Appendix 1 to Wang JM, Hou X, Adeosun S, et al. ERβ agonist alters RNA splicing factor expression, and has a longer window of antidepressant effectiveness than E2 after long-term ovariectomy. J Psychiatry Neurosci 2018.

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Supplementary Materials and Methods

Tissue collection and preparation

Before euthanasia, rats were anesthetized with 100mg/kg ketamine and 10mg/kg xylazine. After blood collection, the vasculatures were flushed by cardiac perfusion with saline. The midbrain and frontal cortex from right brain hemispheres were collected immediately for protein and mRNA isolation. TPH2-positve serotonergic neurons are primarily located in the dorsal raphe, a rather small structure in the midbrain. Therefore, we collected the entire midbrain for TPH2 measurements to ensure the consistency and accuracy throughout all brains. The tissues were weighed and homogenized in RNase free water (10ml/mg tissue) for 1min with a Bullet Blender (NextAdvance, Averill Part, NY), which homogenizes 24 samples simultaneously using 0.1mm RNase-free glass beads (NextAdvance, Averill Part, NY). The homogenate from each sample was divided and stored in two aliquots for further protein and total mRNA extraction respectively. The left hemispheres were preserved in 4% paraformaldehyde at 4°C overnight and then stored in PBS containing 0.09% sodium azide until ready for sectioning.

RNA extraction and Real-time qPCR

Total RNA was extracted from the midbrain and frontal cortex of tested rats using ToTALLY RNA Kit (Ambion, Austin, TX). Primers used in real-time qPCR are: ***TPH2*** (NM_173839.2) forward primer 5'-GCATCTTGGAAGGTGGTGAT-3' and reverse primer 5'-CCTTTGCAAGCAAGAAGGTC-3'; ***SFRS7*** (NM_001039035) forward primer 5'-TGCAGAGGATGCAGTTCGAG-3' and reverse primer 5'-CAGGTGGCCTATCAAACCGA-3'; ***SFRS16*** (NM_001024294) forward primer 5'-GCCGTAAGATTAGCCCTCCC-3' and reverse primer 5'- CGGGACTCGGAACTGGATTC-3' ;

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ZRSR2 (XM_001067753.2) forward primer 5'-TCGAGCCAACTGTCCATTC-3' and reverse primer 5'-GCAAGGTGGGACTTGATGTG-3'; ***CTNNB1*** (NM_053357) forward primer 5'-ATCATTCTGGCCAGTGGTGG-3' and reverse primer 5'-GACAGCACCTTCAGCACTCT-3'; ***18S rRNA*** (NR_046237.1) forward primer 5'-AAACGGCTACCACATCCAAG-3' and reverse primer 5'-CCTCCAATGGATCCTCGTTA-3'. All primers were purchased from Life Technology, Grand Island, NY.

cDNA synthesis and PCR amplification were carried out in duplicates using an iScript RT-PCR kit with SYBRgreen (Bio-Rad, Hercules, CA) under optimized conditions according to the instructions provided by the manufacturer. Briefly, the complete reaction mix was incubated for: 10min at 50°C for cDNA synthesis; 5min at 95°C to inactivate reverse transcriptase; 40 cycles of 10sec at 95°C, followed by 30sec at 60°C, 1min at 95°C, and 1min at 55°C, with 18S rRNA as an internal control. The average cycle number (CT) of duplicates at which signals crossed a threshold set within the logarithmic phase was recorded. Differences in cycle threshold (ΔΔCT) were used to analyze gene expression according to the study of Livak & Schmittgen (1), and results are presented by fold changes between different treatment groups.

Protein extraction and immunoprecipitation

Protein aliquots from brain tissue homogenates were mixed 1:1 with RIPA buffer mix (Sodium orthovanadate 1%, Protease inhibitor 0.1% and PMSF 1% in RIPA buffer) followed by 30min incubation on ice. The homogenate-RIPA-buffer mixes were centrifuged at 11,000rpm for 12min at 4°C. Supernatants were then transferred into fresh tubes and centrifuged again at 11,000 rpm for 15min at 4°C.

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After the second centrifugation, supernatants were transferred into final protein tubes. Protein concentration of each sample was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL).

Protein extracts (200µg in 500µl lysis solution) were first precleared by addition of 15µl protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA). After centrifugation, resulting supernatants were incubated with 2.5µg/ml anti-TPH2 polyclonal antibody (PA1-778, Thermo scientific, Rockford, IL) for 1hr at 4°C. Then 20µl of A-agarose protein was added to each sample and incubated at 4°C on a rocker for 1hr. Samples were centrifuged again at 1,000xg at 4°C for 5min and washed 4 times with 1ml cold PBS. The pellets were boiled for 5min in 20ul Laemmli buffer (Boston BioProducts, Ashland, MA) for electrophoresis. Whole cell lysate from TPH2-expressing SH SY5Y neuroblastoma cells was used as a positive control (2). A sample with agarose beads but without TPH2 antibody was used as a negative control.

Western blot

The heated protein-Laemmli mixes were allowed to cool down on ice for 2min. Samples (30µg each) were loaded on a 10% polyacrylamide electrophoresis gel and were run at 120V for 10min followed by 90V for 2hrs to separate proteins using Mini-PROTEAN Tetra cell (Bio-Rad, Hercules, CA). After electrophoresis, proteins on the gel were transferred to a PVDF membrane in Mini Trans-Blot Electrophoretic Transfer cell system (Bio-Rad, Hercules, CA) in voltage of 36V at 4°C overnight. Proteins were then blotted on the PVDF membrane with Fast Western Blot Kits, ECL Substrate (Pierce Biotechnology, Rockfod, IL), which contained washing buffer, antibody diluent, secondary antibody and

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ECL substrate. The membrane was washed with washing buffer and incubated with antibodies against TPH2 (PA1-778, Thermo scientific, Rockford, IL, 1:1,000), ERβ (H-150, Santa Cruz Biotechnology, Santa Cruz, CA, 1:100) (PA1-310B, Thermo sciencetific, Rockford, IL, 1:500), ERβ2 (gift from Dr. Robert Handa, 1:5,000) and β-actin (ab6276, Abcam Inc., Cambridge, MA, 1:10,000) for 30min at room temperature. The primary antibody incubation was followed immediately by a 10-min secondary antibody incubation at the concentration of 1:10. After washing 3 times with washing buffer, the membrane was incubated with ECL substrate for 5min, and the chemiluminiscent signal was detected using Bio-Rad ChemiDoc™ XRS+ system (Bio-Rad, Hercules, CA) for 10min (3). The anti-TPH2 antibody and ERβ2 antibody were validated in previous studies (4, 5). Data were presented as relative optical densities of the individual bands normalized by β-actin expression ± SEM.

Immunofluorescence and quantification of the intensity

The left hemisphere of the brain was transferred into 30% sucrose PBS at 4°C until it sank to the bottom of the solution. After embedded in OCT Embedding Matrix, brains were sectioned coronally at 40μm thickness throughout the rostral-caudal length with a Leica CM3050 S Cryostat, and were collected in 24-well plates containing PBS with 0.99% sodium azide. Brain sections containing the dorsal raphe were blocked with 5% goat serum and incubated with primary antibodies against MAOA (1:100; sc-20156, Santa Cruz, CA), NeuN (1:400; MAB377, Millipore, Billerica, MA) and GFAP (1:1000; Cat# AB4647, Abcam, Cambrage, England) antibodies. On the following day, sections were incubated with secondary antibodies anti-rabbit Cy5 IgG (1:1000, Vector Laboratories, Inc., Burlingame, CA), anti-chicken Cy3 (1:1000, Jackson ImmunoResearch, West Grove, PA), and anti-mouse FITC (1:1000, Vector

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Laboratories, Inc., Burlingame, CA). Lastly, all sections were rinsed with PBS and coverslipped using Vectashield mounting medium containing DAPI (Vector Labortories, Burlingame, CA).

Fluorescent images were captured and visualized using a Zeiss Observer Z1 (Oberkochen, Germany) with a CSU-W1 T1 Spinning Disk confocal imaging system (Yokogawa, Tokyo, Japan). For each section, a montage picture was obtained using a 10× magnification field. The dorsal raphe was then masked to allow random assignment of 6-9 sampling frames onto the masked area by SlideBook 6.01 software (Innovative Intelligence, Inc., Denver, CO). The dimensions used for sampling frame volume were set to 100µm (x-axis) × 100µm (y-axis) × 10µm (z-axis), yielding a total sampling volume of 100,000 μm^3 per sampling site. Within each optical dissector frame, a series of z-stage images were taken at 1µm intervals using a 63× objective with numerical aperture of 1.42. All photomicrographs from the individual dissector frame were then compiled and stacked for analysis. Next, a separate mask was generated for each sampling site, which allowed unbiased, quantitative assessment of fluorescence intensity.

Forced Swim Test

Forced swim test was conducted to assess the antidepressant effect of E2, DPN and PPT in early and late ET. The forced swim test was performed twice for each animal. The pre-test was performed eight days before ovariectomy. The experimental test was performed on the day following the completion of ER ligand treatments as previously described (3). In each test, the rat was placed in a cylindrical container (40cm deep, 27cm in diameter) filled with 30cm of 30°C water. The amount of time the rat spent swimming or immobile was video-recorded in a 10-min test. Two rats that sunk below the surface during the test were removed from the water immediately and data was excluded. After the swim test, the rat was

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towel dried and kept in a warm environment for 2hr before being returned to its home cage. Swimming was defined as movement of the forelimbs and hind limbs with the front paws breaking the surface of the water. Immobility was recorded as the absence of any movement other than that necessary to keep the head and nose above the water (rats were floating in a vertical position). The video recording was analyzed manually. Before testing, all rats were coded by group; these codes were not broken until all video analyses had been completed.

Elevated Plus Maze

The Elevated Plus Maze apparatus consists of two alleys 60cm long and 5cm wide crossing each other in the middle at right angles (leaving a 5cmX5cm) center and forming a plus sign. One of the alleys was covered with 15cm high walls on either side of the center while the other one is open; thus making two open and two closed arms. The apparatus was elevated on a 30cm high base. Rats were placed one at a time at the end of one of the open arms and were allowed to freely explore the maze for 5minutes. The apparatus was cleaned with 50% ethanol to prevent odor cues. The activity of the animal was digitally recorded and later analyzed offline with the Noldus system. Measures scored included time spend in the open arms, time spent in the closed arms, entries into open arms and into closed arms, distance travelled, time moving/not moving, speed (total distance/time moving) .

Figure legends for Supplementary Figures

Supplementary Figure 1. The expression profiles of splicing factors (RT2 Profiler) in rats receiving vehicle or E2 6 days after OVX. Clustergram was generated from average Ct values of each treatment group. For each group, average Ct values were normalized to a panel of five different housekeeping

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genes. The range of $2^{-\Delta Ct}$ values was given below in the color scale. Genes listed on the left of the clustergram are related genes from the heterogeneous nuclear ribonucleoprotein family (**A**), the arginine/serine-rich splicing factor family (**B**) and RNA binding motif proteins (**C**). n=3 for each group, SV = Sham+vehicle, OV = OVX+vehicle, OE = OVX+E2.

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Supplementary Table 1 List of genes in the custom RT2 Profiler PCR Array

Gene Symbol	Alias	Refseq #	Official Full Name
Fusip1	MGC112885	NM_001025738	FUS interacting protein (serine-arginine rich) 1
Hnrnpa1	Hnrpa1	NM_017248	Heterogeneous nuclear ribonucleoprotein A1
Hnrnpa3	Hnrpa3	NM_001111294	Heterogeneous nuclear ribonucleoprotein A3
Hnrnpab	A1F-C1/Hnrpab	NM_031330	Heterogeneous nuclear ribonucleoprotein A/B
Hnrnpa2b1	Hnrpa2/Hnrpa2b1/ hnRNP	NM_001104613	Heterogeneous nuclear ribonucleoprotein A2/B1
Hnrnpc	Hnrpc/MGC114359	NM_001025633	Heterogeneous nuclear ribonucleoprotein C (C1/C2)
Hnrpd	Auf1	NM_001082539	Heterogeneous nuclear ribonucleoprotein D
Hnrpdl	MGC125262	NM_001033696	Heterogeneous nuclear ribonucleoprotein D-like
Hnrnpf	Hnrpf/MGC125106	NM_001037285	Heterogeneous nuclear ribonucleoprotein F
Hnrph1	Hnrnph1/Hnrph/ MGC124573	NM_080896	Heterogeneous nuclear ribonucleoprotein H1
Hnrnph2	Hnrph2	NM_001014019	Heterogeneous nuclear ribonucleoprotein H2 (H')
Hnrnph3	Hnrph3	NM_001108532	Heterogeneous nuclear ribonucleoprotein H3 (2H9)

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Hnrnpk	Csbp/Hnrpk	NM_057141	Heterogeneous nuclear ribonucleoprotein K
Hnrnpl	Hnrpl/hnrnp-L	NM_001134760	Heterogeneous nuclear ribonucleoprotein L
Hnrnpr	Hnrpr	NM_175603	Heterogeneous nuclear ribonucleoprotein R
Hnrnpu	Hnrpu/SN1	NM_057139	Heterogeneous nuclear ribonucleoprotein U
Hnrnpul1	Hnrpul1	NM_001108477	Heterogeneous nuclear ribonucleoprotein U-like 1
Igf2bp2	RGD1305614	XM_221343	Insulin-like growth factor 2 mRNA binding protein 2
Igfbp4	IBP4/IGF-BP4	NM_001004274	Insulin-like growth factor binding protein 4
Khdrbs3	Etle/Slm2	NM_022249	KH domain containing, RNA binding, signal transduction associated 3
Khsrp	Marta1	NM_133602	KH-type splicing regulatory protein
Mapk10	Jnk3/SAPKC/SAPb/Serk2	NM_012806	Mitogen activated protein kinase 10
Nolc1	Nopp140	NM_022869	Nucleolar and coiled-body phosphoprotein 1
Nrip1_predicted	RIP140	XM_221724	Nuclear receptor interacting protein 1
Pabpn1	-	NM_001135008	Poly(A) binding protein, nuclear 1
Pcbp2	-	NM_001013223	Poly(rC) binding protein 2
Pcgf6	Rnf134	NM_001013154	Polycomb group ring finger 6
Pqbp1	-	NM_001013957	Polyglutamine binding protein 1
Prpf4b	-	NM_001011923	PRP4 pre-mRNA processing factor 4

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			homolog B (yeast)
Prmt1	Hrmt1l2	NM_024363	Protein arginine methyltransferase 1
Ptbp1	Ptb/Pybp/Pybp1/ Pybp2	NM_022516	Polypyrimidine tract binding protein 1
Raly	-	NM_001011958	RNA binding protein, autoantigenic (hnRNP-associated with lethal yellow homolog (mouse))
Rbm10	-	NM_152861	RNA binding motif protein 10
Rbm11_predicted	-	NM_001105898	RNA binding motif protein 11
Rbm12	-	NM_001037657	RNA binding motif protein 12
Mak16	MGC109625/RGD1 311297/Rbm13	NM_001014002	MAK16 homolog (S. cerevisiae)
Rbm14	CoAA	XM_001072105	RNA binding motif protein 14
Rbm17	-	NM_001013058	RNA binding motif protein 17
Rbmx	MGC114544	NM_001025663	RNA binding motif protein, X-linked
Safb	-	NM_022394	Scaffold attachment factor B
Sept6_predicted	MGC189443	XM_001077761	Septin 6
Sf1	Zfp162	NM_001110793	Splicing factor 1
Sfrs3_predicted	-	NM_001047907	Splicing factor, arginine/serine-rich 3
Sfrs5	HRS/SRp40	NM_019257	Splicing factor, arginine/serine-rich 5
Sfrs6	-	NM_001014185	Splicing factor, arginine/serine-rich 6
Sfrs7	MGC116344	NM_001039035	Splicing factor, arginine/serine-rich 7
Sfrs9	MGC105562	NM_001009255	Splicing factor, arginine/serine rich 9
Sfrs10	-	NM_057119	Splicing factor, arginine/serine-rich 10 (transformer 2 homolog, Drosophila)
Sfrs11	Sfrs11	NM_001035255	Splicing factor, arginine/serine-rich 11
Sfrs12	Srrp86	NM_020092	Splicing factor, arginine/serine-rich 12

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Sfrs14_predicted	-	NM_001108397	Splicing factor, arginine/serine-rich 14
Sfrs16	-	NM_001024294	Splicing factor, arginine/serine-rich 16
Sfrs18	MGC109235/ RGD1307395	NM_001025274	Splicing factor, arginine/serine-rich 18
Sfrs2ip	-	XM_231361	Splicing factor, arginine/serine-rich 2, interacting protein
Sh3glb2	MGC105723	NM_001009692	SH3-domain GRB2-like endophilin B2
Snrp70_predicted	-	NM_001108483	U1 small nuclear ribonucleoprotein polypeptide A
Srpk1	MGC116186	NM_001025726	SFRS protein kinase 1
Srrm2_predicted	-	XM_220207	Serine/arginine repetitive matrix 2
Syncrip	Ab2-339	NM_001047916	Synaptotagmin binding, cytoplasmic RNA interacting protein
Tnpo1	-	XM_001070389	Transportin 1
Tnpo2_predicted	-	NM_001107166	Transportin 2 (importin 3, karyopherin beta 2b)
Tnpo3	-	NM_001106587	Transportin 3
U2af2	-	XM_001077658	U2 small nuclear ribonucleoprotein auxiliary factor (U2AF) 2
Ylpm1	RGD1564946	XM_234416	YLP motif containing 1
Zrsr1	-	NM_001017504	Zinc finger (CCCH type), RNA binding motif and serine/arginine rich 1
Zrsr2	RGD1559763	XM_217612	Zinc finger (CCCH type), RNA binding motif and serine/arginine rich 2
Ctnnb1	Catnb	NM_053357	Catenin (cadherin associated protein), beta 1
Tgfb1	-	NM_021578	Transforming growth factor, beta 1

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Actb *	Actx	NM_031144	Actin, beta
Hprt1 *	Hgprtase/Hprt/ MGC112554	NM_012583	Hypoxanthine phosphoribosyltransferase 1
Ldha *	Ldh1	NM_017025	Lactate dehydrogenase A
Rpl13a *	-	NM_173340	Ribosomal protein L13A
Rplp1 *	MGC72935	NM_001007604	Ribosomal protein, large, P1

* Housekeeping genes

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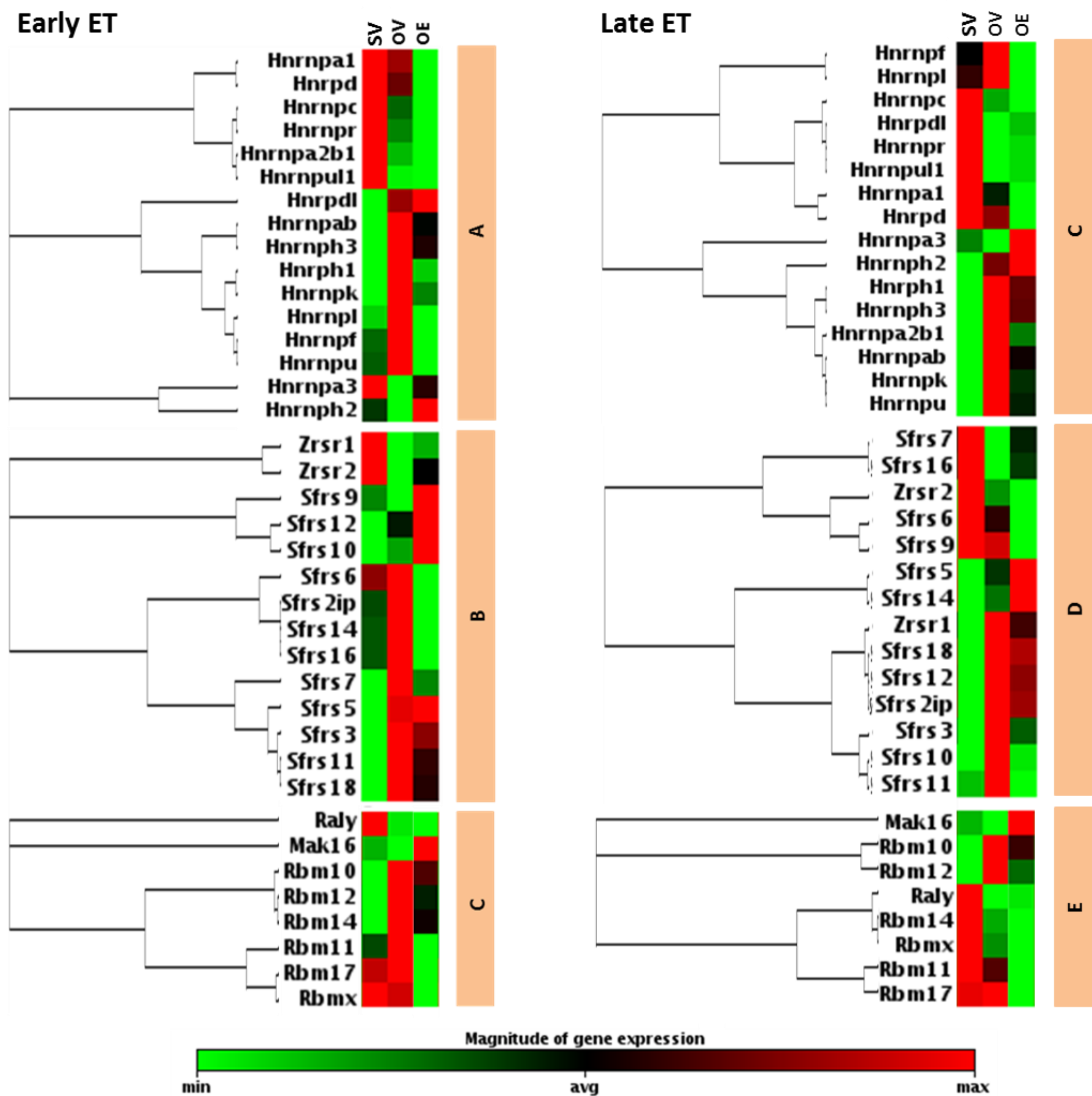
Suppl. Figure 1



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