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Sex and estrogen receptor expression influence opioid peptide levels in the mouse hippocampal mossy fiber pathway

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Abstract

The opioid peptides, dynorphin (DYN) and enkephalin (L-ENK) are contained in the hippocampal mossy fiber pathway where they modulate synaptic plasticity. In rats, the levels of DYN and L-ENK immunoreactivity (-ir) are increased when estrogen levels are elevated (Torres-Reveron et al. 2008 and 2009). Here, we used quantitative immunocytochemistry to examine whether opioid levels are similarly regulated in wildtype (WT) mice over the estrous cycle, and how these compared to males. Moreover, using estrogen receptor (ER) alpha and beta knockout mice (AERKO and BERKO, respectively), the present study examined the role of ERs in rapid, membrane-initiated (6 hr), or slower, nucleus-initiated (48 hr) estradiol effects on mossy fiber opioid levels. Unlike rats, the levels of DYN and L-ENK-ir did not change over the estrous cycle. However, compared to males, females had higher levels of DYN-ir in CA3a and L-ENK-ir in CA3b. In WT and BERKO ovariectomized (OVX) mice, neither DYN- nor L-ENK-ir changed following 6 or 48 hrs estradiol benzoate (EB) administration. However, DYN-ir significantly increased 48 hours after EB in the dentate gyrus (DG) and CA3b of AERKO mice only. These findings suggest that cyclic hormone levels regulate neither DYN nor L-ENK levels in the mouse mossy fiber pathway as they do in the rat. This may be due to species-specific differences in the mossy fiber pathway. However, in the mouse, DYN levels are regulated by exogenous EB in the absence of ER α possibly via an ER β -mediated pathway requiring new gene transcription.

Keywords

opioids; dentate gyrus; CA3 region; synaptic plasticity

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INTRODUCTION

The mossy fiber pathway of the hippocampus is important for cognitive processes [26], including those associated with drug use [25], and its dysfunction has been implicated in epilepsy [27], Alzheimer's disease [16;19], and several psychiatric diseases [14]. Although opioid peptides are not constitutively released from the mossy fibers, endogenous opioid peptides are critical for hippocampal long term potentiation (LTP) and excitability [37]. Opioid peptide release following high frequency stimulation as induced by stress [2] or seizure [15] modulates cell excitability. In rats, both exogenous and endogenous estrogens regulate the levels of dynorphin (DYN) and leu-enkephalin (L-ENK) in the granule cell mossy fiber pathway [35;36]. Given that high estrogen levels are known to increase opioid-dependent hippocampal processes, understanding the interaction between estrogens and opioids would elucidate mechanisms underlying LTP, seizure susceptibility, and drug-seeking behavior [4;13].

Estrogen binds to two classical estrogen receptors (ERs), α and β , which signal through genomic and non-genomic mechanisms [31]. Studies in subtype selective ER knock-out mice have shown that both ERs are important in hippocampal-dependent learning [7;9;29]. Our electron microscopic studies have revealed that both ER α and ER β are primarily extranuclear in the hippocampus and their complementary distributions suggest that they have different roles modulating synaptic plasticity [20;21;24]. Indeed, we have recently shown temporally distinct ER α and ER β effects of estradiol on two molecular markers of synaptic plasticity, phosphorylated Akt and phosphorylated TrkB, in the mouse hippocampus [32]. However, it is not known whether ER α and ER β also differentially regulate opioid peptide levels in the mossy fiber pathway. Furthermore, it is unknown whether the cyclic changes in DYN and ENK observed in rats are maintained in mice.

Thus, the present study used quantitative densitometry first to examine sex and estrous cycle effects on the levels of DYN and L-ENK in the mouse hippocampus. Second, we used quantitative densitometry to determine the effects of estradiol benzoate (EB) injections on DYN and L-ENK levels in the mossy fiber pathway of the dorsal hippocampus from ovariectomized wild type (WT) and knockout mice missing either ER α (AERKO) or ER β (BERKO). Mice were analyzed 6 or 48 hours after EB since these two time points are consistent with relatively rapid, membrane initiated events, or slower, nucleus-initiated events requiring new gene transcription, respectively [32]. The results of this study not only provide new information regarding the effect of exogenous estrogen on levels of DYN and L-ENK in mice, but also elucidate a differential role for ER α and ER β in regulating hippocampal opioid levels in response to estrogens.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Tsukuba and the Rockefeller University and were in agreement with the NIH guide for the Care and Use of Laboratory Animals. Two groups of animals were used. Group 1: The WT C57Bl/6 male and cycling female mice (N = 24) used in this study were the same mice used in our recent studies [24;33]. Vaginal smear cytology was used to assess estrous cycle stage and females were assessed for at least 3 full weeks. Group 2: The WT (N=38; combined from both phenotypes), AERKO (N=23) and BERKO (N=25) female mice used in this study were the same animals used in our recent study [32]. Briefly, one week after their ovaries were removed under isoflurane anesthesia, the mice from each genotype were divided into four groups: 1) 6 hr EB – mice killed 6 hours after one injection EB (5 μ g in 100ul sesame oil, subcutaneous); 2) 6 hr oil – mice killed after one injection of

sesame oil (100 μ l); 3) 48 hr EB – two injections of EB were given 24 hr apart, and the mice were killed 24 hr after the second injection; and 4) 48 hr oil – two injections of oil were given 24 hr apart, and the mice were killed 24 hr after the second injection.

Tissue Preparation

Group 1: Mice were anesthetized with an overdose of sodium pentobarbital (150 mg/kg i.p.) and their brains perfused fixed with 2% paraformaldehyde and 3.75% acrolein in 0.1M sodium phosphate buffer (pH 7.4; PB) [24;33]. Coronal sections through the hippocampus were cut (40 μ m thick) on a Leica vibratome, placed into cryoprotectant [23] and stored at -20°C until use. Group 2: Mice were anesthetized with an overdose of sodium pentobarbital (150 mg/kg i.p.) and their brains perfused fixed with 4% paraformaldehyde in PB [32]. After sinking in 30% sucrose in PB, the brains were frozen (-80°C), and blocks containing the dorsal hippocampus were sliced (40 μ m thick) on a freezing microtome (Microm HM440E) into PB. Sections then were stored at -20°C in cryoprotectant solution [23] until use.

To ensure identical labeling conditions [28], sections from each animal were matched with regard to rostrocaudal level (approximate Bregma: -2.00 to -2.70) and marked with identifying hole punches; each set (Group 1: 1 section from proestrus, estrus, diestrus and male; Group 2: 1 section from WT, AERKO and BERKO) then was pooled into a single container. Tissue sets from each group were processed together through all immunocytochemical steps.

Antibodies

Serial dilution tests for each antibody established that the labeling density was linear. Final antibody dilutions produced slightly less than half-maximal labeling intensity to optimize the detection of intensity variations [5]. The guinea pig polyclonal antibody to dynorphin B was purchased from Peninsula Laboratories (Belmont, CA) and has been previously characterized for specificity using preadsorption [34]. The mouse monoclonal antibody L-ENK was purchased from Sera Labs (Crawley Down, UK) and has been previously characterized using immunoblots and preadsorption controls [6;22].

Immunocytochemistry

Sections were labeled for DYN and L-ENK using the immunoperoxidase method [23]. Sections (group 1 only) were incubated in 1% sodium borohydride in PB and rinsed in PB until the gaseous bubbles disappeared. Sections were washed in Tris-Saline (TS; pH 7.6), blocked in 0.5% bovine serum albumin (BSA) in TS, and incubated in primary antiserum to either DYN (Group 1: 1:20,000; Group 2: 1:4000) or L-ENK (1:1000, both groups) diluted in 0.25% Triton-X in 0.1% BSA/TS for 24 hrs at room temperature followed by 2 days at 4°C . Sections then were incubated in either biotinylated anti-guinea pig immunoglobulin (IgG) or biotinylated horse anti-mouse IgG for 30 min in 0.1% BSA/TS (1:400; Jackson ImmunoResearch, Westgrove, PA). With TS rinses in between each step, sections were incubated in avidin-biotin complex (ABC) for 30 min and then 3, 3'-diaminobenzidine with H_2O_2 (Sigma Aldrich, Milwaukee, WI) for 6 min. The tissue sections then were rinsed in PB, mounted on gelatin-coated slides, dehydrated and coverslipped in DPX (Sigma Aldrich).

Analysis

Details of the densitometric analysis have been described previously [11]. Briefly, an experimenter who was blind to the treatment group took photographs and collected through the regions of interest using a Dage MTI CCD-72 camera on a Nikon Eclipse 80i

microscope and converted to gray-scale values using MicroComputer Imaging Device (MCID) software. The mean gray value (of 256 levels) was selected using ImageJ64 (NIH) for each region of interest and the average pixel density from 3 regions lacking labeling was subtracted to control for variations in background staining. In group 1, one-way ANOVA was used to determine the effect of estrous status (proestrus, estrus, diestrus, and males) on L-ENK immunoreactivity (-ir) or DYN-ir. As there was no significant effect of estrous cycle stage, data from proestrus, estrus, and diestrus were pooled to compare females to males using the Student's t-test. In group 2, one-way ANOVA was used to determine the effect of estrogen treatment within each genotype, and in each region. AERKO-WT and BERKO-WT animals initially were compared to ensure no differences between WT mice of the different genotypes. As there were no differences by genotype, data were pooled and analyzed as WT. Significant differences were further analyzed by Tukey HSD post-hoc analysis ($p < 0.05$). Statistical analysis was performed using JMP 8 (SAS Institute Inc.) and graphs were prepared using GraphPad Prism6 (V6.0b, GraphPad Software Inc.).

RESULTS

In agreement with previous studies in mice and rats [10;36], DYN-ir was limited to the mossy fiber pathway in the dorsal hippocampus (Fig. 1A). At higher magnification, diffuse DYN-ir was concentrated in the hilus of the dentate gyrus (DG) and in stratum lucidum (slu) of CA3 (Fig. 1A). Like DYN, and similar to rats [35], L-ENK-ir was found in the mossy fiber pathway in the dorsal hippocampus (Fig. 2A). Levels of DYN-ir and L-ENK-ir were determined using quantitative densitometry of the mossy fiber of the hilus of the DG, and the CA3, which for analysis was divided into three subregions (Fig. 1a, 2a) reflecting neuroanatomical differences [35]. Notably, in rats, CA3a projects via the lateral septum to the ventral tegmental area [17] and contains high levels of pDOR [3], while in males CA3b is known to be particularly and preferentially affected by stress and hormonal status relative to other hippocampal subregions [18]. Neither DYN-ir nor L-ENK-ir differed significantly by hormonal status (proestrus, estrus, diestrus, or males). However, there was a significant effect of sex on DYN-ir in CA3a ($t(22) = -2.095$; $p = 0.0479$) with females having more DYN-ir than males (Fig. 1D). Similarly, L-ENK-ir levels in CA3b ($t(20) = -2.136$; $p = 0.0453$) were significantly greater in females than males (Fig. 2D).

To determine the role of ER α and ER β in regulating DYN and L-ENK levels in the mossy fiber pathway, we analyzed WT, AERKO, and BERKO ovariectomized mice that had been injected with either EB or oil, and were sacrificed either 6 or 48 hours post-injection. In WT and BERKO mice, neither 6 nor 48 hr EB administration resulted in any significant difference in density of DYN-ir ($p > 0.05$). In contrast, in AERKO mice there was a main effect of EB administration in CA3b ($F(3,10) = 3.9173$; $p = 0.0436$) and DG ($F(3,10) = 6.7385$; $p = 0.0091$) (Fig. 3). In CA3b, 48 hr EB group showed increased DYN-ir density relative to 48 hr and 6 hr oil, however, post-hoc analysis only revealed significance at the 6 hr time point ($p < 0.05$). In the DG, post-hoc tests showed that DYN-ir increased at the 48 hr EB time point relative to 6 hr EB, oil, and 48 hr oil ($p < 0.05$; Fig. 3C). Thus, AERKO mice showed higher levels of DYN-ir 48 hours after EB administration relative to oil administration at the 6 hr time point in CA3b, and relative to both oil time points and 6hr EB administration in the DG (Fig. 3). There was no significant effect ($p > 0.05$) of estradiol treatment on L-ENK-ir in any genotype.

DISCUSSION

This study demonstrates that estradiol's effect on the opioid system in the mouse hippocampal formation is unmasked after the removal of ER α . Despite no effect of the estrous cycle on levels of DYN or L-ENK-ir in WT, exogenous EB administration in

ovariectomized mice increased DYN-ir in the mouse mossy fiber pathway only in the absence of ER α . This regulation likely occurs through slower events requiring new gene transcription, as increased DYN-ir was only observed 48 hr after EB administration. This result is also consistent with recent findings that increased estradiol levels, in the absence of ER α , results in putative ER β -mediated transcription to maintain hippocampal function [12].

These findings agree with our electron microscopic studies in mice demonstrating that ER α and ER β have overlapping distribution in the hippocampal mossy fiber zone. Both ER α and ER β are found in mossy fiber axons, spines and dendrites, and are prominent on the membranes of pyramidal cells [24]. The effects of estrogen treatment on DYN responses at 48 hr, but not after 6 hr, are consistent with genomic regulation. Given the localization of ERs not only in the nucleus but also in other parts of both neuronal and glial cells, these effects may represent either direct genomic actions or indirect actions through signaling pathways that result in genomic regulation [31]. However, since the majority of ERs in the hippocampus are extranuclear [24], indirect genomic actions are more likely.

Previous studies in these AERKO and BERKO mice showed differential roles of ER α and ER β in modulating endpoints important for hippocampal synaptic plasticity at both rapid, membrane-initiated time points, and slower time points consistent with genomic regulation [32]. This work demonstrated that while both ER α and ER β are required to increase levels of phosphorylated AKT and phosphorylated TrkB, ER β mediates the increase in BDNF mRNA in response to EB. Our findings demonstrate yet another estrogen-responsive mechanism modulating hippocampal plasticity with differential dependence on ER α and ER β .

Although the mossy fiber opioid systems of mice and rats share much in common, this study found several differences from the rat literature. First, contrary to rats [35;36], we found no variation in DYN and L-ENK-ir across the estrous cycle. Second, although rats show increases in opioid levels 24 and 72 hours after estradiol administration [35;36], we found no increase in DYN-ir in WT mice 48 hours after EB. While there are similarities in the distribution of hippocampal ERs in the mouse and rat [24], subtle differences in the mossy fiber pathway may explain a putative species difference observed in this study. Given that we and others have shown that the absence of ER α unmasks a potential ER β -mediated genomic response to EB [12], the lack of ER β -ir in mossy fiber terminals in mice relative to rats may underlie the lack of 48 hr response to EB as well as the absence of estrous cycle dependent changes in DYN and L-ENK-ir in WT mice [20;24]. However, it should be noted that in addition to ER α and ER β , another ER, G-protein-coupled estrogen receptor 1 (GPER1), is also found in the mouse mossy fiber pathway (Waters, Akama, McEwen and Milner, unpublished). Moreover, post-synaptic density-95 (PSD-95) binding capacity of GPER1, can be identified in rat hippocampal dendritic spines [1]. The relative contribution of GPER1 to the observed effects on DYN and L-ENK is unknown at this time.

CONCLUSIONS

Together with our previous study [32], these results further elucidate mechanisms through which differential ER signaling contributes to synaptic plasticity in mice. These changes in plasticity may contribute to the effects of estrogens on cognition [8], epilepsy [30], and drug-seeking behaviors [4;25].

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ABBREVIATIONS

AERKO	estrogen receptor alpha knock out mice
BDNF	brain derived neurotrophic factor
BERKO	estrogen receptor beta knock out mice
BSA	bovine serum albumin
CA1	cornu ammonis 1 region
CA3	cornu ammonis 3 region
DG	dentate gyrus
DYN	dynorphin
EB	estradiol benzoate
ER	estrogen receptor
glc	granule cell layer
GP1B1	G-protein-coupled estrogen receptor 1
Hil	hilus of the dentate gyrus
IgG	immunoglobulin
ir	immunoreactivity
L-ENK	leu-enkephalin
LTP	long term potentiation
OVX	ovariectomy
PB	phosphate buffer
pcl	pyramidal cell layer
slu	stratum lucidum
sr	stratum radiatum
TS	tris-saline
WT	wild-type mice

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Highlights

- Unlike rats, mossy fiber DYN and L-ENK levels do not change over the estrous cycle.
- Relative to males, females have higher DYN and L-ENK levels in CA3.
- DYN regulation by estradiol is likely via nucleus-initiated, ER β -mediated pathway.

Fig. 1. The level of DYN-ir is greater in females in CA3a

In the hippocampal formation, DYN-ir is exclusively found in the DG and the CA3 region (A). Bar, 50 μ m. Representative examples of DYN-ir in the CA3a of male (B) and female (C) mice at higher magnification show diffuse DYN-ir in stratum lucidum (slu). Bar, 10 μ m. DYN-ir optical density is greater in females (proestrus, estrus, diestrus; N=6 per stage) than males (N=6) in CA3a (D). Dotted circles indicate where densitometry measurements were taken in each subregion. CA3a, b, c, cornu ammonis 3a; DG, dentate gyrus; sr, stratum radiatum; pcl, pyramidal cell layer.

Fig. 2. The level of L-ENK-ir is greater in females in CA3b

In the hippocampal formation, LENK-ir is exclusively found in the DG and CA3 region (A). Bar, 50 μ m. Representative examples of L-ENK-ir in the CA3b of male (B) and female (C) mice at higher magnification show diffuse LENK-ir in slu. Bar, 10 μ m. L-ENK-ir optical density is greater in females (proestrus, estrus, diestrus, N=5-6 per stage) than males (CA3a, N=4; CA3b,c, DG, N=6) in CA3b (D).

Fig. 3. DYN-ir in the mouse dorsal hippocampus is affected by genotype and estradiol
 Higher magnification of the DG shows diffuse DYN-ir in the hilus (hil) (A, B).
 Representative examples of DYN-ir in the DG of an oil (A) and a 48 hr EB administered (B) AERKO mouse. In AERKO mice, the level of DYN-ir was significantly elevated ($p < 0.05$) in the CA3b and DG after 48 hr estradiol (CA3a, DG, N=3; CA3b N=4) relative to 6 hr oil (CA3a,b N=4, DG N=3), and in the DG, elevation relative to 48 hr oil (CA3a,b N=3; DG N=5) and 6 hr estradiol mice (CA3a,b, DG N=3) was also significant (C). Bar, 20 μ m. gcl, granule cell layer.