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Review

Defining a neuron: neuronal ELAV proteins

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Abstract. Neuronal cells strongly depend on the control exerted by RNA-binding proteins (RBPs) on gene expression for the establishment and maintenance of their phenotype. Neuronal ELAV (nELAV) proteins are RBPs able to influence virtually every aspect of the postsynthesis fate of bound mRNAs, from polyadenylation, alternative splicing and nuclear export to cytoplasmic localization, stability and translation. They enhance gene expression through the last two, best documented activities, increasing mRNA half-life and promoting protein synthesis by a still-unknown molecular mechanism. Developmentally, nELAV proteins have been shown to act as inducers of the transition between neural stem/progenitor cells and differentiation-committed cells, also assisting these neuroblasts in the completion of their maturation program. In brain physiology, they are also the first RBPs demonstrated to have a pivotal role in memory, where they probably control mRNA availability for translation in subcellular domains, thereby providing a biochemical means for selective increase in synaptic strength.

Keywords. nELAV proteins, AREs, neuronal differentiation, neuronal plasticity, mRNA stability, translation, memory.

Introduction

Life of an organism continuously depends on the ability of its molecular circuits to detect, amplify and integrate a multiplicity of incoming external signals and to convert them in the modulation of specific cellular programs to adapt to environmental changes. The majority of these processes finally involve a change in the rate of translation of a number of proteins. Historically, efforts aimed at deciphering the molecular basis of gene expression regulation have been largely focused on transcriptional control. Post-transcriptional processing of mRNAs, from splicing to translation, is now increasingly recognized as a step in the flow of genetic information offering substantial regulative opportunities, which appear to be widely implemented in key cellular programs and responses [1–3]. For example, cytoplasmic regulation of mRNA fate is largely involved in the first stages of embryogenesis [4, 5] but is also increasingly described as a major determinant in the differentiation and maintenance programs of germ cells [6, 7] and of other highly polarized cell types, such as neurons [8, 9]. This is not surprising, as specific functions and modifications take place in precise subcellular compartments, such as the axonal growth cone or the developing dendritic tree, giving rise to the complex neuronal

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architecture and sustaining microdomains characterized by the presence of specialized protein assemblies in the basal activity of terminally differentiated neurons. This temporal compartmentalization of protein functions is obviously not easily obtained by transcriptional modulation of gene expression, while posttranscriptional controls acting subcellularly at the level of mRNA splicing, localization, stability and translation can provide fast and effective means to change the architecture of local protein complexes.

Neuronal RNA-binding proteins

Posttranscriptional regulation is dependent on the activity of a number of *trans*-acting factors, RNA-binding proteins (RBPs) that bind in a sequence-dependent manner to *cis* consensus elements present mainly in the 3′-untranslated region (3′-UTR) of mRNAs but that can also be located in the 5′-untranslated region (5′-UTR), in the coding sequence, in intronic sequences or even in non-coding alternatively spliced variants of mRNAs. A number of RBPs are uniquely, or almost uniquely, expressed in neuroblasts and in differentiating or fully differentiated neurons, suggesting a function in neuronal programs of gene expression. These proteins often belong to gene families whose other members have a wider tissue representation, implying that the neuronal function could be evolved from a less specialized activity, even if, in principle, the opposite transition, from nervous system-related to more general roles, is also conceivable. A recent systems-oriented study [10] reported that out of 380 putative RBP-coding genes in the mouse genome, at least 323 were detected by *in situ* hybridization in the brain of developing mice at embryonic day 13.5 (E13.5) and postnatal day 0 (P0). Of them, 221 (68%) showed regionally localization, while 16 (7%) were strictly neuron-specific, suggesting, from a genome-wide perspective, that RBP-mediated posttranscriptional controls occurring in selected brain regions or cell types are indeed major determinants of brain development. A number of well-described RBPs are known to play specific roles in neuronal developmental programs or postmitotic activity. Among them, Nova-1 and Nova-2 are RBPs convincingly associated with regulation of neuronal alternative splicing of a subset of defined pre-mRNA targets, whose encoded proteins have demonstrated functions at the synapse or in axon guidance. Since the majority of these proteins are also known to physically interact, they have been suggested to form a protein network involved in synaptic plasticity shaped by a brain-specific splicing pattern controlled by the Nova RBPs [11]. Other RBPs recognized to act neuronally as splicing controllers are KSRP [12] and CUGBP2 [13], and for both proteins other activities – activation of degradation and translational repression, respectively [14] – have been suggested. Zip code-binding proteins (ZBPs; ZBP1 and ZBP2) are instead involved in the regulation of mRNA transport in dendrites [15] and again in translational repression [16]. A similar dual role of dendritic transport and translational repression of mRNAs is played by FMRP, the protein whose gene is mutated in the fragile X mental retardation syndrome [17], while another mRNA transport protein, CPEB, can act positively on translation of the bound targets [18], and two others, Stau1 and Stau2, can possess complex activities in addition to microtubule-associated dendritic transport [19], since Stau1 enhances translational initiation [20] but also triggers mRNA decay [21]. The general picture arising from these examples of functional characterization of RBPs acting in neurons is that each of them is probably involved in more than one step in the life of mRNAs following their birth in the nucleus. This remarkable flexibility of functions could have a structural basis in the aggregation of RBPs in the so-called messenger ribonucleoprotein (mRNP) complexes, structured assemblies of dozens of RBPs and target mRNAs representing a common form of cellular organization of neuronal and non-neuronal RBPs [22]. A fascinating hypothesis is that these mRNP particles function as discrete modules of coordinated metabolism of mRNAs encoding proteins involved in related functions, replacing in eukaryotes the operon-based network of concerted gene expression found in prokaryotes [23]. As we will see, ELAV proteins are mostly neuronal RBPs whose established molecular activities and phenotypic functions fit precisely into this scheme of multipurpose, networking proteins endowed with necessary roles in the execution of primary cell programs.

mRNA stability and translational control: the AU-rich elements

Modulation of mRNA decay is implicated in determining changes of the gene expression profile in response to incoming intracellular and extracellular stimuli. A change in mRNA stability can induce a corresponding variation in the translational window, providing a rapid way to modify protein abundance, which can be either cell-wide or confined to subcellular compartments [24]. The rate of mRNA degradation is controlled by both *cis*-acting motifs, evolutionarily conserved sequences present in the mRNA, and by the *trans*-acting factors interacting with them.

The mRNA 3′-UTR seems to play a major role in bearing key sequence determinants controlling mRNA decay. The best characterized of these motifs is the ARE (adenine-uridine-rich element) found in mRNAs endowed with a rapid response to cell environmental stimuli [25].

AREs were at first operationally defined as sequences able to confer mRNA instability when inserted into the 3′-UTR of reporter genes [26]. These destabilizing motifs range in size from 50 to 150 bases, are often characterized by the presence of a different number of copies of an AUUUA pentamer and have a high content of U residues. Functional AREs could be very common in vertebrate genomes. Recently, a computational compilation of mRNAs comprising the 13-base WWWU(AUUUA)UUUW consensus (W = A or U), which is defined somewhat arbitrarily but is seen to be restricted to 3′-UTR sequences, identified more than 4000 mRNAs in human expressed sequences, representing the products of up to 5–8% of human genes [27].

Despite the difficulty in deriving defined comprehensive consensi, a classification scheme has been attempted for AREs, recognizing three groups of sequences [28, 29]. This difficulty probably reflects the absence of information on the three-dimensional structure of such sequences and of specific binding patterns of associated RBPs. However, some functional correlates can be derived by this sequence-based ARE classification [30]. Overall, ARE-bearing mRNAs come from genes with functional over-representation in the gene ontology categories related to the flow of genetic information in cells and its regulation (cell communication, signal transduction, nucleic acids metabolism, cell proliferation), to the response to environmental stimuli (stress response, immune response, response to pathogens and parasites) and to developmental programs (morphogenesis, organogenesis and, important for our perspective, neurogenesis) [27]. This could suggest the preferential location of AREs as defined by the reported consensus in the 3′-UTR of genes whose products play an "informational" role in cells, in the general sense of signal processing or in execution of genetic programs, where fast and precise control of changes in protein abundance is mostly required.

Even if a constant scheme does not emerge, there is no doubt that AREs are docking sites for RBPs**.** Up to now, several ARE-binding proteins have been identified. The interaction of RBPs can correlate negatively or positively with the stability of each ARE-bearing mRNA and can also have consequences on its translation process, an ARE function less studied but probably equally important. AUF1 was the first ARE-binding protein proved to favor mRNA degradation [31], possibly acting by recruiting the exosome, the protein complex responsible for mRNA catabolism [32]. Another RBP involved in ARE-mediated mRNA destabilization is TTP (tristetraprolin), a zinc finger protein that seems to act with a mechanism similar to the one described for AUF1 [32, 33]. Other studied proteins interacting with AREs are TIA-1 and TIAR, two paralogues with translation-repressing function and, much less characterized, a number of other RBPs, including KSRP, AUH, GAPDH, Hsp70, Hsp110, hnRNP A1, hnRNP A2, hnRNP A3, hnRNP C, hnRNP L and nucleolin (for a review see [30]). By far the best studied among the ARE-binding proteins are the ELAV (or Hu) proteins.

nELAV proteins

From a substantial body of evidence coming from different model organisms, ELAV protein orthologues appear to be involved in the regulation of all the fundamental steps of mRNA metabolism, from pre-mRNA splicing to mRNA transport, stability and translation [34–36]. In vertebrates, HuB (a.k.a. Hel-N1), HuC and HuD represent the neuron-specific members of the family (nELAV proteins); HuB is also present in the gonads [37], while HuR (a.k.a. HuA) is ubiquitously expressed.

Human nELAV proteins were first reported as the targets of autoantibodies detected at high titer in patients with paraneoplastic neurological disorder [38]. The presence of certain types of tumors, especially small cell lung carcinoma (SCLC), ectopically expressing antigenic nELAV proteins on the cell surface elicits an autoimmune response involving the production of antibodies that cross the blood-brain barrier and lead to the so-called anti-Hu syndrome, characterized by subacute sensory neuropathy, dementia and/or encephalomyelopathy [39]. The absence of non-neuronal injury in these patients suggests that HuR may not be involved in the pathogenesis of the paraneoplastic disorder. Following cloning and sequencing, ELAV human genes were recognized to be orthologues of the *Drosophila melanogaster elav*, a gene coding for a neuron-specific nuclear RBP involved in the regulation of alternative splicing of some target mRNAs [40, 41]. Recent data are in favor of a more specific nuclear *elav* function in the fly, inhibition of mRNA 3′-end processing by binding at the vicinity of a polyadenylation site, which can result in downstream modification of the splicing pattern [42, 43]. Noteworthy, in this case the bound sequences are not 3′-UTR AREs but AU-rich stretches located in intronic sequences close to a polyadelylation site. Deletion of this gene leads to a phenotype of

embryonic lethality with subtle nervous system malformations, primarily involving massive disruption of the neuropil [44], from which both the acronym of the gene (*embryonic lethal abnormal visual system* [45]) and the notion that it is a key determinant of the development and maintenance of the nervous system originate. Other neural *Drosophila* paralogues of *elav* have been recognized [46, 47], while only one orthologue has been identified in *C. elegans* [48]; a corresponding family is also present in the vertebrates zebrafish [49] and *Xenopus* [37], demonstrating the high evolutionary conservation of the functional solutions provided by nELAV proteins in distantly related phylogenetic branches.

Structurally, the four vertebrate ELAV proteins are characterized by a high degree of sequence homology (70–85 %). They are about 40 kDa in size and contain three ~90 amino acid-long RNA recognition motif-type (RRM) domains [50]. The first two RRMs, positioned at the N-terminal end, are separated from the third one, at the C-terminus, by a hinge region [51]. The hinge regions, which presumably mediate interactions with other cell components [34], and the N-terminal ends are the portions in the family with the highest sequence diversity. The hinge regions contain the *cis* elements responsible for ELAV nucleocytoplasmic shuttling. They have been functionally characterized for the presence of a nuclear localization signal and a nuclear export signal, motifs that are integrated in HuR [52], and are distinct in HuD [53]. *Drosophila elav* has homologous sequences in its hinge region, which dictate the predominantly nuclear localization of the protein [54]. *In vitro* binding studies indicate that the first and second RRMs are primarily implicated in the interaction of nELAVs within the AU-rich sequences present in the bound mRNA [55, 56]. Structural determination of the first two HuC [57] and HuD [58] RRMs bound to AREs indeed show that they form a cleft with their β-sheet-containing surfaces in which the RNA element is inserted. Nothing is known about the mode of binding of the third RRM to RNA, but it has affinity for the poly(A) tail of some target mRNAs and contributes to the stability of the ELAV/mRNA complex [55, 56, 59, 60]. Generally, the poly(A) tail has a timing function for the life of each mRNA, since most of the mRNAs are slowly deadenylated before rapid degradation of the body. This mechanism allows a specific lifetime for cytoplasmic translation, which is approximately constant for a given mRNA. Interestingly, the length of the poly(A) tail also seems to correlate with the efficiency of the binding of ELAVs to their mRNA targets, with the affinity of HuD for the GAP-43 mRNA ~10 fold higher when the mRNA bears long versus short tails [60]. Moreover, expression of the third RRM alone in murine embryos has a dominant negative influence on the neuronal differentiation phenotype induced by coexpressed HuB and HuC [61].

On the cell scale, nELAV proteins have complex cell localization and distribution patterns. In cultured mammalian cells, they appear to be mainly cytoplasmic proteins, with a small fraction of nuclear immunoreactivity. However, cellular distribution may vary among cell types [35]. As for many other RBPs, ELAV proteins are endowed with nucleocytoplasmic shuttling ability [53, 62–64] functionally mapped in HuR and HuD, as mentioned before, in sequences present in the hinge region. A functional role of ELAV in nuclear export has also been suggested by the association of HuR with the SETα, SETβ, pp32 and APRIL proteins, endowed themselves with nucleocytoplasmic shuttling ability [54]. Relevantly, the association state of nELAV can change in the nucleocytoplasmic transition, since in the cytoplasm HuB seems to exist in two forms, the first producing a granular pattern interpreted as isolated mRNP particles and the second, a high molecular weight assembly, consisting of these particles linked to the cytoskeletal network and to polysomes [65]. The cytoplasmic granular mRNPs could coincide with the described multimeric nELAV organization with target mRNAs [66]. An increase in cytoplasmic localization of nELAV proteins can be obtained, in the short term, by stimulating neuroblasts with diacylglycerol-mimicking agents, known to activate protein kinase C (PKC) isoforms [67]. Taking these results together, a model can be proposed in which single ELAV proteins bind borne transcripts as early as in the pre-mRNA form [42, 68–70], influencing polyadenylation and splicing events. Then ELAV proteins participate in the export of the mature mRNAs into the cytoplasm [71], where they are present as oligomeric ELAV-containing mRNPs, after which the bound mRNAs can be directed to the translational apparatus or released to undergo rapid degradation. In this process, specific ELAV posttranslational modifications, such as arginine methylation by CARM1 [72] and threonine phosphorylation by PKCα [67], can be the final events of signaling cascades able to influence, respectively, ELAV association to target mRNAs and ELAV localization and induction of mRNA stabilization (see Fig. 1). This speculative view, although requiring additional experimental validation, is consistent with the data showing that ELAV proteins influence gene expression at different levels and possibly couple the processes of mRNA localization, stability and translation.

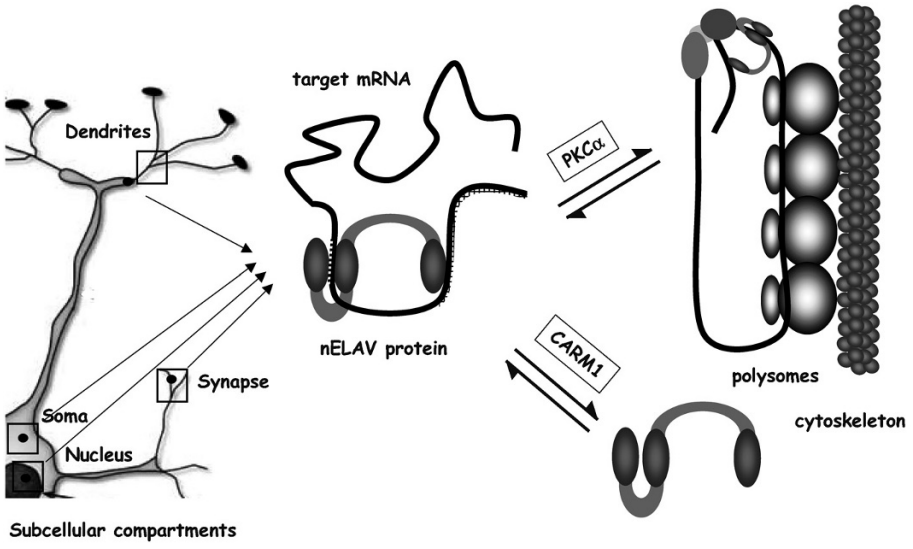

Figure 1. Neuronal subcellular localization and proposed mechanisms of regulation of nELAV proteins. nELAV proteins bind an array of target mRNAs and are localized in differentiated neurons both in the nucleus and the cytoplasm. Cytoplasmic nELAV proteins have been observed in the cell soma and in neural processes, axons and dendrites as well as at the synapse. In these different compartments, nELAV proteins form messenger ribonucleoproteins (mRNPs) putatively composed of different subsets of other RNA-binding proteins (RBPs) and target mRNAs. Translocation of nELAV-containing mRNPs from nucleus to cytoplasm and to the cytoskeleton (possibly associated to polysomes) is triggered by threonine phosphorylation of nELAV mediated by Protein Kinase C α (PKCα), while dissociation of nELAV proteins from specific target mRNAs is triggered by arginine methylation of nELAV mediated by CARM1.

nELAV proteins as determinants of the neuronal lineage in the developing nervous system

In the developing nervous system, progenitor cells committed to give rise to neurons stop proliferating and undergo a postmitotic differentiation program. Posttranscriptional regulation occurring in the early phase of neuronal development is critical to trigger neuronal diversity [73, 74], and a substantial body of evidence indicates that nELAV are centrally involved in this process.

nELAV proteins are reported to be early markers of neuronal commitment and have a specific temporal order of expression in the developing nervous system of vertebrates. *In situ* hybridization experiments performed in murine embryos show that, in the neocortex and cerebellum, HuB expression is present in very early postmitotic neurons within the outer cell layer of the ventricular zone, continuing in the intermediate zone and diminishing in the cortical plate; HuD is more expressed in the intermediate zone and less in the cortical plate, while HuC is absent in ventricular and intermediate neurons but robustly expressed in the cortical plate [75]. In the chick embryo, HuC is delayed in expression with respect to HuD in at least one developmental stage [76], confirming the possibility that the HuB/HuD/HuC mRNA sequence of appearance in differentiating neurons of developing nervous system structures could have mechanistic significance. In the adult mouse central and peripheral nervous system, specific patterns of expression of individual nELAV members are seen in the hippocampus, cerebellum, olfactory cortex, enthorinal cortex and neocortex, as well as in the ventral motoneurons of the spinal cord and dorsal root ganglia, again suggesting for different nELAV expression patterns a role in the maintenance of various types of postmitotic neurons. For example, within the neocortex, HuC is strongly expressed in all neurons, while HuD is prevalent in the large projection cells of the internal pyramidal layer, and HuB is detectable only in scattered neurons [75, 77].

The subset of well-demonstrated nELAV-bound and nELAV-regulated mRNAs represents another indication of the role of these proteins in neuronal differentiation programs (see Table 1). Together with developmentally regulated transcription factors active in the neuronal lineage (c-fos, c-myc, N-myc, Id), we can find microtubule markers of neuronal development (tau, neurofilament M), determinants of neurite outgrowth and synapse formation and function (GAP-43, acetylcholinesterase, neuroserpin, CGRP/calcitonin), determinants of the neural proliferation/differentiation boundary (p21, p27, musashi-1) and the potent neurogenetic factor VEGF.

A direct implication for nELAV proteins in vertebrate neuronal differentiation comes from experiments of perturbation of nELAV gene expression in primary cells and cell line models able to recapitulate *in vitro* neuronal differentiation. Overexpression of HuD has

Table 1. Some described nELAV target mRNAs.

nELAVs	target mRNAs	binding to AU-rich sequences	Activity
HuB	c-myc	[119]	
	c-fos	[119]	
	GM-CSF	[119]	
	GLUT1	[120]	Increased stability and translation [121]
	NF-M		Increased translation [82]
	Id	[122]	
HuC	c-myc	[123]	
	VEGF	[123]	
HuD	c-myc	[123, 124]	
	N-myc		Increased stability [70] and expression [110]
	c-fos	[125]	
	VEGF	[123]	
	$p21^{waf1}$	[126]	Increased stability and expression [72]
	p27		Inhibition of translation [127]
	acetylcholinesterase	[128]	Increased stability [128]
	GAP-43	[129]	Increased stability [78, 79]
	tau		Increased stability [81]
	neuroserpin	[130]	Increased expression [130]
	musashi-1	[84]	Increased stability [84]
	MARCKS	[131]	Increased stability [131]
	CGRP/calcitonin	[69]	Regulation of alternative splicing [69]

been shown *per se* to trigger the neurite outgrowth process in PC12 cells [61, 78, 79] and also to accelerate the rate of the same process in isolated primary rat cortical neurons and retinoic acid-induced embryonic stem cells [80]. Conversely, down-regulation of HuD by antisense RNA or oligonucleotides in PC12 cells blocks the morphological differentiation induced by NGF or PKC activators but not by dibutyryl-cAMP [79, 81]. Overexpression of HuC and HuB in PC12 cells also seems to induce neuronal differentiation [61], and HuB ectopically transfected into human teratocarcinoma NT2 cells induces them to form neurites [82]. These phenotypic changes are accompanied by an increase in levels of nELAV-bound mRNAs and proteins known to be determinants and markers of neuronal differentiation such as tau, neurofilament M and GAP-43 (see above). In a primary cell model, the cultured chicken neural crest cells [76], overexpression of HuD also induces a dramatic increase in the proportion of those cells exhibiting a neuronal phenotype in terms of molecular markers and neurotrophin dependence. Of relevance, this phenomenon was only observed in cells committed to the neuronal lineage. Ectopically localized misexpression of HuB and HuC in the neural tube of whole mouse embryos determines the appearance of neuronal markers, while their specific knocking down by dominant-negative forms suppresses the differentiation of central nervous system motor neurons [61]. Taken together with the "classical" description of the *Drosophila elav* phenotype, these data clearly establish a primary, necessary role for the nELAV proteins in the process of neuronal commitment, which is possibly finely modulated in different cell types by spatiotemporal patterns of expression, likely induced by specific upstream pathways (see later) and mediated by necessary downstream effector genes. Notably, when tested in the same models, HuR was unable to induce neurite extension, identifying functional segregation between the ubiquitous member of the family and the nELAV proteins despite the several demonstrated shared target mRNAs.

The recent availability of the first nELAV knockout (for HuD [83]) allowed both the confirmation of a role in neuronal differentiation and the extension to a more complex function in the neural commitment programs played by nELAV. No gross morphological abnormalities were found in HuD-null mice, but, physiologically, some specific motor/sensory deficits were described, such as an abnormal hindlimb clasping reflex following suspension by the tail and poor performance in a motor coordination assay, the rotarod test. During embryonic development, the neurite extension of several cranial nerves was only transiently impaired, probably suggesting compensation by other nELAV proteins during the sequential onset of their expression in the developing brain. When embryonic cells were cultured clonally to selectively isolate neural stem cells in the neurosphere assay, HuD-deficient cells showed an increased self-renewal activity and a corresponding reduced ability to generate neurons, while the oligodendrocyte and astrocyte lineages were maintained unaffected. Confirmation of this kinetic alteration in neuronal lineage commitment was found by mitotic labeling in the neurogenic supraventricular zone of adult animals.

Therefore, HuD seems to be able to negatively regulate the proliferation of neural stem/progenitor cells, to promote the exit of neuronally committed progenitor cells from the cell cycle and to differentiate postmitotic neurons. The role of nELAV extended back to the transition between neural stem/progenitor cell proliferation and neuronal differentiation has also been confirmed by a recent study performed in collaboration with us [84]. We show nELAV protein expression to be present both in murine neurosphere-forming stem/progenitor cells *in vitro* and in the proliferating supraventricular zone *in vivo*, where they bind and stabilize the ARE-containing mRNA of the stem cell marker *musashi-1*. Posttranscriptional enhancement of *musashi-1* expression by nELAV could be a way to drive musashi-1 protein activity in the transition from proliferating, symmetrically dividing stem cells to asymmetrical division. This event gives rise to the initially differentiated, still slowly proliferating and neuronally committed progenitor cells.

A recent model of mice overexpressing HuD in the forebrain [85] again showed no evident morphological brain abnormalities and allowed confirmation *in vivo* of the increased stabilization activity of GAP-43 mRNA. GAP-43 protein is expressed during both neuronal development and plasticity [86] and is specifically involved in axonal growth [87] and in the establishment of neural connections.

Considering the possible influence of nELAV proteins in non-developmental plasticity programs, we decided to extensively explore their role in memory, a process in which localized neuronal remodelling activities are certainly required.

Memory: a new key involvement of nELAV proteins

Generally speaking, learning and memory mechanisms are complex and dynamic processes affecting synaptic strength and the morphology, biochemistry and physiology of selected regions of the brain, ultimately resulting in persistent changes in the efficacy of cell-to-cell communication [88]. Subcellular, localized changes in gene expression cannot be easily explained, as detailed before, by a purely transcriptional control, while the implication of post-transcriptional mechanisms can better offer a molecular basis to justify those long-term changes taking place at neuronal specialized microdomains.

With this in mind, we investigated the role of nELAVs in spatial memory, adopting as a paradigm of nELAV activity the well-established effects on the GAP-43 mRNA. We demonstrated that nELAV genes undergo sustained up-regulation in expression in hippocampal pyramidal cells (the hippocampus is the cerebral area mainly implicated in spatial learning) only in rodents that have learned different spatial discrimination paradigms, strongly suggesting the generality of the involvement of nELAV proteins in spatial memory formation. This learning-specific increase in nELAV proteins was localized within cytoplasmic compartments of the somata and proximal dendrites and was particularly associated with the cytoskeleton. The CUGBP2 gene, belonging to the BRUNO/CELF RNA-binding protein family (the closest to the ELAV family among RRM-bearing RBPs), showed no change in expression, further indicating the selective involvement of nELAVs in spatial memory trace formation. In parallel, we also observed enhanced expression of the GAP-43 gene and the absence of these learning-triggered events in a brain area, the retrosplenial cortex, involved in memory path integration. The cause-effect relationship between nELAV proteins and learning was additionally demonstrated by data showing significant impairment in spatial memory performance and concomitant down-regulation of GAP-43 expression in mice treated with an antisense oligonucleotide specifically raised against HuC, strongly indicating the need for activation of downstream pathways uniquely induced by nELAV proteins in learning mammals [89, 90]. These studies established for the first time a role for posttranscriptional control of gene expression in memory and suggested a model in which specific RBPs locally positively control the mRNA stability and translation of an array of target genes whose products could cooperatively establish the biochemical and morphological substrates for memory storage.

As already mentioned, in rodents HuD is strictly expressed in neurons and with maturity persists in great amounts, especially in pyramidal-like neurons of the hippocampus and in layer 5 of the cortex [91], indicating a post-developmental specialized role in these neurons. From our data, we also suggest specific involvement of HuD during the encoding phase of spatial learning [90], based on the finding that in rats, 24 h after but not 1 month after a 5-day training session in the water-maze spatial learning paradigm, a learning-induced increase in HuD expression in the whole hippocampal pyramidal cell layer, from the CA1 to the CA4 region, is evident. The increase in protein content was much more prominent in the cytoskeleton, which, paralleling the same phenomenon reported *in vitro* for HuB [65], suggests that the model by which nELAV may bind to microtubules and to the target mRNAs in mRNP particles, which, in turn, associate with polysomes to form a translationally competent complex connected to the cytoskeletal network, may be extended to learning. Following this model, the increased cytoskeletal association of HuD

could be interpreted as an engagement of the complex containing HuD and the bound mRNAs for translation. The persistence of elevated HuD mRNA levels only in the CA1 hippocampal region 1 month after learning suggests that HuD may also be involved in long-term memory storage and in a more rapid activation of the system in response to a recalling stimulus [90].

The involvement of nELAV in memory establishment has been confirmed in another non-spatial paradigm, rat single-trial contextual fear conditioning [92], which specifically induces increased expression of HuD in the hilar region of the dentate gyrus and the CA3. A recent paper links a classical invertebrate model of associative learning, the marine mollusc *Aplysia*, to the ELAV proteins. In *Aplysia* the C/EBP gene, which is a key determinant of serotonin-induced long-term facilitation – an electrophysiological correlate of learning [93] – bears a 3′-UTR with an ARE that is bound and stabilized by an *Aplysia* ELAV orthologue [94].

Gaining insights within the pathways controlling nELAV function

A substantial body of evidence supports a crucial role for the PKC family (serine/threonine-phosphorylating enzymes ubiquitously expressed and implicated in multiple cellular functions) in invertebrate and mammalian models of memory [95, 96]. ARE-dependent mRNA decay can be influenced by PKC-dependent pathways, since in different cell lines, treatment with phorbol esters (PMA; a PKC agonist that mimics the physiological activator diacylglycerol) and/or calcium ionophores induces the stabilization of ARE-bearing mRNAs [97–103]. In addition, in PC12 cells HuD knock-down by an antisense construct blocks PKC-dependent neurite outgrowth [79].

We proposed that nELAV proteins could represent the final target of a cellular cascade involving PKC, inducing the downstream stabilization of specific mRNAs implicated in memory trace formation. According to this hypothesis, we documented that in a human neuroblast cell model, SH-SY5Y neuroblastoma cells, activation of PKCα, a calcium- and diacylglycerol-dependent PKC, promotes (as early events) nELAV nuclear export, nELAV up-regulation and nELAV cytoskeletal colocalization with PKCα itself, resulting also in nELAV threonine phosphorylation. These changes are associated with both increased stabilization of GAP-43 mRNA and higher GAP-43 protein levels [67]. Considering the very short time of PKC stimulation in this experimental setting (15 min), we are tempted to speculate that, at least in our system, these PKC-induced effects on nELAV are exerted at the translational level, which would be in agreement with the reported polysomal localization of nELAV proteins in the cytoplasm of cultured cells [65, 82] and brain tissue [92] and with their proposed activity of translational enhancement. Furthermore, we also demonstrated that nELAV proteins are up-regulated *in vivo* following treatment with the PKC agonist bryostatin-1 [67], a compound reported to improve cognitive abilities in Alzheimer's disease transgenic mice [104]. All these findings suggest that nELAV proteins recruitment and activation can be induced by a branch of the pathways triggered by PKCα stimulation in a molecular cascade that ultimately can positively influence the expression of genes essential for neuronal programs such as neurogenesis, memory formation and, possibly, nerve regeneration. Moreover, our *in vivo* demonstration represents the experimental evidence that the role of nELAV proteins in these programs can be pharmacologically modulated by acting on PKC. Another mechanism of nELAV regulation was recently described, consisting of methylation of HuD by the coactivator-associated arginine methyltransferase 1 (CARM1). In PC12 cells, CARM1-induced methylation of the Arg236 residue of HuD was convincingly associated with a decrease in the HuD-binding and stabilizing activities of the p21 mRNA, a nELAV target whose elevation drives cell cycle exit and promotes neuronal differentiation. The interference with HuD methylation exerted by CARM1 produces an increased association of HuD with the mRNA of p21, and a facilitation of NGF-induced neurite outgrowth of PC12 cells, but no effect was seen on the other mRNAs (GAP-43, tau and p27) targeted by HuD [72]. Notably, in contrast to the PKC-induced effects, HuD nucleocytoplasmic shuttling is not influenced by CARM1. Therefore, PKCα and CARM1 represent enzymes differentially regulating nELAV proteins in the two activities that seem to be necessary to function, subcellular localization and association with mRNAs, probably in a target-specific way.

nELAV proteins in disease

A postmitotic program in which nELAV could have an important role is axon regeneration after injury. HuD, which in mature neurons, as mentioned, is expressed specifically in pyramidal cells and is also present in the axon growth cone [105], undergoes sustained up-regulation of expression following sciatic nerve crush in the rat dorsal root ganglia sensory neurons [106], which lasts for up to 3 weeks after the injury. This event is accompanied by colocalization of

HuD with ribosomal RNA and increased expression of GAP-43, thus providing convincing correlative evidence for the functional involvement of the best characterized of the nELAV proteins in the nerve regeneration process.

Evidence of direct involvement of nELAV proteins in disease comes, as mentioned, from their initial description as autoantigens in paraneoplastic sensory neuropathies and encephalomyelitis, syndromes associated mostly (90 % of cases) with a specific tumor type, SCLC (for a review see [107]). Since the presence of these autoantibodies correlates with a more indolent tumor growth, nELAV proteins could in principle be exploited to develop cancer vaccines. Immunization of mice, with delivery *via* a plasmid expressing a secreted form of HuD, induced a specific anti-HuD response, and indeed HuD-expressing neuroblastoma cells implanted subcutaneously as xenografts in these mice (given the lack of graftable SCLC cells) underwent significantly slower growth than in controls [108]. These results are encouraging for the development of nELAV-based tumor vaccination strategies.

HuD could also be involved in the molecular pathogenesis of neuroblastoma, the most common childhood tumor deriving from a defect in the developmental program of sympathetic ganglia. HuD levels positively correlate with a clinically favorable outcome of the disease [109], while loss of one HuD allele due to the deletion of a large portion of the short arm of chromosome 1, a common karyotypic alteration found in a subset of neuroblastomas, is associated with another genetic hallmark of the disease, amplification of the MYCN gene [110]. Since MYCN mRNA is a demonstrated HuD target [111, 112], and down-regulation of HuD *in vitro* gives rise to a marked increase in the events of MYCN amplification, a mechanistic relationship can be suggested between the two genomic alterations, which are characteristic of high-risk patient groups. An insufficient level of HuD expression caused by loss of one HuD allele in a neural stem cell clone committed to differentiation in the sympathetic nervous system lineage could be a cause of the unscheduled "locking" of these neuroblasts in the stem cell state, predisposing them to neuroblastoma development. In this context it should also be mentioned that overexpression of HuR, the ubiquitous member of the ELAV family, has been suggested to be involved in number of solid tumor types, including colorectal, breast, ovarian and lung cancer, possibly through stabilization and increased translation of the mRNAs of a variety of downstream genes involved in key tumor cell activities (reviewed in [113]).

Given again their established role in neuronal differentiation and plasticity, nELAV proteins could also become interesting candidates for association with neurodegenerative and psychiatric diseases. A first indication for this possibility has come from haplotype analysis of the HuD locus, which demonstrated an association of a haplotype of two single nucleotide polymorphisms with the age-at-onset of Parkinson's disease [114]. Association between these same polymorphisms and susceptibility to Parkinson's disease has been found in a group of Irish case-control samples [115], opening an investigation of a role for HuD in the process leading to this form of neurodegeneration.

Conclusions

What is clear up to now about nELAV proteins is their involvement in different stages of neuronal commitment and final differentiation and their subsequent activity as "plasticity proteins" involved in the post-mitotic neuronal activity of memory encoding. The ability of nELAV proteins to bind and stabilize an array of target mRNAs, possibly in specific neuronal compartments, as well as their cellular localization appear to be crucial for the successful completion of these functions. nELAV proteins are required for basically all stages of the posttranscriptional fate of mRNAs, including mRNA neural splicing, since this activity, previously thought to be restricted to Drosophila *elav*, has now also been demonstrated for mammalian nELAV genes [69]. This heterogeneity of functions could be achieved by the sequential establishment of different nELAV-including mRNP complexes on each mRNA species along its journey in the cell. The contextualization of nELAV in different mRNPs should become a preferred topic for future investigation, stimulated also by the finding that HuD is a component of the cytoplasmic stress granules [116] and of the so-called neuronal granules [117]. Moreover, the ubiquitously expressed ELAV member HuR was recently shown to be necessary for the relief of microRNA-induced translational repression in cytoplasmic processing bodies, establishing an ELAV protein as the first RBP functional modifier of microRNA activity and opening the field of ELAV-microRNA interactions [118]. Two other major points to be convincingly addressed in the future are the identification of the signal transduction pathways leading to nELAV modulation and the characterization on a genomic scale of the array of functional target mRNAs. With the progressive establishment of a systems biology-based perspective on nELAV proteins and with the description of the networks and pathways in which they play a role, it will be possible to shed light on the molecular mechanisms by which they

exert their nucleocytoplasmic shuttling, mRNA stabilization and translation enhancement activities as well as on the mechanistic relationships among these activities. The extent of nELAV involvement in pathological derangements such as the cited paraneoplastic syndromes and neurodegenerative conditions as Parkinson's disease will also become clearer. Hopefully these advancements can soon provide firm evidence of the suitability of nELAV proteins as targets for the pharmacological therapy of conditions related to altered neural tissue homeostasis (principally neural tumors such as neuroblastoma) as well as chronic or acute neurodegeneration.

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