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CUG-BP, Elav-like family (CELF)-mediated alternative splicing regulation in the brain during health and disease

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Abstract

Alternative splicing is an important mechanism for generating transcript and protein diversity. In the brain, alternative splicing is particularly prevalent, and alternative splicing factors are highly enriched. These include the six members of the CUG-BP, Elav-like family (CELF). This review summarizes what is known about the expression of different CELF proteins in the nervous system and the evidence that they are important in neural development and function. The involvement of CELF proteins in the pathogenesis of a number of neurodegenerative disorders, including myotonic dystrophy, spinocerebellar ataxia, fragile X syndrome, spinal muscular atrophy, and spinal and bulbar muscular atrophy is discussed. Finally, the known targets of CELF-mediated alternative splicing regulation in the nervous system and the functional consequences of these splicing events are reviewed. This article is part of a Special Issue entitled “RNA and splicing regulation in neurodegeneration”.

Keywords

alternative splicing; CUG-BP, Elav-like family; brain; neurons; neurological disorders; myotonic dystrophy

Introduction

It is estimated that more than 90% of human genes produce alternatively spliced transcripts, with the majority exhibiting cell type-specific regulation (Pan et al., 2008; Wang et al., 2008). Alternative splicing is particularly prevalent in the brain (de la Grange et al., 2010; Yeo et al., 2004), where alternative splicing of neuronal RNAs has been implicated in the control of axon guidance, synaptogenesis, and excitation (Lee and Irizarry, 2003; Li et al., 2007). Splice site choice is most often the result of combinatorial regulation by multiple factors acting on a transcript (Hertel, 2008; Smith and Valcárcel, 2000). The high degree of alternative splicing in the brain is associated with expression of a larger number of splicing regulators than most other tissues (de la Grange et al., 2010). These splicing regulators include members of the CUG-BP, Elav-like family (CELF), also known as Bruno-like (Brunol) proteins.

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The human CELF family has six members, which can be divided into two subfamilies based on their phylogeny: CELF1-2 and CELF3-6 (Good et al., 2000; Ladd et al., 2001; Ladd et al., 2004). All six family members are expressed in the brain, and all six possess alternative splicing activity (Ladd et al., 2001; Ladd et al., 2004). CELF proteins have been shown to regulate the alternative splicing of transcripts involved in neuronal function, and the dysregulation of CELF-mediated alternative splicing in the brain has been implicated in the pathogenesis of some neurological disorders. It should be noted that like many RNA binding proteins, CELF proteins have been shown to have cytoplasmic roles in addition to regulating alternative splicing in the nucleus, including regulating mRNA adenylation status, stability, and translation in various cell types (Dasgupta and Ladd, 2012). Although alternative splicing regulation is the only function demonstrated for CELF proteins in the brain thus far, the presence of CELF proteins in both the nucleus and cytoplasm in many cell types makes additional cytoplasmic functions likely. Here, the literature regarding CELF proteins in the nervous system is reviewed, with particular emphasis given to the impact of CELF-mediated alternative splicing in the brain on neurological function and disease.

CELF expression in the nervous system

Transcripts for all six human *CELF* genes are strongly expressed in the nervous system. *CELF1* and *CELF2* are broadly expressed in many tissues, including a high level of expression in brain and spinal cord (Choi et al., 1998; Good et al., 2000; Ladd et al., 2001; Ladd et al., 2004). *CELF3* and *CELF5* are detected exclusively in the nervous system, with high expression throughout the brain, but low expression in the medulla oblongata and spinal cord (Ladd et al., 2004; Loria et al., 2003). *CELF3* is also highly expressed in the pituitary gland (Ladd et al., 2004). Although one study found *CELF4* transcripts in many human tissues (Ladd et al., 2004), others detected *CELF4* only in the nervous system (Loria et al., 2003; Meins et al., 2002). Consistent with the reports of more restricted expression, *Celf4* is brain-specific in adult mice (Gabut et al., 2008; Meins et al., 2002; Yang et al., 2007). Like *CELF3* and *CELF5*, *CELF4* expression is high in the brain, but is low in the spinal cord (Gabut et al., 2008; Loria et al., 2003; Meins et al., 2002). *CELF6* is highly expressed throughout the brain, as well as in kidney and testes (Ladd et al., 2004).

CELF expression in the brain is not uniform in humans and rodents. Although *CELF2* is detected in all areas of the brain, it is particularly enriched in the forebrain, including the cerebral cortex, hippocampus, and amygdala (Choi et al., 1998; Naha et al., 2009; Otsuka et al., 2009; Pacini et al., 2005; Zhang et al., 2002). *CELF4* is also detected in all brain regions, but is strongest in cerebral cortex, hippocampus, amygdala, and cerebellum (Gabut et al., 2008; Loria et al., 2003; Meins et al., 2002). *CELF3* and *CELF5* are highly expressed in fore-, mid-, and hindbrain, but are low in the corpus callosum and pons (Ladd et al., 2004; Loria et al., 2003).

All six human *CELF* genes are expressed in fetal as well as adult brain (Ladd and Cooper, 2004; Loria et al., 2003; Meins et al., 2002). Expression of the *CELF* genes in the developing nervous system is highly conserved across species, including rat, mouse, chicken, frog, zebrafish, and worms (Anderson et al., 2004; Brimacombe and Ladd, 2007; Choi et al., 2003; Knecht et al., 1995; Loria et al., 2003; McKee et al., 2005; Naha et al., 2009; Suzuki et al., 2000; Wu et al., 2010). As in the adult, regional differences in expression of the various family members in the developing nervous system have been reported. In the embryonic rodent brain, *Celf2* is expressed in fore-, mid-, and hindbrain, but the highest expression is in the cerebral cortex and hippocampus (Choi et al., 1999; Levers et al., 2002; Naha et al., 2009). In the spinal cord, *Celf2* exhibits a dorsal-ventral gradient of expression with the highest level of expression in neurons around the ventral horn (Anderson et al., 2004). In chick, *CELF1/CELF2* and *CELF4-6* have reciprocal dorsal/

medial and ventral/lateral domains of expression, respectively, in the neuroepithelium of the developing brain and neural tube (Brimacombe and Ladd, 2007). In *Xenopus*, the *celf* genes are also broadly expressed in the developing nervous system, but exhibit family member-specific biases in intensity along the dorsal-ventral and anterior-posterior axes (Knecht et al., 1995; Wu et al., 2010).

CELF proteins in neural development and function

Evidence from both invertebrate and vertebrate species supports the hypothesis that CELF proteins play important roles in neural development and function. The *C. elegans* homolog of the *CELF3-6* subfamily, *unc-75*, is specifically expressed in all neurons and neurosecretory gland cells (Loria et al., 2003). Mutations in *unc-75* cause defects in motor neuron axon sprouting, synaptic transmission, and behavior (Loria et al., 2003). Three of the mammalian *CELF* genes have been knocked out in mice: *Celf1*, *Celf3*, and *Celf4* (Table 1). Thus far, a neurological phenotype has not been reported for either *Celf1* or *Celf3* knockout mice. *Celf1*-null mice have severely impaired fertility and a high rate of perinatal mortality and growth retardation, perhaps due to placental defects (Kress et al., 2007). *Celf3*-null mice exhibit defects in spermatogenesis, but are fertile and apparently healthy (Dev et al., 2007). CELF4 deficiency, however, has been linked with seizures in both mice and humans.

The first loss-of-function model for *Celf4* was the Frequent flier (*Ff*) mouse, in which a transgenic insertion in the *Celf4* gene resulted in haploinsufficiency in *Ff/+* heterozygotes and a *Celf4*-null in *Ff/Ff* homozygotes (Yang et al., 2007). *Ff* mice were so named because they have handling-associated convulsive seizures, often followed by a wild running-bouncing phase of activity and tonic-clonic hindlimb extension (Yang et al., 2007). Brain morphology in *Ff/+* mice is normal, but markedly reduced seizure thresholds were observed. Few homozygous *Ff/Ff* mice survived on the original C57BL/6J background, but on a mixed background homozygotes and some heterozygotes exhibited spike-wave discharges, the hallmark of absence epilepsy. Recently, a second *Celf4*-null mouse model was generated by gene targeting (Wagnon et al., 2011). Like the *Ff* mice, $Celf4^{null}$ mice have a seizure disorder with recurrent limbic and tonic-clonic grand mal-like seizures. Using a tamoxifen-inducible ER-Cre system, Wagnon, et al. showed that deletion of *Celf4* in the germline, adult animal, or specifically in excitatory neurons all induced a convulsive phenotype, whereas deletion specifically in inhibitory neurons did not (Wagnon et al., 2011). A human patient was recently identified with a translocation event that truncated the *CELF4* gene (Halgren et al., 2012). This patient has a complex neurological phenotype, including a history of febrile seizures in childhood. Notably, seizures and other clinical features of this patient (intellectual disability, behavioral disorders, eye problems, and obesity) are shared with patients who have deletions at the 18q12.2 locus, which includes the *CELF4* gene (Halgren et al., 2012). Taken together with the mouse models, this suggests that CELF4 plays an important role in neuronal excitation and perhaps development.

Although loss-of-function models have not yet been reported for CELF2, CELF5, and CELF6, their embryonic and postnatal expression patterns suggest that they, too, have roles in neural development and function. Before the CELF proteins were first described as a family (Good et al., 2000; Ladd et al., 2001), *CELF2* was identified in a screen of genes up-regulated during apoptosis in neuroblastoma cells, where it was called neuroblastoma apoptosis-related RNA binding protein (*NAPOR*) (Choi et al., 1998). It was subsequently observed that *Celf2* expression in the developing nervous system is highest in regions with a high occurrence of apoptosis, prompting speculation that CELF2 controls developmentally regulated programmed cell death (Choi et al., 1999; Naha et al., 2009). Other studies, however, report *Celf2* expression is highest not in proliferating neural precursors (the population in which apoptosis is most prevalent), but rather in differentiating neurons (Choi

et al., 2003; Levers et al., 2002), suggesting a possible developmental role in establishing or maintaining neuronal identity. Consistent with this, there is a dramatic increase in the number of *celf2*-expressing cells in zebrafish *mind bomb* mutants, which exhibit an overproduction of differentiating neurons due to deficits in Notch signaling (Choi et al., 2003).

Changes in CELF2 have been reported in rodent models of brain injury (Table 1). *Celf2* RNA increased, but CELF2 protein decreased, in the hippocampus following global ischemia and reperfusion in mice (Otsuka et al., 2009). The decrease in CELF2 coincided with the degeneration of neurons in the CA1-CA3 region of the hippocampus, where CELF2 is normally highly expressed. Strikingly, in hypothermic mice neither neuronal injury nor loss of CELF2 protein expression was observed in the post-ischemic hippocampal region (Otsuka et al., 2009). In a rat model of fetal alcohol syndrome, *Celf2* expression decreased throughout the fetal brain as well as in the maternal hippocampus following ethanol exposure (Naha et al., 2009). Whether these changes in CELF2 expression are causative (e.g., inducing neuron cell death) or the consequence of brain injury (i.e., resulting from loss of CELF2-expressing neurons) remains unclear. *Celf2* mRNA and protein levels increase in rat hippocampal slices treated with staurosporine, a broad spectrum inhibitor of protein kinases that induces apoptosis (Pacini et al., 2005). Induction of CELF2 preceded caspase-3 activation, and knockdown of CELF2 blocked caspase-3 activation during oxygen-glucose deprivation-induced apoptosis in rat hippocampal cultures (Pacini et al., 2005). It has similarly been reported that in intestinal epithelial cells, CELF2 is up-regulated during radiation injury, and knockdown of CELF2 inhibits radiation-induced apoptosis (Mukhopadhyay et al., 2003a). Together, these data suggest that regulating programmed cell death may be a general role of CELF2.

CELF proteins and neurological disorders

Given that all six human *CELF* genes are expressed in the brain, and the evidence linking multiple members of the CELF family to neural development and function, it is no surprise that dysregulation of CELF proteins and polymorphisms in *CELF* genes have been linked with a number of neurological and neurodegenerative disorders (Table 1).

CELF proteins in the myotonic dystrophy brain

CELF proteins have been implicated in the pathogenesis of several trinucleotide repeat expansion disorders affecting the nervous system. The best-studied example is myotonic dystrophy type 1 (DM1). DM1 is an inherited disorder that affects several systems, including skeletal muscle, heart, and brain (Schoser and Timchenko, 2010). Primarily an adult-onset disorder, neurological symptoms include cognitive impairment, some psychological dysfunction and abnormal personality traits (such as apathy and avoidant personality), and excessive daytime sleepiness (de Leon and Cisneros, 2008; Meola and Sansone, 2007; Schoser and Timchenko, 2010). These symptoms are accompanied by structural abnormalities in the brain including reduced brain volume, increased ventricular space, focal white matter lesions, and cortical atrophy (de Leon and Cisneros, 2008; Schoser and Timchenko, 2010). A congenital form of the disease also exhibits a variable degree of mental retardation and behavioral problems including attention deficit hyperactivity (Meola and Sansone, 2007; Schoser and Timchenko, 2010).

DM1 arises from the expansion of CTG repeats in the 3' untranslated region of the *DMPK* gene (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). The expanded CUG repeat-containing transcripts accumulate in nuclear foci and disrupt the appropriate expression of other genes (Lee and Cooper, 2009; Schoser and Timchenko, 2010). In addition to changes in transcription and translation, the alternative splicing of a set of

transcripts is dysregulated such that fetal variants are aberrantly expressed in adult tissues (Lee and Cooper, 2009; Schoser and Timchenko, 2010). This occurs via perturbation of alternative splicing regulators, including members of the CELF and muscleblind-like (MBNL) families (Lee and Cooper, 2009; Schoser and Timchenko, 2010). MBNL1 binds to and is sequestered in the mutant RNA foci, leading to a loss of MBNL1 activity (Fardaei et al., 2002; Jiang et al., 2004; Mankodi et al., 2001). *Mbnl1*-null mice exhibit many of the features of the disease, but do not recapitulate the entire DM1 phenotype (Kanadia et al., 2003; Suenaga et al., 2012). Although CELF1 was first identified as a CUG repeat-binding protein (Timchenko et al., 1996), unlike MBNL1, CELF1 does not interact with long CUG repeats or co-localize with the mutant *DMPK* RNA in cells (Fardaei et al., 2001; Michalowski et al., 1999). Rather, CELF1 is up-regulated in affected tissues in DM1 patients (Dhaenens et al., 2011; Savkur et al., 2001; Timchenko et al., 2001). The up-regulation of CELF1 has been attributed to protein kinase C (PKC)-mediated phosphorylation, which stabilizes the protein (Kuyumcu-Martinez et al., 2007). In striated muscle, changes in CELF-mediated alternative splicing have been directly linked to disease symptoms (Charlet-B. et al., 2002; Savkur et al., 2001). Over-expression of CELF1 in heart or skeletal muscle in transgenic mice recapitulates many of the alternative splicing defects and clinical features of DM1 in those tissues (Ho et al., 2004; Koshelev et al., 2010; Timchenko et al., 2004; Ward et al., 2010).

Transcripts that are dysregulated in the DM1 brain have been shown to be targets of CELF-mediated alternative splicing regulation, including several exons of the microtubule binding protein tau (*MAPT*) and N-methyl-D-aspartate receptor (*NMDAR1*) exon 5 (Jiang et al., 2004; Leroy et al., 2006a; Leroy et al., 2006b; Sergeant et al., 2001; Vermesch et al., 1996). The degree to which CELF1 is responsible for dysregulation of alternative splicing in the DM1 brain, and to what extent other CELF proteins may be involved, remains unclear. Both CELF1 and CELF2 are up-regulated in the brains of DM1 patients (Dhaenens et al., 2011), and the levels of other CELF proteins have not yet been evaluated. At least one mis-regulated exon, *MAPT* exon 10, responds to over-expression of CELF2, but not over-expression of CELF1 or loss of MBNL1 (Dhaenens et al., 2011), suggesting there are family member-specific contributions.

As many of the mis-regulated alternative splicing events in striated muscle and brain are regulated both by CELF and MBNL proteins (usually in an antagonistic manner), it is now generally accepted that the alternative splicing defects in DM1 result from a combination of elevated CELF activity and loss of MBNL activity (Lee and Cooper, 2009; Schoser and Timchenko, 2010). Notably, different members of the MBNL family also make distinct contributions to DM1 pathogenesis. Like MBNL1, MBNL2 co-localizes with expanded CUG repeat-containing RNA foci (Fardaei et al., 2002; Jiang et al., 2004). While many of the alternative splicing events that are mis-regulated in the DM1 brain are normal in the brains of *Mbnl1*-null mice (Suenaga et al., 2012), *Mbnl2*-null mice recapitulate both splicing defects and neurological symptoms observed in DM1 patients (Charizanis et al., 2012). Conversely, whereas *Mbnl1*-null mice exhibit many of the skeletal muscle features of DM1 patients, *Mbnl2*-null muscle is relatively normal (Charizanis et al., 2012; Kanadia et al., 2003; Lin et al., 2006). Thus it seems likely that different combinations of CELF and MBNL family members are responsible for different aspects of DM1 pathogenesis.

CELF proteins in other trinucleotide repeat expansion disorders

In addition to DM1, a number of neurodegenerative disorders arise from the expression of mutant RNAs containing expanded trinucleotide repeats (Ranum and Cooper, 2006). Among these are different types of spinocerebellar ataxia (SCA), neurodegenerative disorders characterized by progressive motor defects. Like DM1, SCA8 results from the expression of transcripts containing untranslated expanded CUG repeats (Koob et al., 1999). Also an adult

onset disorder, SCA8 is though to follow a similar mechanism to DM1, in which CELF/MBNL-mediated alternative splicing is disrupted in neurons (Daughters et al., 2009; Mutsuddi and Rebay, 2005). MBNL1 has been shown to co-localize with expanded CUG repeat-containing foci in the brain in a SCA8 mouse model, but CELF proteins have not yet been evaluated in this model or in SCA8 patients (Daughters et al., 2009). SCA3, also known as Machado-Joseph disease, results from the expansion of CAG repeats in the coding region of the ataxin-3 protein, resulting in the expression of an expanded polyglutamine repeat-containing protein (Riess et al., 2008). The mutant protein forms intracellular aggregates in the brain, and is associated with neuronal cell loss (Riess et al., 2008). Although its contribution to disease pathogenesis is unclear, CELF2 is down-regulated in the brain in a SCA3 mouse model (Menzies et al., 2010).

Fragile X syndrome is a hereditary form of mental retardation arising from a CGG repeat expansion in the 5' untranslated region of the *FMR1* gene that leads to silencing of the gene and loss of the FMRP protein (Ranum and Cooper, 2006). Over-expression of CELF1 suppresses a neurodegenerative eye phenotype in a transgenic fly model of fragile X syndrome that expresses 90 CGG repeats (Sofola et al., 2007). CELF1 does not bind directly to the CGG repeats, but rather interacts with them by forming a complex with the CGG-binding protein hnRNP A2/B1. The interaction of CELF1 and hnRNP A2/B1 with the CGG repeat-containing RNA and the rescue of the disease phenotype by addition of more CELF1 suggests that sequestration of these factors by the mutant RNA causes a loss of function (Sofola et al., 2007). It should be noted, however, that although hnRNP A2/B1 is also present in the nucleus, it interacts with the CGG-containing RNA primarily in the cytoplasm (Sofola et al., 2007). Thus in this case, it may be cytoplasmic functions of CELF1, not alternative splicing, that are compromised during pathogenesis.

CELF proteins in disorders affecting the peripheral nervous system

In addition to diseases affecting the brain, CELF2 has been implicated in genetic disorders specifically affecting neurons in the peripheral nervous system. Spinal muscular atrophy (SMA) is a neurodegenerative disease in which progressive loss of motor neurons in the spinal cord results in muscular atrophy (Coady and Lorson, 2011). SMA is caused by loss of the survival motor neuron (SMN) protein, which is important in small nuclear RNA/protein complex biogenesis and axonal mRNA transport (Coady and Lorson, 2011). In humans, SMN is encoded by two genes, *SMN1* and *SMN2*, which differ by a single nucleotide that affects the alternative splicing of exon 7 (Coady and Lorson, 2011). SMA patients lack functional copies of *SMN1*, which normally includes exon 7 and produces full-length SMN protein. *SMN2* transcripts predominantly skip exon 7, leading to the production of a truncated SMN protein. Although loss of *SMN2* has no clinical consequences by itself, the copy number of the *SMN2* gene is an important modifier of disease severity when *SMN1* is absent (Coady and Lorson, 2011). CELF2 is up-regulated in motor neurons from an SMA mouse model and in human SMA patients (Anderson et al., 2004). Although the role of CELF2 in SMA pathogenesis is unclear, CELF2 and SMN co-localize in the nucleus in neuronal cell lines, and SMN can be co-immunoprecipitated with CELF2 from mouse brain, suggesting a functional interaction (Anderson et al., 2004).

Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, is an adult-onset neurodegenerative disease with progressive muscle atrophy and weakness that affects primarily males (Katsuno et al., 2010). SBMA is caused by the expansion of a trinucleotide CAG repeat encoding a polyglutamine tract in the androgen receptor (*AR*) gene (La Spada et al., 1991). Although other neuronal tissues are involved, the predominant cells affected are the motor neurons in the anterior horn of the spinal cord and brain stem (Katsuno et al., 2010). Treatment of an SBMA mouse with a virally-expressed microRNA that silences CELF2 ameliorated the disease phenotype (Miyazaki et al., 2012). CELF2 binds to and

stabilizes both wild type and mutant *AR* transcripts (Miyazaki et al., 2012), suggesting that in addition to its nuclear roles in spinal motor neurons CELF2 also has an important cytoplasmic role in regulating transcript turnover during pathogenesis.

Polymorphisms in CELF genes associated with neurological disorders

Genome-side association studies have also linked *CELF* genes with inherited forms of neurological disease. In a study of late-onset Alzheimer's disease, polymorphisms in *CELF2* were identified that are significantly associated with high-risk alleles of *APOE* (Wijsman et al., 2011). In addition to being linked to seizure disorders (Halgren et al., 2012), polymorphisms in the *CELF4* gene are associated with autism spectrum disorders and myopia (Gilling et al., 2008). Further studies are required to determine if changes in CELF protein expression or function underlie or contribute to the risk or severity of these conditions.

Targets of CELF-mediated alternative splicing in the brain

An important step in elucidating the roles and mechanisms of CELF action in the normal and diseased brain is identifying the targets of CELF-mediated regulation. Dozens of endogenous transcripts have been shown to respond to changes in CELF activity in living cells or animals in the heart, skeletal muscle, and liver (Dasgupta and Ladd, 2012), but CELF-regulated alternative splicing events in the brain have been less well studied. As mentioned above, the alternative splicing of transcripts that are dysregulated in the brains of DM1 patients have been shown to respond to changes in CELF protein levels (Dasgupta and Ladd, 2012). Crosslinking immunoprecipitation (CLIP), a method used to identify direct targets through co-purification of a protein from cells with fragments of bound RNA (“CLIP tags”), has only been performed in the brain for one CELF protein, CELF1. CLIP analysis on juvenile mouse brain identified tags for CELF1 in 206 genes (Daughters et al., 2009). Of these, 64% were intronic, consistent with a role in alternative splicing regulation. Thus far, however, only one, *Gabt4*, has been validated. Known targets of CELF-mediated alternative splicing regulation in the brain are summarized in Figure 1.

Gamma-aminobutyric acid transporter 4 (GABT4)

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in mammals (Roth and Draguhn, 2012). GABAergic neurotransmission is terminated by the uptake of GABA by membrane-bound, sodium-dependent transporters (Roth and Draguhn, 2012). A CELF1 CLIP tag was identified in mouse hindbrain that overlaps with the 5’ splice site of exon 7 of *Gabt4* (also known as *Slc6a11*) (Daughters et al., 2009). This region of the transcript is highly conserved between mouse and human. Skipping of *Gabt4* exon 7 leads to the inclusion of a premature termination codon, which is predicted to target the transcript for nonsense mediated decay (NMD). Consistent with this, increased levels of exon 7 inclusion are accompanied by elevated *GABT4* transcript and protein levels in SCA8 patients and in a SCA8 mouse model (Daughters et al., 2009). The increase in GABT4 expression could reduce the amount of GABA at the synapse, explaining a loss of GABAergic inhibition that has been observed in SCA8 mice (Moseley et al., 2006). Expression of an expanded CUG repeat-containing SCA8 RNA or over-expression of CELF1 in a neuroblastoma cell line induced inclusion of exon 7 in endogenous transcripts, as well as an increase in mRNA and protein levels (Daughters et al., 2009). Despite having elevated levels of CELF1, however, *GABT4* exon 7 alternative splicing, transcript and protein levels are normal in the DM1 brain (Daughters et al., 2009). This suggests the regulation of *GABT4* alternative splicing is more complex *in vivo*, likely involving additional factors.

N-methyl-D-aspartate receptor 1 (NMDAR1)

In addition to regulating inhibitory neurotransmission, CELF-mediated alternative splicing has also been implicated in neuronal excitation. NMDAR proteins are ligand-gated ion channels that bind glutamate and promote excitatory synaptic transmission (Zorumski and Izumi, 2012). Glutamate-mediated excitatory signaling is important for synaptic plasticity, learning, and memory. *NMDAR1* transcripts have two alternative exons, 5 and 21, that are regulated by CELF proteins. Exon 5 encodes a 21-amino acid cassette (N1) in the large extracellular amino-terminal domain. When included, this cassette reduces the receptor's affinity for agonists, increases current amplitude, and modulates receptor potentiation (Zukin and Bennett, 1995). *NMDAR1* exon 21 encodes a 37-amino acid cassette (C1) in the carboxy-terminus that contains several PKC phosphorylation sites (Zukin and Bennett, 1995). Inclusion of the C1 cassette directs the NMDAR1 protein to the plasma membrane, mediates receptor clustering via interactions with neurofilament proteins, and modulates receptor signaling (Grabowski and Black, 2001). CELF1 has been shown to bind to an intronic splicing silencer upstream of *NMDAR1* exon 5 in rat brain nuclear extracts (Zhang et al., 1999). CELF2 and CELF4 both promote exon 5 skipping in *NMDAR1* minigenes in transient transfection assays (Han and Cooper, 2005; Zhang et al., 2002). Whereas CELF activity promotes exon 5 skipping, CELF2 binds to an element in intron 21 and induces exon 21 inclusion in rat and human *NMDAR1* minigenes (Han and Cooper, 2005; Zhang et al., 2002). Regional differences in the alternative splicing of endogenous *Nmdar1* correlates with the level of *Celf2* expression in the adult rat brain (Zhang et al., 2002). Skipping of exon 5 and inclusion of exon 21 coincide with high *Celf2* expression in the rat cerebral cortex. Inversely, inclusion of exon 5 and skipping of exon 21 associates with low *Celf2* expression in the cerebellum and spinal cord. Exon 5 inclusion is increased in the brain in DM1 patients (Jiang et al., 2004), however, where CELF1 and CELF2 levels are known to be elevated (Dhaenens et al., 2011), indicating the levels of CELF activity alone are not determinative for *NMDAR1* exon 5 alternative splicing.

Microtubule-associated protein tau (MAPT)

Tau is a microtubule-associated protein encoded by the *MAPT* gene that promotes neurite outgrowth, organizes axonal microtubules, and participates in kinesin-dependent axonal transport (Andreadis, 2012). Hyper-phosphorylated, microtubule-dissociated tau protein is a major component of the neurofibrillary tangles that form in a number of neurodegenerative disorders and dementias, including Alzheimer's disease (Andreadis, 2012). *MAPT* transcripts contain eight alternatively spliced exons that together can generate 30 different tau isoforms (Andreadis, 2005, 2012). Of these, four exons (2, 3, 6, and 10) have been shown to respond to one or more CELF proteins.

Inclusion of exon 3 never occurs without inclusion of exon 2, but exon 2 can be included with or without exon 3 (Andreadis, 2005). Exon 2/3 skipping is induced in endogenous *MAPT* and minigene transcripts in glioblastoma cells by over-expression of CELF2, and to a lesser extent by CELF4 (Leroy et al., 2006a). This is in contrast to experiments in which CELF4 was shown to be a mild activator of exon 2 inclusion when co-expressed with a *MAPT* exon 2 minigene lacking exon 3 in fibroblasts (Li et al., 2003). Over-expression of CELF1 and CELF3 had no effect on exon 2 inclusion using this reporter in transient transfection assays (Li et al., 2003).

Exon 6 alternative splicing involves selection between a canonical 3’ splice site that generates a full-length protein (6c) and two alternative 3’ splice sites (6p and 6d) that each result in a frameshift that produces a tau protein lacking the microtubule domains (Andreadis, 2005). Using an exon 6 minigene, it has been demonstrated that CELF5 and CELF6 mildly increase complete inclusion (6c) or skipping of exon 6 altogether at the

expense of the 6p variant (Leroy et al., 2006b). CELF1-4 have no effect on this minigene (Leroy et al., 2006b).

Exon 10 is perhaps the most-studied *MAPT* alternative exon, as mutations in exon 10 have been linked with a number of neurodegenerative diseases (Andreadis, 2012). Many of these mutations affect the splicing of exon 10 without changing the encoded protein sequence. Exon 10 encodes one of four microtubule-binding domains, and its inclusion increases the affinity of tau for microtubules. CELF3 and CELF4 have both been shown to strongly induce exon inclusion when co-expressed with an exon 10 minigene (Chapple et al., 2007; Wang et al., 2004). In *Celf3*-null mice, *MAPT* exon 10 inclusion is reduced in the testes, but surprisingly is unaffected in the brain (Chapple et al., 2007). This may be due to compensation for loss of CELF3 in the brain, but not testes, by CELF4, other members of the CELF family, or other splicing factors.

All four of the *MAPT* alternative exons regulated by the CELF proteins are also dysregulated in DM1, but the alternative splicing patterns in the DM1 brain are not always consistent with an increase in CELF activity. CELF proteins negatively regulate exons 2 and 3, and indeed inclusion of these exons is decreased in DM1 brain and skeletal muscle (Jiang et al., 2004; Leroy et al., 2006a). In contrast, CELF proteins promote use of the canonical 6c 3' splice site, but in the DM1 brain the 6d variant increases at the expense of 6c (Leroy et al., 2006b). Likewise, CELF activity promotes exon 10 inclusion, but exon 10 skipping increases in the DM1 brain (Jiang et al., 2004). Thus, the contribution of CELF proteins to the dysregulation of tau expression in DM1 or other tauopathies is unclear.

Amyloid beta precursor protein (APP)

APP is a large transmembrane protein that is proteolytically cleaved into a number of peptides, including amyloid beta peptide (Aβ), the major component of extracellular amyloid plaques found in the brains of Alzheimer's disease patients (Di Carlo et al., 2012). Exon 7, which encodes a protease inhibitor domain, and exon 8, which encodes a 12-amino acid cassette on the amino-terminal side of the transmembrane domain, are included in glial cells, but are predominantly skipped in neurons (Tsukahara et al., 1995). Using an intronic splicing enhancer upstream of exon 8 as bait in a yeast three-hybrid system, a CELF homolog (later recognized as CELF2) was identified from a postnatal day 5 rat brain library (Poleev et al., 2000). CELF1 was subsequently shown to promote exon 8 skipping in an *APP* minigene (Poleev et al., 2000). Whereas inclusion of exon 8 was not detected in normal or DM1 adult brains, skipping of exon 7 increases in the DM1 brain (Jiang et al., 2004). The CELF-responsive *APP* minigene used to investigate exon 8 alternative splicing lacks exon 7 (Poleev et al., 2000), however, and a role for CELF proteins in exon 7 alternative splicing has not yet been tested.

Neurofibromin 1 (NF1)

NF1 is a GTPase activating protein (GAP) that negatively regulates Ras signaling by accelerating the conversion of active, GTP-bound Ras to inactive, GDP-bound Ras (Barron and Lou, 2012). By inhibiting the oncogenic activity of Ras, *NF1* is considered a tumor suppressor gene. Loss-of-function mutations in *NF1* result in neurofibromatosis type 1, a hereditary disorder characterized by neurofibromas. The NF1 GTPase activating protein-related domain (GRD) is encoded by several exons (Barron and Lou, 2012). Exon 23a is an alternative exon that lies within the GRD and modulates its activity. *NF1* transcripts lacking exon 23a produce an NF1 isoform with approximately ten times more Ras-GAP activity than an NF1 protein containing this cassette (Barron and Lou, 2012). Exon 23a is predominantly included in most tissues, but skipped in neurons (Barron and Lou, 2012). Skipping of exon 23a correlates with high total levels of CELF protein expression in

neurons, and over-expression of CELF1-5 proteins in non-neuronal cells promotes skipping of exon 23a in endogenous and *NF1* minigene transcripts (Barron et al., 2010). CELF6 has little effect on exon 23a inclusion. Conversely, knockdown of CELF2, or expression of a dominant negative CELF protein, promotes exon 23a inclusion in a neuronal cell line (Barron et al., 2010). CELF1 and CELF2 were both shown to bind to *in vitro* transcribed *NF1* RNA in nuclear extracts, and CELF protein binding blocked the binding of the spliceosomal factor $U2AF^{65}$, suggesting a potential mechanism for CELF-mediated repression of this exon (Barron et al., 2010). In addition to the CELF proteins, *NF1* exon 23a is also known to be negatively regulated by members of the Hu protein family, and positively regulated by T-cell intracellular antigen 1 (TIA-1) and TIA-1-related protein (TIAR) (Barron and Lou, 2012).

Future directions

In this review, we summarized the current literature regarding CELF proteins in the brain, with particular emphasis on the potential roles of CELF proteins in brain function, injury, and disease. A number of transcripts encoding proteins important for neuron function and health have been identified that are subject to CELF-mediated alternative splicing. Nonetheless, it is important to realize that in most cases, clinical symptoms cannot yet be fully explained by known alternative splicing events, and may have other underlying causes. As mentioned at the beginning of this review, in other tissues CELF proteins have been implicated in several aspects of post-transcriptional regulation, including mRNA stability and translation in the cytoplasm. Cytoplasmic functions of CELF proteins have already been implicated in pathogenesis of fragile X syndrome and SBMA (Miyazaki et al., 2012; Sofola et al., 2007). In DM1, changes in CELF-mediated alternative splicing in the brain have been well documented (Jiang et al., 2004; Leroy et al., 2006a; Leroy et al., 2006b; Sergeant et al., 2001; Vermesch et al., 1996), but this does not preclude additional mechanisms. Dysregulation of both CELF-mediated alternative splicing in the nucleus and CELF-mediated translation in the cytoplasm have been implicated in pathogenesis in DM1 skeletal muscle (Schoser and Timchenko, 2010). Likewise, the mechanism by which CELF2 regulates apoptosis in neurons is not understood, but may involve a cytoplasmic activity. In breast and colon cancer cells, CELF2 induces apoptosis by translationally silencing *COX-2* and *MCL1* mRNAs, which encode anti-apoptotic factors (Mukhopadhyay et al., 2003a; Mukhopadhyay et al., 2003b; Subramaniam et al., 2008). CELF1 and CELF2 have both been found predominantly in the nucleus in cultured neurons (Anderson et al., 2004; Jiang et al., 2004). It is possible, however, that CELF protein localization varies depending on specific neuronal subtypes, developmental stage, or pathogenic condition. CELF3 was seen in both the nucleus and cytoplasm when over-expressed in non-neuronal cell lines, while CELF4 and CELF5 were found predominantly in the cytoplasm (Chapple et al., 2007; Wu et al., 2010). The cytoplasmic roles of CELF proteins in the brain will be an important area for future investigations.

The involvement of CELF proteins (whether nuclear or cytoplasmic) in numerous neurodegenerative disorders makes them attractive targets for the development of new therapies. Preliminary studies showing proof-of-concept have already been conducted in cell culture and mouse models of diseases in which CELF activity is pathogenic. MicroRNA-mediated silencing of CELF2 diminished motor impairment, reduced muscular atrophy, and prolonged survival in an SBMA mouse model (Miyazaki et al., 2012). Although not yet tested in the nervous system, CELF proteins have also shown promise as candidates for therapeutic intervention in heart and skeletal muscle models of DM1. Administration of a PKC inhibitor blocked the stabilizing hyperphosphorylation and accumulation of CELF1, restored normal alternative splicing of some transcripts, and improved cardiac function in a heart-specific mouse model of DM1 (Wang et al., 2009). Inhibition of nuclear CELF activity

using a dominant negative CELF protein likewise reduced pathogenic alternative splicing in C2C12 skeletal muscle cells expressing expanded CUG repeat-containing RNA and a skeletal muscle-specific mouse model of DM1 (Berger and Ladd, 2012). It is important to note that in DM1 and other trinucleotide repeat expansion disorders, pathogenic changes in alternative splicing are not due solely to dysregulation of CELF activity, but also other RNA binding proteins such as MBNL1 (Lee and Cooper, 2009; Schoser and Timchenko, 2010). In these cases, therapies targeted specifically against CELF proteins are unlikely to completely alleviate all disease symptoms. Indeed, reduction of CELF activity in the presence of expanded CUG repeat-containing RNA has been shown to rescue the alternative splicing of some, but not all, dysregulated transcripts in DM1 mouse models (Berger and Ladd, 2012; Wang et al., 2009). Nonetheless, as there is currently no cure or treatment for many of these disorders, the development of a therapy that even partially mitigates pathogenesis has the potential to greatly improve clinical outcomes and the quality of life for patients. Moving forward, researchers trying to make the transition from bench to bedside will face several challenges. Issues that will need to be addressed in the development of clinically viable therapies include the delivery, duration, specificity, and side effects of treatments that target CELF proteins. Elucidating the specific contributions of different family members, as well as potential interactions between CELF proteins and other splicing factors expressed in the brain, may also help tailor therapies to target specific aspects of CELF-mediated regulation.

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Abbreviations

CELF	CUG-BP, Elav-like family
DM1	myotonic dystrophy type 1
MBNL	muscleblind-like
SCA	spinocerebellar ataxia
SMA	spinal muscular atrophy
SMN	survival motor neuron
SBMA	spinal and bulbar muscular atrophy
CLIP	crosslinking immunoprecipitation
GABT4	gamma-aminobutyric acid transporter 4
NMDAR1	N-methyl-D-aspartate receptor 1
MAPT	microtubule-associated protein tau
APP	amyloid precursor protein
NF1	neurofibromin 1

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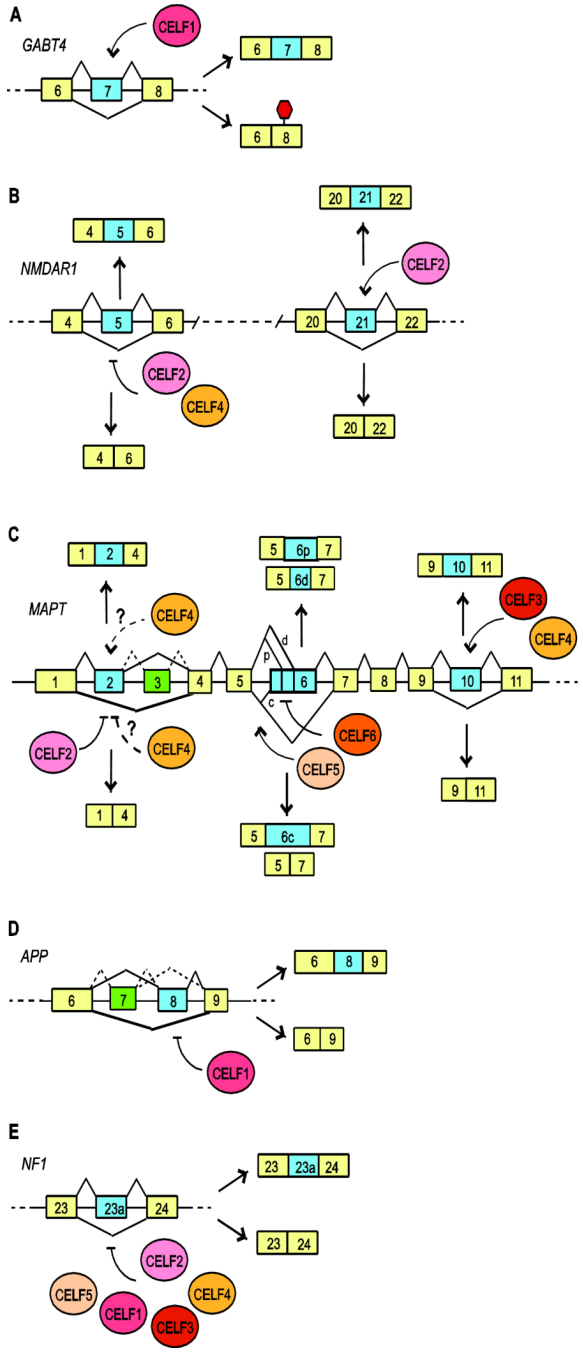

Figure 1. Known targets of CELF-mediated alternative splicing regulation in the brain

A) Skipping of gamma-aminobutyric acid transporter 4 (*Gabt4*) exon 7 leads to the inclusion of a premature termination codon. Over-expression of CELF1 in a neuroblastoma cell line induced inclusion of exon 7 in endogenous transcripts. B) CELF2 and CELF4 promote N-methyl-D-aspartate receptor 1 (*NMDAR1*) exon 5 skipping, whereas CELF2 induces exon 21 inclusion. C) Microtubule-associated protein tau *(MAPT)* exon 2/3 skipping is induced by over-expression of CELF2. CELF4 has been shown be an inhibitor or mild activator of exon 2 inclusion, depending on the cell type used in the experiment; activation of exon 2 inclusion was demonstrated using a minigene lacking exon 3. *MAPT* exon 6 has three alternative 3' splice sites. CELF5 and CELF6 mildly increase complete inclusion (6c) or

skipping of exon 6 altogether at the expense of the 6p variant. CELF3 and CELF4 have been shown to strongly induce *MAPT* exon 10 inclusion. D) CELF1 promotes amyloid beta precursor protein (*APP*) exon 8 skipping. Regulation by CELF1 was tested in the absence of exon 7, which is also alternatively spliced. E) Over-expression of CELF1-5 proteins in non-neuronal cells promotes skipping of Neurofibromin 1 (*NF1*) exon 23a, whereas repression of CELF activity promotes exon 23a inclusion. Exons and introns are not drawn to scale.

Table 1

Loss of function or dysregulation of CELF proteins in the nervous system in animal models and human diseases with neurological symptoms

Family member	Animal model phenotype	Human neurological disease/disorder
CELF1	• Knockout mouse has infertility, perinatal lethal, growth retardation, likely placental defects; no neurological phenotype reported (Kress et al., 2007) • Over-expression rescues neurodegenerative phenotype in fruit fly model of fragile X syndrome (Sofola et al., 2007)	• Up-regulated in DM1 brain (Dhaenens et al., 2011) • Regulates alternative exon that is dysregulated in SCA8 (Daughters et al., 2009)
CELF2	• Down-regulated during brain injury in mouse model of ischemia (Otsuka et al., 2009) and in rat model of fetal alcohol syndrome (Naha et al., 2009) • Down-regulated in brain of SCA3 mouse model (Menzies et al., 2010) • Up-regulated in SMA mouse model (Anderson et al., 2004)	• Up-regulated in DM1 brain (Dhaenens et al., 2011) • Regulates alternative exons that are dysregulated in DM1 brain (Dhaenens et al., 2011; Leroy et al., 2006a; Zhang et al., 2002) • Up-regulated in SMA patients (Anderson et al., 2004) • Polymorphisms associated with high risk alleles for Alzheimer's disease (Wijsman et al., 2011)
CELF3	• Knockout mouse has spermatogenesis defect; no neurological phenotype reported (Dev et al., 2007)	• None described
CELF4	• Knockout mice have complex seizure disorders (Wagnon et al., 2011; Yang et al., 2007)	• Loss of function associated with seizure disorders and other neurological symptoms (Halgren et al., 2012) • Polymorphisms associated with autism spectrum disorders (Gilling et al., 2008)
CELF5	• None described	• Regulates alternative exon that is dysregulated in DM1 (Leroy et al., 2006b)
CELF6	• None described	• Regulates alternative exon that is dysregulated in DM1 (Leroy et al., 2006b)