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Dopamine-induced interactions of female mouse hypothalamic proteins with progesterin receptor-A in the absence of hormone

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Abstract

Neural progesterin receptors (PR) function in reproduction, neural development, neuroprotection, learning, memory, and anxiety response. In the absence of progestins, PR can be activated by dopamine (DA) in the rodent hypothalamus to elicit female sexual behavior. This study investigated mechanisms of DA activation of PR by testing the hypothesis that proteins from DA-treated hypothalami interact with PR in the absence of progestins. Ovariectomized, estradiol-primed mice were infused with a D1-receptor agonist, SKF38393 (SKF), into the 3rd ventricle 30 minutes prior to euthanasia. Proteins from SKF-treated hypothalami were pulled-down with GST-tagged mouse PR-A or PR-B and the interactomes were analyzed by mass spectrometry. The largest functional group to interact with PR-A in a DA-dependent manner was synaptic proteins. To test the hypothesis that DA activation of PR regulates synaptic proteins, we developed estradiol-induced PR-expressing hypothalamic-like neurons derived from human-induced pluripotent stem cells (hiPSCs). Similar to progesterone (P4), SKF treatment of hiPSCs increased synapsin-1/2 expression. This SKF-dependent effect was blocked by the PR antagonist RU486, suggesting that PR are necessary for this DA-induced increase. The second largest DA-dependent PR-A protein interactome comprised of metabolic regulators involved in glucose metabolism, lipid synthesis, and mitochondrial energy production. Interestingly, hypothalamic proteins interacted with PR-A, but not PR-B, in an SKF-dependent manner, suggesting that DA promotes interaction of multiple hypothalamic proteins with PR-A. These *in vivo* and *in vitro* results indicate novel mechanisms by which DA can differentially activate PR isoforms in the absence of P4 and provide

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a better understanding of ligand-independent PR activation in reproductive, metabolic, and mental health disorders in women.

Keywords

progesterone; synapse; energy homeostasis; induced pluripotent stem cells; metabolism

INTRODUCTION

Neural progesterin receptors (PR) are important regulators of neuroprotection^{1–5}, learning and memory^{6–8}, anxiety and stress response^{9–12}, energy homeostasis and reproduction^{13–16}. Many of these effects are mediated by PR expressed in the hypothalamus^{14,17–19}. In rodents and humans, PR are expressed as two isoforms, an N-terminally truncated PR-A and a full-length PR-B^{18,20–22}, which are transcribed from alternate transcription start sites of the same *pgr* gene^{20,23}. While both PR isoforms share a C-terminal ligand binding domain, a DNA binding domain, and two transactivation domains, differences between the two isoforms are contributed by a PRB-specific transactivation domain, and a PRA-specific inhibitory region^{18,20,23–26}. These structural differences contribute to the differential transcriptional activity of PR-A and PR-B *in vitro* and *in vivo*^{27–32}. For example, using PR isoform-specific knockout mice, Mani and colleagues have shown that PR-A is the primary mediator of female reproductive behavior, although both PR isoforms are essential for the full behavioral response³³. These dominant effects of PR-A on behavior may be partially due to differential expression of the isoforms in the hypothalamus¹⁷. Similarly, the PR isoforms have distinct functions in peripheral tissues, such that PR-A is primarily responsible for follicular maturation and embryo implantation whereas PR-B is the major mediator of mammary gland growth and proliferation^{34,35}.

As with other steroid receptors, PR are transcription factors that directly bind to DNA and initiate gene transcription. This PR-mediated transcription is enhanced by interaction with coregulator proteins, including steroid receptor coactivator-1 (SRC-1), SRC-2 and CBP (CREB-binding protein), which are necessary for PR-dependent gene expression and female reproductive behavior^{36–38}. Recently, using proteomics-based approaches, we identified a repertoire of hypothalamic proteins, including synaptic proteins, kinases, and transcriptional, translational and metabolic regulators that associate with PR in a hormone- and isoform-dependent manner¹⁶. In addition, we found that progesterone (P4) enhanced synaptic density by increasing synapsin 1 (SYN 1)-positive synapses in rat primary cortical neurons, suggesting that progestins function in synaptic plasticity¹⁶. In support, PR are expressed in extra-nuclear compartments, including synapses^{39,40} and alter dendritic spine densities in hippocampus^{41,42}, cortex^{43,44}, and hypothalamus⁴⁵. These findings provide insights into multiple pathways and novel functions mediated by progestin-activated PR in brain.

While it is well established that PR acts as a ligand-induced transcription factor to elicit gradual and long-lasting changes^{18,38,46} PR-A and PR-B can also be activated in the absence of hormone by the neurotransmitter dopamine (DA)^{14,47–49}. DA activation of PR in rat and mouse hypothalamus acutely induces female reproductive behavior in the absence of

P4^{33,50}. However, there is a lack of understanding of the mechanisms for this DA-dependent PR activation in the hypothalamus and the induction of female reproductive behavior. One possible mechanism for this activation of hypothalamic PR is a DA-induced cascade resulting in the phosphorylation of hypothalamic proteins that interact with PR in the absence of P4. These DA-induced interactions of hypothalamic proteins with PR could elicit changes in the receptor function, including transcription, that modify behavior.^{16,51–53}. Therefore, in the present study, using pull-down assays combined with mass spectrometry, we tested the hypothesis that proteins from female mouse hypothalamus treated with a D1 receptor (D1R) agonist would interact with PR in the absence of progestins. Following identification of a large set of synaptic proteins, we developed GnRH-positive hypothalamic-like neurons derived from human-induced pluripotent stem cells (hiPSCs) that express E2-induced PR to test the hypothesis that DA activation of PR contributes to an increase in synaptic protein expression.

METHODS

Animals

Female C57/BL6 mice were bred in the Wellesley College Animal Facility and group-housed (3–6/cage) prior to surgery. A 12-hr light/dark cycle was maintained and food and water were provided *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee of Wellesley College and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines.

Ovariectomy and Stereotaxic Surgery

Eight-week old C57/BL6 mice (n=18) were bilaterally ovariectomized under 1.5% isoflurane. Stereotaxic surgery was performed to implant cannula (22 gauge, #81C313GS5SPC, Plastics One, Roanoke, VA, USA) aimed at the 3rd ventricle in the midline, anteroposterior: –1.5 mm from bregma, dorso-ventral: –5mm, as described previously⁵⁴. A dummy cannula was inserted into the guide cannula to keep the tract intact until drug infusion. Mice were individually housed following stereotaxic surgery to prevent cannula displacement by cagemates and to recover from surgery for one week. Then, all mice were injected subcutaneously with estradiol benzoate (EB, 1 µg in sesame oil, #E-8515, Sigma) to induce PR expression in the hypothalamus¹⁷. Forty-eight hours after EB administration, mice were infused with a D1R agonist, SKF 38393 (SKF; #D047, Sigma-Aldrich), or vehicle (Veh, 0.9% sterile saline), through the injector cannula (28 gauge) into the 3rd ventricle. SKF (100 ng in 2µL of 0.9% sterile saline) was infused at a rate of 1µL/min using a 22 gauge Hamilton syringe attached to a stereotaxic instrument (Stoelting, IL)⁵⁴. Thirty mins after the infusion, mice were euthanized by asphyxiation under CO₂ (Figure 1). This dose and time frame for SKF treatment has been previously shown to be sufficient for the PR-dependent expression of sexual receptivity, selectively through D1R activation^{33,54–56}.

Tissue Collection and Protein Extraction

Hypothalamic tissue was dissected using coordinates from the mouse brain atlas¹¹⁷, immediately frozen on dry ice, and stored at -80°C until protein extraction. Hypothalami from 9 mice were pooled to generate each sample. Tissue was added in a tube with Lysing Matrix D (#6913, MP Bio) and lysis buffer (10 mM Tris, 10% glycerol, 400 mM NaCl, 1 mM dithiothreitol (DTT) with EDTA (1 mM, pH 7.4), phosphatase inhibitors (50 mM NaF and 1 mM Na₃V04), and protease inhibitors (1:10 dilution, #P2714, Sigma-Aldrich). The tissue/lysis buffer was added at a 2:1 ratio. The mixture was homogenized at 4 m/s at four 5-sec cycles using Fast Prep 24 (MP Bio), with a 5-sec incubation on ice between every cycle to avoid sample degradation from heating. Lysates were incubated on ice for 30 min followed by centrifugation at 13,200 g for 30 min at 4°C . Supernatants containing proteins were stored at -80°C .

GST-tagged Mouse Progesterin Receptors

Mouse PR-A and PR-B cDNAs were cloned into the CMV-based mammalian cell expression plasmid pcDNAI (Invitrogen, CA), as described previously¹⁶. The DNA construct sequences were verified from the start of the GST to the end of the mouse PR (Genewiz, South Plainfield, NJ). The recombinant proteins containing full-length mouse PR-A or PR-B fused to a glutathione S-transferase (GST) tag were expressed in *Spodoptera frugiperda* (Sf9; RRID:CVCL_0549) insect cells at the Baculovirus/Monoclonal Antibody Facility of the Baylor College of Medicine as described previously^{16,57}. Insect cell cultures for GST-PRA or GST-PRB were grown in the absence of PR ligand. The Sf9 cell pellets containing PR were homogenized in buffer containing 10 mM Tris, 10% glycerol, 400 mM NaCl, 1 mM DTT, 1 mM EDTA (pH 7.4) with protease inhibitors (1:10 dilution, #P2714, Sigma). Lysates were incubated on ice for 30 min, followed by centrifugation at 106,000 g for 30 min at 4°C . Supernatants containing receptors were stored at -80°C .

GST-PR Pull-down Assays

Pull-down assays were done using GST-PRA or GST-PRB with either SKF-, or Veh- treated hypothalamic lysates (Figure 1), as described previously¹⁶. In brief, glutathione sepharose 4B packed resin (50 μL , 0.05 mg/mL, Amersham Biosciences) was pretreated with ovalbumin (1 mg/mL, Fisher Scientific) for 15 min in siliconized centrifuge tubes on an end-over-end rotator at 4°C . Resin was rinsed three times with TG buffer (20 mM Tris-HCl, 10% glycerol) containing 1M urea and 100 mM NaCl (TG-NaCl-U, pH 8.0). Unliganded GST-PRA or GST-PRB, in equal amounts, were suspended in TG buffer with 1M urea (TG-U, pH 8.0) and were incubated with resin on a rotator at 4°C for 1 hr. The immobilized GST-PR in resin was washed four times with TG-NaCl-U (with gradient urea concentrations of 1M, 0.5M, 0.25M and 0M). Equal amounts of pooled hypothalamic homogenates from each treatment condition were added to immobilized GST-PR and incubated on a rotator for 1 hr at 4°C , followed by four washes with TG-NaCl (without urea). Proteins bound to GST-PR resin were eluted by incubating in 250 mM glutathione elution buffer (50 mM Tris-HCl, pH 8.0) at 4°C for 10 min. Any remaining proteins in the resin were eluted by boiling for 5 min in 2X Laemmli sample buffer (Biorad, Hercules, CA) with 35 mM of DTT (Sigma, Saint

Louis, MO), which was used for confirmation of representative mass spectrometry results by western analysis.

Nano-Liquid Chromatography (LC) Mass Spectrometry (MS)/MS Analysis

Tryptic peptides from pull-down assays with unliganded GST-PRA or GST-PRB, were prepared as previously described¹⁶. Samples were block randomized and analyzed in triplicates⁵⁸ and peptide mixtures were analyzed by nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) using a nano-LC chromatography system (UltiMate 3000 RSLCnano, Dionex), coupled on-line to a Thermo Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) through a nanospray ion source (Thermo Scientific). A trap and elute method was used with a C18 PepMap100 trap column (300um X 5mm, 5um particle size; Thermo Scientific). The analytical column was an Acclaim PepMap 100 (75um X 25 cm; Thermo Scientific). After equilibrating the column in 98% solvent A (0.1% formic acid in water) and 2% solvent B (0.1% formic acid in acetonitrile (ACN), the samples (1 µL in solvent A) were injected onto the trap column and subsequently eluted (400 nL/min) by gradient elution onto the C18 column as follows: isocratic at 2% B, 0-5 min; 2% to 45% B, 2-37 min; 45% to 90% B, 37-40 min; isocratic at 90% B, 40-45 min; 90% to 2%, 45-47 min; and isocratic at 2% B, 47-60 min.

All LC-MS/MS data were acquired using XCalibur, version 2.1.0 (Thermo Scientific) in positive ion mode using a top speed data-dependent acquisition (DDA) method with a 3-sec cycle time. The survey scans (m/z 370-1570) were acquired in the Orbitrap at 120,000 resolution (at m/z = 400) in profile mode, with a maximum injection time of 56 msec and an AGC target of 600,000 ions. The S-lens RF level was set to 60. Isolation was performed in the quadrupole with a 1.6 Da isolation window, and HCD MS/MS acquisition was performed in centroid mode using rapid scan rate with detection in the ion trap, with the following settings: collision energy = 32%; maximum injection time 56 msec; AGC target 200,000 ions. Monoisotopic precursor selection (MIPS) and charge state filtering were on, with charge states 2-6 included. Dynamic exclusion was used to remove selected precursor ions, with a $\pm$ 10 ppm mass tolerance, for 15 sec after acquisition of one MS/MS spectrum.

Proteomic Data Analysis

Tandem mass spectra were extracted and charge state deconvoluted by Proteome Discoverer (Thermo Fisher, version 1.4.1.14). Deisotoping was not performed. All MS spectra were searched against a UniProt murine database (version 08-11-2014) using Sequest. Searches were performed with a parent ion tolerance of 5 ppm and a fragment ion tolerance of 0.60 Da. Trypsin was specified as the enzyme, allowing for two missed cleavages, fixed modification of carbamidomethyl (C) and variable modifications of oxidation (M). False discovery rate (FDR) estimation was allowed wherein the peptide-spectrum matches above a 1% FDR were used to generate a spectral library for the quantitative analysis. Data files for each pair of samples were imported into Skyline⁵⁹ for label-free quantitative analysis⁶⁰. Following manual verification of all peaks, triplicate peptide intensities were used to calculate fold changes. The cleaned data were analyzed using Student's *t*-test to determine the effect of SKF within each PR subtype. Proteins from SKF-treated brains that had a 2-

fold association with GST-PR, compared to the Veh-treated brains, and a p-value <0.05 were considered to show an SKF-dependent interaction.

Ingenuity Pathway Analysis of the PR Interactome—Proteins were classified into functional groups based on their primary functions in UniProt database (<http://uniprot.org>). Of the identified proteins, those that belonged to the two largest functional groups, 1) synaptic proteins and 2) regulators of cellular and energy metabolism, were interactively modeled using the Ingenuity Analysis Application (IPA, Qiagen). The relationships depicted in the generated pathway are based on the literature-based interconnectivity data stored in the Ingenuity Knowledge Base Repository. In the network pathway, the types of biological relationships between two proteins are represented using different types of lines.

Western Blot Analysis

Western blot analysis was done as described previously¹⁶ to qualitatively confirm mass spectrometry results for proteins involved in synaptic plasticity (SYN 2 and protein phosphatase 1 regulatory subunit IB, DARPP-32) and metabolism (creatine kinase B). SYN 2 and creatine kinase B were chosen because they were identified to interact with liganded PR in our earlier study¹⁶, while DARPP-32 was shown to be an obligatory mediator of dopamine (DA)-induced female reproductive behavior⁵⁵. Pull-down samples were probed with the following primary antibodies: SYN 2, targeted against domains A and B (mouse monoclonal, 0.3 µg/mL, #mabn1573, Millipore; RRID:AB_2828028); DARPP-32, targeted against the N-terminus (aa 1-100; rabbit monoclonal, 1:100, #ab40801, Abcam; RRID:AB_731843); and creatine kinase B (mouse monoclonal, 10 µg/mL, #sc-373686, Santa Cruz; RRID:AB_10918186). Western blot analysis using a PR antibody (rabbit polyclonal, 1:250, #A0098, DAKO; RRID:AB_2315192) was used to determine equal amounts of GST-PR inputs for pull-down assays. GST-PR was further probed as a reference control in pull-down samples. Fluorescent secondary antibodies donkey anti-mouse AF647 (1:10,000, #A31571, Thermo Fisher; RRID:AB_162542) were used for SYN 2 and creatine kinase B, and donkey anti-rabbit AF546 (1:10,000, #A10040, Thermo Fisher; RRID:AB_2534016) for DARPP-32 and PR. Images of immunoreactive bands were taken using a GelDoc Imager (Bio-Rad, CA).

In vitro Analysis Using Human Induced Pluripotent Stem Cells

Reagents: Progesterone (P4; #P6149), 17P-estradiol (estradiol, or E2; #E8875) were from Merck; SKF 38393 hydrobromide (#0922) and RU486 (mifepristone; #1479) were from Tocris. Pharmacological treatment of cells was performed directly into B27 medium. Drugs were diluted from stock solutions to final concentration in B27 medium; final solvent amount was <0.01%. Vehicle condition consisted of diluting DMSO in the same manner as drugs.

Human Induced Pluripotent Stem Cells (hiPSCs): HiPSC lines were generated from primary keratinocytes as described previously⁶¹. Participants were recruited and methods were carried out in accordance to the 'Patient iPSCs for Neurodevelopmental Disorders (PiNDs) study' (REC No 13/LO/1218). Informed consent was obtained from all subjects for participation in the PiNDs study. Ethical approval for the PiNDs study was provided by the

NHS Research Ethics Committee at the South London and Maudsley (SLaM) NHS R&D Office. HiPSC lines were derived by reprogramming keratinocytes from healthy males and reprogramming was validated (Table 1) as previously described^{61–64}. Briefly, reprogramming was validated by genome-wide expression profiling using Illumina Beadchip v4 and the bioinformatics tool ‘Pluritest’. Additionally, the tri-lineage differentiation potential was established by embryoid body formation. Immunocytochemistry (ICC) was used to validate the expression of different pluripotency markers including Nanog, OCT4, SSEA4 and TRA1-81⁶³ and the alkaline phosphatase activity was assessed by Alkaline phosphatase expression kit (Milipore). The Illumina Human CytoSNP-12v2.1 BeadChip array and KaryoStudio analysis software (Illumina) were used to assess genome integrity (Table 1). HiPSCs were cultured in StemFlex media (#A3 349401, Gibco) on 6-well plates (#140675, Thermo Fisher) coated with Geltrex basement membrane matrix (#A1413302, Thermo Fisher). Cells were passaged upon reaching 60–70% confluency, by incubation with a calcium chelator, EDTA (#15040-33, Thermo Fisher), followed by detachment with a cell lifter to maintain intact iPSC colonies.

Neuronal Differentiation: Neuronal differentiation of hiPSCs was induced by using a modified dual SMAD inhibition protocol⁶⁵. Neural induction was carried out by replacing StemFlex media with a 1:1 mixture of N2 and B27 (Life Technologies) supplemented with 10 μ M SB431542 (Merck), 1 μ M Dorsomorphin (Merck), and 2 μ M XAV939 (Merck) (N2:B27+++ medium). Cells were maintained at 37°C in normoxic conditions in N2:B27+++ medium for 7 days with media replacement every 24 hours. At day (D) 7 cells were dissociated using Accutase (#A11105-01, Life Technologies) and re-plated at a 1:1 ratio into N2:B27+++ medium, supplemented with 10 μ M Rock Inhibitor Y-27632 (Merck) to prevent apoptosis. From D8 onwards, SMAD and WNT inhibitors were excluded from the medium and cells received daily N2:B27 medium alone. Cells were dissociated and re-plated on D12, D16 and D19 and D21. From D9, a uniform sheet of Sox2 and Nestin-positive cells was observed and at D17, cells were observed to self-organize into neuroepithelial rosettes⁶³. On D21, cells were plated into Poly-D-Lysine (5 μ g/ml, # P6407, Merck) and laminin (20 μ g/ml, #L2020, Merck) coated 8-well μ -slides (#80826, Ibd) at a density of 50,000 cell/cm² in B27 medium supplemented with 200 μ M L-ascorbic acid and 10 μ M DAPT (Calbiochem) for 7 days (on D27) to block Notch signaling. Subsequently, neurons were grown in B27 medium with 200 μ M L-ascorbic acid until D50 when they were used for experimentation. The cells were treated with either E2 (10 nM), P4 (1 nM), or RU486 (10 nM) for 24 hrs prior to harvest. The D1/5 receptor agonist SKF38393 (10 μ M) was applied 30 mins prior to the cells harvest. The six treatment groups were: 1) Veh only, 2) E2 only, 3) E2 + P4, 4) E2 + RU486, 5) E2 + SKF, and 6) E2 + SKF + RU486.

qRT-PCR: Total RNA was harvested and lysed with Trizol reagent (Life Technologies) and isolated by centrifugation with 100% chloroform, followed by 100% isopropanol, then with 75% ethanol. The RNA was purified by precipitation with 100% ethanol and sodium acetate (Life Technologies). RNA quantity and quality were first analyzed with the NanoDrop 1000 Spectrophotometer (Thermo Scientific). The quality of RNA was further analyzed for each sample using the Agilent RNA 6000 Nano Kit (#5067-1511, Agilent Technologies) in combination with the Agilent 2100 Bioanalyzer system according to the manufacturer

protocol. The RNA integrity number was measured using Agilent 2100 Bioanalyzer. Residual genomic DNA was removed by addition of TURBO DNA-free (Life Technologies) and incubation at 37°C for 30 minutes. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using random hexamer primers and Superscript III (Life Technologies) following the manufacturer's recommendations. Quantitative real-time PCR (qPCR) was performed with HOT FIREPol EvaGreen qPCR Mix Plus ROX (Solis Biodyne) according to the manufacturer's instructions, in a total volume of 20 µl, containing 1:5 diluted cDNA, qPCR mix, and primers at to a final concentration of 0.3 µM. PCR reaction conditions were 95°C for 15 minutes for the initial denaturation followed by 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds during 33 cycles. The melting curve analyses was preformed from 60°C to 95°C with readings every 1°C. The 2^{-CT} comparative method for relative quantification was used to quantify the genes expression. In order to determine the appropriate housekeeping genes for normalization of the qPCR data, *ACTB*, *B2M*, *GAPDH*, *HMBS*, *HPRT1*, *RPL13A*, *RPL27*, *SDHA*, *UBC*, and *YWHAZ* were screened for efficiency and specificity in the hiPSC lines at multiple times over neuronal differentiation towards a GnRH-neuronal identity. All genes were assayed by determining the relative quantification of the gene expression between hiPSC samples. Based on this analysis, *GAPDH* was chosen as the reference gene as it displayed the lowest variability in expression levels between multiple (x4) hiPSC lines and across all time points assessed.

Immunocytochemistry: Treated neurons were fixed with 4% formaldehyde plus 4% sucrose in PBS. Fixed neurons were permeabilized in 0.1% Triton-X-100 in PBS for 15 minutes and blocked in 4% normal goat serum in PBS for 1 hour at room temperature. Cells were incubated overnight in appropriate primary antibodies at 4°C. The rabbit polyclonal primary antibodies for GnRH (1:200, #HPA027532, Atlas; RRID:AB_10612111), tyrosine hydroxylase (TH; 1:200, #ab152, Millipore; RRID:AB_390204), and vasopressin (1:100, #ab6869, Abcam; RRID:AB_2062094) were used to confirm the hypothalamic-like phenotype of the hiPSC neurons. A chicken polyclonal Tuj1 antibody (1:500, #ab41489, Abcam; RRID:AB_727049) was used as a neuronal marker and a rabbit polyclonal MAP2 antibody (1:1000, #ab32454, Abcam; RRID:AB_776174) was used to outline neuronal morphology. Progesterone receptor and synapsin1/2 expression in the hiPSC neurons were confirmed using a mouse monoclonal (1:500, #MA5-12658, Invitrogen; RRID:AB_11000632) and a chicken polyclonal (1:1000, #106006, Synaptic Systems; RRID:AB_2622240) primary antibodies, respectively. Immunoreactivity was achieved by incubating the cells with 1:500 concentration of Alexa Fluor 594 anti-mouse, Alexa Fluor 594 anti-goat and Alexa Fluor 488 anti-rabbit IgGs in blocking buffer. For nuclei staining, a 1:2000 concentration of DAPI (Thermo Fisher) was used.

Microscopy and Image Analysis: Confocal images were taken as z-stacks with a Leica SP5 laser scanning confocal microscope equipped with 405/488/594nm lasers and using a 63x NA1.4 objective. Identical laser gain and offset settings were used within each biological replicate to enable direct comparison between conditions. Two-dimensional maximum projection reconstructions of images were generated and intensity analyses were performed using ImageJ (rsb.info.nih.gov/ij/). In all images, MAP2 was used to identify neurons and outline neuronal morphology. Mean gray value of PR intensity was measured within the cell

body and along the first 10 μm of each neuron analyzed. SYN 1/2 intensity (mean gray value) was measured along 50 μm of dendrite from each neuron analyzed. In all experiments, 3 clones per hiPSC line were used; each clonal line was used in at least 2 independent differentiations for each condition imaged and analyzed: 18-30 neurons per condition were analyzed.

Statistical Analysis: All statistical analyses were performed in GraphPad (RRID:SCR_000306). All data sets were tested for normality using the D'Agostino & Pearson normality test prior to inferential statistical analyses (D'Agostino & Belanger, 1990). Datasets were found to be normally distributed, thus were measured by one-way-ANOVAs with Tukey's post-hoc test to correct for multiple comparisons. Differences were considered significant if $p < 0.05$. Error bars represent either standard deviation or SEM, as stated in each bar graph.

Results

Mass spectrometry reveals an interaction between PR-A and hypothalamic proteins from SKF-treated mice

To test if PR-A or PR-B, in the absence of P4, interact with proteins from the female mouse hypothalamus in a DA-dependent manner, a DIR agonist, SKF, was infused into the 3rd ventricle. Mouse GST-PRA and GST-PRB were separately used to pull-down hypothalamic proteins from SKF or Veh-treated female mice. Prior to statistical analyses, 525 and 281 proteins were detected to interact with unliganded PR-A and unliganded PR-B, respectively. Given that the focus of the present study was the identification of the SKF-dependent PR interactome, applying a cutoff of $p < 0.05$ and at least a 2-fold change yielded 316 hypothalamic proteins from SKF-treated mice that interacted with GST-PRA compared to Veh mice (Table 2). Interestingly, none of the 281 proteins that interacted with unliganded PR-B did so in an SKF-dependent manner, suggesting limited involvement of PR-B in DA activation of PR. Although many identified proteins are known to exert multiple functions, proteins were functionally grouped based on their primary functions in UniProt database (<http://uniprot.org>). As shown in Table 2, the largest number of proteins are important for synaptic structure and function (62 out of 316; 20%). Other groups, including members of cytoskeleton, calcium regulation, electron transfer/ion channel, and cell signaling, all known to be classically involved in synaptic transmission and plasticity, were also identified. The second largest functional group of proteins that interacted with PR-A in an SKF-dependent manner are involved in cellular and energy metabolism (57 out of 316; 18%). In addition, regulators of transcription, translation, ubiquitination, protein folding/molecular chaperones, and cell proliferation were identified in the SKF-dependent PR-A interactome (Table 2).

Synaptic proteins from SKF-treated female mouse hypothalamus interact with PR-A

Extending our earlier findings that showed that synaptic proteins interacted with ligand-activated PR¹⁶, in the current study, we identified a large number of synaptic proteins that interacted with PR-A in a ligand-independent, but DA-dependent manner (Table 2). For example, many synaptic terminal proteins such as synaptophysin, β -synuclein, γ -synuclein, and synaptotagmin-1 from SKF-treated hypothalamus interacted with PR-A. In addition,

proteins implicated in synaptic vesicle exocytosis, such as synapsin 2 (SYN2), synapsin 3 (SYN3), synaptic vesicle membrane protein VAT-1 homolog-like (VAT1L), synaptosomal-associated protein 25 (SNAP25), amphiphysin, vesicle-associated membrane protein 1 (VAMP1), and VAMP2 also showed DA-dependent interactions with PR-A. Moreover, proteins necessary for neurotransmitter synthesis (e.g. aspartate aminotransferase (AATC), proSAAS), neurotransmitter turnover (e.g. glutamate decarboxylase 2 (DCE2), glutamine synthetase (GLNA), 4-aminobutyrate aminotransferase (GABT), succinate-semialdehyde dehydrogenase (SSDH), and glutamate dehydrogenase 1 (DHE3)), and neurotransmitter transport (excitatory amino acid transporter 1 (EAA1) and EAA2) from SKF-treated hypothalamus preferentially interacted with PR-A compared to the Veh-treated mice. Consistent with the role of Ca^{2+} regulation on synaptic neurotransmitter release, a number of Ca^{2+} -dependent proteins also showed a DA-dependent interaction with PR-A (Table 2). In addition, transporter proteins interacted with PR-A suggesting a role for the receptor in synaptic transmission (Table 2). Other functional groups, including kinases and phosphatases, and their diverse substrates (e.g. those belonging to cytoskeletal proteins, ubiquitination enzymes, ion channel proteins, and molecular chaperones) interacted with PR-A in a DA-dependent manner.

Regulators of energy metabolism from mouse hypothalamus interact with PR-A in an SKF-dependent manner

The current interactome data suggest that PRA-dependent regulation of energy metabolism is extensive and involves enzymes necessary at multiple steps of energy metabolism, including glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation, gluconeogenesis, and glycogenolysis (Table 2). The mediators of glycolysis (including glyceraldehyde-3-phosphate dehydrogenase (G3P), glucose-6-phosphate isomerase, glucose-6-phosphate isomerase (G6PI), phosphoglycerate kinase 1, pyruvate kinase PKM, 6-phosphofructokinase (type C and muscle type)) from SKF-treated, but not Veh-treated, mouse hypothalamus interacted with PR-A. The enzymes required for TCA cycle, such as citrate synthase, malate dehydrogenase, aconitate hydratase, fumarate hydratase, and isocitrate dehydrogenase a (IDH3A), interacted with PR-A in a DA-dependent manner. Proteins integral in oxidative phosphorylation, including dihydrolipoyl dehydrogenase, cytochrome C, ATP synthase A, ATP synthase B, succinyl-CoA ligase A, and malate dehydrogenase, also showed DA-dependent interactions with mouse PR-A. In addition, proteins that protect cells against oxidative stress, such as peroxiredoxin1 (PRDX1), PRDX2, PRDX5, and PRDX6 from SKF-treated hypothalamus, preferentially interacted with PR-A. Additionally, proteins required for gluconeogenesis (e.g. L-lactate dehydrogenase A (LDHA) and LDHB), glycogen utilization (e.g. glycogen phosphorylase brain form (PYGB) and phosphoglucomutase-1 (PGM1), and pyruvate metabolism (e.g. pyruvate dehydrogenase E1B), all showed an SKF-dependent interaction with PR-A. Other proteins, such as apolipoprotein E, acetyl-CoA acetyltransferase, fatty acid synthase, and enoyl-CoA hydratase that are integral for lipid metabolism also preferentially interacted with PR-A in a SKF-dependent manner.

PRA-interacting mouse hypothalamic protein networks

Using Ingenuity Pathway Analysis, the network pathway for synaptic proteins (Figure 2) and metabolic regulators (Figure 3) revealed that the majority of these proteins form complexes with, or directly act (solid lines) on other identified proteins, whereas a smaller subset of these proteins act indirectly (dotted lines) with other proteins in the network. In addition, many proteins in these functional groups possess auto-regulatory mechanisms (indicated by an incomplete circle in Figures 2 and 3) by direct auto-regulation (arrow) or by forming complexes with themselves (no arrow). The networks provided further evidence of the functional interactions of the synaptic proteins and metabolic regulators identified in the current study.

PR-A interaction with select hypothalamic proteins were verified by western blotting

For the qualitative validation of findings from mass spectrometry, proteins from SKF- or Veh-treated hypothalami pulled-down using PR-A were analyzed by western blot. Consistent with mass spectrometry results, a more intense creatine kinase B immunoreactive band (~45 kDa) was detected in pull-downs with SKF-treated samples compared with Veh-treated tissue (Figure 4). DARPP-32 (~35kDa), a protein critical for DA-mediated activation of PR in brain and for mediation of female sexual receptivity⁵⁵, from SKF-treated hypothalamus also interacted strongly with PR-A compared to Veh-treated hypothalamus. Moreover, SYN 2b, and to a lesser extent SYN 2a, interacted with unliganded PR-A in an SKF-dependent manner, together confirming DA-dependent interactions of these proteins with PR-A. Similar intensities of PR-A bands were observed in SKF and Veh lanes, confirming equal amounts of protein loaded in each pull-down assay (Figure 4).

Generation of hypothalamic-like neurons from human induced pluripotent stem cells

Because the largest group of hypothalamic proteins that interacted with PR were synaptic proteins, including SYN1/2, we investigated if PR and SYN1/2 were co-expressed and regulated in neuronal processes, including dendrites. The present PR interactome data, taken together with previous findings¹⁶, indicate that DA-dependent modulation of synaptic protein expression requires E2-induced PR. Therefore, we generated a population of olfactory placode progenitor cells from hiPSCs, which subsequently could be differentiated into a population of hypothalamic-like GnRH-positive neurons that express E2-induced PR^{63,66–68}, to test the DA-dependent functional interaction between the PR and synaptic proteins.

HiPSC lines were derived by reprogramming keratinocytes from healthy males (Table 1) as previously described^{61–64}. HiPSC lines were differentiated into neuroepithelial cells using a modified dual SMAD inhibition differentiation protocol (Figure 5A) as previously described⁶³. QPCR analyses demonstrated that the neuroepithelial marker *SOX2* was highly expressed at day (D)7 and was gradually downregulated during the 50 days of neuronal fate acquisition (Figure 5B). *PAX6*, a marker of forebrain development, was highly expressed from D7 to D20, when cells were terminally differentiated. After this time point, *PAX6* levels were greatly reduced in the cells (Figure 5C). A hypothalamic-like identity of these cells was confirmed by the high expression of the transcription factor NK2 homeobox 1 (*NKX2.1*) in D12 and D18 neural progenitor cells (NPCs) (Figure 5D)^{69,70}. We also found

that *LHX6*, a factor known to be involved in hypothalamic specification^{71,72} was highly expressed in NPCs (Figure 5D).

We next confirmed the generation of GnRH-positive hiPSC neurons by qPCR; *GnRH* was expressed highly in D26 and D50 neurons (Figure 5E). Interestingly, the expression of estrogen receptor genes *ESR1* and *ESR2* increased between the early time point and older, D50, neurons (Figure 5E). Immunocytochemical analysis at D26 further confirmed that the majority of cells generated were positive for β -III tubulin (Tuj1), a marker of neuronal fate, and highly expressed GnRH, tyrosine hydroxylase (TH) and vasopressin (Figure 5F). Taken together, these data suggest the successful generation of GnRH-positive hypothalamic-like neurons (hiPSC neurons).

Estradiol induces PR expression in GnRH-expressing hiPSC neurons

GnRH-positive hiPSC neurons generated from male hiPSCs responded to E2 treatment with a significant increase in PR expression (Figures 6A and 6B). Since a large number of synaptic proteins from SKF-treated hypothalamus interacted with PR-A, we next investigated if PR is localized in the dendrites, the functionally relevant regions of these synaptic proteins, and if the dendritic fraction of PR is also altered by treatments. PR expression was observed in the nucleus, cell bodies, and along MAP2-positive dendrites (Figure 6A). E2 increased both the nuclear and extra-nuclear PR expression in these neurons (Figure 6C). Despite the increase in nuclear expression, PR was predominately expressed in extra-nuclear locations, consistent with previous studies^{39,40,73}. The E2-induced increase in both nuclear and non-nuclear PR expression was not altered by co-treatment with either P4 or SKF. Similarly, PR antagonism with RU486 also had no effect on E2-induced PR expression ($F_{(5,93)}=5.467$, $p = 0.0002$, Tukey post-hoc, one-way ANOVA; $n = 12$ -20 cells from 3 independent hiPSC lines; Figures 6B and 6C).

After confirming PR expression in the dendritic processes of these hiPSC neurons, we then asked if PR and SYN1/2 are co-expressed in dendrites to verify the functional interactions of PR with these hypothalamic synaptic proteins. Indeed, dendrites positively labeled for both E2-induced PR and SYN1/2 were detected in all E2-treated conditions (Figure 7A). Taken together, these data demonstrate that these hiPSC-derived neurons are GnRH-positive and express E2-induced PR, including in the SYN1/2-positive dendrites and thus serve as an appropriate cell model to test the effects of SKF on synapses mediated by E2-induced PR.

Dopaminergic D1R activation increases synapsin1/2 expression in GnRH-positive hiPSC neurons in a PR-dependent manner

We have previously shown that activation of E2-induced PR by P4 increases synaptic protein expression¹⁶. To determine if SKF activation of PR can also regulate SYN 1/2 expression, we examined their expression in D50 hiPSC neurons following treatment (Figure 7A). All neurons were treated with E2 to induce PR expression. Interestingly, E2 alone significantly increased SYN 1/2 expression over vehicle condition (Figure 7B). Moreover, co-treatment with E2 and P4 induced a further increase in SYN 1/2 expression compared to E2 alone (Figure 7A and 7B). This was also mimicked by co-treatment with E2 and SKF (Figure 7A and 7B). Critically, the effect of E2 and SKF on SYN1/2 expression was attenuated by

RU486, indicating the requirement of PR in mediating this effect ($F_{(5,142)}=29.34$, $p < 0.0001$, Tukey post-hoc, $n = 15$ -34 cells from 3 independent hiPSC lines; Figure 7B).

Discussion

In the absence of P4, DA activation of PR in rodent hypothalamus induces female reproductive behavior^{33,50,54}. In the current study, we tested the hypothesis that proteins from female mouse hypothalamus treated with SKF, a D1R agonist, interact with mouse PR in the absence of P4. Using pull-down assays, we identified many proteins from SKF-treated mouse hypothalami that interact with mouse PR-A, but not PR-B, in the absence of P4, suggesting these proteins function in ligand-independent activation of PR-A. The majority of the DA-dependent PR-A interactome was comprised of synaptic proteins. To investigate the functional implications of these DA-dependent interactions of synaptic proteins with PR-A, hiPSC neurons that exhibit hypothalamic-like phenotype and express GnRH and E2-induced PR were used to test PR-dependent effects on synaptic proteins. PR was co-expressed with SYN1/2 in the dendrites of hiPSC neurons, providing evidence for the functional interaction between the two proteins. Furthermore, we found that SYN1/2 expression is upregulated in a PR-dependent manner. In these neurons, P4 increased SYN 1/2 expression, confirming our previous findings that P4 functions in synaptic plasticity¹⁶. Moreover, in the absence of P4, DA activation of PR also increased SYN 1/2 expression, which was blocked by the PR antagonist RU486, suggesting this effect of SKF is PR-dependent. Taken together, the present findings suggest that DA activates hypothalamic PR via phosphorylation of proteins, such as DARPP-32⁵⁵ and facilitates the interaction of PR with additional neuronal proteins. The activated PR can further induce synaptic protein expression that could be important in the regulation of brain function and behavior. This ligand-independent activation of PR by SKF could mediate genomic or non-genomic changes^{33,50,54}. Proteins that are integral in energy metabolism comprised the second largest functional group that complexed with PR-A in a DA-dependent manner, suggesting PR-A functions in energy homeostasis. Furthermore, PR-A interacted with cytoskeletal proteins, ion channels, calcium sensors, and cell signaling proteins, supporting a variety of important functions of PR-A in brain. Proteins required for DNA synthesis and protein synthesis, folding, and transport also complexed with PR-A in a DA-dependent manner in the absence of P4, as demonstrated by mass spectrometry analysis of hypothalamic proteins pulled-down using unliganded PR. In addition, proteins integral for posttranslational modifications (*e.g.* phosphorylation, dephosphorylation, and ubiquitination) interacted with mouse PR-A. The multiple proteins identified suggest that PR function can be regulated in a tissue- and context-specific manner depending on their binding partners and that the interaction of PR and DA systems affect multiple cellular processes, including metabolism and synaptic plasticity.

DA-dependent interactions of hypothalamic synaptic proteins with PR-A

Progestins act via PR to strengthen synapse structure and function in rodent brain^{16,41–45,74}. PR are expressed in dendritic spines and axon terminals in the hypothalamus and hippocampus of female rodents^{39,40,75}. Moreover, PR activation increases dendritic spines and synapse density^{16,43}. Synaptic vesicle-associated proteins identified in the current study, including syntaxin-1, SNAP25, synaptotagmin-1, SYN2, SYN3, VAMP1, VAT1L are

important for vesicle docking and neurotransmitter release in a Ca^{2+} -dependent manner^{76,77}. In particular, synapsins (*e.g.* SYN2 and SYN3) are phosphoproteins essential for synapse formation, maturation, transmission, and remodeling^{78,79}. Our previous findings reveal that SYN1, SYN2 and SYN3 interact with PR in a P4-dependent manner¹⁶. Moreover, P4-induced synapse formation is dependent on SYN1 in rat primary cortical neurons¹⁶. Notably, an SKF-mediated increase in SYN1/2 expression in GnRH-positive hiPSC neurons suggests that PR alters synaptic plasticity by upregulating synaptic proteins and facilitating their interactions with activated PR^{16,45}. In the present studies, additional synaptic proteins from SKF-treated brain that function in the synthesis, transport and clearance of neurotransmitters (EAA1, EAA2, AATC, DCE2, GLNA, GABT, SSDH, DHE3, and PCSK1) interacted with PR-A^{80–83}. Taken together, these findings suggest that PR regulates synaptic physiology to elicit profound effects in brain function and behavior^{14,18}.

Phosphorylation of PR is an important step in DA-mediated PR activation of female reproductive behavior in rodents^{33,49,54,84}. Kinases, phosphatases and their substrates that were identified to interact with PR-A in the present study can directly act on synapses. In particular, DARPP-32, a substrate of D1R required for PR function⁸⁵, was identified to associate with activated PR-A in the current study. DARPP-32 is expressed in PR-positive regions of the female hypothalamus^{58,86,87} and is required for DA-mediated PR induction of reproductive behavior in female rodents^{55,87}. There was little to no SKF induction of sexual behavior in mice treated with antisense to DARPP-32 and in DARPP32-KO mice⁵⁵. DA-dependent sexual behavior and SKF-mediated changes required for sexual receptivity were also blocked by the D1R antagonist SCH 23390, suggesting D1R is required for SKF-mediated hypothalamic PR activation^{56,88}. While we did not detect D1R in the hypothalamic PR interactome, PR interacted with DARPP-32, an obligatory protein for DA-dependent PR activation and reproductive behavior in female rodents^{55,86,87}. Some phosphorylating partners, such as Src kinase from female mouse hypothalamus interact with mouse PR-A, whereas activated human PR-B interacts with Src kinase in breast cells, suggesting tissue- and/or species-specific interactions of the PR isoforms^{16,51}. Taken together, the DA-dependent phosphorylation of DARPP-32 and other synaptic proteins are required for PR activation in the absence of P4.

Another enriched functional group in the DA-dependent PR-A interactome is the calcium signaling proteins. A tight regulation of Ca^{2+} influx is critical to synaptic physiology⁸⁹. Excess Ca^{2+} influx can result in glutamate-induced excitatory damage, a common feature of traumatic brain injury (TBI) and other neurological disorders⁹⁰. Progesterone provides neuroprotection in TBI^{2,3,91} by blocking calcium channels in synapses⁹² and attenuating glutamate excitotoxicity⁸³. The calcium regulators that are known to have protective effect against TBI, including calcineurin B1⁹³ and calcium/calmodulin-dependent protein kinase type II (KCC2/CAMKII)⁹⁴, glutamate transporters EAA1 and EAA2, and glutamate-degrading enzymes GLNA and DHE3, all interacted with PR-A from SKF treated hypothalamus, suggesting that PR-A activated by DA provides neuroprotection through Ca^{2+} -dependent regulation of synaptic glutamate levels. Excess Ca^{2+} accumulation can also compromise mitochondrial function and induce oxidative stress⁹⁵, which is a hallmark of many neurodegenerative disorders^{96,97}. Progestins improve mitochondrial functions, including ATP production and protection from oxidative damage in rat brain⁴. In the current

study, the mitochondrial peroxiredoxins, PRDX1, PRDX2 and PRDX6, from SKF-treated hypothalamus associated with PR-A, suggesting PR mediates neuroprotection against oxidative insults in a DA-dependent manner.

PR-A interacts with energy metabolism-related hypothalamic proteins in a DA-dependent manner

Progestins regulate energy homeostasis in women, non-human primates and rodents ^{16,98–101}. In support, PR are expressed in adipocytes and muscles and P4 increases glycogen breakdown and decreases glucose transport to muscles ^{102,103}. Isoform-specific interaction assays showed that PR-A, but not PR-B, associates with hypothalamic metabolic regulators when activated by P4 ¹⁶. In the present study, several key metabolic mediators (*e.g.* regulators of glycolysis, TCA cycle, glycogen utilization, and oxidative phosphorylation) associated with PR-A in a DA-dependent manner, strongly supporting a role for PR in energy metabolism in the hypothalamus. For example, creatine kinase B, a major catalyzer of ATP production in brain, complexed with PR-A, but not PR-B, in the presence of P4 ¹⁶ or in the absence of P4 in a DA-dependent manner, as confirmed by mass spectrometry and western blotting. Consistent with P4-dependent activation in rat skeletal muscle ¹⁰³, we identified an increased interaction with PR-A of a rate limiting enzyme for glycogenolysis, PYGB from SKF-treated brain, suggesting that DA activation of PR-A increases energy availability to neurons. PR can also alter energy metabolism in non-neuronal tissues. Elevated levels of PR in breast tumors in obese rats correlate with increased expression of proteins involved in carbohydrate and lipid metabolism, including glycolysis (G6PI and G3P), TCA cycle (IDH3A and PGK1), and glycogen utilization (PYGB and PGM1) ¹⁰⁴, which all associated with PR-A following DA activation in the present study. The present findings extend these previous studies in breast tumors to suggest that DA-activated PR (in the absence of P4) contribute to the metabolic functions of PR in brain. The identification of these PR-dependent pathways that are mediated by DA and conserved in brain and breast tumors could enable the development of common therapeutic targets for these tissues.

Because synaptic proteins were previously identified as the largest functional group that interact with P4-activated PR ¹⁶, in the current study we sought to determine if the synaptic proteins also function in ligand-independent PR activation. In particular, SYN1/2, the synaptic proteins that complexed with PR in both ligand-bound and unliganded conditions, are increased along dendrites of hiPSC neurons following DA-dependent PR activation. This increase was blocked by RU486, suggesting this effect is PR-specific. Furthermore, ICV administration of SKF 30 minutes prior to tissue collection increased SYN1/2 expression compared to the E2-treated group. This SKF-induced increase in dendritic SYN1/2 was also effectively blocked by the PR antagonist RU486 suggesting the effect is PR-dependent. While RU486 can inhibit both transcriptional activity of PR ¹⁰⁵ and block the SKF-dependent PR facilitation of reproductive behavior in female rodents ¹⁰⁶, our present data are limited in providing specific mechanisms by which SKF increases, and RU486 inhibits, this PR-mediated SYN1/2 expression. Taken together, these data suggest that PR can affect expression of proteins regulating synaptic function in both a ligand-dependent and -independent manner.

Interestingly, there was a lack of interaction of PR-B with proteins from SKF-treated hypothalamus. It is possible that the PRB-interactome signals were not strong enough to be detected by mass spectrometry. In addition, it should be noted that the mouse PR-B used in the present pull-down assays were not endogenously expressed in mouse hypothalamus, however Sf9 insect cells are a well-established system for the expression of tagged proteins, including GST-PRA and GST-PRB^{16,107}. In support of the present findings, a dominant role for ligand-dependent or ligand-independent activation of PR-A over PR-B has been reported in brain, breast and ovarian tissue^{108,109}.

GnRH-positive hiPSC neurons as an *in vitro* model for the study of PR function in synapses

In this study, GnRH-positive hiPSC neurons are provided as a suitable *in vitro* model to investigate the DA-dependent effects of PR in synapses. These neurons exhibit E2 and P4-dependent regulation of GnRH expression and function^{66–68}. PR are co-expressed with SYN1/2 in these neuronal processes, providing evidence for the significance of the interactions detected via pull-down assays. Furthermore, SKF upregulated SYN1/2 in a PR-dependent manner in these neurons, suggesting that these neurons are an appropriate *in vitro* model for studying ligand-independent activation of PR.

However, it should be noted that there are substantial differences between these hiPSC-derived neurons and rodent GnRH neurons found in the hypothalamus. GnRH neurons originate from outside the hypothalamus, from a subset of neural progenitors found within the olfactory placode, which subsequently migrate into the hypothalamus. There is also evidence that GnRH neurons may descend from neural crest progenitors¹¹⁰. The differentiation protocol used in this study best recapitulates the generation of GnRH neurons from placode progenitors^{111,112}. In humans, GnRH neurons that emerge from the olfactory placode migrate to the hypothalamus, as well as other brain regions¹¹³. It is possible that we have modeled the effects of E2 and P4 on GnRH neurons that are found outside of the hypothalamus. Based on RNA sequencing studies, the GnRH-positive hiPSC neurons express a number of genes that have not been previously described in GnRH neurons from animal models¹¹⁴, but instead best resemble human fetal GnRH neurons. These differences could explain the disparities between the present study and those from GnRH neurons in adult rodents¹¹⁴.

Conclusions and Future Directions

An enhanced representation of synaptic proteins and metabolic regulators in the ligand-independent PR interactome provides novel mechanisms for activation of PR by DA in the brain. In particular, DA-dependent interactions of hypothalamic proteins with PR-A, but not PR-B, is supported by previous findings that PR-A exerts a dominant role in ligand-independent activation by DA in inducing female reproductive behavior³³. Using physiologically relevant brain tissue, the present study identified novel interactions of DA-activated hypothalamic proteins, including a large set of synaptic proteins, with PR-A in the absence of P4. Although pull-down assays are a powerful approach to study protein-protein interactions *in vitro* due to its high sensitivity, it will be important in future studies to

confirm the present findings using co-immunoprecipitation to identify hypothalamic PR protein interactions *in vivo* and to investigate the direct functional outcomes of these interactions in brain function and behavior. Using a cell model of GnRH-positive hypothalamic-like neurons derived from human-induced pluripotent stem cells (hiPSCs) that contain E2-induced PR, we found that DA activation of PR upregulates proteins important for synaptic plasticity. Notably, no interactions of hypothalamic D1R with PR, regardless of SKF treatment, were detected. To determine if DA can act directly on PR in neurons, it will be important in future studies to investigate if D1R and PR are co-expressed in mouse PR-expressing hypothalamic and extra-hypothalamic regions, such as the ventral tegmental area (VTA) ¹¹⁵, posterodorsal medial nucleus of the amygdala (MePD) ⁸⁸, and cortex ¹¹⁶. The present PR-protein interaction findings also suggest important functions of PR-A in metabolic homeostasis. These novel findings of protein complexes involved in DA activation of PR in the absence of hormone provide potential candidates for therapeutic targets for a variety of disorders of reproduction, mental health and metabolism in women.

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Mouse Procedures & Treatments

Figure 1.

Experimental outline. A) Timeline of procedures and treatments of mice prior to collection of the hypothalamic tissue and *in vitro* protein-protein pull-down assays with GST-tagged mouse PR and hypothalamic lysates treated with (+) or without (–) SKF. OVX, ovariectomy; ICV, intracerebroventricular; 3V, 3rd Ventricle; GST, glutathione S-transferase tag; mPR, mouse progesterin receptor.

Figure 2.

Ingenuity Pathway Analysis (IPA) network reveals hypothalamic synaptic proteins that associate with unliganded PR-A in an SKF-dependent manner. Proteins shaded in pink were identified in this study, while those in white were added to the network based on curated relationships in the IPA database.

— :direct interaction; ----- : indirect interaction; ACTR1A: Alpha-centractin; CNTN1: Contactin-; CPLX2: Complexin-2; DNM1: Dynamin-1; NAPB: Beta-soluble NSF attachment protein; NSF: Vesicle-fusing ATPase; RAB1A: Ras-related protein Rab-1A; RAB3A: Ras-related protein Rab-3A; SEPT (5, 6, 7, 11): Septin-(5, 6, 7, 11); SLC1A2:

Excitatory amino acid transporter 2; SLC1 A3: Excitatory amino acid transporter 1; SNAP25: Synaptosomal-associated protein 25; SNCB: Beta-synuclein; SNCG: Gamma-synuclein; STX1B: Syntaxin-1B; STXBPI: Syntaxin-binding protein 1; SYN (2,3): Synapsin (2, 3); SYP: Synaptophysin; SYT1: Synaptotagmin-1; TNR: Tenascin-R; VAMP2: Vesicle-associated membrane protein 2

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Figure 3.

Ingenuity Pathway Analysis (IPA) network shows proteins involved in cellular and energy metabolism that associate with unliganded GST-PRA in an SKF-dependent manner. Proteins shaded in blue were identified in the present study, while those in white were added to the network based on curated relationships in the IPA database.

— :direct interaction; ----- : indirect interaction; ACO2: Aconitate hydratase, mitochondrial; AMPK: 5'-AMP-activated protein kinase; CS: Citrate synthase; DLAT: Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase

complex, mitochondrial; DLD: Dihydrolipoyl dehydrogenase, mitochondrial; EGLN: Egl nine homolog; FH: Fumarate hydratase; IDH3A: Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial; LDH (A, B): L-lactate dehydrogenase (A, B); MDH1: Malate dehydrogenase, cytoplasmic; MDH2: Malate dehydrogenase, mitochondrial; ME1: NADP-dependent malic enzyme; PCSK1N: ProSAAS; PDHB: Pyruvate dehydrogenase E1 component subunit beta, mitochondrial; PFKM: ATP-dependent 6-phosphofructokinase, muscle type; PFKP: ATP-dependent 6-phosphofructokinase, platelet type; PGD: 6-phosphogluconate dehydrogenase, decarboxylating; PGM1: Phosphoglucomutase-1; PI3K: Phosphatidylinositol 3-kinase; PKM: Pyruvate kinase PKM; PRDX (1,2,5,6): Peroxiredoxin (1,2,5,6); Srebp: Sterol regulatory element-binding protein; T3-TR-RXR: T3-Thyroid hormone receptor-Retinoid-X receptor; TXNRD1: Thioredoxin reductase 1, cytoplasmic

Figure 4.

Hypothalamic proteins interact with mouse PR-A in an SKF-dependent manner. Western blots (n=9 samples pooled/ treatment condition) show that creatine kinase B, DARPP-32, and synapsin 2a and synapsin 2b from hypothalami treated with the DIR agonist SKF strongly associate with PR-A in the absence of progesterone as compared to Veh samples. Input (0.25% of total) from hypothalamic extracts from Veh (lane 1) and SKF- (lane 2) treated mice.

Figure 5.

Generation of GnRH-positive hypothalamic-like neurons (hiPSC neurons). (A) Schematic of hiPSC differentiation protocol used. (B and C) Expression profile of cell fate markers determined between day 7 (D7) and D50 following induction of neural differentiation; error bars represent SD. Neuroepithelial marker *SOX2* (B) and forebrain marker *PAX6* (C) are both highly expressed during D7-D20, but their expressions are greatly reduced thereafter. (D) Expression levels of *NKX2.1* and *LHX6*, markers of hypothalamic-like identity were highly expressed in D12 and D18 neural progenitor cells (NPCs); error bars represent SEM. (E) Assessment of mRNA levels in immature (D26) and developing (D50) neurons revealed an increase in expression levels for *GnRH* as well as estrogen receptors *ESR1* and *ESR2*, error bars represent SEM. (F) Representative images of hiPSC-neurons (D26) immunostained for markers of specific cell fate. This revealed that D26 immature hiPSC-neurons were positive for the neural marker Tuj 1, GnRH, tyrosine hydroxylase (TH) and vasopressin. Scale bar = 100 μ m.

Figure 6.

Induction of progesterin receptor (PR) expression by estradiol (E2) in GnRH-positive hiPSC neurons. (A) Representative confocal images of day 50 hiPSC neurons immunostained for MAP2 (morphological marker) and PR; DAPI was used to identify nuclei of cells. HiPSC-GnRH-neurons from 3 hiPC-lines were neutralised and subsequently treated with 10 nM E2, 1 nM progesterone (P4), or 10 nM RU486 for 24 hours. For the groups that received SKF38393 (SKF; D1-dopamine receptor agonist), a 30-min incubation with SKF followed 24 hrs of the respective hormone treatment. Red arrowheads indicate PR expression within cell body; yellow arrows indicate elevated PR expression along MAP2-positive neurites. Pseudo images reference PR abundance: darker colours indicate lower expression, whereas red/yellow pseudocolouring indicate increased abundance. Scale bar = 10 μ m. (B) Quantification of PR expression along MAP2-positive dendrites. (C) Quantification of nuclear PR expression. Error bars represent SEM and symbols display data points from individual cells analysed; n = 12-20 cell from 3 independent hiPSC-lines. (* p < 0.05, ** p < 0.01, *** p < 0.001; compared to Veh; one-way ANOVA with Tukey post-hoc correction).

Figure 7.

Regulation of synapsin1/2 expression by D1 receptor activation in GnRH-positive hiPSC neurons. (A) Representative confocal images of cells co-stained for MAP2 (blue), progesterin receptor) PR (magenta) and synapsin1/2 (green). Neurons were treated with 10 nM estradiol (E2), 1 nM progesterone (P4), or 10 nM RU486 for 24 hours. For the groups that received SKF (D1-receptor agonist), a 30-min incubation with SKF followed 24 hrs of the respective hormone treatment. PR and synapsin 1/2 expression can be seen in the same cell, including along MAP2-positive dendrites of day 50 hiPSC neurons. Dashed yellow rectangles show regions magnified in (B). Scale bar = 10 μ m. (B) Yellow arrowheads indicate synapsin 1/2 expression along MAP2-positive neurites. Pseudo images reference synapsin 1/2 abundance: darker colours indicate lower expression, whereas red/yellow pseudocolouring indicate increased abundance. Scale bar = 5 μ m. (C) Quantification of synapsin 1/2 expression along MAP2-positive dendrites following treatments. Error bars represent SEM and symbols display data points from individual cells analysed; n = 15-34 cells from 3 independent hiPSC-lines (different letters denote differences among groups; one-way ANOVA with Tukey post-hoc correction).

Table 1.Overview of hiPSC quality control ^{61,64}.

hiPSC Line	Age (years)	Sex	Reprogramming method	CytoSNP-12 v2.1	EB Differentiation	Expression of pluripotency markers
CTR_M2_42	30-40	M	Lentivirus	Passed	Passed	Passed
CTR_M3_15	30-40	M	Lentivirus	Passed	Passed	Passed
CTR_M3_22	30-40	M	Lentivirus	Passed	Passed	Passed

Table 2.

Proteins from female mouse hypothalamus associate with unliganded PR-A in an SKF-dependent manner as identified by mass spectrometry. Proteins are assigned functional groups based on their known primary function. A total of 316 proteins that associate with unliganded GST-PRA with an SKF/Veh ratio ≥ 2 and $p < 0.05$ by t -test are included.

UniProt ID	P-values	Fold change SKF/Veh	Protein Name
Synaptic proteins			
SYUG	0.00006	9.40	Gamma-synuclein
EAA1	0.00001	6.71	Excitatory amino acid transporter 1
EAA2	0.00002	5.89	Excitatory amino acid transporter 2
NCAM1	0.00156	5.78	Neural cell adhesion molecule 1
RAC1	0.01584	5.61	Ras-related C3 botulinum toxin substrate 1
GABT	0.00001	5.58	4-aminobutyrate aminotransferase, mitochondrial
VAT1L	0.00105	5.35	Synaptic vesicle membrane protein VAT-1 homolog-like
DNM1L	0.00274	4.64	Dynamin-1-like protein
SCG2	0.00589	4.57	Secretogranin-2
PCSK1	0.00236	4.50	ProSAAS
DLG2	0.00993	4.35	Disks large homolog 2
SSDH	0.00009	4.34	Succinate-semialdehyde dehydrogenase, mitochondrial
PAK1	0.01048	4.27	Serine/threonine-protein kinase PAK 1
SNP25	0.00911	4.20	Synaptosomal-associated protein 25
SYN3	0.01122	3.94	Synapsin III
ANXA6	0.00058	3.93	Annexin A6
NFM	0.00001	3.87	Neurofilament medium polypeptide
SH3G2	0.00018	3.79	Endophilin-A1
AMPH	0.00162	3.77	Amphiphysin
SEPT5	0.01209	3.71	Septin-5
AATC	0.00000	3.60	Aspartate aminotransferase, cytoplasmic
STMN1	0.00029	3.55	Stathmin
STX1B	0.00205	3.53	Syntaxin-1B
NEUM	0.00238	3.37	Neuromodulin
SYUB	0.01278	3.33	Beta-synuclein
VAMP1	0.00304	3.32	Vesicle-associated membrane protein 1
RAB3A	0.00022	3.27	Ras-related protein Rab-3A
SYNJ1	0.00150	3.25	Synaptojanin-1
STXB1	0.00024	3.24	Syntaxin-binding protein 1
ACTZ	0.00731	3.23	Alpha-centractin
NCDN	0.00058	3.16	Neurochondrin
VAMP2	0.02680	3.14	Vesicle-associated membrane protein 2
RAB1A	0.00356	3.12	Ras-related protein Rab-1A

UniProt ID	P-values	Fold change SKF/Veh	Protein Name
PACN1	0.00030	3.05	Protein kinase C and casein kinase substrate in neurons protein 1
SEPT7	0.00134	2.98	Septin-7
SEPT6	0.01040	2.98	Septin-6
DHE3	0.00181	2.89	Glutamate dehydrogenase 1, mitochondrial
1433B	0.00320	2.86	14-3-3 protein beta/alpha
SYPH	0.01134	2.85	Synaptophysin
DPYL5	0.00006	2.82	Dihydropyrimidinase-related protein 5
SYN2	0.00005	2.82	Synapsin II
SYT1	0.00457	2.74	Synaptotagmin-1
1433T	0.00069	2.74	14-3-3 protein theta
DPYL1	0.00041	2.71	Dihydropyrimidinase-related protein 1
DCE2	0.00681	2.65	Glutamate decarboxylase 2
THY1	0.02158	2.65	Thy-1 membrane glycoprotein
1433E	0.00003	2.61	14-3-3 protein epsilon
1433Z	0.00075	2.61	14-3-3 protein zeta/delta
1433F	0.00515	2.60	14-3-3 protein eta
DYN3	0.04836	2.59	Dynammin-3
GLNA	0.00032	2.57	Glutamine synthetase
DYN1	0.00035	2.56	Dynammin-1
ASGL1	0.03685	2.51	Isoaspartyl peptidase/L-asparaginase
FMR1	0.00819	2.51	Fragile X mental retardation protein 1 homolog
NSF	0.01810	2.49	Vesicle-fusing ATPase
SNAB	0.04630	2.48	Beta-soluble NSF attachment protein
SEPT11	0.01801	2.41	Septin-11
TENR	0.00098	2.40	Tenascin-R
CPLX2	0.02331	2.18	Complexin-2
1433G	0.00202	2.17	14-3-3 protein gamma
DYL2	0.02540	2.13	Dynein light chain 2, cytoplasmic
PPR1B/ DARPP32	0.02664	2.13	Protein phosphatase 1 regulatory subunit 1B
Cytoskeletons			
CAPZB	0.00240	8.20	F-actin-capping protein subunit beta
SRR	0.00379	6.35	Serine racemase
TYB4	0.00001	5.25	Thymosin beta-4
AINX	0.00794	4.31	Alpha-intermexin
ARP3	0.00154	4.24	Actin-related protein 3
LASP1	0.00187	4.21	LIM and SH3 domain protein 1
PROF1	0.00317	4.09	Profilin-1
ADDA	0.00114	4.08	Alpha-adducin
SPTB2	0.00312	4.05	Spectrin beta chain, non-erythrocytic 1

UniProt ID	P-values	Fold change SKF/Veh	Protein Name
DREB	0.04358	3.96	Drebrin
PROF2	0.02067	3.96	Profilin-2
NFL	0.00029	3.59	Neurofilament light polypeptide
ATOX1	0.00041	3.48	Copper transport protein ATOX1
SPTN1	0.00065	3.42	Spectrin alpha chain, non-erythrocytic 1
CAZA2	0.01274	3.34	F-actin-capping protein subunit alpha-2
COR1C	0.00030	3.30	Coronin-1C
MARE3	0.02173	3.22	Microtubule-associated protein RP/EB family member 3
COF1	0.00014	3.21	Cofilin-1
DPYL3	0.00002	3.12	Dihydropyrimidinase-related protein 3
ACTS	0.01717	2.95	Actin, alpha skeletal muscle
ACTG	0.00004	2.90	Actin, cytoplasmic 2
TAU	0.00002	2.81	Microtubule-associated protein tau
CAP1	0.00010	2.67	Adenylyl cyclase-associated protein 1
TPPP	0.00001	2.60	Tubulin polymerization-promoting protein
COF2	0.00123	2.55	Cofilin-2
E41L1	0.00222	2.52	Band 4.1-like protein 1
TBB3	0.00009	2.51	Tubulin beta-3 chain
FSCN1	0.00301	2.38	Fascin
TPPP3	0.01691	2.32	Tubulin polymerization-promoting protein family member 3
TBB4B	0.00104	2.32	Tubulin beta-4B chain
MAP1B	0.00004	2.28	Microtubule-associated protein 1B
TBB5	0.00098	2.23	Tubulin beta-5 chain
MTPN	0.04620	2.17	Myotrophin
TBB2B	0.00003	2.06	Tubulin beta-2B chain
TBA4A	0.00492	2.06	Tubulin alpha-4A chain
Calcium Regulation			
CALR	0.00020	4.51	Calreticulin
PCP4	0.00042	4.24	Purkinje cell protein 4
S100B	0.00007	4.14	Protein S100-B
CALB2	0.00021	4.10	Calretinin
KPCB	0.03848	4.04	Protein kinase C beta type
PGCB	0.00021	3.65	Brevican core protein
EPDR1	0.01366	3.65	Mammalian ependymin-related protein 1
SCRN1	0.00021	3.65	Secernin-1
ANK2	0.00047	3.57	Ankyrin-2
KCC2B	0.00215	2.78	Calcium/calmodulin-dependent protein kinase type II subunit beta
CANB1	0.01520	2.26	Calcineurin subunit B type 1

UniProt ID	P-values	Fold change SKF/Veh	Protein Name
PP2BA	0.00123	2.25	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform
KCC2A	0.01272	2.24	Calcium/calmodulin-dependent protein kinase type II subunit alpha
CALM	0.00072	2.23	Calmodulin
AT2B2	0.00438	6.86	Plasma membrane calcium-transporting ATPase 2
Electron Transfer/Ion Channel			
ETFa	0.00037	8.23	Electron transfer flavoprotein subunit alpha, mitochondrial
VATE1	0.03893	7.37	V-type proton ATPase subunit E 1
AT1A1	0.00258	7.24	Sodium/potassium-transporting ATPase subunit alpha-1
AT1A2	0.00039	6.59	Sodium/potassium-transporting ATPase subunit alpha-2
AT2A2	0.00813	6.35	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
ETFB	0.00561	6.27	Electron transfer flavoprotein subunit beta
VATG2	0.00186	4.89	V-type proton ATPase subunit G 2
AT1B1	0.00252	4.35	Sodium/potassium-transporting ATPase subunit beta-1
AT1A3	0.00011	4.32	Sodium/potassium-transporting ATPase subunit alpha-3
VPP1	0.03177	4.10	V-type proton ATPase 116 kDa subunit a isoform 1
VATH	0.00005	4.01	V-type proton ATPase subunit H
Cell Signaling			
PEA15	0.00889	9.81	Astrocytic phosphoprotein PEA-15
CNTN1	0.02587	4.29	Contactin-1
MP2K1	0.00984	3.50	Dual specificity mitogen-activated protein kinase kinase 1
NDKA	0.00011	3.49	Nucleoside diphosphate kinase A
KAP3	0.01949	3.32	cAMP-dependent protein kinase type II-beta regulatory subunit
GLOD4	0.00364	3.27	Glyoxalase domain-containing protein 4
MYPR	0.00025	3.13	Myelin proteolipid protein
GNAO	0.01483	3.02	Guanine nucleotide-binding protein G(o) subunit alpha
CNRP1	0.00814	2.90	CB1 cannabinoid receptor-interacting protein 1
GBB1	0.00024	2.64	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1
DUS3	0.01265	2.61	Dual specificity protein phosphatase 3
2AAA	0.00042	2.24	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform
Transporters			
7B2	0.00022	7.45	Neuroendocrine protein 7B2
TRFE	0.01112	5.32	Serotransferrin
THTR	0.02611	4.12	Thiosulfate sulfurtransferase
RAB14	0.00553	3.94	Ras-related protein Rab-14
LIS1	0.00016	3.89	Platelet-activating factor acetylhydrolase IB subunit alpha
ALBU	0.00018	3.79	Serum albumin

UniProt ID	P-values	Fold change SKF/Veh	Protein Name
GDIB	0.00005	3.62	Rab GDP dissociation inhibitor beta
ACBP	0.00308	3.49	Acyl-CoA-binding protein
GDIA	0.00001	3.32	Rab GDP dissociation inhibitor alpha
VATA	0.00000	3.27	V-type proton ATPase catalytic subunit A
AP180	0.00020	3.26	Clathrin coat assembly protein AP180
RAN	0.00012	3.20	GTP-binding nuclear protein Ran
VPS35	0.00400	3.20	Vacuolar protein sorting-associated protein 35
GDIR1	0.00461	3.20	Rho GDP-dissociation inhibitor 1
VATB2	0.00164	2.91	V-type proton ATPase subunit B, brain isoform
HBB1	0.00402	2.72	Hemoglobin subunit beta-1
HBA	0.00010	2.61	Hemoglobin subunit alpha
CLH1	0.00063	2.49	Clathrin heavy chain 1
AAK1	0.01983	2.15	AP2-associated protein kinase 1
DYL2	0.02540	2.13	Dynein light chain 2, cytoplasmic
Cellular & Energy Metabolism			
TRXR1	0.00478	14.02	Thioredoxin reductase 1, cytoplasmic
ECHM	0.02149	8.45	Enoyl-CoA hydratase, mitochondrial
MAOX	0.00189	7.74	NADP-dependent malic enzyme
GRHPR	0.03647	6.60	Glyoxylate reductase/hydroxypyruvate reductase
SERC	0.00060	5.67	Phosphoserine aminotransferase
FABP5	0.00342	5.60	Fatty acid-binding protein, epidermal
FA49B	0.01836	5.60	Protein FAM49B
FAS	0.00002	5.51	Fatty acid synthase
FABPH	0.00607	5.51	Fatty acid-binding protein, heart
LDHA	0.00001	5.39	L-lactate dehydrogenase A chain
ACYP1	0.00257	5.29	Acylphosphatase-1
KAD1	0.00036	4.84	Adenylate kinase isoenzyme 1
PLPP	0.04142	4.68	Pyridoxal phosphate phosphatase
IDH3A	0.00101	4.35	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial
BACH	0.00000	4.33	Cytosolic acyl coenzyme A thioester hydrolase
PRDX6	0.00528	3.88	Peroxiredoxin-6
PGM1	0.00012	3.87	Phosphoglucomutase-1
PDXK	0.00009	3.82	Pyridoxal kinase
PGK1	0.00027	3.64	Phosphoglycerate kinase 1
6PGD	0.02056	3.49	6-phosphogluconate dehydrogenase, decarboxylating
G6PI	0.00007	3.49	Glucose-6-phosphate isomerase
DLDH	0.00196	3.48	Dihydrolipoyl dehydrogenase, mitochondrial
ENOA	0.00000	3.45	Alpha-enolase

UniProt ID	P-values	Fold change SKF/Veh	Protein Name
TPIS	0.00001	3.34	Triosephosphate isomerase
AATM	0.00279	3.31	Aspartate aminotransferase, mitochondrial
PP1G	0.00009	3.31	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit
ALDOC	0.00000	3.28	Fructose-bisphosphate aldolase C
PEBP1	0.00127	3.22	Phosphatidylethanolamine-binding protein 1
DHPR	0.01434	3.22	Dihydropteridine reductase
ALDOA	0.00033	3.12	Fructose-bisphosphate aldolase A
KPYM	0.00000	3.11	Pyruvate kinase PKM
PRDX2	0.00478	3.03	Peroxiredoxin-2
KCRB	0.00004	3.00	Creatine kinase B-type
FUMH	0.00182	2.99	Fumarate hydratase, mitochondrial
G3P	0.00065	2.99	Glyceraldehyde-3-phosphate dehydrogenase
IPYR	0.01978	2.97	Inorganic pyrophosphatase
MDHC	0.00074	2.93	Malate dehydrogenase, cytoplasmic
ATPA	0.00151	2.92	ATP synthase subunit alpha, mitochondrial
ODP2	0.00713	2.89	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial
CISY	0.00139	2.88	Citrate synthase, mitochondrial
CYC	0.00306	2.88	Cytochrome c, somatic
HXK1	0.00059	2.87	Hexokinase-1
PYGB	0.01142	2.80	Glycogen phosphorylase, brain form
K6PF	0.00324	2.77	6-phosphofructokinase, muscle type
MDHM	0.00011	2.76	Malate dehydrogenase, mitochondrial
ODPB	0.01496	2.73	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial
APOE	0.03085	2.68	Apolipoprotein E
TALDO	0.00223	2.66	Transaldolase
LDHB	0.00001	2.66	L-lactate dehydrogenase B chain
THIL	0.00005	2.62	Acetyl-CoA acetyltransferase, mitochondrial
PRDX5	0.00062	2.60	Peroxiredoxin-5, mitochondrial
ATPB	0.00023	2.54	ATP synthase subunit beta, mitochondrial
PRDX1	0.00048	2.51	Peroxiredoxin-1
SUCA	0.00700	2.44	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial
ACON	0.00059	2.42	Aconitate hydratase, mitochondrial
K6PP	0.00023	2.38	6-phosphofructokinase type C
ACLY	0.03720	2.35	ATP-citrate synthase
Other Enzymes			
ESTD	0.00970	8.57	S-formylglutathione hydrolase
SPRE	0.03523	6.71	Sepiapterin reductase

UniProt ID	P-values	Fold change SKF/Veh	Protein Name
ADHX	0.01662	6.39	Alcohol dehydrogenase class-3
CAH2	0.00003	4.13	Carbonic anhydrase 2
DDAH1	0.00042	3.99	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1
CRYM	0.00005	3.97	Ketimine reductase mu-crystallin
HDHD2	0.00043	3.96	Haloacid dehalogenase-like hydrolase domain-containing protein 2
PGAM1	0.00005	3.86	Phosphoglycerate mutase 1
AL7A1	0.00408	3.81	Alpha-aminoadipic semialdehyde dehydrogenase
PSA	0.00002	3.74	Puromycin-sensitive aminopeptidase
AK1A1	0.01651	3.74	Alcohol dehydrogenase [NADP(+)]
TKT	0.00097	3.68	Transketolase
ASSY	0.02263	3.37	Argininosuccinate synthase
DPYL4	0.01193	3.30	Dihydropyrimidinase-related protein 4
ENOG	0.00005	3.24	Gamma-enolase
SODC	0.00050	3.15	Superoxide dismutase [Cu-Zn]
LGUL	0.00840	3.15	Lactoylglutathione lyase
PPCEL	0.00363	2.91	Prolyl endopeptidase-like
DPYL2	0.00020	2.89	Dihydropyrimidinase-related protein 2
GUAD	0.00334	2.60	Guanine deaminase
CN37	0.00801	2.27	2',3'-cyclic-nucleotide 3'-phosphodiesterase
Transcription			
KCY	0.00520	6.56	UMP-CMP kinase
NT5C	0.00342	6.31	5'(3')-deoxyribonucleotidase, cytosolic type
AN32A	0.00020	4.75	Acidic leucine-rich nuclear phosphoprotein 32 family member A
AP1B1	0.00034	4.18	AP-1 complex subunit beta-1
TAGL3	0.00019	4.00	Transgelin-3
EFTU	0.02217	3.56	Elongation factor Tu, mitochondrial
ARF3	0.00418	3.47	ADP-ribosylation factor 3
NDRG2	0.00055	3.45	Protein NDRG2
NSF1C	0.00509	3.34	NSFL1 cofactor p47
AP2A2	0.00067	3.04	AP-2 complex subunit alpha-2
HMGB1	0.01268	2.88	High mobility group protein B1
BASP1	0.00021	2.52	Brain acid soluble protein 1
CSRP1	0.00668	2.45	Cysteine and glycine-rich protein 1
AP2A1	0.00001	2.22	AP-2 complex subunit alpha-1
HNRPK	0.00124	2.09	Heterogeneous nuclear ribonucleoprotein K
AP2B1	0.01957	2.09	AP-2 complex subunit beta
DDX17	0.00002	2.08	Probable ATP-dependent RNA helicase DDX17
Translation			

UniProt ID	P-values	Fold change SKF/Veh	Protein Name
IF5A1	0.00534	6.70	Eukaryotic translation initiation factor 5 A-1
UK114	0.00157	4.40	Ribonuclease UK114
SYWC	0.00209	3.97	Tryptophan--tRNA ligase, cytoplasmic
IMPCT	0.00090	3.92	Protein IMPACT
TSR2	0.01589	3.69	Pre-rRNA-processing protein TSR2 homolog
RL23	0.00001	3.26	60S ribosomal protein L23
IF4H	0.00011	3.04	Eukaryotic translation initiation factor 4H
RS23	0.01866	2.66	40S ribosomal protein S23
RL9	0.03345	2.53	60S ribosomal protein L9
RS8	0.04217	2.29	40S ribosomal protein S8
RS11	0.02283	2.25	40S ribosomal protein S11
FBLL1	0.00730	2.23	rRNA/tRNA 2'-O-methyltransferase fibrillarin-like protein 1
PABP1	0.04778	2.20	Polyadenylate-binding protein 1
FBRL	0.00028	2.04	rRNA 2'-O-methyltransferase fibrillarin
IF4A2	0.03558	2.01	Eukaryotic initiation factor 4A-II
Ubiquitination			
PSA5	0.00714	4.27	Proteasome subunit alpha type-5
UBP5	0.00247	4.20	Ubiquitin carboxyl-terminal hydrolase 5
PIMT	0.03321	9.71	Protein-L-isoaspartate(D-aspartate) O-methyltransferase
NEDD8	0.00568	7.10	NEDD8
THOP1	0.02828	4.67	Thimet oligopeptidase
UBA1	0.00002	3.98	Ubiquitin-like modifier-activating enzyme 1
UB2L3	0.00005	3.66	Ubiquitin-conjugating enzyme E2 L3
UCHL1	0.00013	3.46	Ubiquitin carboxyl-terminal hydrolase isozyme L1
PCBP2	0.00116	3.39	Poly(rC)-binding protein 2
UB2D2	0.00642	3.39	Ubiquitin-conjugating enzyme E2 D2
PCBP1	0.02341	3.07	Poly(rC)-binding protein 1
OTUB1	0.02514	3.04	Ubiquitin thioesterase OTUB1
CAND1	0.00895	3.00	Cullin-associated NEDD8-dissociated protein 1
UBE2N	0.00618	3.00	Ubiquitin-conjugating enzyme E2 N
CYTB	0.00919	2.95	Cystatin-B
PSA1	0.01733	2.93	Proteasome subunit alpha type-1
PSA7	0.00042	2.88	Proteasome subunit alpha type-7
HINT1	0.00011	2.86	Histidine triad nucleotide-binding protein 1
WDR1	0.00569	2.73	WD repeat-containing protein 1
BIN1	0.01992	2.70	Myc box-dependent-interacting protein 1
UBC	0.00113	2.25	Polyubiquitin-C
MIF	0.00056	2.39	Macrophage migration inhibitory factor
Cell proliferation			

UniProt ID	P-values	Fold change SKF/Veh	Protein Name
TCTP	0.00338	12.87	Translationally-controlled tumor protein
GUAA	0.00426	5.96	GMP synthase [glutamine-hydrolyzing]
TPRGL	0.00790	5.02	Tumor protein p63-regulated gene 1-like protein
HPRT	0.00115	4.50	Hypoxanthine-guanine phosphoribosyltransferase
SAHH2	0.00194	3.84	Putative adenosylhomocysteinase 2
TERA	0.00003	3.67	Transitional endoplasmic reticulum ATPase
E41L3	0.00370	2.96	Band 4.1-like protein 3
DTD1	0.00042	2.49	D-tyrosyl-tRNA(Tyr) deacylase 1
Protein Folding/ Molecular Chaperones			
TCPA	0.00211	6.77	T-complex protein 1 subunit alpha
COTL1	0.00194	5.63	Coactosin-like protein
PTPA	0.01297	4.08	Serine/threonine-protein phosphatase 2A activator
FKB1A	0.00136	3.86	Peptidyl-prolyl cis-trans isomerase FKBP1A
CYTC	0.01739	3.63	Cystatin-C
TCPZ	0.01058	3.57	T-complex protein 1 subunit zeta
PDIA3	0.00294	3.29	Protein disulfide-isomerase A3
TCPB	0.00363	3.24	T-complex protein 1 subunit beta
CH10	0.00034	3.21	10 kDa heat shock protein, mitochondrial
PARK7	0.00603	3.16	Protein DJ-1
TEBP	0.00124	2.94	Prostaglandin E synthase 3
TCPD	0.00091	2.92	T-complex protein 1 subunit delta
TCPQ	0.00508	2.79	T-complex protein 1 subunit theta
PDIA1	0.00773	2.78	Protein disulfide-isomerase
TCPE	0.01844	2.76	T-complex protein 1 subunit epsilon
PPIA	0.00483	2.62	Peptidyl-prolyl cis-trans isomerase A
F10A1	0.01592	2.57	Hsc70-interacting protein
HS90A	0.00037	2.52	Heat shock protein HSP 90-alpha
HS90B	0.00195	2.45	Heat shock protein HSP 90-beta
STIP1	0.00782	2.44	Stress-induced-phosphoprotein 1
CH60	0.00250	2.21	60 kDa heat shock protein, mitochondrial
Total: 316			