RNA binding proteins: a common denominator of neuronal function and dysfunction

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In eukaryotic cells, gene activity is not directly reflected by protein levels because mRNA processing, transport, stability, and translation are co- and post-transcriptionally regulated. These processes, collectively known as the ribonome, are tightly controlled and carried out by a plethora of *trans*-acting RNA-binding proteins (RBPs) that bind to specific *cis* elements throughout the RNA sequence. Within the nervous system, the role of RBPs in brain function turns out to be essential due to the architectural complexity of neurons exemplified by a relatively small somal size and an extensive network of projections and connections. Thus far, RBPs have been shown to be indispensable for several aspects of neurogenesis, neurite outgrowth, synapse formation, and plasticity. Consequently, perturbation of their function is central in the etiology of an ever-growing spectrum of neurological diseases, including fragile X syndrome and the neurodegenerative disorders frontotemporal lobar degeneration and amyotrophic lateral sclerosis.

Keywords: alternative polyadenylation; alternative splicing; amyotrophic lateral sclerosis; anti-Hu syndrome; CPEB; ELAV; fragile X syndrome; FMRP; FUS; HU; HuB; HuC; HuD; HuR; neuron; neurodegeneration; Nova-1; Nova-2; paraneoplastic opsoclonus-myoclonus ataxia; PTBP-2; PTBP-1; TDP-43; FTL

Introduction: RBPs as Multi-tasking Modulators of Protein Output

The timing and dosage of gene expression are fundamental determinants of cellular phenotype and organismal complexity. Consequently, the regulation of gene expression is highly coordinated at multiple levels by ubiquitous and cell-specific *trans*-acting factors. Whereas for many years the specific focus has been on basal transcriptional regulation, post-transcriptional mechanisms regulating RNA metabolism have increasingly emerged as major determinants of gene output. The main reason is the high sequence plasticity, structural diversity, and agility of mRNA molecules that makes them ideal hubs for partners to bind and modulate protein output. RNA binding proteins (RBPs) are *trans*-acting factors that reversibly bind to these RNAs either alone or in conjunction with non-coding RNAs,

particularly microRNAs (miRs)^[1]. The transit interaction of RBPs and miRs with the RNAs results in the formation of ribonucleoprotein complexes that ultimately determine the fate of RNAs.

Post-transcriptional regulation confers several advantages to cells, some of which are particularly essential for neurons (summarized in Fig. 1). First and foremost, alternative pre-mRNA splicing allows the functional proteome to qualitatively expand; new proteins are generated from the same pre-mRNA with different binding partners and functions^[2]. Then, there is alternative polyadenylation (APA) that allows either the formation of different proteins from the same pre-mRNA (if APA occurs in an internal exon), or, more often, the generation of transcripts with different 3'UTR sizes that can be quantitatively regulated by additional RBPs and/or miR complexes^[3]. Modulation of RNA stability is

Fig. 1. Diverse mechanisms of RNA binding protein function.

another key target of post-transcriptional mechanisms. In this case, different RBPs stabilize or destabilize mRNA transcripts by binding to intronic and/or exonic sequences to quantitatively regulate protein output^[4, 5]. Further, RBPs may also be involved in translation regulation by enhancing or reducing the translation efficiency of mRNAs^[6, 7]. Finally, RBPs may be involved in mRNA transport along axons and dendrites, subcellular localization, or activity-dependent local translation^[8]. Importantly, different RBPs can interact with the same RNA at different binding sites or compete for the same binding site, increasing the complexity of RNA regulation^[9]. Not surprisingly, deregulation of RBPs leads to impaired protein homeostasis and cellular function. This may trigger the development of disease, especially in tissues where cells are long-lived, highly differentiated, and poorly replenished throughout the organism's life^[10].

This review focuses on the multifunctional roles of

RBPs in neurons, with special emphasis on those RBPs that are strongly associated with neuronal function and dysfunction. It concludes with the emerging view that RBPs may serve as nucleation centers for neurodegenerative processes, based on their requisite role in RNA metabolism and their strong intrinsic propensity for protein aggregations aggravated by stress.

RBPs and Pre-mRNA Splicing: Driving Phenotypic Diversity

In higher eukaryotes, alternative mRNA splicing is a key mechanism that allows expansion of the functional proteome from a genome of limited size depending on cell type, developmental stage, and stimuli. Alternative transcripts are generated by a series of splicing events that include exon skipping, intron retention, alternative first/

last exons, and mutually-exclusive exons. This happens in the nucleus and relies on the interaction between the spliceosome components, the splicing regulator proteins, and the pre-mRNA. The spliceosome is an RNA-protein complex consisting of the small nuclear RNAs U1-6 and several RBPs that catalyze splicing^[11]. These core components are common to all cells, and their function is to bind at intron-exon boundaries and catalyze intron removal and exon joining. In addition, specific *cis* elements on these pre-mRNAs and cell-type specific splicing regulators that recognize them drive cell-specific alternative splicing^[12]. Splicing events are highly prevalent since 92%–94% of human genes undergo alternative splicing^[13], 86% of which express minor isoforms that amount to 15% or more of the total gene expression^[14]. The majority of splicing events (88%) take place in the coding region and alter the protein products^[15]. mRNA transcript diversity is most prevalent in the brain, in part as a result of high gene expression^[13,16], with brain tissues expressing the greatest number of tissue-specific exons^[17, 18]. Besides generating diversity, alternative pre-mRNA splicing indirectly influences the stability, transport, localization, and translation of mRNA transcripts. Even minor changes, not immediately appreciated, like the use of a longer 5'UTR, may lead to either reduced protein translation^[19, 20] or altered subcellular distribution and enhanced translation activity under non-cap-dependent conditions as shown for postsynaptic proteins bearing IRES *cis* elements^[21, 22]. In the nervous system, alternative splicing has been implicated in the control of neuron specification, differentiation, and the modification of synaptic strength. Five of the most relevant cell-specific splicing regulators in the brain are the PTBP-2, HU, NOVA, TDP-43 and FUS proteins, all of which are discussed below. Of note, besides their main role in pre-mRNA splicing, these RBPs have additional RNA regulatory functions that are described in other sections of this review (Fig. 1).

PTBP-2

Polypyrimidine tract binding protein 2 (PTBP-2) is expressed in early post-mitotic neurons, as well as muscle and testis, and has 73% homology to the ubiquitously-expressed PTBP-1^[23–25]. Like PTBP-1, it contains four RNA recognition motifs (RRMs), nuclear import/export signals, and recognizes UCU-rich targets to regulate

alternative splicing^[26–29]. A recent high-throughput study has shown that as much as 96% of PTBP-2 binding sites are found in introns, consistent with a role in pre-mRNA processing^[27]. Conforming to the splicing events of other splicing regulators (e.g. HU, Nova, and TDP-43), upon binding to downstream introns of pre-mRNA, PTBP drives exon inclusion, while upon binding to upstream introns, it drives exon exclusion. In most cases, PTBP acts as a repressor of alternative splicing^[26–29]. To discern its physiological role, a series of elegant experiments has shown that undifferentiated neural cells express high levels of PTBP-1 protein that alternatively splices the *ptbp-2* pre-mRNA to generate a nonsense-mediated decay isoform that fails to translate into a mature protein. During neuronal differentiation, however, the increase in miR-124 expression reduces PTBP-1 levels and allows *ptbp-2* pre-mRNA to be efficiently spliced and translated^[26, 30]. Subsequent, detailed analysis by Licatalosi *et al.* (2012) showed that the precise role of PTBP-2 is to maintain neural progenitor pools and prevent premature neurogenesis in the developing brain. They based this assessment on (1) the finding that *ptbp-2*-null mice display ectopic nests of neuronal progenitors, and (2) cross-linking immunoprecipitation high-throughput sequencing (HITS-CLIP) assays showing that PTBP-2 inhibits the incorporation of adult-specific alternative exons in mRNAs that encode proteins associated with the control of cell fate, proliferation, and the actin cytoskeleton^[27].

HU

The mammalian homologs of the *Drosophila* embryonic lethal abnormal vision (ELAV) protein, also known as HU proteins (HuR, HuB, HuC and HuD), are by far the best characterized RNA-binding proteins with roles that span all stages of mRNA metabolism including pre-mRNA splicing, mRNA transport, stability, and translation^[31–35]. HU proteins are 70% homologous at the protein level and contain three RRM^[36]. HuR is ubiquitously expressed, while HuB, HuC, and HuD are neuron-specific members of the family although HuB is also expressed in the gonads^[37]. Each displays a characteristic expression pattern during development. Using *in situ* hybridization in the mouse brain, Okano *et al.*, have shown that HuB is expressed in early post-mitotic neurons in the outer layer of the ventricular zone, continuing in the intermediate zone, and diminishing in the cortical plate. HuD is predominantly expressed in

the intermediate zone and less in the ventricular zone and cortical plate, while HuC is expressed in the cortical plate and is absent from the other two zones^[36]. In the adult brain, all neurons express some set of *hu* mRNAs with different neuronal tissues expressing from one to all *hu* mRNAs. In the neocortex, for instance, all neurons express HuC but few express HuD or HuB mRNA. Overall, HuB and HuD show similar distributions and display similar or opposing functions during development. Both stimulate neurite outgrowth and neuronal differentiation *in vitro*, but HuB potentiates neural stem cell proliferation while HuD has a negative impact on this process^[38-41]. Accordingly, *hud*-null mice contain increased numbers of self-renewing cells in the subventricular zone, indicating that HuD is required for the exit of neural stem cells from the cell cycle^[38]. These mice also revealed a transient impairment in the neurite extension of cranial nerves during early embryonic development. Moreover, they displayed an abnormal clasping defect and poor performance on the rotarod test, suggesting a sensory/motor defect^[38]. A study of *huc* null mice revealed significant defects on the rotarod test. However, when they were tested for tail-twitching they showed no defect, likely due to functional redundancy as dorsal root ganglia robustly express all HU proteins^[36, 42]. Despite the fact that both *huc*- and *hud*-null mice (*hub*-null mice have not been generated yet) do not show any gross anatomical defects, *huc/d* double-nulls die shortly after birth, further supporting the idea of functional redundancy^[42]. The most recent evidence suggests that HU proteins play important roles in neuronal plasticity. They are significantly upregulated in hippocampal neurons after contextual or spatial learning tasks and after glutamate receptor activation^[43-47]. In addition, *hud* transgenic mice exhibit aberrant acquisition and retention of memories^[48], while *huc*-null mice display spontaneous epileptic seizure activity as a result of reduced glutamate expression^[42]. In humans, HU proteins have been associated with the anti-HU syndrome that resembles the phenotype of *hu*-null mice and is characterized by sensory neuropathies, autonomic, brain stem, and cerebellar dysfunctions, short-term memory loss, and epileptic seizures. This syndrome is the outcome of an immune response to neuronal HU proteins that are ectopically expressed in certain tumors such as small-cell carcinomas and neuroblastomas. These auto-

immune responses involve the production of antibodies that cross the blood-brain barrier and injure neurons in a yet poorly characterized manner^[49]. At the molecular level, HU proteins bind to AU- and GU-rich elements to stabilize mRNA and/or promote translation. Three recent high-throughput studies have shown that as much as 30% of HU binding sites are found in introns, answering the long-unresolved question of why the nuclear abundance of HU is high^[4, 42, 50]. Further, these studies showed that intronic binding to regulate splicing is often coupled in *cis* with 3'UTR binding to enhance pre-mRNA stability^[4]. In addition to these findings, HU proteins have been shown to bind nascent pre-mRNAs co-transcriptionally to modulate the speed of transcription and, thus, the inclusion of certain exons in a process that involves protein-protein interaction with RNA pol II and HDAC II^[51]. Moreover, HU proteins influence alternative splicing indirectly, by enhancing the mRNA stability and translation as well as modulating the splicing activity of another neuronal splicing regulator, NOVA-1^[52].

NOVA

As with the neuronal HU proteins, neuro-oncological ventral antigen (NOVA)-1 and -2 proteins were originally discovered as target antigens in the auto-immune neurological disorder paraneoplastic opsoclonus-myoclonus ataxia. In this disorder, patients with lung or fallopian tumors develop excessive motor movements as a result of impaired motor inhibition in the nervous system^[53-56]. NOVA-1 and NOVA-2 are expressed in differentiated neurons with largely reciprocal expression in the central nervous system. NOVA-1 is expressed primarily in the hindbrain and spinal cord while NOVA-2 occurs in the neocortex^[53, 57, 58]. Both NOVA proteins bind to clusters of a minimum of three YCAY (Y is either a C or U) motifs on target mRNAs and can tolerate variable spacing between these^[58, 59]. *Nova-1*-null mice are born indistinguishable from their littermates but die after 2–3 weeks with profound motor failure that correlates with apoptotic death of motor neurons in the spinal cord and brainstem^[60]. Similarly, *nova-2*-null mice die a couple of weeks after birth and are characterized by aberrant migration of cortical and Purkinje neurons, whereas the neural progenitor cell fate remains intact^[61]. *Nova* double-null mice are born alive, but they do not move, even after noxious sensory stimuli (tail pinch),

and die immediately after birth. These mice are born stiff but otherwise have normal gross morphology with a beating heart. Histological analysis indicated that these animals never inhaled, because diaphragmatic muscle atrophy occurs and the lung alveoli fail to expand, pointing to a lack of functional motor innervation^[62]. At the molecular level, Nova proteins possess three K-homology (KH) domains for RNA-binding and dimerization and shuttle between the nucleus and the cytoplasm with ~60% of Nova proteins residing in the nucleus^[57, 59]. HITS-CLIP analysis revealed that NOVA crosslinks to both intronic and 3' UTR clusters in many transcripts, suggesting, similar to HU, an ordered set of *cis*-actions on target mRNAs^[63]. More recently, a study of >200 transcripts displaying significant steady-state changes between wild-type and *nova*-null mice revealed that NOVA binding is primarily to intronic rather than stability-associated 3'UTR elements. Further analysis indicated that binding of NOVA to intronic sequences of these pre-mRNAs regulates the inclusion of cryptic exons that trigger nonsense-mediated decay leading to the reduced synthesis of functional proteins^[64]. Interestingly, most of these NOVA targets encode for synaptic proteins, including several implicated in familial epilepsy. Accordingly, NOVA was found to shift from the neuronal nucleus to the cytoplasm in response to seizure treatment with pilocarpine. Moreover, *nova* haplo-insufficient mice display spontaneous epilepsy^[64].

TDP-43

Transactive response DNA-binding protein of 43 kDa (TDP-43) is a predominantly nuclear protein, ubiquitously expressed and highly conserved. Its mis-localization in the cytoplasm is a hallmark of sporadic and familial amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and some synucleinopathies. In affected neurons and glia, TDP-43 is bound in the cytoplasm in the form of biochemically-insoluble inclusion bodies^[65]. At the molecular level, TDP-43 contains two RRM motifs and shows clear preference in binding to at least five UG repeats^[66, 67]. It contains a Gly-rich domain that mediates protein-protein interactions and all but one of the 48 identified ALS mutations occur in this domain^[68]. This domain also contains a Q/N-rich region, described as prion-like domain that mediates co-aggregation with poly-glutamine misfolded proteins^[69]. TDP-43 interacts with proteins in the spliceosome machinery and it is largely thought to be

an important component of pre-mRNA splicing^[70]. Recent high-throughput studies revealed that most TDP-43 binding occurs in introns (~70%) and to a lesser extent in 3'UTR and non-coding RNA (~10%)^[71]. Binding of TDP-43 to long/deep (>2 kb from the nearest intro-exon junction) intronic sequences correlates positively with protein expression, suggesting that it may suppress cryptic splice site expression and/or regulate mRNA stability^[5]. TDP-43 influences alternative splicing in a position-dependent manner, similar to the other RBPs. Hence, TDP-43 binding further upstream of an alternatively-spliced exon promotes its exclusion, while binding to proximal intronic sequences downstream of the alternatively-spliced exon promotes its inclusion^[71]. With respect to TDP-43 binding to 3'UTR sequences, the great majority has been detected in the cytoplasm, indicating that this RBP also regulates post-splicing events such as stabilization and/or transport^[71]. This view is reinforced by earlier studies showing that (1) TDP-43 enhances the stability of several mRNAs^[72, 73], (2) it is present in RNA-transporting granules^[74], and (3) it affects motoneuron terminal synapses in animal models^[75]. *Tdp-43*-null mice are embryonic lethal due to peri-implantation defects, while hemizygotes exhibit motor defects^[75, 76].

FUS

Like TDP-43, mutations in the fused in sarcoma (FUS) gene cause familial ALS and FTL-D-FUS. FUS is a ubiquitously-expressed RNA-processing protein and is predominantly localized in the nucleus. It contains a single RRM, a Gly-rich domain, three RGG domains that are also implicated in RNA binding, and a zinc-finger domain that binds GGUG RNA sequences^[77, 78]. The vast majority of mutations associated with ALS are missense, occurring in the Gly-rich and nuclear localization signal (NLS) motifs. NLS mutations disrupt the nuclear import of FUS, resulting in a reduction of nuclear function and an increase in the cytoplasmic portion rendering it prone to aggregation, which is likely the first step in the pathophysiological cascade that leads to FUS-associated neurodegeneration^[79-82]. Like TDP-43, FUS is thought to be an important component of the spliceosome machinery^[83, 84]. It also regulates transcription by binding to RNA pol II, affecting its phosphorylation^[85]. Because transcription and pre-mRNA splicing are tightly coupled^[86], FUS may function like HU to integrate these processes through RNA-protein and protein-protein interactions.

High-throughput studies have revealed that some 60% of FUS binding occurs in distal intronic regions, ~30% in proximal introns, and ~10% in 3'UTRs^[87]. Further, ~30% of literature-curated lncRNAs contain FUS binding sites^[87]. Interestingly, FUS is often bound to the antisense RNA strand at the promoter regions and downregulates sense-strand transcription^[88]. Importantly, FUS tags most often cluster in alternative splice sites rather than constitutively-spliced splice sites, also suggesting a role in alternative splicing^[88]. Gene ontology analysis revealed that FUS splice targets are predominantly involved in axonogenesis, axon guidance, cell adhesion, and other cytoskeleton-associated pathways^[87, 89, 90]. Finally, comparison of TDP-43 and FUS targets detected only a few RNAs bound by both proteins^[87]. Inbred *fus*-null mice die perinatally^[91] and exhibit dendritic spine defects compatible with its role in local mRNA transport^[92] and translation^[92]. Transgenic *fus* mice succumb to progressive paralysis and die after ~12 weeks. These mice show FUS-positive inclusions in spinal motor neurons and therefore replicate some aspects of human pathology^[93].

RBPs and Local mRNA Translation: Spatiotemporal Control of Protein Expression

Neurons develop and maintain not only elaborate but also distinct types of processes, the axon and the dendrites, that extend to great distances. These processes or compartments are then engaged in synapses with hundreds to thousands of counterparts in other neurons. Such synaptic contacts, which represent the minimal storage unit of information in the nervous system, are maintained through structural and functional coupling of a repertoire of the same and different proteins in these distinct compartments^[94]. Many of these proteins are transported to terminals on kinesin motors, particularly during the initiation phase of synapse formation, while a great number of other proteins are locally translated during differentiation and maturation^[95, 96]. In the latter case, the asymmetric localization of mRNAs helps to limit protein expression to these compartments. Stimulus-induced remodeling of synaptic strength, also known as synaptic plasticity, occurs at each individual synaptic terminal, in part as a result of rapid translation of these localized mRNAs. Consequently,

dynamic regulatory mechanisms for transport and the quantitative and qualitative translation of these mRNAs are in place with RBPs playing a central role. Here, we focus on two relevant RBPs, fragile-X mental retardation protein (FMRP) and cytoplasmic polyadenylation element-binding (CPEB) protein.

FMRP

FMRP is an RBP that is highly expressed in the brain and critically contributes to mRNA transport as well as translational control at the synapse^[97, 98]. As the name suggests, it is responsible for fragile-X syndrome (FXS), the first neurological disease clearly linked to a dysfunction of RNA metabolism. FXS is caused by a CGG triplet repeat expansion within the 5'UTR of the FMR1 gene, resulting in abnormal DNA methylation and transcriptional silencing^[99, 100]. FXS patients suffer from intellectual disability and autism. In neurons, FMRP is associated with polyribosomes in the cytosol and dendrites (no polyribosomes have been detected at presynaptic terminals as yet) and has also been detected in axons and growth cones^[98, 101-106]. FMRP is a multi-domain protein harboring two KH domains and a single Arg-Gly-Gly-rich (RGG-type) RNA-binding domain^[107, 108] of which KH2 is perhaps the most critical for function^[109, 110]. In marked contrast to other RBPs, FMRP preferentially binds to coding sequences with no discernible preference for sequence or structural motif^[103] despite earlier reports^[107, 111]. HITS-CLIP analysis revealed that its mRNA targets are highly enriched in both pre- and post-synaptic terminals and some 30 of these targets have been linked to autism spectrum disorders, possibly explaining the etiology of FXS. Nearly all investigations have shown that FMRP represses translation by causing ribosome stalling^[103] and trapping mRNAs in cytoplasmic granules^[112]. This mechanism appears to be selective and reversible, involving the phosphorylation of FMRP and its subsequent interaction with the miRISC complex. Thus, it has been delineated that phosphorylation of FMRP at serine 499 suppresses translation, while activity-dependent dephosphorylation by protein phosphatase 2A allows translation of bound mRNAs^[8]. Mechanistically, when FMRP is phosphorylated it recruits Argonaute 2 miRISC complexes loaded with miRs to repress translation, while it releases miRISC from target mRNAs upon its dephosphorylation, allowing their translation to occur^[113]. An additional function of FMRP

was recently proposed by Dictenberg *et al.*, who showed that FMRP directly associates with kinesin motors and likely serves as an adaptor for microtubule-based mRNA transport in an activity-dependent manner in dendrites^[114].

CPEB

The CPEB protein family is comprised of four paralogous members, CPEB1–4, all of which are widely expressed, sometimes with overlapping patterns^[115]. CPEB1, the best characterized member, binds to cytoplasmic polyadenylation element (CPE) sites (UUUUUAU or UUUUAAU) in the 3' UTR of target mRNAs and modulates poly(A) tail length *via* interaction with other proteins^[116]. On the other hand, CPEB2–4 do not bind CPE or regulate polyadenylation^[117]. CPEBs harbor two RRM and two zinc finger motifs by which they exert their effects^[118]. The mechanism of action of CPEB1 was originally delineated in *Xenopus* oocytes^[119], but more recently, most of the auxiliary components have been identified in neuronal dendrites too^[120]. Following transcription, most mRNAs acquire long poly(A) tails of 200–250 nucleotides. After export to the cytoplasm, however, the CPE-containing mRNAs are bound by CPEB1 and its interacting partners that include the poly(A)-specific ribonuclease (PARN) and the poly(A) polymerase germ-line development 2 (GLD2) proteins. When both are bound to CPEB1, PARN activity predominates resulting in a shortened poly(A) tail of 20–40 nucleotides^[121, 122]. Stimuli that promote CPEB phosphorylation lead to the expulsion of PARN from the RNP complex and allow an increase in the poly(A) tail by GLD2 polymerase. The elongated poly(A) tail then serves as a platform for the poly(A)-binding protein (PABP) to recruit the 40S ribosomal subunit to the 5'UTR of the mRNA and start translation. Phosphorylation of CPEB1 in dendrites is thought to be mediated by aurora kinase A and/or calcium/calmodulin-dependent protein kinase type II alpha^[120, 123–125]. CPEB1 has, in addition, been shown to repress translation by recruiting the 4E-BP protein neuroguidin that interacts with the cap-binding protein eukaryotic translation initiation factor 4E (eIF4E) to prevent its association with eIF4G to initiate translation^[6]. Of note, CPEB is highly enriched at post-synaptic densities, indicating that it is important for local translation^[116, 124]. Accordingly, *cpeb1*-null mice have memory deficits and reduced long-term potentiation (LTP)^[126–128]. In addition, *cpeb1* mice mutated at phosphorylation sites T171 and S177 in cerebellar Purkinje neurons display significant

impairment of motor coordination and motor learning delay, reinforcing the overall importance of CPEB1 for synaptic function^[129].

RBPs and Alternative Polyadenylation: Fine-tuning mRNA Translation

Polyadenylation is a two-step process that involves endonucleolytic cleavage of the pre-mRNA, followed by the synthesis of a polyadenylation tail at the 3' end. The target selectivity of cleavage is mediated by four sequence elements in the 3'UTR. Foremost is a polyadenylation signal containing the canonical AAUAAA or AUUAAA sequence (also known as poly(A) signals or PAS), located 10–35 nucleotides upstream of the cleavage site, and serves as the binding site for the cleavage and polyadenylation specificity factor (CPSF1). Downstream of PAS is a less well-defined region rich in U or GU nucleotides referred to as downstream element (DSE) that constitutes the binding site for cleavage-stimulating factor (CSTF). The interaction of CPSF1 and CSTF proteins is thought to be the most important determining factor for the selection of a cleavage site. Then, there is an element upstream of PAS that contains U(G/A)UA nucleotides and is the binding site of the cleavage factor I complex (CPSF5 plus CPSF6 or 7). This element is thought to promote recognition of the cleavage site. A fourth sequence rich in G nucleotides downstream of DSE has also been proposed to play a role in polyadenylation (pA), but the protein(s) that bind it has yet to be identified.

The presence of non-canonical PAS sequences together with the tissue-specific distribution of auxiliary RBPs that recognize/compete for binding onto the polyadenylation elements is thought to determine alternative polyadenylation (APA). Generally, two types of APA are distinguished. One in which APA sites are located in introns/internal exons, resulting in the production of different protein isoforms (qualitative change), and one in which APA sites are located in the 3'UTR region, giving rise to transcripts encoding the same protein isoform but with different 3'UTR lengths. Given that 3'UTRs are the main targets of miRs and regulatory RBPs, APA is expected to modify gene expression quantitatively in the latter case. In this regard, Legendre *et al.*, (2006) carried out a systematic examination of 3'UTRs produced by APA and found that 52% of miR target sites are located downstream of the

main PAS site^[130]. It is estimated that half of human genes undergo alternative cleavage and polyadenylation to generate transcripts with variable 3'UTR lengths^[131]. A close connection between gene transcription and pA site choice has been demonstrated, in which highly-expressed genes transcribe mRNAs with shorter 3'UTRs, while transcripts that are expressed at lower levels are associated with longer 3'UTR isoforms^[132]. Along with this, higher gene expression is tightly linked to cell division, where short 3' UTR isoforms with fewer miR sites are abundant in proliferating cells^[133]. In contrast, differentiated cells possess longer 3'UTRs^[132]. With respect to the nervous system, 3'UTR analysis of the longest and shortest human mRNA transcripts revealed that pre-synaptic mRNAs have significantly longer 3'UTRs compared to all other transcripts, including post-synaptic ones. The tendency of pre-synaptic mRNAs to have relatively longer 3'UTRs remained when analysis of the shortest 3'UTR isoforms was carried out. In contrast, post-synaptic transcripts revealed a significant drop in 3'UTR length between the longest and shortest 3'UTR isoforms. These results indicated that pre-synaptic mRNAs maintain a relatively long 3'UTR for enhanced *trans* regulation, irrespective of 3'UTR length fluctuations, while post-synaptic proteins possess a broader spectrum of 3'UTR lengths to avert *trans* regulation under specific conditions^[134].

Several examples illustrate the role of APA in mRNA localization. Perhaps the best-studied molecule is brain-derived neurotrophic factor (BDNF) that is processed to two transcripts with either a long or a short 3'UTR, both encoding the same protein. The short 3'UTR mRNA is restricted to the soma whereas the long 3'UTR mRNA is preferentially targeted to dendrites. Mutant mice lacking the long 3'UTR isoform show little expression of BDNF in dendrites, despite normal levels of total BDNF. As a result, these mice exhibit deficits in the pruning and enlargement of spines, as well as impairment in LTP in dendrites but not in the soma of hippocampal neurons^[135]. Phenotypically, they develop severe hyperphagic obesity^[136]. Furthermore, BDNF transcripts are differentially regulated, with the long 3'UTR isoform being translated under stimulation with pilocarpine, insulin, or leptin. The short BDNF 3'UTR isoform on the other hand, displays constitutive translation^[136, 137]. Dendritic targeting of BDNF is thought to be in part mediated by the binding of CPEB1 to a CPE-like element in the 3'UTR after

KCl-induced depolarization in hippocampal neurons^[138]. Further, the stability of BDNF long 3'UTR mRNA is mediated by HuD binding to a highly conserved AU-rich element, specifically located in the long 3'UTR^[139].

Computational predictions have indicated that variations of the canonical PAS sequence are relatively frequent, occurring in >30% of the ends^[140]. Interestingly, while the canonical sequence predominates in genes with a unique PAS site, the less-conserved variant PAS sites occur with higher frequency in genes with multiple PAS sites. Moreover, these variant sites tend to be located upstream of the more canonical PAS site, indicating that APA is regulated by the abundance of polyadenylation complex proteins or the existence of cell-type specific *trans* auxiliary proteins^[141]. Indeed, evidence exists for both mechanisms. Ji and Tian have revealed that CPSF and CSTF components are strongly upregulated during the generation of induced pluripotent stem cells from different tissues and this is associated with the usage of proximal PAS sites, while longer 3'UTR isoforms appear with aging as a result of weakened mRNA polyadenylation activity^[132, 142]. Moreover, genome-wide analysis of existing mRNA-sequencing data revealed that a third of non-canonical proximal PAS sites tend to possess a higher frequency of U and GU nucleotides downstream of the pA site compared with canonical pA signals, implying that a strong CSTF binding site might compensate for the absence of a consensus hexanucleotide^[143]. Interestingly, these U/GU sequences are also prime binding sites for HU proteins. Hence, Zhu *et al.* have shown that all HU proteins selectively block both cleavage and poly(A) addition at these sites, possibly by interfering with CSTF^[144]. More recently, further support to this came from the study of transgenic ELAV flies that display ectopic synthesis of long mRNAs, indicating that ELAV binds directly to proximal PAS sites to suppress cleavage and pA in the brain^[145]. Remarkably, the mRNAs of HU proteins also code for different APA variants displaying both differential expression and stability mediated by family members, indicating that HU proteins also have auto-regulatory functions^[146-148].

Recently, a link between RBP and miRs sites has emerged. Initially, it was reported that destabilization mediated by a transfected miR is generally attenuated by the presence of destabilizing AU-rich motifs and augmented

by stabilizing U-rich motifs, the binding sites of translation- and turnover-associated RBPs such as HU, AU-binding factor 1, and tristetrapolin (TTP)^[149, 150]. Subsequently, transcriptome-wide analysis of HuR revealed that most miR sites are in the immediate vicinity of HuR sites^[4, 50, 151]. The authors elaborated that when miR and HU sites overlap the transcripts are preferentially regulated by HU proteins, but when they do not overlap the transcripts are regulated by miRs. Interestingly, *hu* transcripts are themselves direct targets of miRs and concurrently, directly regulate the stability and/or maturation of other miRs, pointing to a vast repertoire of different regulatory loops^[4, 50, 152–154].

Like HU proteins, NOVA proteins appear to be an important component of APA in the brain. HITS-CLIP analysis of the genomic position of NOVA clusters revealed that 23% of tags map to intergenic regions that likely correspond to previously-undescribed isoforms of RefSeq genes with alternative terminal exons^[63]. To further delineate this, the same group used exon array screening of altered 3'UTR length between NOVA-2 wild-type and null brains to identify ~300 mRNA transcripts with such differences. The data suggested that NOVAs bind YCAY elements flanking regulated pA sites, and that the position of NOVA binding may determine whether it acts to promote or inhibit pA site use. In transcripts in which NOVA enhances the use of a pA site, it binds to more distal elements and may antagonize the action of auxiliary factors. In cases where NOVA suppresses pA site use, binding sites are located within 30 nucleotides of the pA site and overlap with the canonical CPSF and/or CSTF binding sites, likely interfering with the formation of the cleavage complex^[63].

Another example of a splicing factor multi-tasking at the 3'UTR of mRNAs is PTBP. PTBP-1 has been shown to either compete with CSTF for recognition of the pA signal's pyrimidine-rich DSE reducing 3'end cleavage^[155] or induce 3' processing and polyadenylation by directly recruiting the splicing factor heterogeneous nuclear ribonucleoprotein H to G-rich sequences, which then stimulates pA through direct interaction with CSTF^[156].

RBPs and Neurodegeneration: Nucleation Centers for Neurodegenerative Processes in the Aging Brain

The molecular and cellular bases of neurodegenerative

diseases are poorly understood. Traditionally, they are described as protein disorders in which misfolded monomeric proteins initially oligomerize and then aggregate to form fibrils^[157, 158]. These processes are largely thought to be unidirectional and detrimental, with no biological function. Their kinetics is dependent on the amount of starting proteins, their aggregation propensity or hydrophobicity, and the ability of the mechanisms of cell clearance - chaperones, proteasomes, and autophagy - to minimize their rate of assembly. The recent finding that the pathological redistribution of some RBPs from nucleus to cytoplasm is a hallmark feature of a wide spectrum of neurological disorders, however, has highlighted the involvement of the very dynamic RBPs and/or RNAs in the development of these processes. The importance of RNAs is underlined by the fact that mutations that disrupt the RNA binding ability of RBPs, like in the case of FUS or TDP-43, reduce or prevent their toxicity^[159–162]. Further evidence comes from the finding that some RNAs, such as the products of mutated *c9orf72* and *fmr1* genes, lead to neurodegeneration by a poorly-characterized mechanism that is likely to involve the accumulation and sequestration of RBPs to nuclear foci^[163–166].

The recently appreciated importance of RBPs in neurodegeneration is reflected in their highly-conserved protein structure. Apart from containing RNA recognition motifs, they all possess a glycine-rich hydrophobic domain that mediates self-dimerization and non-self protein-protein interactions. In some RBPs, like T-cell intracellular antigen-1 (TIA-1), the hydrophobic domain further shares homology with prion proteins^[167]. The protein-protein interactions mediated by RBPs are normally reversible and tightly linked to the fate of their mRNA target. In general, when mRNAs are not engaged in translation, they assemble with RBPs in RNA granules. These granules are of three main types: (1) ribonucleoprotein particles (RNPs) that function in mRNA nucleocytoplasmic shuttling and axonal and dendritic transport, (2) processing bodies that also contain translation repressors and components of the mRNA decay machinery, and (3) stress granules (SGs) that sequester non-essential capped mRNAs in response to stress, promoting the translation of essential stress response proteins such as HSP70. As expected, RNA granules are highly dynamic structures constantly

exchanging mRNA transcripts and proteins through all stages of mRNA processing^[168, 169]. Of these, SGs have recently received particular attention as most if not all of the RBPs linked to neurodegeneration associate with them in cell culture. Thus, TDP-43, FUS, ataxin-2 (ATXN2), survival motor neuron 1, and angiogenin have all been shown to co-localize with classic SG markers (TIA-1, TIAR, TTP, and G3BP) in cells undergoing stress. Further, disease-linked mutations of *tdp-43*, *fus*, and *atxn2* genes promote agglomeration in SGs, either by directly increasing the tendency of the protein to aggregate or by preventing nuclear translocation^[79, 160, 170-175]. Of interest, examination of the brains of boxers and head injury patients also revealed accumulation of TDP-43 in cytoplasmic aggregates that may be remnants of trauma-induced SGs^[176, 177]. Moreover, SG proteins such as TIA-1 and PABP co-localize with neuropathology in the brains of patients with Alzheimer's disease, FTDP-17, FTLDP, and ALS^[175, 178].

Thus, an alternative view of the neurodegeneration processes could be the formation of prolonged assemblies of RNPs in cytoplasmic granules, particularly SGs, that lose their dynamic disassembly over time and can only be cleared by autophagic mechanisms^[179]. Since autophagy declines with aging^[180], the clearance of these assemblies slows down, allowing some to be retained or grow by sequestering incoming RBPs. This ultimately alters mRNA metabolism, resulting in the production of aberrant mRNAs that either further potentiate RBP assembly or are mis-expressed, disrupting protein homeostasis and leading to cell death. Extracellularly released RBP complexes could then be endocytosed by surrounding cells, perpetuating the toxic effect^[181].

One of the most puzzling questions that arise from neurodegeneration studies is why neurons are so profoundly affected by aging. Moreover, it is intriguing that pathology is only detected in distinct populations of neurons in the brain, despite the fact that the RBPs associated with neurodegeneration are ubiquitously expressed. A possible answer may lie in the unique features of neurons that include longevity, poor supply of progenitors, polarization, and degree of interconnection. Neurons are the longest-living cells in the body that, in time, could accumulate toxic protein aggregates that subsequently derail homeostatic mechanisms and drive cell death. The neurogenic niches supplying progenitors are limited and located in distinct

areas of the brain, thus, most neurons are never replaced, allowing deficits to persist and grow over time. Further, by being highly polarized, neurons are greatly dependent on RBPs for function and are therefore highly vulnerable to RBP defects, whether these are inherited or caused by environmental input. It is known that the entire translation machinery is present at synaptic terminals, and at least in dendrites, all three types of RNA granules have been detected^[182, 183]. Confined by space and the need to rapidly respond to synaptic stimuli, the different types of granules and their constituting RBPs are in close physical proximity, allowing enhanced ribonucleoprotein interactions. It is possible that over time or under stress, cycling of RBPs between these granules may start to lose coherence and initiate the formation of aggregates with other RBPs and mRNAs/proteins to disrupt synaptic function. It is conceivable that neurodegenerative pathology is initiated at these sites, a view supported by findings showing that some synaptic degeneration precedes neuronal loss^[184-186]. Finally, because neurons are highly interconnected and spread out, there is a greater chance of receiving toxic RNP assemblies by endosomes from neighboring derailed cells. These toxic RNPs disrupt host RNA metabolism and disperse the defect in a manner resembling prion propagation.

Conclusion

RBPs are important mediators of qualitative and quantitative protein expression in neurons. Their role spans all stages of neuronal development including neurogenesis, differentiation, and synaptic plasticity. Deficits in RBP expression and/or distribution disrupt mRNA metabolism, leading to intellectual disabilities, motor impairments, and neurodegeneration. High-throughput sequencing studies have greatly advanced our understanding of their interactions with mRNA targets. In future, a major challenge is to better discern their roles in development and disease and how they fine-tune the expression of key neuronal proteins, given the multitude of RBP-mRNA and RBP-RBP interactions in the different RNP-processing granules.

ACKNOWLEDGMENTS

I thank Maria Paschou for assistance with the preparation of the figure. I also apologize to colleagues whose work was not cited due to space limitations. Work in my laboratory is funded

by grants from the Greek General Secretariat for Research and Development, Ministry of Education.

Received date: 2014-01-23; Accepted date: 2014-02-28

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