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Structural and functional characteristics of estrogen receptor beta (ER β) splice variants: implications for the aging brain

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Abstract

Estrogen receptor beta (ER β) is a multifunctional nuclear receptor that mediates the actions of estrogenic compounds. Despite its well defined role in mediating the actions of estrogens, a substantial body of evidence demonstrates that ER β has broad range of physiological functions that are independent of those normally attributed to estrogen signaling. These functions can, in part, be achieved by the activity of several alternatively spliced isoforms that have been identified for ER β . This short review will describe structural differences between the ER β splice variants that are known to be translated into proteins. Moreover, we discuss how these alternative structures contribute to functional differences in the context of both healthy and pathological conditions. This review also describes the principal factors that regulate alternative RNA splicing. The alternatively spliced isoforms of ER β are differentially expressed according to brain region, age, and hormonal milieu underscoring the likelihood that there are precise cell-specific mechanisms that regulate ER β alternative splicing. However, despite these correlative data, the molecular factors regulating alternative ER β splicing in the brain remain unknown. Here, we review the basic mechanisms that regulate alternative RNA splicing and use that framework to make logical predictions about ER β alternative splicing in the brain.

INTRODUCTION

Aging represents a continuous spectrum of phenotypic and behavioral changes that are ultimately driven by inefficient cellular functioning that contributes to increased cell damage over time. Detrimental changes in cellular function include telomere shortening, increased missense/nonsense mutations, translational errors, and organelle dysfunction, all of which can result in protein dysfunction and the accumulation of cytotoxic protein aggregates. Concurrent with these age-related abnormalities is an increased incidence of global alternative RNA splicing (1). Alternative RNA splicing is heralded as a means of expanding the cellular proteome beyond the constraints of strict genetic coding. As such, it is widely considered to be both beneficial and detrimental depending on the structural and functional aspects of the alternatively expressed isoforms. In young healthy tissues, it is likely that one

dominant isoform is expressed (2), despite the availability of the molecular machinery required to achieve the expression of multiple isoforms. However, human studies demonstrate that aging increases the incidence of alternative splicing independent of disease, thereby increasing the abundance of alternatively spliced isoforms that are expressed in a variety of tissues. One of the highest rates of alternative splicing events occurs in the brain, and increased expression of alternatively spliced isoforms can contribute to age-related deficits in many neurobiological processes (1, 3–5)

Menopause is a unique biological process in women that is concurrent with aging. A physiological hallmark of menopause is the sharp decline in circulating levels of 17 β -estradiol (E2), the primary circulating gonadal estrogen in reproductive age women. The rapid decline of E2 at menopause further complicates the natural aspects of aging, as E2 is key for the maintenance of healthy organs and tissues in young women. The beneficial actions of E2 on various organs are achieved through the high affinity binding of E2 to its cognate receptors: estrogen receptor (ER) α (NR3A1/ESR1) and ER β (NR3A2/ESR2), which function as both transcription factors and mediators of intracellular signaling cascades (6–8). Over the past decade, many alternatively spliced transcripts for ER α and ER β have been identified using RNA deep sequencing platforms, but few have been shown to translate into functional protein products (9–13). Notably, several of these translated alternatively spliced isoforms appear to have constitutive transcriptional activity and function largely independent of ligand (i.e. E2) binding (14–17). Further, the concurrent age-related increase in global alternative splicing events provides a molecular framework to allow for the increased expression of these variants during this critical life transition. This intriguing hypothesis suggests that alternatively spliced forms of ER may be more biologically relevant post-menopause, whereas the dominant isoform may be more active during times of high circulating estrogen (18, 19).

This review will focus on the structure, function, and regulation of validated ER β splice variants in humans and rodents. ER β was originally cloned in 1996, and since then has been characterized as mediating many of the non-reproductive actions of E2. For example, ER β may function alone or in concert with other estrogen receptors (ER α and GPR30) to carry out the numerous cardio- and neuroprotective effects of E2. Moreover, ER β is expressed in a wide range of brain regions and does not exhibit global alterations in expression through aging. However, its expression has been found to be significantly decreased in some brain regions, independent of E2 deprivation (20, 21). Additionally, ER β subcellular localization may be altered during aging (22), which could impact the efficacy of canonical ER β signaling pathways. Together, these distinctions from ER α make ER β , and its splice variants, an attractive candidate for therapeutic strategies aimed at alleviating negative symptoms associated with decreased circulating E2 and improving cognitive function in post-menopausal women.

ER β : BASIC STRUCTURAL ORGANIZATION

Estrogen receptors are classified as ligand-activated transcription factors belonging to a large superfamily of nuclear receptors (23, 24). The two primary receptors, ER α and ER β are encoded by distinct genes, and the mature transcripts contain 8 exons. Similar to other

receptors belonging to the nuclear receptor superfamily, the ER β protein is composed of 6 functional domains denoted by letters A-F extending from the N- to C- terminus of the protein (Fig. 1) (25–27).

The N-terminal A/B domain contains the activation function-1 (AF-1) region, characterized by its cooperativity in ligand dependent transcriptional activation and its synergistic interactions with the C-terminal ligand binding domain (LBD) (28). Post-translational modifications in the AF-1 region of both ERs can also initiate ligand independent transcription through the recruitment of select co-regulatory proteins (29). An important distinction for ER β is the apparent inhibitory effects of the AF-1 domain on its transcriptional activity. For instance, experimental deletions of the ER β AF-1 region increased dimerization and subsequent transcriptional activity, suggesting that specific amino acid residues in this region are critical for keeping the receptor transcriptionally inactive (30).

Next, the C domain is the most highly conserved region among all members of the nuclear receptor family and contains the residues responsible for recognizing specific DNA enhancer regions, such as estrogen response elements (EREs). Stable DNA binding is achieved by the coordinated interactions of two perpendicular α -helices (6). The first helix contains the residues responsible for DNA recognition and is situated between two zinc finger motifs, which are composed of 8 cysteine residues surrounding two zinc ions. The second helix is generally thought to confer stability to the complex, binding non-specifically to the DNA backbone and thereby allowing the temporal resolution necessary for the recruitment of additional co-regulatory proteins and basal transcriptional machinery. Notably, ER β has been shown to interact with DNA through a wide variety of mechanisms that are not limited to the presence of canonical EREs or direct DNA binding, for example by tethering to other basic transcription factors (31–33). In both humans and rats, the DNA-binding domains (DBD) of ER α and ER β show over 95% sequence homology. Therefore, it is not surprising that ER α and ER β can form functional heterodimers (34), and recent *in silico* modeling demonstrated that the heterodimer is more stable than the ER β homodimer (35).

The D domain is a flexible hinge region that provides a functional link between the DBD and the LBD. This region harbors the nuclear localization signal, which, upon ligand binding, allows the receptor to translocate from the cytoplasm to the nucleus where it can interact with DNA. The hinge region is the least characterized portion of ER β and is highly variable among members of the nuclear receptor superfamily. Therefore, some studies suggest this region is multifunctional, yet highly specific to individual receptor subtypes, and possibly confers cell-type specific functions (36).

The E domain, or ligand binding domain (LBD), has been the most thoroughly characterized, both functionally and structurally, for all nuclear receptors. This domain is comprised of 12 α -helices that together form a ligand binding pocket and, upon ligand binding, expose a co-regulatory protein binding surface. The orientation of helix 12 is critical to the conformation ERs adopt once bound to ligand, and ultimately influence the ability of the receptor to bind other proteins and activate gene transcription. Helix 12 contains the core residues of the activator function-2 (AF-2) domain, a short amphipathic

conserved alpha-helix that interacts with coregulatory proteins through an LxxLL motif. The LBD domain shares only 55% sequence homology between ER α and ER β , which has enabled the development of selective estrogen receptor agonists and antagonists.

Finally, at the C-terminal end of the receptor lies a short F domain whose function has been largely uncharacterized for ER β and has almost no sequence homology with ER α (37, 38). This domain is typically truncated or absent in the human ER β splice variants studied to date, making its functional properties particularly relevant, as described more fully in the next section. The highly defined functional domains of ER β underscore the importance of each domain in facilitating estrogens' actions in a given cell or tissue. Alternative splicing results in the altered structure of one or more of these domains, making it reasonable to predict that the ER β splice variants could have distinct interacting protein partners and perhaps even target genes that are not traditionally ascribed as being estrogen-regulated.

ER β SPLICE VARIANTS

Shortly after the discovery of ER β , several alternatively spliced ER β variants were identified in both humans and rodents (39–43). Although the human and rodent ER β splice variants differ in structure, they demonstrate remarkably similar functions. Most notably, many of the variants appear to have constitutive, or ligand independent, actions on downstream target genes. The biological consequences of this constitutive activity are predictably different for various tissue types, yet our understanding of the biological function for many of these variants is still in its infancy.

Rodent splice variants

To date, there have been 5 experimentally validated functional ER β splice variants in rodents. From this point on, the dominant isoform will be referred to as ER β 1. The first variant discovered in rodents was the rat ER β 2 splice variant (rER β 2). This variant contains an in-frame insertion of 54 nucleotides between exons 5 and 6, which results in a novel 18 amino acid sequence the ligand binding domain (39, 42, 44). The origin of these extra nucleotides was initially unclear; however, we have predicted that these extra nucleotides represent an alternative exon based on the presence of upstream binding motifs for the neuronal specific RNA binding protein Nova1 (neuro-oncological ventral antigen 1; Fig. 2).

The elongated LBD of rER β 2 results in decreased binding affinity for E2 and many other estrogenic compounds, such as xenoestrogens and phytoestrogens, compared to rER β 1. Because of this decreased affinity for ligand, rER β 2 seems have primarily ligand independent activity in most cell types. For example, reporter assays in hippocampal-derived HT-22 cells demonstrated that rER β 2 exhibits ligand independent activation of ERE-driven promoters and repression of AP-1-driven promoters; these effects are only modestly enhanced by the addition of E2 (17). These reporter assays demonstrated that, compared to rER β 1, the ligand independent action of rER β 2 at both ERE and AP-1 promoters is lower, but rER β 1 exhibits a much larger response to ligand than rER β 2 (38). Furthermore, the observed differences in transcriptional activity were not due to changes in DNA binding affinity, but could be explained by the inability of rER β 2 to recruit specific steroid receptor coactivators and/or differential posttranslational modifications of the splice variants (14, 17).

These data provided useful mechanistic information that detailed the differential transcriptional potential of ER β splice variants at discrete regulatory elements; however, most endogenous gene promoters are highly complex and contain multiple regulatory regions that function in concert. Studies using more complex promoters, such as arginine vasopressin (AVP), demonstrated that rER β 2 robustly induced AVP transcriptional activity in the presence of E2 and 3 β Adiol, a metabolite of the potent androgen dihydrotestosterone (DHT) (45). These results suggest that rER β 2 can act equivalently to ER β 1 to regulate complex gene promoters, despite its lower ligand binding affinity, but this is likely dependent on cell type and the presence of other co-regulatory factors. Similarly, the rER β 2 LBD structure does not appear to affect its binding affinity for the ER antagonist 4-OH-tamoxifen, a phenomenon which could also be cell-type specific (42).

Next, it was discovered that exon 3 may be deleted from either rER β 1 or rER β 2, removing 39 amino acids that would normally encode the second zinc finger of the DNA-binding domain; these variants are referred to as rER β 1 δ 3 and rER β 2 δ 3, respectively (42). These are analogous to some ER α variants, which also lack exon 3 and therefore, a portion of its DBD. The lack of a second zinc finger in the DBD results in an unstable association with EREs on gene promoters, thereby reducing or eliminating any transcriptional activation (42). However, these variants can still tether to other DNA-bound transcription factors to elicit a physiological response, such as activation of AP-1-mediated promoter activity in the presence of E2. This contrasts with rER β 1 and rER β 2, as they both repress AP-1-mediated promoter activity except in the presence of tamoxifen (33, 46). Notably, rER β 1 δ 3 does not show any ligand independent or ligand dependent activity in neuronal cell lines at the rat AVP promoter, which contains a combination of AP-1 and ERE motifs (45). The δ 3 variants also organize into highly punctate subnuclear domains in response to E2 in a manner that is distinct from that of rER β 1 or rER β 2 (46). Moreover, rER β 1 δ 3 variants colocalized with the nuclear receptor coactivator GRIP-1 in these same subnuclear punctate domains (46), suggesting that rER β 1 δ 3 and rER β 2 δ 3 are functionally responsive to ligand through their interactions with coregulatory proteins, despite an inability to bind DNA.

Finally, exon 4 (99 amino acids) may be deleted from rER β 1, yielding a splice variant known as rER β 1 δ 4, resulting in deletion of the D domain of the protein (43). This variant lacks the ability to localize to the nucleus, consistent with the deletion occurring in the nuclear localization sequence of the hinge region. Somewhat surprisingly, rER β 1 δ 4 also lacks the ability to bind E2, which indicates the hinge region is important for conferring a conformational structure that is favorable for creating a stable ligand binding pocket (43). Overall, rER β 1 δ 4 is expressed at very low levels in most brain regions tested with the exception of the hippocampus (43). The relatively high expression of rER β 1 δ 4 in the hippocampus suggests that it may play a role in memory formation or other functions regulated by the hippocampus, but these proposed functions have not been validated experimentally, as rER β 1 δ 4 remains the least well-studied of all the rodent ER β splice variants.

Human ER β splice variants

To date, there have been 5 experimentally validated functional human ER β isoforms termed hER β 1-hER β 5. The splicing of hER β 3 appears to be under special regulatory control, because its expression is restricted to the testes (41). Therefore, its functional properties are less studied and will not be further discussed in this review. Unlike rat ER β splice variants which include insertions and deletions throughout the middle regions of the protein, human ER β splice variants arise from the specific alternative splicing of exon 8, thereby resulting in sequence variations, or truncations, at the C-terminal E and F regions of the protein (Fig. 1) (40, 41).

Consequently, the LBD of the protein is the primary functional component that is altered in these splice variants. Indeed, all of the hER β isoforms can bind to DNA and form dimers with ER β 1 in the presence of E2 (15, 16). Interestingly, heterodimers between hER β 1 and any of the other splice variants enhanced transactivation compared to hER β 1 homodimers in an ERE-dependent reporter assay in the presence of E2 and other estrogenic compounds in HEK293 cells (15). However, only the full length hER β 1 can interact with SRC-1 (steroid receptor coactivator-1) to induce transactivation, highlighting the importance of the hER β C-terminus for coregulatory protein binding (15). These data suggest that only one coregulatory protein binding site is needed in a hER β dimer, and indeed, the absence of the second binding site seems to facilitate hER β actions.

Human ER β 2 (also called hER β cx) is the most characterized of the human variants and is widely considered to function as a dominant negative receptor for ER α (47). Molecular modeling demonstrated that hER β 2 has altered (more restricted, “tight”) 3D structures in both the ligand binding pocket and coregulator binding surface, which impairs its ability to bind ligands (15, 16). Hence, hER β 2 has no ligand dependent transcriptional activity, yet it retains some ligand independent activity consistent with its intact AF-1 region (16).

Structurally, hER β 4 and hER β 5 are the smallest, as they completely lack helix 12 and the entire F domain, resulting in a more open conformation of the ligand binding pocket (41, 48). This conformation creates an unstable association with ligands and consequently lower binding affinities; however, unlike hER β 2, they are both able to bind ligands to some extent. In reporter gene assays, these variants also exhibit ligand independent activity similar to hER β 2 due to the intact AF-1 domain. For instance, in ERE-driven reporter assays in COS-7 cells, hER β 4 and hER β 5 only demonstrated ligand independent increases in promoter activity, while hER β 1 appeared to drive both ligand dependent and ligand independent promoter activity (48).

All of the apo-hER β splice variants can constitutively activate ERE promoter activity and repress AP-1 promoter activity in reporter assays using HT-22 cells (mouse-derived hippocampal neuronal cell line), with E2 treatment only affecting the full-length hER β 1 isoform (16). At the more complex AVP promoter, all of the hER β isoforms showed ligand independent repression of AP-1-mediated promoter activity, yet only hER β 1-mediated activity was abolished using the antagonist ICI 182 780 (16). Decreased ligand binding affinities of hER β splice variants have important implications for therapeutic strategies that employ ER agonists or antagonists, as the success of these therapies must rely on the ligand

dependent activation of the dominant ER β 1 isoform. Therefore, an increase in alternative splicing that shifts the expression profile of ER β isoforms could render hormone-based therapies completely ineffective, or potentially amplify the effects if there is an increase in ER β 1 :splice variant heterodimerization.

To our knowledge, there is no experimental evidence to suggest that alternative splicing of ER β alters its heterodimerization with ER α . However, based on the sequences of the rodent ER β splice variants in particular, it is reasonable to predict that dimerization with ER α would be altered. Current therapeutic interventions might not account for variations in dimerization proficiency, but future compounds could be designed to exploit this different heterodimerization profile and develop more precisely targeted therapeutic strategies.

REGULATION OF ER β ALTERNATIVE SPLICING

Alternative splicing represents a mechanism by which the cell can increase proteomic and, consequently, functional diversity from a single pre-mRNA transcript. Normally, splicing is achieved by the coordinated actions of RNA:protein complexes called the spliceosome, which are composed of both small nuclear RNAs (snRNAs) U1-U6 and associated small nuclear ribonucleoproteins (snRNPs). The spliceosome, when positioned on specific nucleotide recognition sites, such as the polypyrimidine tract (PPT) and the branchpoint sequence (BPS), functions to remove non-coding introns from the pre-mRNA transcript and ligate the remaining exons to a cohesive coding sequence mRNA (49–52). Conversely, alternative splicing occurs when variable compositions of exons and introns of a particular gene are irregularly excluded or included in the mature mRNA product, a process which is often regulated by tissue-specific RNPs (53, 54). The selective exclusion or inclusion of exons can result in the formation of premature stop codons and untranslatable protein products, or translated protein products with widely divergent functional properties compared to the dominant isoform.

The five human ER β variants (hER β 1-5) result from exon exclusion or exon skipping, which ultimately give rise to protein structures with variable length C-terminal truncations in the F and E domains (15). Exon skipping can occur through the concerted actions of *trans*-acting factors differentially binding to *cis*-acting elements on the DNA sequence. *Cis*-acting elements include exonic splicing enhancers (ESE) and exonic splicing silencers (ESS). These sequence motifs, complexed with SR (serine arginine rich) proteins or snRNPs, can then facilitate or inhibit the assembly of the spliceosome around weak splice sites. Alternative splicing events at weak splice sites can then result in mature mRNAs with various exon compositions, as in the case of the ER β isoforms. As previously discussed, these receptor isoforms have functionally unique roles in both E2-mediated and ligand independent signaling processes compared to the dominant ER β 1 isoform (14–17, 45, 48, 55). This raises the possibility that increased alternative splicing events will significantly impact normal cellular and physiological processes, leading to an increased risk for various pathologies. Therefore, it is important to determine how ER β alternative splicing is carefully regulated to produce cell-type specific expression profiles of ER β isoforms, and how these expression profiles relate to diverse cellular functions.

There is little known about how the splice variants are selectively processed by specific cell types, but evidence shows that there are discrete spatial and temporal expression patterns for ER β splice variants in the brain (43, 46, 56). For instance, an abundance of rER β 2 protein expression was detected in rat hypothalamic nuclei, such as the paraventricular nucleus (PVN) and the supraoptic nucleus (SON), whereas expression levels were low in the dentate gyrus (DG) (56). Analysis of a wide variety of brain regions showed that overall rER β 2 was the most highly expressed rER β splice variant in the rat brain, except in the hippocampus where rER β 164 levels were most abundant (43). These data suggest that the expression of rER β splice variants is differentially regulated by brain region and likely coupled to specific neurological and/or behavioral functions. However, it remains to be seen whether specific rER β splice variant expression is initiated upstream of cellular differentiation, priming the progression of neural stem cells to their respective fates, or whether specific rER β splice variant expression is a consequence, or perhaps even an artifact, of already differentiated brain regions.

Cis- and trans-regulatory elements

Distinct *cis*-regulatory sequences in the human ER β gene confer a molecular architecture that facilitates alternative splicing in prostate and mammary glands, raising the possibility that these same molecular mechanisms contribute to brain-region specific splicing. For instance, full length hER β transcription can be initiated from 2 different promoters characterized as 5' untranslated exons, 0K and 0N, in the prostate and mammary glands (57–59). In the prostate gland, hER β 1 and hER β 2 were predominantly transcribed from the 0N promoter, whereas hER β 5 was uniquely transcribed from the 0K promoter (58). In contrast, both hER1 and hER β 5 were transcribed from the 0K promoter in benign and malignant mammary epithelial cells (60). Depending on the site of transcription initiation, the nascent RNA transcript will include different lengths of 5' untranslated sequences (i.e. UTRa and UTRc), which can have stable secondary structures preventing translation of the RNA product (60). Moreover, these 5' UTRs can dictate differential expression of ER β splice variants through disruption or recruitment of spliceosome components. Specifically, the presence of UTRa corresponded to hER β 1 and hER β 5 expression, whereas the presence of UTRc corresponded to hER β 2 expression in MCF-7 cells (60). Importantly, translation efficiency varied even for individual UTRs depending on cell type, suggesting that other cellular components, such as *trans*-acting factors, interact with the 5' UTR sequence and dictate cell specific expression. There is also evidence that the translational efficiency of the ER β splice variants depends, in part, on crosstalk between the 5' UTR sequence and the length of the 3' UTR, with shorter lengths promoting translation, despite the presence of a typically inhibitory 5' UTRa (61). To our knowledge, these same alternative promoter elements have not yet been defined for the rodent ER β isoforms.

The alternative hER β promoters are also subject to epigenetic modifications, such as DNA methylation. DNA methylation can regulate alternative splicing by recruiting methyl-binding domain proteins, such as MeCP2, that then associate with splicing factors (62). For instance, MeCP2 has been shown to associate with splicing factors such as the pre-mRNA processing factor 3 (Prpf3), which presumably could affect splicing dynamics (63). In ovarian carcinoma cells, an interesting correlation was observed in which the hypermethylation of

the ON promoter resulted in the decreased expression of hER β 1, hER β 2, and hER β 4 mRNA, and increased expression of hER β 5 mRNA. These findings suggest that ON promoter hypermethylation could promote the assembly of specific spliceosome components through methyl-binding domain proteins, ultimately influencing the differential alternative splicing of hER β (64). However, the mechanism behind the selective enhancement of specific hER β splice variant expression (namely hER β 1, hER β 2, and hER β 4) is still unclear, since all four human splice variants including hER β 5 are the result of similar exon exclusion events.

It is important to note that another layer of complexity is added when considering the role of *trans*-acting factors that could potentially interact with the abovementioned *cis*-regulatory elements. The molecular mechanisms of ER β splice variant regulation in the brain are still largely uncharacterized; however, a possible explanation of the unique expression profiles can be attributed to the brain region-specific expression of RNPs (i.e. *trans*-acting factors) that comprise the spliceosome. Indeed, recent unpublished work in our lab has identified putative consensus binding sites for NOVA-1 (neuro-oncological ventral antigen-1), a neuron-specific RNP, upstream of the 54bp insert in ER β 2, as well as correlative changes in NOVA-1 expression with ER β 2 in several brain regions (65). Aside from these preliminary studies, there have been no publications to date identifying specific *trans*-acting factors regulating ER β alternative splicing in the brain.

Activity dependent splicing

Neurons are intrinsically excitable cells, and evidence suggests that brain region-specific expression profiles of ER β splice variants could be due to regional differences in neuronal firing activity. A recent study demonstrated that neuronal activity induced by calcium influx via *N*-methyl-*D*-aspartic acid receptors (NMDARs) and L-type voltage-dependent calcium channels (L-VDCC) could regulate alternative splicing processes (66). Transient increases in intracellular calcium concentrations can activate calcium-sensitive kinases, such as protein kinase C (PKC) and Ca²⁺/calmodulin-dependent kinase IV (CaMKIV). These kinases, once activated, can then alter the phosphorylation status and function of RNA binding proteins, ultimately resulting in alternative splicing of the nascent RNA transcript (67). This mode of regulation would represent a mechanism whereby the abundance of RBPs would not necessarily change in concordance with splice variant expression; instead, RBP activity would simply be turned “on” or “off” by a phosphorylation event. For example, Iijima and colleagues demonstrated that neurexin (Nrxn) could be alternatively spliced by CaMKIV-dependent phosphorylation of the ribonucleoprotein SAM68 (Src-associated substrate in mitosis of 68 kDa) (68). Although there has been no evidence for the activity dependent splicing of ER β up to this point, Ratti and colleagues demonstrated that NOVA1 activity could be affected by PKC-dependent signaling pathways (69). These data, in conjunction with findings that NOVA1 can alter ER β splice variant expression, suggest that the alternative splicing of ER β could also be regulated by fluctuations in intracellular calcium concentrations mediated by neuronal activity (69). Since hypothalamic neuronal firing rates are distinct from other brain regions such as the cortex, activity-dependent splicing of ER β itself, or even *trans*-acting factors that affect ER β splicing, could potentially explain the unique expression of ER β splice variants that was observed in discrete brain regions.

RNAPII kinetics

Splicing can occur co-transcriptionally, and an essential component to this process is RNA polymerase II (RNAPII) (70). Various splicing factors such as SR proteins have been shown to associate with the C-terminal domain (CTD) of RNAPII (52). These RNA binding proteins can then promote the assembly of spliceosome components to allow for the physiological processing of nascent mRNA transcripts. Therefore, it is not surprising that altering the rate of RNAPII elongation can result in alternatively spliced mRNAs, as splice sites can either be passed over or recognized depending on the pace of elongation. Generally, factors that slow down RNAPII elongation result in exon inclusion, as there is a longer time interval available for the assembly of the spliceosome around a weak splice site (71). In agreement with these findings, our lab previously showed that inhibiting topoisomerase I (TOP1) activity to slow splicing kinetics allowed for the aberrant recognition of weak splice sites present in the rER β pre-mRNA. As expected, rER β 2 transcripts, with characteristic exon inclusions, were significantly increased when TOP1 was inhibited in a hypothalamic derived GT1-7 cell line (72).

Aging and E2 deprivation

The number of alternative splicing events in the human brain have been shown to increase with age; therefore, it can be speculated that ER β splice variant expression is also increased during the aging process (1). Consistent with this hypothesis, our lab demonstrated that rER β 2 expression was significantly increased in the hypothalamus of 18-month-old (aged) female rats compared to their 3-month-old (young) counterparts (72). However, the other rat ER β splice variants were not detected in significant concentrations in the brain regions tested, and therefore, it cannot be concluded that all ER β splice variants increase with age. Additionally, another layer of complexity must be considered in females, as aging is closely associated with the onset of menopause, during which there is a loss of circulating E2. Interestingly, age and E2 deprivation together have been shown to alter rER β 2 expression in a brain-region specific manner, suggesting that the normal aging process and the absence of E2 could have both independent and coordinated effects on the alternative splicing of ER β (72). These findings were also consistent with a separate study showing that the deprivation of E2 corresponded to the increased expression of rER β 2 in both the rat brain and circulating white blood cells (73). Moreover, increased rER β 2 expression correlated with a reduction in the effectiveness of E2 treatment on depressive behaviors using a forced swim test paradigm (73). Together, these data suggest that the presence of E2 inhibits the expression of rER β 2 splice variants in the brain; however, the mechanism for how this occurs is still unclear.

ER β SPLICE VARIANTS AND AGE-RELATED DISEASE

To date, the majority of studies implicating ER β splice variants in disease have focused on the role of these variants in the context of human prostate, breast, ovarian and thyroid cancers. However, as we have described above, alternative splicing of ER β can change the functional domains which are present in the protein. Deletions or insertions within these functional domains can impact ER β -DNA binding, ligand binding affinity, dimer formation, and/or interactions with co-regulatory proteins. Therefore, it is logical to predict that

alternative ER β splicing can impact a wide variety of pathological conditions that have yet to be defined.

Clinical studies have shown that the expression of hER β splice variants can vary dramatically between healthy and cancerous tissues in age-matched patients. For example, hER β 5 was overexpressed in endometrial carcinoma tissues compared to normal endometrial tissue, and the increased expression of hER β 5 was strongly correlated with an increased expression of several oncogenes (11). An enrichment of hER β 5 was also observed in patients with HER2 positive and triple-negative breast cancer and predicted poorer prognostic outcomes (74). By contrast, another study reported that increased hER β 2 and hER β 5 expression was correlated with a better relapse-free survival rate in 105 post-menopausal breast cancer patients; however that study did not report the HER2 status in those tumors, suggesting that even within breast cancers, there are context specific functions of ER β splice variants (75). The development of malignant prostate tumors also revealed a dramatic shift in the ratio of the dominant hER β 1 isoform to its alternatively spliced variants. Specifically, the expression of hER β 1, which is normally anti-proliferative in prostate tissue, gradually declines and then becomes undetectable coincident with an increased expression of hER β 2 during the progression of prostate cancer (76, 77). Moreover, stable overexpression of hER β 2 in prostate tumor-derived PC-3 cells resulted in increased expression of several oncogenic genes (78). Taken together, these data suggest that some hER β splice variants can increase oncogene expression leading to carcinogenesis in certain tissues.

Interestingly, the hormonal milieu appears to also play a role in determining the carcinogenic potential of hER β splice variants. For instance, hER β 2 was expressed in 100% of patients with papillary thyroid cancers (PTC), and its expression was correlated with increased Ki-67 and VEGF expression in both postmenopausal women and men (i.e. low E2) (18). In contrast, hER β 2 expression was lower in pre-menopausal women with PTC and concurrent lymph node metastasis, suggesting that higher E2 levels prevented the progression of PTC by regulating the expression of hER β 2 (18). These data are consistent with those from our laboratory and others demonstrating increased expression of ER β 2 in the brain of aged ovariectomized female rats (i.e. low E2), and the subsequent decrease of ER β 2 following E2 treatment (72, 73). Overall, evidence suggests that there is a correlation between the expression of ER β alternative splice variants and cancer progression. Although to our knowledge, no studies have demonstrated a direct causal role of ER β splice variants in tumor development.

CONCLUSIONS

In summary, there have been remarkably few studies investigating the consequences of increased ER β alternative splicing in the context of neurological diseases (79–81). The ER β splice variants that have been identified in humans and rodents are diverse, not only in their biochemical structure, but also in their cellular and physiological function, allowing predictable consequences for age-related shifts in the ratios of alternatively spliced variants in the brain. Different cell types and tissues exhibit distinct ER β splice variant expression profiles, further underscoring the probability that each variant has a unique physiological

function in a given cell type. The precise molecular mechanisms regulating ER β alternative splicing is still largely unknown, and are further complicated by the likelihood of tissue-specific factors that direct alternative splicing events. Dysregulation of ER β splicing events can contribute to disease progression, as characterized in various cancer paradigms, yet many of these studies have been correlative and do not address whether alternative ER β splice variants are causal factors for disease progression.

As discussed throughout this review, several studies have focused on determining the functional role of ER β in the brain. However, few have differentiated between the dominant isoform and the alternatively spliced variants, or examined them in a cell-type or brain-region specific manner (65, 81). Moreover, clinical studies examining patient samples in various disease states have not taken splice variant expression into consideration (79). These oversights could severely limit our understanding of aging and associated disease states, as these alternatively spliced variants can have widely divergent functional properties compared to the parent isoform. Technical limitations often preclude the assessment of all possible isoforms, because structural and sequence properties of alternatively spliced variants are not conducive to the creation of highly specific antibodies. Therefore, the development of novel antibody-free techniques for rapid detection of alternatively spliced variants *in situ*, as opposed to relying on mass spectrometry detection from tissue homogenates, is an important future direction. Failure to account for alternative splicing in could impede the development of more precisely targeted therapeutics, as the field of personalized medicine moves forward.

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Rat variants

Human variants

Figure 1. Schematic diagram of ERβ splice variants

Rat (top) and human (bottom) ERβ isoforms including the coding exons and corresponding proteins domains of rat and human ERβ splice variants. The asterisk in exon 8 of the human variants denotes regions of amino acid sequence variation between the isoforms.

Figure 2. Nova-1 consensus sequences located in exon 5 of rat ERβ

The full-length rat ERβ sequence was analyzed for RNA binding protein sites using RBPmap (<http://rbpmap.technion.ac.il/index.html>). Nova1 YCAAY 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.