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17 β -Estradiol Regulation of the mRNA Expression of T-type Calcium Channel subunits: Role of Estrogen Receptor α and Estrogen Receptor β

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Abstract

Low voltage-activated (T-type) calcium channels are responsible for burst firing and transmitter release in neurons and are important for exocytosis and hormone secretion in pituitary cells. T-type channels contain an $\alpha 1$ subunit, of which there are three subtypes, Cav3.1, 3.2 and 3.3, and each subtype has distinct kinetic characteristics. Although 17 β -estradiol modulates T-type calcium channel expression and function, little is known about the molecular mechanisms involved. Presently, we used real-time PCR quantification of RNA extracted from hypothalamic nuclei and pituitary in vehicle and E2-treated C57BL/6 mice to elucidate E2-mediated regulation of Cav3.1, 3.2 and 3.3 subunits. The three subunits were expressed in both the hypothalamus and the pituitary. E2 treatment increased the mRNA expression of Cav3.1 and 3.2, but not Cav3.3, in the medial preoptic area and the arcuate nucleus. In the pituitary, Cav3.1 was increased with E2-treatment and Cav3.2 and 3.3 were decreased. In order to examine whether the classical estrogen receptors (ERs) were involved in the regulation, we used ER α - and ER β -deficient C57BL/6 mice and explored the effects of E2 on T-type channel subtypes. Indeed, we found that the E2-induced increase in Cav3.1 in the hypothalamus was dependent on ER α , whereas the E2 effect on Cav3.2 was dependent on both ER α and ER β . However, the E2-induced effects in the pituitary were dependent on only the expression of ER α . The robust E2-regulation of the T-type calcium channels could be an important mechanism by which E2 increases the excitability of hypothalamic neurons and modulates pituitary secretion.

Keywords

Cav3.1; Cav3.2; Cav3.3; $\alpha 1$ subunits; hypothalamus; pituitary

Introduction

In the guinea pig 17 β -estradiol (E2) treatment increases the mRNA expression of T-type calcium channel subunit, Cav3.1 (α_{1G}), in hypothalamic nuclei, including the arcuate nucleus (Qiu et al., 2006a). Moreover, the increased Cav3.1 mRNA expression is associated with

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increased excitability in arcuate neurons (Qiu et al., 2006a). Increased excitability with E2 treatment has also been observed in ventromedial hypothalamic (VMH) neurons (Lee et al., 2008). Voltage-gated Ca^{2+} channels represent a heterogeneous family of calcium-selective channels that are critical for controlling calcium entry and neuronal firing in a number of brain regions including the hypothalamus (Erickson et al., 1993; Perez-Reyes et al., 1998; Niespodziany et al., 1999; Whitaker et al., 2000; McRory et al., 2001; Perez-Reyes, 2003; Catterall et al., 2003; Kato et al., 2003; van den Top et al., 2004). These different channels can be distinguished by their distinct molecular, electrophysiological and pharmacological characteristics. Among this family, the low voltage-activated (LVA) T-type for “transient” current, as opposed to L, for “Long-lasting” current, are thought to be involved in pacemaker activity, neuronal oscillations and resonance and rebound burst firing (Kim et al., 2001; Perez-Reyes, 2003; van den Top et al., 2004; Qiu et al., 2006a). The calcium channels are complex proteins composed of 4-5 distinct subunits, $\alpha 1$, $\alpha 2$, β , δ and γ (Catterall et al., 2003). However, the pharmacological and electrophysiological diversity of calcium channels are primarily due to the existence of multiple $\alpha 1$ subunits (Catterall et al., 2003). The $\alpha 1$ subunit is the largest and contains the conduction pore, as well as known binding sites for regulation by drugs and second messengers (Catterall et al., 2003; Catterall et al., 2005). T-type channels are shown to contribute to a transient inward current, and several subtypes of the T-type channel $\alpha 1$ subunit have been cloned (Cav 3.1, 3.2, 3.3) (Perez-Reyes et al., 1998; Lee et al., 1999). The mRNA and protein of the $\alpha 1$ subunit of the Cav 3.1 ($\alpha 1\text{G}$) channel are highly expressed in the hypothalamus (Craig et al., 1999; Talley et al., 1999; Qiu et al., 2006a). The Cav 3.2 ($\alpha 1\text{H}$) and Cav 3.3 ($\alpha 1\text{I}$) subtypes are also present but at much lower levels (Craig et al., 1999; Talley et al., 1999). T-type calcium channels are also expressed in pituitary cells including melanotropes, somatotropes, lactotropes and gonadotropes (Keja and Kits, 1994; Tomic et al., 1999; Mansvelder and Kits, 2000; Van Goor et al., 2001). Within the pituitary, these channels are critical for controlling calcium entry and hormone secretion (Van Goor et al., 2001; Stojkovic et al., 2005). However, in spite of the compelling evidence for a physiological role of T-type calcium channels in hypothalamic and pituitary functions, there have been few studies on the physiological regulation of these channels by steroid hormones (Qiu et al., 2006a; Lee et al., 2008). Therefore, we have used wildtype and transgenic mice to investigate the mRNA expression and E2 regulation of Cav3.1, Cav3.2 and Cav3.3 subtypes in the hypothalamus and pituitary. In particular, we explored E2 regulation of these channel subtypes in estrogen receptor α (ER α) and ER β knock out animals (αERKO and βERKO , respectively). Our findings suggest that mRNA expression of Cav3.1 and Cav3.2, but not Cav3.3 subunits are significantly increased in hypothalamic nuclei in E2-treated animals. In the pituitary, Cav3.1 is increased in response to E2-treatment, whereas Cav3.2 and 3.3 are decreased. The E2-dependent up-regulation of Cav3.1 and 3.2 in hypothalamic nuclei is dependent on ER α and ER α /ER β , respectively, whereas in the pituitary E2 regulation of Cav3.1, 3.2 and 3.3 are all dependent on ER α . Therefore, it appears that in the hypothalamus both ER α and ER β are needed for E2 regulation of Cav3 channel subtypes, whereas in the pituitary primarily ER α is involved.

Materials and Methods

Animal treatment and Experimental Procedures

Adult female mice (C57BL/6) were maintained under constant temperature and light (light on between 0600 h and 1800 h local time) with food and water provided *ad libitum*. Animal care and use were approved by an institutional committee and were according to NIH and International guidelines. The animals were ovariectomized under ketamine/xylazine anesthesia (10/2 mg/kg i.p.) and were implanted with silastic capsules containing oil or 6.2 μg 17 β -estradiol (E2) in oil. Six days later oil- and E2-implanted mice were injected, respectively, with oil or E2 (1-2 μg) and were euthanized 24h later at 0900-1000h. This E2 treatment paradigm was designed to induce a luteinizing hormone (LH) surge during the afternoon of day 7

(Bronson and Vom Saal, 1979). α ERKO mice (C57BL/6 from P. Chambon) were generated from breeding pairs heterozygous for the disrupted ER α gene, and offspring were screened by PCR amplification of tail DNA using primers as described previously (Dupont et al., 2000; Qiu et al., 2006b). β ERKO mice (C57BL/6 from K. S. Korach) were generated from breeding pairs heterozygous for the disrupted ER β gene as described previously (Krege et al., 1998). Offspring were screened with two sets of primers as described previously (Qiu et al., 2006b): the first primer pair (5'ACATCCATACACCCCCACTCAACC3' and 5'AAAGAAACATGTCCTGGCAAATCA3') produced a 600 bp product for the WT allele and no product for the KO allele. The second primer pair (5'TTCTGAGGGATCCGCTGTAAG3' and 5'AGGCTGCTGATCTCGTTCT 3') produced a 450 bp product for the KO allele and no product for the WT allele.

Tissue Preparation

A brain slicer (EM Corporation, Chestnut Hill, MA) was used to produce 1 mm frontal blocks, which were placed in RNAlater (Ambion, Austin, TX) for tissue RNA preservation. Care was taken to place each brain in the brain slicer in the same orientation, and to use the same slots for producing a preoptic -, anterior hypothalamic - and basal hypothalamic block (see Fig. 1). Each block was examined using a dissecting microscope, and the rostral and caudal border of each block were determined according to a mouse brain atlas (Franklin and Paxinos, 1997) and our own histological brain sections (see Fig. 1). Thereafter hypothalamic areas including the dorsal and ventral medial preoptic area (mPOAd and mPOAv, respectively), paraventricular nucleus (PVH), the dorsomedial and ventrolateral parts of the ventromedial nucleus (VMHdm and VMHvl, respectively), as well as dorsomedial (DMH) and arcuate nuclei were dissected using a dissecting microscope (Fig. 1). The pituitary was also harvested. Tissues were snap frozen and then stored at -80°C.

For illustration of the tissue blocks as well as the nuclear groups within these blocks the following was done: Photomicrographs were taken of the fresh 1 mm tissue blocks on a Nikon Eclipse TE200-S microscope (Figs 1A,B,C). The images were captured using the Nikon DS-Qi1Mc digital camera and Nikon NIS Elements AR 3.0 software and the contrast adjusted to bring out the different structures. Representative 15 μ m sections from the rostral and caudal parts of the 1 mm blocks in Figures 1A, B, and C were stained with cresyl violet (Figures a1, a2, b1, b2, c1, c2, respectively) and photographed using a Nikon Eclipse E800 microscope. Brightness and contrast of the photomicrographs were adjusted using Adobe Photoshop CS.

RNA Extraction and Reverse Transcription

Total RNA was extracted from hypothalamic nuclei and the pituitary under RNase-free conditions using the Ambion RNAqueous Micro kit (Ambion, Austin, TX) according to the manufacturer's protocol and quantified using the NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA was DNase-1 treated (DNasefree, Ambion) at 37° C for 30 min to minimize genomic DNA contamination. cDNA was synthesized from 200 ng of total RNA using 50 U murine leukemia virus reverse transcriptase (Applied Biosystems Inc. (ABI), Foster City, CA), 4 μ l 5 $\times$ Go Taq Flexi Buffer (Promega), 5 mM MgCl₂, 0.625 mM dNTP, 100 ng random hexamer primers (Promega, Madison, WI) 15 U Rnasin (Promega) and 10 mM dithiothreitol (DTT) in diethyl pyrocarbonate (DEPC)-treated water (Ambion) in total volume of 20 μ l. Reverse transcription was conducted using the following protocol: 42° C for 60 min, 99° C for 5 min and 4° C for 5 min. The cDNA was diluted to 1:20 with nuclease-free water (Ambion) for a final estimated cDNA concentration of 0.5 ng/ μ l and stored at -20° C. Basal hypothalamic test tissue RNA was used for positive and negative controls (no reverse transcriptase) and processed simultaneously with the experimental samples.

Real-time PCR Quantification Assays

Quantitative real-time PCR analysis was performed on the ABI 7500 Fast System using Taqman universal PCR master mix according to manufacturer's specifications. Taqman gene expression assays containing primers and probes for mouse Cav3.1 ($\alpha 1G$) (assay ID- mM00486549_m1) and mCav3.2 ($\alpha 1H$) (assay ID- mM00445369_m1) subunits and mouse β -actin (assay ID-4352341E) were prepared by ABI. The target gene-specific probes were labeled using the reporter dye FAM, and the β -actin internal control probe was labeled with reporter dye VIC. Multiplex PCR was performed with Cav3.1 or Cav3.2 and β -actin primers and probes using two-step PCR protocol: 95° C for 10 min (initial denaturing) followed by 40 cycles of amplification at 94° C for 15 sec (denaturing), 60° C for 1 min (annealing). All samples were run in triplicate, and amplification data were analyzed with ABI 7500 v1.3.0 software. For the mCav3.3 ($\alpha 1I$) subunit, qPCR was performed using Power SybrGreen PCR mastermix (ABI) on the 7500 Fast System. At the time when this study was initiated, a Taqman gene expression assay for this subunit was not available. Therefore, primers for Cav 3.1 and Cav 3.2 were also designed for SybrGreen qPCR to confirm the results of the Taqman gene expression assays and to compare the relative amounts of the three transcripts. Primer pairs were designed to cross at least one intron-exon boundary using Primer Express version 3.0 (ABI) or Clone Manager (Scientific and Educational Software, Cary, NC). The primers were as follows: mCav 3.1 (144 nt product, accession number NM_009783, forward primer 2935-2954 nt, reverse primer 3059-3078 nt); mCav3.2 (84 nt product, accession number NM_021415, forward primer 2709-2728 nt, reverse primer 2773-2709 nt); mCav3.3 (129 nt product, accession number NM_001044308, forward primer 965-983 nt, reverse primer 1076-1093 nt; beta actin (63 nt product) accession number NM_007393; forward primer 849-867 nt, reverse primer 890- 911 nt. The qPCR reaction contained 10 μ l 2 $\times$ mastermix, 0.5 μ M forward and reverse primers, 3 μ l cDNA and nuclease-free water to a 20 μ l final volume. Real-time qPCR was performed on samples in triplicate under the following conditions: Initial denaturing at 95° C for 10 min followed by 40 cycles of amplification at 94° C for 15 sec (denaturing), 60° C for 1 min (annealing), and completed with a dissociation step for melting point analysis with 35 cycles of 95° C for 15 sec, 60° C to 95° C (in increments of 1° C) for 1 min and 95° C for 15 sec.

Primer Efficiency

Standard curves for Cav3.1, Cav 3.2, Cav3.3 and β -actin were prepared using serial dilutions of basal hypothalamus cDNA in triplicate to determine the percent efficiency [$E = 10^{(-1/m)}$ -1; m =slope] of each amplification (Pfaffl, 2001) (Fig. 2). For the SybrGreen assay, the standard curves produced similar high efficiencies (Cav 3.1, 100%; Cav 3.2, 100%; Cav 3.3, 96%; β -actin, 100%) and high linearity (Pearson correlation coefficient $r^2 = 0.97$ for Cav 3.1; 0.98 for Cav 3.2; 0.99 for Cav 3.3 and 0.99 for β -actin) (Fig. 2). For the Taqman assay, the linearity correlation coefficient, r^2 , was 0.99 for Cav3.1, Cav3.2 and beta actin, and the efficiencies were 95% for Cav3.1, 91% for Cav3.2 and 93% for beta actin. Therefore, the amplification efficiencies for the target and reference genes in both assays were within the range (90%-100%) needed for utilization of the $\Delta\Delta C_T$ method for quantification purposes (Livak and Schmittgen, 2001; Pfaffl, 2001).

Real-time PCR Analysis

Relative quantification analysis was done using the comparative $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). The data were expressed as an n-fold change in gene expression normalized to a reference gene and relative to a calibrator sample. The reference gene β -actin was used to normalize the target genes for the amount of total RNA in the reverse transcription reaction and was found to be unresponsive to the E2-treatment. Two analyses are presented here using the $\Delta\Delta C_T$ method; one comparing different quantities of Cav3.1, Cav3.2

and Cav3.3 across different brain regions and the other comparing differences in oil-treatment versus estrogen-treatment in individual tissues. Hippocampal cDNA was used as an external calibrator when comparing the relative amounts of Cav3.1, Cav3.2 and Cav3.3 across different brain regions because it had a similar expression level in all three transcripts. The mean ΔC_T of the oil-treated samples was used as an internal calibrator when comparing oil-treatment versus E2-treatment. To determine the C_T (cycle threshold) for each transcript, the threshold was set at the lowest point of the exponential curve where the slope of the curve was the steepest and above the baseline of the first 15 cycles (horizontal arrows, Fig. 2). The $\Delta\Delta C_T$ method normalized the C_T from each sample for each target gene by subtracting the C_T of the reference gene (β -actin) (ΔC_T). The $\Delta\Delta C_T$ values were calculated using the hippocampus ΔC_T or the mean ΔC_T of the oil-treated samples as the calibrator ΔC_T ($\Delta\Delta C_T = (C_T \text{ target gene} - C_T \text{ reference gene}) - C_T \text{ of calibrator}$). The relative linear quantity of the target gene was calculated using the formula $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001). Therefore, all data are expressed as an n-fold change in gene expression relative to the calibrator. The data are reported as relative mRNA expression. For the distribution analysis, the n-fold difference was averaged for each individual tissue and was analyzed using a one-way ANOVA with a Neuman Keuls post hoc test. For analysis of E2-induced effects, the mean $\pm$ SEM of the n-fold difference was determined for oil-treated and E2-treated tissues and each tissue analyzed statistically using a two-tailed Student's t-test ($p < 0.05$ was considered significant).

Estrogen response elements

The mouse Cav3.1, 3.2 and 3.3 genes were analyzed to look for estrogen response elements (EREs) using the Alibaba2.1 program (Grabe, 2002). This program accesses the TRANSFAC database (BioBase, Germany) of known transcription elements to create individual matrices for each analyzed sequence. The consensus ERE is 5'-GGTCAnnnTGACC-3', where n can be any nucleotide. We examined 4 kilobases (kb), 3 kb of untranscribed and 1 kb of the transcribed sequences, flanking the transcriptional start sites of the mouse Cav3 genes for the presence of classical EREs.

Results

Effects of 17 β -estradiol on the uterine weights

At the time of harvesting the brains for mRNA analysis, the uteri from WT, α ERKO and β ERKO animals were dissected and weighed. As expected, the uterine weights were greatly increased ($p < 0.001$) in E2-treated ($n=6$) versus oil-treated ($n=6$) WT animals (Fig. 3). The uterine weights were also significantly increased in E2 treated ($n=6$) versus oil treated ($n=5$) β ERKO females ($p < 0.001$), but significantly decreased in E2-treated ($n=5$) as compared to oil-treated ($n=6$) α ERKO animals (4.6 ± 0.7 mg and 8.6 ± 0.6 mg, respectively; $p < 0.01$) (Fig. 3). These findings support previous work that the stimulatory actions of E2 on the uterus are primarily via ER α (Couse and Korach, 1999; Dupont et al., 2000).

Real time PCR assay evaluation

Our quantitative analysis of T-type calcium channel Cav3.1 and 3.2 subtypes were obtained using either the Taqman or the Sybr Green assay procedure (see Fig. 2), and the Cav3.3 subtype was analyzed using the Sybr Green assay since the Taqman assay for this transcript was not available when these experiments were initiated. Therefore, we compared Taqman versus Sybr Green assays for Cav3.1 and 3.2 mRNA analysis in both the brain and pituitary, and obtained for the most part similar results with both assays (data not shown). We confirmed, however, our previous findings (Zhang et al., 2007) that the Sybr Green assay was more sensitive for measuring low levels of transcripts, potentially because of the greater efficiency of the primers: 100% for Cav3.1, 3.2 and beta-actin in the Sybr Green assay versus 95, 91 and 93%, respectively, in the Taqman assay.

Cav 3.1, 3.2 and 3.3 subtype mRNA distribution in brain and pituitary

Using quantitative real-time PCR (qPCR; Fig.2) we found that Cav3.1, 3.2 and 3.3 mRNA subtypes were all expressed in the brain and the pituitary in ovariectomized, oil-treated animals (Figs. 4, 5). For Cav3.1, the highest mRNA expression was observed in the ventral and dorsal parts of the medial preoptic area (mPOAv and mPOAd, respectively), followed by the dorsomedial part of the VMH (VMHdm) (Fig. 4A). Cav3.1 mRNA levels were the lowest in the arcuate nucleus and the ventrolateral part of the VMH (VMHvl). The mRNA expression of Cav3.2 in ovariectomized, oil-treated animals was the highest in the mPOAd and the lowest in the mPOAv (Fig. 4B). Moderate, but equal expression levels of Cav3.2 were found in the arcuate nucleus, and the two components of the VMH (Fig. 4B). Cav3.3 mRNA expression was the highest in the mPOAd and VMHdm, with lower levels of mRNA in the mPOAv and VMHvl (Fig. 4C). The lowest expression level of Cav3.3 mRNA was observed in the arcuate nucleus (Fig. 4C).

In the pituitary from ovariectomized, oil-treated females, the mRNA expression of Cav3.3 was the most abundant, followed by moderate levels of Cav3.2 and the lowest expression of Cav3.1 mRNA (Fig. 5).

17 β -Estradiol regulation of Cav 3.1, 3.2 and 3.3 subtypes

Based on our findings previously that E2 treatment increases the mRNA expression of Cav3.1 in the mPOA and arcuate nucleus in guinea pig (Qiu et al., 2006a), we measured Cav3.1 mRNA levels and compared it to the expression of Cav3.2 and 3.3 in hypothalamic nuclei in ovariectomized oil- and E2-treated mice. As illustrated in Fig. 6, we found that the mRNA expression of both Cav3.1 and Cav3.2, but not Cav3.3, was significantly increased in hypothalamic nuclei in E2-treated animals. Specifically, Cav3.1 mRNA levels were significantly increased in the arcuate nucleus with E2- as compared to oil-exposure ($p < 0.001$), and less, but still significantly up-regulated in the mPOAv ($p < 0.02$) and VMHvl ($p < 0.05$) (Fig. 6A). E2 treatment did not increase the Cav3.1 mRNA expression in mPOAd, PVH, VMHdm and the dorsomedial nucleus (DMH) (Fig. 6A). E2 treatment significantly up-regulated ($p < 0.003$) Cav3.2 mRNA expression in the mPOAv and also significantly increased this expression in the arcuate nucleus ($p < 0.02$) (Fig. 6B). There were no significant changes of Cav3.2 mRNA expression in the other hypothalamic areas (Fig. 6B). In contrast to Cav3.1 and 3.2, Cav3.3 mRNA levels were not significantly altered by E2 treatment in any of the hypothalamic regions that were analyzed (Fig. 6C).

17 β -Estradiol regulation of Cav 3.1, 3.2 and 3.3 subtypes in ERKO animals

In order to begin to elucidate the mechanism by which E2 alters the mRNA expression of T-type calcium channel genes, we treated ovariectomized wild-type, α ERKO and β ERKO females with E2 and measured the mRNA expression of Cav3.1, 3.2 and 3.3 subtypes in the mPOAv, arcuate nucleus and the pituitary (Figs. 7, 8, 9). In the mPOAv, E2 treatment increased the mRNA expression of Cav3.1 in WT ($p < 0.02$) and β ERKO ($p < 0.05$) animals, but did not alter its expression in α ERKO animals (Fig. 7A). Also, E2 treatment up-regulated Cav3.2 mRNA expression in the mPOAv in WT ($p < 0.003$), but this increase was lost in both α ERKO and β ERKO animals (Fig. 7B). The steroid treatment did not alter Cav3.3 mRNA expression in the mPOAv in any of the experimental groups (Fig. 7C).

In the arcuate nucleus, estrogen treatment increased Cav3.1 mRNA levels in WT ($p < 0.001$) and β ERKO ($p < 0.01$) females, but not in the α ERKO female mice (Fig. 8A). In contrast, E2 treatment up-regulated Cav3.2 mRNA expression in WT mice only ($p < 0.02$), and not in similarly treated α ERKO or β ERKO animals (Fig. 8B). As in the POA, E2 exposure did not alter Cav3.3 mRNA expression in the arcuate nucleus in any of the three groups of animals (Fig. 8C). This would suggest that in the hypothalamus, E2-regulation of Cav3.1 mRNA

expression is primarily through an ER α -dependent mechanism, whereas E2-regulation of Cav3.2 is dependent on both ER α and ER β .

We also explored E2 regulation of T-type calcium channel subtypes in the pituitary from WT, α ERKO and β ERKO animals. These studies revealed that E2-treatment as compared to oil-treatment increased many fold the pituitary mRNA expression of Cav3.1 in WT ($p < 0.001$) and β ERKO ($p < 0.001$) animals, but E2 had no effect in α ERKO animals (Fig. 9A). In contrast to Cav3.1, both Cav3.2 and 3.3 mRNA expression was decreased in the pituitary in E2-treated WT animals (Fig. 9B,C). The E2-induced decrease in Cav3.2 and 3.3 mRNA expression that was observed in WT ($p < 0.02$ and 0.001 , respectively) was also observed in β ERKO animals ($p < 0.02$ and 0.001 , respectively). As with Cav3.1, the effects of E2 on Cav3.2 and 3.3 were completely lost in α ERKO animals (Fig. 9), suggesting that in the pituitary only ER α is necessary for E2's action on T-type calcium channel subunits.

Estrogen Response Elements of Cav3.1, 3.2 and 3.3 genes

Using the transcription factor binding site prediction program Alibaba2.1 (Grabe, 2002), we searched for estrogen response elements (EREs) in 4 kb of mouse Cav3.1, 3.2 and 3.3 sequences. The program predicted 3 EREs in the 5' untranslated region and 1 ERE in the 5' translated region of the Cav3.1 gene, as well as 5 activator protein-1 (AP-1) and many more specificity protein-1 (Sp1) transcription sites. There was only one ERE, but 7 AP-1 and many Sp1 sites in the upstream region of the Cav3.2 gene. The program predicted three EREs in the 5' untranslated region and one ERE in the 5' transcribed region of the Cav3.3 gene, as well as 7 AP-1 and numerous Sp1 sites. Therefore, predictive findings suggest that the Cav3 genes all express EREs and associated Sp1 and AP-1 transcription sites.

Discussion

Using quantitative real-time PCR, we have found that the T-type calcium channel α 1 subunits, Cav3.1 (α 1G), 3.2 (α 1H) and 3.3 (α 1I), are differentially expressed in hypothalamic nuclei and in the pituitary. Moreover, in the hypothalamus, E2 increases the mRNA expression of the Cav3.1 and 3.2 subunits in an ER α - or ER α /ER β dependent manner, respectively. Interestingly, in the pituitary, Cav3.1 is increased with E2-treatment, whereas Cav3.2 and 3.3 are both decreased, and these E2-induced effects are lost in α ERKO, but not β ERKO animals. Therefore, the hypothalamic actions of E2 on Cav3 gene expression are both ER α and ER β -dependent, whereas the actions of E2 on the Cav3 genes in the pituitary appear to be dependent on ER α only.

According to our present quantitative analysis of T-type calcium channel subunit expression in the mouse preoptic area and basal hypothalamus, Cav3.1 mRNA is the most abundant within the POA as compared to Cav3.2 and 3.3 subunits. In contrast, Cav3.3 mRNA is the most abundant in the VMH, whereas in the arcuate nucleus the three T-type calcium channel subunits are about equally expressed. Previous publications based primarily on *in situ* hybridization analysis of Cav3.1 mRNA distribution in the male rat and female guinea pig, have illustrated that Cav3.1 is highly expressed in hypothalamic nuclei (Craig et al., 1999; Soong et al., 1993; Kase et al., 1999; Talley et al., 1999; Qiu et al., 2006a). In comparison, Cav3.2 and 3.3 are only slightly expressed (Talley et al., 1999). The different results are most likely because of the different animal models and different methods used.

Although anatomical mRNA and protein distribution studies are limited, there is overwhelming physiological evidence for the expression of T-type calcium currents in the hypothalamus (Poulain and Crette, 1987; Erickson et al., 1993; Kelly and Rønnekleiv, 1994; Sundgren-Andersson and Johansson, 1998; Niespodziany et al., 1999; Kato et al., 2003; van den Top et al., 2004; Brown et al., 2004; Nahm et al., 2005; Qiu et al., 2006a; Lee et al., 2008). All of

these studies support the idea that T-type channels are critical for neuronal excitability. In particular, these channels can generate burst firing through calcium currents that are activated at more hyperpolarized potentials than the normal resting potential (Huguenard, 1998; Kim et al., 2001; Qiu et al., 2006a). This important characteristic ensures that hyperpolarizing inputs, which would otherwise be inhibitory, can lead to rebound excitation and neuronal burst firing (Huguenard and McCormick, 1992; Erickson et al., 1993; Huguenard, 1998; Kelly and Wagner, 2002).

The kinetics of Cav3.1, 3.2 and 3.3 are strikingly different when expressed in cell lines (Klockner et al., 1999; Kozlov et al., 1999). In particular, the currents induced by the expression of Cav3.3 channels activate and inactivate with much slower kinetics as compared to the other subunits (McRory et al., 2001). More recently it has been documented in cerebellar neurons expressing either Cav3.1 or 3.3 subtypes that robust bursting activity is associated with Cav3.1, whereas weak bursting activity is associated with Cav3.3 expression (Molineux et al., 2006). These data are consistent with the observation that rebound excitation and burst firing are lost in thalamocortical relay neurons from Cav3.1 deficient animals (Kim et al., 2001). However, the specific physiological role of the different T-type calcium channel $\alpha 1$ subunits that are expressed in the hypothalamus is currently not known.

Similar to what we reported in the guinea pig (Qiu et al., 2006a), the expression of Cav3.1 is significantly increased in the E2-treated mouse hypothalamus as compared to vehicle controls. The E2 effects were found specifically in the ventral POA, arcuate nucleus and ventrolateral VMH. Interestingly, Cav3.2 mRNA expression, but not that of Cav3.3, was also positively regulated by E2 in these same brain regions with the exception of the VMHv1. The E2-induced augmentation of mRNA expression of T-type calcium channel subtypes leads to increased excitability (rebound excitation) in arcuate and VMH neurons (Qiu et al., 2006a; Lee et al., 2008). These findings are important for they explain a mechanism by which E2 increases hypothalamic neuronal excitability and cellular function through increased mRNA expression. For example, POMC neurons within the arcuate nucleus, which are crucial for maintaining energy homeostasis, express functional T-type calcium channels and exhibit increased excitability in E2-treated animals (Qiu et al., 2006a). Since increased POMC tone leads to reduced appetite (Balthasar et al., 2004; Gao et al., 2006), this may explain in part the effectiveness of estrogen in attenuating body weight gain in ovariectomized animals (Butera and Czaja, 1984; Asarian and Geary, 2002; Qiu et al., 2006b; Qiu et al., 2007).

T-type calcium channels may also play a significant role in generating membrane oscillations underlying pacemaker activity in NPY/AgRP neurons within the hypothalamic arcuate nucleus (van den Top et al., 2004). It is well known that these neurons are important for maintaining energy homeostasis and increased activity would lead to increased food consumption (Smith and Grove, 2002; Gropp et al., 2005). However, the specific role of E2 in regulating T-type calcium currents in NPY/AgRP neurons is currently not known. The ventromedial nucleus of the hypothalamus is another area important for maintaining energy homeostasis. In this study we have documented E2-mediated increase in T-type Cav3.1 mRNA expression in the lateral part of the VMH. These findings are consistent with recently published data that E2-treatment increases low voltage activated (T-type) calcium currents, but not high voltage activated currents in this hypothalamic nucleus (Lee et al., 2008). The VMH is important for sexual receptivity as well as the maintenance of normal body weight in part through an ER α -dependent mechanism (Musatov et al., 2007).

We have found that T-type calcium channel subtypes are highly expressed in the mPOA and the mRNA expression is regulated by E2 also in this brain region confirming previous findings in the guinea pig (Qiu et al., 2006a). Moreover, whole cell and sharp electrode intracellular recordings have documented the presence of T-type calcium channels in mPOA neurons

(Sundgren-Andersson and Johansson, 1998; Wagner et al., 2000). Since these channels play a critical role in sculpturing burst firing activity (Erickson et al., 1993; Sundgren-Andersson and Johansson, 1998; Huguenard, 1998), it will be important to further elucidate the specific role of T-type channels in POA neurons. It is well documented that the mPOA and the adjacent supraoptic nucleus control a number of important homeostatic functions including reproduction, water balance and temperature regulation, and E2 is an important modulator of many of these functions (Rapkin, 2006; Wintermantel et al., 2006; Wechselberger et al., 2006; Kauffman et al., 2007; Sladek and Somponpun, 2008).

We searched for EREs in the Cav3 genes using Alibaba2.1 program (Grabe, 2002) because the areas that exhibited increased mRNA expression of Cav3.1 and 3.2 after E2 treatment coincided with the previously reported distribution of ER α and ER β (Mitra et al., 2003; Merchenthaler et al., 2004). We found putative ERE sites in the mouse Cav3 genes, which argues for an ERE-dependent effect. Subsequently, we explored the effects of E2 in ER α or ER β knockout animals. Indeed, we found that the E2-induced increased expression of Cav3.1 in the mPOA and arcuate nucleus was lost in α ERKO animals, suggesting that this effect of E2 was primarily through ER α . We also found that the E2-induced up-regulation of Cav 3.2 mRNA expression in the mPOA and the arcuate nucleus was abrogated in both α ERKO and β ERKO animals, suggesting that this effect of E2 was through both receptors. Interestingly, animals treated with the mER ligand, STX (Qiu et al., 2006b), also exhibit increased Cav3.1 mRNA expression in the arcuate nucleus (Roepke et al., 2008). These findings suggest that estrogen may signal not only through ER α and ER β , but also through a membrane-initiated signaling pathway (mER) to increase T-type calcium channel expression and excitability of hypothalamic neurons.

In the pituitary, we found the expression of all three Cav3 genes although Cav3.3 was the most abundant, at least in ovariectomized animals. With E2 treatment the mRNA expression of Cav3.1 increased many fold, whereas that of Cav3.2 and 3.3 decreased. We reported previously in guinea pig that E2 increases Cav3.1 mRNA expression in the pituitary (Qiu et al., 2006a). To our knowledge, the effects of E2 on Cav3.2 and 3.3 T-type calcium channel subunit expression in the pituitary has not been reported. Therefore, the mechanism by which E2 induces these differential responses on Cav3 subtype expression, is currently unknown but may be related to the different cell types. It has, however, been reported that E2 inhibits T-type calcium current in spermatogenic cells, which also expresses all of the T-type channel subtypes (Espinosa et al., 2000; Treviño et al., 2004). Again, the mechanism of action of estrogen is unknown.

It is well documented that low-voltage activated (T-type) channels are involved in triggering exocytosis in pituitary cells (Keja and Kits, 1994; Tomic et al., 1999; Mansvelder and Kits, 2000; Stojkovic et al., 2005). In particular, T-type calcium channels play an important role in initiating the rising phase of the action potential to stimulate exocytosis (Mansvelder and Kits, 2000). The T-type calcium channels are expressed in melanotropes, gonadotropes, lactotropes and somatotropes, and most of these pituitary cells also express ER α (Couse et al., 1997; Mitchner et al., 1998; Shen et al., 1999; Mansvelder and Kits, 2000; Van Goor et al., 2001). Therefore, estrogen through ER α may regulate Cav 3 expression in most, if not all of the different pituitary cells. Our GnRH/LH surge-inducing E2-treatment paradigm would prime the pituitary for increased LH and prolactin secretion. However, further studies are needed to determine the differential role of Cav3.1, 3.2 and 3.3 in the pituitary, as well as cell-specific effects of E2.

In summary, the present study shows that Cav3.1, 3.2 and 3.3 are differentially expressed in the hypothalamus and pituitary. In the hypothalamus, E2-treatment leads to increased mRNA expression of Cav3.1 in an ER α -dependent manner, whereas the E2-induced increase in Cav3.2 is dependent on both ER α and ER β . In the pituitary E2-treatment increases Cav3.1, but

decreases Cav3.2 and 3.3 in an ER α -dependent manner. Therefore, the robust E2-regulation of the T-type calcium channel mRNA expression could be an important mechanism by which E2 increases the excitability of hypothalamic neurons and modulates pituitary secretion.

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Figure 1.

Preoptic and basal hypothalamic tissue microdissections. Tissues were dissected from female mouse coronal blocks (1 mm) as indicated by the stippled outlines (A-C). The regions dissected included the ventral and dorsal parts of the medial preoptic area (mPOAv and mPOAd, respectively), the paraventricular nucleus (PVH), the arcuate nucleus (Arc), dorsomedial and ventrolateral parts of the ventromedial nucleus (VMHdm and VMHvl, respectively) and the dorsomedial nucleus of the hypothalamus (DMH). The nuclear groups within the rostral (a1, b1, c1) and caudal (a2, b2, c2) regions of each hypothalamic block are illustrated in cresyl violet-stained brain sections (15 μ m) from wildtype C57 mice. The dissected areas are indicated by the stippled lines in a-c. Abbreviations: ac, anterior commissure; fx, fornix; oc, optic chiasm; opt, optic tract. Scale bars = 500 μ m.

Figure 2.

Primer efficiencies and quantitative real-time PCR (qRT-PCR) assays for Cav3.1 ($\alpha 1G$), 3.2 ($\alpha 1H$) and 3.3 ($\alpha 1I$) transcripts using the Sybr Green method. **A, B, C**, Standard curves were prepared using cDNA serial dilutions as follows: 1:5, 1:10, 1:50, 1:100, 1:500, 1:1000. Cycle number was plotted against the normalized fluorescence intensity (Delta Rn) to visualize the PCR amplification. The cycle threshold (CT; arrow) is the point in the amplification at which the sample values were calculated. **D, E, F**, Representative dissociation (melting) curves, showing single-product melting at 80° C for Cav3.1, 79° C for Cav3.2 and 82° C for Cav3.3. Single peaks illustrate that only one product was formed and the lack of any other peaks illustrates that the primers did not form primer dimers. **Insets** in A, B and C show standard

curve regression lines and slope using serial dilution data. The primer efficiencies calculated from the slopes were 100% for Cav3.1 and Cav3.2 and 96% for Cav3.3. Beta-actin was used as control and also exhibited 100% efficiency (data not shown). These efficiencies allowed us to use the $\Delta\Delta CT$ method for quantification purposes.

Figure 3.

The E2 treatment increased the uterine weights in ovariectomized WT and βERKO females, but decreased the uterine weights in αERKO females. After the treatment period, the uteri of the different groups of mice were harvested and examined. Note that there was a significant increase in uterine size after E2 in WT and βERKO mice. In contrast, the uterine size was significantly decreased in αERKO females. Bar graphs represent mean ± SEM of uterine weights. ** $p < 0.01$; *** $p < 0.001$; $n = 5-6$ mice/group.

Figure 4.

Cav3.1, 3.2 and 3.3 mRNA expression levels in different hypothalamic areas. Relative mRNA abundance of Cav3.1 (**A**), Cav3.2 (**B**) and Cav3.3 (**C**) in ovariectomized mouse hypothalamus using real-time PCR. Values represent the relative abundance of each transcript in microdissected hypothalamic nuclei (n=5-6). For each T-type calcium channel subtype, relative abundance was quantified using the $\Delta\Delta\text{CT}$ method and normalized to each transcript value in the hippocampus as this structure had about equal levels of Cav3.1, 3.2 and 3.3 mRNAs. Values represent mean $\pm$ SEM, and bars with equal letters are significantly different. In **A**, a and b represent $p < 0.001$; In **B**, a represents $p < 0.01 - 0.001$, b and c represent $p < 0.05 - 0.001$; In **C**, a represents $p < 0.01 - 0.001$, b represents $p < 0.001$, c represents $p < 0.01$.

Figure 5.

Cav3.1, 3.2 and 3.3 mRNA expression levels in the pituitary. Relative abundance of Cav3.1, 3.2 and 3.3 mRNA in the pituitary from ovariectomized mice using real-time PCR. For each T-type calcium channel subtype, relative abundance was quantified using the $\Delta\Delta CT$ method and normalized to each transcript value in the hippocampus. Values represent mean $\pm$ SEM. Bars with equal letters are significantly different. a represents $p < 0.01$; b and c represent $p < 0.001$.

Figure 6.

17 β -estradiol (E2) upregulates Cav3.1 and 3.2 mRNA expression in hypothalamic nuclei. **A**, **B**, **C**, Quantitative real-time PCR measurements of Cav3.1, 3.2 and 3.3 mRNAs in microdissected brain nuclei from oil- and E2-treated mice (n=4-6 per group). The expression values were calculated using the $\Delta\Delta\text{CT}$ method ($\Delta\Delta\text{CT} = \text{CT target gene} - \text{CT reference gene} - \Delta\text{CT of calibrator}$), where the calibrator was the mean ΔCT of the oil-treated samples. Bar graphs represent the mean $\pm$ SEM. Statistics were calculated by two-tailed Student's *t* test of oil-E2 groups: *, $p < 0.05$; ***, $p < 0.001$.

Figure 7.

The E2 induced increase in Cav 3.1 and 3.2 subunit mRNA in the POA are dependent on ER α and ER α /ER β , respectively. **A, B, C**, The relative mRNA expression of Cav3.1, 3.2 and 3.3 in the mPOAv from oil- and E2-treated ovariectomized WT, α ERKO and β ERKO C57BL/6 mice (n = 5-6 per group). The relative quantity of target mRNA was calculated by the $\Delta\Delta$ CT method ($\Delta\Delta$ CT = CT target gene – CT reference gene) – Δ CT of calibrator), where the calibrator was the mean Δ CT of the oil-treated samples. Bar graphs represent the mean $\pm$ SEM. Statistics were calculated by two-tailed Student's *t* test of oil-E2 groups: *, p < 0.05; ***, p < 0.001.

Figure 8.

The E2 induced increase in Cav 3.1 and 3.2 subunit mRNA in the arcuate nucleus are dependent on ER α and ER α /ER β , respectively. **A, B, C**, The relative mRNA expression of Cav3.1, 3.2 and 3.3 in the arcuate nucleus from oil- and E2-treated ovariectomized WT, α ERKO and β ERKO C57BL/6 mice (n = 5-6 per group). The relative quantity of target mRNA was calculated by the $\Delta\Delta$ CT method ($\Delta\Delta$ CT = CT target gene – CT reference gene) – Δ CT of calibrator), where the calibrator was the mean Δ CT of the oil-treated samples. Bar graphs represent the mean $\pm$ SEM. Statistics were calculated by two-tailed Student's *t* test of oil-E2 groups: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 9.

The E2 induced increase in Cav 3.1 and decrease in Cav3.2 and 3.3 subunit mRNAs in the pituitary are dependent on ERα only. **A, B, C,** The relative mRNA expression of Cav3.1, 3.2 and 3.3 in the pituitary from oil- and E2-treated ovariectomized WT, αERKO and βERKO C57BL/6 mice (n = 5-6 per group). The relative quantity of target mRNA was calculated by the $\Delta\Delta\text{CT}$ method ($\Delta\Delta\text{CT} = \text{CT target gene} - \text{CT reference gene} - \Delta\text{CT of calibrator}$), where the calibrator was the mean ΔCT of the oil-treated samples. Bar graphs represent the mean $\pm$ SEM. Statistics were calculated by two-tailed Student's *t* test of oil-E2 groups: *, $p < 0.05$; ***, $p < 0.001$.