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Estrogen Regulation of Proteins in the Rat Ventromedial Nucleus of the Hypothalamus

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Abstract

The effects of estradiol (E₂) on the expression of proteins in the pars lateralis of the ventromedial nucleus of the hypothalamus (VMNpl) in ovariectomized rats was studied using 2-dimensional gel electrophoresis followed by RPLC-nanoESI-MS/MS. E₂ treatment resulted in the up-regulation of 29 identified proteins. Many of these proteins are implicated in the promotion of neuronal plasticity and signaling.

Keywords

Estradiol; Rat; Ventromedial Hypothalamus

Introduction

Sexually receptive female rats display a stereotypical behavioral response when mounted by a male. This behavior, termed lordosis, is characterized by a pronounced dorsiflexion of the spinal cord and elevation of the head and rump in response to stimulation by the male.¹ The expression of lordosis is dependent on the presence of estrogen which acts to prime specific regions of the central nervous system for subsequent behavioral facilitation by progesterone.^{2,3} The central regulatory circuit governing female receptivity, including critical estrogen-concentrating brain nuclei such as the ventromedial nucleus of the hypothalamus (VMN), is well established.³⁻⁶ Furthermore, steroid hormones can exert physiological effects by binding intracellular receptors that function as nuclear transcription factors at hormone responsive elements of DNA,^{7,8} and estrogen-induced transcription in the VMN is required to activate lordosis.³ Thus, female rat sexual behavior provides an excellent model to study how the ovarian steroid-induced signals are translated into a behavior response. A key element in understanding how steroid signals are converted into a behavioral output involves the identification of proteins induced or post-translationally modified in the brain in response to the hormones. Based in part on genomic studies, ovarian steroids have been proposed to systematically regulate multiple genes which serve to synchronize ovulation and receptivity in order to maximize reproductive success.^{9,10} These genes have been clustered into functional groups in the central nervous system (CNS) and are believed to act cooperatively in the activation and facilitation of sexual behavior.^{9,10} As a complementary approach to genomic

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studies, a global view of steroid-mediated effects on proteins in discrete brain nuclei, such as VMNpl, is necessary for understanding the mechanism underlying E₂ activation of female sexual receptivity. The proteins are the final effectors underpinning the behavior response and the relation between mRNA levels and protein expression are not always consistent.^{11,12} Furthermore, post-translationally modified proteins can only be detected at the protein level. Such information has not yet been documented in the literature, although several proteins under E₂ regulation in VMNpl have been individually identified.¹⁰

Recently we combined microdissection¹³ and proteomic analysis to construct a partial database for the pars lateralis of the VMN obtained from ovariectomized rats.¹⁴ In this study, two-dimensional electrophoresis (2-DE) was used to separate proteins extracted from VMNpl tissue punches obtained from brains of ovariectomized (ovx) rats treated with either E₂ or sesame oil vehicle (V), followed by nanoRPLC-ESI-MS/MS analysis to identify proteins regulated by E₂ that could potentially impact the expression of progesterone facilitated female sexual behavior.

Experimental Section

Reagents

Proteases inhibitor cocktails for mammalian tissue, 17 β -estradiol benzoate (E₂), sesame oil and (Glu¹)-Fibrinopeptide B human were purchased from Sigma-Aldrich (St. Louis, MO). Immobilized pH-gradient (IPG) ready strip (pH 3-10, nonlinear, 11 cm), and all other reagents were purchased from Bio-Rad Laboratories (Hercules, CA).

Animals

Adult female Sprague-Dawley rats were raised in the animal colony at the University of South Dakota. The rats were housed in groups of four in plastic cages with food and water available ad lib. The animal room was maintained on a reversed 12L:12D photoperiod with lights off at 09:00 h. The rats were ovariectomized (ovx) under ketamine/xylazine anesthesia and allowed to recover for 7 days prior to any additional treatment. Ovariectomy removes the endogenous source of the sex hormones and abolishes the expression of female sexual behavior. The behaviors can be reinstated in a controlled and predictable manner by treating the ovx rats with exogenous hormone replacement.¹⁵ After recovery, the rats were randomly divided into an E₂ treatment group (n=9) or a V group (n=9) which were injected sc with E₂ (5 μ g/0.1 ml sesame oil) or V (0.1 ml sesame oil), respectively. This dose of E₂ is effective in priming females for the facilitatory behavioral effects of progesterone but, in the absence of progesterone, results in relatively low levels of lordosis during behavioral testing.^{16,17} After 48 hours, the rats were rapidly decapitated 4 to 5 h after lights off. The brains were removed and immediately frozen on dry ice. The brains were stored at -80 °C until microdissection for the proteomic analysis. The University of South Dakota IACUC approved the protocols used in these experiments.

Microdissection, sample preparation and two-dimensional gel electrophoresis (2-DE)

The experimental process was essentially same as described previously.¹⁴ Briefly, serial 300 μ m slices were cut from the frozen brains in a Leica Jung CM1800 (Wetzlar, Germany) cryostat at -8 °C and from the slices, anterior and posterior aspects of the pars lateralis of the VMN (plates 27-31; punch numbers 145, 147)¹³ were dissected using a 500 μ m id punch needle. The tissue punches obtained from the VMNpl, pooled from three rats of the same treatment group, were dissolved in 250 μ l sample buffer (9.5 M urea, 4% CHAPS, 50 mM DTT, 0.2% Bio-Lyte ampholytes, pH 3-10 and 1% protease inhibitor cocktail) and disrupted by sonication in an ice bath. After determining soluble protein concentrations using the Bradford assay,¹⁸ a 200 μ l aliquot of the dissolved proteins containing 180 μ g protein was used for overnight in-

gel rehydration of the dry read IPG strip (pH 3-10, nonlinear, Bio-Rad Laboratories, Hercules, CA) at room temperature (16 hr). Isoelectro-focusing was run using an IEF cell (Bio-Rad Laboratories, Hercules, CA). Focusing started at 250 V for 2 hr, slowly ramped to 8,000 V in 2.5 hr, and then kept at 8,000 V until 24,000 Vh was reached. The focused strips were equilibrated sequentially in equilibration buffer I (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol and 130 mM DTT) and equilibration buffer II (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 135 mM iodoacetamide), each for 15 min. The equilibrated strips were placed on top of 12 % Tris-HCl SDS-polyacrylamide gels and run at 35 mA/gel using a PROTEAN II xi electrophoresis cell (Bio-Rad Laboratories, Hercules, CA) until the dye front reached the bottom of the gels. The gels were then stained in SYPRO Ruby (Bio-Rad Laboratories, Hercules, CA) and visualized using the Typhoon 9410 Workstation Fluorescence Scanner (Amersham-GE, Piscataway, NJ).

Protein expression profile analysis

Spot detection and normalization at local regression mode (for details see PDQuest user manual at <http://www.bio-rad.com>), and gel matches were automatically conducted by the 2-DE analysis software, PDQuest 8.0 (Bio-Rad Laboratories, Hercules, CA) on imported 2-DE images from the scanner, followed by manual fine editing. In order to identify the spots that were undetectable on the 2-DE maps of V groups but up-regulated on the 2-DE maps of E₂ groups, quality analysis was performed using the built-in function of PDQuest software, followed by visual verification. The selected protein spots were excised using a spotcutter (Bio-Rad Laboratories, Hercules, CA).

Protein identification by mass spectrometry analysis

The excised spots were in-gel digested using trypsin sequencing grade (Promega, Madison WI) and nanoRPLC in-line desalted as described previously.¹⁴ The eluted ions were analyzed by one full precursor MS scan (400-1500 m/z) followed by four MS/MS scans of the most abundant ions detected in the precursor MS scan while operating under dynamic exclusion or direct data acquisition system (DDAS, please see Supplementary file DDAS_experiment.pdf for additional details). Spectra obtained in the positive ion mode with nano ESIQ-ToF micro mass spectrometer (Micromass, UK) were deconvoluted, and analyzed using the MassLynx software 4.0 (Micromass, UK). A peak list (PKL format) was generated to identify +1 or multiple charged precursor ions from the mass spectrometry data file (processing parameters are detailed in Supplementary Table 1). The instrument was calibrated in MS/MS mode using 500 fmole of (Glu¹)-Fibrinopeptide B human with a root mean square residual of 3.495 e⁻³ amu or 7.722 e⁰ ppm. Parent mass (MS) and fragment mass (MS/MS) peak ranges were 400-1500 Da and 65-1500 Da, respectively.

Mascot server v2.2 (www.matrix-science.com, UK) in MS/MS ion search mode (local licenses) was applied to conduct peptide matches (peptide masses and sequence tags) and protein searches against NCBI nr v20080718 (6833826 sequences; 2363426297 residues) using taxonomy filter Rodentia (Rodents) (221999 sequences). The following parameters were set for the search: carbamidomethyl (C) on cysteine was set as fixed; variable modifications included asparagine and glutamine deamidation and methionine oxidation. Only one missed cleavage was allowed; monoisotopic masses were counted; the precursor peptide mass tolerance was set at 1 Da; fragment mass tolerance was 0.3 Da and the ion score or expected cut-off was set at 5. Known keratin contaminants ions (keratin) were excluded. The MS/MS spectra were searched with MASCOT using a 95% confidence interval (C.I. %) threshold (p<0.05), with which minimum score of 41 was used for peptide identification. The protein redundancy that appeared at the database under different gi and accession numbers were limited to Rodentia with the first priority assigned to *Rattus norvegicus* and the second priority assigned to *Mus musculus*. All of the proteins identified in the current study were found these domains.

Results

In order to survey the E_2 - regulated proteins in VMNpl, a proteomics approach was applied. In this study, each 2-DE gel was generated from punch-dissected samples of the VMNpl pooled from three rats/treatment. Thus, each treatment (E_2 or V) consisted of 3 independent replicates that were used to produce three gels/treatment. Gel-to-gel correlation coefficients were obtained using the scatter plot tool in PDQuest (Bio-Rad Laboratories, Hercules, CA). Correlations for the three gels generated from the V-treated rats were 0.880, 0.905, 0.881, respectively, with an overall mean coefficient of variation (CV) of 24.2%. For the E_2 -treated rats, gel-to-gel correlation coefficients were 0.821, 0.885, 0.867, respectively, with a mean CV of 30.6%. Representative gels are shown in Figure 1.

We focused on proteins that were undetectable on 2-DE maps obtained from groups without hormone replacement but were dramatically up-regulated by E_2 replacement. These proteins represented a greater than 10-fold increase in stain intensity when compared to background as defined by PDQuest 8.0 (Bio-Rad Laboratories, Hercules, CA). Thus, the difference in expression of these proteins between E_2 and V treatments is unequivocal and robust, and should represent prominent effects of E_2 in the VMNpl. Comparisons of the protein spot intensities between the E_2 and V groups revealed that 30 spots fit into this category. Of these spots, 23 were successfully identified as 29 non-redundant proteins assigned with 95 % confidence (Mascot score 41, $p < 0.05$; some spots contained more than one protein). The parent mass, charge state and the results from MS/MS analysis error for these proteins are reported in Supplementary Table 2. The other seven spots were below the significant threshold for identification. A total of six proteins from the 29 reported proteins returned single peptide match identification. The sequences, precursor m/z and charge for these proteins are detailed in Supplementary Table 3. The identified spots are labeled on the 2-DE maps with randomly assigned numbers (Figure 1) and representative spots are shown in higher magnification in Figure 2.

The identified proteins were roughly categorized into four groups. The first group includes proteins that have been linked to vesicular transport and membrane protein trafficking (Table 1). Among the proteins are alpha- and beta- soluble N-ethylmaleimide-sensitive factor (NSF) attachment proteins (SNAPs), rab guanine dissociation inhibitor (GDI) alpha, chromatin modifying protein 4B (CHMP4B) and vesicle associated membrane protein 2 (VAMP 2).

Treatment with E_2 also resulted in the up-regulation of proteins that have been implicated in neuronal plasticity. This group occupies the biggest fraction of the VMNpl proteins up-regulated by E_2 (Table 2). Proteins grouped in this cluster include: cofilin2, Ulip2, also referred to as TOAD-64 (turn on after division protein 64 kD)/CRMP2 (collapsing response mediator protein-2), cytoplasmic dynein intermediate chain 2B, glial fibrillary acidic protein delta (GFAP; detected in two spots), cytoskeleton-associated protein 1, similar to tubulin 2 alpha chain (detected in three spots, implying post-translational modification), tubulin alpha 1, tubulin beta 2C, and N-myc downstream regulated gene 2 (NDRG2).

The third group of proteins up-regulated by E_2 includes proteins involved in cellular energy metabolism, including F1-ATPase, NADH dehydrogenase Fe-S protein 1, aldolase A, enolase-1, voltage dependent anion channel (VDAC) and ATP synthase alpha (Table 3). The last group of proteins that were up-regulated following E_2 treatment, shown in Table 4, includes heat-shock proteins and proteins involved in signal transduction pathways. Three members of the heat shock protein (Hsp) family including Hsp90 alpha, Hsp72-ps1, a dnaK type molecular chaperone, and an unnamed protein similar to Hsp8, were markedly increased in the VMNpl following E_2 treatment. These multifunctional proteins were detected in three different spots (spots 2, 3 and 20, respectively Figure 1). Proteins linked to signaling processes that were up-

regulated include: alpha isoform of regulatory subunit A of protein phosphatase 2 (PP2A), ubiquitin aldehyde binding 1 (Otub1 protein) which is a member of the OUT (ovarian tumor) domain, and nucleosome assembly protein 1-like 5 (NAP1L5).

Discussion

During the past decade progress has been made in the identification of E₂-regulated genes in the VMNpl using molecular approaches. A number of these E₂-induced genes, including those which code for the expression of progesterin receptors, neuropeptide Y receptors, galanin, oxytocin, GnRH, mu opioid receptors, pro-opiomelanocortin, glutamic acid decarboxylase, glutamate receptors and tyrosine hydroxylase, are believed to be important in the regulation of lordosis.¹⁰ As a complementary approach, we used proteomic measurements to simultaneously assess multiple proteins regulated by E₂ in the VMNpl. The application of proteomic analysis in this study resulted in the identification of multiple proteins in the VMNpl that were up-regulated in response to E₂. These proteins are discussed with respect to their potential participation in the priming effects of E₂ that regulate female sexual receptivity in the rat.

E₂ affects proteins linked to vesicular transport and membrane protein trafficking

The expression of Hsp90 alpha was increased in the VMNpl following E₂ treatment (Figure 2 and Table 4). This result is consistent with previous reports based on western blot analysis.^{19,20} Hsp90 chaperones are associated with multiple functions including the regulation of exocytosis and endocytosis.^{21,22} Of particular interest are studies indicating that Hsp90 combines with cysteine-string protein, a synaptic vesicle associated chaperone protein,²³ to form a complex with Rab-specific α -GDP-dissociation inhibitor (α -GDI).²¹ We found that α -GDI was also up-regulated by E₂ in the VMNpl (Figure 1). This complex is believed to modulate neurotransmitter release by regulating recycling of Rab3A, a G protein implicated in exocytosis.^{24,25}

Other vesicle trafficking regulation proteins that were up-regulated by E₂ in the VMNpl include alpha- and beta- N-ethylmaleimide-sensitive factor attachment proteins (SNAPs), vesicle associated membrane protein 2 (synaptobrevin/VAMP 2), cytoplasmic dynein intermediate chain 2B and chromatin modifying protein 4B (CHMP4B). The synaptobrevin/VAMP 2 isoform is a widely distributed synaptic vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) in the rat central nervous system.²⁶ SNARE mediates Ca²⁺-triggered exocytosis by forcing the membranes into close proximity for fusion.²⁷ After membrane fusion, SNAPs disassemble SNARE complexes to release SNARE and sustain exocytosis.²⁸ Estradiol treatment up-regulated both the ubiquitous alpha-SNAP and the neuron specific beta-SNAP²⁹ isoforms of SNAP in the VMNpl. These two SNAP isoforms may have different roles in the regulation of exocytosis. Alpha-SNAP has been proposed to function in the high-affinity form of Ca²⁺-dependent exocytosis that occurs at low resting intracellular Ca²⁺ concentrations and is believed to set the tone for synaptic communication.³⁰ In contrast, beta-SNAP has been shown to promote exocytosis at elevated intracellular Ca²⁺ concentrations.³⁰ Additionally, beta-SNAP enhances endocytosis of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors to down-regulate the density of AMPA receptors on synaptic membranes.³¹ Since recent work suggests that activation of AMPA receptors in the VMN suppress lordosis and proceptive behaviors,^{32,33} it is possible that the E₂-induced up-regulation of beta-SNAPs down-regulates synaptic AMPA receptors in the VMN to contribute to the expression of lordosis. Such an effect, if present, may be coordinated with E₂-induced up-regulation of CHMP4B (Table 1) which has been proposed to modulate cell surface receptor down-regulation by controlling the trafficking of plasma membrane proteins from endosomes to lysosomes.³⁴

E₂ affects proteins linked to neuronal plasticity

Steroid-mediated rewiring of the synaptic connectivity within the VMN has been proposed as another important mechanism in the regulation of female sexual behavior.³⁵⁻³⁷ Previous studies found that E₂ promotes increases in dendritic spine density in some neurons in the VMN^{35,37} while stimulating dendritic spine retraction to eliminate other synapses.³⁶ In agreement with reports that E₂ regulates dendritic spine density in the VMN, we detected the up-regulation of multiple isoforms of tubulin in the VMNpl after E₂ treatment (Table 1 and Figure 1), indicating active cytoskeleton reorganization. Correspondingly, cytoskeleton-associated protein 1, a protein required for tubulin folding (Rat Genome database (RGD) 1309965, <http://rgd.mcw.edu>), was up-regulated. In addition, E₂ up-regulated cofilin 2, an actin depolymerizing factor which is likely related to dendritic spine retraction,³⁸ and the cytoplasmic dynein intermediate chain 2B, a subunit in the cytoplasmic dynein complex that functions in axonal transport (Table 1 and Figure 1). Cytoplasmic dynein is integral in the retrograde transport of membranous organelles from synapses back to the cell body.³⁹ This protein consists of multiple subunits⁴⁰ including intermediate chains that directly bind the membranous organelles. Moreover, an isoform shift from cytoplasmic dynein intermediate chain 2C to cytoplasmic dynein intermediate chain 2B has been shown to precede neurite outgrowth.⁴¹ Such an isoform shift may explain the up-regulation of cytoplasmic dynein intermediate chain 2B detected in this study and would be consistent with the presence of active neurite outgrowth as reported previously in the VMN of E₂-treated animals.^{35,37} The E₂-induced up-regulation of Ulip2/TOAD-64/CRMP2, a protein that regulates axon outgrowth and path-finding^{42,43} and N-myc downstream regulated gene 2 (NDRG2), a protein reported to promote neurite outgrowth,⁴⁴ in the VMNpl also suggest the presence of active neurite outgrowth.

The marker for astrocytes, glial fibrillary acidic protein delta (GFAP) was also up-regulated in the VMNpl following E₂ treatment. GFAP is the major intermediate filament protein that supports astrocyte processes. The dynamic expansion or withdrawal of astrocyte processes has been suggested to regulate the available surface on neurons to form synapses.⁴⁵ Previous work has linked estrogen responsive astrocytes to changes in synaptic plasticity and glutamate neurotransmission based on findings that E₂ alters GFAP expression in the hypothalamus and affects both astrocyte morphology and function as indicated by increased expression of glutamine synthetase^{46,47}

E₂ promotes energy production

Glucose metabolism is the sole energy source of brain tissue and high levels of neuroactivity elevates energy consumption. In the VMNpl of E₂-treated animals, key enzymes in energy metabolism, including F1-ATPase, NADH dehydrogenase Fe-S protein 1, aldolase A, enolase-1 and ATP synthase alpha, were up-regulated (Table 2). In addition, E₂ up-regulated voltage-dependent anion channel (VDAC), pore forming proteins on mitochondria outer membranes that function in controlling the flux of metabolites between mitochondria and the cytosol.⁴⁸ In neuronal presynaptic terminals, mitochondria have been proposed to modify synaptic transmission through the control of ATP and calcium release through VDAC to the cytosol.⁴⁹ Thus, up-regulation of VDAC in VMNpl by E₂ may play a role in the enhancement of synaptic transmission and/or supplying energy to support synaptic remodeling. Taken together, these effects suggest that E₂ promoted increased ATP production to satisfy elevated energy consumption, possibly in support of E₂-influenced changes in dendritic spine density.³⁵⁻³⁷

E₂ regulation of heat-shock proteins and proteins involved in signal transduction pathways

The first protein in this group is the alpha isoform of regulatory subunit A of protein phosphatase 2 (PP2A). PP2A is a major protein phosphatase that dephosphorylates

phosphoserine and phosphothreonine residues in eukaryotic cells and regulates diverse signaling pathways in coordination with protein kinases.⁵⁰ Estradiol has been reported to rapidly activate PP2A in rat cerebellar cells⁵¹ and PP2A has been implicated in E₂ neuroprotection of cultured neurons to excitotoxic insult.⁵² In addition to these rapid non-genomic effects, E₂ up-regulation of the regulatory subunit A of PP2A in the VMNpl indicates a potential role of PP2A in modulating signaling pathways that may be related to the expression of female sexual behavior. PP2A has been shown to regulate the mitogen-activated protein kinase (MAPK) pathway⁵³ and forms complexes with p21- activated kinases 1 and 3 (PAK1 and PAK3, respectively⁵⁴). This is of interest because estrogen activation of MAPK signaling has been proposed to facilitate female receptivity based on the demonstration that MAPK inhibitors suppress lordosis in steroid-primed ovariectomized rats.⁵⁵ Furthermore, MAPK phosphorylation of estrogen-induced progesterone receptors has been linked to enhanced transcriptional activity and behavioral facilitation by progesterone.⁵⁶ Finally, E₂ induced activation of MAPK is reported to regulate synaptic plasticity in a number of brain regions.⁵⁷ PAK1 and PAK3 are also believed to contribute to neurite outgrowth and synaptic plasticity.^{58,59} Thus, the activation of PP2A may play a role in the regulation of neuronal plasticity in the VMNpl by modulating the MAPK and PAK signaling pathways. What is perhaps most remarkable is that PP2A can alter the expression of estrogen receptor alpha (ER α) by regulating the stability of ER α mRNA in breast cancer cell lines⁶⁰ and a direct interaction between PP2A and ER α has been shown to regulate ER α -mediated transcription activities.⁶¹ It would be intriguing to determine if a similar interaction between PP2A and ER α occurs in the VMNpl.

A second protein up-regulated by E₂ in the VMNpl is nucleosome assembly protein 1-like 5 (NAPIL5). NAP1-like proteins are implicated in the formation and maintenance of the nervous system.⁶² NAPIL5 is expressed in the CNS but its function is not known.⁶³ This protein shares sequence homology with nucleosome assembly protein 1, a multiple function protein that serves as a chromatin-assembly factor and histone storage protein, and may regulate transcription.^{63,64}

The third protein in this group is a deubiquitylating enzyme, ubiquitin aldehyde binding 1 protein (Otub1 protein), which is a member of the OUT (ovarian tumor) domain.⁶⁵ Protein ubiquitylation is controlled through the cooperation between ubiquitylating and deubiquitylating enzymes and is involved in wide range of cellular processes, including transcriptional control and signal transduction.^{67,68} Interestingly, Otub1 was detected in the same spot with CHMB4B (Figure 1 and Tables 1 and 3), a protein that is also up-regulated by E₂ and was suggested to regulate the plasma membrane protein trafficking from endosomes to lysosomes (discussed above). Considering that ubiquitylation of plasma membrane proteins provide the signal for triggering internalization and sorting of proteins into multivesicular endosomes,⁶⁸ we speculate that Otub1 may form protein complexes with CHMB4B to regulate plasma membrane proteins, such as receptors or ion channels for either reinsertion into the plasma membrane or sorting to lysosomes for degradation.

Heat shock proteins also play roles in signal transduction pathways. The E₂ induction of progesterone receptors and their relevance to sexual behavior is well established.⁶⁹⁻⁷² The E₂ up-regulation of Hsp90 alpha may be related to the chaperone complex formed with progesterone receptors that allows progesterone binding *in vitro*.^{73,74} In addition, Hsp90 alpha enhances nitric oxide synthesis from neuronal nitric oxide synthase⁷⁵ and inhibits superoxide generation from nitric oxide synthase⁷⁶ Such an effect, if present in the VMN, may be relevant to the behavioral priming actions of E₂ since previous work suggests that nitric oxide stimulates lordosis.^{77,78} Less can be inferred about potential effects resulting from the estrogen-induced expression of an unnamed protein similar to Hsp8 and Hsp72 in the VMNpl. Both proteins are members of the heat shock protein 70 family of molecular chaperones. Stress induction of Hsp72 protects a variety of cell types from damage through the reinstatement of folding in

damaged proteins and prevention of apoptotic pathway activation.⁷⁹ Previous work has shown that both Hsp72 and Hsp90 are also markedly increased in uterine tissue in response to E₂ activation of estrogen receptors.⁸⁰

It is worth pointing out that in this study we did not detect corresponding changes at the protein level of some previously detected genes that are regulated by E₂ at the mRNA level in the VMN.¹⁰ The most likely explanation for these differences is that we focused on proteins that were dramatically and unambiguously up-regulated by E₂ replacement. In addition, some E₂-regulated genes may be translated into low abundance and/or highly hydrophobic proteins that were beyond the detection limits allowed by the current 2-DE electrophoresis. Finally, some of these discrepancies might also reflect changes in mRNA levels that are not necessarily expressed as alterations in protein abundance.^{11,12} More sensitive and comprehensive proteomics techniques may need to be applied to detect these proteins from microdissected brain samples.

Conclusions

In conclusion, this study demonstrates that E₂ markedly up-regulates a number of proteins in the pars lateralis of the VMN. The effects of E₂ on protein expression in the VMNpl, based on the current proteomic analysis, appear to be the up-regulation of proteins which have been implicated in stimulating neuronal plasticity and altering signal transduction. Concurrently, E₂ treatment resulted in the up-regulation of proteins involved in energy production that may be related to the metabolic requirements for the above processes. Future studies, which target specific proteins identified in the current report, may be rewarding in elucidating the possible functional significance and/or relevance of these E₂-induced proteins to the role of E₂ in priming female sexual behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DEDICATION. This manuscript is dedicated to our friend and colleague Mo Bing (1963-2007). Bing was an outstanding person and we miss him.

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Figure 1.

Representative 2-DE maps of the VMN pars lateralis generated from microdissected tissue pooled from 3 rats treated with vehicle (V) or 17 β-estradiol (E₂). The proteins were separated on a nonlinear IPG strip (pH 3 - 10), followed by separation on 12% SDS-gel and staining with SYPRO Ruby. The numbers indicate proteins up-regulated by E₂ and correspond to the numbers used with the spot identifications listed in Tables 1-4.

Figure 2.

Higher magnification of selected spots labeled in Fig. 1. For each pair, the left panel is from the VMNpl map generated from vehicle-treated rats (V). The right panel shows corresponding magnification of spots obtained from the VMNpl of rats treated with 17β -estradiol (E_2). The assigned numbers correspond with the protein identifications listed in Tables 1-4.

Estradiol up-regulation of proteins linked to vesicular transport and membrane protein trafficking in the VMHpl

Table 1

Spot number	name ^a	Accession number	% ^b	Mol. mass	pI	Score ^c	Matches ^d	Sequences
1	rab GDI alpha	CAA52413	13	51058	5.05	206	6	KLYSESLLARY KVVEGSFVYKG RKQNDVGEADQ- KFLMAMGQLVKM KMILLYTEVTRY RFQLLEGPPESMGRG
4	alpha-soluble NSF attachment protein	NP_542152	15	33627	5.30	222	4	KTIQGDDEEDLR KQAEAMALLAEAEK KVAGYAAQLEQYQKA RAIEYTDMDGRF
5	similar to N-ethylmaleimide sensitive fusion protein attachment protein beta	XP_575249	7	40322	5.88	132	2	KSIQGDGEGDGLK-KVAAAYAAQLEQYQQA
12	vesicle associated membrane protein 2B	CAB43509	24	14557	5.44	78	2	RADALQAGASQFETSAAKL RLQQTQAQVDEVVDIMRV
15	similar to charged multivesicular body protein 4b (chromatin modifying protein 4Bb) (CHMP4B)	XP_001065258	10	29240	4.76	106	2	KGGPTPOEAIORL KQLAQIDGTLSTIEFQRE

^aFunction annotations were retrieved from NCBItr (<http://www.ncbi.nlm.nih.gov/>)

^brepresents % coverage

^cThreshold was set up by the server at the significance level $P \geq 0.05$ for random hit; scores greater than threshold were taken as a significant match (<http://www.matrixscience.com>)

^dMatches is the number of matching peptides to the target proteins

Table 2 Estradiol up-regulation of proteins implicated in neuronal plasticity in the VMNpl

Spot number	name ^a	Accession number	% ^b	Mol. mass	pI	Score ^c	Matches ^d	Sequences
6	Tubulin beta 2c	NP_954525	7	50225	4.79	145	3	RPPQLNADLRK KLA VNMVPEPRL RAVLVDLEPGTMDSVR
7	Tubulin, alpha 1	AAH62238	13	50816	4.94	192	4	KDVNAAIATIKT RAVFVDLEPTVIDEVRT (2X) KVGINYQPPTVVPGGDLAKV KTGGDDSDSNTFESSETGAGKH
8	Tubulin alpha	0812252A	9	50894	4.94	152	3	KDVNAAIATIKT RAVFVDLEPTVIDEVRT KVGINYQPPTVVPGGDLAKV
12	unnamed protein, similar to tubulin-2	CAA24536	19	16448	4.76	73	2	KDVNAAIATIKT KVGINYQPPTVVPGGDLAKV
13	N-myc downstream regulated gene 2	NP_598267	10	39587	5.30	196	3	KLDPTQTSTFLKM RTASLTSAASIDGSR KMADSGGQQLTQPGKL
13	glial fibrillary acidic protein delta	AAD01874	4	48809	5.72	113	2	KFADLTDVASRN KALAAELNQLRA
14	cytoplasmic dynein intermediate chain 2B	AAA89164	6	70748	5.11	138	3	RTLAENASRA RADAEAEAAATRI KSVSTPSEAGSEAGSQDSGDGAVGSR T
15	cytoskeleton-associated protein 1	NP_001035270	6	27515	5.06	55	1	RLSEEKAQASAIISVGSRC
16	similar to Cofilin-2	XP_001078378	9	13581	8.99	66	2	RYALYDATYETKE KLGGSVVVSLEGKPL
18	ulip2 protein	CAA71370	8	62531	5.95	172	4	KSAAEVIAQARK RGSPLVVISQSKI KQIGENLIVPGGVKT KMDENQFVAVTSTNAAKV
19	glial fibrillary acidic protein delta	AAD01874	9	48809	5.72	152	4	RFLQQNKA KFADLTDVASRN KLADVYQAELE RESASYQEALRL
21	similar to Tubulin alpha 2 chain	0812252A	6	50894	4.93	73	2	KDVNAAIATIKT RAVFVDLEPTVIDEVRT

^aFunction annotations were retrieved from NCBItr (<http://www.ncbi.nlm.nih.gov/>)

^b represents % coverage

^c Threshold was set up by the server at the significance level $P \geq 0.05$ for random hit; scores greater than threshold were taken as a significant match (<http://www.matrixscience.com>)

^d Matches is the number of matching peptides to the target proteins

Table 3 Estradiol up-regulated proteins that function in energy production in the VMNpl

Spot number	name ^a	Accession number	% ^b	Mol. mass	pI	Score ^c	Matches ^d	Sequences
9	voltage dependent anion channel (VDAC)	AAD02476	3	32672	8.30	52	1	RVTQSNFAVGKYT
10	Chain A, F1-ATPase	1MAB_A	2	55361	8.28	93	1	RILGADTSVDLEETGRV
11	aldolase A	AAA40715	17	39691	8.39	267	5	KGILAADESTGSIKR KAAQEEYIKRA RLQSIGTENTENRRF KGVVPLAGTNGETTOGLDGLSERC KVDKGVVPLAGTNGETTTQGLDGLSE RC
14	NADH dehydrogenase (ubiquinone) Fe-S protein 1	AAH81892	10	80331	5.65	250	6	RFEAPLFNARI KSATYVNTEGRA KVALIGSPVDLTTRY RFASEIAGVDDLGTGTRG RVAGMLQSFEGKA RGNDMQVGTYIEKM
22	ATP synthase alpha	AAA40784	16	58904	9.22	394	7	KAVDSLVPIGRG RVVDALGNADGKG KTSIADITINQKR RILGADTSZVDLEETGRV RTGAIVDVPVGDELLGRV KVL SIGDGIARV KTGTAE MSSILEERI
23	enolase 1, alpha	NP_036686.2	2	47437	6.37	41	1	RGNPTVEVDLYTAKG

^aFunction annotations were retrieved from NCBIInr (<http://www.ncbi.nlm.nih.gov/>)
^brepresents % coverage
^cThreshold was set up by the server at the significance level $P \geq 0.05$ for random hit; scores greater than threshold were taken as a significant match (<http://www.matrixscience.com>)
^dMatches is the number of matching peptides to the target proteins

Table 4
Estradiol up-regulation of proteins linked to signal transduction pathways in the VMNpl

Spot number r	name ^a	Accession number	% ^b	Mol. mass	pI	Score ^c	Matches ^d	Sequences
1	unnamed protein, similar to alpha isoform of regulatory subunit A, protein phosphatase 2, isoform 2	BAC40565	8	66077	5.00	160	5	RDKAVESLRA KVLELDNVKS KLTQDDVDVKY KLSTIALALGVERT RMAGDPVANVRF
2	unnamed protein, similar to heat shock protein 8	BAE42451	5	50547	6.17	63	2	KDAGTIAGLNVLRI RTTPSYVAFDTERL
3	dnaK-type molecular chaperone hsp72-ps.1	S31716	8	71112	5.43	222	4	KDAGTIAGLNVLRI RTTPSYVAFDTERL KVAGYAAQLEQYQKA RAIEIYTDMGFR
15	Oub1 Protein	AAH54410	8	31205	4.85	120	2	RLLTSGYLQRE RIQQEIAVQNPLVSERL
17	similar to nucleosome assembly protein 1-like 5	AAH87702	7	17019	4.15	50	1	KNDFIESLPNPVKC
20	heat shock protein 1 hsp90 alpha	NP_786937	1	85161	4.93	42	1	KDQVANSFAFVERL

^aFunction annotations were retrieved from NCBIInr (<http://www.ncbi.nlm.nih.gov/>)

^brepresents % coverage

^cThreshold was set up by the server at the significance level $P \geq 0.05$ for random hit; scores greater than threshold were taken as a significant match (<http://www.matrixscience.com>)

^dMatches is the number of matching peptides to the target proteins