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REVIEW

Pre-mRNA splicing modulations in senescence

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Summary

Aging and associated diseases involve multilevel changes in the complex phenomenon of alternative splicing. Here, we review the potential genomic and environmental origins of such changes and discuss the research implications of these findings.

Key words: aging; alternative splicing; pre-mRNA; SR proteins; hnRNPs.

Introduction

From the viewpoint of a molecular biologist, aging reflects gradual deterioration of the molecular components, checkpoints and/or events, the concerted functioning of which are vital for cell viability and proliferation. The complexity of alternative splicing of pre-mRNA makes this process particularly vulnerable to senescence, leading to both transient changes and chronic aging-related diseases.

With the emergence of microarray technologies, changes in gene expression profiles during aging have recently become the focus of extensive research. However, relatively little is yet known about changes in alternative splicing, a key pre-mRNA processing mechanism during cellular and organismal senescence.

The progressive nature of the aging process is often ascribed to passive accumulation of late-onset mutations due to lack of natural selection beyond the age of reproduction (Kirkwood, 2002). Alternatively, aging is viewed as the outcome of active selection of traits that are beneficial at an early stage of life but have deleterious effects later on (reviewed in Partridge & Gems, 2002). The idea that aging is a genetically programmed mechanism has also been subject to some debate. Planned or sporadic, passive or active, aging also involves changes in the pathways of gene expression that are not necessarily associated with mutation. This is exemplified in most aging-related diseases, such as Alzheimer's and Parkinson's diseases and tauopathies, which represent the best studied cases of aging, involving aberrations in the alternative splicing of pre-mRNA. Here, we describe this emerging concept and discuss its research implications. We present examples of alternative splicing events during aging, and propose that these examples reflect a generally modified state of the pre-mRNA processing machinery in senescent cells, leading to altered expression levels of pre-mRNA processing factors and their downstream target mRNAs and corresponding proteins during aging.

Aging-related modifications in the alternative splicing of specific gene products were sought, primarily as a means to search for the molecular origin(s) of aging-related deterioration, for example, neural adhesion processes, dopaminergic neurotransmission, insulin responses and gastric cytoprotection (Table 1).

Together, the picture that emerges from these studies is of a general control switch that causes all of these aging-induced impairments in alternative splicing. For example, these may all reflect one or a few changes in regulatory factor(s), such as splicing-related proteins, which modify the delicate regulation of the splicing process. Support for this concept can be found in recent microarray screens for changes in gene expression during aging (Lee *et al*., 1999; Shelton *et al*., 1999; Cao *et al*., 2001; Tollet-Egnell *et al*., 2001). Significant age-related tissue-specific changes were found in the expression levels of several RNA processing genes in aged, compared to young, gastrocnemius muscle, neocortex and cerebellum of C57BL/6 mice (Lee *et al*., 1999). Changes spanned serine, arginine-rich (SR) proteins, heterogeneous nuclear ribonucleoproteins (hnRNPs), as well as 3′-end processing factors (Table 2). We present a scheme of the key processes in the pathway of pre-mRNA splicing (Fig. 1), highlighting those elements in which age-related changes have been reported.

Pre-mRNA processing during aging

SR proteins contain at least one RNA recognition motif (RRM) and an arginine, serine-rich (RS) domain, allowing them to bind both the pre-mRNA and additional proteins. They bind the nascent pre-mRNA at specific sites, which can act as both splicing enhancers and splicing suppressors. These *cis*-acting motifs are usually degenerate and can be found within both exons and introns, exonic splicing enhancers (ESEs) being the more prevalent. Through such interactions with the pre-mRNA, SR proteins can regulate splicing and alternative splicing in a concentration-dependent manner (Manley & Tacke, 1996).

hnRNPs serve as a crucial checkpoint in pre-mRNA processing. They include a diverse group of proteins containing RNA binding motifs as well as several auxiliary domains. This allows them to simultaneously bind pre-mRNA and other proteins (Krecic & Swanson, 1999). Misdirected regulation of the expression levels

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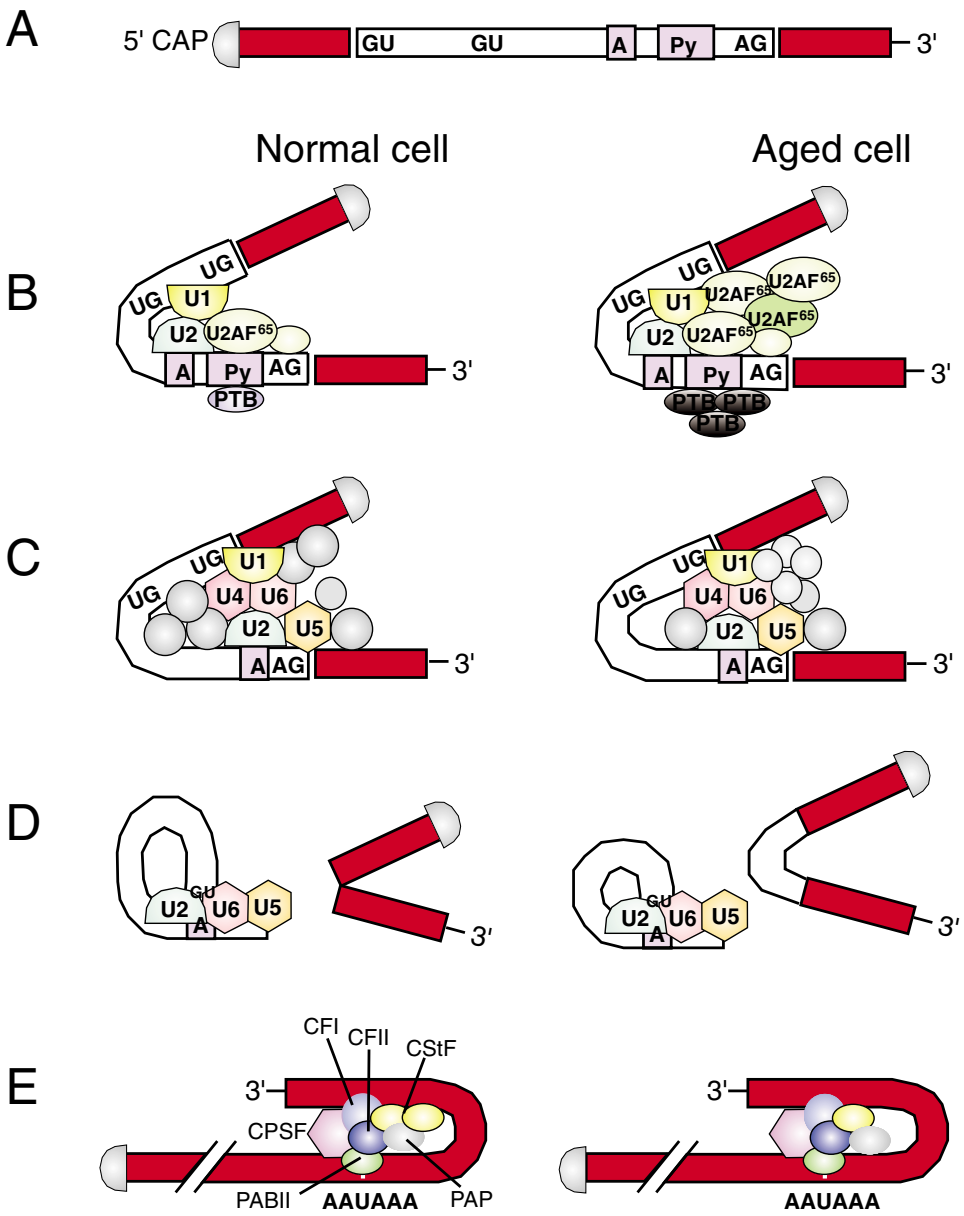

Fig. 1 Misdirected regulation of pre-mRNA processing during aging. Factors reported to change in level during aging are depicted, along with the different steps of pre-mRNA processing in which they are involved in normal and aged cells. The representations are not to scale; only one intron is shown, and in E, the spliced exons are extended to allow representation of the cleavage and polyadenylation factors. A. The 'naked' pre-mRNA transcript. Exons are marked red and introns white. Shown are two alternative 5′ (GU) splice sites and a single 3′ (AG) splice site, the branch point (A) and the polypyrimidine tract (Py). The 5′ end of the pre-mRNA is capped shortly after initiation of transcription. (The 3′ end, A–D, does not represent the physical end of the pre-mRNA). B. Initial snRNP complex formation. The U1 small nuclear RNA and associated proteins comprise the U1 snRNP, which binds the 5′ splice site. Likewise, the U2 snRNA forms snRNP complexes, which are recruited to the branch point by U2AF. U2AF binds the Py tract through its heavy ($U2AF^{65}$) subunit. PTB also binds the Py tract and regulates splicing. $U2AF^{65}$ and PTB were shown to be significantly over-produced in the gastrocnemius muscle and cerebellum, respectively, of aged C57BL/6 mice, thus potentially affecting splicing (the stoichiometry presented is speculative). C. Spliceosome assembly and attraction of SR proteins. The remaining spliceosomal components (U4, U5, U6) assemble together with additional SR proteins, shaded grey. These splicing factors bind simultaneously exonic or intronic splicing enhancer sequences on the pre-mRNA through their RRM and additional proteins through their RS domain. Several of these components were shown to be modified in aged tissues. The splicing-related factor/RNA helicase PRP16, for example, was ca. two-fold decreased in the cerebellum. D. Intron release and exon joining. Splicing involves two sequential transesterification reactions, which result in the release of the intron in the shape of an RNA 'lariat', and ligation of the flanking exons to form the mRNA. Altered expression levels of the participating factors may lead to the alternatively spliced transcript shown. E. 3′ end processing. The cleavage and polyadenylation complex is shown. This complex consists of CPSF, which recognizes and binds the poly(A) site (AAUAAA), together with CStF, CFI, CFII and PAP, the combined complex of which catalyses the first, slow, polyadenylation step. PABII then binds the short, newly formed, poly(A) tail and rapidly adds additional 200–250 adenines. CStF and PAP were both ca. 1.5-fold decreased in the neocortex of C57BL/6 aged mice, potentially harming the 3′-end processing pathway. Abbreviations: Py, polypyrimidine tract; snRNP, small nuclear ribonucleoprotein particle; U2AF, U2 auxiliary factor; PTB, polypyrimidine tract binding protein; SR-proteins, serine, arginine-rich proteins; CPSF, cleavage and polyadenylation specificity factor; CStF, cleavage stimulation factor; CF, cleavage factor; PAP, poly(A) polymerase; PAB, poly(A)-binding protein.

Table 1 Aging-associated changes in alternative splicing

Gene	Organism	Tissue	Affected process	Reference
APP, APLPs	rat	brain	amyloid plaque formation	(Sandbrink *et al*., 1997)
Dopamine D2 receptor	rat	neostriatal subregions	dopaminergic neurotransmission	(Merchant *et al*., 1993)
N-CAM	rat	heart	neural adhesion	(Andersson *et al*., 1993)
Insulin receptor	rat	liver, muscle, heart	insulin responses	(Vidal *et al*., 1995)
MGF	rat	skeletal muscle	insulin responses	(Owino *et al*., 2001)
COX-1	rat	stomach	gastric cytoprotection	(Vogiagis *et al*., 2000)
NF1	human	blood	rRas signalling, neural architecture	(Wimmer *et al*., 2000)

Table 2 Aging-associated alterations in the expression of murine pre-mRNA processing genes

Gene	Fold change*	Tissue	Affected pre-mRNA processing step	Reference to reported function
SF3A2 (SAP62, PRP11)	3.4	cerebellum	U2 snRNP binding	(Ruby *et al*., 1993)
$U2AF^{65}$	3.2	gastrocnemius muscle	Splice site recognition, splicing	(Wang *et al*., 1995)
Sox17	2.4	gastrocnemius muscle	Transcription, splicing	(Ohe *et al*., 2002)
hPRP22 (HRH1)	2.5	neocortex	Spliceosome disassembly, 2nd catalytic step, mRNA release	(Company *et al*., 1991); (Schwer & Gross, 1998)
PTB	2.3	cerebellum	Alternative splicing	(Wagner & Garcia-Blanco, 2001)
PolyA + RNA export protein	2.1	gastrocnemius muscle	3′ end processing, mRNA export	
hnRNP H3 (hnRNP 2H9)	2.0	cerebellum	Splicing, heat-shock splicing arrest	(Mahe *et al*., 1997)
CStF	0.7	neocortex	Transcript cleavage	
DDX18	0.7	neocortex	DEAD-box protein, specific function unknown	
hPRP16	0.6	cerebellum	Aberrant lariat discard	(Burgess & Guthrie, 1993)
PABI	0.4	cerebellum	mRNA stability	
hPRP22	0.4	cerebellum	Spliceosome disassembly, 2nd catalytic step, mRNA release	(Company *et al*., 1991); (Schwer & Gross, 1998)

*From Lee *et al*. (1999).

of SR-proteins and hnRNPs can influence splice site selection or lead to alternative or aberrant splicing of many downstream target sequences. hnRNP A1, for example, was shown to suppress splicing of exon 3 of the HIV-1 tat gene through high-affinity binding to an exonic splicing silencer (ESS) and recruitment of additional hnRNP A1 molecules, while the SR protein ASF/SF2 prevents this hnRNP A1 accumulation at the ESS and antagonizes the splicing arrest (Zhu *et al*., 2001).

One of the first essential splicing factors to be identified was the 65-kDa subunit of the U2 auxiliary factor ($U2AF^{65}$). $U2AF^{65}$ binding to the conserved poly pyrimidine (Py) tract at the 3′ end of introns is required for the subsequent binding of U2 snRNA to the 5′ splice site (usually a GU dinucleotide). The other subunit, $U2AF^{35}$, binds the 3′ splice site (usually an AG). $U2AF^{65}$ mRNA levels were found to increase by over three-fold in the gastrocnemius muscle of aged mice (see References cited in Table 2). As $U2AF^{65}$ contains both the RNA recognition motif and the RS domain of SR-proteins, it can regulate splicing in a concentration-dependent manner, as do the SR proteins (Manley & Tacke, 1996). Uncontrolled regulation of the expression level of $U2AF^{65}$ may therefore lead to aberrant splicing activity and alternative splicing of numerous target genes (Wang *et al*., 1995).

The Py tract-binding protein (PTB, hnRNP I) also binds intronic Py tracts. PTB was shown to play a direct role in pre-mRNA alternative splicing through antagonistic effects on exon definition, repressing, for example, the inclusion of exon 7 in the β-tropomyosin gene (Wagner & Garcia-Blanco, 2001). Its expression levels were reported to increase over two-fold in the cerebellum of aged mice, which can potentially harm finely regulated alternative splicing of many pre-mRNAs by repressing exon inclusion and promoting aberrant splicing.

Other factors that may have a direct or indirect role in influencing splicing during aging are hPRP22, hPRP16, SF3A2, DDX18 and hnRNP H3 (Table 2). The expression level of the splicing factor PRP22, for example, was 2.5-fold increased in the neocortex of aged C57BL/6 mice, while the same factor displayed 2.7-fold decrease in the cerebellum of these mice (Lee *et al*., 1999). Although the function of several of these factors is largely obscure, this expanded list points to a central involvement of splicing changes in the aging process.

Initiation and efficacy of transcription

Another checkpoint, which may affect aging-related changes in pre-mRNA splicing, occurs prior to the splicing process, at the earlier phases controlling the initiation and efficacy of transcription. This involves the expression levels of a large number of transcription factors, also shown to be modified in aged tissues (Lee *et al*., 1999; Cao *et al*., 2001; Tollet-Egnell *et al*., 2001). Since transcription and splicing are tightly coupled

processes, such changes are likely to affect the splicing machinery as well. The transcription factor SOX17 (D49473), for example, with a direct role in pre-mRNA splicing (Ohe *et al*., 2002), exhibited 2.4-fold over-expression in the aged gastrocnemius muscle. While changes in expression levels may not correlate directly with changes in activity, altered expression of transcription-associated genes during aging adds further support to the concept of pre-mRNA splicing as an age-sensitive process. Indeed, aging-related diseases often reflect abnormal upstream factors, including impaired regulation or aberrant fine-tuning of mRNA processing in general and pre-mRNA splicing in particular.

The 3′-end processing complex of pre-mRNA is also subject to aging-related changes. This complex consists of cleavage factors 1 and 2 (CF1, 2), cleavage stimulation factor (CStF), cleavage and polyadenylation specificity factor (CPSF), poly(A)-polymerase (PAP), and poly(A)-binding protein II, which acts after the initial, slow polyadenylation phase is complete (Minvielle-Sebastia & Keller, 1999). CStF was decreased in the neocortex of aged mice (Lee *et al*., 1999), perhaps indicating a depression of the 3′-terminal processing of nascent mRNAs in conjunction with the aberrant splicing that accompanies aging. The cytoplasmic poly(A)-binding protein (PABI) was decreased to less than half its normal level in the cerebellum of aged mice, while the poly(A)+export protein was similarly increased in the gastrocnemius muscle of these mice. This points to the tissue specificity of age-dependent changes in 3′-end processing and mRNA export, again, keeping in mind that changes in expression levels may not necessarily directly correlate with changes in activity.

Age-related diseases

Age-related, late-onset diseases are especially vulnerable to splicing variations of different origins. These include mutations affecting the correct splicing of a particular gene. Age-related macular degeneration (AMD) is an example of an aging-related disease that is associated with a late-onset splicing mutation (Allikmets *et al*., 1997). AMD is the most common cause of acquired visual impairments in the elderly (Stone *et al*., 2001). The occurrence of AMD is often associated with mutations within the Stargardt disease gene (STGD1 or ABCR), coding for a photoreceptor-specific member of the ATP-binding cassette (ABC) superfamily of transporter proteins. One of these mutations, a G for A substitution at position 5196, was found to be a donor splice site mutation (Allikmets *et al*., 1997). The late onset of the disease phenotype, in this case, may be explained by age-related modifications in the efficacy and/or composition of pre-mRNA processing factors, since the disease is associated with the consequences of a genomic splice-site mutation that for unknown reasons are apparent only at a later stage in life.

Aging-related impairments in pre-mRNA processing need not necessarily involve a protein-modifying mutation as they may be caused by an altered upstream splicing regulator. Amyotrophic lateral sclerosis (ALS) serves as an example.

ALS is a late-onset neurodegenerative disease that is characterized by selective degeneration of spinal cord motor neurones. Most ALS patients suffer from a significant loss of the astroglial excitatory amino acid transporter 2 (EAAT2, previously known as GLT-1) in the motor cortex and spinal cord (Lin *et al.*, 1998). Affected tissues are highly populated by abnormal EAAT2 mRNA species resulting from aberrant alternative splicing. Since the EAAT2 gene is not mutated in these patients, it was suggested that a protein involved in pre-mRNA processing is the culprit (Bai & Lipton, 1998). Furthermore, alternative splicing of neuronal nitric oxide synthase (nNOS) was demonstrated in the spinal cord of ALS patients: nNOSβ and nNOSγ, but not nNOSα, were up-regulated (Catania *et al*., 2001). This demonstrates modified alternative splicing of nNOS, in addition to EAAT2, and strengthens the hypothesis that an as yet unknown aberrant upstream splicing regulator is involved in ALS pathology.

Alzheimer's disease (AD) is the most common neurodegenerative disorder of aging, characterized by progressive memory loss and cognitive deterioration. AD involves premature death of selected cholinergic neurones, associated with the formation of amyloid plaques, the appearance of which in the brain of patients is facilitated by mutations in several different genes (e.g. APP, PS1, PS2). Misdirected splicing regulation of relevant gene products either due to specific mutations or to aberrant processing of such products with no known mutation were both demonstrated in AD. Presenilins provide examples of both types of alteration: mutations within the fourth intron of presenilin 1 (PS1) were shown to impair PS1 splicing and cause early onset AD, while an exon 5-deficient splice variant of apparently normal PS2, which accumulates following hypoxia in cultured neuroblastoma cells, was found to be prevalent in the brains of AD patients, indicating that this unique splice variant is inducible, rather than being produced at a constant level due to mutation in the corresponding gene (Sato *et al*., 1999).

Tauopathies are a family of late-onset neurodegenerative diseases associated with mutations within the microtubule-associated protein (MAP) tau gene (Lee *et al*., 2001). Progressive accumulation of filamentous tau inclusions causes neural degeneration in specific brain regions of patients with tauopathies. The late onset of this phenotype suggests that an age-related molecular change is responsible. Tau is alternatively spliced in the adult human brain (Lee *et al*., 2001) and transgenic mice over-expressing the shortest human tau variant display age-dependent CNS deterioration reminiscent of human tauopathies (Ishihara *et al*., 1999). Thus, as yet unspecified impairments in pre-mRNA processing and/or splicing may contribute to these neurodegenerative conditions.

Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) is an example of a tauopathy associated with changes in alternative splicing. Several mutations within the tau gene were demonstrated for FTDP-17, a large proportion of which affect the splicing pattern of tau exon 10 by influencing exonic/intronic splicing enhancers/suppressors (D'Souza *et al*., 1999). The aberrant splicing of tau exon 10 alters the ratio of the tau isoforms incorporated into the neuronal tangles that are the neuropathological hallmark of the demented brain. Excessive tau accumulation results in filamentous inclusions

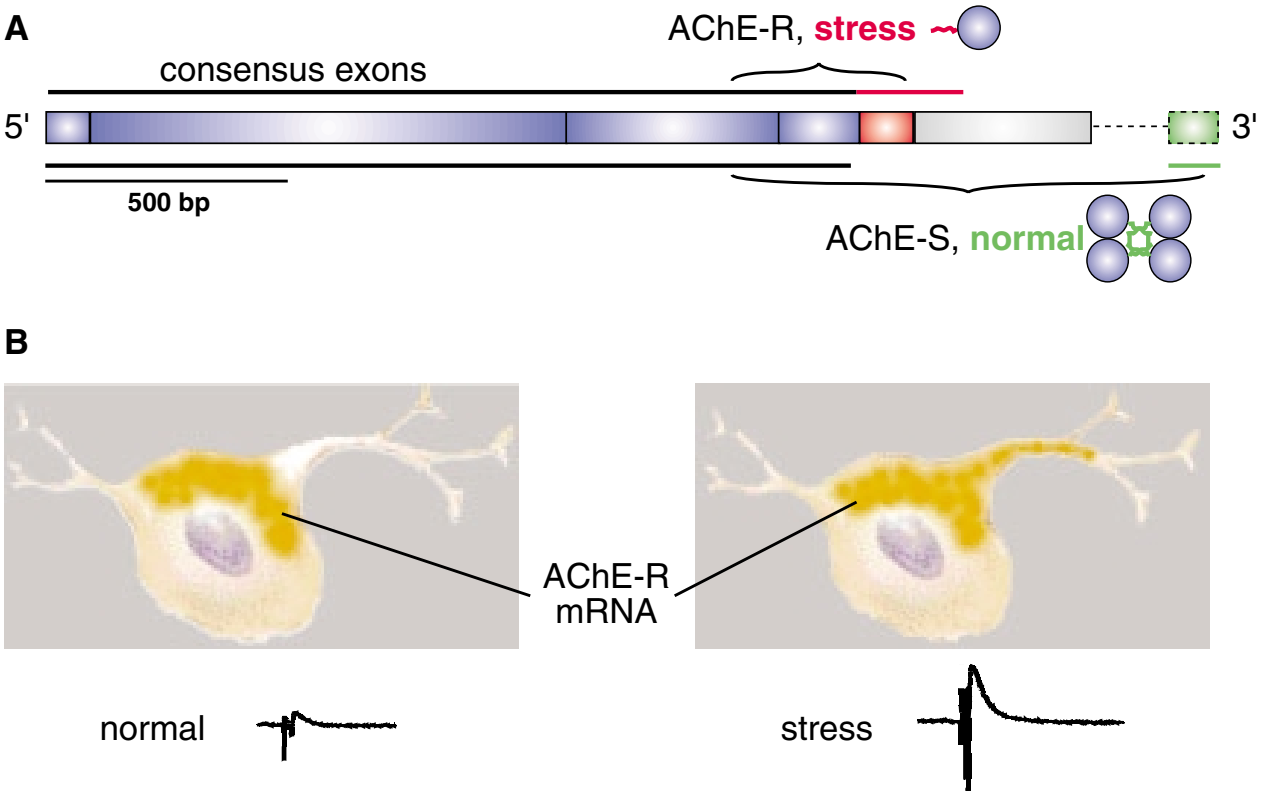

Fig. 2 Stress-induced alternative splicing in neuronal acetylcholinesterase mRNA. A. The AChE mRNA transcript includes 5′-consensus exons as well as variant-3′ exons, which are subject to alternative splicing under the influence of various psychological, physical and chemical stressors (Soreq & Seidman, 2001; Meshorer *et al.*, 2002). The primary transcript (normal) encodes a protein with a C-terminal sequence, which enables multimerization and binding to a non-catalytic structural subunit, allowing attachment to the synaptic membrane. The stress-induced alternative transcript encodes a protein with a distinct C-terminal sequence, which does not allow multimerization, leading to the soluble, secretory isoform. B. Under normal conditions, AChE-R mRNA resides in the perinuclear area, but stress induces its dendritic translocation. The AChE-R protein product fails to adhere to the synaptic membrane, resulting in exaggerated field potentials under stress, measured as extracellular recordings on the CA1 areas of hippocampus slices in response to stimulation of stratum oriens, shown here as representative voltage traces (Meshorer *et al.*, 2002).

within these tangles. In this case as well, the mutated genotype leads to an abnormal phenotype with a delayed onset, hinting at a change in an unidentified age-related factor involved in pre-mRNA processing.

Stress and splicing

Another consideration with regard to late-onset phenotypes involves the interrelationships between individual genotypes and the environment. Stress-induced changes in the pathway to gene expression, for example, may change the age of onset of an aberrant pattern of pre-mRNA splicing, initiating a cascade of events that expedite the onset of an aging-related disease. Such conditions are evident primarily in the central nervous system because of its role in the initiation of stress responses and because of the range of cell types and complex gene expression profiles in the mammalian brain. Examples include the aberrant splicing of K^+ channels (Xie & McCobb, 1998), of the Tra1 splicing regulator (Daoud *et al.*, 1999) and of acetylcholinesterase (AChE) mRNA (Meshorer *et al.*, 2002). The latter example is particularly relevant to the issue at hand, as it directly relates to the behavioural changes and the cognitive impairments which are characteristic of old age and which are implicated in age-related neurodegenerative diseases.

The *ACHE* gene gives rise to several mature transcripts. Of these, the primary 'synaptic' AChE-S mRNA constitutes the preponderant AChE mRNA under normal conditions and is translocated into neuronal processes. Under the influence of various stressors, however, a splicing shift occurs which leads to overproduction and neurite translocation of the normally rare 'readthrough' AChE-R mRNA transcript (Fig. 2). It is not yet clear why AChE-R mRNA stays in the cell body under normal conditions but travels to neuronal processes under conditions of stress. However, this change alters both the nature of the AChE protein product and its subcellular location (Fig. 2). Thus, neuritic AChE-S forms synaptic membrane-associated tetramers; in contrast, AChE-R forms soluble, secretory monomers that cannot adhere to the membrane and hence fail to address incoming cholinergic stimuli, leading to prolonged neuronal hypersensitivity (Meshorer *et al.*, 2002). Large excesses of AChE-R, such as those that accumulate in the injured brain, are detrimental: massive overproduction of this variant in the brain of head-injured transgenic mice over-expressing human AChE reduces survival and slows the recovery, whereas antisense intervention with AChE-R

production improves survival and recovery of both transgenic mice and their parent strain (reviewed in Soreq & Seidman, 2001). As head injury is the largest risk factor known for non-familial AD, the splice shift leading to AChE-R overproduction seems to be causally involved in an increased risk for neurodegeneration. That the AD brain displays increases in AChE-R monomers (Darreh-Shori *et al*., in press) as opposed to decreases in AChE-S tetramers supports this notion.

Cell proliferation

One final key process, which is now known to be affected by aging and which depends on the regulation of pre-mRNA processing, is cell proliferation. Pre-mRNA processing events, which are subject to aging dependent impairment, participate in control of cell proliferation (Verdi *et al*., 1999), and aging has long been known to be associated with aberrant cell proliferation (Cameron, 1972; Xiao *et al*., 2001). Therefore, although neuronal stem cells maintain into old age some capacity to proliferate (van Praag *et al*., 2002), the efficacy of cell proliferation is likely affected by damaged splicing, with consequences for many physiological functions. The proliferation of neuronal stem cells in the murine hippocampus, for example, emerges as an essential prerequisite for the preservation of newly acquired trace memories (Shors *et al*., 2001). This physiological process is impaired even in non-demented, normal older individuals (West & Covell, 2001). Our current considerations therefore imply that the memory impairments of old age may reflect detrimental changes in pre-mRNA processing and consequent impairment in stem cell proliferation.

Most of the examples presented above refer to post-mitotic tissues such as brain and muscle, in which the relevant cells are terminally differentiated. In view of this line of speculation, it would be most interesting to explore the changes during aging that take place in the pre-mRNA processing machinery of proliferating cells of these organs, particularly of the neuronal stem cells.

Concluding remarks

Because microarray screening for age-related changes will identify genes and regulatory factors only if there happens to be a substantial change in their expression, advances by this approach alone may be limited. Based on the evidence we have presented, advances in the molecular biology of aging may well come about by screening for alternative splicing options of key genes that encode factors that contribute to pre-mRNA processing.

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