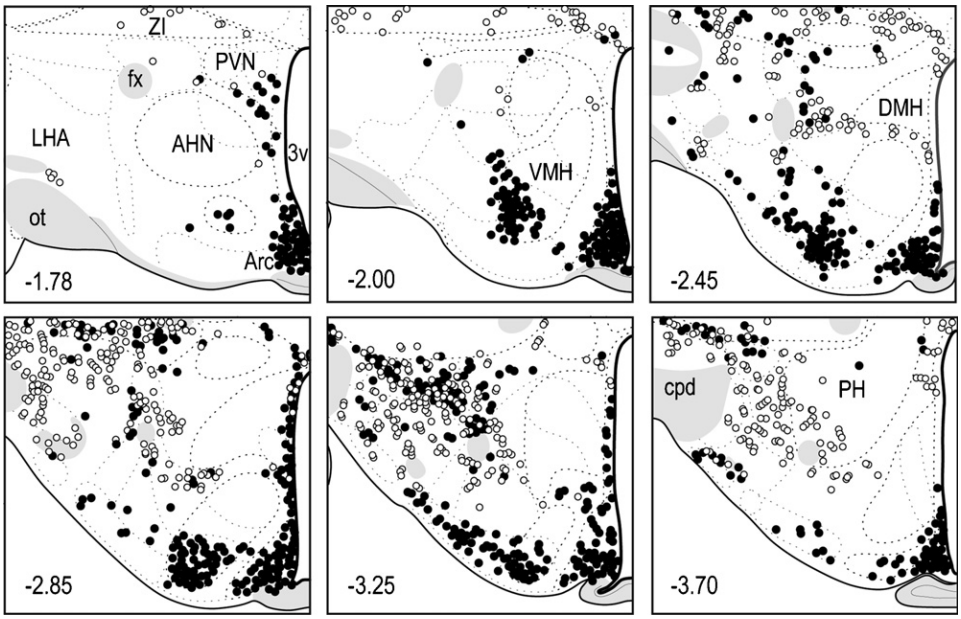

Fig. 2. Representative map of MCH- (open circles) and ERα- (closed circles) labeled cells in male rat hypothalamus. Numbers in lower left are mm caudal to bregma. AHN, anterior hypothalamic nucleus; cpd, cerebral peduncle; PH, posterior hypothalamic nucleus; PVN, paraventricular hypothalamic nucleus; ZI, zona incerta.

While these data suggest an indirect mechanism for the previously described regulation of appetite and *Mch* expression by E_2, we have not overlooked the possibility that more direct action of E_2 on MCH neurons may occur via novel, non-genomic means. In recent years, evidence has accumulated to support the existence of extra-nuclear, membrane-bound forms of estrogen receptors [12]. Such receptors can exert potent effects, not only on neuronal excitability, but also transcriptional activity [25]. Though rapid effects of E_2 have not been described in MCH neurons, and we report no extranuclear labeling by the ERα antibody we used, this mechanism cannot be ruled out.

In summary, our data suggest that the most likely substrates for the regulation of ingestive behavior by MCH and ERα are structures where those substances are found in close association (e.g. LHA). Additionally, estrogenic regulation of MCH-induced ingestive behavior appears to occur at least one synapse removed from MCH neurons themselves, as these neurons do not express ERα.

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