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17β-estradiol regulates the RNA binding protein Nova1, which then regulates the alternative splicing of estrogen receptor-β in the aging female rat brain

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VERIFICATIONS

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CS, CD, CK, EP, YR, and TP performed the experiments, analyzed and interpreted the data.
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Abstract

Alternative RNA splicing results in the translation of diverse protein products arising from a common nucleotide sequence. These alternative protein products are often functional and can have widely divergent actions from the canonical protein. Studies in humans and other vertebrate animals have demonstrated that alternative splicing events increase with advanced age, sometimes resulting in pathological consequences. Menopause represents a critical transition for women, where the beneficial effects of estrogens are no longer evident, therefore factors underlying increased pathological conditions in women are confounded by the dual factors of aging and declining estrogens. Estrogen receptors are subject to alternative splicing, the spliced variants increase following menopause, and they fail to efficiently activate estrogen-dependent signaling pathways. However, the factors that regulate the alternative splicing of estrogen receptors remain unknown. We demonstrate novel evidence supporting a potential biological feedback loop where 17β-estradiol regulates the RNA binding protein Nova1, which, in turn, regulates the alternative splicing of estrogen receptor β. These data increase our understanding of estrogen receptor alternative splicing and could have potential implications for women taking hormone replacement therapy post-menopause.

INTRODUCTION

Alternative RNA splicing increases the diversity of proteins that can be translated from the limited set of genes found in eukaryotes. Over 95% of multi-exonic genes are alternatively spliced and that number is likely underestimated due to current technical limitations in high resolution deep sequencing platforms (Pan et al., 2008). Recent evidence revealed that alternative splicing events increase in the brain with advanced age in both humans and non-primate animals (Tollervey et al., 2011). Moreover, increased alternative splicing events in the brain have generally been correlated with negative outcomes and are predicted to lead to neurodegeneration, possibly as a result of altered protein function and/or impaired protein translation of the spliced variants (Anthony and Gallo, 2010; Twine et al., 2011). For instance, alternative splicing events in several RNA transcripts increased with age in the human brain and many of the alternatively spliced transcripts in patients over 82 years of age were also detected in younger patients with neurodegenerative diseases (Tollervey et al., 2011).

The effects of aging in women are compounded by the menopausal transition and concomitant decline in circulating estrogens, specifically 17β-estradiol (E2), the major estrogen produced by the ovaries. The effects of E2 are mediated primarily by two nuclear steroid receptors (estrogen receptor (ER) α and ERβ), which are encoded by distinct genes located on different chromosomes (Kuiper et al., 1996). Classical intracellular signaling involves ER:ligand binding, followed by the formation of receptor dimers (hetero- or homodimers) and translocation to the nucleus where they function as transcription factors on ER target genes (Jensen et al., 1968). Our previous studies using a rat model of surgically-induced menopause “estropause” demonstrated that declining levels of E2 correlated with

the expression levels of ERβ splice variants in a brain region specific manner (Shults et al., 2015a). Notably, we detected increased expression of the dominant negative form of ERβ (ERβ2), which has reduced ligand binding affinity due to a spliced 54 bp insert within the ligand-binding domain (LBD) and preferentially forms heterodimers with ERα (Lu et al., 2000; Maruyama et al., 1998; Petersen et al., 1998). These functional differences result in decreased efficacy of estrogen signaling when ERβ2 levels are elevated. Our previous results were consistent with others that have observed a concordant increase in ERβ2 protein levels following prolonged periods of E2 deprivation in rats and this increase correlated with poor performance on cognitive behavior tests (Wang et al., 2012). Clinically, the efficacy of hormone replacement therapy (HT) could be affected by altered expression of the ERβ splice variants, or ERα splice variants, due to their divergent effects on E2 signaling (Hattori et al., 2016; Leung et al., 2006; Mott and Pak, 2012; Ogawa et al., 1998; Pak et al., 2007). Despite the correlative evidence for increased splicing associated with aging, the molecular factors regulating alternative splicing of ERβ in the brain remain unknown.

RNA-binding proteins (RBPs) are key regulators of post-transcriptional alternative splicing. These *trans*-acting factors bind to *cis*-sequences contained within the pre-mRNA transcript to coordinate and facilitate alternative splicing events. RNA binding proteins not only regulate pre-mRNA splicing, but also coordinate 5′ end capping, cleavage, polyadenylation, nuclear mRNA export, localization, translation, and degradation of mRNA (Glisovic et al., 2008). These critical RNA binding proteins can also have tissue-specific roles, which is evidenced by the high number of alternative splicing events that occur in the brain (Black, 2003; Xu et al., 2002). The central nervous system enriched RNA binding protein Nova1 (neuro-oncological ventral antigen 1) binds YCAY elements coded in pre-mRNA transcripts to selectively enhance or block exon exclusion in a location-dependent manner (Ule, Jernej et al., 2006; Zhang et al., 2010). Like many RNA binding proteins, Nova1 works in concert with heterogeneous nuclear ribonucleoproteins (hnRNPs), serine/arginine-rich (SR) proteins, and other factors to regulate splicing (Hanamura et al., 1998). Nova1 regulates exon splicing through differential binding of the pre-mRNA transcript. Nova1 often promotes exon exclusion if it binds upstream of an alternatively spliced exon, but it can also promote inclusion of exons if it binds downstream of the exon (Ule, Jernej et al., 2006; Zhang et al., 2010). Notably, the ERβ pre-mRNA transcript contains three Nova1 RBP consensus sequences flanking the ERβ2 LBD insert that we predicted may promote exon exclusion based on their location (Fig. 1A). Therefore, it is possible that Nova1 can regulate ERβ alternative splicing through RBP interactions post-transcriptionally.

This study tested the hypothesis that Nova1 mediates age-related increases in ERβ splice variant expression. To that end, our goals were to determine 1) if age and/or prolonged periods of E2 deprivation differentially alter Nova1 expression in the aging female brain; 2) if ERβ is a direct binding target for Nova1; and 3) if Nova1 regulates the alternative splicing of ERβ. We used an *in vivo* rat model of surgically-induced menopause “estropause”, as described previously (Mott et al., 2014; Pinceti et al., 2016; Rao et al., 2015; Shults et al., 2015a). Specifically, we analyzed three brain regions that express ERβ and are functionally distinct: the hypothalamus, dorsal hippocampus, and ventral hippocampus. The hypothalamus is a major regulator of core body temperature and the stress response, which are modulated by E2 and often dysregulated in postmenopausal women (Liu et al., 2012; Liu

et al., 2011; McGregor et al., 2014). The hippocampus is divided into two distinct functional regions: dorsal, which mediates cognitive functions, and ventral, which mediates emotional memory (Fanselow and Dong, 2010). Together, our results implicate Nova1 as an important regulator of ERβ alternative splicing in the aged brain.

MATERIAL AND METHODS

Animal experiments and estrogen deprivation paradigm

Female Fischer 344 rats (3 mo. and 18 mo. of age) were obtained from the NIH aging colony (Taconic, Germantown, NY, USA). In this strain of rat, these ages equate to young adult and postmenopausal ages as related to humans based on calculations from rodent survival curves, reduced frequency of proestrous cycles, decreased circulating estradiol concentrations, and increased cycle durations (Shults et al., 2015b). All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Loyola University Chicago, permit number 2009018. Experiments were carried out in accordance with the guidelines set forth by the IACUC and all appropriate measures were taken to minimize pain and suffering.

Experiment #1: Comparison of Nova1 splicing factor expression in young (3 mo.) and aged (18 mo.) female rats—Animals were bilaterally ovariectomized (OVX) under vaporized isoflurane anesthesia, as previously described ((Mott et al., 2014; Rao et al., 2015; Shults et al., 2015b). One week (7 d.) following OVX, animals were administered a subcutaneous injection of vehicle (safflower oil) or 2.5 μg/kg E2 (Sigma, Cat. No. E8875) (N=6–10/treatment group) based on previous studies (Mott et al., 2014). Animals receiving E2 treatment achieved circulating E2 levels similar to women receiving HT as reported previously (OVX-Veh (23.2 ± 2.7 pg/mL; OVX-E2 (56.5 ± 6.3 pg/mL) (Mott et al., 2014; Rao et al., 2015; Shults et al., 2015b). Treatments were administered once/day x 3 consecutive days. Animals were humanely euthanized 24 hours after the last treatment and brains were rapidly removed and flash frozen for tissue processing.

Experiment #2: Effects of varying lengths of E2 deprivation on Nova1 mRNA and protein expression—Aged (18 mo.) female rats were OVX as described above and then separated into 4 groups that would undergo increasingly longer periods of time without E2: 1, 4, 8, and 12 weeks (N = 16–20/group; Figure. 2) (Rocca et al., 2010, 2011; vom Saal and Finch, 1988). After the assigned E2 deprivation period, animals received a subcutaneous injection of either vehicle (safflower oil) or 2.5 μg/kg E2 (Sigma, Cat. No. E8875) (N=6–10/treatment group). Treatments were administered once/day x 3 consecutive days. Animals were humanely euthanized 24 hours after the last treatment and brains were rapidly removed and flash frozen for tissue processing.

Experiment #3: Effects of ERβ and ERα selective agonists on Nova1 expression in aged (18 mo.) female rats—Aged (18 mo.) female rats were OVX as described above. One week (7 d.) following OVX, animals were administered a subcutaneous injection of vehicle (safflower oil), 2.5 μg/kg E2 (Sigma, Cat. No. E8875), 1 mg/kg diarylproprionitrile (DPN, Tocris, Cat. No. 1494), or 0.5 mg/kg propyl pyrazole triol

(PPT, Tocris, Cat. No. 1426) (N=6–10/treatment group). These doses were based on previous studies that demonstrated selective activation of their respective receptors (Frasor et al., 2003; Harris et al., 2002). Treatments were administered once/day x 3 consecutive days. Animals were humanely euthanized 24 hours after the last treatment and brains were rapidly removed and flash frozen for tissue processing.

Quantitative RT-PCR (RT-qPCR)

Flash frozen brains were sectioned at 200 μm on a freezing microtome and regions of interest were microdissected utilizing 0.75 mm Palkovit's brain punch tool (Stoelting Co., Wood Dale, IL). The hypothalamus (−0.8 mm to −3.8 mm relative to bregma), dorsal hippocampus (−2.30 to −4.16 mm relative to bregma), and ventral hippocampus (4.30 to 6.04 mm relative to bregma) were all microdissected for RNA and protein isolation (Watson, 2014). Brains were split sagittally for tissue collection where one hemisphere was used for RNA isolation and the other for protein isolation in a non-biased manner. RNA isolation was performed on sonicated tissue samples using Trizol reagent (Invitrogen, #15596-026) according to the manufacturer's specifications. All RNA samples were quantified using Nanodrop spectrophotometry and analyzed for quality by visualization of the RNA on 1.0% agarose gel. cDNA was reverse transcribed from 1.0 μg RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814) according to manufacturer's specifications. Nova1 RT-qPCR was performed with FastStart Universal SYBR Green Master Mix (Roche, #04913914001) on an Eppendorf Realplex4. HPRT was used as a reference gene did not change with hormone treatments. Nova1 and HPRT primer sequences were designed and commercially manufactured (Integrated DNA Technologies, Coralville, IA):

Nova1 Forward- TTACCCAGGTACTACTGAGCG, *Nova1 Reverse*- CCCATCAGGTTTCTGGGA,

HPRT Forward- AGCAGTACAGCCCCAAAATGG, *HPRT Reverse*- TGCGCTCATCTTAGGCTTTGT.

ERβ RT-qPCR was performed with TaqMan Gene Expression MasterMix (Applied Biosystems, #4369016) and TaqMan custom FAM-probes that are specific for ERβ1 (i.e. wtERβ) and ERβ2 splice variant on an Eppendorf Realplex4. HPRT was measured using TaqMan Gene Expression Assay Rn01527840_m1. ERβ1 was measured by TaqMan Gene Expression Assay Rn00562610_m1. The following custom probes sequence was designed and used to detect rat ERβ2 splice variant: *ERβ2 – TCCTCAGAAGACCCTCAC*. The following primer sequences were used to detect the custom probe listed previously: *ERβ2 forward – AGCCTGTTGGACCAAGT, reverse – GCACTCTTCATCTGCGCAAC.* Data was analyzed by delta delta ct method as previously described (Lava and Schmitt, 2001).

Protein isolation and western blotting

Hypothalamic, dorsal hippocampus, and ventral hippocampus tissue isolated from aged female rat brains were placed in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, #78510) supplemented with 7x Protease and Phosphates Inhibitor cocktail (Thermo Fisher Scientific, #88668). Tissue was sonicated and insoluble material including

DNA was polluted and excluded from the soluble portion of the extracts. 10 μg protein was reduced in 4X lamely buffer (Bio-Red, #161-0747) at 95°C for 5 min. and run on 10% S DS-PAGE gel. Nova1 protein expression was detected with anti-Nova1 (Upstate/Millipore, Cat. No. #07-637) antibody. This antibody has 80% cross-reactivity for Nova2, which was detected in all brain tissue samples tested and in the hypothalamic IVB cell line. Blots were imaged using a Chasidic (Bio-Red, Hercules, CA, USA) and Nova 1 bands were quantified using Image Lab 3.0 Software (Bio-Red, Hercules, CA, USA). Nova1 protein expression was normalized to β-acting expression with anti-β-acting (13E5) antibody (Cell Signaling, Cat. No. #4970).

Cell culture experiments

ERβ-positive hypothalamic-derived cells (IVB, generously provided by John Krakow, University of Cincinnati) and human embryonic kidney cells (HEK) were used for all *in vitro* experiments. Cells were maintained in Dubach's Minimum Essential Medium (DMEM) media containing glucose, L-glutamine, sodium private and 10% fetal bovine serum (FBS). IVB cells were grown to confluence (70–80%) and were transected with GFP-tagged Nova1 in a dose-dependent manner (0.1 – 2.0 μg DNA). pedant Nova1 elf was a gift from Nicolas Charlet-Berguerand (Addgene, Plasmid # 61275). For RNA immunoprecipitation (RIP) experiments, HEK cells were grown to confluence in 10cm plates, then transected with 3 μg Nova1 elf in combination with either 3 μg of ERβ2 plasmid (previously characterized and provided as a gift from Dr. Robert Handa (Price et al., 2001) or ERβ2 mutant plasmid. pcDNA3.1 empty vector was used as a negative control. Transient transfections of plasmid DNA were performed using a lipid-mediated transfection reagent according to manufacturer instructions (Gemini Bio-Products, #400–700). All cell line experiments were done at least using triplicate technical replicates and a minimum of 6 separate experiments to ensure reproducibility. Cells were then collected 24–48 hours later for RNA isolation, cDNA synthesis, and RT-qPCR as described above.

Site-Directed Mutagenesis

The pcDNA3.0 plasmid expression vectors (Invitrogen) containing a cDNA insert coding rat ERβ1 (i.e. wtERβ) and ERβ2 were mutated using the Quick Change II XL site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA) to create the Nova1 binding site mutants. Primers were designed using the QuickChange primer design available from the Agilent website (http://www.genomics.agilent.com/primerDesignProgram.jsp) and sequential deletions of the three putative binding sites located in exon 5 of the gene were made in accordance with the manufacturer's instructions. Vectors were validated by DNA sequencing (ACGT, Inc., Wheeling, IL, USA) to confirm successful site-directed mutagenesis.

RNA immunoprecipitation (RIP)

RIP experiments were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (EMD Millipore, #17-700) following manufacturer's specifications. Briefly, HEK cells overexpressing Nova1 and ERβ1/ERβ2 or ERβ1/ERβ2 mutant were washed and isolated with 10 ml ice-cold PBS, then centrifuged at 1500 RPM for 5 minutes. An equal volume of RNA Lysis Buffer was used to resuspend pellet, then incubated on ice for 5 minutes in order to lyse the cells. Magnetic beads were pre-incubated

with 2.5 μg Nova1 antibody and 2.5 μg rabbit IgG (EMD Millipore, Cat. No. PP64B) (negative control). Antibody-coated beads were resuspended and then incubated with cell lysates rotating overnight at 4°C. Supernatant was removed by centrifugation at 1 4K RPM for 30 min. and beads were washed 6 times with RIP wash buffer. Following proteinase K treatment at 55°C for 30 min., supernatant was removed from magnetic beads and placed in a new tube for RNA isolation and cDNA synthesis. RIP analysis used the −2 delta delta CT values for determining enrichment fold-change relative to IgG controls as previously described (Atasoy et al., 2003).

Statistical Analysis

Significant interactions were assessed by two-way ANOVA with age x treatment as factors (Experiment 1: young vs. aged), or time x treatment (Experiment 2: E2 deprivation paradigm) using Systat 13 software (Systat Software Inc, San Jose, CA, USA), followed by Tukey's Honestly-Significant-Difference post hoc analysis to determine significant differences among groups where $p<0.05$. Different letters and/or symbols denote statistically significant differences between groups. A separate Tukey's Honestly-Significant-Difference post hoc test was utilized within groups that showed a statistically significant main effect of age and/or treatment. A one-way ANOVA followed by Tukey's Honestly-Significant-Difference post hoc analysis was conducted to determine significant difference ($p<0.05$) between selective ER agonists (DPN and PPT; Exp. #3) where treatment was the main effect in the hypothalamus of 18-mo. animals and *in vitro* studies. Student's t-test was conducted in other experiments where noted. All data are presented as mean +/− SEM.

RESULTS

Nova1 expression decreased in the aging female brain (Experiment #1)

Age is associated with an increase in alternative splicing events in the brain and decreased circulating E2 alters splicing of the ERβ transcript (Shults et al., 2015a; Tollervey et al., 2011; Wang et al., 2012). Expanding on this observation, we examined the effects of age and E2 deprivation on mRNA expression of the neuron-specific RNA binding protein NOVA1. Our results revealed a significant main effect of treatment and age in the hypothalamus ($F(1,22)=11.708$, $p<0.005$), dorsal hippocampus ($F(1,21)=5.892$, $p<0.05$) and ventral hippocampus ($F(1,21)=14.064$, $p<0.005$). E2 treatment in the young animals either significantly increased (hypothalamus, dorsal hippocampus, Fig. 2A, B) or decreased (ventral hippocampus, Fig. 2C) Nova1 mRNA, whereas E2 treatment had no effect in the aged animals. Consistent with previous reports, we also observed a significant age-related decline in Nova1 mRNA expression that was evident in both the aged vehicle- and E2-treated animals in all brain regions (Fig. 2).

Expression of Nova1 is altered by E2 deprivation in a brain-region specific manner in aged female rats (Experiment #2)

Next, we focused on the aged animals only to determine how E2 administration affected Nova1 mRNA following increased time after OVX (i.e. "estrogen deprivation paradigm", Fig. 1B).

Hypothalamus—Most striking, E2 significantly increased Nova1 mRNA 4 and 8 weeks post-OVX to a maximum of approximately 13-fold (Fig. 3A). Also, age alone (vehicle-treated) increased Nova1 mRNA, with statistical significance being observed by 8 weeks post-OVX, although the fold changes in vehicle-treated animals were modest by comparison to the E2 effects. Notably, Nova1 mRNA reached a nadir to levels nearly undetectable in both vehicle and E2-treated animals by 12 weeks post-OVX (Fig. 3A). A two-factor ANOVA demonstrated a significant interaction between length of E2 deprivation (time post OVX) and E2 treatment on Nova1 mRNA in the hypothalamus (Supp. Table 1).

Protein levels of NOVA1 in the hypothalamus were not always consistent with changes in mRNA, but the general direction of change was the same. For instance, similar to the observed mRNA increase in E2-treated animals at 4 weeks post-OVX, there was also a significant increase in NOVA1 protein (Fig. 3B). However, this increase was evident in both the vehicle and E2-treated groups, which was not observed at the mRNA level. Similarly, there was no corresponding statistically significant increase in NOVA1 protein at the 8-week time point. Protein levels were also not different at the 12-week time point, despite observations of very low mRNA levels (Fig. 3B). A two-factor ANOVA of NOVA1 protein confirmed a statistically significant main effect of time (post-OVX), but not treatment, in the hypothalamus (Supp. Table 1).

Hippocampus—The effects of both E2 treatment and E2 deprivation were more modest in the dorsal and ventral hippocampus compared to what we observed in the hypothalamus. In the dorsal hippocampus, there was a significant main effect of time on Nova1 mRNA expression post-OVX (Supp. Table 1). Specifically, an age-related decline in Nova1 mRNA was observed at 4 and 8 weeks post-OVX, and this effect was prevented by E2 treatment (Fig. 4A). However, significant changes in NOVA1 protein levels only corresponded to mRNA at the 4-week time point (Fig. 4B).

There was a significant interaction in the ventral hippocampus between time and treatment on Nova1 mRNA expression (Fig. 5A, Supp. Table 1). In addition, there was a main effect of time post-OVX (“E2 deprivation”), as Nova1 mRNA steadily increased during the E2 deprivation period independent of E2 treatment (Fig. 5A; Supp. Table 1). Similarly, there was a significant main effect of time in NOVA1 protein, which also steadily increased with age independent of E2 treatment (Fig. 5B; Supp. Table 1).

E2-induced increases in hypothalamic Nova1 expression are mediated by both ERα and ERβ (Experiment #3)

To determine which E2 receptor mediated the observed increases in hypothalamic Nova1, acute administration of the ERβ-specific agonist diarylproprionitrile (DPN) or the ERα-specific agonist propyl pyrazole triol (PPT) was given to 18-mo. old female OVX rats. Our results demonstrated that both DPN and PPT significantly increased expression of Nova1 mRNA in the hypothalamus, yet the magnitude was much greater in animals treated with PPT (3.5-fold compared to 2.25-fold, respectively, Fig. 6). There were insufficient tissue samples to measure Nova1 protein levels in these animals.

Overexpression of Nova1 decreases ERβ splice variant expression through direct binding of ERβ mRNA

Nova1 regulates exon splicing through differential binding of the pre-mRNA transcript. Nova1 promotes exon exclusion if it binds upstream of an alternatively spliced exon (Ule, Jernej et al., 2006; Zhang et al., 2010). The nucleotide sequence for ERβ exon 5 has a cluster of putative Nova1 binding sites located upstream of the sequence that differentiates ERβ2 from ERβ1 (Fig. 1A). Therefore, NOVA1 would be predicted to increase the exclusion of the ERβ2-specific sequence resulting in increased processing of ERβ1 transcripts. In order to determine if NOVA1 regulates ERβ splicing, Nova1 was overexpressed in a dose-dependent manner (0.01 μg – 2 μg DNA) *in vitro* in the IVB rat hypothalamic cell line. Overexpression of Nova1 at all concentrations resulted in abolishment of ERβ2 mRNA expression when compared to pcDNA, while ERβ1 expression increased in a dose-dependent manner concomitantly with Nova1 expression (Fig. 7). We then tested whether NOVA1 directly interacts with ERβ mRNA and if the interaction was dependent on the identified Nova1 binding sites located in exon 5. RNA immunoprecipitation demonstrated a 75-fold enrichment of NOVA1 protein with wtERβ1 mRNA in HEK-293 cells (Fig. 8). Conversely, ERβ mRNA was undetectable in NOVA1 precipitate when the putative binding sites in exon 5 were mutated (Fig. 8) demonstrating that the NOVA1 association with ERβ mRNA was specific to these sites.

DISCUSSION

Our results revealed the novel finding that hypothalamic Nova1 mRNA and protein expression is regulated by E2 and age in female rats. Further, low expression levels of Nova1 were sufficient to completely abolish ERβ2 splice variant expression in hypothalamic-derived cell lines and Nova1 directly interacted with ERβ mRNA through specific binding sites in the transcript. Together, these findings provide strong evidence that Nova1 likely plays an important role in regulating the alternative splicing of the ERβ transcript in the brain.

The NOVA1 splicing factor is an important RNA binding protein (RBP) responsible for regulating the alternative splicing of many different RNA transcripts in the brain. Here, we demonstrated that E2 regulated the expression of Nova1 mRNA in young, but not old, female brains. However, this pattern was dramatically altered after prolonged periods of E2-deprivation (i.e. akin to a post-menopause state), suggesting that E2 replacement therapy after menopause could shift the pattern of alternative RNA splicing for Nova1 target genes. We also showed that one of these target genes, ERβ, is directly spliced by NOVA1 binding to exon 5, which resulted in the decreased production of ERβ2. Together, these data suggest that there could be a biological feedback loop wherein E2 regulates Nova1, which, in turn, determines the ratio of ERβ variants in the cell. These initial findings provide strong support for this hypothesis, but more studies are required to confirm that such a feedback system exists in the brain. Moreover, E2-induced regulation of Nova1 mRNA can be mediated through ERα, as demonstrated by our data using the specific ERα agonist PPT, suggesting the exciting possibility of a dynamic relationship whereby ERα is able to indirectly dictate the alternative splicing of ERβ in the brain.

We and others previously demonstrated that ERβ2 expression increased in the aged female brain, and its expression varied depending on the presence or absence of E2 (Shults et al., 2015a; Wang et al., 2012). Moreover, increased ERβ2 was correlated with poor cognitive performance and increased stress-related behaviors (Wang et al., 2012). Consistent with those findings the current study demonstrates that the RBP, Nova1 mRNA, decreased in the hypothalamus in aged female rats, thereby providing a possible molecular mechanism for the observed age-related increase in alternative splicing of ERβ. Specifically, the highest levels of ERβ expression were observed after 1 and 12 weeks of E2 deprivation in the hypothalamus (Shults et al., 2015a), and the data reported herein show that Nova1 expression is coincidently lowest at those same time points. Further evidence supporting Nova1 as a molecular mechanism regulating ERβ splicing was our observation that Nova1 directly and specifically interacted with ERβ mRNA transcripts, and consequently, Nova1 overexpression abolished ERβ2 splice variant expression. The brain regions tested (hypothalamus, dorsal hippocampus, and ventral hippocampus) endogenously express Nova1, as previously observed by immunohistochemistry with a Nova1 specific antibody, and also express important Nova1-regulated genes (Dredge and Darnell, 2003; Li et al., 2013; Shults et al., 2015a; Ule et al., 2005a). E2 signaling is physiologically important in these regions, and the actions of E2 may work in conjunction with Nova1 in regulating neurogenesis and synaptic plasticity (Park et al., 2009; Racca et al., 2010; Ratti et al., 2008; Ule et al., 2005b; Walf and Frye, 2007; Walf et al., 2008; Walf et al., 2004).

Notably, a lack of correlation between Nova1 mRNA and protein was observed at certain time points. This was especially evident in the hypothalamus at 4 and 8 weeks post-OVX. Quite often mRNA and protein levels do not correspond due to the kinetics of mRNA translation and turnover. However, we can also attribute these findings to technical issues in the way the samples were collected. The animals were euthanized and both mRNA and protein were isolated at the same time from the brain tissues samples. Physiologically, there is a time lapse from transcriptional expression to protein translation, which is different for every gene. Nevertheless, the protein levels follow the same directional trend as the mRNA in about half of the time points tested for the different brain regions and we are confident that given additional time points a better correlation between the two would have been evident.

Analysis of the ERβ pre-mRNA nucleotide sequence revealed many RBP consensus sites that could regulate its alternative splicing (RBPmap; rbpmap.technion.ac.il). Among these are several NOVA1 consensus sites clustered upstream of the ERβ2 54 bp insert. However, previous high throughput studies mapping all NOVA1 pre-mRNA interactions failed to detect an interaction between ERβ and NOVA1 (Ule et al., 2005a; Ule et al., 2003; Ule, J. et al., 2006). One possible explanation for the discrepancy between those studies and our current observations might be that NOVA1:ERβ interactions were enriched in the brain regions we analyzed, and therefore the sensitivity in our assay was increased, whereas the high throughput studies measured interactions in the whole brain. Our data suggests that NOVA1 mediated an exclusion of the ERβ2-specific nucleotide sequence (54 bp insert), which resulted in significantly more wild-type ERβ1 expression. However, the presence of other RBP binding sites suggest that other splicing factors could also play a role in ERβ

alternative splicing, especially when considering the presence of other described ERβ splice variants in the brain, such as ERβ1δ3 (Price et al., 2001).

One major objective of this study was to analyze Nova1 expression in aged animals that underwent a surgically-induced menopause paradigm ("estropause"), which was designed to mimic hormone replace therapy (HT) occurring at increasingly longer time points following menopause in humans (Pinceti et al., 2016; Rao et al., 2015; Shults et al., 2015a). This paradigm has previously shown to achieve clinically relevant levels of E2 similar to those reported in postmenopausal women receiving hormone replacement therapy (Pinceti et al., 2016; Schmidt et al., 1994; Shults et al., 2015a; Talboom et al., 2008). Overall, our data suggest that Nova1 mRNA and protein expression varies by brain region following longer periods of ovarian hormone deprivation. This pattern of expression could promote splicing events associated with age-related neurodegeneration and disease (Lu et al., 2004; Serrano and Klann, 2004; Tollervey et al., 2011). Specifically, in the hypothalamus Nova1 mRNA transcription was increasingly sensitive to E2 treatment up to 8 weeks following OVX, but these effects did not persist and Nova1 levels became nearly undetectable by 12 weeks post-OVX. Logically, it can then be reasoned that ERβ2 would be highest at that same time point. Indeed, our previous study showed that ERβ2 levels were increased 12 weeks following OVX, but only after E2 treatment (Shults et al., 2015a). These data indicate that other splicing factors could be more important after prolonged periods of E2 deprivation in the hypothalamus, or that the efficacy of E2 at later time points is altered by global ER expression changes in these regions.

Indeed, age-related changes ERα or ERβ expression likely play a pivotal role in E2 regulation of Nova1. Our lab previously demonstrated that ERβ expression, and its alternatively spliced variants, are altered with age and E2 deprivation in a brain-region specific manner (Mott et al., 2014; Shults et al., 2015a). Similarly, others have shown that ERα expression is altered with age and neurodegeneration (Chakraborty et al., 2003; Ishunina and Swaab, 2012; Mehra et al., 2005; Yamaguchi and Yuri, 2014; Yamaguchi-Shima and Yuri, 2007). Analysis of the Nova1 promoter region revealed the presence of multiple ½ ERE (estrogen response element) and AP-1 (activator protein 1) sites, which indicate that E2 most likely regulates Nova1 through genomic ER-mediated pathways. ERs can act at these two distinct sites in different manners. For instance, ERα enhances and ERβ inhibits, AP-1-mediated gene transcription (Paech et al., 1997; Webb et al., 1999). Therefore, E2-induced increases in Nova1 mRNA expression could be mediated by ERα and/or ERβ acting independently of each other (Cowley et al., 1997), perhaps through homodimerization of the receptors binding directly to ERE sites on the Nova1 promoter. On the other hand, heterodimerization could have inhibitory effects on Nova1 transcription (Ogawa et al., 1998). Our results demonstrated that the specific agonists for both ERα and ERβ increased Nova1 expression in the hypothalamus after just one week post-OVX in the aged animals, and these agonists only activate the homodimer configuration (Carroll et al., 2012; Stauffer et al., 2000). By contrast, E2 had no effect at this same time point in the hypothalamus, suggesting that the effects of E2 were likely mediated by heterodimers. Another possibility is that activation of membrane and/or non-classical estrogen receptors might contribute to the complexity of our *in vivo* results. For instance, G-protein coupled estrogen receptors GPER and GqmER have been shown to have non-genomic effects in

response to ligand. Activation of these receptors by E2 may affect the expression of Nova1 through activation of MAPK signaling pathways (Bertelsen et al., 2004; Filardo and Thomas, 2005). Future studies are required to determine the exact mechanism by which ERs regulate Nova1.

Previous studies demonstrating that Nova1 decreased with age were limited to samples taken from male cortex (Tollervey et al., 2011). In agreement with those findings, our results showed similar decreases in Nova1 mRNA expression with age in all three brain regions we studied. However, the effects of E2 in young animals differed between brain regions: E2 significantly increased Nova1 mRNA expression in the hypothalamus and dorsal hippocampus, whereas it significantly decreased Nova1 mRNA in the ventral hippocampus. Decreased Nova1 in the aged brain might be a consequence of general neuronal aging, as neurons are subjected to increased oxidative stress (Serrano and Klann, 2004), perturbed energy homeostasis (Ames, 2004), DNA damage (Lu et al., 2004), and accumulation of misfolded/aggregated proteins (Trojanowski and Mattson, 2003). The 18-mo. old animals in our studies equate to an approximate age of 55 human years old (Shults et al., 2015b) however, the advanced ages in previous Nova1 studies were conducted in males between 82–86 years old. Therefore, our data suggest the novel conclusion that Nova1 mRNA and protein expression may decline in the brain at a younger age than previously reported. Another possibility is that there is a sex difference in Nova1 regulation in the brain, which is supported by our data showing that E2 regulates Nova1.

CONCLUSIONS

Overall, the results of the present study provide crucial insight into the effects of aging and prolonged ovarian hormone deprivation on Nova1. We also provide a novel mechanism by which ERβ is alternatively spliced, and how observed changes in Nova1 expression may alter expression of not only ERβ splice variants but could also alter a variety of Nova1-regulated proteins in the brain. Consequences of these changes in expression may further explain the effects of reproductive senescence on cognitive function and memory, and Nova1 may be a novel therapeutic target in maintaining efficacy in hormone replacement therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Estradiol regulates Nova1 in the aged female rat brain
- Duration of estradiol deprivation differentially alters Nova1 expression
- Estradiol acts through both ERα and ERβ to regulate Nova1
- The splicing factor Nova1 regulates ERβ alternative splicing
- Nova1 associates with ERβ RNA transcripts at specific sites

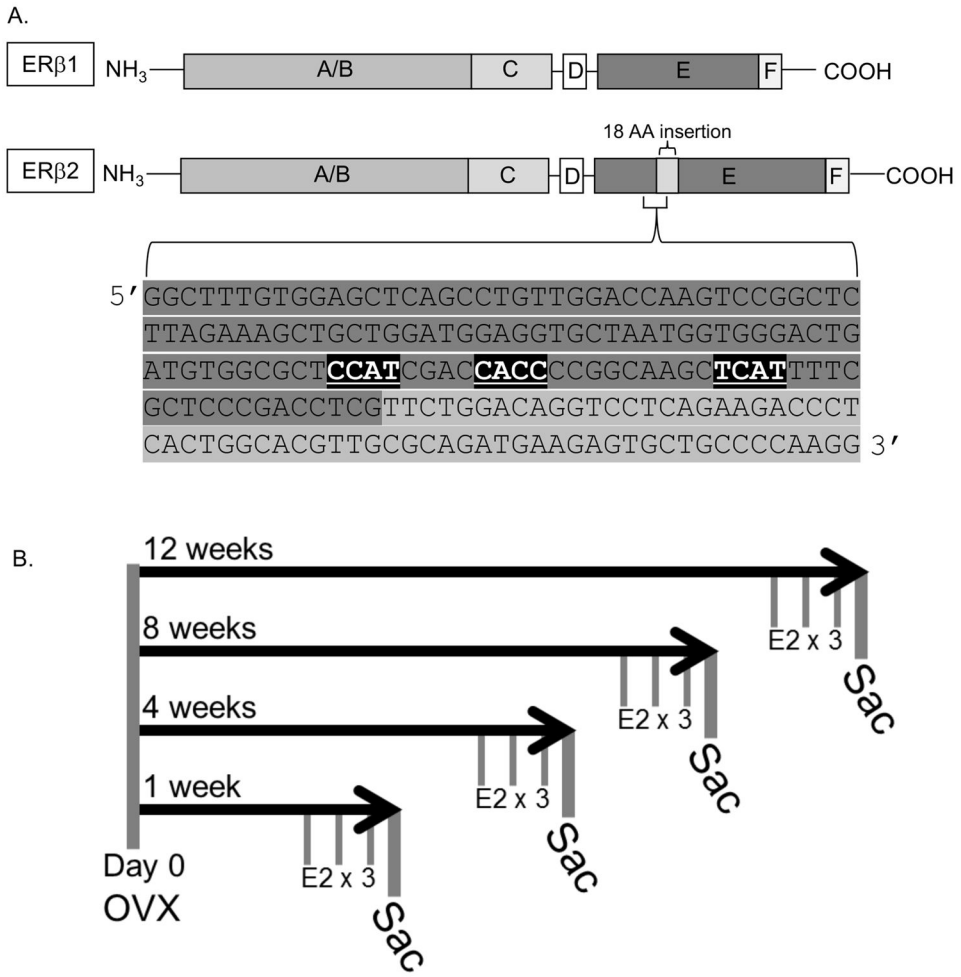

Figure 1. A) Nova1 consensus sequences in ERβ exon 5 and B) animal paradigm

A) The full length rat ERβ sequence was analyzed for RNA binding protein sites using RBPmap (http://rbpmap.technion.ac.il/index.html). Nova1 YCAY consensus sites are highlighted (black boxes, white text) in ERβ exon 5 (highlighted in dark gray). The downstream 54bp ERβ2 insert is shown in the light gray box. Scores assigned to Nova1-binding probabilities must be greater than 0.895 (out of 1.00) based on weighted rank in order to be detected. B) Female rats were ovariectomized (OVX) at 18 months of age (Day 0). Treatment with vehicle (safflower oil) or 2.5 μg E2 (dissolved in safflower oil) was given by i.p. injection once per day at indicated time points following OVX. Animals were humanely euthanized (SAC) 24 hours after the last treatment.

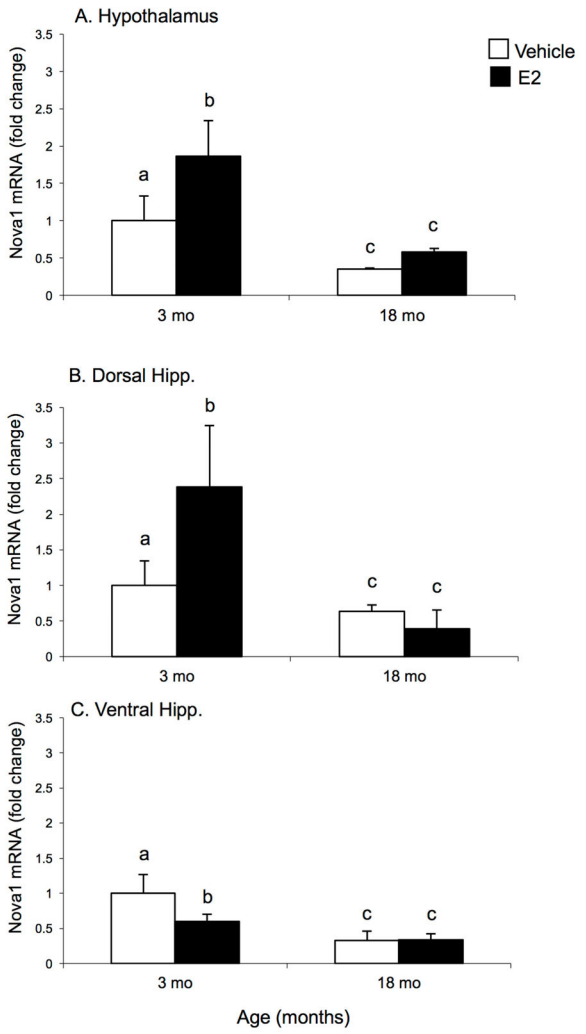

Figure 2. Nova1 mRNA decreased in the brain with age and was altered by E2 treatment

Nova1 mRNA expression was measured in the (A) hypothalamus, (B) dorsal hippocampus, and (C) ventral hippocampus at 1 week post-OVX in 3- and 18-month old female rats (N = 6/age/treatment group). Animals were OVX and 1 week later treated with either vehicle (safflower oil) or 2.5 μg E2 (dissolved in safflower oil). Dissimilar letters indicate statistically significant differences between groups ($p<0.05$) by Tukey's Honestly-Significant-Difference Test following two-factor ANOVA. Data are expressed as mean ± SEM.

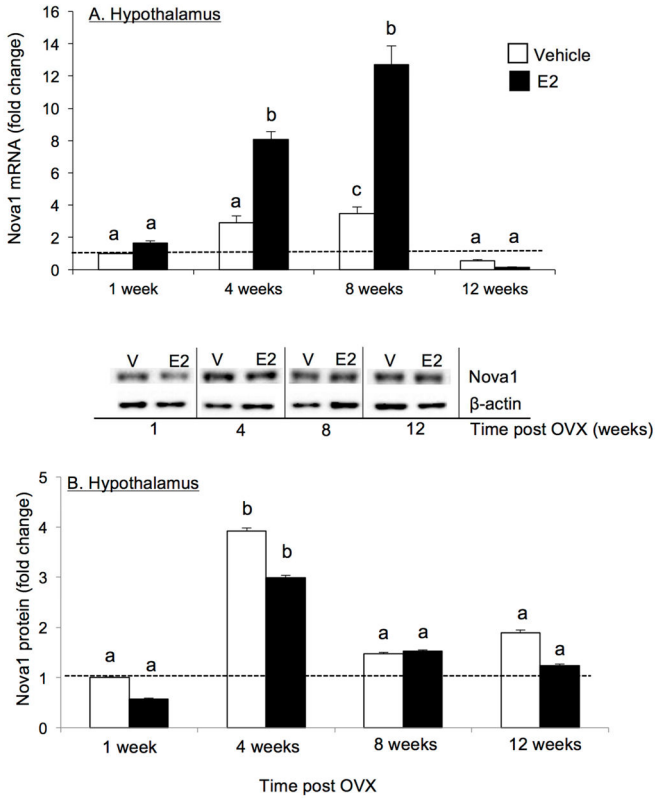

Figure 3. Nova1 mRNA and protein levels are altered by E2 treatment and length of E2 deprivation in the hypothalamus of aged (18-mo. old) female rats following varying lengths of time post-OVX

Nova1 mRNA expression (A) and protein levels (B) were measured in the hypothalamus at 1, 4, 8, and 12 weeks post-OVX (N = 6–10/age/treatment group). Prior to euthanasia, animals were treated with either vehicle (safflower oil) or 2.5 μg E2 (dissolved in safflower oil) once/day for 3 consecutive days. A two-factor ANOVA demonstrated a significant interaction between length of E2 deprivation (time post OVX) and E2 treatment on Nova1 mRNA in the hypothalamus (A). Panel B shows a representative western blot (top) using anti-Nova1 primary antibody for each treatment group at the various time points, normalized to β-actin, with quantification shown in the histogram determined by densitometry from multiple western blots. A two-factor ANOVA of NOVA1 protein confirmed a statistically significant main effect of time (B). Dissimilar letters indicate statistically significant differences between groups ($p<0.05$) as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

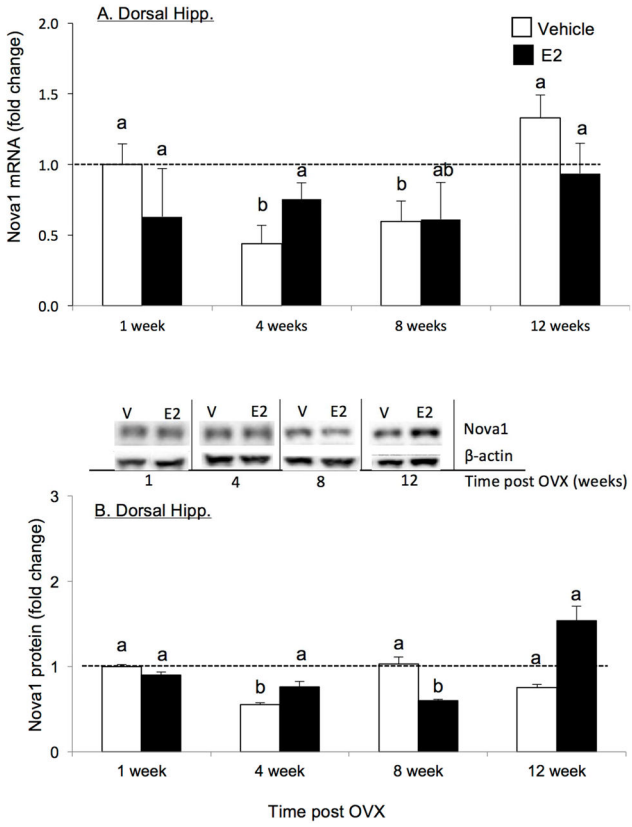

Figure 4. Nova1 mRNA and protein levels are altered by length of E2 deprivation in the dorsal hippocampus of aged (18-mo. old) female rats following varying lengths of time post-OVX

Nova1 mRNA expression (A) and protein levels (B) were measured in the dorsal hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6–10/age/treatment group). Prior to euthanasia, animals were treated with either vehicle (safflower oil) or 2.5 μg E2 (dissolved in safflower oil) once/day for 3 consecutive days. A two-factor ANOVA of NOVA1 mRNA confirmed a statistically significant main effect of time. Panel B shows a representative western blot using anti-Nova1 primary antibody for each treatment group at the various time points, normalized to β-actin, with quantification shown in the histogram determined by densitometry from multiple western blots. Dissimilar letters indicate statistically significant differences between groups ($p<0.05$) as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

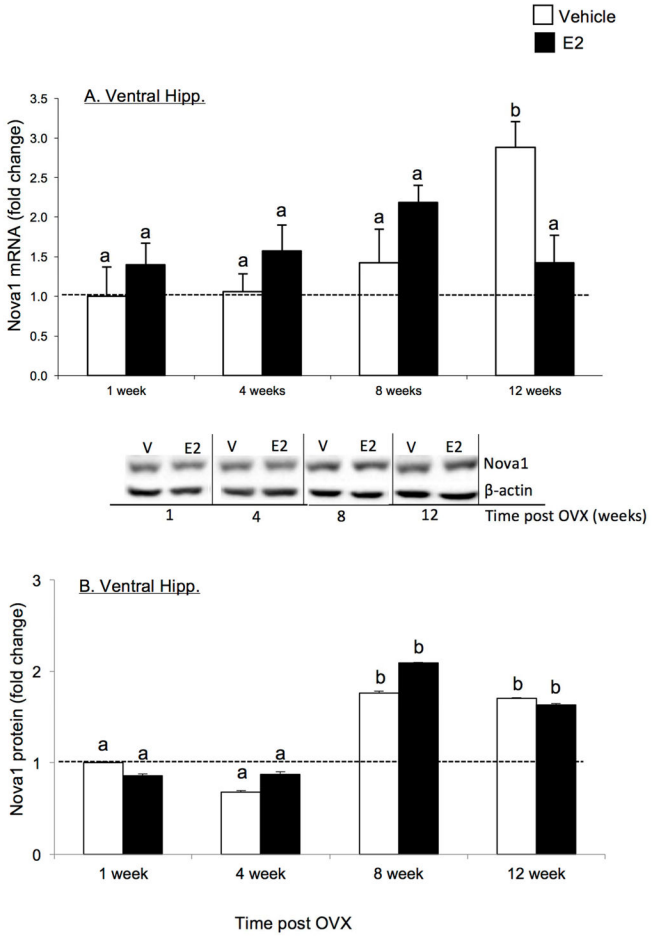

Figure 5. Nova1 mRNA and protein levels are altered by length of E2 deprivation in the ventral hippocampus of aged (18-mo. old) female rats following varying lengths of time post-OVX

Nova1 mRNA expression (A) and protein levels (B) were measured in the ventral hippocampus at 1, 4, 8, and 12 weeks post-OVX (N = 6–10/age/treatment group). Prior to euthanasia, animals were treated with either vehicle (safflower oil) or 2.5 μg E2 (dissolved in safflower oil) once/day for 3 consecutive days. A two-factor ANOVA demonstrated a significant interaction between length of E2 deprivation (time post OVX) and E2 treatment on Nova1 mRNA in the ventral hippocampus (A). Panel B shows a representative western blot using anti-Nova1 primary antibody for each treatment group at the various time points, normalized to β-actin, with quantification shown in the histogram determined by densitometry from multiple western blots. A two-factor ANOVA of NOVA1 protein confirmed a statistically significant main effect of time (B). Dissimilar letters indicate statistically significant differences between groups ($p<0.05$) as determined by Tukey's Honestly-Significant-Difference Test following two-way ANOVA. Data are expressed as mean ± SEM.

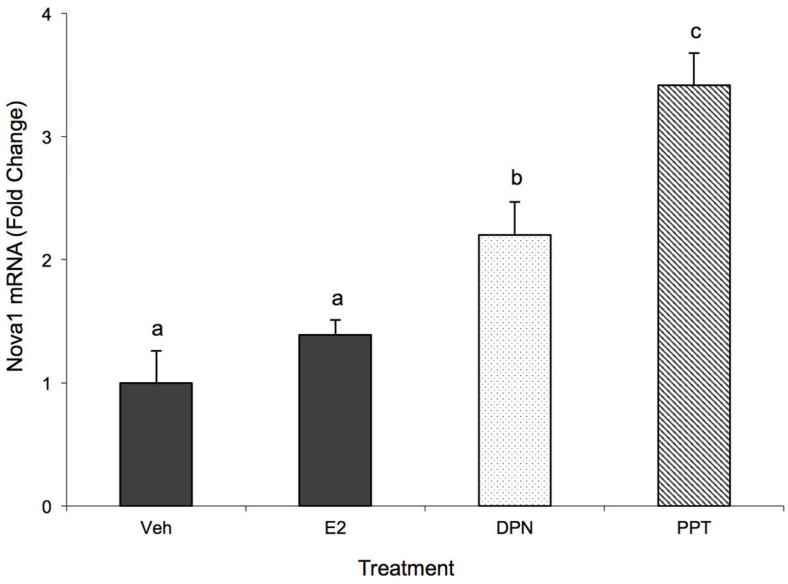

Figure 6. ERα- and ERβ-selective agonists increase Nova1 mRNA expression

Nova1 mRNA expression was measured in the hypothalamus of 18-month old female rats that were OVX and 1 week later treated with either vehicle, E2, 1mg/kg DPN (ERβ-selective agonist), or 0.5 mg/kg PPT (ERα-selective agonist) once/day for three consecutive days (N = 6–8/treatment group). Dissimilar letters indicate statistically significant differences between groups ($p<0.05$) as determined by Tukey's Honestly-Significant-Difference Test following one-way ANOVA. Data are expressed as mean ± SEM.

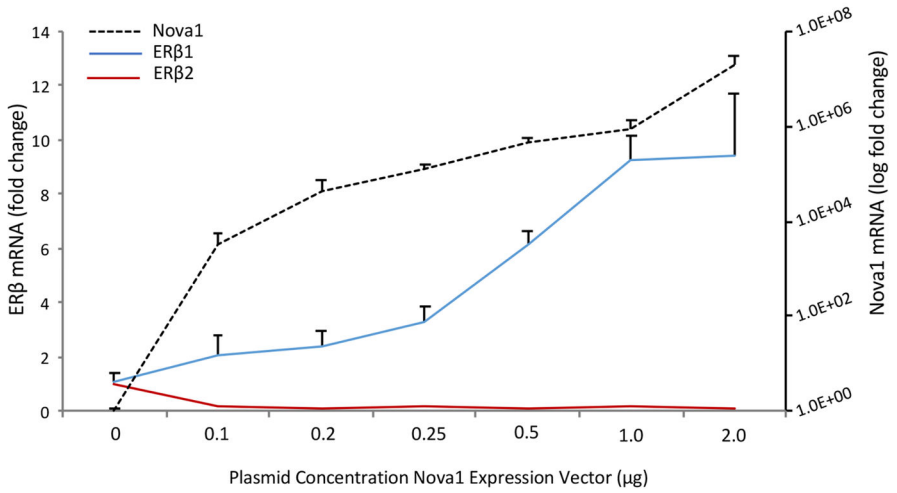

Figure 7. ERβ alternative splicing decreases with Nova1 overexpression

ERβ1 (blue line) and ERβ2 (red line) mRNA expression was measured in hypothalamic-derived IVB cells transfected with increasing concentrations of Nova1 eGFP plasmid (0.1–2ug DNA) (N=5–6 technical replications/concentration from 6 independent experiments). Left Y-axis indicates the fold change in ERβ1 and ERβ2 mRNA expression as determined by ΔΔCT. Right Y-axis indicates increased Nova1 mRNA (dashed line) dependent on plasmid concentration.

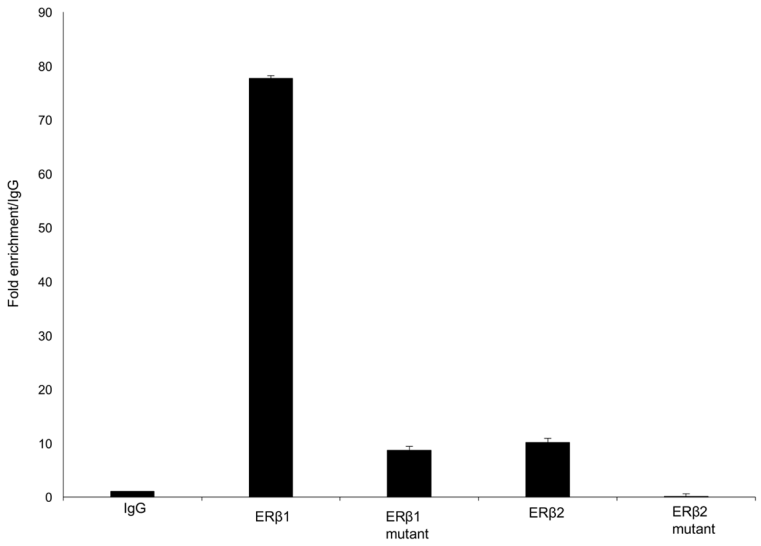

Figure 8. NOVA1 directly interacts with ERβ mRNA at YCAY sites located in exon 5

RNA immunoprecipitation results demonstrating fold-enrichment of ERβ2 mRNA associated with Nova1 protein. HEK cells were transfected with an expression vector containing Nova-1-GFP and WT ERβ1/β2 or mutated ERβ1/β2, which had deletions in the 3 YCAY consensus Nova1 binding sites located in ERβ exon 5. Data are expressed as mean fold-enrichment ± SEM (N = 8–15 technical replicates from 4–6 independent experiments).