Mini-Symposium

The Role of RNA and RNA Processing in Neurodegeneration

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From its transcription from DNA, RNA undergoes a complex processing, including splicing and editing, and associates with specific proteins that determine its subcellular localization and stability. It is now clear that many cellular activities involve some aspects of RNA biology; in neurons, synaptic activity is a typical process in which RNA plays a critical role. For instance, proper targeting of specific mRNAs to dendrites allows rapid local protein translation at postsynaptic sites in response to stimulation by neurotransmitters or neurotrophic factors, an important component of synaptic plasticity. Repression of local translation at the postsynaptic level by noncoding RNAs, including microRNAs, offers another level of regulation mediated by RNA (Kosik and Krichevsky, 2005). Increased complexity of the proteome and functional diversity can be generated from a limited number of genes (~20,000–25,000 in the human genome) by including or skipping specific exons through pre-mRNA alternative splicing. In neurons, alternative splicing generates neurotransmitter receptors with different specificities and coordinates the activity of protein networks at the synapse (Ule et al., 2005). RNA processing participates in cellular surveillance by degrading mRNA containing premature termination signals through nonsense-mediated decay, hence avoiding their translation into truncated proteins. Proteins binding to defined RNA elements control RNA metabolism at all levels. Of particular interest are proteins binding to the 5′- and 3′-untranslated regions (UTRs) of the mRNA that regulate translation and mRNA stability.

Given the importance of RNA processing in governing neuronal function, it will come as no surprise that its impairment can lead to neuronal dysfunction or, in extreme cases, neuronal degeneration. A prime example of this is spinal muscular atrophy (SMA), an autosomal recessive lower motor neuronopathy disorder of childhood. SMA is caused by mutations in the *SMN1* gene leading to the loss of its product, the survival of motor neuron (SMN) protein (Frugier et al., 2002). The product of a second copy of the gene, *SMN2*, cannot compensate the loss of SMN because of a mutation in the last exon that affects its splicing, resulting in predominantly truncated, unstable forms of SMN. Interestingly, SMA is a disease of RNA processing in a second way as the SMN protein is itself an RNA-binding protein involved in various aspects of RNA function such as splicing (Gubitz et al., 2004) and mRNA localization to the axon (Rossoll et al., 2003). New evidence has emerged recently for a widespread involvement of RNA-mediated mechanisms in a number of degenerative pathologies of the CNS. RNA-based mechanisms have been implicated in conditions ranging from triplet repeat expansion diseases, amyotrophic lateral sclerosis (ALS), and dementias, leading to the recognition of new pathogenic pathways in which pathogenicity is initiated at the RNA level, hence challenging the traditional view that pathogenesis is triggered by abnormal protein processing, be it aberrant posttranslational modifications, proteolytic processing, or misfolding. The following examples illustrate two major RNA-dependent mechanisms involved in neurodegeneration: *trans*-dominant effect of RNA on the activity of RNA-binding proteins and aberrant alternative splicing.

Trans-dominant pathogenic role of RNA: abnormal activity of UTR-binding proteins

A number of human diseases are caused by triplet repeat expansions in nontranslated regions of the responsible genes (Ranum and Day, 2004). Fragile X syndrome (FXS) is caused by a CGG expansion in the 5′-UTR of the *Fmr1* gene, consistent with a loss-of-function mechanism in which these expansions prevent the expression of the normal protein. Conversely, the discovery of the dominant mutation in myotonic dystrophy type 1 (DM1) results in a long expansion of 300–12,000 nucleotides of CUG repeats in the 3′-UTR of the mRNA encoding myotonic dystrophy protein kinase did not fit into a loss-of-function category. A second form of myotonic dystrophy, DM type 2 (DM2), also is caused by a very large expansion of a repeat, although the offending RNA sequence in this case is a CCUG tetramer repeat located in an intron.

FXS carriers have *Fmr1* alleles, called premutations, with an intermediate number of CGG repeats between patients (>200 repeats) and normal individuals (<55 repeats). Some *Fmr1* premutation carriers aged in their 50s and older develop a distinct tremor/ataxia syndrome, designated "fragile-X-associated tremor/ataxia syndrome" (FXTAS), and consisting of progressive intention tremor, gait ataxia, parkinsonism, and autonomic dys-

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function (Hagerman and Hagerman, 2004). The most striking neuropathological feature associated with FXTAS is the presence of eosinophilic, intranuclear inclusions in both neurons and astrocytes (Hagerman and Hagerman, 2004). However, no evidence of neurodegeneration has been found in either full mutation patients or in the *Fmr1* knock-out mouse model, indicating that modulation of the levels of the *Fmr1* gene product, the translational repressor FMRP (fragile X mental retardation protein), is not likely to account for the neurodegeneration. The fact that *Fmr1* premutation alleles differ from both normal and full mutation alleles by producing more *Fmr1* transcripts with lengthy rCGG repeats raises the possibility that the neurodegeneration is RNA mediated (Tassone et al., 2000). This notion is further supported by the observation of intranuclear inclusions in a "knock-in" mouse model in which the five endogenous CGG repeats had been replaced with an ~100 CGG-repeat fragment within the *Fmr1* gene (Willemsen et al., 2003). To directly test the toxicity of rCGG repeats, Jin et al. (2003) have established a *Drosophila* model that ectopically expresses the human *Fmr1* 5′-UTR containing either normal- or premutation-length rCGG repeats. These experiments showed that fragile X premutation rCGG repeats alone are sufficient to cause neurodegeneration in a dosage- and repeat-length-dependent manner and to induce the formation of inclusions. Using rCGG oligoribonucleotide capture from brain lysates followed by mass spectrometry, an rCGG-repeat-binding protein (rCGGBP) has been identified. The interaction with rCGG repeat is conserved between mammals and *Drosophila* and is sequence specific. Furthermore, overexpression of rCGGBP in *Drosophila* suppresses rCGG-mediated neurodegeneration in a dose-dependent manner. These data support the disease mechanism of the fragile X premutation rCGG repeat sequestering specific rCGGBPs, leading to neuronal cell death.

DM1 and DM2 are primarily muscle diseases but also involve CNS pathology. The disease-causing potential of these mutant RNAs probably derives from the length of the repeat sequences and their interaction with RNA-binding proteins of the muscleblind-like (MBNL) family. MBNL proteins bind to expanded CUG and CCUG repeats, and the mass of repeat expansion RNA in DM cells is sufficient to sequester MBNL proteins and interfere with their function as regulators of alternative splicing. Several neuronal transcripts have been identified that show misregulated splicing in DM1, including tau, amyloid precursor protein, and NMDA NR1 receptor, but their connection with the CNS symptoms remains uncertain (Jiang et al., 2004). As evidence for such a protein sequestration mechanism, disruption of *Muscleblind 1* in mice induces abnormalities of alternative splicing that are strikingly similar to those observed in human DM1 skeletal muscle (Kanadia et al., 2003). An interesting feature of RNAs with highly expanded CUG or CCUG repeats is that they are retained in nuclear foci. In cortical neurons and striated muscle, muscleblind proteins are recruited into the nuclear foci and depleted elsewhere from the nucleoplasm.

Similarly, other neurodegenerative conditions caused by nontranslated repeat expansions might involve pathogenic RNA mechanisms. These include spinocerebellar ataxias (SCA) type 8, 10, and 12 and Huntington's disease-like 2 (Ranum and Day, 2004). Particularly noticeable is SCA8, which is linked to a CUG repeat expansion in an untranslated transcript that induces progressive neurodegeneration in *Drosophila* (Mutsuddi et al., 2004). Because untranslated triplet repeats can cause neurodegeneration, a question that comes immediately to mind is whether RNA-based mechanisms also play a role in diseases caused by translated CAG repeats, which are all neurodegenerative, the best example being Huntington's disease. A strong argument against such a possibility is that polyglutamine sequences encoded by CAG or CAA repeats cause indistinguishable phenotypes in *Drosophila* and untranslated CAG repeats do not cause a phenotype in this system (McLeod et al., 2005). However SCA12 is caused by a CAG expansion in the 5′-UTR of the *PPP2R2B* gene and is phenotypically very similar to other SCAs caused by translated CAG repeats (Holmes et al., 1999). Expansions in CAG repeat disorders are more modest that in FXTAS and DM1, but they could potentially sequester RNA-binding proteins that may play a modulating role.

The potential implication of UTR-binding proteins in neurodegeneration is not limited to diseases caused by repeat expansions. For instance, overexpression of the 3′-UTR of the light neurofilament subunit (NF-L) mRNA causes motor neuron disease in mice (Nie et al., 2002) and accumulation of ubiquitinated aggregates in degenerating motor neurons in culture (Lin et al., 2003). The cognate RNA-binding protein p190RhoGEF binds to the 3′-UTR of NF-L mRNA and stabilizes it (Canete-Soler et al., 2001). Neurofilaments are particularly relevant to ALS because they accumulate in the perikarya of dying motor neurons in sporadic as well as familial forms of ALS caused by missense mutations in the superoxide dismutase 1 (*SOD1*) gene (Bruijn et al., 2004). In G93A mutant SOD1 transgenic mice, which develop a motor neuron disease phenotype reminiscent of the human disease, aggregates of NF-L, p190RhoGEF, and mutant SOD1 are very prominent in the vacuolated neuropil in the vicinity of degenerating motor neurons. Aggregates of NF-L are potentially neurotoxic in that they have synergistic effects on the aggregation of mutant, but not wild-type, SOD1 (Lin et al., 2004). Interestingly, similar aggregates of p190RhoGEF and NF-L occur in neurites surrounding degenerating motor neurons of mice expressing untranslated NF-L RNA in the 3′-UTR of a green fluorescent protein reporter transgene, suggesting that the aggregates themselves may be a triggering event in the pathogenesis of motor neuron degeneration (Lin et al., 2005). The findings support a model whereby interactions of p190RhoGEF and NF-L protein lead to aggregation of NF-L protein with neurotoxic effects on motor neurons in motor neuron disease. The participation of p190RhoGEF in the pathogenesis of motor neuron degeneration is believed to derive from its prospective role in regulating steady-state levels of NF-L expression. p190RhoGEF binds and stabilizes NF-L mRNA and also interacts with unassembled NF-L protein and links stabilization of NF-L mRNA with the disposition of NF-L subunits in the cell. Interactions of p190RhoGEF lead to coaggregation with unassembled NF-L protein and downregulation of NF-L mRNA. However, p190RhoGEF does not interact with assembled NF-L subunits so that assembly of NF-L prevents the interactions of p190RhoGEF and NF-L protein that downregulate NF-L mRNA. Alteration of this regulatory pathway may lead to excessive aggregation of NF-L and p190RhoGEF with neurotoxic effects on motor neurons and could account for the selective loss of NF-L mRNA that occurs in motor neurons of mice bearing mutant *SOD1* transgenes and of patients with familial and sporadic ALS.

Alternative splicing in neurodegeneration: mechanisms and RNA-based therapies

Disruption of the exquisite functional diversity provided by isoforms arising from a single gene by alternative pre-mRNA splicing can also have neurotoxic consequences. The selection of specific 5′ and 3′ splice sites in alternative splicing events involves exonic and intronic splicing silencer and enhancer elements and

splicing factors binding to these elements, especially members of the serine–arginine (SR)-rich protein family. Alternative splicing can be disrupted through stress from a primary condition modifying the activity of splicing factors or by mutations affecting splicing elements.

The first example comes from ALS in which, in addition to neurofilaments, another neuronal intermediate filament protein, peripherin, forms intraneuronal proteinaceous aggregates and is overexpressed in motor neurons. Three splice variants of peripherin have been identified in the mouse: Per 58, which is encoded by all nine exons of the peripherin gene; Per 56, generated by the use of a cryptic acceptor site at the beginning of exon 9 that results in a frame shift and replacement of the C-terminal 21 amino acids of Per 58 with a unique eight amino acid sequence; and Per 61, which is generated by the in-frame retention of intron 4 leading to a 32 amino acid insertion within a domain of peripherin crucial for intermediate filament assembly. Per 58 and Per 56 appear to be normal isoforms in motor neurons of wild-type mice, whereas Per 61 is not. However, Per 61 is expressed in motor neurons of mutant *SOD1* transgenic mice, indicating that mutant *SOD1* induces differential splicing of the peripherin pre-mRNA (Robertson et al., 2003). Per 61 is toxic to primary motor neurons in culture and also induces peripherin and neurofilament aggregate formation. It is likely that alternative splicing of the peripherin pre-mRNA also occurs in humans; indeed, several expressed sequence tags representing alternatively spliced variants of peripherin can be found in the National Center for Biotechnology Information database. Finding ALS-specific splicing events involving peripherin, and perhaps other genes, will open the way to identifying new pathogenic mechanisms in ALS.

The mechanistic importance of impaired alternative splicing in neurodegeneration has been ascertained by the discovery of mutations in the *MAPT* gene, encoding the microtubule-associated protein, tau, in frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Hutton et al., 1998). FTDP-17 is part of the group of dementias characterized by intraneuronal deposition of tau and collectively referred to as tauopathies that include Alzheimer's disease and also DM1. Exon 10 (E10) of the *MAPT* gene encodes the second of four imperfect microtubule-binding repeats in the C-terminal half of the tau protein. Tau pre-mRNA is alternatively spliced to produce $E10^+$ and $E10^-$ tau isoforms that are expressed in approximately equal amounts in adult human brain (Avila et al., 2004). Sixteen missense, silent, deletion, and intronic FTDP-17 mutations affect 7 of 10 splicing regulatory sequences, which include both tau E10 splice sites as well as several splicing enhancer and silencer elements in E10 and in the flanking introns (D'Souza and Schellenberg, 2002). E10 retention in FTDP-17 results in a twofold to sixfold excess of $E10^+$ tau over $E10^-$ tau (Hutton et al., 1998; Connell et al., 2005). The identity and function of *trans*-factors that interact with *cis*-regulatory sequences offer clues to normal and aberrant tau E10 splicing. Using *in vitro* binding assays and *in vivo* knock-down and overexpression assays, the SR splicing factor splicing factor 2 (SF2)/alternate splicing factor (ASF) and the SR-like splicing factor Tra2β that bind the normal E10 polypurine enhancer (PPE) element were identified (Jiang et al., 2003). FTDP-17 mutations Δ280K and N279K disrupt the PPE and inhibit or enhance E10 splicing, respectively, through altered associations with SF2/ASF and Tra2β. Mutation Δ280K abolishes all *in vitro* interactions, whereas mutation N279K increases Tra2β binding. Interestingly, overexpression only of SF2/ASF restores splicing in Δ280K. The general involvement of other SR and non-SR splicing factors is currently being studied using knock-down assays. Transgenic mice that express normal and mutant human tau genes have been generated to determine how FTDP-17 splicing mutations alter the spatiotemporal expression pattern of E10 in the developing CNS. The expression of critical splicing factors in relation to E10 levels in different brain regions will be instrumental in understanding how subtle changes in E10 expression contribute to the clinical variability seen not only in FTDP-17 but also in other related dementias.

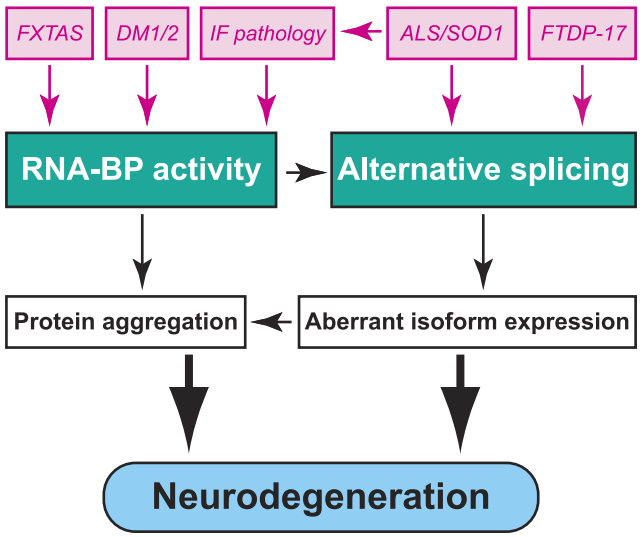

Figure 1. Diagram summarizing the involvement of RNA-mediated mechanisms in the neurodegenerative process in the nontranslated triplet repeat expansion diseases, FXTAS and DM1 and DM2, FTDP-17, and disorders with intermediate filament (IF) pathology. The latter refer to motor neuron diseases with accumulations of neurofilaments or peripherin, which include sporadic and *SOD1*-linked amyotrophic lateral sclerosis (ALS/SOD1). In this diagram, aggregation is used *sensu lato*, encompassing microscopically visible inclusions as well as smaller oligomers.

From a therapeutic perspective, correcting isoform imbalance, be it tau or another transcript, would require ideally direct intervention at the RNA level, hence maintaining endogenous transcriptional control. Reduction of E10 inclusion has been achieved by using oligonucleotides binding to E10 splice junctions in PC12 cells that express predominantly $E10^+$ tau (Kalbfuss et al., 2001). Another, more versatile strategy is to reprogram tau mRNA using spliceosome-mediated RNA *trans*-splicing, or SMaRT (Puttaraju et al., 1999). SMaRT creates a chimeric mRNA through a *trans*-splicing reaction mediated by the spliceosome between the 5′ splice site of an endogenous target pre-mRNA and the 3′ splice site of an exogenously delivered pre-*trans*-splicing RNA molecule. Tau pre-mRNA is a suitable target for SMaRT at the level of E10 splicing, and the 3′end of the tau message can be replaced by the 3′end of a pre-*trans*-splicing molecule containing human tau exons 10–13 and a targeting domain binding to intron 9. Furthermore, *cis*-splicing exclusion of E10 can be bypassed by *trans*-splicing, and conversion of $E10^-$ tau RNA into $E10^+$ RNA can be achieved with an efficiency of ~30% (Rodriguez-Martin et al., 2005). Thus, *trans*-splicing strategies can be used to replace efficiently alternatively spliced exons and suggest a potential therapeutic application of SMaRT not only for tauopathies but also for other disorders linked to impairment of alternative splicing.

Concluding remarks

The above examples illustrated the implication of RNA-based neurotoxic mechanisms in a number of neurodegenerative conditions. The putative mechanisms involved are summarized in

Figure 1. Disruption of alternative splicing regulation, resulting directly from either mutations affecting splicing regulatory elements or abnormal activity of RNA-binding proteins, can upset a tightly regulated tissue and developmentally regulated expression program leading to imbalance or inappropriate expression of isoforms of key proteins. Novel *trans*-dominant mechanisms offer a new perspective on the role of RNA in neurodegenerative processes. Surprisingly, both aspects of RNA-based mechanisms are linked to protein misfolding and aggregation, a hallmark of neurodegeneration. The realization that aberrant RNA processing is a mediator of at least some neurodegenerative processes point to specific RNAs as targets for novel therapeutic interventions.

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