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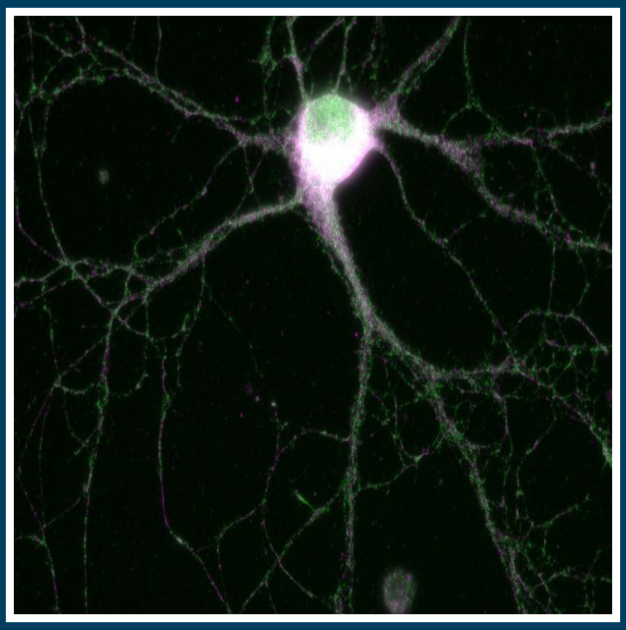

mRNA LIFE CYCLE IN BRAIN FUNCTION AND MALFUNCTION

Topic Editors
Jernej Ule, Peter Scheiffele
and Michael A. Kiebler

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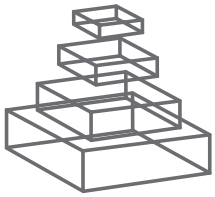

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mRNA LIFE CYCLE IN BRAIN FUNCTION AND MALFUNCTION

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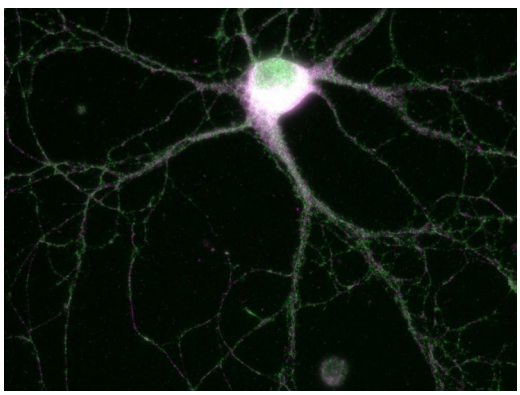

Double-immunofluorescence stainings from a 9 day in vitro old primary hippocampal neuron using anti-rabbit Barentsz (labeled in green with Alexa-Fluor 488) and anti-mouse monoclonal Staufen2 (labeled in red with Cy3) antibodies. The picture shows the localization of two distinct RNA-binding proteins, Barentsz and Staufen2, in the cell body as well as in dendrites. For experimental details, please see Fritzsche, Karra et al., Cell Reports, in press. Picture from Sabine Thomas and Michael A Kiebler, Ludwig-Maximilians-University, Munich, Germany.

Neurons and glia are highly polarized cells that achieve their specialized functions largely due to their high complexity of regulating gene expression. In particular, these cells critically contribute to every step in the regulation of the mRNA life cycle in a unique manner. For example, the brain has the highest levels of alternative splicing and RNA editing. Many of the alternatively spliced isoforms of a given protein produced in brain are required for neuronal development, synaptic transmission and plasticity. However, the effects of RNA processing on the functions of resulting protein isoforms are still poorly understood. Further layers of regulation are added by control of mRNA transport, translation or degradation. Most importantly, these regulatory processes can take place within neuronal axons and dendrites, due to the unique ability of neurons to transport mRNAs far from the cell body. Regulation of mRNA translation in specific neuronal compartments is crucial for neuronal functions that take place far from cell body, such as axon guidance and synaptic plasticity.

The mRNA life cycle is orchestrated by a complex interplay of RNA binding proteins (RBPs) and non-coding RNAs, which can interact at many different positions on pre-mRNAs and mRNAs, and often form larger particles that mediate mRNA transport or degradation.

Mutations in several RBPs, toxic RNA repeats, or other defects in post-transcriptional regulation contribute to a variety of neurologic diseases. In most cases, the mechanisms leading to the neurologic defects are poorly understood. To understand the full complexity of post-transcriptional regulation, and how it can go awry in the brain, new experimental and computational approaches are being developed. In this volume, we have asked leaders in the field to overview the literature of published research, present results of their current research, and provide their thoughts on the new developments and future directions of the field.

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EDITORIAL
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What, where, and when: the importance of post-transcriptional regulation in the brain

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Keywords: alternative splicing, RNA editing, RNA methylation, translation, mRNA trafficking, axon guidance, synaptic plasticity, neurological disease

INTRODUCTION

Until recently, RNA metabolism has been considered a purely academic topic that kept a small group of molecular biologists busy. Thanks to many emerging techniques—mainly, but not exclusively—systems approaches that employ high-throughput sequencing, such as UV cross-linking and immunoprecipitation (CLIP), ribosome profiling and RNAseq, we gained major new insights into the importance of RNA metabolism for brain function, as well as malfunction (Darnell, 2013). In that context, the brain presents a particularly fascinating diversity of post-transcriptional gene regulation through many new and recently discovered mechanisms. Neurons exhibit remarkably rich molecular repertoires that match their complex morphologies and functions. RNA is often localized to various subcompartments in order to exert specific local functions. Moreover, dynamic changes in RNA processing and turnover provide powerful mechanisms for neuronal plasticity. The central importance of these mechanisms is highlighted by the severe neurological disorders associated with defects in post-transcriptional processing functions in the brain.

EMERGING TECHNIQUES

The last decade marked an expansion of genome-wide experimental and computational techniques that provide unprecedented insights into the mechanisms and physiological relevance of post-transcriptional regulation in the brain, and how it can go awry in disease (Darnell, 2013; Modic et al., 2013). The new methods enable to study protein-RNA and miRNA-RNA interactions with high specificity and positional resolution (Gascon and Gao, 2012; Konig et al., 2012). Moreover, the development of RNAseq, ribosome profiling and related emerging functional genomic and computational methods, has enabled global studies of alternative splicing, RNA editing, methylation, stability and translation (Ingolia et al., 2009; Norris and Calarco, 2012; Tariq and Jantsch, 2012; Trivedi and Deth, 2012).

MOLECULAR DIVERSITY FOR DIVERSE CELLULAR FUNCTIONS

A remarkable feature of neuronal and glial cells is their morphological and functional diversity. Such specialized functions are achieved through highly complex gene expression programs. The brain exhibits the highest levels of alternative splicing and RNA editing (Norris and Calarco, 2012; Tariq and Jantsch, 2012). Even though functions of individual alternative protein isoforms are understood for only a few cases, it is clear that intact regulation of alternatively splicing is required for the development of neurons or glia, and for the formation of functional synapses (Norris and Calarco, 2012). Moreover, A to I editing often alters the critical properties of neuronal receptors and channels, and is thereby required for synaptic transmission (Tariq and Jantsch, 2012). Thus, pre-mRNA processing and editing greatly enhances proteome diversity, and thus the functional complexity of the nervous system.

mRNA TRANSPORT AND LOCAL TRANSLATION

In addition to molecular diversity, post-transcriptional mechanisms also are key contributors to spatial-temporal control of neuronal mRNA functions. In neuronal precursor cells, RNA localization is required for asymmetric divisions of neuronal progenitor cells. Knockdown of certain key RBP regulators of mRNA localization causes premature differentiation of radial glial cells into neurons (Kusek et al., 2012; Vessey et al., 2012). Later on, control of mRNA translation or degradation can take place within neuronal axons and dendrites, due to the unique ability of neurons to transport mRNAs far from the cell body. Local translation of mRNAs within axonal growth cones or within dendritic spines enables neurons to remodel these critical structures. Thus, the local proteome and function of neuronal sub-compartments can be acutely and selectively modified in response to specific signals. This enables rapid and selective control of processes such as axon guidance and synaptic plasticity at sites that are remote from the cell body.

A recent study identified as many as 2550 transcripts that are transported to either axons or dendrites (Cajigas et al., 2012). mRNA transport and local translation depend on cis-acting regulatory elements that are recognized by RBPs, forming a ribonucleoprotein complex (RNP) that directs mRNA transport and translation (Doyle and Kiebler, 2011). In navigating axons, RNPs control the choice of mRNAs that are translated in response to extrinsic cues, which in turn determines the direction of axon growth (Hornberg and Holt, 2013). Similarly, mRNA transport to neuronal dendrites is controlled by specific cis-acting elements. Here, Tongiorgi and colleagues

(Baj et al., 2013) present a hypothesis suggesting how a common single nucleotide polymorphism in the human brain-derived neurotrophic factor gene (*BDNF*) gene may affect the dendritic transport of BDNF mRNA, and thereby cause deficits in memory. This remains an area of intense research, with a recent study suggesting an anterograde, rather than retrograde mode of BDNF action (Dieni et al., 2012).

SYNAPTIC PLASTICITY

mRNAs localized to dendrites have a key function in synaptic plasticity. In response to synaptic stimuli, local control of mRNA translation near synapses is required to facilitate long-lasting forms of synaptic plasticity, the cellular basis for learning, and memory formation (Kapeli and Yeo, 2012; Fernandez et al., 2013). This does not only involve local control of mRNA polyadenylation and translation, but also protein degradation via the proteasome (Cajigas et al., 2010; Udagawa et al., 2012). Moreover, all aspects of mRNA regulation, from nuclear RNA editing to local control of mRNA translation, play crucial roles in the alteration of the synaptic proteome that is required to maintain synaptic homeostasis and prevent pathological recurrent network excitation (Turrigiano, 2011; Penn et al., 2013). In this context, a new hypothesis is being proposed for the methyltransferase PRMT, which is regulated by redox status and can methylate the RGG domain of RBPs such as FUS, which could modulate regulatory functions of RNPs and thereby affect synaptic function (Trivedi and Deth, 2012).

ALTERED RNA METABOLISM IN DISEASE

Mutations in RBPs, toxic RNA repeats, or other defects in post-transcriptional regulation contribute to a variety of neurologic diseases, especially motor neuron diseases (Ramaswami et al., 2013). This is corroborated by changes in pre-mRNA processing or RNA editing of important neuronal receptors or channels, which were observed neurodegenerative and psychiatric disorders, as well as epilepsy (Tariq and Jantsch, 2012). Moreover, it was recently proposed that specific RBPs or miRNAs might be secreted from stressed motoneurons to stimulate defence mechanisms in astrocytes or endothelial cells (Aparicio-Erriu and Prehn, 2012; Gascon and Gao, 2012). This indicates that perturbed homeostasis of RBPs or miRNAs, and the consequent changes in RNA metabolism may play a central role in neurodegenerative processes (Aparicio-Erriu and Prehn, 2012; Gascon and Gao, 2012; Kapeli and Yeo, 2012).

Taken together, we feel that the present collection of reviews on the mRNA life cycle in normal brain function and malfunction provides a timely update by leading researchers to reflect recent developments in key technologies, and summarizes the current understanding and future directions for the studies of mRNA metabolism in the brain.

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Emerging roles of alternative pre-mRNA splicing regulation in neuronal development and function

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Alternative pre-mRNA splicing has the potential to greatly diversify the repertoire of transcripts in multicellular organisms. Increasing evidence suggests that this expansive layer of gene regulation plays a particularly important role in the development and function of the nervous system, one of the most complex organ systems found in nature. In this review, we highlight recent studies that continue to emphasize the influence and contribution of alternative splicing regulation to various aspects of neuronal development in addition to its role in the mature nervous system.

Keywords: alternative splicing, genomics, nervous system, RNA processing, gene regulation

INTRODUCTION

The nervous system is a uniquely complex structure, composed of diverse classes of neuronal cells found in close proximity to one another. These many classes of neurons must form precise synaptic connections with other neurons that can often be separated by large distances, establishing the circuitry governing the ability to sense, interpret, and appropriately respond to stimuli. It is of great interest to understand how the developmental steps of neurogenesis, migration, pathfinding, synapse formation, and maintenance are controlled with such precision. Defects in any of these processes in humans lead to numerous cognitive and motor disabilities (Mitchell, 2011). It is also of importance to better grasp the mechanisms governing the modulation of synaptic strength and plasticity in the mature nervous system, which plays a key role in sensory adaptation, learning and memory, and other behaviors.

Diverse spatio-temporal gene regulatory mechanisms have proven vital for the control of patterning in the nervous system, including regulation of mRNA synthesis by transcription factors (West and Greenberg, 2011), the dynamic alteration of chromatin states through modifying enzymes (Ooi and Wood, 2008; Yoo et al., 2009), turnover or translational repression by microRNAs (Meza-Sosa et al., 2012), RNA decay pathways, and regulation by post-translational modifications (Fukushima et al., 2009). Alternative splicing, the process in which multiple mRNA isoforms can be generated through differential splice site selection in precursor transcripts, is an additional important mechanism of gene regulation, contributing to transcriptomic and proteomic diversity in metazoans (Nilsen and Graveley, 2010). Greater than 95% of human genes undergo alternative splicing (Pan et al., 2008; Wang et al., 2008), and disruption of splicing contributes to a number of genetic diseases (Chakarova et al., 2002; Briese et al., 2005; Winkler et al., 2005; Mordes et al., 2006; Wang and Cooper, 2007). The nervous system exhibits particularly high levels of alternative splicing (Yeo et al., 2004; Grosso et al., 2008). Indeed, a recent large scale study of human tissues found that the cerebellum exhibited the highest degree of alternative splicing among 11 tested tissue samples, containing 50% more differentially expressed alternative exons than the next highest tissue (the testes; de la Grange et al., 2010). These results suggest that regulated splicing can serve as a potential mechanism for generating the high levels of molecular and cellular diversity observed in the nervous system (Lipscombe, 2005; Li et al., 2007).

The fidelity and efficiency of splicing depends on the action of five small nuclear RNAs (snRNAs) functioning as components of ribonucleoprotein particles called snRNPs, in conjunction with up to hundreds of additional auxiliary proteins (Wahl et al., 2009). This elaborate and highly dynamic complex known as the spliceosome regulates splicing with single nucleotide precision (Will and Luhrmann, 2011). Decades of research have begun to elucidate the "splicing code," the complete set of *cis*-acting RNA features (for example, sequence motifs, exon and intron length, secondary structure) and *trans*-acting splicing factors that dictate where and in what context differential splicing will occur in transcripts (Wang and Burge, 2008; Barash et al., 2010). The combination of detailed biochemical experiments with more recent genome-wide approaches and computational analyses have revealed diverse mechanisms by which alternative splicing can occur, and have been described in greater detail in several recent excellent reviews (Blencowe, 2006; Chen and Manley, 2009; Licatalosi and Darnell, 2010; Han et al., 2011; McManus and Graveley, 2011; Irimia and Blencowe, 2012).

In this focused review, we examine the role of alternative splicing during neuronal development and in response to neuronal activity. Although a large number of alternative isoforms derived from important neuronal genes have been reported in the literature, special emphasis is given here to recent findings illuminating the role of specific *trans*-acting splicing factors and select target splicing events they regulate in the biogenesis and function of neurons.

ALTERNATIVE SPLICING IN NEURONAL DEVELOPMENT AND MAINTENANCE

Alternative splicing plays an important role in generating diversity and specificity in the developing and mature nervous system. In this section we describe several recent examples demonstrating the role of alternative splicing in neuronal differentiation, migration and pathfinding, and synapse formation and function (**Figure 1**).

THE RS DOMAIN-CONTAINING SPLICING FACTOR nSR100/SRRM4 CONTROLS NEUROGENESIS

The neural-specific SR-related protein of 100 kDa (nSR100/SRRM4) was identified in a computational and expression based survey of genes encoding RS domain-containing proteins (Calarco et al., 2009). In a similar manner as other members of the SR and SR-related family of proteins, nSR100 was found to regulate alternative splicing decisions. Microarray profiling experiments in mouse neuroblastoma cells and tissues revealed that depletion of nSR100 results in increased skipping of alternatively spliced exons normally included in the brain, suggesting that it mainly acts to promote the inclusion of alternative exons. A significant fraction of genes containing these regulated exons are known to be important for regulating neuronal differentiation, raising the possibility that specific splice variants modulated by nSR100 could contribute to this process. Consistent with this notion, nSR100 was found to play a critical role in neuronal differentiation and neurite extension *in vitro* as well as nervous system and sensory organ development in zebrafish embryos *in vivo* (Calarco et al., 2009). The specific mechanism by which nSR100 regulates alternative splicing in the nervous system remains to be elucidated. However, nSR100 was shown to be required for proper inclusion of a target neural-specific exon using *in vitro* splicing extracts, indicating that it plays a direct biochemical role in promoting exon inclusion (Calarco et al., 2009). It was also found that the introns flanking alternative exons regulated by nSR100 are enriched in pyrimidine rich motifs (Calarco et al., 2009). The majority of these motifs are likely recognized by the polypyrimidine tract binding protein PTBP1 and its tissue-specific paralog PTBP2 (also called neural- or brain-enriched n/brPTB). Consistent with a link between these regulators in modulating neural-specific alternative splicing, many nSR100-dependent alternative splicing events are also regulated by PTBP1 and PTBP2.

More recently, it was discovered that nSR100 indirectly controls the steady-state abundance of a network of transcripts in neuronal cells distinct from the population of mRNAs that it regulates at the level of splicing. Depletion of nSR100 in mouse neuroblastoma cells led to decreased levels of hundreds of transcripts, and a subset of these changes were shown to be dependent on repressor element 1 silencing transcription factor (REST, also known as NRSF), a transcriptional repressor of genes involved in neurogenesis (Raj et al., 2011). In neuronal cells, REST transcripts include an additional exon that results in the introduction of a stop codon and production of a truncated protein lacking domains required for its repressive activity. Raj et al. (2011) found that nSR100 plays a critical role in promoting the inclusion of this alternative exon, suggesting that the expression of nSR100 in neurons contributes to the reduced activity of REST upon differentiation to the neural lineage. Importantly however, REST was also found to directly repress nSR100 transcription in non-neuronal cells and thus indirectly inhibit neural-specific alternative splicing. This negative feedback loop between two gene regulatory levels was found to be important for developmental outcomes in the nervous system, as inhibiting nSR100 expression in mouse brain disrupted cortical neurogenesis, preventing neuronal precursor cells from committing to a neuronal fate (Raj et al., 2011). These results are in agreement with

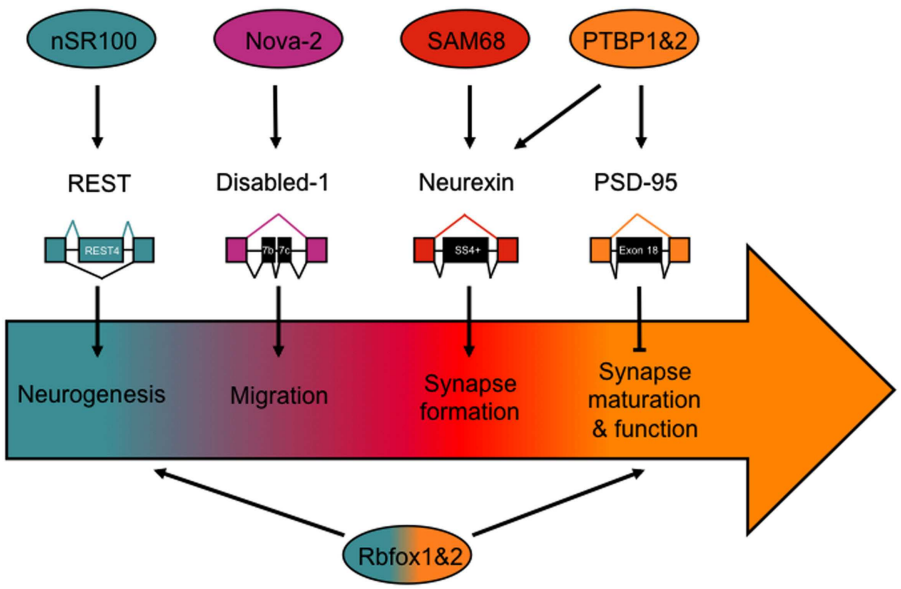

FIGURE 1 | Notable alternative splicing events important for nervous system development, and the factors implicated in regulating their splicing patterns. Developmental stages are represented by the arrow, beginning with neurogenesis, and ending with synapse maturation and function. Splicing factors and target transcripts are correspondingly color coded to the developmental stage in which they are known to be important.

previous studies showing that loss of REST de-represses neuronal transcripts in non-neuronal tissues, while REST overexpression inhibits the expression of transcripts in neuronal tissues, which in one study was shown to result in axon pathfinding errors in chick embryos (Chen et al., 1998; Paquette et al., 2000).

THE SPLICING FACTOR NOVA-2 CONTROLS NEURONAL MIGRATION

The Nova RNA binding proteins were among the first tissue-specific regulators of alternative splicing to be identified. Initial studies in knockout mice indicated that Nova-1 plays a critical role in the maintenance of brainstem and spinal cord neurons, likely through the regulation of alternative splicing in these cells (Jensen et al., 2000). Subsequent studies utilizing splicing-sensitive microarray profiling in mice lacking Nova proteins identified a network of brain-specific splicing events coordinated by these factors (Ule et al., 2005). Importantly, transcripts with Nova-regulated exons encoded proteins that were significantly enriched in functions associated with the synapse (Ule et al., 2005). Integrating this alternative splicing regulatory network with genome-wide cross-linking and immunoprecipitation (CLIP; Licatalosi et al., 2008; Zhang et al., 2010) studies identifying *in vivo* Nova binding sites in the transcriptome has provided two key advances. First, these datasets have enabled the formulation of "RNA maps" correlating cognate Nova YCAY *cis*-element locations in pre-mRNA transcripts with effects on splicing regulation, leading to mechanistic insights into how the Nova proteins modulate alternative splicing (Ule et al., 2006). Second, these networks identify target isoforms and pathways that likely contribute to aspects of neuronal physiology. In agreement with this latter point, subsequent phenotypic exploration of the role of target isoforms in Nova knockout mice has identified a role for Nova in modulating slow synaptic inhibition and in neuromuscular junction formation (Huang et al., 2005; Ule et al., 2005; Ruggiu et al., 2009).

Recently, Nova-2 was demonstrated to be important for the migration of late-born cortical and Purkinje neurons in mice (Yano et al., 2010). These migration defects were due largely to the aberrant splicing of transcripts from a single gene, the Reelin signaling adaptor Disabled-1. Reelin signaling is an important pathway regulating neuronal migration in the cortex, cerebellum, and hippocampus. Binding of the Reelin ligand to its receptors ApoER2 and Vldlr leads to phosphorylation of Disabled-1, which recruits various adaptor proteins that mediate cytoskeletal rearrangements and appropriate neuronal migration and positioning (Bar et al., 2000; Ayala et al., 2007). Disabled-1 is thus a critical effector protein in the Reelin signaling pathway.

Nova-2 suppresses the inclusion of Disabled-1 exons 7b and 7c (7bc+), which encode an additional 33 amino acid peptide of unknown function, the inclusion of which could produce a protein isoform with dominant negative activity. Expression of Disabled-1 (7bc+) in the E14.5 mouse cortex was sufficient to cause migration defects similar to the Nova-2 knockout, while Disabled-1 (7bc−) substantially rescued the Nova-2 knockout migration defects (Yano et al., 2010). Thus, Nova-2 controls the sensitivity of neurons to the Reelin signaling pathway, presumably by affecting the balance of Disabled-1 (7bc−), which is activated by the Reelin signaling pathway, and Disabled-1 (7bc+), which, through a currently unknown mechanism, impairs the Reelin signaling pathway. The alternative splicing of Disabled-1 is developmentally regulated, with Nova2-dependent suppression of Disabled-1 (7bc+) highest during the critical window of migration for late-born neurons (E14.5–E16.5), suggesting Nova2-mediated alternative splicing of Disabled-1 as a mechanism to control neuronal sensitivity to Reelin signaling throughout development (Yano et al., 2010).

PTBP1 AND PTBP2 REGULATE SYNAPSE FORMATION AND MAINTENANCE

PTBP1 and PTBP2 display mutually exclusive patterns of expression in the developing brain, with PTBP1 found in glial and non-neuronal cells, and PTBP2 in neurons (Boutz et al., 2007). This non-overlapping pattern of expression is established by an elegant cross-regulatory network where PTBP1 normally suppresses the inclusion of an exon in PTBP2 transcripts, leading to a non-functional isoform degraded by the nonsense-mediated mRNA decay (NMD) pathway (Boutz et al., 2007; Spellman et al., 2007). In neurons however, PTBP1 is silenced by miR-124, a neuron-specific microRNA, leading to the de-repression of PTBP2 (Makeyev et al., 2007). The consequences of modulating the relative levels of PTBP1 and PTBP2 in neuronal cells have been initially revealed through splicing-sensitive microarray profiling of mouse neuroblastoma cells depleted of these factors (Boutz et al., 2007). Analogous to the Nova-regulated alternative splicing network, PTBP1- and PTBP2-dependent alternative splicing events are frequently found in transcripts expressed from genes with known roles in neuronal differentiation and physiology.

A role for PTBP1 and PTBP2 in regulating the expression of PSD-95, an important scaffolding protein essential for synaptic maturation and plasticity of excitatory neurons, has recently been identified. Overexpression of PTBP1 and PTBP2 in cultured hippocampal neurons was shown to repress synaptic activity, dendritic spine formation, and reduce levels of PSD-95 transcripts (Zheng et al., 2012). This reduced mRNA abundance is caused by PTBP1 and PTBP2 binding to a pyrimidine rich *cis*-element upstream of PSD-95 exon 18, leading to increased exon skipping and the production of a transcript containing a premature termination codon that is targeted for degradation by the NMD pathway. Importantly, the increased expression of PSD-95 in developing neurons in the cortex was found to correlate with three distinct phases of PTBP1 and PTBP2 expression. At the neural progenitor stage, when PTBP1 levels are high, PSD-95 expression is at its lowest. In embryonic neurons, the weaker repressor PTBP2 is more highly expressed while PTBP1 expression is lost, leading to intermediate levels of PSD-95. Finally, in post-natal cortical neurons, PTBP2 is no longer expressed, allowing PSD-95 abundance to reach its highest levels (Zheng et al., 2012). These results indicate that the sequential changes in relative expression of PTBP1 and PTBP2 can allow for distinct splicing regulatory programs to be established at different stages in neuronal maturation.

ALTERNATIVE SPLICING OF NEUREXINS AND NEUROLIGINS IN SYNAPSE FORMATION AND MAINTENANCE

A number of studies in recent years have demonstrated the importance of alternative splicing of neurexins and their binding partners neuroligins in establishing and/or maintaining synapses

(Boucard et al., 2005; Chih et al., 2006; Graf et al., 2006). Neurexins and neuroligins function as adhesion proteins across the synaptic cleft, and increasing evidence suggest that these factors are central organizing proteins at both glutamatergic and GABAergic synapses in the brain (Craig and Kang, 2007). The Neurexin gene loci are highly complex, with the capacity of generating thousands of potential transcript variants pre-synaptically in mammals through the use of alternative promoters and alternative splicing (Boucard et al., 2005; Chih et al., 2006). The post-synaptic neuroligins also undergo alternative splicing, but to a lesser degree. Several key variants from each of these factors have been functionally characterized in cell culture, leading to the proposal of a trans-synaptic adhesive splicing "code" in which particular neurexin isoforms have specific affinity to particular neuroligin isoforms, and the isoforms utilized in neurons affect the functional properties of the synapse (Boucard et al., 2005; Chih et al., 2006; Graf et al., 2006). For instance, the addition of an alternative exon (B+) to neuroligin 1 decreased its ability to recruit GABAergic synaptic components but increased its glutamatergic synaptic recruitment. This change in activity was due to the reduced binding of neuroligin 1 (B+) isoforms to neurexin variants with splice site #4 selected (SS4+; Chih et al., 2006). Neuroligin (B+) bound neurexin (SS4−) strongly but exhibited only weak binding with neurexin (SS4+), while neuroligin (B−) had strong interaction with both neurexin (SS4+) and (SS4−) isoforms. These results point toward a role of neurexin and neuroligin alternative splicing in shaping the strength and class of synapses (Chih et al., 2006).

Several factors involved in the splicing of neurexin transcripts have been identified. The first was PTBP2, which was demonstrated to suppress selection of SS4 in neurexin-2α (Resnick et al., 2008). More recently it has been demonstrated that the KH domain RNA binding protein SAM68 regulates selection of the SS4 in neurexin 1 and neurexin 3, and that it does so in a neuronal activity-dependent fashion (further discussed below; Iijima et al., 2011).

Rbfox-1/A2BP1 AND Rbfox-2/Rbm9 PLAY A ROLE IN NEURONAL DEVELOPMENT AND FUNCTION

Members of the Rbfox family of RNA binding proteins display enriched or highly specific expression patterns in the neuromuscular system, and regulate alternative splicing decisions through interactions with the highly conserved *cis*-element (U)GCAUG (Underwood et al., 2005; Zhang et al., 2008; Zhou and Lou, 2008; Sun et al., 2012). Focused biochemical studies and several genome-wide analyses have demonstrated that the Rbfox proteins can function as activators or repressors of splicing, depending on the location of (U)GCAUG elements in target pre-mRNA transcripts (Jin et al., 2003; Zhang et al., 2008; Zhou and Lou, 2008; Sun et al., 2012). Together, these studies have begun to shed light on the relevant networks of transcripts modulated by these factors, although the role of the Rbfox proteins in nervous system development and function *in vivo* has remained somewhat unclear.

Two recent studies from the Black laboratory using Rbfox knockout mice have provided further insight toward the functional importance of these proteins in the nervous system (Gehman et al., 2011, 2012). Deletion of *Rbfox1* specifically in the nervous system of transgenic mice did not seem to have any effects on neuronal development or morphology in the brain. However, loss of *Rbfox1* did lead to spontaneous seizures, increased sensitivity to induced seizures, and increased excitability in neurons of the dentate gyrus. Integration of splicing-sensitive microarray profiling and CLIP-Seq datasets identified alternative splicing events differentially regulated in the brains of *Rbfox1*$^{-/-}$ mice, several of which were linked to genes known to be associated with epilepsy and others with roles in synaptic function (Gehman et al., 2011).

In contrast to loss of Rbfox1 in the nervous system, deletion of the gene encoding Rbfox2 in the nervous system led to pronounced defects in cerebellar development. *Rbfox2*$^{-/-}$ animals have much smaller cerebella than wild-type littermates, defects in Purkinje cell migration and dendritic arborization, and reduction in the migration and number of granule cells (Gehman et al., 2012). Again, splicing-sensitive microarray profiling experiments were performed, revealing alternative splicing events displaying significant changes upon loss of Rbfox2. Genes with affected exons were associated with neuronal development and function, and a subset of Rbfox2-dependent alternative splicing events were also regulated by Rbfox1, suggesting partial redundancy between the two factors. In agreement with these data, double knockout mice displayed far more severe phenotypes than those observed in either single knockout mutant (Gehman et al., 2012). Finally, in an attempt to separate a possible role for both Rbfox proteins in the mature nervous system from their collective role in development, transgenic animals were generated that deleted these two factors specifically in Purkinje cells. Intriguingly, these double knockout mice possess no gross morphological or developmental abnormalities, but display impaired motor skills and significant reductions in spontaneous firing frequency of Purkinje cells, demonstrating that the Rbfox proteins also play an important role in mature neural circuitry in addition to their contribution to development (Gehman et al., 2012).

NEURONAL ACTIVITY-DEPENDENT ALTERNATIVE SPLICING REGULATION

DEPOLARIZATION CAUSES CHANGES IN SPLICING OF NEURONAL TRANSCRIPTS

Calcium signaling has long been recognized to play an important role in various cellular processes such as muscle contraction and gene transcription, and in neurons it is critical for modulating neuronal activity and for learning and memory (West et al., 2001). Increasing evidence suggests that depolarization-induced calcium influx can also regulate alternative splicing in neurons. One of the early studies showing splicing differences in response to neuronal depolarization utilized the cholinergic agonist pilocarpine administered to the brains of rats. Chronic induction of depolarization with pilocarpine caused altered alternative splicing patterns in a number of key neuronal transcripts in the rat hippocampus and cortex, including tra2-beta, clathrin light chain B, NMDAR1, and *c-src* (Daoud et al., 1999).

A number of further studies using chemical treatments to induce or inhibit calcium signaling in neurons have revealed additional calcium-dependent alternative splicing events. For example, Ania-6, an RNA polymerase II-associated cyclin, exhibited increased inclusion of intron 6 upon glutamate stimulation, but decreased inclusion when stimulated by depolarizing

concentrations of KCl. Increased intron inclusion leads to altered protein localization such that the longer isoform is found in nuclear speckles and is associated with hyperphosphorylated RNA Pol II (Berke et al., 2001; Sgambato et al., 2003). In a separate study, mature transcripts encoding SNAP25, a membrane-bound component of the SNARE complex essential for synaptic vesicle fusion, were found to include one of two mutually exclusive alternative exons (5a or 5b). Chronic depolarization of PC12 cells or of cerebellar granule cells by exposure to elevated extracellular K^+ resulted in altered splicing in which the abundance of the 5b isoform is increased (Hepp et al., 2001).

MODULATION OF NEURONAL ACTIVITY DURING CIRCADIAN RHYTHMS

Another potentially interesting physiological process that influences neuronal activity over sufficiently long timescales to involve new gene synthesis and RNA processing lies in the regulation of circadian rhythms. The connection between regulated splicing and circadian rhythms in the nervous system has been supported by a recent study using RNA-Seq in the *Drosophila* brain, which identified numerous splicing events regulated in response to circadian time or period, including splicing of key circadian genes (Hughes et al., 2012). Furthermore, Sanchez et al. (2010) identified the arginine methyl transferase PRMT5, which methylates arginine residues in the spliceosomal Sm proteins, in a screen for novel genes affecting circadian clock regulation in *Arabidopsis*. Mutations in PRMT5 were found to affect both transcription and alternative splicing of many transcripts, including several components of the circadian clock (Sanchez et al., 2010). The authors further demonstrated that a mutation in the *Drosophila prmt5* ortholog causes aberrant circadian-dependent behavior as well as altered mRNA splicing patterns. In both organisms, loss of PRMT5 led predominantly to increased intron retention. Taken together, these findings suggest that PRMT5 directly methylates splicing factors, though an alternative model in which PRMT5 leads to epigenetic changes cannot be ruled out (Sanchez et al., 2010). Continued exploration of the mechanisms controlling circadian regulation of alternative splicing will undoubtedly reveal novel insights.

MECHANISMS OF ACTIVITY-DEPENDENT ALTERNATIVE SPLICING

Although key neuronal transcripts undergoing depolarization-dependent alternative splicing have been discovered, our understanding of the mechanisms controlling this phenomenon is still in its infancy. In the sections that follow, we will highlight current progress in elucidating the role of *cis*-elements, chromatin states, and RNA binding protein modification as regulators of activity-dependent splicing (see **Figure 2** for an outline of examples).

mRNA ELEMENTS NECESSARY FOR ACTIVITY-DEPENDENT SPLICING

Several *cis*-elements regulating alternative splicing changes in response to neuronal depolarization have been identified. The BK (Big Potassium) channel encoded by the *Slo* gene in mammals is important for determining calcium and voltage sensitivity in neurons. The STREX exon of *Slo*, which contributes to enhanced neuronal sensitivity to Calcium when included in transcripts (Saito et al., 1997; Xie and Black, 2001), undergoes increased exon exclusion in response to KCl-mediated depolarization in cultured cells (Xie and Black, 2001). This depolarization-dependent alternative splicing required the Ca^{2+}/calmodulin-dependent protein kinase CaMKIV. The *cis*-elements that conferred responsiveness to CAMKIV signaling were identified, and came to be known as CaRREs (CaMKIV-responsive RNA elements; Xie and Black, 2001). Since then, a number of additional depolarization-dependent alternatively spliced transcripts have been shown to contain CaRREs and to be responsive to CaMKIV (Xie et al., 2005; Lee et al., 2007). Candidate RNA binding proteins that act downstream of CaMKIV signaling and bind to the CaRREs had remained elusive until recent years, when the heterogeneous ribonucleoprotein hnRNP L was identified to interact with CaRRE1 at the

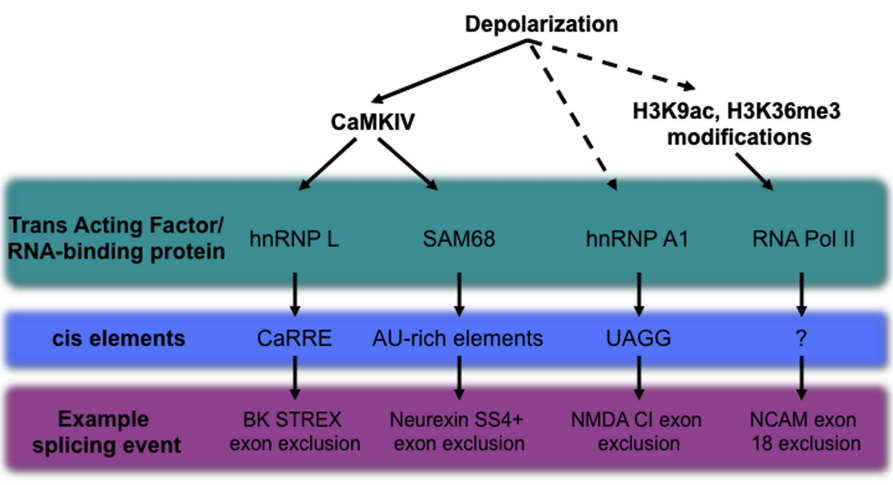

FIGURE 2 | Mechanisms of depolarization-dependent alternative splicing, including known trans-acting factors, the *cis*-elements with which they interact, and a representative alternative splicing event regulated by each specific pathway. Established mechanisms are shown as solid arrows, while activities with unknown intermediates are shown with dashed lines.

upstream 3′ splice site of the STREX exon (Yu et al., 2009). Knockdown of hnRNP L led to increased inclusion of the STREX exon prior to depolarization. However, upon treatment with KCl, cells with reduced hnRNP L levels had smaller STREX exon inclusion changes relative to control cells, but still showed some response to depolarization (Yu et al., 2009). These results suggest that other factors in addition to hnRNP L play a role in the observed depolarization-mediated effects. Indeed, a more recent study has now demonstrated that hnRNP L-like (hnRNP LL) acts redundantly with hnRNP L for the complete modulation of the STREX exon in response to depolarization (Liu et al., 2012).

An additional *cis*-element involved in activity-dependent splicing was uncovered in experiments demonstrating that K^+-induced alternative splicing of the CI cassette exon of the NMDA R1 receptor relied on the presence of two exonic UAGG silencing motifs. These motifs were previously identified as part of a multicomponent regulatory code involving 5′ splice site proximal GGGG elements in coordinating tissue-specific splicing regulation of the CI exon (Han et al., 2005). Introduction of the UAGG silencing motif into a constitutively spliced exon in an unrelated gene led to an increase in exon skipping, and importantly, further increased skipping in response to extracellular K^+-induced depolarization (An and Grabowski, 2007). Biochemical studies demonstrated that the association of the heterogeneous nuclear ribonucleoprotein hnRNP A1 with these UAGG motifs was increased in response to cellular excitation (An and Grabowski, 2007). Although it is currently unclear how this increased association is induced, these results suggest that a signaling cascade must exist that connects responses to depolarization at the plasma membrane and in the cytoplasm with subsequent effects on activity in the nucleus. Interestingly, hnRNP A1 has been demonstrated to alter its shuttling state between the nucleus and cytoplasm in response to osmotic shock (Allemand et al., 2005). It will be interesting to determine whether depolarization leads to increased shuttling of hnRNP A1 to the nucleus, which would offer a possible explanation for the elevated association with UAGG elements observed.

MODIFICATION OF CHROMATIN

Nucleosome positioning and chromatin modification have been recognized as important factors in memory formation and consolidation (Guan et al., 2002; Levenson et al., 2004). More recently, chromatin state and epigenetic marks, including post-translational histone-tail modifications and DNA methylation, have been found to play an important role in alternative splicing regulation (Hnilicova and Stanek, 2011; Luco and Misteli, 2011). Genome-wide analyses have indicated that nucleosomes have increased occupancy in exons compared to flanking intronic regions, and that local enrichment of certain histone modifications can facilitate alternative exon inclusion (see below; Nahkuri et al., 2009; Schwartz et al., 2009; Tilgner et al., 2009; Huff et al., 2010). Conversely, splicing has also been found to be important for the establishment of histone H3 lysine 36 methylation in intron-containing genes, suggesting a bi-directional communication (de Almeida et al., 2011; Kim et al., 2011). Additionally, and not mutually exclusive with the influence of chromatin state, the rate of RNA polymerase II (pol II) elongation during nascent transcript synthesis has also been found to affect alternative splicing (de la Mata et al., 2003). Two models have been proposed to possibly account for how pol II elongation can regulate alternative splicing. First, the recruitment model suggests that splicing factors can directly associate with pol II, likely via the C-terminal domain (CTD) of its largest subunit. These pol II-recruited splicing factors would then be available to recognize cognate *cis*-elements found in nascent pre-mRNA transcripts. Second, the kinetic model posits that chromatin structure influences the local rate of pol II transcription elongation, potentially exposing normally weak splice sites for extended periods of time, thereby allowing them to be more efficiently recognized by the spliceosome (Kornblihtt, 2007; Munoz et al., 2010).

Several recent studies have reinforced the hypothesis that local enrichment of distinct histone marks and DNA methylation status at alternative exons and flanking sequences can modulate pol II elongation rate and alternative splicing. First, Luco et al. (2010) have demonstrated using chromatin-immunoprecipitation assays that H3-K36me3 marks are enriched around a set of alternative splicing events regulated by PTBP1. The histone-tail binding protein MRG15, which specifically recognizes H3-K36me3, was also found to be in a physical complex with PTBP1, thus providing a link between histone modifications and the potential recruitment of splicing factors (Luco et al., 2010). In a separate study, Shukla et al. (2011) discovered that the DNA-binding protein CCCTC-binding factor (CTCF) can mediate local pausing of pol II and inclusion of weak alternative exons. These authors further revealed that the action of CTCF is inhibited by DNA methylation at these regulated exons, suggesting a mechanistic link between elongation rate, methylation, and CTCF binding in modulating alternative splicing. Finally, Close et al. (2012) have identified a novel polymerase-associated complex called DBIRD that was found to promote exon skipping. Depletion of components of the DBIRD complex was found to predominantly increase pol II occupancy surrounding regulated exons, leading to more inclusion. In a search for *cis*-elements associated with these DBIRD-sensitive exons, the authors identified enrichment of (A + T) rich sequences, which have been shown in previous studies to act as pol II elongation pause sites *in vitro* (Close et al., 2012).

An intriguing study has also implicated neuronal depolarization in the control of alternative splicing by affecting RNA Pol II transcription kinetics. Exon 18 of Neural Cell Adhesion Molecule (NCAM) transcripts undergoes developmentally regulated alternative splicing in which the exon-excluded isoform (NCAM140) is abundant in neuronal precursors while the exon-included isoform (NCAM180) is increasingly expressed throughout the process of neuronal differentiation (Pollerberg et al., 1985, 1986; Cunningham et al., 1987). Schor et al. (2009) demonstrated that NCAM exon 18 skipping increased in response to neuronal depolarization with KCl. This depolarization-mediated increase in exon skipping was not dependent on CaMKIV (Schor et al., 2009), but instead involved histone modification changes specifically in the vicinity of exon 18. Depolarization led to an increase in histone H3-K9 acetylation and H3-K36 tri-methylation exclusively in the region between exons 17 and 19, as well as a local increase in chromatin relaxation and accessibility. Furthermore, exon 18 inclusion could be artificially recapitulated by either using a mutant "slow" pol II or

by applying the drug trichostatin which inhibits histone deacetylation. These results are consistent with a kinetic coupling model in which depolarization leads to specific local histone modifications in the region of the alternative exon causing local chromatin relaxation, in turn increasing the speed of pol II transit through the exon and facilitating increased exon 18 exclusion (Schor et al., 2009).

REGULATION OF RNA BINDING SPLICING FACTORS

The activity or levels of RNA binding proteins that regulate alternative splicing can be modified in response to neuronal activity. For instance, phosphorylation of hnRNP L on serine 513 by CaMKIV was found to play a crucial role in the differential regulation of STREX exon inclusion upon treatment of cells with KCl (Liu et al., 2012), providing a molecular link between signaling downstream of the stimulus and subsequent effects on alternative splicing. Two recent studies have implicated the RNA binding protein Rbfox-1/A2BP1 as an important splicing factor mediating activity-dependent alternative splicing (Lee et al., 2009; Amir-Zilberstein et al., 2012). First, transcripts encoding Rbfox-1 itself were identified as a target of depolarization-dependent splicing in mouse cells, where exon 19 was found to be excluded in response to depolarization. Exclusion of exon 19 led to the accumulation of a Rbfox-1 protein isoform targeted to the nucleus, where it re-activated the inclusion of target exons initially displaying more skipping upon depolarization (Lee et al., 2009). Thus, Rbfox-1 lacking exon 19 counteracted the effects of depolarization-dependent exon exclusion, suggesting a novel feedback-based mechanism for adapting to chronic neuronal depolarization. A second study implicated Rbfox-1 as an important downstream target of the hypothalamic transcription factor Orthopedia (Otp) in response to stress (Amir-Zilberstein et al., 2012). Rbfox-1 was demonstrated to be a transcriptional target of Otp, and Rbfox-1 transcript levels were upregulated in response to stress in mice. Rbfox-1 in turn was responsible for an increase in exon 14 inclusion in the pituitary adenylate cyclase-activating peptide (PACAP) receptor PAC1. Inclusion of PAC1 exon 14 led to a decrease in the levels of stress-induced corticotropin-releasing hormone (CRH). These results suggest that Rbfox-1-mediated inclusion of PAC1 exon 14 creates an isoform that helps terminate stress-induced transcription of CRH. In agreement with this model, zebrafish injected with morpholinos inhibiting PAC1 exon 14 inclusion exhibited abnormal "anxiety like" behavior and sustained expression of CRH transcription (Amir-Zilberstein et al., 2012).

Another recent study has provided insight into the mechanism controlling depolarization-dependent splicing of the neurexin SS4+ alternative isoform. As discussed above, alternative splicing of SS4 can modulate the affinity of neurexins for their post-synaptic ligands. Iijima et al. (2011) have now shown that SS4 selection can be suppressed in response to induced depolarization by various methods in cultured mouse neurons. This suppression of exon inclusion is dependent on CaMKIV and results in altered *trans*-synaptic signaling in response to depolarization. The STAR family RNA binding protein SAM68 was shown to be required for depolarization-dependent splicing of SS4, and to bind directly to AU-rich response elements in the neurexin pre-mRNA. Although SAM68 protein levels and localization were not affected by depolarization, a serine residue within a consensus CaMKIV recognition motif was found to be more highly phosphorylated following depolarization. These results suggest a model where neuronal depolarization affects CaMKIV due to increased intracellular calcium, leading to the phosphorylation of SAM68, which then alters neurexin splicing. Interestingly, the neurexin pre-mRNA does not contain recognizable CaRRE sequences, and a number of transcripts containing CaRRE sequences were not affected by loss of SAM68 (Iijima et al., 2011). Thus, it appears that CamKIV-dependent alternative splicing regulation depends on multiple downstream RNA binding proteins binding to distinct *cis*-elements.

PERSPECTIVES AND FUTURE DIRECTIONS

Neuronal depolarization can affect the splicing of many transcripts in the nervous system, but the mechanisms by which it does so still remain largely unknown. A major future challenge will be to identify the signaling cascades in addition to the CAMKIV pathway linking cellular excitation to alternative splicing via changes in the activity of splicing factors, chromatin state, and perhaps additional mechanisms. Moreover, experiments thus far revealing a role of depolarization in nervous system alternative splicing have relied on *in vitro* cell culture models requiring chronic depolarization for many hours to modulate the firing activity of neurons. It will now be important to understand how chronic neuronal depolarization affects alternative splicing regulation *in vivo*, and what consequences the affected isoforms have on neuronal physiology. Many depolarization-responsive alternative splicing events that have been identified are found in transcripts encoding channel proteins, neurotransmitter receptors, and other modulators of synaptic strength (Xie and Black, 2001; Lee et al., 2007; Iijima et al., 2011). Phenomena such as synaptic gain control and homeostasis, where synapses can alter their sensitivity in response to chronic hyper- or hypo-stimulation (Burrone and Murthy, 2003), are thought to occur over the course of hours. This time-frame overlaps well with the temporal dynamics of depolarization-induced alternative splicing changes observed *in vitro*. While it may be a technically challenging feat, genome-wide analyses of splicing changes in organisms maintained under differing stimuli or behavioral paradigms inducing such synaptic gain control or homeostatic maintenance would provide further insight into the mechanisms and relevance of neuronal activity in regulating alternative splicing *in vivo*.

The fact that the activity of master regulators of gene expression such as transcription factors can be modified by alternative splicing has blurred the lines of how differentiation programs in cell lineages are established (Gabut et al., 2011; Raj et al., 2011). In a broader sense, these results raise an interesting implication, namely, that regulation of alternative splicing events by RNA binding proteins can play a causal rather than simply consequential role in developmental transitions and activity states of neurons. Future experiments establishing the contribution of splicing and other RNA binding regulators to the identity and fate of neuronal lineages represents an important goal in basic research but also in biomedical applications such as regenerative medicine. While a handful of neuronal-specific splicing regulators have been discovered, it is unlikely that the current repertoire of known factors is

sufficient to account for the remarkable degree of splicing complexity observed in the nervous system. Hundreds of RNA binding proteins have been identified in metazoan genomes, and many of them remain uncharacterized. As such, identification and characterization of novel regulators of splicing will be important. Large scale RNAi and/or cDNA overexpression studies in cell culture models will be useful for identifying factors whose inhibition or overexpression affect splicing in the nervous system. Importantly, invertebrate model organisms provide a valuable platform for performing forward and reverse genetic screens to identify previously uncharacterized factors affecting splicing. In addition to identifying RNA binding proteins, such unbiased screens may also uncover novel classes of genes not previously known to regulate alternative splicing, such as non-coding RNAs and chromatin regulators (Luco et al., 2010; Tripathi et al., 2010). Furthermore, it will be important to understand splicing regulation in the nervous system in a more spatially resolved manner, for example, by identifying brain sub-region and neuronal-subtype specific alternative splicing events, the factors that control these events, and the effects they have on specification and function of individual classes of neurons.

Although progress has been made toward understanding splicing changes in the development of the nervous system, much less is known about the interplay between alternative splicing regulation and aging in the brain. It has been known for some time that aberrant splicing of the LMNA gene leads to an accelerated aging phenotype found in individuals with Hutchinson-Gilford Progeria Syndrome (HGPS; Todorova et al., 2003). Recent studies using patient cell lines and HGPS mouse models have identified candidate regulators involved in the cryptic splicing of LMNA (Lopez-Mejia et al., 2011). It is tempting to speculate that analysis of the aging brain will also implicate splicing factors in both aging-related splicing changes and the gradual deterioration of the nervous system. Indeed, a recent study has identified dynamic alterations in splicing during normal brain aging consistent with an increase in PTB-dependent splicing, as well as splicing changes in diseased brain consistent with decreased NOVA-dependent splicing (Tollervey et al., 2011). In the future, it will be important to more directly understand the mechanisms and the consequences of splicing in the aging brain.

Finally, mutations in several RNA binding proteins expressed in the nervous system have been associated with neurodevelopmental disorders (Grabowski and Black, 2001; Wang and Cooper, 2007; Morikawa and Manabe, 2010). Advances in genome-wide approaches to globally monitor transcripts bound by these proteins and their effects on aspects of mRNA metabolism, including alternative splicing, are beginning to shed light on underlying mechanisms of action and provide a more detailed understanding of disease etiology. Deeper investigation of the transcript networks regulated by these RNA binding proteins will hopefully provide promising new insight into the development of treatments for some of these disorders.

As demonstrated by some of the examples described above, alternative splicing has the potential to generate multiple protein isoforms, but can also modulate other properties of mRNA transcripts, including their stability. Two recent studies have suggested that tissue-specific alternative exons can frequently encode structurally disordered regions in proteins and have the potential to influence post-translational modification and protein–protein interaction interfaces (Buljan et al., 2012; Ellis et al., 2012). These observations collectively indicate that it remains an important goal to develop additional techniques and approaches that will facilitate the characterization of the functional consequences of alternative splicing events in biological pathways.

We are embarking on an exciting time where techniques for large scale analysis of nervous system transcriptome dynamics in distinct cellular subtypes, multiple developmental states, and in response to environmental cues, are constantly improving. These approaches are already revealing a previously unappreciated role for post-transcriptional gene regulatory mechanisms in almost all aspects of nervous system physiology. Continued research taking advantage of these techniques, coupled with emerging computational approaches and more traditional biochemical and molecular genetic assays, will produce a more comprehensive understanding of alternative splicing regulation. These integrative analyses should also shed further light on the interplay between alternative splicing and other layers of gene regulation in generating the constellation of neuronal subtypes and their diverse functional properties.

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Transcript diversification in the nervous system: A to I RNA editing in CNS function and disease development

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RNA editing by adenosine deaminases that act on RNA converts adenosines to inosines in coding and non-coding regions of mRNAs. Inosines are interpreted as guanosines and hence, this type of editing can change codons, alter splice patterns, or influence the fate of an RNA. A to I editing is most abundant in the central nervous system (CNS). Here, targets for this type of nucleotide modification frequently encode receptors and channels. In many cases, the editing-induced amino acid exchanges alter the properties of the receptors and channels. Consistently, changes in editing patterns are frequently found associated with diseases of the CNS. In this review we describe the mechanisms of RNA editing and focus on target mRNAs of editing that are functionally relevant to normal and aberrant CNS activity.

Keywords: calcium channel, glutamate receptor, inosine, potassium channel, RNA modification, RNA editing, serotonin receptor

RNA EDITING

RNA editing is a site specific, post transcriptional modification of RNA. Two types of RNA editing can be distinguished. On the one hand, insertion-deletion type editing inserts or removes single or multiple nucleotides from an RNA molecule (Simpson et al., 2000). This type of editing is mostly found in organelles of various phyla. Deamination type editing, on the other hand, changes the identity of a base by deaminating cytidine to uracil or adenosine to inosine, respectively (Carter, 1995). Deamination type editing has been found in all kingdoms of life. Deamination of cytidines was first discovered in the mRNA encoding apolipoprotein B which is deaminated by Apobec1 a member of the apobec/AID cytidine deaminase family that mostly target cytidines in DNA. Recently, abundant cytidine deamination by Apobec1 was identified in the 3′ UTRs of many mouse mRNAs (Rosenberg et al., 2011). The function of these editing events remains to be determined, however.

Adenosine deamination by adenosine deaminases that act on RNA (ADAR) seemingly only affects metazoan nuclear encoded RNAs. Deamination of adenosines leads to the formation of inosines which are recognized as guanosines by most cellular machineries (Bass and Weintraub, 1988). Editing in coding regions of mRNAs can therefore lead to a codon exchange and the subsequent translation of a protein that differs from the genomically encoded version. Moreover, editing can also introduce or remove splice sites and thereby lead to the formation of novel mRNAs (Rueter et al., 1999). Finally, inosines in RNA can change the base-pairing propensity of an RNA and therefore alter their folding and change their signature for RNA-binding proteins (Nishikura, 1992). Thus, also editing in non-translated regions of an mRNA may have profound impact on the fate of the affected RNA. Besides mRNAs, also primary and precursor miRNAs can be targets for RNA editing by ADARs. Editing of pri- and pre-miRNAs can alter their processing but also their base-pairing potential with target mRNAs. Therefore, editing of miRNAs can indirectly change the abundance and translatability of their target mRNAs.

Editing of mRNAs was originally believed to be a rare event. In recent years, however, editing was found to be widespread in mRNAs of higher eukaryotes (Athanasiadis et al., 2004; Levanon et al., 2004). In all organisms editing by ADARs is most abundant in the nervous system. The profound alterations of the transcriptome and proteome introduced by RNA editing may thus help to solve a long lasting biological paradigm, namely, how biological complexity can be achieved with an almost constant number of genes: editing-induced alterations of splice patterns and coding potential of mRNAs may, together with alternative splicing, contribute to the formation of a complex proteome from a limited number of genes. Consistently, alterations in the editing patterns or loss of editing is accompanied by pathologic conditions and disease (Morabito and Emeson, 2009).

THE ADAR PROTEIN FAMILY

ADARs were first discovered in *Xenopus laevis* as an unwinding activity that destabilizes RNA duplexes upon A to I editing (Bass and Weintraub, 1987; Rebagliati and Melton, 1987). ADARs have been well characterized in many organisms including insects, worms, and vertebrates (Bass, 2002). The first ADAR gene identified was vertebrate ADAR1 harboring three double-stranded RNA-binding domains (dsRBDs) and a conserved deaminase domain with zinc binding motifs. Subsequent screens led to the identification of ADAR2 (Melcher et al., 1996; O'Connell et al., 1997). Recent analyses have shown the presence of ADAR1 and ADAR2 in many species including sea urchin and sea anemones (Jin et al., 2009). The vertebrate genome encodes two additional, ADAR proteins.

ADAR3, which presumably arose from ADAR2 by gene duplication, contains all functional domains. However, no function has been ascribed to this isoform (Chen et al., 2000). The fourth ADAR-like gene, termed TENR, is expressed in the male germ line and has one dsRBD. TENR lacks conserved zinc chelating residues in the deaminase domain thus explaining its inactivity (Hough and Bass, 1997). ADARs are related to the tRNA editing family of ADATs which are found in all kingdoms of life (Jin et al., 2009).

PHENOTYPES OF ADAR DEFICIENCY

Different phenotypes are associated with the lack of individual ADAR isoforms. ADAR2 null mice have episodes of epileptic seizures and show subsequent postnatal death. A key substrate of ADAR 2 is the mRNA encoding GluA2. Underedited GluA2 allows increased influx of Ca^{2+} leading to death of neurons (Brusa et al., 1995). Consistently, *ADAR2*$^{-/-}$ mice can be rescued by replacing the genomic, unedited GluA2 copy with a "preedited" gene copy (Higuchi et al., 2000). Still, even the rescued *ADAR2*$^{-/-}$ mice display a range of subtle phenotypes ranging from a decreased acoustic startle response to decreased blood glucose level. The molecular mechanisms underlying these changes are still to be determined (Horsch et al., 2011). ADAR2 overexpressing mice, in contrast, display hyperphagia and obesity (Singh et al., 2007). This phenotype can be reproduced by a catalytically inactive version of ADAR2 that retains its RNA-binding ability. This suggests that RNA binding of ADARs can lead to editing-independent phenotypes (Singh et al., 2007).

Mice lacking ADAR1 die during embryonic development, show defective hematopoiesis, widespread apoptosis, and liver disintegration (Hartner et al., 2004). Molecularly, ADAR1 deficient mice show an increase in interferon signaling with the precise molecular mechanisms leading to death remaining unknown (Hartner et al., 2009).

In *Drosophila melanogaster* inactivation of the single ADAR gene causes tremors, lack of coordination, mating defects, and neurodegeneration presumably resulting from underediting of important dADAR target genes such as Na^{+} (*para*), Ca^{2+}(*cac*), and glutamate-gated Cl^{-} channels (*DrosGluCl-α*; Palladino et al., 2000). *Caenorhabditis elegans* strains with homozygous deletions in either of the two ADARs *adr-1* or *adr-2* show chemotactic defects, also indicating a role in the editing of neuronally expressed substrates (Tonkin et al., 2002).

SUBSTRATES OF ADAR

RNA editing by adenosine deaminases can affect coding and non-coding RNA sequences. Substrate RNAs are recognized by the dsRBDs located in ADARs. These domains bind to A-form helices formed by double-stranded RNAs. Thus, editing sites are defined by base-paired regions of 20 or more nucleotides in length. A-form helices display a wide minor groove and a narrow major groove. Sequence specific information of the bases cannot be easily contacted making a sequence specific positioning of ADARs difficult (Ryter and Schultz, 1998). Still, various mechanisms can contribute to substrate and editing specificity. Multiple dsRBDs found in ADARs can coordinately bind to substrates (Stefl et al., 2005). Most double-stranded structures formed by endogenous RNAs are disrupted by bulges. These bulges set natural boundaries for the binding of dsRBDs (Lehmann and Bass, 1999). If two or more dsRBDs need to bind to a double-stranded region of limited length they can help to position each other. Structural analysis of ADAR2 bound to a stem loop substrate shows nicely that some dsRBDs can also bind to terminal loops thus helping to increase substrate specific binding (Stefl et al., 2006). Recently, specific minor groove interactions between dsRBDs and nucleotides have been identified. These interactions can increase sequence specificity dramatically, therefore aiding in selecting specific adenosines within a stretch of double-stranded RNA (Stefl et al., 2010). Finally, also the deaminase domains of ADAR1 and ADAR2 display substrate specificities that preferentially select certain adenosines depending on their local sequence context (Polson and Bass, 1994; Eggington et al., 2011). As the adenosine to be edited typically lies within a double-stranded structure the target adenosine needs to be accessed through a base flipping mechanism (Stephens et al., 2000; Yi-Brunozzi et al., 2001).

The altered base-pairing potential of inosines can lead to an alteration of the RNA secondary structure. Thus, editing in the non-translated regions of mRNAs may alter their localization, stability, and translatability. However, the biological consequences of editing in these targets is still under debate. The consequences of adenosine deamination in coding regions of mRNAs and pri-miRNAs on the other hand are more easy to understand. As inosines are read as guanosines during translation, inosines can alter the coding potential or targeting specificity of mRNAs and miRNAs, respectively (Vesely et al., 2012).

The proteins encoded by edited pre-mRNAs vary widely in their function. However, frequently editing-induced amino acid exchanges affect receptors and ion channels expressed in the brain. Another class of proteins affected by RNA editing play a role in cytoskeletal remodeling which also plays an important role in neuronal outgrowth and plasticity. In the following, representative examples of both classes of proteins and the functional implications of their editing will be described.

GLUTAMATE-GATED ION CHANNELS

Five subunits of the glutamate receptor (GluA2, GluA3, GluA4, GluK1, and GluK2) are found to undergo ADAR-mediated RNA editing (Bass, 2002). A total of four editing sites that result in amino acid changes have been identified, namely glutamine to arginine (Q/R), arginine to glycine (R/G), isoleucine to valine (I/V), and tyrosine to cysteine (Y/C; see **Table 1**).

AMPA GluA2 subunit mRNA was the first target discovered. It is edited mainly at two coding sites leading to a glutamine to arginine and arginine to glycine conversion (Sommer et al., 1991; Lomeli et al., 1994; see **Figure 2**).

Two additional editing sites are found in intron 11 of GluA2 mRNA, called hotspot 1 (or +60 site) and hotspot 2 (or+262/263/264 site), respectively (Higuchi et al., 1993). Editing at the Q/R site reduces Ca^{2+} permeability (see **Figure 1**). The edited $GluA2^{R}$ isoforms also show reduced endoplasmic reticulum (ER) exit efficiency, whereas unedited $GluA2^{Q}$ isoforms readily tetramerize and are transported to the synaptic membrane (Greger et al., 2002, 2003). GluA2 in the unedited Q form leads to epileptic seizures and subsequent postnatal death. This toxic effect has been attributed to increased calcium influx (Higuchi et al., 2000).

Table 1 | Selected editing events in the CNS.

Target	Editing site	Function	Diseases
GluA2	Q607 R	Calcium impermeable ER exit efficiency reduction	ALS, epilepsy, glioblastomamultiforme, pediatric astrocytoma
GluA2	R764G	Enhanced rate of desensitization Modulation of alternative splicing	Spinal cord injury (SCI), epilepsy Schizophrenia on drug administration
Serotonin receptor 5HT2C	I156V I156M N158S N158G N158D I160V	Modulation of surface expression of the receptor Reduced G protein coupling Decreased Erk signaling	Schizophrenia Bipolar disorder Depression Anxiety Prader–Willi syndrome
Kv1.1	I400V	Faster recovery from inactivation Reduced potency of channel blockers	Epilepsy
$GABA_A$	I342M	Reduced stability of α3 subunit	Migraine
FLNa	Q2341R	Binds to: Kv4.2 K^+ Channel	
		Presenilins metabotropicmGlu5a/b, mGlu7b, mGlu8a, weak: mGlu7a mGlu4a	
CyFIP2	K320E		
Nova-1	S383G	Increase in protein stability	
Ca(v)1.3	I1606M Q1607R Y1609C	Decrease in calmodulin mediated calcium dependent inhibition (CDI) and faster recovery from inactivation	

Proposed physiological and pathological consequences.

Additionally increased receptor density due to faster ER exit may also contribute to this effect (Greger et al., 2002; see **Figure 1**).

The R/G conversion reduces the assembly of homomeric receptors and slows down receptor maturation in the ER (Greger et al., 2006). Additionally, R/G site editing results in enhanced recovery from desensitization (Lomeli et al., 1994). Editing events in the GluA2 pre-mRNA also affect splicing of nearby introns. Editing at the R/G site of GluA2 takes place two nucleotides upstream of the 5′ splice site in intron13. The Q/R site is located in exon 11, 25 nucleotides upstream of the 5′ splice site of intron 11 (Higuchi et al., 1993). Editing at the Q/R site and the intronic hotspot enhances splicing of the nearby intron, while editing at the R/G site represses splicing at the downstream intron (Schoft et al., 2007). Editing at the R/G site may affect base-pairing of the pre-mRNA with the U1snRNA (Schoft et al., 2007). R/G site editing also influences the alternative splicing of the two downstream exons as editing promotes inclusion of exon15 (flip) over exon14 (flop). GluA2 protein with an edited G and the flip variant undergoes rapid maturation in the ER relative to the flop form. The flip variant also stimulates dendritic growth (Hamad et al., 2011). The flop isoform, in turn, promotes assembly of heteromeric AMPA receptors (Penn and Greger, 2009).

Also kainate receptor subunits GluK1 and -2 are edited at the Q/R position. GluK2 undergoes additional editing at the I/V and Y/C sites, located at positions 621, 567, and 571 respectively, which may lead to higher calcium permeability (Kohler et al., 1993).

$GABA_A$ RECEPTOR

$GABA_A$ receptors are ligand gated chloride channels consisting of five subunits: 2 α subunits, 2 β subunits, and either a γ or a δ subunit (Hevers and Luddens, 1998). The existence of 6 α, 3 β, 3 γ, and 4 δ subunits allows for the assembly of a wide variety of stoichometries. Position 342 of the α_3 subunit is highly edited, resulting in an isoleucine (AUA) to methionine (AUI) codon change (Ohlson et al., 2007; see **Figure 2**). The editing site is defined by a specific RNA structure marked by bulges at a defined distance from the editing site as well as a specific terminal loop structure (Tian et al., 2011). With age, the two α subunits show opposing expression patterns. While α_1 expression increases with age the α_3 subunit is predominant at embryonic level (Hutcheon et al., 2004). Moreover, editing is developmentally regulated. The pre-mRNA is mostly found unedited around day e15 but is edited from 80% to 100% at postnatal day 7 (p7; Ohlson et al., 2007; Rula et al., 2008)

The I/M change in $GABA_A$ receptor causes a delay in currents and faster deactivation upon stimulation by GABA (Rula et al., 2008). Expression of unedited $GABA_A$ receptor in the developing brain is crucial for synapse formation (Ben-Ari et al., 2007). Recently, editing has been proposed to affect the stability of the α_3 subunit as the edited version displays low cell surface expression. The M version of the receptor maintains the hydrophobic environment but can influence the interaction between α and γ subunits or ligand interaction (Daniel et al., 2011; see **Table 1**; **Figure 1**).

VOLTAGE-GATED POTASSIUM CHANNELS

Neuronal Kv1.1 channels are built of a tetramer of pore forming α subunits along with four regulatory beta subunits and accessory subunits. The channels regulate action potential and modulate neuronal excitability by opening and closing of a potassium

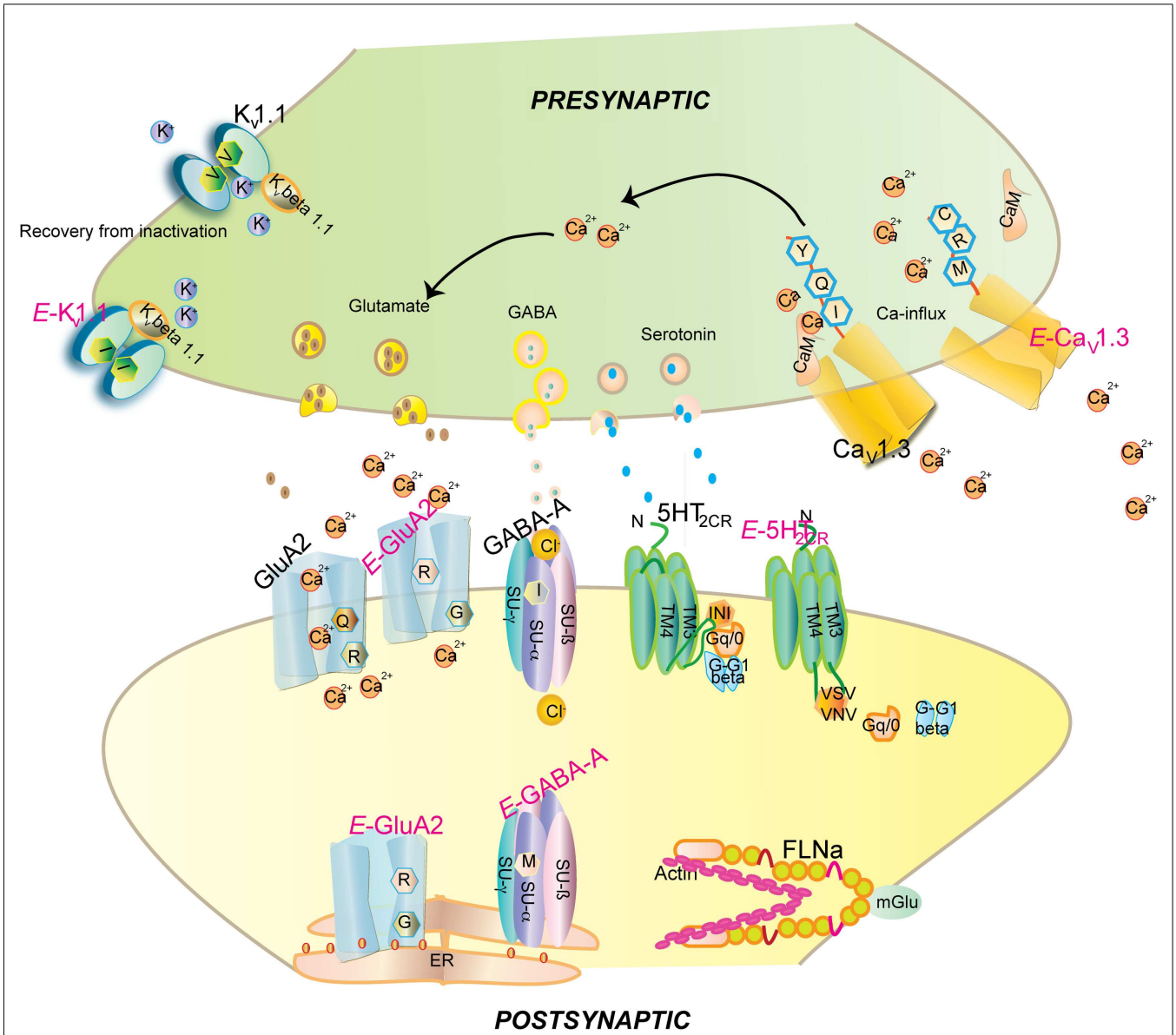

FIGURE 1 | Impact of editing on selected neuronal receptors and proteins. Shown are several receptors and channels in their unedited (black) and edited (E, in pink) versions. Editing at the Q/R site of ionotropic glutamate receptor GluA2 subunit decreases Ca^{2+} permeability and endoplasmic reticulum exit efficiency. Membrane trafficking of the $GABA_A$ receptor is reduced by editing of I to M in the alpha3 subunit. Editing of the serotonin 5-HT_{2c} receptor converts the amino acids I-N-I to V-S-V or V-N-V. This reduces G-protein coupling in the receptor. The editing-induced I to V exchange in $K_v1.1$ (I/V) alters the interaction with Kv β1.1 (see **Figure 3** for detail). Editing of the IQ motiv in $Ca_v1.3$ to MR abolishes calmodulin binding. Filamin alpha (FLNa) is edited in a region that is known to interact with metabotropic glutamate receptor mGlu7b and some of its relatives.

selective pore. The human Kv1.1 (*KCNA1)* gene is intronless and undergoes A to I RNA editing leading to an isoleucine to valine exchange (see **Figure 1**). The amino acid exchange is located within the sixth transmembrane segment (S6) which lies at the ion conducting pore (Bhalla et al., 2004; see **Figure 2**). Kv1.1 channels are edited up to 65–80% in medulla, thalamus and spinal cord (Decher et al., 2010). The I–V change is evolutionarily conserved and also occurs in Kv2 (DmShab Shaker) channels in *Drosophila melanogaster* together with four other editing events (Bhalla et al., 2004; Ryan et al., 2008).

Kv1.1 associates with the redox sensor Kv β1 in the ER (Pan et al., 2008). Kv β harbors an N-terminal inactivation domain that controls inactivation and lag time of Kv1.1. The edited Kv1.1 shows a 20 fold higher recovery from Kv β1 mediated inactivation than the unedited version of the channel (Bezanilla, 2004; Bhalla et al., 2004; see **Figure 3**).

The Kv channel blocker 4-aminopyridine (4-AP) has been shown to induce epileptic seizures. RNA editing makes the channel insensitive to 4-AP by disrupting the interaction between the pore lining and the channel blocker (see **Table 1**; Streit et al.,

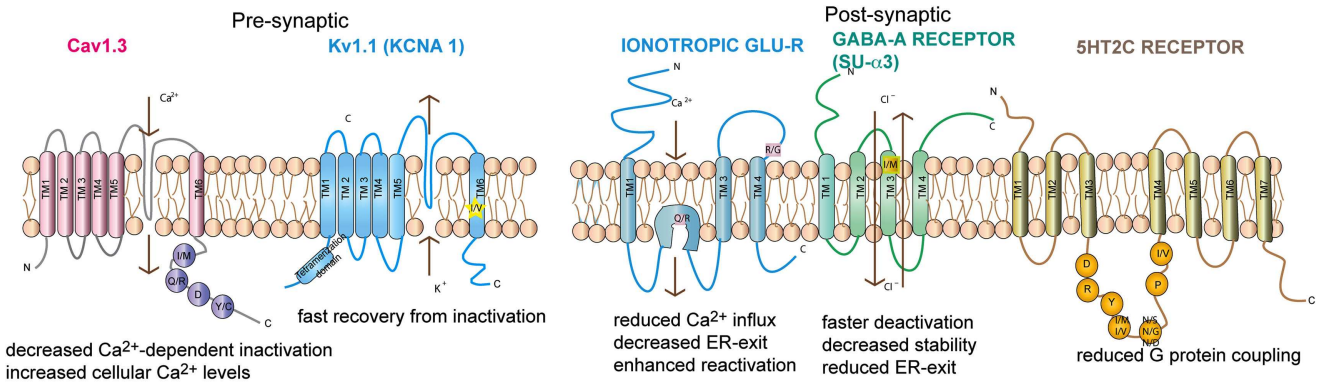

FIGURE 2 | Topography of the neuronal channels containing editing sites Ionotropic glutamate receptor, $GABA_A$ receptor subunit α3, Kv1.1, Cav1.3, and 5-HT_{2c} receptors are shown. The relative position of the editing sites within these receptors are highlighted. Consequences of editing are indicated.

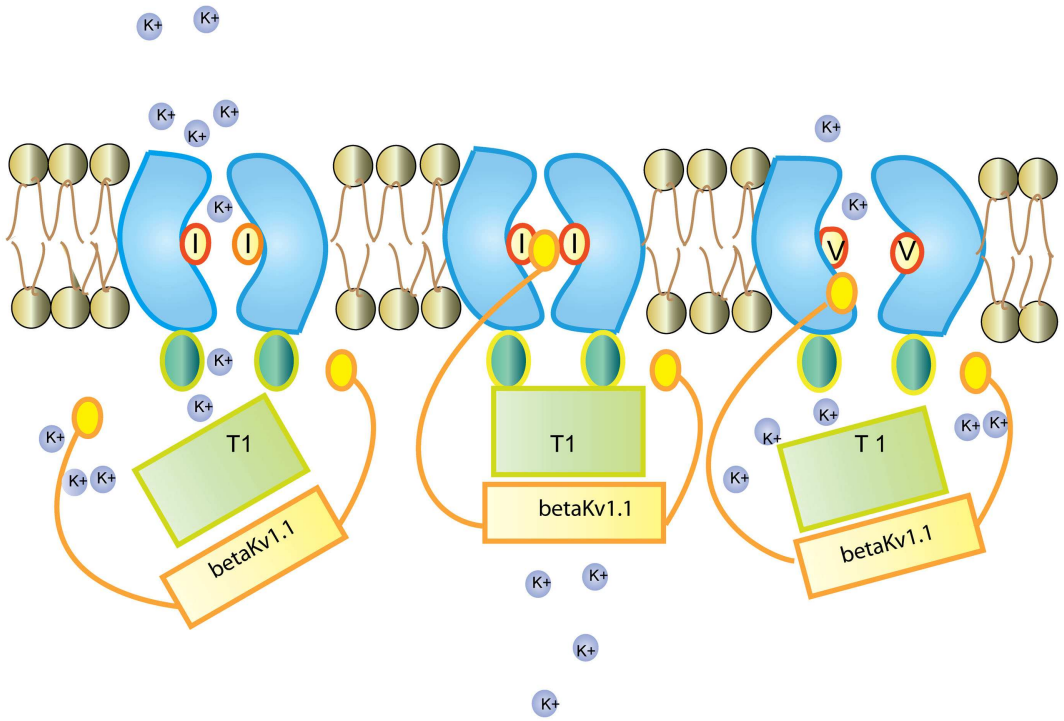

FIGURE 3 | Regulation of the Kv1.1 channel by K_v β1.1 K_v β1.1 has an inactivation gate which interacts with the unedited (I) form of the K_v1.1 receptor. Editing of K_v1.1 changes the isoleucine to valine (V). This reduces the affinity for K_v β1.1 and enhances recovery from inactivation. Figure adapted from (Bezanilla, 2004).

2011). A similar insensitivity was observed against arachidonic acid (Decher et al., 2010).

Squid Kv 1.1A has also been shown to be extensively edited (Rosenthal and Bezanilla, 2002). Here editing not only affects channel gating but also influences the tetramerization of the channel.

VOLTAGE-GATED CALCIUM CHANNELS

Voltage-gated calcium channels (VGCC) are classified into two types: Low voltage activated (LVA) and High voltage activated (HVA) channels (Lacinova, 2005). LVA L-type calcium channels are involved in a broad range of neuronal processes such as neuronal pacemaking, secretion of neurotransmitters, synaptic transmission, mRNA stability, and modulation of other ion channels (Singh et al., 2008). The opening of these channels permits calcium influx. The channels are inactivated by voltage dependent inhibition (VDI) and intracellular calcium dependent inhibition (CDI). The pore forming α1 subunit contains four domains (I–IV), each domain consisting of six transmembrane segments (S1–S6; Catterall et al., 2005; see **Figure 2**). S5 and S6 of all four domains form

the central pore with S6 lining the inner surface of the pore and occluding the pore in the inactive state. S1 to S4 from each domain form the voltage sensing domain and on activation the S4 segment moves outward triggering S6 movement leading to gate opening (Swartz, 2004).

Calmodulin (CaM) binds to the IQ domain located at the C-terminus of the pore forming α1 subunit. The formation of a Ca^{2+}-CaM complex results in CDI (Tadross et al., 2008). Calcium binding to the N- and C-terminal CaM lobes can induce distinct channel regulation (Dick et al., 2008). Recently, editing of the core sequence of the IQ domain of $Ca_v1.3$ by ADAR2 has been discovered. The core sequence comprises of the 4 amino acids IQDY. Upon editing different isoforms such as MQDY, IRDY, MQDC, MRDY, MRDC, or IQDC can be generated (see **Figures 1** and **4**). This editing event is restricted to the central nervous system (CNS; Huang et al., 2012). Additionally, the pattern of editing is developmentally regulated. It is negligible at e14 and prominently increases at p4. RNA editing of the IQ domain shows spatial distribution being highest at the frontal cortex and hippocampus (Huang et al., 2012). The MQ and IR versions show weaker CDI while the MR variant exhibits up to 50% reduction of CDI and faster recovery from inactivation. Reduction in CDI consequently increases the cellular calcium load (see **Table 1**).

SEROTONIN 2C RECEPTOR

The mammalian 5-hydroxytryptamine receptor subtype 2C (5-HT_{2c}) is found widely distributed in the CNS. 5-HT_{2c}R belongs to the G-protein-coupled receptor superfamily that stimulates phospholipase C (PLC) activity (Hoyer et al., 2002). The 5-HT_{2c} receptor interacts with the multi PDZ-domain bearing protein (MPDZ). Both these proteins co-localize on the apical membrane of choroid plexus cells (Drago and Serretti, 2009). The pre-mRNA encoding serotonin receptor 5-HT_{2c} is edited at five sites termed A, B, C, D, and E. Editing can lead to the exchange of three amino acids that are located within the second intracellular loop of the receptor (see **Figure 2**). This region has been shown to be important for efficient G-protein coupling (Niswender et al., 1998). Editing at the five positions in RNA can, in principle, yield a combination of 32 different mRNAs which encode 24 different protein isoforms (Burns et al., 1997; Niswender et al., 1999). In mouse brain, however, only eight major receptor variants can be detected at significant levels. Also sequencing analysis of human brain samples only detected 12 possible isoforms derived from RNA editing. This suggests that not all possible combinations of editing do exist *in vivo* (Wang et al., 2000; Wahlstedt et al., 2009). Moreover, the repertoire of possible editing combinations varies throughout development (Wahlstedt et al., 2009). Editing at sites A and B is accomplished by ADAR1, sites C, D, and E, however, are preferentially deaminated by ADAR2 (Hartner et al., 2004).

Upon editing, reduced G-protein coupling is observed in the edited states (Burns et al., 1997; see **Figure 1**). Editing not only reduces the constitutive activity of the receptor but also diminishes agonist potency and calcium release (Price et al., 2001). At the cellular level, editing changes the surface expression of the receptor. The subcellular distribution of the receptor depends on β arrestin-2 interaction with inverse correlation to the constitutive

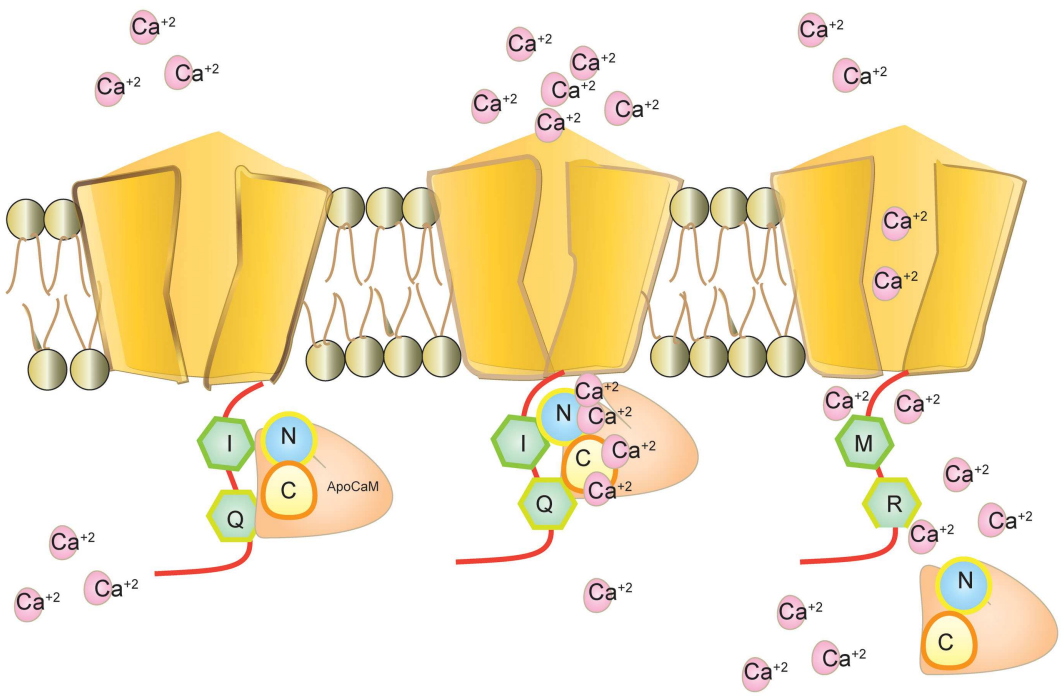

FIGURE 4 | $Ca_v1.3$ and calmodulin interaction. Calmodulin binds to $Ca_v1.3$ without calcium as apoCaM at the IQ motif. Calmodulin binds to calcium through its N-terminal and C-terminal loop and mediates calcium dependent inhibition (CDI). Editing modifies IQ to MR and inhibits calmodulin binding. Intracellular calcium increases in the absence of CDI.

activity of the receptor. Editing decreases the constitutive activity and enhances surface expression. The fully edited VGV isoform displays complete surface expression. The unedited INI isoforms exhibit endosomal accumulation whereas the edited VSV isoforms with moderate activity display vesicular and cell surface expression (Marion et al., 2004). Editing also modulates the expression of the receptor through splicing. Unedited 5-HT_{2c} transcripts result in a splice version that yields a shorter protein, while edited transcripts form the full length receptor (Flomen et al., 2004). However, the underlying factors resulting in alternative splicing are still not entirely clear. It was shown, for instance, that the human and mouse C/D box snoRNAs HBII-52 and MBII-52, respectively, can inhibit site C editing via base-pairing (Vitali et al., 2005). This base-pairing event also seemingly masks a silencer element important for the regulation of splicing (Kishore and Stamm, 2006).

The serotonin 2c receptors can also activate extracellular signal-regulated kinase (ERK) independent of G-protein coupling. Expression of the unedited INI isoform increases ERK1/2 phosphorylation in transfected HEK293 cells while expression of the edited VGV form decreases phosphorylation. However, this activation is significantly reduced upon β-arrestin depletion (Labasque et al., 2010). Editing also decreases downstream ERK signaling. Consistently, a shift toward the edited isoforms leads to reduced ERK signaling in prefrontal cortex of ADAR2 transgenic mice (Singh et al., 2011). Overexpression of ADAR2 and hyperediting of the 5-HT-$_{2c}$ receptor is also correlated with depressive behavior (Singh et al., 2009).

Mice carrying either a completely unedited "INI" version or a completely edited "VGV" version of the 5-HT-$_{2c}$ receptor have been generated (Kawahara et al., 2008). While the INI mice grow normally but are rather immobile in a forced swim assay, resembling a depressive behavior (Mombereau et al., 2010). It has also been shown that a decrease in the INI form of the receptor can lead to a decrease in ERK1/2 phosphorylation in transgenic ADAR2 mice (Singh et al., 2011). Aberrant ERK1/2 phosphorylation in turn is linked to depression and suicidal behavior as ERK1/2 plays a critical role in synaptic plasticity (Dwivedi et al., 2009).

Mice expressing the fully edited VGV version of the receptor, in contrast, have reduced fat mass, growth retardation, and high energy expenditure most likely due to hyperactivation of the sympathetic nervous system (Kawahara et al., 2008). Mutant mice with fully edited VGV isoforms have also been shown to display symptoms resembling those of the Prader–Willi syndrome (PWS; Morabito et al., 2010).

Thus, data from transgenic mice clearly demonstrate that the editing status of 5-HT_{2c}-R can directly influence behavior underscoring the importance of RNA editing for the etiology of psychiatric disorders.

ACTIN ORGANIZATION BY FILAMINS

Two actin cross-linking proteins Filamin A (FLNa) and Filamin B (FLNb) are amongst a group of newly identified mammalian editing targets (Levanon et al., 2005; Nishimoto et al., 2008; Li et al., 2009). The two 280 kDa proteins form homo- and heterodimers and mediate orthogonal branching of actin filaments (Fucini et al., 1997; Sheen et al., 2002; Popowicz et al., 2006). Mammalian filamins are built of 24 immunoglobulin (Ig) like repeats divided into two rod segments. Rod 1 consisting of repeats 1–15 interacts with actin filaments whereas rod 2 is built from repeats 16–23 and interacts with several proteins (Chen et al., 2011). Repeat 24 is required for dimerization. Actin reorganization is essential for cell motility and migration and is an important determinant in dendritic spine and synapse formation (Dillon and Goda, 2005; Popowicz et al., 2006). Depletion of FLNa leads to embryonic lethality with severe cardiovascular and bone development defects (Feng et al., 2006; Hart et al., 2006). Also FLNb deficient mice show defective microvasculature and bone malformation (Zhou et al., 2007). Editing of FLNa or FLNb leads to a conserved glutamine (Q) to arginine (R) codon exchange in repeat 22 (Li et al., 2009) that is developmentally regulated (Wahlstedt et al., 2009). Repeat 22 has been shown to be involved in the interaction with a broad range of proteins (Popowicz et al., 2006; see **Table 1**).

FLNa interacts with the C-terminus of the metabotrobic glutamate receptor mGlu5a, 5b, 7b, and 8a (see **Figure 1**). Moreover, low affinity binding was also detected for mGlu4a and mGlu7a. Repeats 21 and 22of FLNa harboring the edited amino acid represent the minimal region critical for this interaction (Enz, 2002). Editing may thus regulate this interaction, the potential consequences of which remain to be determined.

FLNa also interacts with potassium channel Kv4.2 at filipodial roots and shows overlapping expression in cortical and hippocampal neurons. A "PTPP" amino acid motif in Kv4.2 (AA 601–604) is critical for this interaction. Again, FLNa repeats 21–24 are involved in this interaction. Coexpression of filamin in heterologous cells enhances the whole cell current density by ∼2.7-fold most likely by properly positioning functional K_V4.2 receptors at the cell surface (Petrecca et al., 2000).

Presenilins (PS) belong to a conserved protein family that were the first proteins identified responsible for familial Alzheimer disease (FAD; Nelson et al., 2010). Presenilins harbor eight transmembrane domains. PS1 and PS2 were identified in a yeast two hybrid assay to interact with repeats 21–24 of FLNa. A region between TM6 and TM7 of the presenilins is responsible for this interaction. The same loop harbors 14 different mutations that are associated with FAD. FLNa and PS1 co-localize in astrocytes (Zhang et al., 1998). Moreover, overexpression of PS1 in cultured HEK293 cells redistributes FLN from the cell periphery to the cytoplasm. The FAD-linked mutation PS1M146L induces FLNa expression (Lu et al., 2010). The FLNa PS1interactionis well conserved and could be physically and genetically demonstrated in *Drosophila melanogaster* (Guo et al., 2000).

FLNa also co-localizes with the neuronal microtubule associated protein Tau. Tau is involved in polymerization and stability of microtubules. Tau protein is abnormally phosphorylated and forms neurofibrillary tangles in the hippocampus in Alzheimer patients. It is believed that Tau induced FLNa depletion leads to actin network destabilization and consequently to synaptic loss (Feuillette et al., 2010).

The functional implication of editing-induced Q2341R amino acid exchange in repeat 22of FLNa is still unknown. However, it may have an effect on a broad range of interactions (Chen et al., 2011). One example is the interaction of FLNa with β-integrin. Repeat 21 cannot interact with β-integrin unless repeat 20 disassociates from it (Lad et al., 2007). Similarly, FLNa editing may change

neuronal receptor organization as well as synaptic transmission by altering the interaction profile with binding partners.

CYTOPLASMIC FMRP INTERACTING PROTEIN 2 (CyFIP2)

CyFIP2 was identified as an interaction partner of the fragile X mental retardation protein (FMRP) in a yeast two hybrid screen. The region of interaction between CyFIP2 and FMRP overlaps with the FMRP dimerization site (Schenck et al., 2001). CyFIP2 also interacts with FMRP-related proteins FXR1P and FXR2P (Schenck et al., 2001). The CyFIP2 encoding pre-mRNA is primarily edited by ADAR2 introducing a single K320Eamino acid exchange in mouse and human CyFIP2 (Levanon et al., 2005; Nishimoto et al., 2008).

CyFIP2 is a member of the WAVE/SCAR complex and is involved in actin remodeling. It plays a pivotal role in neuronal wiring as it directly interacts with FMRP and Rac-1 (Schenck et al., 2003). Flies have a single *Cyfip* gene which is 67% identical to human CyFIP1 and CyFIP2. Mutant *Cyfip* flies display shorter synapses and profound axonal path finding, growth, and branching defects (Schenck et al., 2003, 2004). CyFIP2 is mainly involved in maintaining synaptic plasticity as it is involved in translational regulation impeded in fragile X mental retardation. In vertebrates like Zebrafish that harbor both CyFIP1 and CyFIP2, *cyfip2* mutants exhibit dorso-nasal axonal pathfinding defects (Pittman et al., 2010). RNAi of CyFIP2 in murine melanoma cells leads to aberrant lamellipodia proving the functionality of Cyfip2 in actin remodeling and cell motility (Steffen et al., 2004).

Editing at the K/E position of CyFIP2 increases during mouse brain development ranging from 4% at e15 to 75% editing at p21 (Wahlstedt et al., 2009). However, there seems a significant decline in CyFIP2 editing with age in human brain (Nicholas et al., 2010).

The biological significance of Cyfip2 editing is currently not clear. One possibility would be that the migratory behavior of cells is regulated by CyFIP2 by antagonizing Rac-1. However, interactions with FMR1 or the nucleo-cytoplasmic shuttling of Cyfip2 might equally be affected by editing. With the new discovery of modulation of ADAR by FMR1 in flies the possibility of a feed back loop of CyFip2 and ADAR regulation also appears possible as Cyfip antagonizes FMR1 in flies (Schenck et al., 2003).

HU PROTEINS

Hu proteins are RNA-binding proteins which play an essential role in neuronal differentiation and plasticity. HuB, HuC, and Hu D are neuron specific whereas HuR is associated with cell stress responses. Each Hu protein has three RNA recognition motifs (RRM1–3). Hu proteins preferentially bind to AU rich RNA elements (ARE) where they can act as RNA stabilizers and regulators of polyadenylation and translation (Mobarak et al., 2000; Zhu et al., 2006; Hinman and Lou, 2008). Recently, five editing sites were discovered in HuD and HuB in a bioinformatic screen of deep-sequencing data (Enstero et al., 2010). The functional implication of editing is unknown. However, it is likely that editing of Hu proteins can alter the landscape of the brain transcriptome (Paz-Yaacov et al., 2010).

NOVA-1

Recently, another RNA-binding protein, NOVA-1, was found to be edited (Irimia et al., 2012). NOVA-1 is a key regulator of alternative splicing of RNAs encoding synaptic proteins involved in neuronal activity in the CNS. NOVA-1 binds pre-mRNAs in a sequence dependent manner and diversifies proteins by splicing regulation. *Nova-1* null mice die postnatally from motor neuron death due to spinal and brainstem neuron apoptosis (Jensen et al., 2000). The splicing regulation by NOVA-1/2 is well conserved from mammals to insects. Both Nova-1/2 and the *Drosophila melanogaster* ortholog PASILLA (PS) binds to YCAY enriched regions located upstream of repressed exons and downstream of activated exons (Brooks et al., 2011). RNA editing increases the Nova-1 half life by decreasing its susceptibility to proteasome degradation (Irimia et al., 2012). This stabilization of Nova-1 by RNA editing can create another layer of complexity in diversification of brain specific transcripts.

Dysregulation of A to I editing has been found associated with a number of diseases, ranging from mental disorders to cancers (Paz et al., 2007). The following sections will give an overview on diseases that are strongly influenced by ADAR-mediated editing.

ASTROCYTOMA

This glial cell tumor is classified on the basis of malignancy into four grades (I–IV). Glioblastomamultiforme (GBM) is a grade IV tumor with a survival rate of less than 18 months in children and adults (Stupp et al., 2005). Glial cells respond to external stimuli via neuronal receptors (Bergles et al., 2000; Gallo and Ghiani, 2000). Hypoediting of GluA2 at the Q/R site has been observed in GBM leading to increased Ca^{2+} influx and activation of the Akt pathway through phosphorylation (Ishiuchi et al., 2007). Also in pediatric astrocytoma the malignancy increases with a decrease in editing. GBM cells show strong migratory activity which is reduced upon ADAR2 expression. Furthermore a decrease in GluK2 editing at the I/V and Y/C sites is observed in different brain regions (Cenci et al., 2008). Since both GluA2 and GluK2 are edited by ADAR2, ADAR2 overexpression strongly inhibits cell proliferation and slows down the cell cycle. Mutation in the ADAR2 deaminase domain does not affect tumor malignancy proving the necessity of editing in tumor progression (Cenci et al., 2008). In this type of tumor ADAR2 is expressed at a normal level, while ADAR1 and ADAR3 are overexpressed leading to the assumption that higher concentrations of ADAR1 and ADAR3 may inhibit the activity of ADAR2 (Cenci et al., 2008).

In pediatric astrocytoma high levels of interferon induced ADAR1 p150 are found. Overexpression of ADAR1 might again interfere with ADAR2 activity (Chen et al., 2000; Cenci et al., 2008).

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS) is characterized by slow degeneration of upper and lower motor neurons with a consequent loss of voluntary movement (Rothstein, 2009). Different mechanisms are proposed to be the underlying causes of this disease. Decreased editing at the Q/R site leading to increased Ca^{2+} influx has been observed in mice displaying late onset ALS (Kuner et al., 2005). The editing efficiency at the GluA2 Q/R site also decreases dramatically in ALS patients (Kawahara et al., 2004). Consistent with reduced Q/R site editing, a significant decrease in ADAR2 expression has been observed in spinal motor neurons of ALS patients (Hideyama et al., 2011).

However, no decline in editing of Q/R in upper motor neurons was observed.

Additionally, the flip-flop alternative splicing pattern of GluA2, downstream of the R/G editing site is pushed toward flip-bearing transcripts in ALS patients (Kawahara and Kwak, 2005). The flip form of GluA2 promotes assembly of slowly desensitizing AMPA receptors (Tomiyama et al., 2002).

Clearing of glutamate from the synaptic cleft is accomplished through glutamate transporters that prevent repeated firing and excitotoxicity. The astroglial EAAT2 glutamate transport is responsible for clearing glutamate from the cleft. ALS patients show 50% decreased EAAT2 protein levels as editing generates a cryptic polyadenylation site leading to intron 7 retention (Flomen and Makoff, 2011). Depletion of EAAT2 leads to neuronal death in transgenic mice (Rothstein et al., 1996).

PRADER–WILLI SYNDROME

The Prader–Willi locus is genomically imprinted and only expressed from the paternally inherited chromosome, while the maternal copy is transcriptionally silenced (Constancia et al., 2004). Loss of expression or mutation of the paternal 15q11–q13 locus therefore leads to the formation of the Pader–Willi disease phenotype. Patients have growth defects in both sexes due to growth hormone deficiency, and cognition problems (Butler, 2011). Amongst several other transcripts the small C/D box snoRNA MBII-52 is located within the Prader–Willi locus. This snoRNA contains 18 nucleotides that are complementary to the editing site C of the serotonin 5-HT_{2c} receptor. When expressed in nucleoli the 5-HT_{2c} pre-mRNA can even be targeted for 2′-O-methylation (Vitali et al., 2005). Loss of MBII-52 causes an increase in editing. Mice with a deleted PWS imprinted control region show enhanced locomotor activity and aberrant discriminative behavior (Doe et al., 2009). Altered 5-HT_{2C}R editing can also lead to phenotypes that mimic PWS. Mice expressing the fully edited VGV form of the serotonin receptor also exhibit PWS-like phenotypes such as hyperphagia, hypotonia, increased metabolism, and slim stature (Morabito et al., 2010). Molecularly, this isoform exhibits blunted G-protein coupling, reduced constitutive activity and enhanced serotonergic neurotransmission possibly as a consequence of increased surface expression (Kawahara et al., 2008; Morabito et al., 2010).

TRANSIENT FOREBRAIN ISCHEMIA

Cerebral ischemia in CA1 pyramidal neurons is caused by reduced oxygen supply, primarily as a consequence of heart attacks or occlusions of arteries. Neuronal damage is caused due to increased Ca^{2+} influx because of increased $GluA2^{Q}$ expression (Liu et al., 2004). Increase in calcium activates Cdk5 which phosphorylates NMDA receptors (Liu et al., 2004). Phosphorylation, in turn, prolongs opening of NMDA receptors which can activate nitric oxide synthase leading to the formation of toxic peroxynitrite that induces neuronal death (Fiskum et al., 1999; Bossy-Wetzel et al., 2004). During experimental induction of ischemia in rat brain ADAR2 expression is reduced. Consistently, recovery from ischemia can be accomplished through increased ADAR2 expression (Peng et al., 2006).

Downregulation of R/G site editing has been observed during spinal cord injury (SCI). Reduced editing at this site may limit cell death progression by suppressing postsynaptic excitation. Thus, editing might influence recovery after SCI (Barbon et al., 2010). Reduced editing at the R/G site was also observed in rat prefrontal cortex upon treatment with phencyclidene (PCP) that instigates schizophrenia like behavior (Barbon et al., 2007).

EPILEPSY

Epilepsy is a common neurological disorder characterized by seizures caused by neuronal hyperexcitability (Bozzi et al., 2012). Decreased editing of the AMPA receptor Q/R site leads to calcium permeable channels. Mice heterozygous for an editing deficient GluA2 allele develop seizures and die at 3 weeks of age while complete absence of GluA2 expression does not provoke seizures (Brusa et al., 1995).

Increased editing at the R/G site of the GluA2 transcripts and also of $K_v1.1$ have also been linked to seizures (Vollmar et al., 2004). Editing at the R/G site enhances glutamate response of the receptor and modulates neuronal excitability (Lomeli et al., 1994). The editing-induced I/V change in $K_v1.1$ channels lies in the S6 segment. This is the target site of many drugs blocking the channel (Decher et al., 2010). Interestingly, the Kv channel blocker 4-aminopyridine (4-AP) also induces seizure like events in rats. RNA editing, in turn, reduces the affinity of 4-AP and serves as a compensatory mechanism against epileptic seizures (Streit et al., 2011).

PSYCHIATRIC DISORDERS

Changes in the editing pattern of 5-HT_{2C} pre-mRNA have been linked to different psychiatric disorders such as schizophrenia, depression, and bipolar disorder (**Table 1**). Editing leads to reduced G-protein activation resulting in decreased basal activity (Niswender et al., 1999). However, the observed correlations do not allow a clear-cut conclusion. Sample sizes are typically small and the investigated samples are rarely well controlled and matched, therefore giving a heterogeneous picture. For instance, overexpression of the edited VSV receptor isoform has been observed in patients suffering from schizophrenia and bipolar disorders (Dracheva et al., 2008). Previously, in two different studies on suicide victims suffering schizophrenia, a significantly under edited B site and a hyper edited A site has been observed (Niswender et al., 2001; Sodhi et al., 2001). Analysis on suicide victims suffering major depression, in contrast, revealed an increase in editing at the C and C′ site accompanied by decreased D site editing. Treatment with fluoxetine, a serotonin selective uptake inhibitor, causes opposing effects on editing of these sites indicating site specific serotonin dependent regulation (Gurevich et al., 2002). Deregulation of A to I editing in schizophrenia and bipolar disorder (type I) patients and underediting of I/V site in GRIK2 resulting in high calcium influx has also been related to over expression of ADAR2 isoforms with diminished catalytic activity (Silberberg and Ohman, 2011; Silberberg et al., 2012). However, increase in ADAR1 expression has also been suggested as an inhibitor of ADAR2 activity (Simmons et al., 2010).

OUTLOOK

Current studies on RNA editing have clearly shown that adenosine deamination is most abundant in the CNS where it plays a major role in the diversification of the transcriptome. Three major processes seem to be primarily affected by A to I editing: first, many receptors and channels are modulated in their primary response and sensitivity to stimuli. Second, in many cases receptor assembly and retention in the ER seems to be affected by RNA editing. Finally, cytoskeletal components required for both outgrowth of neurons but also to the structuring of the cortical cytoskeleton and the anchoring of receptors is affected by RNA editing. It is one of the challenges to understand how these three processes are interconnected possibly being regulated through neuronal activity that may feed back on the process of RNA editing itself.

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Reciprocal regulation of A-to-I RNA editing and the vertebrate nervous system

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The fine control of molecules mediating communication in the nervous system is key to adjusting neuronal signaling during development and in maintaining the stability of established networks in the face of altered sensory input. To prevent the culmination of pathological recurrent network excitation or debilitating periods of quiescence, adaptive alterations occur in the signaling molecules and ion channels that control membrane excitability and synaptic transmission. However, rather than encoding (and thus "hardwiring") modified gene copies, the nervous systems of metazoa have opted for expanding on post-transcriptional pre-mRNA splicing by altering key encoded amino acids using a conserved mechanism of A-to-I RNA editing: the enzymatic deamination of adenosine to inosine. Inosine exhibits similar base-pairing properties to guanosine with respect to tRNA codon recognition, replication by polymerases, and RNA secondary structure (i.e.,: forming-capacity). In addition to recoding within the open reading frame, adenosine deamination also occurs with high frequency throughout the non-coding transcriptome, where it affects multiple aspects of RNA metabolism and gene expression. Here, we describe the recoding function of key RNA editing targets in the mammalian central nervous system and their potential to be regulated. We will then discuss how interactions of A-to-I editing with gene expression and alternative splicing could play a wider role in regulating the neuronal transcriptome. Finally, we will highlight the increasing complexity of this multifaceted control hub by summarizing new findings from high-throughput studies.

Keywords: AMPA receptor, A-to-I RNA editing, dynamics of RNA editing, R/G editing site, ADAR2

A-TO-I RNA EDITING IN THE VERTEBRATE NERVOUS SYSTEM

Although progress has been made in characterizing the functions of invertebrate editing sites, the challenge of understanding the true scale and roles of RNA editing in regulating neurophysiology in higher vertebrates continues at a somewhat slower pace. In particular, the impact of editing in non-coding regions, which harbor the vast majority of editing sites (see below) is not known. Base changes via RNA editing expand on the central dogma of molecular biology by readjusting the genetic code at the RNA level in order to substitute amino acids (Rosenthal and Seeburg, 2012). Remarkably, this occurs at functionally critical positions in targets mediating synaptic transmission. For example, editing of the α3 subunits of $GABA_A$ receptor ion channels modulates agonist potency and receptor gating properties to tune inhibition (Ohlson et al., 2007; Rula et al., 2008). Similarly, at excitatory synapses A-to-I editing is responsible for a number of recoding events in many of the non-NMDA glutamate receptor ion channel subunits (AMPA GluA2, 3, 4, and kainate GluK1, 2; Sommer et al., 1991; Köhler et al., 1993; Lomeli et al., 1994), including the efficient Q/R site conversion of GluA2, which gates calcium permeability and receptor trafficking that are essential for survival (Sommer et al., 1991; Burnashev et al., 1992; Brusa et al., 1995; Greger et al., 2002). More generally, membrane excitability is modified by A-to-I editing of select subunits of voltage-gated potassium (Kv1.1) and calcium (Cav1.3) channels resulting in altered channel inactivation properties (Bhalla et al., 2004; Huang et al., 2012). Neuromodulatory control by serotonin is also targeted, where A-to-I editing of the metabotropic receptor 5-HT_{2C} attenuates coupling to its G-protein second messenger system (Burns et al., 1997). Furthermore, editing could also regulate serotonin signaling more globally by modifying activity of the enzyme tryptophan hydroxylase-2 (TPH2, Grohmann et al., 2010), which is rate-limiting for serotonin synthesis in the brain (Zhang et al., 2004). As the list of non-synonymous codon-changes in neuron-related transcripts continues to grow (e.g., Danecek et al., 2012), it appears that A-to-I RNA editing is poised to directly tune the function of key nervous system components. This is particularly evident in invertebrates where recoding sites are more frequent and where functional changes have been elucidated (e.g., Rosenthal and Bezanilla, 2002; Hoopengardner et al., 2003; Colina et al., 2010). In fact, a recent study showed that editing of delayed rectifier potassium channels mediates temperature adaptation (Garrett and Rosenthal, 2012) to compensate

for an overall slower signaling at low temperatures, editing accelerates gating kinetics of this potassium channel in Arctic and Antarctic squid species relative to their tropical relatives (Garrett and Rosenthal, 2012). Invertebrate RNA editing is beyond the scope of our discussion and so we refer the reader to some recent reviews (Jepson and Reenan, 2008; Rieder and Reenan, 2012).

DEVELOPMENTAL REGULATION OF A-TO-I EDITING

The deamination reactions responsible for A-to-I editing are catalyzed by a family of "editases": adenosine deaminases acting on RNA (ADARs). The relatively high inosine content of brain mRNA (Paul and Bass, 1998), seizure susceptibility and lethal neurological phenotype of ADAR2 knockout mice (Higuchi et al., 2000), and the overall more selective expression of ADARs in the nervous system suggest that A-to-I editing contributes to refining neuronal function in development and during adult forms of synaptic plasticity. Developmental elevation of editing at various sites for many coding targets has been shown recently using new high-throughput sequencing technologies (Wahlstedt et al., 2009). These findings concur with earlier, more detailed studies on specific sites (e.g., Bernard and Khrestchatisky, 1994; Lomeli et al., 1994; Rula et al., 2008; Huang et al., 2012; Irimia et al., 2012). Age-dependent increases have also been documented for editing of small, non-coding RNA sequences, such as microRNAs (miRNAs), which typically bind to 3′ untranslated regions (UTRs) of transcripts to signal their degradation (Ekdahl et al., 2012). The potential for cross-talk between editing and gene expression control mechanisms in regulating neuronal development is exemplified with the case-study of miRNA cluster 379–410 (Ekdahl et al., 2012; Vesely et al., 2012). Here, editing in the critical seed regions of miRNA-381 and 376b prevents binding to Pumilio 2 (Pum2) mRNA, which codes for a translational repressor serving to negatively regulate outgrowth of neuronal dendrites. Consistent with this, developmental changes in editing of these miRNAs correlated with increased expression of Pum2 (Ekdahl et al., 2012). The increasing discovery of edits in non-coding sequences and the enrichment of some ADARs in the nervous system make it tempting to postulate that some observed tissue-specific expression patterns could result from editing-dependent switches in miRNA seed regions (Kawahara et al., 2007) or 3′-UTRs (Borchert et al., 2009), or from ADAR-modulated processing of microRNAs (Yang et al., 2006; Heale et al., 2009). Indeed, transcription profiling of the brain of ADAR2 knockout mice indicates editing could regulate the expression of a large number of genes (Horsch et al., 2011). Intriguingly, the genetic impact of A-to-I editing may be underestimated from mouse models since a disproportionately large amount of editing in humans also occurs in embedded primate-specific Alu elements that likely function to regulate gene expression (Maas, 2010).

CROSS-TALK BETWEEN A-TO-I EDITING AND ALTERNATIVE SPLICING

In addition to interactions with gene-expression control mechanisms, cross-talk exists between A-to-I editing and alternative splicing. Developmentally regulated, evolutionarily conserved RNA editing of transcripts encoding the central nervous system (CNS)-specific alternative splicing factor Nova1, reduces its degradation by the proteasome thereby increasing Nova1 protein levels (Irimia et al., 2012). Nova1 is expressed most in the ventral spinal cord where it is essential for normal postnatal motor function and notably regulates alternative splicing of multiple inhibitory synaptic targets, including the major scaffold protein gephyrin and the γ2 and α2 subunits of the $GABA_A$ and glycine receptor ion channels, respectively (Jensen et al., 2000; Ule et al., 2005). It remains to be determined how changes in editing of endogenous Nova1 impact on the splicing of its targets, and whether or not aberrant Nova1 editing could aggravate motor neuron demise in sporadic amyotrophic lateral sclerosis (ALS); a condition strongly associated with deficient ADAR2 expression and GluA2 Q/R site editing (e.g., Hideyama et al., 2010, 2012). Feedback regulation of editing exists and occurs directly via alternative splicing: ADAR2 regulates splicing of its own pre-mRNA by creating a new splice acceptor site via its A-to-I editing activity. This causes an insertion of 47 nucleotides into the coding sequence and a frameshift resulting in a truncated, catalytically inactive protein (Rueter et al., 1999; Slavov and Gardiner, 2002; Feng et al., 2006). Another interesting example demonstrating the interaction of A-to-I editing with other RNA processes occurs in the $5\text{-}HT_{2C}$ receptor pre-mRNA. Here, an alternative splice donor site (necessary for the coding of a full-length receptor isoform) is silenced by a sequence element, which is weakened either by RNA editing (Flomen et al., 2004) or by an editing-independent mechanism that involves base-pairing of a small nucleolar RNA (snoRNA) HBII-52 (Kishore and Stamm, 2006). Consequently, neurons employ an unusual mechanism to regulate the editing of full-length $5\text{-}HT_{2C}$ receptors, which is significant in maintaining a normal serotonergic system and its associated impact on cognition and behavior (Kishore and Stamm, 2006; Doe et al., 2009; Morabito et al., 2010). Editing-dependent changes in splicing efficiency are also pivotal for AMPA-type glutamate receptor subunits: the essential Q/R recoding event in the GluA2 subunit, which controls ion channel calcium permeability, is associated with more efficient pre-mRNA splicing (Brusa et al., 1995). As a result, coupled editing and splicing ensures a significantly high fraction of Q/R-edited GluA2 mRNA to tolerate modest changes in ADAR2 activity (Schoft et al., 2007; Hideyama et al., 2012; Penn et al., 2013). Also in GluA2, a correlation between R/G site editing and alternative splice site selection appears to reflect a coupling associated with the homeostatic control of AMPA receptor biogenesis and function selectively in the CA1 region of the hippocampus (Penn et al., 2012; Balik et al., 2013).

NEURONAL ACTIVITY DRIVEN REGULATION OF RNA EDITING

The prospect of activity-dependent changes in A-to-I editing is an exciting recent development. There are various studies describing changes in A-to-I editing in diseases including ALS, epilepsy, and cancer, which mostly involve the GluA2 Q/R site and are associated with Ca^{2+} influx through AMPA receptors (Krestel et al., 2004; Maas et al., 2006). Another target is the serotonin receptor; of which altered G-protein coupling efficiencies

of the 5-HT_{2C} receptor have been implicated in neuropsychiatric disorders (e.g., Gurevich et al., 2002; Bhansali et al., 2007; O'Neil and Emeson, 2012). However, one feature underlying many of these findings is that the pathological insults tend to have a dramatic impact on neuronal activity (e.g., stress, kindling, ischemia,). Some evidence points to a control of editing fundamentally by neuronal signaling. Early reports showed that serotonergic signaling via 5-HT_{2C} receptor could regulate editing of its own transcript to feedback onto the strength of receptor G-protein coupling (Gurevich et al., 2002). The same group later showed that the effect of a serotonin-selective reuptake inhibitor could reverse stress-induced changes in 5-HT_{2C} editing (Englander et al., 2005). Recent work in neuronal cultures derived from cerebral cortex has demonstrated that pharmacologically induced changes in neuronal activity can impact on ADAR targets (Orlandi et al., 2011; Sanjana et al., 2012). Altering neuronal activity in cultured hippocampal slices revealed analogous results, which turned out to be cell-type specific: editing changes occurred in the CA1, but not in CA3 subfield, which are composed of functionally and anatomically diverse neuronal cell types (Balik et al., 2013). Therefore, A-to-I editing has the capacity to fine-tune signaling in select neuronal circuitries. In two independent studies, chronic treatments lead to similar changes in AMPA receptor R/G site editing and concurrent changes in ADAR2 expression levels (Sanjana et al., 2012; Balik et al., 2013), which was also accompanied by regulation of ADAR2 self-editing (Balik et al., 2013). Interestingly, a recent study used a reporter based on the R/G site substrate to screen for repressors of ADAR2-mediated editing and identified three RNA-binding proteins (Tariq et al., 2013). The expression of two of these candidates, the splicing factor SFRS9 and the RNA helicase DDX15, was found to be regulated during mouse development and also responded to activity manipulations in CA1 of cultured hippocampal slices (Tariq et al., 2013). Binding of these factors around the R/G site might inhibit editing either by competing with ADAR2 for the substrate and/or by interacting directly with editase to reduce its activity (Tariq et al., 2013). A characterization of the physiological impact of editing site regulation in the plasticity of neuronal functions as well as an elucidation of cell-type/state specific changes in editing is now crucial and a very exciting prospect.

MECHANISMS UNDERLYING ADAR REGULATION

The mechanisms underlying editing regulation are currently unclear. These partly involve changes in ADAR levels (Balik et al., 2013), which, in the case of ADAR2, are under negative feedback control (Feng et al., 2006). However, this will depend on the efficiency of editing for a given ADAR substrate and is less likely to be relevant for strongly edited sites (Balik et al., 2013), such as the GluA2 Q/R site, for example. The "strength" of editing varies during development (Lomeli et al., 1994; Wahlstedt et al., 2009) and may be regulated in a cell- or tissue-selective manner. High-throughput sequencing data from cell lines imply overall low levels of editing (e.g., Bahn et al., 2012), but how this relates to editing levels in tissue remains to be established. Earlier reports described changes in ADAR expression levels during development (e.g., Paupard et al., 2000; Hang et al., 2008), but have recently been challenged as being responsible for observed editing site changes (Jacobs et al., 2009; Wahlstedt et al., 2009). Over the last decade, a great deal of emphasis has been placed on identifying and characterizing ADAR isoforms arising from alternatively spliced exons and transcription start sites (George et al., 2011). The varying activity of different ADAR isoforms has been described for some editing sites and so has their regulated expression during brain development (George et al., 2011), and the control of ADAR1 transcript levels by microRNAs (miRNA-1, Lim et al., 2005). The enigmatic, brain-specific (but non-catalytic) ADAR3 protein has been proposed to act in a dominant negative fashion on targets of other ADARs *in vitro* (Chen et al., 2000), but still little is known about its role and significance (Nishikura, 2010). More recently, protein structural studies have revealed candidates for the modulation of ADAR protein function. The ADAR2 catalytic domain contains a structurally integral inositol hexakisphosphate (IP_6) required for efficient editing activity (MacBeth et al., 2005). An intriguing postulation is that elevated IP_6 formed from phospholipase C (PLC) following 5-HT_{2C} activation might increase activity of nascent ADAR2 protein and account for some of the feedback onto 5-HT_{2C} receptor editing (Schmauss et al., 2010). However, further work is required to determine whether or not levels of IP_6 in neurons are rate-limiting for ADAR2 activity. Post-translational modifications have also been shown to regulate ADAR protein function or abundance, including SUMOylation, phosphorylation-dependent propyl-isomerization and ubiquitination (Desterro et al., 2005; Marcucci et al., 2011). Furthermore, the control of dynamic associations of ADARs with subcellular compartments has been proposed as a means to sequester functional ADARs away from their targets in the nucleus. For example, induced translocation of ADAR2 (and likely also the short p110 form of ADAR1) from the nucleolus can increase activity at editing sites (Desterro et al., 2003; Sansam et al., 2003). However, contextual examples for this type of regulation in the nervous system remain elusive. Another example is the cytoplasmically localized p150 form of ADAR1, which is transcribed from an interferon-inducible promoter and can undergo regulated expression in some tissues, although not in the brain (Shtrichman et al., 2002; George et al., 2005). Further clues from pathology may reveal more candidate mechanisms relevant to physiological ADAR control. One example is the potential ADAR2 regulation by CA1-specific changes of cAMP-response element-binding protein (CREB) activity that occur following transient ischemic insults (Peng et al., 2006; Kitagawa, 2007). Consistent with these suggestions, the ADAR2 promoter contains a CREB/AP-1 binding site, which incidentally has shown necessary for ADAR2 regulation in glucose-responsive pancreatic cells via the stress-activated protein kinase JNK1 pathway (Yang et al., 2012). Furthermore, a link between calcium signaling via L-type voltage-gated calcium channels and activation of nuclear CREB might be key to understanding activity-dependent changes in ADAR2 expression (Wheeler et al., 2008; Balik et al., 2013). Challenges lie ahead to identify and detail the potential routes of ADAR regulation that are physiologically most relevant in different nervous system contexts.

HIGH-THROUGHPUT SEQUENCING AND EDITING IN NON-CODING REGIONS

The lack of a clear signature for potential editing sites in gene sequence was limiting for the identification of new RNA editing sites (Hoopengardner et al., 2003). Before high-throughput sequencing techniques were available, a systematic search for new sites was based on computational analysis of the available databases containing genomic and transcriptional data. For example, human expressed sequence tags (ESTs) and cDNA data were aligned to genomic sequences to yield the discovery of four new genes subjected to editing (Clutterbuck et al., 2005; Levanon et al., 2005). However, these approaches were clearly limited as evidenced by the fact that they failed to identify all previously known editing sites. What these approaches did reveal though is that recoding sites are just the tip of the iceberg and that the majority of editing occurs in non-coding regions, which are vastly enriched in Alu repetitive elements (Athanasiadis et al., 2004; Blow et al., 2004; Kim et al., 2004; Levanon et al., 2004). These findings explained the abundance of inosine in brain mRNA (Paul and Bass, 1998) and confirmed experimental findings of editing in non-coding regions (Morse et al., 2002). Moreover, since Alu elements are primate-specific and account for >10% of the human genome, A-to-I substitutions are significantly more abundant in primates (Eisenberg et al., 2005). The specific role of these non-coding edits on nervous system operation has not been elucidated. The high abundance of Alus, particularly in gene-enriched regions, will increase the probability for oppositely oriented Alus to anneal into dsRNA secondary structures thus serving as substrates for editing. Alterations of the stability of edited dsRNA structures will affect global RNA metabolism via a link with RNA interference (e.g., Bass, 2006). The advent of high-throughput sequencing technology has led to further advances in our understanding of editing at the genome level and facilitated verification of candidate sites (Li et al., 2009; Wahlstedt et al., 2009), has revealed the interdependence or coupling of multiple editing sites within a transcript (Ensterö et al., 2009), clarified the sequence and structural determinants for editing (Bahn et al., 2012) and enabled a comparison of the sites and frequency of edits between genomes (Danecek et al., 2012). The ongoing efforts of consortia like ENCODE and the 1000 Genomes Project will undoubtedly advance these fronts further (Djebali et al., 2012; Park et al., 2012).

OUTLOOK

As the discovery of new editing sites continues, so does the need to understand their function, and regulation, in maintaining normal neurophysiology and in mediating adaptability during neuronal plasticity. It is increasingly apparent that the impact of ADARs is widespread, diverse, and under dynamic control, thus the need to dissect the functions of individual editing sites is apparent. Animal models are going some way to achieve this and their contribution to our current understanding have been reviewed (e.g., Rula and Emeson, 2007 and references therein). Recently, new manipulations have emerged that could improve the throughput for investigating the functions of A-to-I editing events, such as the use of substrate-specific helix-threading peptides (Schirle et al., 2010) and steric antisense oligonucleotides (Mizrahi et al., 2013; Penn et al., 2013). Recent advances in the delivery of oligonucleotides using cell-penetrating peptides brings researchers closer to applying these manipulations *in vivo* more routinely (Järver et al., 2012; Moulton, 2012). In the future, these tools together with the increasing capacity of high-throughput resources might lead to therapeutic approaches that could correct defective editing associated with neurological diseases in humans.

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Role of a redox-based methylation switch in mRNA life cycle (pre- and post-transcriptional maturation) and protein turnover: implications in neurological disorders

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Homeostatic synaptic scaling in response to neuronal stimulus or activation, and due to changes in cellular niche, is an important phenomenon for memory consolidation, retrieval, and other similar cognitive functions (Turrigiano and Nelson, 2004). Neurological disorders and cognitive disabilities in autism, Rett syndrome, schizophrenia, dementia, etc., are strongly correlated to alterations in protein expression (both synaptic and cytoplasmic; Cajigas et al., 2010). This correlation suggests that efficient temporal regulation of synaptic protein expression is important for synaptic plasticity. In addition, equilibrium between mRNA processing, protein translation, and protein turnover is a critical sensor/trigger for recording synaptic information, normal cognition, and behavior (Cajigas et al., 2010). Thus a regulatory switch, which controls the lifespan, maturation, and processing of mRNA, might influence cognition and adaptive behavior. Here, we propose a two part novel hypothesis that methylation might act as this suggested coordinating switch to critically regulate mRNA maturation at (1) the pre-transcription level, by regulating precursor-RNA processing into mRNA, via other non-coding RNAs and their influence on splicing phenomenon, and (2) the post-transcription level by modulating the regulatory functions of ribonucleoproteins and RNA binding proteins in mRNA translation, dendritic translocation as well as protein synthesis and synaptic turnover. DNA methylation changes are well recognized and highly correlated to gene expression levels as well as, learning and memory; however, RNA methylation changes are recently characterized and yet their functional implications are not established. This review article provides some insight on the intriguing consequences of changes in methylation levels on mRNA life-cycle. We also suggest that, since methylation is under the control of glutathione anti-oxidant levels (Lertratanangkoon et al., 1997), the redox status of neurons might be the central regulatory switch for methylation-based changes in mRNA processing, protein expression, and turnover. Lastly, we also describe experimental methods and techniques which might help researchers to evaluate the suggested hypothesis.

Keywords: alternative splicing, FMRP, glutathione, homeostatis, redox status, Rett syndrome, *S*-adenosylmethionine, synaptic scaling

METHYLATION AFFECTS PRECURSOR-RNA PROCESSING AND mRNA SYNTHESIS

METHYLATION BASED MODULATORY ROLE OF MICRORNAs

Precursor-RNA (Pre-RNA) is the immature and the incompletely processed mRNA molecule in the nucleus and which needs to be processed before exporting it into cytoplasm in fully functional mature mRNA form. The pre-RNA processing is an early yet highly regulated event in protein synthesis, wherein regulatory-RNAs (re-RNA) and RNA binding proteins (RNABPs) exert dynamic control. Micro-RNAs (mi-RNA) and other non-coding RNAs [nc-RNA; e.g., long non-coding RNAs (lnc-RNAs)], are the major re-RNAs involved in pre-RNA processing and are capable of inducing alterations in gene expression. A functional complementation exists between the levels of DNA methylation and mi-RNA function (Su et al., 2011) and expression (Saito et al., 2006; Chuang and Jones, 2007). In addition, mi-RNAs, small nucleolar RNA (sn-RNA), and anti-sense RNA can regulate the levels of DNA methylation (Qureshi and Mehler, 2010). Hence, a highly sophisticated dynamic regulatory network exists, which involves intertwined processes, (1) Methylation and (2) multifaceted actions of various non-protein coding RNAs (Weber et al., 2007). This proposal of an interactive loop corresponds to one discussed by authors Bernstein and Allis (2005), wherein the authors indicate the role of nc-RNAs like mi-RNAs, ribosomal-RNA, and transfer-RNA in DNA methylation and transcription, as well as, how some of these RNA molecules are themselves regulated by levels of DNA methylation and methyl binding proteins like MeCP2. (Mehler and Mattick, 2007).

Apart from a DNA methylation role, the mechanism through which mi-RNA or other nc-RNAs regulate pre-RNA processing

is not clearly identified. However, experts suggest involvement of mi-RNA and anti-sense RNA to sequester the processing site for mRNA transcripts and not allow normal mRNA binding. This competition between pseudogenes and mRNA for regulation of the processing site by mi-RNA has recently surfaced and is termed as "the competitive endogenous RNA" (ce-RNA) theory of mRNA translation and processing (Salmena et al., 2011). Interestingly, this processing site for regulation of mRNA translation has also been suggested to provide an intrinsic layer of control over expression patterns of mRNA (Rigoutsos and Furnari, 2010). In addition, apart from it's role in the nucleus mi-RNA also plays a regulatory role at the synapse. This is exemplified via their influence on the function of proteins like Fragile X mental retardation protein (FMRP; Mehler and Mattick, 2007), which is involved in mRNA translation and synaptic transport of about 400 different transcripts (Santoro et al., 2011) including several synaptic proteins and proteins involved in neural development as suggested by Santoro et al. (2011) Reports suggest a correlation between the decline in synaptic localization of proteins and observed neurological disorders, for example: SHANK3 in autism spectrum disorders (ASD; Durand et al., 2007) Neuroligin, neurexin, and PSD-95 (Warren, 2011) in Rett syndrome (Sudhof, 2008) and Fragile X syndrome (Wang et al., 2007). Interestingly, some of these neurological disorders are also associated with the malfunction of FMRP. (Darnell et al., 2011; Santoro et al., 2011).

RNABP METHYLATION AND REGULATION OF ALTERNATIVE SPLICING PATTERNS

Alternative splicing is one of the earliest phenomena to be identified as a "coding language" used by RNA molecules to generate protein diversity. Alternative splicing occurs by selective and guided skipping of intragenic sequences during transcription and pre-RNA processing. However, over the past decade it has been shown that patterns of alternative splicing are not just involved in protein diversity for evolutionary and developmental purposes, but they also play a major regulatory role in pre-RNA processing. This newly identified role of alternative splicing is especially important in neurons, as it affects cell fate determination, axon guidance, and synaptogenesis (Li et al., 2007). Some experts consider the process of alternative splicing in neurons as an ability of mRNA to adapt and perform differential protein expression in response to local stimulus, neuronal activation, or changes in the neuronal niche, which ultimately promotes homeostasis (Grabowski and Black, 2001; Lipscombe, 2005). Errors of splicing phenomena in neurons have been shown to be involved in several neuromuscular and neurological disorders, including spinal muscular atrophy, fronto-temporal dementia, Fragile X syndrome and Rett syndrome (Li et al., 2007). A similar regulatory role of alternative splicing can be exemplified in FMRP expression and function; for example, in *Drosophila melanogaster* the short isoform of FMRP (without the glutamine-asparagine domain) is inadequate for participating in short and long term memory formation (Banerjee et al., 2010). Deletion of the homologous region (i.e., the C-terminal domain) in human FMRP does not allow binding of kinesin, and thus consequently inhibits dendritic transport of mRNA molecules (Dictenberg et al., 2008), and affects synaptic plasticity.

The methylation status of the RNA-binding domain of RNABP is believed to regulate splicing pattern on mRNA transcript (Young et al., 2005). Methylcytosine binding protein-2 (MeCP2) recognizes 5-methylcytosine on DNA and is a critical transcription factor implicated in neuro-developmental disorders, including Rett syndrome and autism spectrum disorder (Chahrour et al., 2008). *Y-box-binding protein 1 (YBP1)* is a RNA binding protein, which interacts with MeCP2, and this conjugation critically regulates splicing, such that mutations in YBP1 or MeCP2, or alterations in MeCP2 levels (as observed in Rett syndrome), can affect mRNA splicing patterns, and cause aberrant gene expression (Young et al., 2005). Thus RNABP methylation status is an important regulator of alternative splicing phenomena.

EFFECTS OF METHYLATION ON POST-TRANSCRIPTIONAL REGULATION OF mRNA

METHYLATION OF RNA

Similar to various other post-transcriptional modifications, RNA methylation also occurs on different RNA species like tRNA, rRNA, mRNA, tmRNA, sn-RNA, snoRNA, mi-RNA, and viral RNA (Motorin and Helm, 2011). In fact, RNA methylation occurs at different positions and a variety of RNA-methyltransferases are employed for this process. It is a post-transcriptional modification, dependent on the levels of *S*-adenosylmethionine (SAM), which serves as the methyl donor (**Figure 1A**; Martin, 1992). The most common and highly studied RNA methylation is involved in the process of "capping" at the 5′ end. The guanosine nucleotides are methylated and this marking of eukaryotic mRNA allows cells to distinguish host mRNA from other types of RNA molecules including viral mRNA molecules.

Methylation of cytosine (5MeC), well-known for DNA, has also been recently reported for RNA (Rozenski et al., 1999). However, levels of 5MeC in RNA are low, and the major form of methylation in RNA (i.e., about 30–50% of total RNA methylation) is reported to occur at the 6-position on adenine residues (^{m6}A; Martin, 1992). 5MeC has been described in RNA species like rRNA and tRNA (Rozenski et al., 1999), whereas the highly conserved heterogeneous RNAs (hnRNA) show ^{m6}A residues (Yu, 2011). However, the methylated 6-adenine (^{m6}A) residue is localized in a general consensus sequence, G^{m6}AC or A^{m6}AC in almost all RNA transcripts (Wei and Moss, 1977). Some studies discussed in this section also indicate a significant correlation between alterations in ^{m6}A levels and subsequent changes in mRNA processing activity. In particular, two separate studies demonstrated about a 1.5 fold elevation in translation of dihydrofolate reductase (DHFR) RNA transcript in correlation with an increase in its level of methylation on mRNA transcripts (Heilman et al., 1996), whereas inhibition of methylation capacity by depleting SAM levels led to a decrease in DHFR transcript processing (Tuck et al., 1999). Levels of ^{m6}A also regulate the selection of splicing sites, and supporting evidence shows that SAM depletion disrupts splicing patterns, and decreases cytoplasmic and consequently synaptic localization of mRNA molecules (Caboche and Bachellerie, 1977).

BC1, a small non-coding RNA (snc-RNA) is highly expressed in neurons (Muslimov et al., 2002) and enriched at synapses (Chicurel et al., 1993). It forms a ribonucleoprotein (RNP) with several partners including FMRP and acts as a liaison between FMRP

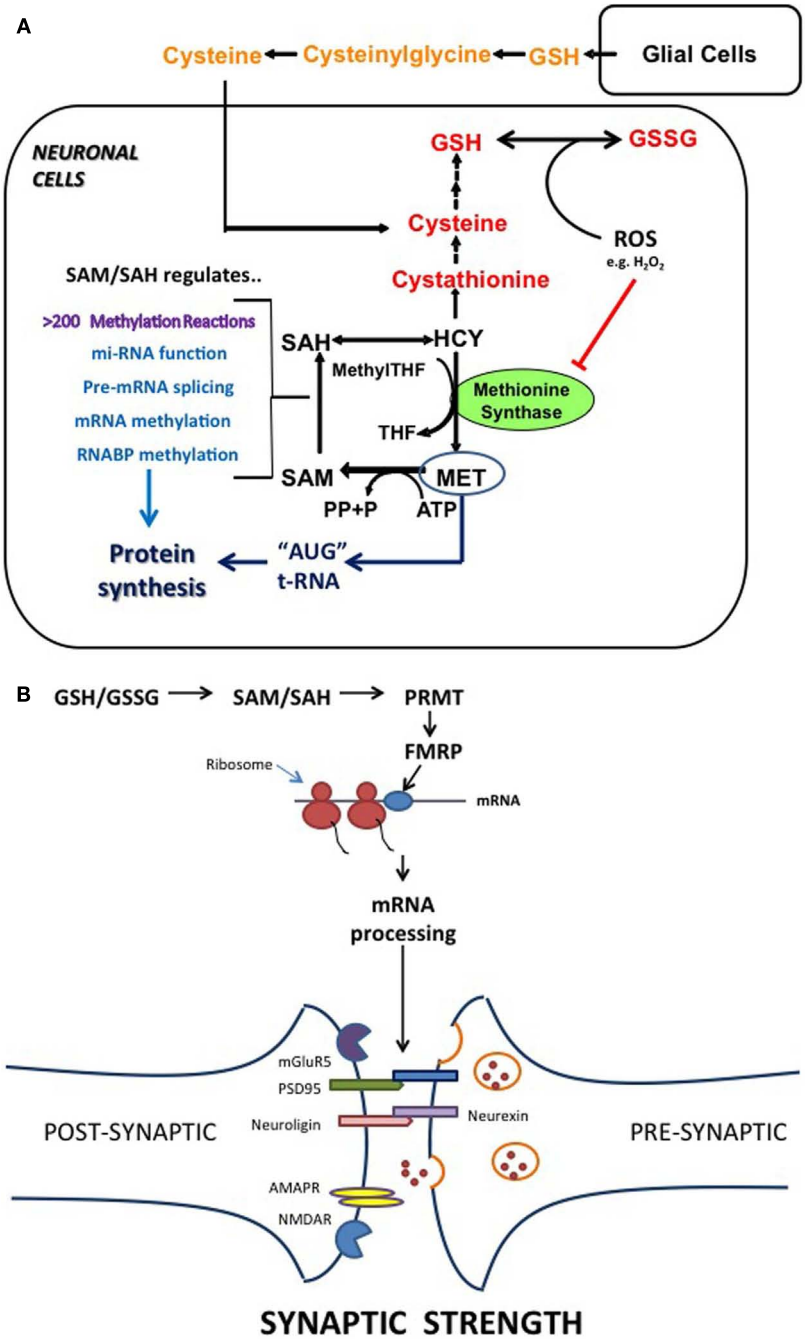

FIGURE 1 | Summary of the hypothesis. (A) *The relationship between the methionine cycle of methylation and the transulfuration pathway which converts HCY to cysteine.* In brain, glial cells are a primary source of released GSH, which is hydrolyzed to cysteine in the extracellular space. (Raps et al., 1989; Hirrlinger et al., 2002) The intracellular availability of cysteine is rate-limiting for GSH synthesis, and the GSH/GSSG-based redox status is regulated through a combination of cysteine uptake and transsulfuration of HCY. Redox status regulates the SAM/SAH level via its influence on methionine synthase. More than 200 methylation reactions are dependent on SAM levels; Key steps in mRNA processing are regulated via SAM-dependent methylation. Levels of intracellular methionine affect protein synthesis, since it is the required amino acid for initiation of translation. **(B)** *An example of a methyltransferase being regulated by redox status which affects neuronal plasticity.* PRMT is a SAM-dependent methyltransferase which methylates the RGG domain of FMRP (a RNABP). FMRP is involved in regulation of about 400 different mRNA transcripts, including NLGN3 (neuroligin-3), PSD-95 (post synaptic density protein-95) and the AMPA-type glutamate receptor. Thus redox status, acting via methylation reactions, can control synaptic strength between neurons, thereby providing a potential molecular mechanism for Hebbian learning and memory formation.

and FMRP's target mRNA molecules (Zalfa et al., 2003). The tudor domain of FMRP selectively binds to non-methylated BC1 (Zalfa et al., 2003, 2005). Recently, it has also been shown that BC1 in intracellular compartments contains 2′-O-methylation in the FMRP binding domain, whereas if present at synapses, BC1 lacks the 2′-O-methylation mark, which allows its FMRP interaction (Lacoux et al., 2012). Thus, the methylation status of BC1 indirectly regulates translation and mRNA processing at synapses by regulating FMRP (Lacoux et al., 2012). Studies show that hypomethylation of precursors and intermediates of ribosomal-RNA in the nucleus inhibits their cytoplasmic export and prolongs nuclear accumulation, thus inhibiting further RNA processing (Dictenberg et al., 2008). In fact, Vaughan et al. (1967) showed that depriving cells of methionine and limiting the methylation capacity leads to a blockade of ribosome production itself. A similar study with cycloleucine (a reversible inhibitor of nucleic acid methylation) showed that hypomethylation affects the RNA maturation process at different stages and results in altered mRNA levels in a cumulative manner (Caboche and Bachellerie, 1977). All of the above effects can result in alterations in mRNA translation, and in neurons it might lead to protein deprivation at synapses and hence hinder changes in synaptic plasticity. However, these are preliminary results and further proof would be required to support this idea.

RNA BINDING PROTEIN METHYLATION

mRNA biogenesis depends upon nuclear formation of a messenger ribonucleoprotein particle (mRNP), which is then exported to the cytoplasmic compartment (Yu, 2011). RNABPs regulate a highly dynamic, yet well-orchestrated molecular organization and recognition pattern for mRNP formation. Arginine methylation, which is a major feature of post-transcriptional regulation, occurs on almost all RNABPs, including heterogeneous RNP (hnRNP) and serine/arginine-rich (SR) proteins (Bedford and Clarke, 2009). Arginine methylation is implicated in various cellular processes including, but not limited, to transcription and RNA processing, which includes nuclear export and synaptic localization of mRNA (Yu, 2011). In mammals, the process of arginine methylation is performed by about 10 known protein arginine methyltransferases (PRMTs), whose activity is dependent on levels of SAM as the methyl group donor (Bedford and Clarke, 2009). Thus depletion of SAM decreases PRMT methylation of FMRP (Santoro et al., 2011), which alters processing of mRNA transcript associated with FMRP.

As mentioned above, RNABP methylation occurs at a specific consensus domain known as "RGG" (arginine flanked by glycine) (Bedford and Clarke, 2009). The RGG domain in RNABP recognizes a particular mRNA transcript and selectively binds to it, which results in mRNA translation and/or transport. Indeed, studies involving FMRP show that alterations in the level of RGG methylation are closely associated with changes in protein-protein and protein-RNA interactions (Dolzhanskaya et al., 2006). In the case of FMRP, it was also suggested that differential RGG methylation levels in FMRP can strongly affect the affinity of FMRP for about 400 different mRNA transcripts, about 95 of which belong to proteins involved in synapse formation, and approximately 28 of these proteins are implicated in autism (Santoro et al., 2011). Preliminary results implicate a similar regulatory role of methylation status of mRNA transcript in the KH2 type RNA-binding domain of RNABP, in RNA-RNABP kissing complex formation (De Boulle et al., 1993). A I304N mutation in the KH2 domain of FMRP blocks its ability to bind to polyribosomes and regulate RNA processing (Feng et al., 1997). Additionally, investigators have generated mouse models for Fragile X syndrome carrying this I304N mutation in the KH2 domain of FMRP, and have showed specifically that lost RNA binding ability (due to mutation) led to decrease in FMRP levels and polyribosome association (Zang et al., 2009). In addition, a similar mutation is observed in the KH2 RNA-binding domain in post-mortem brain samples from Fragile X syndrome patients (De Boulle et al., 1993). Similarly, several other proteins possessing a conserved RGG domain play an important role in pre-RNA processing (e.g., "RGG" methylation in spliceosomal small nuclear ribonucleoproteins (snRNPs) regulates alternative splicing). Hence, binding of RNA to RNABP and formation of mRNPs during transcription is a dynamic yet ordered process, and a number of factors involved in the process, appear to be influenced by methylation capacity and levels of methylation on RNA as well as RNABP.

REGULATION OF PROTEIN TURNOVER VIA METHYLATION CAPACITY

Protein arginine methylation, the process of adding monomethyl or dimethyl groups to arginine residues, is a well-known methylation reaction (Gary and Clarke, 1998). About 12 ATPs are required per methylation cycle and evolutionary retention of such an "expensive" system underscores the biological importance of this post-translational modification (Boisvert et al., 2003). Histone arginine methylation and myelin basic protein were the first proteins known to be methylated (Paik and Kim, 1968; Brostoff and Eylar, 1971). At present, more than 200 proteins are known to contain RG-repeats and can be methylated at arginine residue by different classes of PRMT (Boisvert et al., 2003). Most of these proteins are associated with RNA maturation process as mentioned earlier, and are involved in mRNA translation regulation through RNABP (Boisvert et al., 2003). Thus, all these studies support the general concept that protein arginine methylation regulates localization and turnover of synaptic proteins.

Methionine is the initiating amino acid for protein synthesis, as the starting codon sequence "AUG" on any mRNA molecule corresponds to methionine. Hence, intracellular levels of methionine can regulate initiation of protein synthesis. Lower methionine availability (for methionine-loaded MET-tRNA) would result in decreased initiation of translation, affecting a wide range of cellular functions. In neurons this could decrease the rate of synaptic protein synthesis, limiting the ability to dynamically adjust the composition of the proteome in accordance to changes in neuronal niche. Importantly, protein lifespan depends partly upon the ubiquitinylation of exposed lysine residues at their epsilon amino group in a protein which targets these proteins for proteasomal degradation. However, methylation or homocysteinylation of these sites will block ubiquitination and extend protein lifespan, allowing integration with protein synthesis (Shukla et al., 2009; Williamson and Whetton, 2011). Thus, the equilibrium between levels of methionine (MET) and homocysteine (HCY) is important for

normal translation, and protein turnover. This would act as a regulatory point for modulating protein homeostasis at synapses, thus regulating synaptic plasticity.

CENTRAL REGULATORY REDOX SWITCH

The methylation potential depends on levels of SAM and SAH, as described above and as indicated in **Figure 1A**. However, levels of SAM are in turn dependent upon the levels of methionine, homocysteine, and activity of the folate and vitamin B12 dependent enzyme methionine synthase (**Figure 1A**). Most importantly, methionine synthase activity is highly sensitive to cellular redox status and to fluctuations in the major intracellular anti-oxidant, glutathione (GSH; Waly et al., 2004). The methyl group donated by SAM derives from adenosylation of methionine, and during all SAM-dependent methylation reactions, donation of this methyl group results in *S*-adenosylhomocysteine (SAH), which is an inhibitor of methylation, based upon its competition with SAM for methyltransferase binding (Yi et al., 2000). SAH is reversibly converted to HCY by SAH hydrolase, whose activity affects the rate of methylation reactions (Chiang et al., 1996). Methionine synthase, which forms MET from HCY, also regulates SAM levels, as indicated in **Figure 1A**. More than 200 methylation reactions (including DNA and RNA methylation) are dependent upon the SAM/SAH ratio (Petrossian and Clarke, 2011). Interestingly, one of the most widely accepted causes of DNA and RNA damage in a cell is oxidative stress, which is induced by a decline in levels of the major anti-oxidant GSH. Levels of GSH in neurons can be maintained by HCY and MET through the intermediates cystathionine and cysteine. Abnormal levels of these metabolites, including cysteine, GSH, SAM, and SAH have been extensively demonstrated in ASD (James et al., 2004; Deth et al., 2008). In addition, redox levels have also been directly linked to regulation of mRNA (Ufer et al., 2010) as well as micro-RNA (Wiesen and Tomasi, 2009; Ufer et al., 2010). However, the reader is asked to seek further literature from the references cited above. Thus, anti-oxidant levels can regulate SAM/SAH-based methylation reactions throughout the cell, with implications for the clinical pathophysiology of neurological and neuro-developmental disorders.

The concept of a redox-based methylation switch for mRNA-related events requires validation through experimental investigation of the hypothesis we put forth. Recent technological advancements allow individual mRNA transcript sequencing as well as whole transcriptome sequencing using the SOLiD™system (Lao et al., 2009). Hence, methylation changes on mRNA transcripts in animal models of neurological disorders (e.g., Rett syndrome, ASD, etc.), as well as in post-mortem brain samples of patients suffering from these disorders, can be measured at the individual mRNA transcript level. Bisulfite sequencing can be used to measure methylation status in the whole transcriptome, and population-based transcriptome comparisons can be analyzed (Schaefer et al., 2009). In addition, a cause-effect relationship between levels of methylation in mRNA/RNABP or other such effectors, and resulting neurological or behavioral effects, should also be investigated. This is exemplified by the correlative studies described above, involving the FMRP methylating enzyme PRMT and resulting neurological changes observed with its decreased activity (**Figure 1B**; Santoro et al., 2011). In addition, manipulations of the redox state in neuronal cells can be altered by oxidative insults and/or anti-oxidant interventions (e.g., *N*-acetylcysteine or GSH). Comparisons of subsequent changes in mRNA methylation patterns across the transcriptome or in individual transcripts can be measured by bisulfite sequencing, as described above, as well as by mass spectrometry (Qiu and McCloskey, 1999).

Some of the experiments suggested above are exemplified from studies performed by researchers in Germany (Hermes et al., 2004). In this study, researchers manipulated redox conditions and investigated subsequent effects on methylation potential (SAM/SAH levels) and alterations in levels of mRNA and DNA methylation. They induced hypoxia in HepG2 cell cultures, which led to increased SAM and decreased SAH levels with about four-fold elevation in methylation potential (Hermes et al., 2004). Real-time PCR amplification quantified specific mRNA transcripts, namely VEGF and erythropoietin. Incorporation of radiolabeled L-[methyl-^{3}[H]-methionine] and ^{14}[C]-uridine into mRNA reported that inhibition of SAH hydrolase led to decreased methylation potential and decreased mRNA methylation, which suggested that increased SAH levels led to probable inhibition of mRNA-methyltransferase, which is consistent with reports from other studies (Backlund et al., 1986). Similar studies could be performed for neuronal cell cultures and with advanced techniques like FRAP (fluorescence recovery after photobleaching), the synaptic localization or transport of proteins could be tested (Antar et al., 2005). Additionally, by keeping animals/cell cultures in a hypobaric or hyperbaric oxygen chamber and using optogenetic tools to selectively stimulate a certain population of cells involved in particular brain function, correlations could be made between brain activity, redox status, and synapse formation. For these purposes, redox status could be evaluated by using magnetic resonance imaging (MRI) or single-photon emission computed tomography (SPECT) to image ^{99m}Technetium hexamethylpropyleneamine oxime (HMPAO) conjugated to glutathione (Suess et al., 1991). Post-mortem gene expression analysis could then be performed in addition to quantification of mRNA and DNA methylation status using techniques described above. These and other such studies would allow researchers to test the underlying major hypothesis that redox state is the ultimate source of regulatory control over mRNA methylation, mRNA processing, protein synthesis, and protein turnover.

CONCLUSION

Thus, evidence from a number of studies indicates that, methylation capacity and methylation levels of mRNA play a major role in it's maturation and processing, which further affects protein expression and synaptic localization. Any alterations in these key phenomena can trigger an array of effects which might terminally result in neurological disorders. Redox status adds another intricate layer to the sparsely clarified processes mentioned above. However, redox state should be considered as a powerful tool, which can be manipulated to study mRNA regulation and strengthen our current insights of basic biological processes.

The temporospatial localization of proteins is important for synaptic plasticity and a redox-based methylation switch provides modulation of mRNA maturation and lifespan, which eventually

influences protein homeostasis at synapses and influences higher-order cognitive functions. A "*holonarchy*" for synaptic plasticity can be imagined, beginning at mRNA synthesis, transcription, translation, protein turnover, methylation reactions, and at the highest level redox status serves as the central regulatory switch. All these biological processes are individually highly dynamic and complex, yet they are well-coordinated and interrelated processes which provide feedback regulation to each other in order to control and maintain homeostatic synaptic plasticity. However, significant additional evidence supporting this hypothesis is needed, which will not only help in clarifying the functional linkage between key regulatory factors like mi-RNA, RNABPs, GSH, and SAM/SAH, but will also identify potential targets for treating neurological disorders like ASD, Fragile-X syndrome, and other synaptic protein deficiency disorders which can result from defects in mRNA maturation and processing.

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Genome-wide approaches to dissect the roles of RNA binding proteins in translational control: implications for neurological diseases

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Translational control of messenger RNAs (mRNAs) is a key aspect of neurobiology, defects of which can lead to neurological diseases. In response to stimuli, local translation of mRNAs is activated at synapses to facilitate long-lasting forms of synaptic plasticity, the cellular basis for learning, and memory formation. Translation, as well as all other aspects of RNA metabolism, is controlled in part by RNA binding proteins (RBPs) that directly interact with mRNAs to form mRNA-protein complexes. Disruption of RBP function is becoming widely recognized as a major cause of neurological diseases. Thus understanding the mechanisms that govern the interplay between translation control and RBP regulation in both normal and diseased neurons will provide new opportunities for novel diagnostics and therapeutic intervention. As a means of studying translational control, genome-wide methods are emerging as powerful tools that have already begun to unveil mechanisms that are missed by single-gene studies. Here, we describe the roles of RBPs in translational control, review genome-wide approaches to examine translational control, and discuss how the application of these approaches may provide mechanistic insight into the pathogenic underpinnings of RBPs in neurological diseases.

Keywords: translation, neurological disease, RNA binding proteins, ribosome profiling, CLIP

INTRODUCTION

Analogous to DNA, which is organized and packed via strong associations with histones in the nucleus, precursor, and mature messenger RNAs (mRNAs) never exist as "naked" ribonucleic acid sequences. After transcription in the nucleus, RNA binding proteins (RBPs) recognize *cis*-regulatory RNA elements within precursor mRNA sequence to form messenger ribonucleoprotein (mRNP) complexes. Again, analogous to DNA-binding proteins such as transcription factors that regulate gene expression by binding to DNA elements in the promoters of genes, RBPs regulate the fate of target RNAs by interacting with specific sequences or RNA secondary structural features within the transcribed RNA molecule. These *cis*-regulatory RNA elements can be found in the 5′ and 3′ untranslated regions (UTRs), introns, and exons of all protein-coding genes. RNA elements in 5′ and 3′ UTRs are frequently involved in targeting RNA to specific cellular compartments, affecting 3′ end formation, controlling RNA stability, and regulating mRNA translation. RNA elements in introns and exons are known to function as splicing enhancers or silencers to control the process of precursor mRNA splicing (Jensen et al., 2009).

A genome-wide survey of 323 mouse RBPs by *in situ* hybridization in the developing brain yielded the surprising result that two-thirds of those RBPs are expressed in a cell type specific manner (McKee et al., 2005). Compared to other cells in the body, the complex structure and specialization of neurons explains the need for having many RBPs to maintain proper neural function. Consistent with the crucial roles of RBPs in regulating RNA homeostasis in the nervous system, mutations that impair RBP function have been linked to severe neurological diseases such as Fragile X syndrome (FXS), Fragile X-associated tremor/ataxia syndrome (FXTAS), Amyotrophic lateral sclerosis (ALS), Frontotemporal lobar dementia (FTLD), Spinal muscular atrophy (SMA), and Myotonic dystrophy (Lukong et al., 2008). To understand the impact of mutations within RBPs in neurodegeneration, we need to elucidate the normal activities of RBPs in neurons. It is well-known that RBPs are intimately involved with the regulation of alternative splicing, a process by which numerous isoforms are generated from a single genetic loci, and is in fact, more prevalent in the nervous system than in any other cell types (Yeo et al., 2004; Wang et al., 2008a). RBPs are required to protect mRNAs during their transport from the soma to distal axonal and dendritic locations, and once at these locations, RBPs mediate local *de novo* synthesis of proteins (translation). Local translation at or near axonal and dendritic synapses is the underlying mechanism of synaptic plasticity (Sutton and Schuman, 2006), which refers to the ability of synapses to undergo long-lasting biochemical and morphological changes in response to stimuli (Richter and Klann, 2009). As a result, local translation is critical for cognition and memory. Local synaptic translation is also critical for axon guidance and nerve regeneration (Willis et al., 2005). Accordingly,

pharmacological inhibition of protein synthesis prevents some forms of synaptic plasticity in cultured neurons and attenuates long-term memory in mice (Scharf et al., 2002; Kelleher et al., 2004; Banko et al., 2005; Sutton and Schuman, 2006).

Given the significance of RBP biology and mRNA translation in controlling neuron structure and function, advances in sequencing and microarray technology have sparked the development of genome-wide methods that enable the neuroscience community to dissect the roles that RBPs play in controlling mRNA translation in the brain. Here we review how RBPs associate with different mRNP complexes to regulate translation, summarize emerging genome-wide methods that enable an unbiased examination of translation on a global scale, and discuss how genome-wide studies using these methods have and will continue to aid our understanding of translational control in normal and pathological neurobiology.

MESSENGER RNP COMPLEXES AND TRANSLATIONAL CONTROL

From synthesis to destruction, mRNAs are coated with RBPs that sequester mRNA into mRNP complexes and ultimately influence their cellular fate. These mRNP complexes, as depicted in **Figure 1**, are polysomes, RNA granules, RNA particles, stress granules (SGs), processing bodies (P-bodies), and RNA-induced silencing complexes (RISCs). This section provides a brief description of these complexes and introduces RBPs with roles in translation that associate with these complexes (for more details, see Kiebler and Bassell, 2006; Sossin and DesGroseillers, 2006; Erickson and Lykke-Andersen, 2011).

Polysome complexes are the centers of protein production and are present in the cell body, axon, and dendrites of a neuron (Steward and Levy, 1982; Giuditta et al., 2002). RBPs such as Lin28 have been shown to decorate polysomes and promote translation (Balzer and Moss, 2007). It is important to note, however, that polysome-associated mRNAs can be translationally repressed. Several groups have reported instances where translational inhibition of certain proteins did not correspond to a decrease in ribosome number on the encoding mRNAs (Olsen and Ambros, 1999; Braat et al., 2004; Nottrott et al., 2006; Petersen et al., 2006). Such observations likely captured an event called ribosomal stalling, where ribosomes temporarily or permanently stop elongating along transcripts. The RBPs FMRP and Staufen have been shown to induce ribosomal stalling (Thomas et al., 2009; Darnell et al., 2011). MicroRNA-loaded RISCs (miRISCs), which also associate with polysomes, can repress translation by promoting ribosomal pausing (Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006).

For local translation to occur at synapses, mRNAs must be transported from the soma to synapses. RNA particles and RNA granules function to traffic mRNAs to designated subcellular compartments. These structures are complexes of mRNAs and interacting RBPs, motor proteins, and adaptor proteins that tether the RBPs to motor complexes. While in transit, transcripts are both protected from degradation and are translationally repressed until the appropriate signals are received. The RBP Zip-code binding protein 1 (ZBP1) is a well-known regulator of mRNA transport and translational repression (Huttelmaier et al., 2005). Other RBPs such as Staufen, FMRP, and Pumilio also suppress translation of

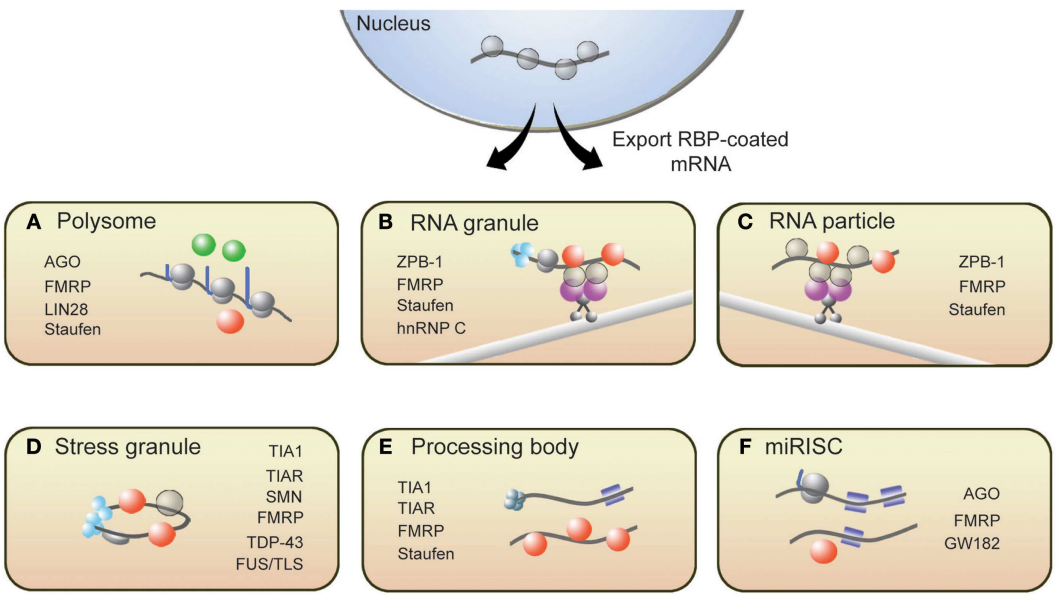

FIGURE 1 | Messenger RNAs associate with several RNP structures that influence their translational state. (A) Polysomes, sites of translation, contain RBPs that activate (green spheres) or repress (red spheres) translation. Following synthesis and processing, mRNA is exported from the nucleus and transported throughout the cell along microtubules via **(B)** RNA granules and **(C)** RNA particles. Repressor RBPs (red spheres) are present within RNA particles to ensure that mRNAs are not translated during transit. Messenger RNAs within RNA granules are associated with translation initiation machinery (light blue spheres) including ribosomes, suggesting that translation has commenced but is halted during transit. The translational fate of mRNA is dictated in part by the RBPs bound to them. If targeted by repressor RBPs or miRISCs (blue squares), mRNAs will associate with **(D)** stress granules, **(E)** processing bodies, or **(F)** miRISC structures resulting in either degradation or translational repression. Some RBPs present in neuronal RNP complexes are listed.

transcripts in RNA particles and RNA granules (Kiebler and DesGroseillers, 2000; Wang et al., 2010). An important distinction between these two transport complexes is that RNA granules contain ribosomes while RNA particles do not (Sossin and DesGroseillers, 2006). The presence of ribosomes within RNA granules suggests that the translation of associated mRNAs is blocked at the step of translational elongation.

RNA binding proteins are present within several other mRNP complexes that contain translationally repressed mRNAs. In response to cellular stress, TIA1, TIAR, G3BP, and other RBPs aggregate to form SGs, which contain untranslated mRNAs (Buchan and Parker, 2009; Kedersha and Anderson, 2009). SGs are proposed to safeguard specific mRNAs from destruction during cellular stress and, upon relief of the stress signal, disassemble to allow translationally repressed mRNAs to re-enter translation. P-bodies are another type of mRNP structure containing non-translating mRNAs that are destined for degradation (Coller and Parker, 2005; Teixeira et al., 2005) or are eventually released to re-associate with polysomes (Brengues et al., 2005). While the composition of P-bodies is not fully characterized, they generally contain decapping enzymes, exonucleases, translational repressors, microRNA (miRNA) silencing machinery, and translation-regulating RBPs, including CPEB, Staufen, and eIF4E (Parker and Sheth, 2007). Lastly, guided by miRNAs, RISCs repress translation of target mRNAs at the stage of translation initiation or elongation (Valencia-Sanchez et al., 2006). RBPs such as FMRP have been shown to associate with miRISC (Caudy et al., 2002; Witold, 2005) and can either promote or antagonize the repressive actions of miRISC (Brodersen and Voinnet, 2009). These translation-silencing mRNPs have been observed to interact with one another, and some components of SGs overlap with P-bodies (Kedersha et al., 2005) and miRISCs with P-bodies (Liu et al., 2005; Edbauer et al., 2010); however, the mechanisms that mediate these interactions remain to be established.

The interactions between RBPs and mRNAs are dynamic, allowing mRNAs to move from one mRNP to another in a controlled, bidirectional manner. This is an important feature of mRNA regulation because it ensures that the post-transcriptional fate of mRNAs is responsive to intracellular and extracellular signals. Signaling pathways, which are stimulated by various intracellular and extracellular cues, largely influence the mRNP distribution, and thus translational status, of mRNAs by regulating the expression and/or function of RBPs. For example, activation of the mTOR signaling results in the phosphorylation of FMRP (Narayanan et al., 2008); this post-translational modification affects the ability of FMRP to regulate translation (Ceman et al., 2003) and associate with RISC complexes (Cheever and Ceman, 2009). In response to signal-induced synaptic activation, the RBP Staufen was shown to activate translation by redistributing target mRNAs from RNA granules to translating polyribosomes (Krichevsky and Kosik, 2001).

GENOME-WIDE APPROACHES TO STUDY TRANSLATION

Advancements in technologies have significantly improved our ability to study translation at a genome-wide scale. Highly parallel techniques such as microarrays and high-throughput sequencing (deep sequencing) have revolutionized approaches to gene discovery, offering unbiased approaches and may be modified to investigate specific aspects of RNA regulation. In this section, we highlight studies that have utilized such technologies to investigate translational control at the genome-wide level both within neural and non-neural contexts (summarized in **Table 1**).

GENE EXPRESSION PROFILING USING MICROARRAYS

With the ability to examine gene expression on a global scale, microarray studies have provided evidence that diverse populations of mRNAs are localized at synapses. Martin and colleagues (Poon et al., 2006) were one of the first groups to examine synaptically localized mRNAs in rat neurons by mechanically separating axonal and dendritic processes from the cell body and performing microarray analysis on the isolated mRNA. Strikingly, they found that a significant proportion of synaptic mRNAs encoded translation factors and regulators, and proposed that this may be a general mechanism to enhance the capacity for local translation at synapses. Zhong et al. (2006) performed microarray studies on rat brain mRNA, which led to the discovery that the repertoire of synaptic mRNAs is more diverse than previously thought. The group not only identified transcripts that encoded translation factors and regulators, but also transcripts that encoded receptor and channel proteins, signaling molecules, cytoskeleton, and adhesion proteins, membrane trafficking proteins, and molecules involved in protein degradation. Additional studies have examined the synaptic transcriptome within other contexts, such as brain-derived nerve growth stimulation (Schratt et al., 2004) or neurons displaying molecular signatures of Alzheimer's disease (Williams et al., 2009). Interestingly, results from these and other microarray studies displayed little overlap, suggesting that a large number of mRNAs can be sequestered at synapses but that their localization largely depends on the cellular context.

POLYSOME PROFILING

A widely held view is that mRNA expression correlates closely with expression of the protein it encodes; this is certainly true in most instances, but is not always the case (Anderson and Seilhamer, 1997; Gygi et al., 1999). Indeed, the lack of correlation between mRNA and protein expression is expected given that multiple mechanisms are in place to control translation of mRNA. In this regard, microarray studies provide limited insight into the translational status of mRNA. An alternative approach called polysome profiling exploits the observation that, in general, polysome-associated mRNAs are translationally active. By separating polysome, monosome, and other mRNP complexes by centrifugation through a sucrose gradient, well-translated mRNAs can easily be distinguished from poorly translated mRNAs (**Figure 2A**). Morris and colleagues were one of the pioneering groups that used polysome profiling to examine the translation state of the transcriptome (Zong et al., 1999). Specifically, total cytoplasmic extracts from cultured human fibroblasts were layered onto sucrose gradients and centrifuged to separate the different mRNP complexes. Transcripts residing in high-density fractions containing polysome species were examined by microarray analysis to identify well-translated mRNAs (**Figure 2A**), while

Table 1 | Genome-wide methods to study translation.

	RNA isolation methodology	Novelty/advantages	Limitations	Reference
Polysome profiling	Purification of polysome-associated mRNAs by centrifugation through a sucrose gradient	Original method to examine translation status of transcriptome	Labor intensive; scaling issues; does not differentiate between active and stalled ribosomes	Zong et al. (1999)
TRAP	Immunoprecipitation (IP) of EFGP-L10a-associated mRNAs from mouse brain tissue	Examines polysome-associated mRNAs within a specific cell type *in vivo*	Each bacTRAP mouse line is limited to surveying one cell type; EGFP antibodies are costly relative to anti-HA antibody; does not differentiate between active and stalled ribosomes	Heiman et al. (2008), Doyle et al. (2008)
RiboTag	IP of Rlp22-HA-associated mRNAs from mouse tissue	Examines polysome-associated mRNAs within a specific cell type *in vivo*; takes advantage of Cre recombinase-expressing mouse lines to expand the range of cell types that can be investigated; commercial anti-HA antibody is less costly than in-house EGFP (see TRAP)	Does not differentiate between active and stalled ribosomes	Sanz et al. (2009)
Ribosome profiling	Nuclease digestion of polysome complexes, followed by centrifugation through a sucrose gradient or cushion to purify ribosome-mRNA complexes; ribosome-protected fragments are deep sequenced	Determines ribosome position and translation efficiency for individual mRNAs; reveals novel translational regulatory features (e.g., uORFs, start and termination sites, ribosome stall position)	May be difficult to apply to mouse models	Ingolia et al. (2009), Ingolia et al. (2011)
CLIP	UV-mediated crosslinking of mRNA-protein complexes, followed by nuclease digestion and IP of RBP of interest to recover RBP-protected mRNA fragments	Demonstrated the feasibility of crosslinking mRNA and protein using UV irradiation, which results in covalent bonds	Generated a limited dataset with a high false positive rate; low crosslinking efficiency	Ule et al. (2003)
CLIP-seq or HITS-CLIP	CLIP coupled with deep sequencing	Identifies direct RBP binding sites at nucleotide resolution	Low crosslinking efficiency	Licatalosi et al. (2008)
iCLIP	HITS-CLIP with modifications whereby a 5′ adapter and random barcode is attached to cDNA molecules after reverse transcription; the former modification allows for circularization of the cDNA	Introduction of a random barcode enables identification and quantification of unique cDNA products; cDNA circularization allows for the capture and sequencing of truncated cDNAs usually lost with standard CLIP, revealing crosslinking sites at nucleotide resolution	Low crosslinking efficiency	König et al. (2010)
PAR-CLIP	Photoreactive ribonucleoside analogs (e.g., 4SU or 6-SG) are incorporated into mRNA; nuclease digestion and IP of RBP of interest isolates RBP-protected mRNA fragments	Use of 4SU or 6-SG increases crosslinking efficiency; exact crosslinking sites are revealed after sequencing by T to C transitions in the cDNA prepared from RBP-bound mRNA	Some RBPs may not be amenable to PAR-CLIP	Hafner et al. (2010), Castello et al. (2012)
iPAR-CLIP	PAR-CLIP method applied to *C. elegans* exposed to 4SU	First demonstration of CLIP in a non-cell line system; allows for physiologically relevant, context-dependent studies of protein-RNA interactions in *C. elegans*	Technique yet to be applied to other *in vivo* models	Jungkamp et al. (2011)

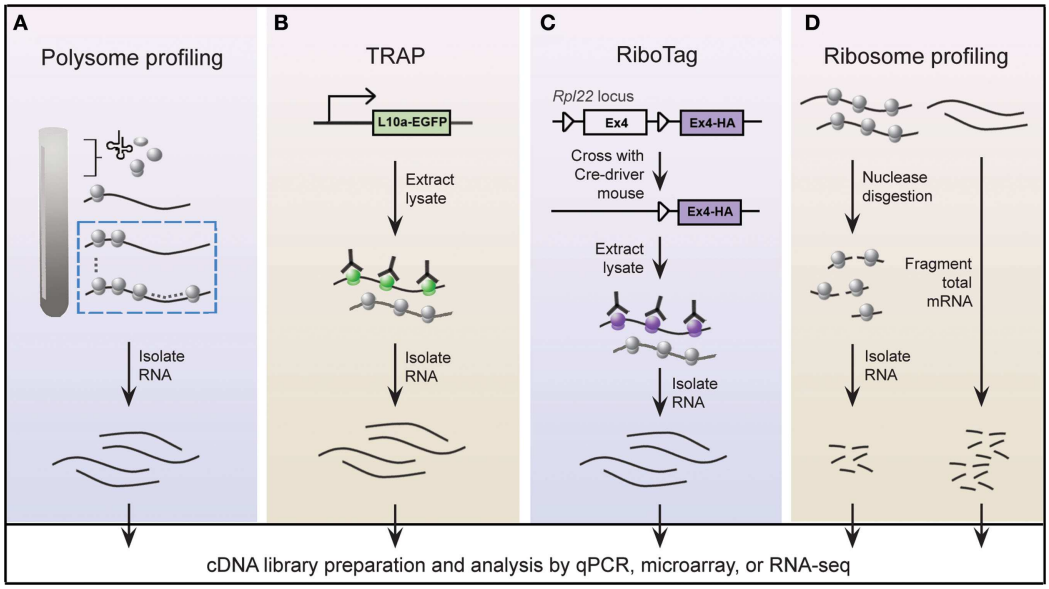

FIGURE 2 | Schematic of genome-wide methods to study polysome-associated mRNAs *in vitro* and *in vivo*. (A) With polysome profiling, cytoplasmic lysates from cells are layered onto a sucrose gradient and undergo centrifugation to separate tRNAs, 40S, 60S, and 80S ribosomes, and polysomes. Messenger RNAs from fractions corresponding to polysomes (dashed blue box) are isolated and identified by various approaches. **(B)** Engineered bacTRAP mice drive expression of EGFP-tagged L10a, a ribosomal protein found in polysomes (green ribosomes), from promoters that are activated in specific cells of the central nervous system. EGFP-L10a-mRNA complexes are immunopurified from brain tissue from bacTRAP mice, and associated mRNAs are identified by various techniques. **(C)** The RiboTag mouse carries an Rpl22 allele with a floxed wild-type C-terminal Exon4 followed by a HA-tagged Exon4. When the RiboTag mouse is crossed with a mouse expressing Cre-recombinase in a cell-type specific manner, Cre-recombinase activates expression of HA-tagged Rpl22, which incorporates into polysomes (purple ribosomes). Homogenized tissues from the offspring are subjected to co-immunoprecipitation using antibodies against HA, and associated mRNAs are identified by various techniques. **(D)** Using ribosome profiling to identify ribosome occupancy on mRNAs, cycloheximide-treated lysates from cultured cells are digested by micrococcal nucleases to remove mRNA sequences that are not bound by ribosomes (left). The resulting monosome complexes are purified by ultracentrifugation through a sucrose gradient or cushion. Ribosome-protected fragments are recovered and deep sequenced. In parallel, total mRNA from a similar preparation of cycloheximide-treated lysate is fragmented and deep sequenced (right), and serves as a normalizing control.

transcripts sequestered within the low-density fractions were also examined to identify poorly translated mRNAs.

Polysome profiling has been readily applied to various cellular contexts and cell types, including neurons (Johannes et al., 1999; Preiss et al., 2003; Rajasekhar et al., 2003; Schratt et al., 2004; Iguchi et al., 2006). A study that nicely demonstrated the utility of polysome profiling examined the widespread inhibition of translation in response to cellular stress, a process that appears to involve movement of mRNAs from polysome complexes to P-bodies. The movement of transcripts from polysomes to P-bodies is thought to be a general phenomenon in eukaryotic cells, yet evidence to support this view is based on single-gene studies. To address whether this view is in fact a general phenomenon or limited to a subset of mRNAs, Arribere et al. (2011) used polysome profiling in combination with translation inhibitors to measure the translational activity and ribosome occupancy upon glucose withdrawal and at different times following glucose re-addition. This study illustrated the power of genome-wide studies: Arribere and colleagues were able to provide mechanistic insight to translational control based on an examination of the entire transcriptome, not just representative transcripts. Notably, they showed that a substantial portion of mRNAs, many of which encode survival factors, is actively translated during stress; this finding disputed the prevailing notion that translational inhibition is widespread during stress. They also found that re-entry of pre-existing mRNAs, presumably from P-bodies, into polysomes is restricted to a subset of mRNAs rather than a general phenomenon as initially proposed (Brengues et al., 2005; Teixeira et al., 2005; Brengues and Parker, 2007; Hoyle et al., 2007).

As with any technique, polysome profiling has several limitations. From a technical perspective, this technique is labor intensive and difficult to scale-up (Larsson and Nadon, 2008). Furthermore, polysome profiling should be performed with uniform cell populations due to the heterogenic nature of transcriptomes between cell types; this presents a significant challenge in performing polysome profiling *in vivo*. A final consideration concerns the underlying assumption of the technique that mRNAs bound by multiple ribosomes are translationally active. Both active and stalled ribosomes have been shown to co-sediment during isolation of polysome complexes through sucrose gradients (Sivan et al., 2007), indicating that polysome profiling does not completely distinguish translationally active from repressed mRNAs. Complementary molecular studies that directly measure *de novo* protein synthesis would be useful to discriminate translating from non-translating polysome-associated mRNAs. Despite these limitations, polysome profiling

has been extremely successful in identifying translationally active mRNAs.

TARGETING RIBOSOMES: IMMUNOPRECIPITATION-BASED METHODS TO ISOLATE POLYSOMES *IN VIVO*

Gene expression patterns vary greatly between cell types, thereby requiring that genome-wide studies be performed using homogeneous cell populations. The difficulty of performing polysome profiling *in vivo* is due to the challenge of extracting homogeneous cell populations in sufficient quantities without affecting the transcriptome (Okaty et al., 2011). The mammalian brain is an especially challenging model because of its immense heterogeneity. Enrichment methods, such as fluorescence-activated cell sorting or laser-capture microdissection, accurately separate genetically and/or morphologically distinct cells; however, manipulation of purified cells during isolation steps may alter the transcriptional profile (Okaty et al., 2011). These challenges must be addressed in order to achieve the full benefits of this powerful technique *in vivo*, giving us a better understanding of translational control within a physiologically relevant setting. Two groups independently tackled this issue by genetically altering mice to express epitope-tagged versions of ribosome protein subunits (Heiman et al., 2008; Sanz et al., 2009). All translated mRNAs are, at one point, associated with ribosomes; thus affinity purification of epitope-tagged ribosomes would allow for isolation of polysome-associated mRNAs. Heiman and colleagues termed their methodology translating ribosome affinity purification, or TRAP, which involves a series of bacterial artificial chromosome transgenic mice, called bacTRAP mice. In these genetically modified mice, expression of EGFP-tagged ribosomal protein L10a is driven by defined promoters that are activated in specific cell types of the central nervous system (CNS; **Figure 2B**; Heiman et al., 2008). Heiman and colleagues used bacTRAP mice that expressed EGFP-L10a from the Drd1a receptor or Drd2 receptor to isolate polysome-associated mRNA from striatonigral or striatopallidal cells, respectively, of the mouse striatum. Cells derived from the brain tissue of these mice were lysed in cycloheximide-spiked buffer to halt elongating ribosomes. Polysome-associated RNA corresponding to the specific cell type was isolated by immunoprecipitating for EGFP-L10a and examined by microarray analysis. The group demonstrated the power of TRAP technology by identifying polysome-associated mRNAs unique to four neuronal populations that are intermixed and morphologically indistinguishable. In an accompanying paper, Doyle et al. (2008) demonstrated the generality of TRAP with a comprehensive study of polysome profiles for 24 additional CNS cell types.

Using a strategy similar to TRAP, Sanz and colleagues engineered a mouse line called RiboTag that contains three HA tags inserted into the locus of *Rlp22*, a gene that encodes a ribosome protein present within polysomes (**Figure 2C**; Sanz et al., 2009). Expression of the $Rlp22^{HA}$ allele depends on Cre recombination such that in the absence of Cre recombinase only endogenous (wild-type) *Rlp22* is expressed. The group crossed RiboTag mice with several neuron-specific Cre recombinase-expressing mice. Brain tissues from the resulting offspring were used to immunopurify HA-tagged polysomes and recover associated mRNAs from specific cell populations expressing Cre recombinase. They demonstrated that the RiboTag system indeed reliably purifies mRNAs associated with ribosomes in a cell type specific manner.

The RiboTag strategy has several advantages over TRAP. First, the RiboTag mouse can be crossed to any Cre recombinase-expressing mouse line, allowing for polysome profiling of a significantly greater variety of cell types. In contrast, the TRAP system requires engineering a separate bacTRAP mouse line for each cell type of interest. Second, $Rlp22^{HA}$ is expressed at levels similar to wild-type Rlp22, thereby maintaining the appropriate stoichiometry of ribosomal subunits and kinetics of translation. The TRAP system instead expresses the EGFP-L10a transgene from an exogenous promoter, which may result in different levels of EGFP-L10a compared to wild-type L10a. Third, the TRAP method recommends using in-house monoclonal antibodies against EGFP, which is costly for multiple experiments. Alternatively, the RiboTag method uses commercial anti-HA antibodies that are far more cost effective. Finally, RiboTag technology has the ability to generate $Rlp22^{HA}$-expressing cells (e.g., $Rpl22^{HA}$-expressing mouse embryo fibroblasts) that are capable of proliferating indefinitely in culture dishes, providing an abundant and renewable model for obtaining ribosome-associated mRNAs by immunoprecipitation rather than by the laborious process of sucrose gradient centrifugation. This ability is severely limited with the TRAP system since many of the current bacTRAP mice activate EGFP-L10a expression in neurons, which do not proliferate *in vitro*. Overall, the TRAP and RiboTag technologies provide an efficient and rapid method of isolating polysome-associated mRNA from a single cell type *in vivo*.

RIBOSOME PROFILING

Many studies that have been designed to investigate global translation typically use genome-wide measurements of mRNA and/or protein expression as indicators of protein synthesis. However, transcripts are subject to multiple levels of translational control rendering mRNA expression an imperfect substitute for protein synthesis. Global protein expression, as measured by mass spectrometry, is also a poor proxy of translation since protein stability also contributes to changes in protein expression. The most precise measurement of translation is direct quantification of protein synthesis. To measure protein synthesis at a genome-wide level, Weissmann and colleagues developed a technique called ribosome profiling (**Figure 2D**) to map the precise positions of ribosomes within the transcriptome (Ingolia et al., 2009, 2012). Ribosome profiling involves nuclease digestion of cell extracts to degrade mRNA that is unprotected by ribosomes, leaving approximately 28 nucleotide RNA fragments. Individual ribosome-RNA complexes are isolated by centrifugation through a sucrose gradient or cushion, followed by a series of purification steps to recover the mRNA fragments, such as fragment size selection by gel electrophoresis and rRNA removal by subtractive hybridization. Ribosome-protected mRNA fragments (ribosome footprints) are identified by high-throughput sequencing, revealing the locations of ribosomes along transcripts at nucleotide-level resolution.

Ingolia et al. (2009) first demonstrated the utility of ribosome profiling for studying translation in *Saccharomyces cerevisiae*. The authors showed that ribosome profiling could quantitatively

measure translational efficiency as defined by the ratio of ribosome footprint density to total mRNA. Ribosome density proved to be a much better predictor of protein production than measurements of mRNA levels. Ribosome density also correlated well, though not perfectly, with protein expression, suggesting that in some contexts mass spectrometry does not accurately measure global protein synthesis. The correlative differences between protein expression and translation efficiency observed by Ingolia et al. (2009) may be attributed to protein degradation and such differences could be exploited to investigate protein stability at a genome-wide level.

In addition to being an optimal tool for genome-wide measurements of protein synthesis, ribosome profiling is capable of discovering novel regulatory mechanisms of translation. While ribosome footprints are expected to map to coding regions of transcripts, a ribosome profiling study in yeast found that a small fraction of footprints (1.2%) mapped to non-coding regions (Ingolia et al., 2009). The majority of this 1.2% footprint fraction mapped to the 5′ UTRs of transcripts, leading to the discovery of 153 upstream open reading frames (uORFs) of which fewer than 30 had previously been described. Because ribosome profiling maps ribosome footprints at a resolution such that a three-base codon periodicity is observed, positional data can be used to identify frame shifts, non-canonical start codons, and stop codon readthrough, all of which would predict novel protein isoforms. As proof-of-principle, Ingolia et al. (2009) uncovered a total of 143 non-canonical (non-AUG) start sites in the yeast transcriptome; this was a substantial contribution to our knowledge of translational initiation sites in yeast, having previously known of only two examples of non-AUG initiation sites (Chang and Wang, 2004; Tang et al., 2004). Sites of premature translational termination can also be identified with this technique. Subsequent ribosome profiling studies have been implemented in other model organisms, including bacterial and mammalian systems (Guo et al., 2010; Ingolia et al., 2011; Oh et al., 2011; Hsieh et al., 2012). Not surprisingly, these studies also produced a long list of candidate initiation start sites, alternative reading frames, and uORFs. A comparison of these features across model systems or among different cell types will be informative in determining whether translational control mechanisms are general or specific to individual organisms and cell types.

Translation is a dynamic process and the kinetics of ribosome movement along transcripts is an important aspect of translational control. While the temporally static nature of ribosome profiling may appear ineffective for the study of translational kinetics, Ingolia et al. (2011) demonstrated otherwise by combining ribosome profiling with run-off elongation assays. First, cells were treated with harringtonine to block additional rounds of translation initiation (Fresno et al., 1977). Cycloheximide was then applied to cells for various time points afterward, freezing all actively translating ribosomes. Ribosome positions were then determined by ribosome profiling and the change in ribosome positioning over the course of cycloheximide treatment was used to generate a moving picture of ribosomes. In log phase growing yeast, the rate of translation appeared to be independent of mRNA class, mRNA length, or whether mRNAs encoded secreted or cytoplasmic proteins. This adaptation of ribosome profiling will prove useful in studying the rate of translation in the context of cellular stress or pathological states where global translation is aberrant (Shenton et al., 2006).

Another critical aspect of translational kinetics is ribosomal pausing. Pausing or stalling of elongating ribosomes is a mechanism of translational repression and may be caused by steric hindrance (due to secondary structure of the transcript or by the exiting nascent peptide), recruitment of low abundance tRNAs to rare codons, or RBPs such as FMRP (Lovett and Rogers, 1996; McNulty et al., 2003; Darnell et al., 2011). Ingolia et al. (2011) reasoned that the ribosome density for a given codon should be commensurate with the average ribosome dwell time, i.e., slower ribosome movement across a codon (longer dwell time) should result in more footprint counts at that codon. Based on this inference, the group used ribosome profiling to identify thousands of ribosome pause sites at the resolution of individual codons in the transcriptome of mouse embryonic stem cells (ESCs). Ribosomal pausing was initially described more than 20 years ago (Wolin and Walter, 1988), yet the mechanisms underlying this process is not well understood. Recently, ribosome profiling in bacteria was used to show that a Shine–Dalgarno-like feature in mRNA facilitates ribosome pausing (Li et al., 2012), demonstrating that this technique provides an efficient method to elucidate the molecular mechanisms of ribosomal pausing.

Similar to polysome profiling, ribosome profiling is technically challenging and labor intensive. Another consideration regarding ribosome profiling, as pointed out by Morris (2009), relates to the use of cycloheximide to halt elongating ribosomes prior to cell lysate preparation. Although characterized as an inhibitor of elongation, cycloheximide has also been shown to block translation initiation at similar concentrations used for ribosome profiling (Obrig et al., 1971) but not at lower concentrations (Lodish, 1971). If the exposure of cycloheximide to cells is low such that translation elongation, but not initiation, is negatively affected, this may allow ribosomes to accumulate at the 5′ end of transcripts and result in spurious ribosome footprinting patterns. This is especially relevant to applications of ribosome footprinting where drug delivery and exposure are difficult to control, for example with studies using mice. Yet despite these drawbacks, ribosome profiling remains a powerful tool to identify novel uORFs, initiation start sites, termination sites, and alternative reading frames, and provide insights on ribosome movement or lack thereof (ribosomal pausing) will most certainly lead to the discovery of new and unexpected modes of translational control.

TARGETING RBPs: IMMUNOPRECIPITATION-BASED METHODS TO ISOLATE RBP-mRNA COMPLEXES

Key to understanding the role of RBPs in translational control, and RNA metabolism in general, is identifying their mRNA targets. Initial genome-wide attempts to identify RBP targets employed a technique called RNA immunoprecipitation (RIP). Much like its DNA counterpart chromatin immunoprecipitation, RIP involves formaldehyde crosslinking of proteins to RNA, followed by immunoprecipitation of the protein-RNA complex. Protein-bound transcripts are then used to make a cDNA library that is subjected to microarray analysis. A major concern with RIP is the low signal to noise ratio: RNA tends to be "sticky" making the technique vulnerable to extracting non-physiological

binding partners (Mili and Steitz, 2004). The Darnell group introduced an alternate approach to RBP target identification termed CLIP for crosslinking and immunoprecipitation (Ule et al., 2003), which is reviewed in greater detail elsewhere (Darnell, 2010a; König et al., 2012). Instead of using formaldehyde, CLIP uses UV irradiation to cement protein-RNA interactions by creating covalent bonds between proteins and RNA that are within distances of a few angstroms. Unprotected RNA is removed by partial digestion with RNase (in the original protocol) or micrococcal nuclease, which is easily inactivated by EGTA to avoid spurious, continual RNA digestion throughout the procedure (Yeo et al., 2009; Zisoulis et al., 2010; Polymenidou et al., 2011). Protein-RNA complexes are recovered by immunoprecipitation with antibodies against the protein of interest. Transcript fragments of approximately 60–100 nucleotides in length are released from proteins and are further processed for sequencing. Several aspects of this technique make it well suited to study RBP-RNA interactions. First, the direct interaction between a RBP and its target mRNA is faithfully preserved, since crosslinking only occurs with RBPs and mRNA that are within angstrom distances. Second, crosslinking of direct RBP-mRNA interactions via strong covalent bonds allows these complexes to be purified under stringent conditions, further reducing background signal. Third, UV irradiation does not preserve protein–protein interactions, thereby avoiding the possibility of indirect protein-mRNA interactions.

The first application of CLIP sought to identify RNA targets of Nova, a neuronal KH-type RBP, which is implicated in paraneoplastic neurologic degeneration (Darnell, 2010b). In this study, Ule, Jensen, and colleagues identified 34 candidate mRNA targets, most of which are involved in neuron function (Ule et al., 2003). The use of low-throughput sequencing to identify Nova targets, however, generated a limited dataset and made it difficult to discern authentic from spurious mRNA targets (50% false positive rate). To remedy this, the same group performed a subsequent study in which CLIP was combined with high-throughput sequencing (HITS-CLIP). This strategy generated a more robust dataset of Nova targets, confirming, and refining their previous assertions about Nova as a splicing regulator (Licatalosi et al., 2008). Further modifications to the standard CLIP protocol have improved the crosslinking efficiency. One such modification called Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation, or PAR-CLIP, uses photoreactive ribonucleoside analogs [e.g., 4-thiouridine (4SU) or 6-thioguanosine], which are incorporated into nascent mRNAs in live cells (Hafner et al., 2010) or whole organisms (Jungkamp et al., 2011). Photoreactive ribonucleoside analogs crosslink with proteins more efficiently than endogenous ribonucleotides, thereby enhancing the signal to noise ratio. During cDNA preparation of labeled mRNA, crosslinked sites are prone to thymidine to cytidine or guanosine to adenosine transitions (when 4-thiouridine or 6-thioguanosine is used, respectively), revealing exact locations of nucleotide-protein interactions. This feature was initially exploited to identify individual ribonucleotides of a small nucleolar RNA that interacted with RBPs (Granneman et al., 2009) and has since been applied to other RBPs (Hafner et al., 2010). It is important to note that not all RBPs may be amenable to PAR-CLIP, such as CUG triplet repeat RNA binding protein (CELF1; Castello et al., 2012). A further modification of CLIP, called individual nucleotide resolution CLIP or iCLIP, provided an alternative approach to locating the exact crosslinking position (König et al., 2010). Ule and colleagues took advantage of the fact that reverse transcriptase arrests at sites of nucleotide-peptide crosslinking (a peptide remnant of the RBP remains after proteinase K digestion). The resulting truncated cDNAs are normally lost during the standard CLIP library preparation, but the iCLIP protocol was designed to recover and sequence these truncated cDNAs to identify exact crosslinking sites. Using CLIP or its variants, genome-wide protein-RNA interaction maps have been assembled for numerous RBPs, including Nova (Ule et al., 2003), RBFOX2 (Yeo et al., 2009), Argonaute proteins (Chi et al., 2009; Hafner et al., 2010; Zisoulis et al., 2010; Leung et al., 2011), TDP-43 (Polymenidou et al., 2011; Tollervey et al., 2011), FMRP (Darnell et al., 2011), and hnRNP proteins (Katz et al., 2010; König et al., 2010; Huelga et al., 2012).

The ability to systematically uncover RBP target sites within the transcriptome has provided insights to the role of RBPs in translational control. Using *in vivo* PAR-CLIP (iPAR-CLIP), Jungkamp et al. (2011) proposed several models by which GLD-1, a conserved germline-specific RBP in *C. elegans*, functioned as a translational repressor. Highly conserved 5′UTR GLD-1 binding sites were discovered near the start codon of target transcripts; this was unexpected, since prior studies indicated that GLD-1 primarily targets the 3′ UTR. Following extensive biochemical validation of their iPAR-CLIP results, Jungcamp and colleagues proposed that GLD-1 dimers, as the protein is known to form, bind to the 5′ and 3′ UTRs to promote circularization of mRNA to block translational initiation (Jungkamp et al., 2011). Alternatively, binding of GLD-1 near start codons at the 5′ UTR may prevent assembly of the ribosome.

Another study examined the mechanisms of translational control by FMRP using CLIP. Darnell et al. (2011) identified FMRP binding sites within the polysome-associated fraction of the transcriptome by HITS-CLIP and found that a significant portion of gene targets were involved in neuronal synaptic plasticity and synaptic-related signaling pathways. The majority of FMRP binding sites (66%) resided within coding sequences. This was unexpected given that translational control mechanisms usually involve binding of RBPs within UTRs. Furthermore, the distribution of FMRP appeared to be uniform along transcripts. Darnell and colleagues extended these observations to demonstrate that FMRP directly stalls ribosomes on polysome-associated transcripts, thereby suppressing translation. Given that many of these FMRP targets are involved in synaptic transmission, it was hypothesized that FMRP functions to represses translation of associated mRNAs during transit from the soma to synapses, to prevent premature translation and/or degradation. Upon release of FMRP from its targets, presumably by signal activation, the transcripts, already loaded with ribosomes, would be rapidly translated. Given that the interplay between different RBPs is important for RNA transport and local synaptic translation, the application of CLIP to neuronal RBPs will be instrumental in defining their individual and combinatorial contributions to translational control.

CLIP is accompanied by limitations that are either unique to particular version or inherent to all (Darnell, 2010a; Ascano et al.,

2012). The ability of CLIP to capture a representative population of RNA targets is influenced by the crosslinking efficiency between RNA and the protein of interest, nuclease digestion conditions (Kishore et al., 2011), and the specificity and reactivity of the antibody used to isolate the RBP-RNA complex; these factors are inherent to all CLIP versions. Crosslinking efficiency will vary depending on the RNA target sequence and the type of amino acids available for crosslinking. Nucleic acids are generally more reactive with cysteine, lysine, phenylalanine, tryptophan, and tyrosine residues, and this residue preference appears to be preserved with 4SU-labeled RNA (Meisenheimer and Koch, 1997; Meisenheimer et al., 2000). 4SU is generally thought to enhance crosslinking efficiency compared to unmodified uridine (Hafner et al., 2010), although this is not always the case (Kishore et al., 2011). A drawback of PAR-CLIP is the difficulty in applying this method to whole animal models other than *C. elegans*, where uniform exposure of 4SU may not be feasible. Until a method for efficient delivery of ribonucleoside analogs to other animal models (namely mice) is established, HITS-CLIP is generally required for *in vivo* studies.

CHALLENGES AND CONSIDERATIONS FOR GENOME-WIDE STUDIES IN NEURONS

The application of genome-wide studies to neurobiology is accompanied by challenges intrinsic to the neural model system and genome-wide method being employed. The mammalian CNS is complex with hundreds of morphologically distinct cell types, each expressing a unique transcriptome. This presents several challenges when performing genome-wide studies: purifying homogenous cell populations, isolating cells in a manner that does not alter gene expression, and obtaining sufficient quantities of cells for analysis. With regard to the latter issue, some of the techniques discussed here require up to several tens of micrograms of RNA for analysis, necessitating a large input of cells. Cultured primary neurons derived from rodent tissue are a well established model for studying neurons; however, they do not obviate some of these challenges, as they may not be completely homogenous and do not expand *in vitro*. Furthermore, establishing and maintaining cultured primary neurons are difficult and require extensive training of the researcher. Neural progenitor cells (NPCs), either differentiated from stem cells or isolated from rodents or humans, are an alternative *in vitro* model system. NPCs can be expanded and differentiated into multiple neuronal cell types, and developing an efficient differentiation process should yield a fairly homogenous cell population. Importantly, NPCs that were differentiated from induced pluripotent stem cells (iPSCs) derived from patient fibroblasts have been shown to recapitulate the molecular phenotypes of corresponding neurological diseases (Thonhoff et al., 2009; Ming et al., 2011), providing valuable models to investigate global translation in neurodegeneration using the methods described herein. The labor intensive, lengthy, and costly nature of establishing iPSCs and differentiating them into NPCs is a major drawback of this system.

Similar to cell culture models, animal models used for genome-wide analyses are fraught with the challenges mentioned above; however, some of these challenges may be circumvent with the use current technologies. To remedy the issue of complex cell heterogeneity, the RiboTag and TRAP technologies were created, both of which expressed tagged versions of ribosomes in a cell type specific manner to isolate mRNA. While the intended application of RiboTag and TRAP technologies is for *in vivo* polysome profiling, conceivably they may be adapted for *in vivo* ribosome profiling studies. The application of HITS-CLIP in heterogenic mouse brain tissue is standard (Licatalosi et al., 2008; Polymenidou et al., 2011), and in theory the technology required to perform HITS-CLIP in a cell type specific manner *in vivo* is obvious. Using transgenic mice that express a tagged version of the RBP of interest in a cell type specific manner, HITS-CLIP could easily be implemented *in vivo* using an antibody that recognizes the tag. Naturally, adaptation of RiboTag or TRAP technologies for ribosome profiling or transgenic mice for HITS-CLIP will require optimization steps to ensure a seamless integration.

Lastly, bioinformatic challenges inherent to genome-wide studies largely center on the issue of interpreting the massive amounts of raw sequencing data. A convergent problem in analyzing sequence reads derived from CLIP and ribosome profiling data is the identification of precise binding sites of RBPs or ribosome footprints within the RNA transcripts. Thus far, the analysis of CLIP data relies on inherent assumptions about the binding kinetics of RBPs with their preferred RNA substrates. The RBPs that have been studied thus far, for example Nova, RBFOX2, Argonaute, and hnRNP family members (Ule et al., 2003; Yeo et al., 2009; Hafner et al., 2010; Zisoulis et al., 2010; Leung et al., 2011; Huelga et al., 2012), tend to interact with several binding sites within a given substrate very strongly. Computational approaches to identify precise binding sites (or clusters of reads), known as cluster- or peak-finding algorithms, have therefore assumed that the majority of reads within an RNA that are below an expected threshold are not true binding sites and represent experimental noise or artifacts. However, other RBPs, such as TDP-43 or FUS/TLS may act in "scanning" mode on some RNA substrates (unpublished observations), interacting with many sites with low affinity, rather than a few sites with high affinity, with nevertheless important biological effects. Most algorithms are conservative in identifying these low affinity sites within RNA substrates. Ribosome footprints fall into this second category of many locations of roughly equal occupancy with mRNA substrates. Thus while ribosome profiling data can be used to measure quantitative differences in ribosome occupancy within RNAs, precise footprint sites such as paused ribosomes are not effectively identified by existing peak-finding algorithms. Another glaring concern with analyzing RNA and ribosome binding data is the dearth of statistical tools to measure whether we have saturated the potential number of binding sites observed (one strategy is suggested in Polymenidou et al., 2011), or techniques to measure the rate of false positives and negatives. Lastly, not all RNA binding sites are functional. It is not unthinkable that while highly expressed RBPs interact with many RNA substrates, many, if not most of these sites may not actually have an impact in regulating the life cycle of the RNA molecule. Integrating other genome-wide assays that reveal the dependence of the RNA on the RBP is important for assigning functions to these binding sites. Thus, computational approaches to integrate these information with CLIP data, and also comparing how multiple RBPs affect the same molecules will be crucial in going forward (Huelga et al., 2012).

Table 2 | List of RBPs involved in translation and implicated in neurological diseases.

RBP	Function	Disease	Reference
FMRP	Repressor	FXS	Darnell et al. (2011)
hnRNP A2/B1	Activator	ALS, FTLD	Kwon et al. (1999)
hnRNP C	Activator	AD	Lee et al. (2010)
IGHMBP2	Regulator	SMA	Grohmann et al. (2001), de Planell-Saguer et al. (2009)
Musashi	Repressor	AD	Okano et al. (2002), Perry et al. (2012)
SMN	Putative repressor	SMA	Piazzon et al. (2008)
TDP-43	Repressor	ALS, FTLD	Lagier-Tourenne et al. (2010)

AD, Alzheimer's disease; ALS, Amyotrophic lateral sclerosis; FTLD, Frontotemporal lobar dementia; FXS, Fragile X syndrome; SMA, Spinal muscular atrophy.

RNA BINDING PROTEINS AND TRANSLATIONAL CONTROL IN NEUROLOGICAL DISEASES

RNA binding proteins have received considerable attention for their roles in neurodegeneration (Lukong et al., 2008; Lui-Yesucevitz et al., 2011). This is not surprising given that many RNA processing events, including local translation, are important for neuronal function (Klann and Dever, 2004; Sutton and Schuman, 2006). Of the handful of RBPs that are associated with neurological diseases, only several have been implicated in the regulation of translation (**Table 2**), most of which were determined by single-gene studies. Presently, there is a lack of genome-wide studies examining the translation functions of RBPs in normal or pathological contexts of neurons, with the exception of a few studies related to FMRP; this leaves much room for investigation. In this section, we review our current knowledge of these RBPs (listed in **Table 2**) with regard to translational control and neurological diseases.

FMRP is one of the more extensively studied RBPs with regard to translational regulation and neurodegeneration. It is essential for proper synaptic function, as loss of FMRP in mice results in aberrant pre- and post-synaptic plasticity (Deng et al., 2011). FMRP is present throughout the neuron (soma, dendrites, and axon) with the majority of FMRP (85–90%) being associated with polysomes (Zhang and Darnell, 2011). Misregulation of FMRP is linked with FXS, a condition characterized by impaired cognitive, physical, emotional, and sensory function (Bagni and Greenough, 2005; Bassell and Warren, 2008). Consistent with this, a genome-wide analysis of FMRP RNA targets using HITS-CLIP revealed that many FMRP targets encode proteins that are implicated in autism spectrum disorders (Darnell et al., 2011). Mutations within the FMRP gene that lead to reduced expression are frequently observed in patients with FXS (De Boulle et al., 1993; Snow et al., 1993). As discussed in this review, FMRP downregulates target gene expression by blocking translation in part by stalling ribosomes. FMRP has also been shown to interact with Ago2 and recruit miRISCs to its mRNA targets, providing another mechanism by which FMRP mediates gene silencing (Muddashetty et al., 2011). Edbauer et al. (2010) demonstrated that FMRP associates with miR-125b and miR-132 in the mouse brain, interactions of which greatly influence dendritic spine morphology and synaptic physiology of hippocampal neurons. In this study, a limited set of miRNAs was examined and it is likely that additional miRNAs interact with FMRP. Genome-wide methods tailored to studying miRNA-protein interactions are well established (reviewed in Wilbert and Yeo, 2011) and would provide an unbiased approach toward identifying miRNAs that interact with FMRP as well as other RBPs.

The evolutionarily conserved Musashi family of RBPs, herein referred to as Musashi, has strong links to both translational control and neurobiology (Okano et al., 2002). Preferentially expressed in the mammalian nervous system, Musashi is a key contributor to maintaining the proliferative capacity of neural stem cells, such that its loss leads to a reduction in the formation of neurospheres, or cell-cultured neural stem cells (Sakakibara et al., 2002). Musashi is thought to maintain progenitor self-renewal by translationally repressing inhibitors of stem cell proliferation. One of these targets, *CDKN1A*, encodes the anti-proliferative cell-cycle inhibitor p21WAF (Battelli et al., 2006). *NUMB* is another Musashi target that is a negative regulator of the Notch signaling pathway. Since Notch signaling is crucial for neural stem cell maintenance (Hitoshi et al., 2002), translational silencing of *NUMB* by Musashi augments this proliferative process (Imai et al., 2001). The mechanism by which Musashi suppresses translation, at least for *NUMB*, involves disrupting the eIF4G binding-Poly(A)-binding protein interaction, thereby preventing assembly of the 80S ribosomal complex (Kawahara et al., 2008). If Musashi is characteristic of most RBPs, then it has hundreds or thousands targets in addition to *NUMB* and *CDKN1A*. Using the iterative *in vitro* selection process SELEX, the RNA binding sequence of Musashi has been identified (Imai et al., 2001). This sequence information can be useful in predicting targets, yet still requires single-gene studies to validate authentic Musashi targets. The application of HITS-CLIP to define Musashi targets and locate discrete binding sites would prove invaluable toward understanding the function of Musashi. This knowledge may also be useful to investigate a potential role of Musashi in Alzheimer's disease (AD). A link between Musashi and AD was established with the observation that Musashi expression was reduced in patients with AD (Perry et al., 2012). Consistent with Musashi being a key regulator of neural stem cells, downregulation of Musashi correlated with a reduction in neural stem cells, the latter event of which is often observed in AD. Whether misexpression of Musashi is a determinant or downstream effect of AD is uncertain. As will be a common theme among the RBPs addressed in this section, a comprehensive understanding of Musashi function as revealed by genome-wide studies will likely identify therapeutic targets implicated in AD.

TAR DNA-binding protein-43 (TDP-43) is another RBP that has recently been recognized as an important contributor to neurological diseases (Lagier-Tourenne et al., 2010). It is well established as a regulator of transcriptional repression and alternative splicing, but may also have a role in translational repression (Wang et al., 2008c). In hippocampal neurons, TDP-43 appears to reside within RNA granules and P-bodies – storage sites of

repressed mRNAs; this observation is consistent with the finding that TDP-43 acts as a translational repressor in an *in vitro* assay (Wang et al., 2008b). A pathological link between TDP-43 and neurodegeneration was initially established with the observation that TDP-43 is present within brain cell inclusions of patients with ALS or FTLD (Arai et al., 2006; Neumann et al., 2006). Dominant mutations in the gene encoding TDP-43 (*TARDBP*) that cause mislocalization of the protein were subsequently identified in cohorts of patients with ALS and FTLD (Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008; Yokoseki et al., 2008). The aberrant activities of disease-associated forms of TDP-43 have been recapitulated in transgenic rats displaying neurological impairments and in reprogrammed pluripotent stem cells, confirming the pathogenicity of these mutations (Bilican et al., 2012; Huang et al., 2012). Findings from genome-wide studies have advanced our understanding of the pathogenic activities TDP-43 by revealing that TDP-43 modulates the levels and alternative splicing of many of its RNA targets, a substantial portion of which encode proteins involved in neuronal development and function (Polymenidou et al., 2011; Tollervey et al., 2011). A connection between the translational silencing and alternative splicing functions of TDP-43 may exist, as demonstrated for the TDP-43 target S6 kinase 1 (S6K1) Aly/REF-like target (SKAR). TDP-43 appears to control alternative splicing of SKAR, which results in the expression of a SKAR isoform that can no longer activate the translation-stimulating SK61-dependent signaling pathway (Fiesel et al., 2012). Therefore loss of TDP-43 resulted in upregulation of the SKAR isoform that increases SK61 activity and consequently stimulated global translation. Whether the translational functions of TDP-43 require its splicing activities for other TDP-43 targets remains elusive. But more pressingly, the role of TDP-43 in global translation remains unknown and will best be addressed by employing the genome-wide methods discussed here.

Two members of the heterogeneous nuclear ribonucleoproteins (hnRNPs) family of RBPs, hnRNP A2/B1, and hnRNP C, have been recognized as translational regulators with links to neurological diseases. As key regulators of RNA metabolism, hnRNP proteins are widely expressed in various tissues, including the mammalian brain (Kamma et al., 1995, 1999). In neuroblastoma cells, hnRNP C was shown to enhance the translation of mRNA that encode amyloid precursor protein (APP), a protein that, when aberrantly processed, is the major constituent of cerebral amyloid plaques found in patients with AD (Lee et al., 2010). By associating with the same region of APP mRNA as FMRP, hnRNP C prevents FMRP from binding to and silencing the translation of APP mRNA. Another hnRNP family member, hnRNP A2/B1, promotes translation through several mechanisms. It has been show to mediate transport of specific mRNAs to distant dendrites where they are translated to produce a local supply of proteins that are required for synaptic plasticity (Gao et al., 2008). HnRNP A2/B1 was also shown to control the translation of mRNAs encoding myelin basic protein and c-Myc through mechanisms that are not well defined (Kwon et al., 1999; Shi et al., 2011). Similar to TDP-43, hnRNP A2/B1 is present within brain cell inclusions of some patients with FXTAS, suggesting that the pathogenic functions of hnRNP A2/B1 involve its mislocalization (Iwahashi et al., 2006).

Several other neurological disease-related RBPs are suggested to have roles in translation, yet direct experimental evidence to support such roles is lacking. One example is survival of motor neuron (SMN), an RBP that is mutated in patients with SMA (Wirth, 2000). SMA is a fatal autosomal recessive disorder characterized by degeneration of lower motor neurons that ultimately results in paralysis with muscular atrophy (Lunn and Wang, 2008). SMN is involved in the assembly of the spliceosome, a complex that carries out gene splicing, and is also required in the formation of SGs (Liu et al., 1997; Pellizzoni et al., 1998; Hua and Zhou, 2004). In addition, SMN is found in complexes with FMRP, and through this association, SMN is hypothesized to mediate translation (Piazzon et al., 2008). Loss-of-function mutations in the gene encoding the RBP immunoglobulin μ-binding protein 2 (IGHMBP2) are also associated with SMA (Grohmann et al., 2001). Biochemical analysis of IGHMBP2 determined that it physically associates with regulators of tRNA transcription and ribosome biogenesis, suggesting strong ties to translational regulation (de Planell-Saguer et al., 2009). The mechanism by which defects in SMN and IGHMBP2 cause SMA or whether either protein has a defined role in translation remains unknown.

CONCLUSION

Although progress has been made in understanding translational regulation in neurons, many questions remain unaddressed. Specifically, our knowledge of RBP function in translation is very limited. We have yet to discover direct targets of many neuron-related RBPs and determine how these RBPs influence ribosome kinetics and the distribution of mRNAs with different mRNP complexes. We also do not know whether misregulation of certain RBPs, an event often associated with neurological diseases, affects global translation and, if so, whether aberrant translational regulation is an underlying mechanism leading to disease. Genome-wide methods are well suited to address these questions, and can provide highly detailed information that will reveal novel translational control mechanisms. The tool set of genome-wide methods to study translational control is extensive, and improvements to both the molecular and computational components of these techniques are ongoing. Since genome-wide studies have the capacity to produce an overwhelming amount of data, it is important that key findings be independently validated by molecular techniques. Such complementation between genome-wide and molecular studies will undoubtedly provide insights to the basis for translational regulation in neurons.

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RNA-binding proteins and translational regulation in axons and growth cones

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RNA localization and regulation play an important role in the developing and adult nervous system. In navigating axons, extrinsic cues can elicit rapid local protein synthesis that mediates directional or morphological responses. The mRNA repertoire in axons is large and dynamically changing, yet studies suggest that only a subset of these mRNAs are translated after cue stimulation, suggesting the need for a high level of translational regulation. Here, we review the role of RNA-binding proteins (RBPs) as local regulators of translation in developing axons. We focus on their role in growth, guidance, and synapse formation, and discuss the mechanisms by which they regulate translation in axons.

Keywords: RNA-binding proteins, local translation, axon outgrowth, axon guidance, synapse formation

INTRODUCTION

Spatial localization of mRNA is a well conserved mechanism for restricting gene expression to a specific subcellular site in many cell types across animal and plant phyla (Condeelis and Singer, 2005; Holt and Bullock, 2009). In neurons, localization and translational regulation of mRNA plays a key function in dendrites and post-synaptic compartments (Bramham and Wells, 2007), and mounting evidence points to a similarly important role in axons (Jung et al., 2012). The response to several guidance cues require local protein synthesis in the tip of the growing axon, the growth cone (GC), (Campbell and Holt, 2001; Wu et al., 2005; Leung et al., 2006; Piper et al., 2006; Yao et al., 2006) and axonal mRNA translation is critical for axon survival (Hillefors et al., 2007; Cox et al., 2008; Yoon et al., 2012) and regeneration (Zheng et al., 2001; Verma et al., 2005). A large number of mRNAs are found in both growing (Andreassi et al., 2010; Zivraj et al., 2010) and mature axons (Taylor et al., 2009; Gumy et al., 2011), with some transcripts restricted to specific neuronal subtypes, axonal compartments (Zivraj et al., 2010), or developmental time points (Zivraj et al., 2010; Gumy et al., 2011).

Different guidance cues ultimately lead to the translation of distinct subsets of mRNAs (Wu et al., 2005; Leung et al., 2006; Piper et al., 2006; Yao et al., 2006), yet, puzzlingly, cause an increase in the activity of markers of global translation in the GC (Campbell and Holt, 2001; Leung et al., 2006; Piper et al., 2006). This begs the question of how translation of specific mRNAs is locally regulated. The specificity is likely mediated, at least in part, via RNA-binding proteins (RBPs). RBPs comprise a large family of proteins that form ribonucleoprotein (RNP) complexes with their target mRNAs and can act as cytoskeletal adaptors and/or translational silencers to transport their cargo to subcellular locations (Besse and Ephrussi, 2008). Once on site, RBPs can either act as translational repressors or activators of their mRNA targets, thus providing a way to control translation spatially and temporally. Here, we review the role of RBPs as regulators of local protein synthesis in the axon during development, from axon elongation, to axon guidance and synapse formation in target-arrived axons. Lastly, we discuss the possible mechanism by which RBPs regulate the specificity of local translation in axons and GCs.

AXONAL GROWTH CONE RBPs REVEALED BY PROTEOMIC ANALYSIS

RBPs are widely expressed in the central nervous system (CNS) and many exhibit region-specific expression in the developing brain, suggesting that RBPs may play a major role in establishing cell-type specific function during development (McKee et al., 2005). However, most of our knowledge of the function of RBPs in neurons stems from distinct cellular or dendritic compartments, and although RBPs have been found in axons (Zhang et al., 2001; Rossoll et al., 2002, 2003; Leung et al., 2006; Price et al., 2006; Yao et al., 2006; Christie et al., 2009; Akten et al., 2011), their full repertoire has not been determined and little is known about their abundance and distribution in axonal compartments.

An unbiased proteomic study has been performed recently on GCs from whole rat embryonic brain (Estrada-Bernal et al., 2012) and we have interrogated this dataset to determine the repertoire of RBPs. Interestingly, our analysis indicates that about 1% of all GC proteins are putative RBPs. This estimate is likely to be an under-representation because the experimental design of the study favors proteins expressed in the majority of GCs without taking into account any regional-or cell-specific expression of RBPs. Consistent with this line of reasoning, RBPs previously identified in axons, such as HuD (Akten et al., 2011; Fallini et al., 2011), the Fragile X mental retardation protein (FMRP) (Antar et al., 2006; Price et al., 2006; Christie et al., 2009; Akins et al.,

2012) and cytoplasmic polyadenylation element-binding protein (CPEB) (Kundel et al., 2009) were not identified in this screen, indicating that these RBPs may only be present in specific subsets of axons. Nonetheless, the study provides unparalleled insights into the repertoire of GC RBPs. Out of the 22 putative RBPs identified, only two, zipcode binding protein 1 (ZBP1, also known as IMP-1 and Vg1RBP) and survival motor neuron 1 (SMN) have previously been identified in GCs (Zhang et al., 2001, 2003, 2006; Leung et al., 2006; Fallini et al., 2011; Welshhans and Bassell, 2011). The single largest group of RBPs, comprising about 50% of all RBPs identified in the GCs, were the heterogenous nuclear ribonucleoprotein family (hnRNP) family of RBPs, a large family of RBPs that varies greatly in both function and structure (Han et al., 2010). To date, only one family member has previously been identified in axons and GCs (Rossoll et al., 2002, 2003; Glinka et al., 2010), but their striking enrichment in the GC proteome suggests that they may have a widespread role in developing axons. Members of the hnRNP family have also been identified in post-synaptic densities (Jordan et al., 2004; Zhang et al., 2012), indicating that they may serve key functions in both pre- and post-synaptic compartments. However, it is worth nothing that the hnRNPs enriched in post-synaptic densities differs from the hnRNPs most abundant in GCs (Zhang et al., 2012).

Of the other RBPs identified in GCs, four were RNA-recognition motif (RRM) containing proteins previously identified for their role in splicing and transcription (Imai et al., 1993; Patturajan et al., 1998; Kataoka et al., 2000; Guo et al., 2003; Cazalla et al., 2005; Chuang et al., 2011; Albers et al., 2012). Many hnRNPs also have known nuclear functions, and it is interesting to note that the majority of RBPs identified in this study, including hnRNP K (Expert-Bezancon et al., 2002; Lynch et al., 2005; Stains et al., 2005), U (Kukalev et al., 2005; Huelga et al., 2012), F (Min et al., 1995; Martinez-Contreras et al., 2006; Huelga et al., 2012), E1 (Kim et al., 2005; Akker et al., 2007), H1 SMN (Pellizzoni et al., 2002) and RNA binding motif protein 8a (RBM8a, also known as Y14) (Kataoka et al., 2000; Chuang et al., 2011; Albers et al., 2012), have well-established nuclear functions as regulators of splicing and transcription. In fact, of all RBPs found in GCs, only ZBP1 is best known for its cytoplasmic function. This raises the intriguing possibility that many neuronal RBPs may have a dual role both in the nucleus and cytosol. Interestingly, both proteins and mRNAs of splicing factors have been found in GCs (Zivraj et al., 2010; Estrada-Bernal et al., 2012), suggesting that axonal mRNA regulation may be more complex than previously thought.

RBP-MEDIATED LOCAL REGULATION OF AXON GROWTH, GUIDANCE, AND SYNAPSE FORMATION

While the role of RBPs in dendrites and post-synaptic compartments has traditionally received more attention (Bramham and Wells, 2007; Swanger and Bassell, 2011), several studies are starting to focus on the role of RBPs in axons. Some of these RBPs, like hnRNP R and SMN, appear to localize mainly in axons outside of the nucleus. Others, such as FMRP and ZBP1, have both dendritic and axonal functions. In this section, we review the role RBPs play as local regulators during axon growth, guidance, and synapse formation (**Table 1**).

AXON GROWTH

Axon outgrowth and the continuous regulation of axon growth are key steps during axon guidance and regeneration. Two RBPs associated with neurodegenerative disorders affecting motor neurons have been implicated as local regulators of axon growth suggesting that translational regulation in axons during this process may be broadly crucial for the survival and health of motor neurons. These RBPs are SMN and TDP-43.

SMN is a ubiquitously expressed RBP most known for its role in assembling small nuclear ribonucleoprotein (snRNP) complexes involved in splicing (Burghes and Beattie, 2009). Depletion of SMN is the cause of spinal muscular atrophy (SMA) and loss of SMN leads to degeneration of motor neurons. However, why the loss of a ubiquitously expressed gene causes a specific defect in motor neurons is not well understood. SMN has been detected in axons (Rossoll et al., 2002; Zhang et al., 2003, 2006; Fallini et al., 2011), and cultured motor neurons from a SMN mouse model display axonal defects including reduced axon growth, smaller GCs and reduced levels of β-*actin* mRNA in the axon and GC (Rossoll et al., 2003). In zebrafish and *Xenopus tropicalis,* knockdown of SMN leads to truncated motor neuron development *in vivo* (McWhorter et al., 2003; Ymlahi-Ouazzani et al., 2010). SMN can interact with several other RBPs (Mourelatos et al., 2001; Rossoll et al., 2002; Wang et al., 2002; Piazzon et al., 2008), and is thought to regulate translation indirectly via these interactions as SMN itself lacks any known RNA-binding domains. One of these RBPs, hnRNP R, is reduced in GCs and axons of cultured motor neurons lacking SMN (Rossoll et al., 2003), and depletion of hnRNP R in zebrafish gives a similar phenotype to SMN knockdown (Glinka et al., 2010). hnRNP R can associate with β-*actin* 3′ UTR and co-localizes with β-*actin* in GCs (Glinka et al., 2010). Knockdown of hnRNP R leads to a decrease in β-*actin* mRNA levels in GCs but no change in total mRNA levels, suggesting that hnRNP R specifically alters the subcellular location of β-*actin* mRNA. Overall, these findings suggest that SMN and hnRNP R co-regulate β-*actin* mRNA localization and translation in the distal axon during axon growth in motor neurons.

The TAR DNA binding protein 43, TDP-43, is also implicated in axonal regulation of motor neuron outgrowth. TDP-43 is mostly a nuclear DNA/RNA-binding protein involved in many parts of mRNA post-transcriptional regulation such as splicing, stability, and transport (Lee et al., 2012). TDP-43 is implicated in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD-U) where TDP-43 is found in large insoluble granules in the cytoplasm, but the pathogenesis of these granules is not clear. Apart from its nuclear location, TDP-43 has been found in axons of motor neurons where it co-localizes with other RBPs (Fallini et al., 2012). Axonal TDP-43 levels increase after BDNF stimulation in cultured motor neurons, and depletion of TDP-43 increases axon length and branching (Fallini et al., 2012). However, in mouse neuroblastoma neuro-2a cells TDP-43 depletion inhibits neurite outgrowth (Iguchi et al., 2009), and in zebrafish embryos zTDP-43 depletion causes reduced axon length in motor neurons (Kabashi et al., 2010), suggesting that TDP-43 may have different roles during neuronal development in different neuronal populations and species.

Table 1 | RBPs in axons.

RBP	Species	Cell type	Function	Target mRNA	References
CPEBs	Rat	Hippocampal neurons	Axon growth, branching	β-*catenin*	Kundel et al., 2009
	X.laevis	RGCs	Axon guidance		Lin et al., 2009
FMRP	Mouse	Hippocampal neurons	GC motility	*map1b*	Antar et al., 2006
			Axon guidance		Li et al., 2009
			Synapse formation		Hanson and Madison, 2007
	Drosophila	Mushroom body motor neurons	Branching, synapse formation	*futsch*	Pan et al., 2004; Tessier and Broadie, 2008
			Branching, synapse formation		Zhang et al., 2001; Gatto and Broadie, 2008
FMRP, FXR2, FXR1	Rat	Developing brain	Synapse formation?		Christie et al., 2009; Akins et al., 2012
hnRNP R	Mouse	Motor neurons	Axon growth	β-*actin*	Rossoll et al., 2003; Glinka et al., 2010
	Zebrafish		Axon growth, synapse formation		Glinka et al., 2010
HuD	Mouse	Motor neurons	Axon growth, branching	*cpg15*	Fallini et al., 2011
SMN	Mouse	Motor neurons	Axon growth	β-*actin*	Rossoll et al., 2003
		Motor neurons	Branching, synapse formation		Kariya et al., 2008; Kong et al., 2009
		RGCs			Liu et al., 2011
	Zebrafish	Motor neurons	Axon growth,		McWhorter et al., 2003
	X.tropicalis	Motor neurons	Branching		Ymlahi-Ouazzani et al., 2010
TDP-43	Mouse	Motor neurons	Axon outgrowth		Fallini et al., 2012
	Drosophila		Synapse formation	*futsch*	Godena et al., 2011; Lin et al., 2011
	Zebrafish		Axon growth, branching, synapse formation		Kabashi et al., 2010
ZBP1	*X.laevis,* chick, mice	RGCs, cortical neurons	Axon guidance	β-*actin*	Zhang et al., 2001; Leung et al., 2006; Yao et al., 2006; Sasaki et al., 2010

AXON GUIDANCE

Local protein synthesis plays a key role during axon guidance *in vitro* (Campbell and Holt, 2001; Wu et al., 2005; Leung et al., 2006; Piper et al., 2006; Yao et al., 2006) and *in vivo* (Leung et al., 2013) and although many mRNAs have been identified in axons (Taylor et al., 2009; Andreassi et al., 2010; Zivraj et al., 2010; Gumy et al., 2011), the identity of those that are actively translated and how they are regulated is less clear. RBPs are needed to transport mRNA to the GCs, but what regulatory role they play in the GCs during axon guidance is not well known. Some RBPs, depicted in **Figure 1**, can mediate the response to guidance cues by regulating local translation of their target mRNAs. ZBP1 was the first RBP found to regulate axon guidance, and its local regulation of β-*actin* mRNA in response to guidance cues is conserved in several species (Zhang et al., 2001; Leung et al., 2006; Welshhans and Bassell, 2011). In *Xenopus laevis*, the ZBP1 ortholog, Vg1RBP, mediates turning toward the attractive guidance cue Netrin-1 (Leung et al., 2006) and to brain-derived neurotrophic factor, BDNF (Yao et al., 2006). Stimulation of retinal ganglion cell (RGC) axonal GCs by a Netrin-1 gradient induces polarized movement of Vg1RBP toward the Netrin-1 source, and this is accompanied by an asymmetrical increase in activated eIF-4E-binding protein 1 (4EBP1) and β-*actin* translation (Leung et al., 2006). A BDNF gradient also leads to asymmetric β-*actin* and Vg1RBP localization in spinal cord neuron GCs, and preventing the β-*actin*-ZBP1 interaction abolishes both Ca^{2+}-mediated attraction and repulsion (Yao et al., 2006). This suggests that ZBP1 is crucial for regulating both the translation and spatial location of β-actin during GC turning. Together these two studies gave the first insight into how an RBP can spatially restrict translation in the GC. Translational dysregulation of β-actin can cause morphological defects in axons of several types of neurons (Zhang et al., 2001; Huttelmaier et al., 2005; Leung et al., 2006; Yao et al., 2006; Welshhans and Bassell, 2011), and several axonal RBPs have β-actin mRNA among their targets (Zhang et al., 2001; Rossoll et al., 2003; Huttelmaier et al., 2005; Leung et al., 2006; Glinka et al., 2010; Welshhans and Bassell, 2011), suggesting that translational regulation of β-actin may be of particular importance in axons. In dendrites, ZBP1-mediated dysregulation of β-actin perturbs branch development (Perycz et al., 2011), but whether or not ZBP1 has a similar function in axonal branching is not known.

FMRP is best known for its role as a translational regulator in the post-synaptic compartment, but it is also gaining attention

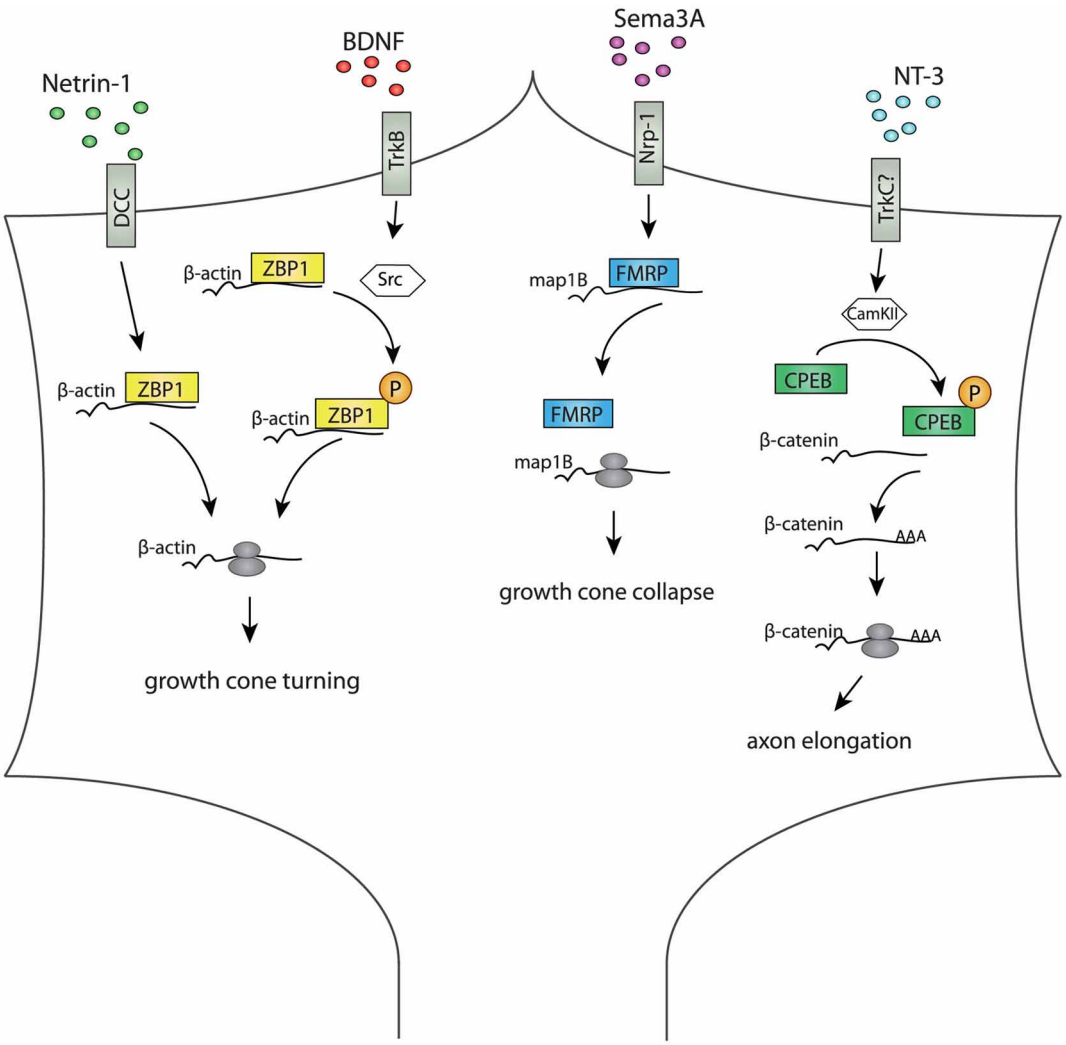

FIGURE 1 | Diagram summarizing RBP-mediated regulation of different cue-stimulated responses in axonal growth cones. In the growth cone, RBPs mediate translation of specific mRNAs after cue stimulation. Netrin-1 induces ZBP1 localization and translation of β-actin close to the source of Netrin-1, and this is crucial for growth cone turning (Leung et al., 2006; Lin and Holt, 2007). BDNF induces Src-mediated ZBP1 phosphorylation, β-actin translation and growth cone turning toward the BDNF source (Yao et al., 2006; Sasaki et al., 2010). Growth cone collapses and Map1B mRNA translation in response to Sema3a is attenuated in axons depleted of FMRP, suggesting a role for FMRP in Sema3A-mediated axon guidance (Li et al., 2009). NT3 induces CamKII mediated phosphorylation of CPEB1, which activates polyadenylation and translation of β-catenin mRNA crucial for axon elongation and branching (Kundel et al., 2009).

for its role in axons (Christie et al., 2009; Deng et al., 2011; Till et al., 2011; Akins et al., 2012). FMRP is present in axons and GCs (Antar et al., 2006), and knockdown of FMRP in hippocampal neurons leads to reduced GC motility, excess filopodia (Antar et al., 2006), and attenuated collapse in response to the repulsive guidance cue Semaphorin 3A (Sema3A) (Li et al., 2009). Sema3A stimulation increases the levels of phosphorylated eukaryotic translation initiation factor eIF4E and MAP1b translation in distal axons, but this increase is abolished in FMRP deficient neurons (Li et al., 2009), suggesting a role for FMRP in axons during Sema3A-mediated GC steering via regulation of MAP1b translation in the GC.

AXON ARBORIZATION AND SYNAPSE FORMATION

Translational regulation is crucial for synaptic function, and a number of cognitive disorders are linked to mRNA dysregulation. For example, Fragile X syndrome (FXS), the most common form of inherited mental retardation, is caused by the loss of FMRP function and subsequent dysregulation of its target mRNAs (Bassell and Warren, 2008). Although FXS is thought to be caused mainly by the loss of FMRP function in the post-synaptic compartment, several lines of evidence suggest that FMRP may also have a pre-synaptic role at the synapse. FMRP binds to many mRNAs encoding pre-synaptic proteins (Akins et al., 2009; Darnell et al., 2011), and several pre-synaptic proteins are differentially regulated in fmr1 knockout (KO) mice

(Klemmer et al., 2011). In *Drosophila*, the mushroom body neurons of dFMRP null mutants have over-branched axonal arbors and form abnormal synapses, (Pan et al., 2004; Tessier and Broadie, 2008), and abnormal pre-synaptic structures at the neuromuscular junction (NMJ) (Zhang et al., 2001; Gatto and Broadie, 2008). Furthermore, in a mosaic mouse model of FXS, neurons lacking FMRP function form fewer synaptic connections than wild type neurons, suggesting that pre-synaptic FMRP function may determine the likelihood of forming a synapse (Hanson and Madison, 2007). Pre-synaptic expression of FMRP appears restricted to a subset of neuronal circuits where it is present in granules (Fragile X granules; FXGs) in association with its paralogs FXR2p and FXR1p (Christie et al., 2009; Akins et al., 2012). The expression of these granules peak during the time of synapse formation and pruning (Christie et al., 2009; Akins et al., 2012), indicating a possible pre-synaptic role for FMRP and its paralogs during synapse formation in a subset of axon population.

Disruption of SMN also causes pre-synaptic abnormalities. In a mouse model of SMA, axons at the NMJ terminals are poorly arborized and display abnormal neurofilament accumulation in the nerve terminals (Kariya et al., 2008). Furthermore, SMN knockdown causes abnormal synaptic transmission (Kariya et al., 2008; Kong et al., 2009), lower synaptic vesicle density at the pre-synaptic site (Kong et al., 2009) and a reduction of $Ca_v2.2$ Ca^{2+} channels at the NMJ (Jablonka et al., 2007). Interestingly, knockdown of hnRNP R leads to a similar phenotype (Glinka et al., 2010), indicating that SMN and hnRNP R may co-regulate translation both during axon growth and synapse formation. SMNs' function has mostly been studied in motor neurons, but similar defects have been reported in the retina of a mouse SMA model (Liu et al., 2011), suggesting that perhaps SMN has a conserved role in axon elongation and connectivity in several axon populations. SMN may regulate synapse formation partially via co-regulation of the candidate plasticity-related gene 15 (cpg15), an activity-regulated protein with key functions during branching and synaptogenesis in the NMJ. SMN can interact with HuD (Akten et al., 2011; Fallini et al., 2011), an RBP known to bind to and regulate cpg15 expression (Wang et al., 2011), and both SMN and HuD co-localize with cpg15 in axons. Disruption of SMN function reduces the amount of *cpg15* mRNA, and overexpression of cpg15 partially rescues the SMA phenotype in a zebrafish model (Akten et al., 2011). Together these studies suggest a crucial role for SMN and HuD mediated *cpg15* mRNA regulation in axons during synapse formation at the NMJ.

TDP-43 has also been shown to cause defects in axonal branching and synapse formation at the NMJ. Depletion of TDP-43 causes an increase in synaptic boutons at the NMJ in *Drosophila* (Lin et al., 2011), and immature and excessive branching in zebrafish (Kabashi et al., 2010). In *Drosophila*, the defects are associated with a decrease in the microtubule stabilizing protein futsch, a MAP1B ortholog. dTDP-43 can interact directly with *futsch* mRNA, and dTDP-43s RNA-binding property is essential for its function in synapse formation (Godena et al., 2011). Furthermore, dTDP-43 depletion decreases futsch protein in distal boutons, but *futsch* mRNA levels was unchanged, suggesting a role for dTDP-43 in translational regulation of futsch (Godena et al., 2011).

TRANSLATIONAL REGULATION BY RBPs IN THE AXON AND GROWTH CONE

How do RBPs repress translation in the GC, and how is translation activated? Translational repressors are found in RNPs (Kim-Ha et al., 1995; Nakamura et al., 2004; Paquin et al., 2007) and when bound to their targets these repressors can regulate translation by either blocking translation elongation, or, most often, translational initiation. ZBP1 can block translation initiation by inhibiting recruitment of the 60S subunit (Huttelmaier et al., 2005), FMRP is thought to block translation elongation by recruiting the eIF4E-binding protein CYFIP1 (Napoli et al., 2008) and the post-synaptic RBP, Pumilio, regulates the abundance of eIF4E at the NMJ (Menon et al., 2004).

RBPs may also regulate translation via modulating the length of the poly(A) tail of mRNA. CPEB controls translation by polyadenylation and directly binds the CPE sequence in the 3′UTR of its target mRNAs (Richter, 2007). Blocking polyadenylation attenuates the collapse response to Sema3A in *Xenopus* retinal axons (Lin et al., 2009), and blocking CPEB1's function in hippocampal neurons causes a reduction in NT3-induced β-actin translation in the GC, possible via Ca^{2+} mediated inositol triphosphate (IP3) and Ca^{2+}/calmodulin-dependent protein kinsase II (CamKII) activation (Kundel et al., 2009). This suggests that regulation of poly(A) tail length may be a common way for guidance cues to regulate translation of specific mRNAs.

Stimulation of GCs with protein synthesis-inducing guidance cues, such as Netrin-1 and Sema3A, leads to the activation of global translation, as indicated by 4EBP1 and mTOR activation, yet they each stimulate the translation of a distinct set of mRNAs (Wu et al., 2005; Leung et al., 2006). Furthermore, guidance cues can stimulate translation globally while repressing specific transcripts (Yoon et al., 2012), and both translation reporters and newly synthesized protein can be localized to specific compartments in the GC (Leung et al., 2006; Yao et al., 2006). How translational specificity is achieved and how it is spatially localized in the GC is largely unknown.

Signal-mediated phosphorylation of RBPss present in the GC may provide a way to regulate translation of specific mRNAs. BDNF induces Src-mediated phosphorylation of ZBP1, and blocking this step attenuates local β-actin translation and GC turning (Sasaki et al., 2010). Src is activated asymmetrically toward the BDNF source (Yao et al., 2006), indicating that localized activation of distinct set of RBPs may provide both spatial and temporal control over translation. FMRP activity is also regulated by phosphorylation (Narayanan et al., 2008; Muddashetty et al., 2011; Coffee et al., 2012), suggesting that phosphorylation could be a common mechanism for releasing RBP-mediated repression upon cue stimulation.

RBPs can also mediate translational regulation via small non-coding RNAs such as microRNAs (miRNAs). miRNAs can associate with RBPs (Schratt et al., 2006; Edbauer et al., 2010), and RBPs are known to regulate the abundance of miRNAs (Michlewski et al., 2008; Xu et al., 2008, 2011; Xu and Hecht, 2011). In dendrites, there is evidence that miRNAs can act locally as translational repressors (Schratt et al., 2006). miRNAs and RNA-induced silencing complex (RISC) components have been

found to associate with FMRP (Caudy et al., 2002; Jin et al., 2004; Muddashetty et al., 2011), and FMRP may depend on miRNAs to repress some of its targets (Muddashetty et al., 2011). HuR can interfere with miRNA-mediated repression in cell culture, both as an antagonist of miRNA repression (Bhattacharyya et al., 2006) and in a cooperative manner to help facilitate repression (Kim et al., 2009). Interaction studies suggest that HuR may regulate the efficiency of several miRNAs (Mukherjee et al., 2011), and it would be interesting to see if its neuronal family member, HuD, can act in a similar fashion. miRNAs have been found in the distal axon (Natera-Naranjo et al., 2010; Han et al., 2011; Dajas-Bailador et al., 2012), and seem to play a role in guidance cue responses. Knockdown of Dicer leads to axon guidance defects in the visual system in mice (Pinter and Hindges, 2010), and knockdown of the miRNA miR-124 leads to guidance defects of RGC axons caused by an attenuated response to Sema3A (Baudet et al., 2012). However, miRNA-RBP mediated regulation is a relatively novel concept and whether or not RBPs can regulate miRNA repression in axons and GCs is not yet known.

Another intriguing possibility is that receptor-ribosome interactions may be used to restrict translation spatially and confer additional translation specificity. The Netrin-1 receptor, DCC, can interact directly with the translational machinery by forming complexes with ribosomal subunits. This interaction is disassociated upon Netrin-1 stimulation to promote DCC-mediated translation (Tcherkezian et al., 2010), suggesting a possible mechanism to spatially restrict cue-induced translation to a specific subcellular compartment.

FUTURE PERSPECTIVES

RBPs are beginning to emerge as important players in the presynaptic compartment during the building of neuronal circuits, but many questions still remain. The list of axonal RPBs is still incomplete, and little is known of their mRNA targets in axons. New techniques such as crosslinking immunoprecipitation (CLIP) (Ule et al., 2005) and high throughput sequencing-CLIP (HITS-CLIP) (Licatalosi et al., 2008) will be valuable in future studies for identifying RBP-mRNA complexes in different axon populations and developmental time points. Moreover, further studies on the interactions of RBPs with other post-transcriptional regulatory pathways are needed to help gain insight into how translational specificity is achieved in the GC. For example, it will be important to investigate if guidance receptor coupling to the translational machinery (Tcherkezian et al., 2010) is a common way of restricting translation locally, and to understand the interplay between other pathways such as miRNAs, RBP phosphorylation and mRNA polyadenylation in regulating the spatiotemporal control of local protein synthesis in response to extrinsic cues.

The observation that many axonal RBPs are best known for their nuclear roles suggests that some axonal RBPs may have dual functions in the nucleus and cytosol. Nuclear mRNA processing is important for subsequent cytosolic localization (Giorgi and Moore, 2007), and the axonal and nuclear localization of some RBPs may provide a platform to coordinate pre-mRNA processing and cytosolic translational regulation (Bava et al., 2013). The presence of splice-regulating RBPs in axons suggests the intriguing possibility that some pre-mRNA processing may occur locally in axons. Indeed, cytoplasmic splicing has been identified in neurons (Bell et al., 2010), and splice components localized to dendrites retain their ability to splice RNA (Bell et al., 2010). Splice factors have been found in GCs (Estrada-Bernal et al., 2012), but whether they are involved in splicing or other processes is not known.

Finally, although it is increasingly clear that local translation occurs in navigating axons and post-synaptic compartments, its role in target-arrived axons is much less understood. Transcripts of synapse-associated proteins are commonly present in axons (Zivraj et al., 2010), and pre-synaptic translation has been implicated in synapse development (Taylor et al., 2013), synaptic plasticity (Yin et al., 2006; Deng et al., 2011; Je et al., 2011; Johnstone and Raymond, 2011; Till et al., 2011) and arborization (Dajas-Bailador et al., 2012; Donnelly et al., 2013). Translational dysregulation is thought to underlie several neurodevelopmental and neurodegenerative disorders (Bear et al., 2004; Liu-Yesucevitz et al., 2011; Santini et al., 2013; Taylor et al., 2013). RBPs such as FMRP, SMN and TDP-43 have all been linked to neurological diseases, and their presence in axons suggests that axonal translation may play a role in disease pathology. Elucidating how presynaptic translation influences synapse formation and the role RBPs play in this process will further deepen our understanding of how neuronal circuits are formed and maintained in the developing brain.

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Toward a unified biological hypothesis for the BDNF Val66Met-associated memory deficits in humans: a model of impaired dendritic mRNA trafficking

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Brain-derived neurotrophic factor (BDNF) represents promotesa key molecule for the survival and differentiation of specific populations of neurons in the central nervous system. BDNF also regulates plasticity-related processes underlying memory and learning. A common single nucleotide polymorphism (SNP) rs6265 has been identified on the coding sequence of human *BDNF* located at 11p13. The SNP rs6265 is a single base mutation with an adenine instead of a guanine at position 196 (G196A), resulting in the amino acid substitution Val66Met. This polymorphism only exists in humans and has been associated with a plethora of effects ranging from molecular, cellular and brain structural modifications in association with deficits in social and cognitive functions. To date, the literature on Val66Met polymorphism describes a complex and often conflicting pattern of effects. In this review, we attempt to provide a unifying model of the Val66Met effects. We discuss the clinical evidence of the association between Val66Met and memory deficits, as well as the molecular mechanisms involved including the reduced transport of BDNF mRNA to the dendrites as well as the reduced processing and secretion of BDNF protein through the regulated secretory pathway.

Keywords: neurotrophins, BDNF, memory deficits, post-traumatic stress disorder, hippocampus atrophy, dendritic mRNA trafficking, regulated protein secretion

BDNF AND ITS Val66Met POLYMORPHISM

The development and plasticity of the nervous system are deeply influenced by a family of neurotrophic factors called neurotrophins. In mammals, the neurotrophin family consists of four related proteins: Nerve growth factor (NGF; Levi-Montalcini and Booker, 1960), Brain-derived neurotrophic factor (BDNF; Barde et al., 1982), neurotrophin-3 (NT3; Hohn et al., 1990) and neurotrophin-4 (NT4; Berkemeier et al., 1991). Since its discovery three decades ago, BDNF has been firmly implicated in the development of the nervous system in vertebrates and in the support of survival and differentiation of specific populations of neurons in the central nervous system (Bibel and Barde, 2000; Binder and Scharfman, 2004; Chao et al., 2006). More recently, BDNF has also emerged in several functions in the adult brain in which it is involved in the main plasticity-related processes including memory and learning (Tyler et al., 2002; Yamada et al., 2002).

A common single nucleotide polymorphism (SNP) rs6265 has been identified on the coding sequence of human *BDNF* located at 11p13 (Hall et al., 2003). This single base mutation, presenting an adenine instead of a guanine at position 196 (G196A), results in the amino acid substitution Val66Met (Hall et al., 2003). The polymorphism Val66Met only exists in humans and has been associated with a plethora of effects ranging from detrimental molecular, cellular and brain structural modifications associated with social and cognitive dysfunction (Dincheva et al., 2012). While the literature on this polymorphism is rapidly increasing, there is little consensus on the pattern of results. In this review, we discuss how this single nucleotide variant affects molecular mechanisms of memory formation and maintenance and summarize clinical evidence on the association between Val66Met SNP and memory deficits. In conclusion, we hypothesize a biological mechanism underlying the memory deficits associated with the Val66Met polymorphism of BDNF.

CLINICAL ASPECTS OF Val66Met

The first association between the BDNF Val66Met SNP and a clinical phenotype was reported in schizophrenic patients, their relatives and healthy controls (Egan et al., 2003). This study showed a specific effect on cognitive functions where the Met BDNF allele reduced the delayed recall of episodic memory in all three groups but had no influence on other cognitive domains or *intelligence quotient* (IQ). Both Egan et al. (2003) and Hariri et al. (2003) demonstrated that Met BDNF carriers displayed reduced hippocampal engagement during encoding and retrieval of a spatial task with respect to Val/Val homozygotes. Two independent MRI investigations (Pezawas et al., 2004; Szeszko et al., 2005) extended the findings of Egan and Hariri (Egan et al., 2003; Hariri et al., 2003) by demonstrating in affectively ill individuals that Val/Met heterozygotes displayed lower hippocampal volumes than their Val/Val counterparts. In addition, there was volume reduction of

the gray matter in the dorsolateral prefrontal cortex (DLPFC), which is implicated in learning and memory processes involving also the hippocampus (Pezawas et al., 2004; Hwang et al., 2006; Benjamin et al., 2010). Further, in a community of elderly Caucasian individuals, BDNF Val/Val homozygotes performed significantly better compared to both Val/Met heterozygous and Met/Met homozygous individuals on a delayed recall task and an alphabet-coding task (ACT), a measure of processing speed. Another study also noted a decrease in hippocampal volume in Met BDNF allele carries, although it did not reach statistical significance (Goldberg et al., 2008) while other authors confirmed the hippocampal volume reduction (Benjamin et al., 2010; Teh et al., 2012; Tost et al., 2013).

In a fascinating study, Schofield et al. (2009) found a significant increase in hippocampal activity in Met BDNF carriers compared to BDNF Val/Val homozygotes during the auditory oddball task. The auditory oddball task has been asserted to measure auditory attention and auditory capacity. It is a task in which the subject must detect a relevant ("oddball") stimulus, which is presented infrequently and randomly within a train of task-irrelevant stimuli. Schofield and colleagues argued that Met BDNF carriers might require greater hippocampal activation than their BDNF Val/Val counterparts to process auditory stimuli presented during the task, inadvertently depleting resources for prefrontal processing of stimuli. Similar circuit dysregulation has been observed previously. A protective role for the Met allele has also been proposed for enhanced verbal reasoning in the elderly (Harris et al., 2006), systemic lupus erythematosus (Oroszi et al., 2006), multiple sclerosis patients (Zivadinov et al., 2007; Cerasa et al., 2010), and Parkinson's disease (Foltynie et al., 2005), although others have challenged these findings (Liguori et al., 2007; Miyajima et al., 2008).

Additional studies found either a gender-specific association (Echeverria et al., 2005) or no association in schizophrenic and bipolar patients (Strauss et al., 2004; Tramontina et al., 2009) as well as in other clinical conditions characterized by cognitive impairment such as multiple sclerosis (Cerasa et al., 2010), HIV-associated neurocognitive disorders (Levine et al., 2012) and Parkinson's disease (Guerini et al., 2009). The disagreement between results could be due to a number of differences in study design (Hong et al., 2011). First, these studies are typically characterized by relatively small sample size. Second, there is a noticeable variability in the phenotypes analyzed in these researches. Specifically, it is striking how many different tasks have been used in the field to assess arguably similar (or the same) cognitive functions. In animal studies, BDNF is studied especially in the hippocampus and therefore, the impact of the Val66Met polymorphism could have been more evident in studies focused on hippocampus-specific tasks. More orchestrated approaches to behavioral phenotyping for cognition in the design of genetic and allelic association studies on BDNF are highly needed and are likely to be fruitful. Otherwise, using the mere Mini Mental State Examination (MMSE), the effects of BDNF Val66Met polymorphism on cognition may not emerge (Zivadinov et al., 2007; Forlenza et al., 2010).

More recently, Mandelman and Grigorenko (2012) conducted a meta-analysis examining the relationship between BDNF Val66Met and cognition, but did not find significant associations between the Val66Met polymorphism and any of the phenotypes that were included. The obvious additional candidates for sources of such between-study heterogeneity are demographic characteristics such as gender, age and phase of illness, ethnicity, physical exercise, cardio-vascular health status and diagnosis (Verhagen et al., 2010; Lu et al., 2012; Martinho et al., 2012; Nagata et al., 2012; Smith et al., 2012). Furthermore, it is possible that the genetic variant may be associated with some intermediate phenotypes, which, in turn, could be related to some but not all of the cognitive phenotypes examined. Given the multitude of BDNF protein isoforms and the diversity of its transcripts in different brain areas, it is conceivable that cognitive phenotypes should be grouped not by their behavioral similarities, but by similarities in the brain activation pathways that underlie these phenotypes.

BIOLOGICAL ASPECTS OF THE Val66Met MUTATION

It is important to highlight that the vast majority of the results reported in the literature and summarized here, were obtained by investigations on homozygous BDNF Met/Met (Met BDNF) mice while the double mutated Met/Met allele is quite rare in humans. Indeed, data from human population are collected mostly from individuals with BDNF Val/Met (single allele mutation) or BDNF Val/Val (Mandelman and Grigorenko, 2012). The biological effects of the SNP rs6265 in the BDNF coding sequence have been studied using several different model systems, starting from cell culture, to animal models or in correlation with diseases affecting the human population. In their initial work, using an *in vitro* neuronal culture system, Egan and coworkers (Egan et al., 2003) demonstrated that Met BDNF in fusion with GFP and transfected in neurons is produced at levels similar to the control Val BDNF-GFP. The knock-in animal model for the Met BDNF allele created by Chen et al. (2006; Dincheva et al., 2012) confirmed that the total BDNF production is not affected by the polymorphism. A recent detailed analysis of BDNF protein in specific brain areas of BDNF Val and Met knock-in mice showed slight but significant reduction of BDNF in the hippocampus (HPC) and in prefrontal (PFC) cortex while no variations were found at the level of amygdala and striatum (Bath et al., 2012; Yu et al., 2012).

Chen and coworkers were first to describe the functional importance of the Met BDNF allele in the brain. They reported alterations in hippocampal anatomy of the Met BDNF knock-in model. The total hippocampal volume of Met BDNF mice was found to be reduced and dentate gyrus (DG) neurons were measured as significantly smaller in total volume and dendritic complexity while the soma size was not affected (Chen et al., 2008). Similar neuronal volumetric reduction was reported also on ventromedial prefrontal cortex cells, while neurons from the striatum were not affected (Yu et al., 2009). The same mouse model was used to establish the effect of Met polymorphism in prefrontal cortex (PFC) where a specific atrophy of apical dendrites of layer 5 pyramidal cells was found (Liu et al., 2012). Furthermore, the induction of synaptogenesis in PFC by ketamine administration on brain slices was almost abolished indicating

impaired synaptic formation/maturation. Met BDNF mice presented alterations in the generation of LTP at the CA3-CA1 synapse. Ninan and colleagues were able to dissect out the mechanism of this deficit revealing that NMDA receptor-dependent LTP was specifically affected while the non-NMDA receptor neurotransmission (i.e., mGluR) was normal (Ninan et al., 2010; Pattwell et al., 2012). Yet, it remains unclear if these BDNF effects on the NMDA receptor are either due to a deficit in the basal regulation of the NMDA receptor trafficking and expression or to an altered acute activity-dependent release of BDNF, in Met BDNF mice. Additional evidence of deficits in neuronal activation was observed in ventromedial prefrontal cortex of Met BDNF mice following fear extinction tests: the Met substitution results in reduced cellular activation (–50%), visualized by cFOS positivity (Yu et al., 2009).

EFFECTS OF Val66Met ON BDNF SECRETION AND TRAFFICKING

Apart from of the evidence of a normal (total brain) or reduced (HPC and PFC) production of BDNF in presence of the Val66Met mutation, several studies reported a differential subcellular distribution of BDNF upon Met substitution. BDNF was shown to regulate synaptic strength in a site-restricted manner and therefore, its subcellular distribution is very important for the functional role of this neurotrophin (Steward and Schuman, 2001; Horch and Katz, 2002; Lu, 2003; Alonso et al., 2004; Horch, 2004). Given the morphological structure of neurons, highly polarized cells with a relatively small soma and long dendritic/axonal processes, the BDNF protein must overcome several challenges to exert its functions correctly. In particular, just like many other synaptic proteins, BDNF is synthesized in response to synaptic stimuli and needs to be delivered at synapses located in specific subcellular districts where it can modify their structure and function (Tongiorgi, 2008; Edelmann et al., 2013). Jiang and Schuman reviewed the three different models that explain how a protein can be selectively delivered at activated synapses (Jiang and Schuman, 2002). In one model, the protein is synthesized in the soma and becomes tagged for a specific group of synapses, where it is subsequently transported. The second model describes the protein as being transported to dendrites without a specific target but is then captured by "tagged" synapses which have been appropriately stimulated. In the third model, which is in contrast with the previous two, it is the mRNA that is transported to dendrites and where it is locally translated by the protein synthesis machinery localized in proximity to synapses. Most researchers in the field agree that these three mechanisms might coexist within the same cell and may even regulate the very same protein.

BDNF represents one clear example of coexistence of multiple mechanisms for dendritic trafficking. The mRNAs encoding for BDNF were found to be transported and actively accumulate along dendrites following stimulation of electrical activity (Tongiorgi et al., 1997, 2004; Jakawich et al., 2010; Baj et al., 2013). BDNF was also shown to be transported to dendrites in large, dense-core secretory vesicles (Edelmann et al., 2013). Accordingly, recent studies have investigated the possible impact of the Met polymorphism on these BDNF sorting mechanisms.

In neurons, Met BDNF seems to be preferentially produced and accumulated within the somatic district and only partially transported in the proximal area of the primary dendrites whereas Val BDNF is produced and transported also in secondary and tertiary dendrites (Egan et al., 2003; Chen et al., 2004, 2006). The abnormal distribution of Met BDNF in cell body and dendrites of hippocampal neurons appears to be accompanied by a significant reduction in the secretion of this neurotrophin. In particular, secretion of BDNF induced in response to electrical stimuli is compromised (30% decrease) while the constitutive secretion of BDNF is not affected (Egan et al., 2003; Chen et al., 2004, 2006). BDNF sorting into the regulated secretory pathway appears to be mediated by two mechanisms. The first, involves interaction of the sorting receptor carboxypeptidase-E (CPE) with a tetrad of aminoacids (I16, E18, I105, D106) in the mature BDNF region forming a specific recognition motif, which is not affected by the Val66Met mutation (Lou et al., 2005). The second sorting mechanism, regards the interaction of sortilin with the pro-BDNF region comprised between aminoacid 44 and 102, wherein the Val66Met occurs (Chen et al., 2005). The current view is that substitution of the Val66 with a Met causes less efficient interaction with sortilin and therefore Met BDNF proteins show decreased targeting to the regulated secretory pathway (Dincheva et al., 2012). Further evidence in support of this hypothesis was provided by experiments in which Met BDNF was able to impair the trafficking of BDNF Val following formation of BDNF Val and Met BDNF heterodimers (Chen et al., 2004).

The combinatorial effect of normal BDNF production but decreased dendritic localization and secretion is correlated with the reduction of BDNF in SecII-positive secretory vesicles and Synaptophysin-positive synapses (Egan et al., 2003). The different biochemical pathways involved in the production and sorting of BDNF was also specified by Del Toro et al. (2006), who not only confirmed the reduction of Met BDNF in SecII vesicles but also found an accumulation of Met BDNF in Giantin-positive Golgi vesicle. Moreover, they found no visible differences between Val BDNF and Met BDNF in calnexin-positive vesicles while the total number of vesicles containing BDNF was strongly reduced by about 30% (Del Toro et al., 2006). It is important to note that the polymorphism at position 66 in the BDNF pro-domain does not affect the cleavage of the pro-BDNF form and the generation of the mature form of BDNF (Chen et al., 2004).

Transport dynamics of Met BDNF and Val BDNF through the secretory pathway are comparable. Indeed, the calculation of the mean translocation velocity of secretory vesicles in dendrites showed no differences between the two alleles (Del Toro et al., 2006), suggesting that the transported BDNF protein may probably account for the constitutive secretion of this neurotrophin which is also not affected by the Val66Met polymorphism. However, the physiological role of BDNF is only partially supported by this basal BDNF secretion. In fact, Bath et al. (2008) showed that TrkB activation in neurogenic regions, revealed by the intensity of pTrkB staining on total TrkB (in SVZ and OB), was reduced by 30% in Met BDNF brains. These data point out that activity-dependent BDNF secretion is required

for a physiological activation of TrkB to sustain neurogenesis and neuroblast survival. Accordingly, a recent characterization of the neurogenesis at the level of the DG in Met BDNF mice, calculated as density of BrdU positive cells, found a considerable reduction (ca. –25%) of proliferating neural progenitors (Bath et al., 2012).

EFFECTS OF BDNF Val66Met: AN mRNA PERSPECTIVE

Among the studies that report a reduced amount of dendritic BDNF protein in presence of the Val66Met polymorphism, one study compared the trafficking of BDNF mRNA with the wild type sequence (Val allele: G196) or with the mutation (Met allele: A196). The results revealed significantly reduced dendritic trafficking of BDNF mRNA bearing the A196 mutation (Chiaruttini et al., 2009). This effect was found to be due to a reduced affinity of the RNA-binding protein Translin for the mutated allele. The RNA-binding domain of Translin contains a pocket made by three aminoacids (His90, Arg21, Glu89), which specifically recognizes a guanosine by forming three hydrogen bonds (**Figures 1A,C**). The transition from Guanine to Adenine at position 196 reduces the number of possible hydrogen bonds to just one, with a dramatic reduction in binding stability of Translin to the mutated BDNF mRNA (**Figure 1B**). Since the minimal tertiary configuration with which Translin can bind to an mRNA is a dimer, the authors hypothesized that there could be a second Guanine on BDNF mRNA that binds to the other Translin participating to the dimer (**Figure 1D**). Indeed, a second Guanine was identified at position 177 and when mutated to an Adenine, it abolished the Translin-mediated trafficking in dendrites exactly like the Guanine at position 196 (Chiaruttini et al., 2009). This mutation of Guanine at position 179 has no effect. Translin is involved in the trafficking of other transcripts associated with synaptic functions such as the mRNA encoding for CamK-II and the small non-coding RNA BC-1, implicated in regulating protein translation in dendrites (Li et al., 2008; Jaendling and McFarlane, 2010). In addition, Translin can associate with a similar protein called TRAX to form a complex known as C3PO, which is highly conserved from fungi to humans (Tian et al., 2011; Ye et al., 2011). This complex is a component of the RISC complex and is required for the mechanisms of RNA interference, which regulates mRNA stability and translation at synapses. Remarkably, Translin KO-mice have several behavioral abnormalities (Li et al., 2008; Jaendling and McFarlane, 2010). Thus, abnormal interactions of an mRNA with Translin may have profound effects on its trafficking, stability and translation (Wu et al., 2011).

A recent study in humans found a strong correlation, defined by linkage disequilibrium, among rs6265 and two other SNPs in the 3′UTR of BDNF (rs11030100 and rs11030099). All these SNPs transform BDNF mRNA into a target for the miR-26s family providing a supplementary component involved in regulation of mRNA stability and translation (Caputo et al., 2011). These pieces of evidence are consistent with findings in BDNF Met/Met mice and suggest that the reduced availability of BDNF mRNA in dendrites of Met BDNF neurons leads to a deficiency of local protein translation and may contribute to deficits in activity-dependent release of Met BDNF from post-synaptic terminals.

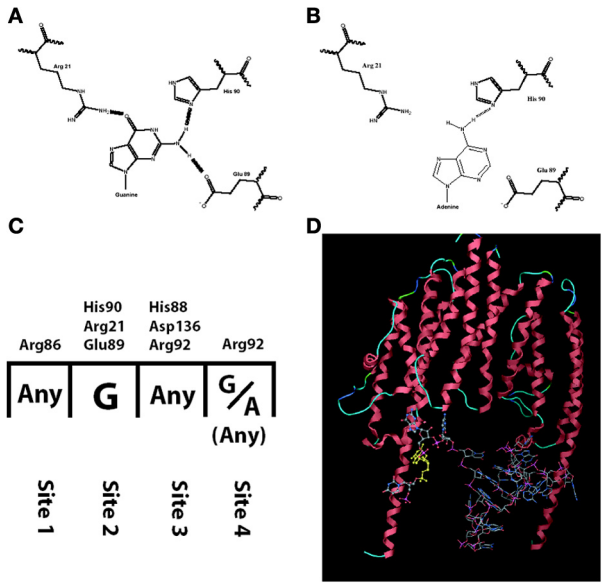

FIGURE 1 | Interaction between translin and human BDNF mRNA is hampered by the mutation G196A. (A) Translin amino acids His-90, Arg-21, Glu-89 form three hydrogen bonds with G196 (normal human BDNF) but only one with A196 (mutated BDNF, in **B**). **(C)** The nucleotide: amino acid selectivity for each of the four binding sites on translin. **(D)** Three-dimensional modeling of translin dimer binding to human BDNF mRNA.

TOWARD A UNIFIED BIOLOGICAL HYPOTHESIS FOR THE Val66Met DEFICIT

At the current state of research, a number of studies support the view that the BDNF Val66Met polymorphism, with one or two copies of the Met BDNF allele, can lead to altered performance of learning and memory functions, especially with impairments in hippocampal and cortical processes, suggestive of reduced neuroplasticity. To better understand how a small change in the BDNF sequence may determine so many effects, we believe that it is worth trying to assemble the large body of data available in the literature into a unified model at the cellular, anatomical, and behavioral level.

From a cellular standpoint, converging studies suggest that neuroplasticity deficits can be accounted mainly by impairment of activity-dependent translation and release of the Met BDNF from post-synaptic sites. We now describe how this deficit can result from a combination of reduced transport of Met BDNF mRNA in dendrites and an altered processing of the mutated protein through the regulated secretory pathway.

The deficit in mRNA trafficking found in BDNF bearing the Met allele (G196A at mRNA level), can be put into the broader perspective of the so called "spatial code model of BDNF transcripts" (Chiaruttini et al., 2008; Tongiorgi, 2008). BDNF is encoded by multiple mRNAs generated by alternative splicing. Eleven non-coding 5′UTR exons are alternatively spliced to a common downstream exon containing the coding region and a 3′UTR with two polyadenylation signals (An et al., 2008) which produce 11 different transcripts in rodents and 14 in

humans, each with two 3′ tails (Aid et al., 2007; Pruunsild et al., 2007). Following a series of studies demonstrating the differential subcellular distribution of the different BDNF transcripts (Pattabiraman et al., 2005; Chiaruttini et al., 2008), a hypothesis was put forward that the multiple transcripts encoding the very same protein are used to generate a spatial code for expressing BDNF at restricted subcellular locations, leading to localized effects (Tongiorgi, 2008). This model was recently completed by real-time PCR analysis of all rodent transcripts *in vivo* (Baj et al., 2013). In untreated rats, various BDNF transcripts were detected in dendritic fields of the hippocampus (exons 6 and 7 in CA1; exons 1, 6, and 9a in CA3; and exons 5, 6, 7, and 8 in DG). However, due to the very low levels of most of these transcripts, the exon 6 was found to be the main transcript present in dendrites at resting conditions. Strong neuronal activation by pilocarpine-induced *status epilepticus* caused an increase in all hippocampal dendritic laminae of BDNF transcripts encoding exons 2, 4, and 6 and also of BDNF exons 3 and 9a in DG molecular layer, whereas the other transcripts were restricted to the soma (Baj et al., 2013). Importantly, overexpression or silencing of the four most abundant brain BDNF transcripts, encoding exon 1, 2, 4, or 6, led to differential effects on dendritic morphology *in vitro*, with exons 1 and 4 affecting only the proximal dendritic domains and exon 2 and 6 being able to shape the distal dendritic district (Baj et al., 2011). This differential effect was demonstrated to be due to localized expression of BDNF and activation of TrkB receptor in the same subcellular domains where the specific mRNAs are localized (Baj et al., 2011). The conversion of the G196 to A196 impairs the transport of BDNF mRNA into dendritic domains (Chiaruttini et al., 2009), and therefore, the mutated BDNF mRNAs can only be translated in the soma. Since the change G196A is located within the coding region, it is present in all BDNF mRNA variants but its effects on mRNA trafficking are only evident for those BDNF transcripts that are actively transported to dendrites (such as exon 2 or 6). However, activity-dependent secretion is also impaired, causing entrapment of the Met BDNF protein within the Golgi apparatus and the observed increase in the soma and depletion from distal dendrites (Egan et al., 2003; Chen et al., 2004) (**Figure 2**). In this model, the converging misplacement of BDNF mRNA and protein can affect the ability of BDNF to maintain dendritic branching in the periphery of the neuron and support plasticity at synapses in distal dendrites which represent, notably, the large majority of glutamatergic excitatory synapses (**Figures 2 A,B**).

Anatomically and physiologically, the available findings are consistent with morphological and functional hippocampal atrophy and subsequent cortico-cortical disconnection syndrome, which involves the disruption of neural networks between the anterior and posterior cerebral areas (Delbeuck et al., 2003; Nagata et al., 2010). Met carriers, rather than subjects with the Val/Val phenotype, may benefit from a protective role on executive function through hippocampus cortical atrophy or other subcortical tract changes, as reported in previous studies on elderly people. This observation may in part, explain the reason why this polymorphism despite its deleterious effects remains highly diffused in the world population. Interestingly, as pointed out by Li et al. (2010), the negative effects of the BDNF polymorphism

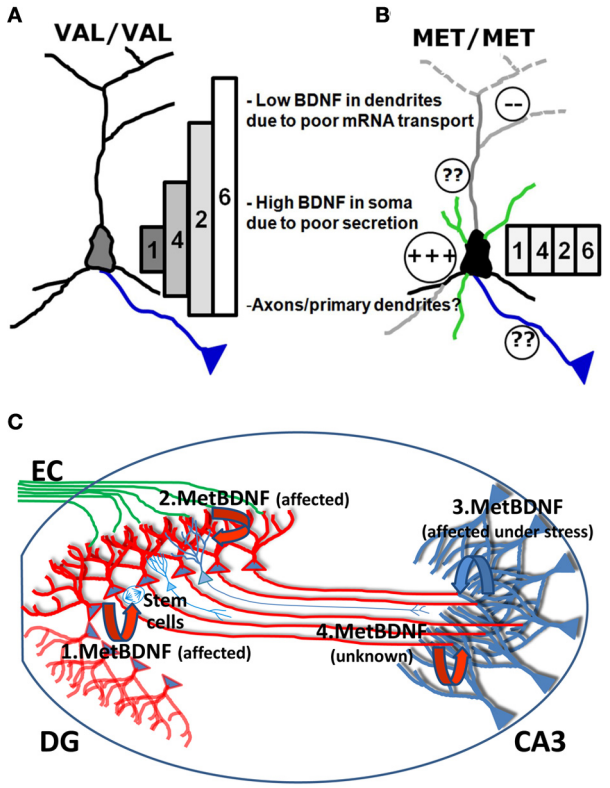

FIGURE 2 | Model of the biological effects of Val and Met BDNF alleles in the hippocampus. (A) In Val/Val neurons, the four most abundant brain BDNF transcripts, encoding exon 1, 2, 4, or 6, are differentially localized in dendrites, forming a gradient with exon 1 being restricted to the soma and exons 2, 4, and 6 in dendrites at increasing distances according to the gradient 4<2<6. Exons 1 and 4 can affect only the morphology of proximal dendrites while exon 2 and 6 are able to shape the distal dendrites. **(B)** In Met/Met neurons, transport of BDNF mRNA in distal dendritic districts is impaired and all BDNF mRNA isoforms accumulate in the cell soma. Met BDNF protein increases in the soma, also due to poor secretion. It is unclear if axonal transport and secretion can be affected by the Met mutation. **(C)** BDNF released from DG granule cells is shown in red, while BDNF released from CA3 neurons is shown in blue. (1.BDNF) Secretion of Met BDNF from the soma of DG granule cells is altered and provides insufficient local trophic support for survival and differentiation of DG subgranular neural stem cells. (2.BDNF) Release of Met BDNF from dendrites of DG neurons is affected and cannot provide sufficient autocrine support to dendrites which show reduced arborization. (3.BDNF) Local production and release of Met BDNF from apical dendrites of CA3 neurons is reduced and cannot provide the target-derived trophic support to promote innervation of CA3 from newly formed mossy fibers and autocrine support to CA3 dendrites. (4.BDNF) It is unknown if anterogradely transported Met BDNF in mossy fibers can support dendrites from CA3 neurons and if anterograde transport of BDNF is affected by the Met mutation.

on episodic memory are most likely observed when associative and executive demands are high. These observations are in line with the hypothesis that the magnitude of genetic effects on cognition is greater when brain resources are reduced, as with old age. Accordingly, the logical consequence of the strong effects of Met BDNF allele on mental performance are pronounced in the elderly, with poor or no impact on reproduction and therefore, transmission of the allele to the following generations can

be ensured thus contributing to the maintenance of this mutation through the population. In addition, carriers of the Met allele are more likely to develop post-traumatic stress disorder after traumatic experiences.

In **Figure 2C** we summarize how deficits in Met BDNF secretion and transport, observed at the cellular level, may affect the hippocampal neuronal circuit. According to this model, since various studies reported that survival and differentiation of DG subgranular neural stem cells are reduced, secretion of Met BDNF from the soma of DG granule cells is altered and provides insufficient local trophic support (**Figure 2**). In addition, since DG dendrites were shown to have reduced arborization, the model predicts that release of Met BDNF from dendrites of DG neurons is affected and cannot provide sufficient autocrine support. Following stressful situations, Met/Met individuals may undergo more easily to atrophy of apical dendrites of CA3 neurons. According to our model, the local production and release of Met BDNF from apical dendrites of CA3 neurons is reduced and thus cannot provide target-derived trophic support to promote the innervation of CA3 from newly formed mossy fibers and autocrine support to CA3 dendrites. It is currently unclear if Met BDNF present in mossy fibers can support dendrites from CA3 neurons and if anterograde transport of BDNF is affected by the Met mutation. One plausible hypothesis is that BDNF released from mossy fiber axons may stimulate dendritic targeting of BDNF mRNA in CA3 apical dendrites, thus contributing to a local autocrine loop which supports the maintenance of apical dendritic arborization.

In conclusion, the view proposed here does not claim to be the ultimate interpretation of the available data but intends to be a stimulus for the scientific community to develop a consensus model on the biological mechanisms of one of the most fascinating human mutations affecting cellular functions, brain morphology, and cognition.

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The FMRP regulon: from targets to disease convergence

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The fragile X mental retardation protein (FMRP) is an RNA-binding protein that regulates mRNA metabolism. FMRP has been largely studied in the brain, where the absence of this protein leads to fragile X syndrome, the most frequent form of inherited intellectual disability. Since the identification of the FMRP gene in 1991, many studies have primarily focused on understanding the function/s of this protein. Hundreds of potential FMRP mRNA targets and several interacting proteins have been identified. Here, we report the identification of FMRP mRNA targets in the mammalian brain that support the key role of this protein during brain development and in regulating synaptic plasticity. We compared the genes from databases and genome-wide association studies with the brain FMRP transcriptome, and identified several FMRP mRNA targets associated with autism spectrum disorders, mood disorders and schizophrenia, showing a potential common pathway/s for these apparently different disorders.

Keywords: fragile X syndrome, autism, schizophrenia, major depressive disorders, FMRP, RNA-binding proteins, synaptic plasticity, local protein synthesis

INTRODUCTION

Protein synthesis at subcellular sites is a well-conserved mechanism that allows the rapid expression of specific genes in response to localized cues (Xing and Bassell, 2013). During transport, mRNAs are stabilized via association with multiple and different *trans*-acting factors, such as RNA-binding proteins (RBPs) and non-coding RNAs, forming ribonucleoparticles (RNPs) that vary in size and composition during cell cycle and development.

In highly polarized cells, such as neurons, mRNAs are transported from the nucleus to dendrites and axons where these molecules undergo local translation and degradation (Steward and Schuman, 2003; Bramham, 2008; Cajigas et al., 2010; Doyle and Kiebler, 2012; Hornberg and Holt, 2013) according to their subcellular localization and cellular inputs (Bramham, 2008).

RNA-binding proteins recognize and bind mRNA targets through regulatory elements in the 5′ and 3′ untranslated regions (UTRs) (Pichon et al., 2012), and in some cases the coding regions are also involved in these interactions (Anko and Neugebauer, 2012). Binding to mRNAs is mediated through well-known RNA-binding motifs, which are often present in multiple copies (Clery et al., 2008) and typically bind short RNA sequences (Anko and Neugebauer, 2012). Several RBPs cooperate for the binding of mRNA, thereby increasing the specificity of this interaction (Matlin et al., 2005; Ule and Darnell, 2006). The actin cytoskeleton might well facilitate RNA recognition, as this structure associates with RBPs and coordinates the binding of these proteins to mRNA (Percipalle, 2009). However, individual RBPs bind to several mRNAs. The multi-targeted binding property of RBPs has led to a model of regulated gene expression in eukaryotes termed "*the post-transcriptional operon*" (Keene, 2007).

The fragile X mental retardation protein (FMRP) is a widely studied RBP in the brain. Silencing of the *FMR1* gene encoding FMRP leads to fragile X mental retardation syndrome (FXS), the most common cause of inherited intellectual disability (Bagni et al., 2012). A majority of the clinical cases of FXS reflect a lack of FMRP due to a large trinucleotide CGG-repeat expansion in the 5′ UTR of the gene, resulting in *FMR1* gene silencing. Rare cases have been reported to carry partially deleted or mutated FMRP (De Boulle et al., 1993; Mila et al., 2000; Coffee et al., 2008; Collins et al., 2010). The *FMR1* gene and FMRP have also been associated with the pathogenesis of other disorders, such as fragile X-associated tremor ataxia syndrome (FXTAS), premature ovarian failure (POF), and autism spectrum disorder (ASD) (Bagni et al., 2012).

Here, we briefly reviewed the structure and function of FMRP, a multifunctional RBP that regulates the transport, stability and local protein synthesis of hundreds of RNAs in the brain. We further discuss how anomalies in the expression of FMRP alter the condition of its targets and ultimately, highlight a subset of FMRP target mRNAs dysregulated in autism spectrum disorders (ASDs), mood disorders (MDs) including bipolar disorder (BD), major depressive disorder (MDD), attention deficit hyperactive disorder (ADHD), and schizophrenia (SCZ).

FMRP STRUCTURE, RNA TARGETS AND PROTEIN PARTNERS

The human *FMR1* gene is ubiquitously expressed (https://www.genevestigator.com/gv/), with higher abundance in some tissues

(Kaufmann et al., 2002; Xie et al., 2009). The gene comprises 17 exons spanning 38 kb of Xq27.3 (Eichler et al., 1993). Alternative splicing of the gene results in the generation of 12 protein isoforms (De Boulle et al., 1993; Brackett et al., 2013).

In the mammalian brain, FMRP targets hundreds of mRNAs (Miyashiro et al., 2003; Darnell et al., 2011; Bagni et al., 2012; Gross et al., 2012; Wang et al., 2012) and non-coding RNAs, such as the brain cytoplasmic RNA BC1/BC200 *in vitro* and *in vivo* (Zalfa et al., 2003, 2005; Gabus et al., 2004; Johnson et al., 2006; Lacoux et al., 2012) and a few microRNAs (Jin et al., 2004; Edbauer et al., 2010; Gessert et al., 2010; Muddashetty et al., 2011; Tian et al., 2013).

Structural studies of the FMRP domain have contributed to the understanding of the molecular function/s of this protein. The N-terminal region, characterized by the presence of two Tudor domains (TD)(Ramos et al., 2006), binds *in vitro* RNA homopolymers and the small non-coding *BC1* RNA (Gabus et al., 2004; Zalfa et al., 2005; Lacoux et al., 2012) (**Figure 1**). The central region contains two K homology domains (KH) and a nuclear export signal (NES) (Valverde et al., 2008). The most severe single point mutation identified in a patient with FXS is an Ile367Asn, located on helix α2 of the KH2 domain (De Boulle et al., 1993). The murine FMRP, carrying the corresponding mutation (Ile304Asn), loses the ability to bind RNA (Zang et al., 2009), likely reflecting the destabilization of the hydrophobic core, which partially unfolds the domain (Di Marino et al., 2013). A recent study in non-neuronal cells has shown that the FMRP Ile304Asn mutation reduces the binding affinity of a subset of mRNAs, such as *neurofibromatosis type 1* (*NF1*), *FMR1*, *bifunctional glutamate/proline-tRNA ligase* (*EPRS*), *serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform* (*PPP2CA*), *ubiquitin-protein ligase E3A (UBE3A), structural maintenance of chromosomes protein 1A* (*SMC1A*) and *cohesin subunit SA-2* (*STAG2*) (Ascano et al., 2012).

The C-terminal region, containing an RGG box, is involved in the interaction of well-characterized FMRP mRNA targets (Darnell et al., 2001; Menon and Mihailescu, 2007; Westermark and Malter, 2007; Zalfa et al., 2007; Menon et al., 2008; Blackwell et al., 2010). The C-terminal region of FMRP binds *post synaptic protein-95* (*PSD-95*) *mRNA* (Zalfa et al., 2007), *microtubule associated protein 1B* (*MAP1B*) (Darnell et al., 2001; Zalfa et al., 2003), *semaphorin 3F* (*SEMA3F*) (Menon and Mihailescu, 2007), *extracellular matrix protein 2* (*SC1*), *brain acid soluble protein 1 (NAP22) (Darnell et al., 2001)* and *serine/threonine-protein kinase LMTK1 (AATYK)* (Blackwell et al., 2010) mRNAs, a few of which are depicted in **Figure 1**.

FMRP homodimerises and interacts with several cytoplasmic and nuclear proteins involved in mRNA metabolism and cytoskeleton-remodeling proteins (Bagni and Klann, 2012). Among the best characterized FMRP-interacting proteins are

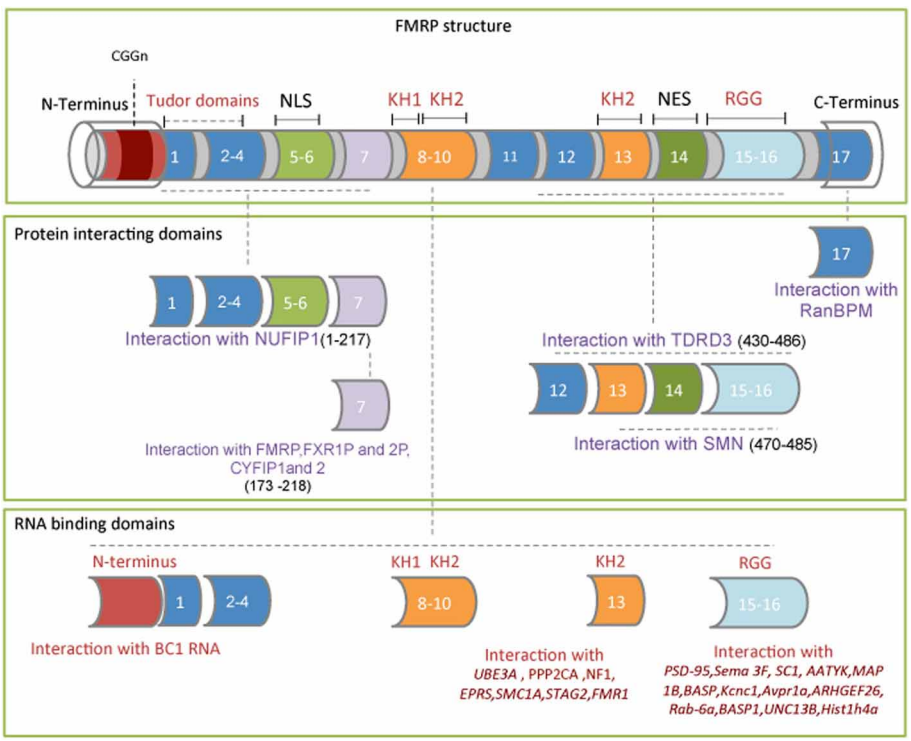

FIGURE 1 | FMRP exon structure comprising its functional domains. **Upper frame:** The red box at the N-terminus of exon 1 indicates the location of the CGG triplet repeat within the 5′ UTR of the mRNA. The four RNA binding domains are: the N-terminus, the two K homology domains (KH1 and KH2) and the RGG box. **Middle frame:** FMRP domains interacting with NUFIP1, CYFIP1, CYFIP2, FXR1P, FXR2P, TDRD3, and SMN proteins. The FMRP amino acid sequence involved in these interactions is shown between the brackets. The nuclear localization signal (NLS) and the nuclear export signal (NES) are also indicated. **Lower frame:** The FMRP RNA binding domains and the RNA/mRNA targets directly bound are indicated.

protein argonaute-2 (Ago2) (Muddashetty et al., 2011), 58 kDa microspherule protein (MSP58) (Davidovic et al., 2006), nuclear fragile X mental retardation-interacting proteins 1 and 2 (NUFIP1 and 2) (Bardoni et al., 2003), the survival of motor neuron (SMN) (Piazzon et al., 2008), the Tudor domain-containing protein 3 (Linder et al., 2008), nuclear export factor 2 (NXF2) (Zhang et al., 2007), dicer (Cheever and Ceman, 2009), cytoplasmic interacting protein CYFIP1 (Schenck et al., 2001, 2003; Napoli et al., 2008; De Rubeis et al., 2013) and the two paralogs, fragile X-related proteins 1 and 2 (FXRP1 and FXRP2) (Tamanini et al., 1999) (**Figure 1**).

CELLULAR AND MOLECULAR FUNCTIONS OF FMRP

Although in neurons, FMRP has been localized in the nucleus, cell body and dendrites (Willemsen et al., 1996), the cytoplasmic function of FMRP has been the most studied. FMRP forms large cytoplasmic RNPs containing several proteins and RNAs, and this protein is involved in the transport, stability and translation of several mRNAs (Bagni et al., 2012). One report suggested FMRP might also function as splicing enhancer (Didiot et al., 2008). Additionally, Drosophila FMRP has been related to the RNA-editing pathway (Bhogal et al., 2011).

REGULATION OF mRNA TRANSPORT

FMRP transports RNA/mRNAs from the cell body to synapses in an activity-dependent manner and through a dynamic association with microtubule motors (Kanai et al., 2004; Antar et al., 2005; Ferrari et al., 2007; Dictenberg et al., 2008; Charalambous et al., 2013). FMRP granules transport mRNA including its own (Antar et al., 2004; Ferrari et al., 2007; Kao et al., 2010), and the absence of FMRP impairs the localization of *Map1b* and *SAP90/PSD-95-associated protein 4* (*Sapap4)* mRNAs, thus altering the proper synthesis of these proteins at synapses (Dictenberg et al., 2008; Kao et al., 2010).

REGULATION OF mRNA STABILITY

Initial studies performed in *Fmr1* KO mice have revealed that the absence of FMRP alters the abundance of hundreds of mRNAs in the brain (Brown et al., 2001; Miyashiro et al., 2003; Gantois et al., 2006); a few mRNAs were found to be down regulated in all three studies. Further analyses on specific mRNAs showed that dysregulation occurred in specific brain areas and/or subcellular compartments, suggesting that FMRP might regulate the same mRNA in multiple ways (Miyashiro et al., 2003). FMRP modulates the stability of certain mRNAs by preventing or sustaining mRNA decay (De Rubeis and Bagni, 2010). As an example of the two opposite activities on different mRNAs, it has been shown that hippocampal FMRP protects *PSD-95* mRNA from decay (Zalfa et al., 2007) in an activity-dependent manner; however, FMRP protein also facilitates the decay of *nuclear RNA export factor 1 (NXF1)* mRNA in mouse neuroblastoma (N2a) cells (Zhang et al., 2007). Furthermore, FMRP regulates *PSD-95* mRNA stability in the hippocampus (Zalfa et al., 2007) and regulates translation at cortical synapses (Muddashetty et al., 2007). *PSD-95* mRNA is an important player in synaptic plasticity and is affected in ASD (Feyder et al., 2010) and SCZ (Toro and Deakin, 2005).

The cortical region of the *Fmr1* KO mouse brain shows the reduced expression of different $GABA_A$ receptor subunits (El Idrissi et al., 2005; Gantois et al., 2006), consistent with evidence of imbalanced GABAergic signaling in FXS patients. Taken together, FMRP-RNPs might play different roles in several brain regions and regulate mRNAs through different mechanisms according to the developmental stage and subcellular localization.

REGULATION OF mRNA TRANSLATION

The translational dysregulation of FMRP mRNA targets significantly contributes to the FXS phenotype (Bagni et al., 2012; Darnell and Klann, 2013). Initial studies performed in lymphoblastoid cells derived from FXS individuals showed an increased translation rate in several FMRP targets (Brown et al., 2001). The increased translation of FMRP mRNA targets was also observed in *Fmr1* KO mice specifically at synapses, consistent with the idea that FMRP functions as a repressor of translation (Muddashetty et al., 2007; Narayanan et al., 2007; Napoli et al., 2008; De Rubeis et al., 2013).

FMRP activity is regulated in response to different receptor signaling cascades, i.e., type I metabotropic glutamate receptors (mGluRs) (Huber et al., 2002), the 2-amino-3-(5-methyl-3-oxo-1,2-ox-azol-4-yl) propanoic acid (AMPA) receptors (Nakamoto et al., 2007), the γ-aminobutyric acid (GABA) receptors (Centonze et al., 2008; Curia et al., 2009; Shang et al., 2009), the N-methyl-D-aspartate (NMDA) receptors (Suvrathan et al., 2010; Yun and Trommer, 2011; Eadie et al., 2012), the tyrosine kinase or BDNF/NT-3 growth factor (TrkB) receptors (Napoli et al., 2008; Louhivuori et al., 2011; De Rubeis et al., 2013), the dopamine (DA) receptors (Wang et al., 2008) and recently the cannabinoid receptors (Maccarrone et al., 2010; Busquets-Garcia et al., 2013).

One of the most affected and best characterized signaling cascades in fragile X is the mGluR (Bear et al., 2004). Upon mGluR receptor activation, FMRP-mediated translational block is released and protein synthesis can ensue. In the absence of FMRP, the increase in protein synthesis results in a receptor imbalance; an increase in the mGluR1 and mGluR5 activity and the reduced insertion of AMPA receptors at the surface that leads to enhanced mGluR long-term depression (mGluR-LTD) (Bear et al., 2004).

mGluR-LTD is a form of synaptic plasticity that involves mRNA targeting and local protein synthesis and degradation (Bear and Malenka, 1994), and this condition can be induced through the application of (S)-3,5-dihydroxyphenylglycine (DHPG) (Wisniewski and Car, 2002) in a protein synthesis-independent manner (Huber et al., 2002). In *Fmr1* KO mice, DHPG-induced LTD is strongly increased and these electrophysiological phenotypes established the "mGluR theory" in FXS (Bear et al., 2004).

FMRP activity is regulated through posttranslational modifications. DHPG-induced LTD also activates FMRP synthesis at synapses (Antar et al., 2004; Ferrari et al., 2007; Kao et al., 2010), which in turn is quickly degraded through the ubiquitin-proteasome system (Hou et al., 2006). The effect of FMRP on protein synthesis is influenced by the phosphorylation status of FMRP (Ceman et al., 2003), via the mTOR pathway (Narayanan

et al., 2007): phosphorylated FMRP represses translation, while dephosphorylated FMRP releases the inhibition, allowing protein synthesis to ensue, a mechanism similarly shown for previously characterized eukaryotic initiation factor 4E binding proteins (eIF4E-BPs) in non-neuronal cells (Richter and Klann, 2009).

FMRP has also been detected in P bodies (PB), stress granules (SG) (Kedersha et al., 2005), and cytoplasmic structures, containing translationally silent pre-initiation complexes. FMRP is part of mRNPs (Siomi et al., 1996; Laggerbauer et al., 2001; Ishizuka et al., 2002; Zalfa et al., 2003; Anderson and Kedersha, 2006; Monzo et al., 2006; Papoulas et al., 2010; Charalambous et al., 2013), supporting the function of FMRP as a translational repressor at the initiation level, as observed at synapses both *in vitro* (Laggerbauer et al., 2001) and *in vivo* (Napoli et al., 2008; De Rubeis et al., 2013).

We have shown that FMRP represses translation through its binding to CYFIP1, a neuronal eIF4E-BP (Napoli et al., 2008). CYFIP1 binds to eIF4E, blocking the initiation of translation. Subsequently, the synaptic stimuli CYFIP1-FMRP complex is released from eIF4E and translation ensues (Napoli et al., 2008). Notably, CYFIP1 is also implicated in actin cytoskeleton remodeling (Kobayashi et al., 1998; Eden et al., 2002; Schenck et al., 2003; Stradal et al., 2004; Chen et al., 2010). We have recently shown that CYFIP1 links local protein synthesis and actin dynamics (De Rubeis et al., 2013). FMRP has also been proposed to regulate mRNA elongation (Darnell et al., 2011).

FXS AND COMMONALITIES WITH OTHER DISEASES

FXS is the most common monogenic cause of ASD, and 30% of patients with FXS present autistic behaviors (Bagni et al., 2012). Early studies performed on heterozygous females carrying the fragile X mutant gene showed a greater frequency of psychopathologies associated with schizophrenia spectrum diagnoses (Reiss et al., 1988). Furthermore, carriers of premutated *FMR1* alleles (reduced FMRP levels) have been associated with a significant degree of psychiatric disorders (Bourgeois et al., 2009). Recently, low FMRP levels have been detected in the postmortem brain from subjects with SCZ, BD and MDD (Fatemi et al., 2010; Kelemen et al., 2013; Kovacs et al., 2013) and in blood samples from schizophrenia patients (Kovacs et al., 2013). Some individuals that display psychoses also carry FMR1 full and pre-mutations (Jonsson et al., 1995; Ashworth et al., 1996; Khin et al., 1998).

It is not known whether decreased levels of FMRP are the cause or the consequence of the development of these disorders. However, it is tempting to speculate that the loss or reduced function of FMRP might lead to a dysregulation of particular FMRP target genes associated with ASD, SCZ, and MD, suggesting the correlation of certain FXS features with these neuronal disorders. Because the GABAergic system is dysfunctional in these disorders (Kelemen et al., 2013) and the lack of FMRP affects the expression of some GABA receptor subunits (D'Hulst and Kooy, 2007), it is reasonable to hypothesize that FMRP reduction might explain the alterations of proteins associated with the GABAergic system in these different neurological diseases. Indeed, recent findings showed that a selective activator of $GABA_B$ receptor reversed some FXS associated pathologies (Henderson et al., 2012).

It cannot be ruled out that certain proteins, which are risk factors for ASD, SCZ and/or MD, work together with FMRP and might disrupt the function of this protein in a disease context. Recently, it has been observed that topoisomerase Top3β, a risk gene for SCZ and ASD (Iossifov et al., 2012; Xu et al., 2012; Stoll et al., 2013), binds to FMRP and modifies the function of this protein *in vitro*, thereby supporting normal neurodevelopment and averting mental disorders (Xu et al., 2013). In addition, the authors observed that the disruption of either *Top3β* or *Fmr1* genes in Drosophila led to a dysregulation of *ptk2*, which is genetically associated with SCZ (Walsh et al., 2008). Notably, CYFIP1 has been associated with ASD (Sahoo et al., 2006) (Doornbos et al., 2009; Van Der Zwaag et al., 2010; Von Der Lippe et al., 2010; Talebizadeh et al., 2013), SCZ and epilepsy (Sahoo et al., 2006; Tam et al., 2010; Zhao et al., 2012). In addition, we have recently shown that the CYFIP1 interactome contains many novel proteins associated with ASD, SCZ, and MDD, providing new perspectives to define the regulatory pathways shared by neurological disabilities characterized by spine dysmorphogenesis (De Rubeis et al., 2013), a common feature of several neuropsychiatric disorders (Penzes et al., 2011).

Over the last 10 years, several hundred putative FMRP mRNA targets have been identified in the brain

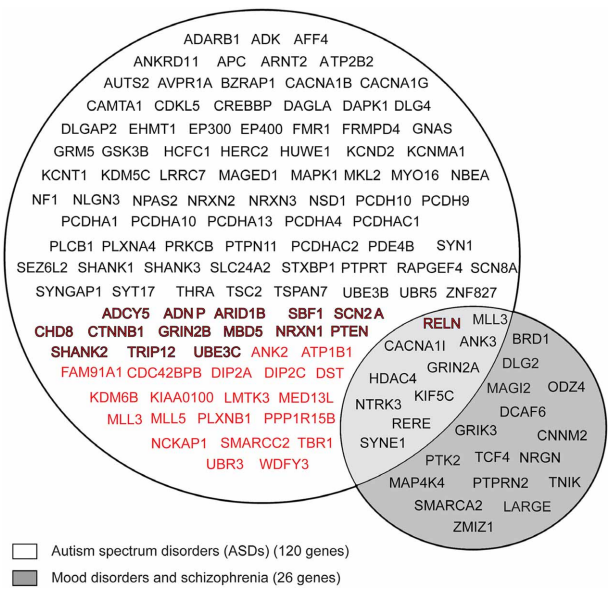

FIGURE 2 | Venn diagram of FMRP mRNA targets associated with autistic spectrum disorder (ASD), schizophrenia (SCZ) and mood disorder (MD). FMRP neuronal target genes (1169) were compared with *de novo* gene disruptions (nonsense, splice and frameshifts) reported in GWAS are associated with ASD, as previously described (Iossifov et al., 2012; Neale et al., 2012; O'roak et al., 2012b; Sanders et al., 2012), the 528 genes from the SFARI (https://sfari.org/resources/sfari-base) and the 304 genes from the AutDB databases (http://autism.mindspec.org/autdb/Welcome.do). Approximately 120 FMRP target genes were associated with ASD (in black). Among the 120 target genes, included 35 genes were identified from the GWAS (in red) and 100 genes were identified from the two databases (in black). Fifteen genes showed overlap between the GWAS, the SFARI, and AutDB databases (in black/red color). The 1169 FMRP target genes were compared with the genes associated with SCZ and MD in the NHGRI GWAS database (http://www.genome.gov/gwastudies/) and 26 common FMRP targets were shown.

(Brown et al., 2001; Chen et al., 2003; Miyashiro et al., 2003; Zalfa et al., 2003, 2007; Muddashetty et al., 2007; Darnell et al., 2011), and more than 6000 targets have been identified in non-neuronal cells (Ascano et al., 2012). While these analyses have expanded the number of FMRP targets, further studies are required to elucidate the extent to which each mRNA contributes to the FXS clinical phenotype/s. There is substantial evidence that individuals with intellectual disabilities are prone to psychological profiles independently of the genetic and/or environmental cause (Turk, 2011).

Based on several large-scale studies, the number of FMRP neuronal target mRNAs is approximately 1,400 (Brown et al., 2001; Chen et al., 2003; Miyashiro et al., 2003; Darnell et al., 2011). We compared 1,169 unique (non-overlapping in the mentioned studies) FMRP mRNA targets with *de novo* ASD associated genes identified through recent genome-wide association studies (GWAS)(Iossifov et al., 2012; Neale et al., 2012; O'roak et al., 2012a,b; Sanders et al., 2012), obtained from the SFARI (http://gene.sfari.org) and the Autism databases (AutDB (http://www.mindspec.org/autdb.html).

As represented in **Figure 2**, according to the GWAS, 35 FMRP target mRNAs are associated with ASD (in red), while the SFARI and AutDB databases have revealed that 100 FMRP target mRNAs are candidate genes for ASD (in black). Fifteen genes overlap between the results obtained in the GWAS and the SFARI and AutDB databases (in black/red color). This analysis shows that approximately 10% of the neuronal FMRP targets identified, in the above-mentioned studies, overlap with the genes associated with ASDs (120 out of 1169).

We also compared the 1,169 FMRP target mRNAs with 176 genes associated with BD, attention deficit-hyperactivity disorder (ADHD), mood disorder (MD), and SCZ (GWAS compiled by the National Human Genome Research Institute catalog http://www.genome.gov/). Twenty-six (out of 176) FMRP target mRNAs were also identified in this cohort (**Figure 2**, in gray). Because a few genes in this group (10) were also detected among the FMRP targets in the ASD group, it is reasonable to hypothesize that ASD, SCZ, and mood disorders (BD, MDD, ADHD) share certain common signaling pathways.

FUTURE PERSPECTIVES

Post-transcriptional studies have revealed that the FMRP regulon controls disease-related proteins that affect both neurodevelopment and adult brain plasticity and homeostasis. The emerging wave of genetic association studies has revealed a large number of risk genes for several neurodegenerative diseases and neurodevelopmental disorders, such as SCZ, ASD, and BD (http://www.genome.gov/gwastudies).

The risk genes for neurodevelopmental disorders, identified through GWAS, were compared with the list of the FMRP targets, and the results suggest that several pathways are dysregulated in FXS and might account for specific FXS phenotypes.

As the FMRP acts as a protein synthesis repressor, it is reasonable to propose that the FXS phenotype might reflect the overexpression of specific genes. However, FMRP not only regulates gene expression at the translational level, but it also influences the stability of several mRNAs. Furthermore, to determine the functional association of the FMRP regulon with the repertoire of genes altered in individuals carrying ASD, SCZ, and MD, it is important to investigate the dosage of these genes in individuals with FXS. Moreover, FXS is a neurodevelopmental disorder, and the absence of FMRP could affect the expression of specific targets at different developmental stages and in different brain areas. Further studies on FMRP targets and the FMRP interactome at specific developmental stages would help to determine the cause of these disorders and develop further strategies to ameliorate FXS.

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Molecular mechanisms in amyotrophic lateral sclerosis: the role of angiogenin, a secreted RNase

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Amyotrophic lateral sclerosis is a fatal neurodegenerative disease caused by the loss of motoneurons. The precise molecular and cellular basis for neuronal death is not yet well established, but the contemporary view is that it is a culmination of multiple aberrant biological processes. Among the proposed mechanisms of motoneuron degeneration, alterations in the homeostasis of RNA binding proteins (RBP) and the consequent changes in RNA metabolism have received attention recently. The ribonuclease, angiogenin was one of the first RBPs associated with familial and sporadic ALS. It is enriched in motoneurons under physiological conditions, and is required for motoneuron survival under stress conditions. Furthermore, delivery of angiogenin protects cultured motoneurons against stress-induced injury, and significantly increases the survival of motoneurons in SOD^{G93A} mice. In this overview on the role of angiogenin in RNA metabolism and in the control of motoneuron survival, we discuss potential pathogenic mechanisms of angiogenin dysfunction relevant to ALS and other neurodegenerative disorders. We also discuss recent evidence demonstrating that angiogenin secreted from stressed motoneurons may alter RNA metabolism in astrocytes.

Keywords: amyotrophic lateral sclerosis, RNA metabolism, angiogenin, RNA binding proteins, stress signals

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is fatal neurodegenerative disease with a late-onset, where motoneurons in the spinal cord and brainstem die. After diagnosis, only about 25% of patients survive beyond 5 years, with the majority suffering a fatal respiratory failure within 3–5 years. Most cases are believed to be sporadic, with only about 10% of patients having a confirmed family history.

Several genetic alterations have been linked with ALS. Mutations in the copper/zinc superoxide dismutase 1 (SOD1) gene, responsible for *circa* 20% of the familial ALS forms and 1% of "sporadic" cases, have been considered the major genetic cause of ALS (Rosen et al., 1993). Recently, however, an expanded non-coding GGGGCC repeat in *C9ORF72* has been identified, which seems to be responsible for about 24% of familial ALS (DeJesus-Hernandez et al., 2011). In a Finnish cohort, the percentage of linkage for this mutation was as high as 46% of ALS, putting this as the most common genetic cause of ALS known to date (Renton et al., 2011). Other important genes linked to ALS include two RNA binding proteins, transactive response (TAR) DNA-binding protein (TDP-43), and fused in sarcoma/translocated in liposarcoma (FUS/TLS), which are associated with *circa* 4% of familial ALS (reviewed by Lagier-Tourenne et al., 2010).

Still, the large majority of sporadic cases have no known genetic component (reviewed by Valdmanis and Rouleau, 2008). These observations have led to the hypothesis of ALS being an oligogenic or polygenic disorder, a hypothesis that could also explain the large number of familial ALS-associated gene mutations that exhibit a relatively low penetrance (Valdmanis and Rouleau, 2008).

ANGIOGENIN IN ALS

A clinical study initiated in Ireland has identified several mutations in the angiogenin (*ANG*) gene in ALS patients of Irish and Scottish background, both in familial and sporadic cases (Greenway et al., 2006). Subsequent clinical studies confirmed the association of these mutations with ALS, and identified new mutations in backgrounds from Brazil, China, France, Germany, Italy, Netherlands, Sweden, and USA (**Table 1**). Only one clinical study so far failed to find a link between *ANG* gene mutations and ALS in an Italian population (Corrado et al., 2007), but one can arguably reason that such study was small (262 ALS patients) in comparison with other reports (with an average of approximately 1,500 ALS patients per study), and that some of the *ANG* mutations identified may have a lower disease penetrance, similar to other ALS-associated mutations (Valdmanis and Rouleau, 2008). More recently, a link between angiogenin mutations and Parkinson's disease has also been demonstrated (Steidinger et al., 2011; van Es et al., 2011).

Angiogenin, firstly isolated from the conditioned medium of colon carcinoma cells (Fett et al., 1985), is a member of the pancreatic RNase A superfamily, recently renamed as vertebrate secreted RNases (Li and Hu, 2012). This RNase is characterized by an unusual low catalytic activity, but has a significant biological ability to induce angiogenesis – hence its name (reviewed by Tello-Montoliu et al., 2006; **Table 2**). Most of the ALS-linked *ANG* mutations are predicted to affect the catalytic activity or cellular localization of the enzyme (summarized in **Table 1**), suggesting that the associated phenotype in ALS is mainly caused by loss of activity. The mutants identified by Greenway and colleagues were biochemically characterized (Crabtree et al., 2007), and all but the

Table 1 | Angiogenin mutations associated with ALS.

Mutation	Origin of disease	Ethnicity	Possible/known effect on function	Oligogenic model	Association with other neurodegenerative conditions
M(−24)S (Wu et al., 2007; Gellera et al., 2008)	Sporadic	Europe /America	Affect correct translation		
M(−24)I (van Es et al., 2011)	Sporadic	Europe	Affect correct translation		Parkinson's disease (van Es et al., 2011)
F(−13)L (Fernández-Santiago et al., 2009; van Es et al., 2011)	Sporadic	Europe	Affect processing/traffic		
F(−13)S (Gellera et al., 2008; van Es et al., 2011)	Familial	Europe	Affect processing/traffic		
G(−10)D (van Es et al., 2011)	Sporadic	Europe	Affect protein function		
P(−4)Q	Sporadic	Europe	Affect processing/traffic		
P(−4)S (Wu et al., 2007; van Es et al., 2011)	Sporadic	America	Affect processing/traffic		Parkinson's disease (van Es et al., 2011)
Q12L (Greenway et al., 2006; van Es et al., 2011)	**Sporadic**	**Europe**	**Loss of activity**		
K17I (Greenway et al., 2006; Wu et al., 2007; Millecamps et al., 2010; van Es et al., 2011; van Blitterswijk et al., 2012)	**Sporadic/ familial**	**Europe/ America**	**Loss of activity**	**TDP-43 FUS/TLS**	**Frontotemporal dementia** (van Es et al., 2009)
K17E (Greenway et al., 2006; van Es et al., 2009; van Es et al., 2011)	**Sporadic**	**Europe**	**Loss of activity**		
S28N (Wu et al., 2007; van Es et al., 2011)	Sporadic	America	Impaired nuclear translocation/loss of activity		
R31K (Greenway et al., 2006; van Es et al., 2011)	**Sporadic**	**Europe**	**Impaired nuclear translocation**		
C39W (Greenway et al., 2006; van Es et al., 2011)	**Familial**	**Europe**	**Loss of activity**		
K40I (Greenway et al., 2006; van Es et al., 2011)	**Sporadic**	**Europe**	**Loss of activity**		
I46V (Greenway et al., 2006; Gellera et al., 2008; Conforti et al., 2008; Paubel et al., 2008; Fernández-Santiago et al., 2009; van Es et al., 2011)	**Familial/ sporadic**	**Europe**	**Loss of activity**		
K54E (Fernández-Santiago et al., 2009; Millecamps et al., 2010; van Es et al., 2011)	Sporadic/ familial	Europe	Affect interaction with nucleic acids/proteins	FUS/TLS	
T80S (van Es et al., 2011)	Sporadic	Europe	Tolerated/affect protein function		
F100I (van Es et al., 2011)	Sporadic	Europe	Tolerate/benign		
V103I (Zou et al., 2012)	Sporadic	Asia	n.a.		
P112L (Wu et al., 2007; van Es et al., 2011)	Sporadic	America	Impaired nuclear translocation/loss of activity		
V113I (Gellera et al., 2008; van Es et al., 2011)	Sporadic/ familial	Europe	Tolerated/affect protein function		
H114R (Gellera et al., 2008; van Es et al., 2011)	Familial	Europe	Loss of activity		
R121H (Paubel et al., 2008; Millecamps et al., 2010; van Es et al., 2011)	Sporadic/ familial	Europe	Loss of activity		
R145C (van Es et al., 2011; Luigetti et al., 2011)	Sporadic	Europe	n.a.	SOD1	
g.446C→T (Gellera et al., 2008; UTR region)	Sporadic	Europe	Affect gene expression		

n.a., not available/analyzed; Bold, These mutations have been biochemically characterized by Crabtree et al., 2007.

Table 2 | Functions associated with angiogenin.

Function	Mechanism	RNAse activity-dependent	Reference
Angiogenesis/wound healing	Activation of PLC signal pathway Activation of Erk1/2 signal pathway rRNA synthesis	Yes, depends on nuclear translocation	Fett et al. (1985); Bicknell and Vallee (1988); Moroianu and Riordan (1994); Liu et al. (2001); Pan et al. (2012)
Neurite growth and pathfinding	Unclear	Yes – angiogenin inhibitor blocks function	Subramanian and Feng (2007); Subramanian et al. (2008)
Neuroprotection	Activation of PI3K/Akt signal pathway Engagement of HIF-1α Paracrine signaling	Yes – loss of protection with inactive ALS-associated mutants	Kieran et al. (2008); Sebastia et al. (2009); Steidinger et al. (2011); Skorupa et al. (2012)
Response to stress	Inhibition of protein translation Assembly of stress granules	Yes – cleavage of rRNA Yes – cleavage of tRNA	Emara et al. (2010); Fu et al. (2009); Yamasaki et al. (2009); Ivanov et al. (2011)

PLC, Phospholipase C; PI3K, Phosphatidylinositol 3-kinase.

ANG^{R31K} mutant showed a marked reduction in catalytic activity as predicted from structural studies.

Angiogenin has long been associated with different pathological conditions, such as cancer and angiogenesis, neovascularization associated with diabetic retinopathy and ischemia, as well as rheumatoid arthritis (reviewed by Adams and Subramanian, 1999). Early studies with endothelial cells have identified angiogenin as a hypoxia-inducible, secreted protein which acts as a potent inducer of rRNA transcription and neovascularization (Moroianu and Riordan, 1994). The potential involvement of angiogenin as a neuronal signaling molecule relevant to ALS therefore came as a surprise. However a previous study has associated another hypoxia-inducible, angiogenic, and neurotrophic factor, VEGF, to play a role in ALS (Lambrechts et al., 2003).

Angiogenin is expressed at high levels in the developing nervous system both in the brain and spinal cord, predominantly in neurons, and its activity has been shown to be necessary for neurite extension/pathfinding in differentiated motoneuron-like cells derived from pluripotent P19 carcinoma cells (Subramanian and Feng, 2007; **Figure 1A** and **Table 2**). Interestingly, ALS-associated angiogenin mutants failed to show the same activity (Subramanian et al., 2008). Angiogenin is also expressed and enriched in adult motoneurons (Greenway et al., 2006), and has been shown to protect mature, cultured motoneurons against different ALS-associated insults, such as excitotoxicity (Ca^{2+} mediated injury resulting from glutamate receptor overactivation), hypoxia, and endoplasmic reticulum stress. Angiogenin has been shown to promote and sustain cell survival signaling through AKT and ERK kinase pathways (Kieran et al., 2008; Sebastia et al., 2009). In addition, angiogenin delivery significantly increased the life-span and improved motor function in $SOD1^{G93A}$ mice, an established mouse model of ALS when delivered post-symptom onset (Kieran et al., 2008).

PARACRINE ACTIVITY OF ANGIOGENIN

Recently our group has provided compelling evidence of a new signaling pathway between motoneurons and astroglia mediated by angiogenin (Skorupa et al., 2012). Our data indicate that angiogenin is a neuronally produced protein which may constitutively regulate RNA cleavage in motoneurons (**Figure 1B**). However both transcription and secretion of angiogenin by motoneurons is potently activated in response to stress, and motoneuron-derived, secreted angiogenin is subsequently taken up nearly exclusively by astroglia (Skorupa et al., 2012). This process involves syndecans as astrocyte receptors and clathrin-mediated endocytosis as key uptake mechanism. Uptake of angiogenin into astrocytes subsequently modifies the RNA profile of astroglia (Skorupa et al., 2012). Furthermore, uptake of angiogenin into astrocyte was shown to be required for the protection of angiogenin from stress-induced motoneuron injury (Skorupa et al., 2012). An attractive hypothesis derived from these studies is that angiogenin may represent a "help me" signal secreted from stressed motoneurons that stimulates defense mechanisms in astrocytes (**Figure 1B**). Likewise, it is possible that secreted angiogenin may act on endothelial cells to promote angiogenesis, thereby increasing blood supply to "stressed" motoneurons (**Figure 1B**).

RNA METABOLISM IN ALS AND OTHER NEURODEGENERATIVE DISEASES

RNA cleavage in motoneurons, astrocytes, or other target cells by angiogenin may significantly alter their RNA metabolism. The current knowledge about RNA metabolism in neurons has been comprehensively reviewed by Strong (2010). Neurons present asymmetrical protein translation, i.e., neurons are able to direct a site-specific protein translation by "packaging" and transporting quiescent mRNA through the cell within ribonucleoprotein (RNP) complexes, also known as *RNA granules*. There are three main types of RNA granules in a mature neuron: (a) transport granules, which contain translationally silent RNA; (b) P-bodies or degradative granules, responsible of mRNA decay; and (c) stress granules (SG), which sequester mRNA in a translationally silent state at times of neuronal injury.

Stress granules assemble transiently under stressful conditions such as hypoxia, starvation, or exposure to radiation and are able to reprogram RNA translation. Interestingly, angiogenin has also been linked to SG assembly (Emara et al., 2010). Moreover, both TDP-43 and FUS are known to associate with SG (Colombrita et al., 2009; Bosco et al., 2010; Liu-Yesucevitz et al., 2010; reviewed in Dewey et al., 2012). TDP-43 and FUS seem to be implicated in

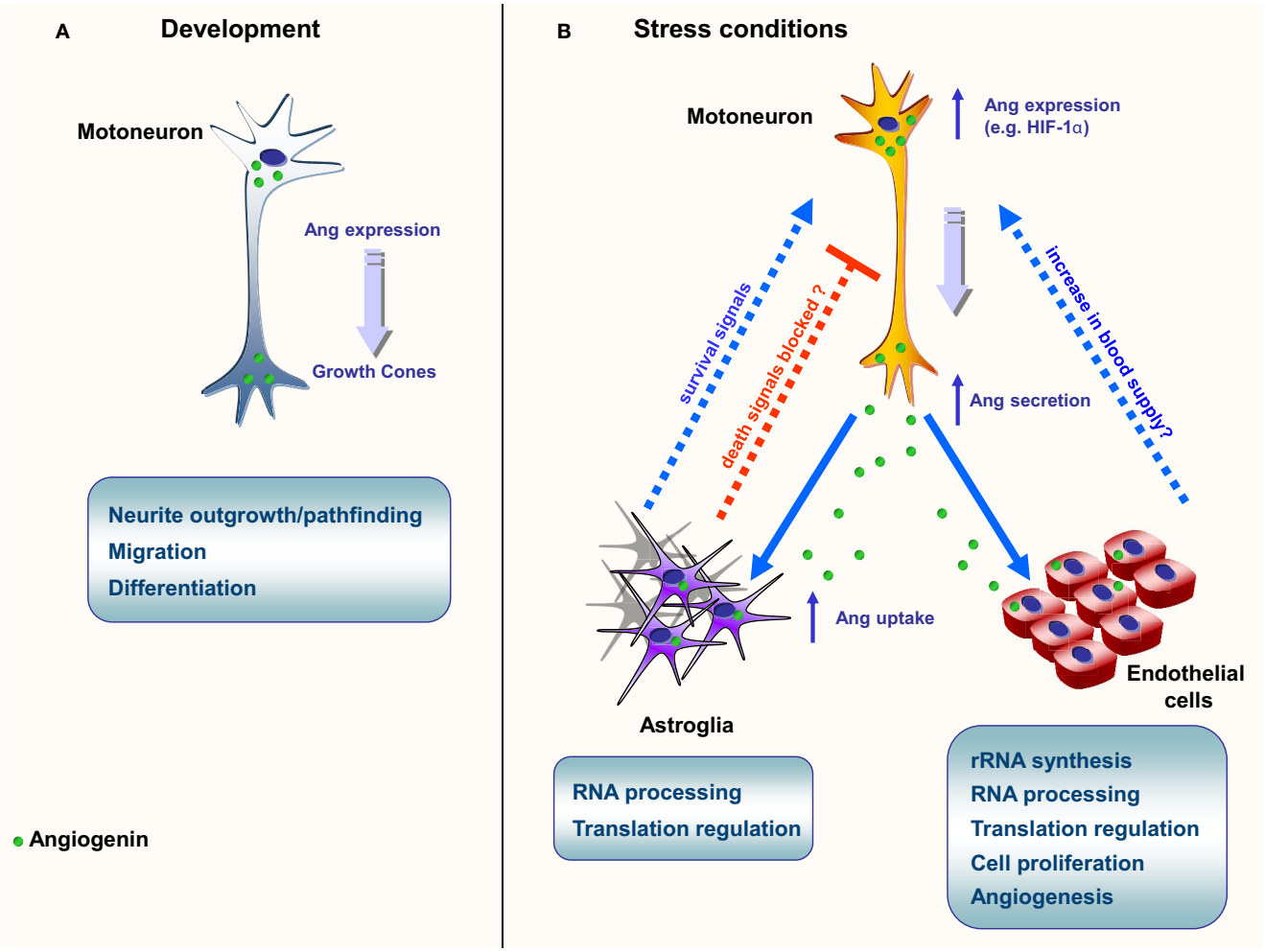

FIGURE 1 | Schematic representation of the main known functions of angiogenin. (A) Neurite growth and pathfinding. Angiogenin is found in high levels during embryogenesis, both on the brain and spinal cord; **(B)** Neuroprotection in ALS models. In situations of stress, such as starvation and hypoxia, angiogenin expression is up-regulated in motoneurons. Angiogenin is secreted and endocytosed by surrounding astroglia and close endothelial cells. In astroglia, angiogenin processes RNA, possibly altering the protein translation profile. A similar phenomenon is proposed to happen in endothelial cells, culminating in the production of survival signals (astroglia) and angiogenesis (endothelial cells), possibly resulting in increase of blood flow in affected areas.

transcription regulation, splicing regulation, miRNA processing, mRNA transport, translation, and decay (reviewed by Lagier-Tourenne et al., 2010; **Figure 2**). The detailed role of angiogenin in the regulation of these processes therefore warrants further investigations.

Angiogenin is known to stimulate the transcription of rRNA (Li and Hu, 2010) and represents the ribonuclease responsible for the generation of tRNA-derived, stress-induced small RNAs, also known as tiRNAs (Fu et al., 2009; Yamasaki et al., 2009; Ivanov et al., 2011). These tiRNAs are capable of inhibiting protein translation when cells are submitted to stress conditions, such as heat shock, hypothermia, hypoxia, starvation, and radiation. Furthermore, in an elegant study, Emara et al. (2010) demonstrated that angiogenin-generated tiRNAs are able to stimulate the formation of SG. This observation potentially puts angiogenin in interaction with other SG-related proteins, such as TDP-43 and FUS/TLS in the context of ALS.

In our model of angiogenin-mediated neuroprotection, we observed the intraneuronal generation of RNA fragments of consistent size to tiRNAs, however angiogenin internalized by astrocytes generates RNA fragments of different sizes, suggesting that it processes different substrates (Skorupa et al., 2012). RNA processing in astroglia may therefore specifically alter the translational output of astroglia. Two possible mechanisms of action emerge from these observations: (1) angiogenin could *inhibit* the astrocytic production of toxic molecules, or (2) angiogenin would *induce* the astrocytic production of protective molecules. In both hypotheses, angiogenin actions could be mediated by the reprogramming of the protein profile of astrocytes. A third possibility, where angiogenin would be both down-regulating

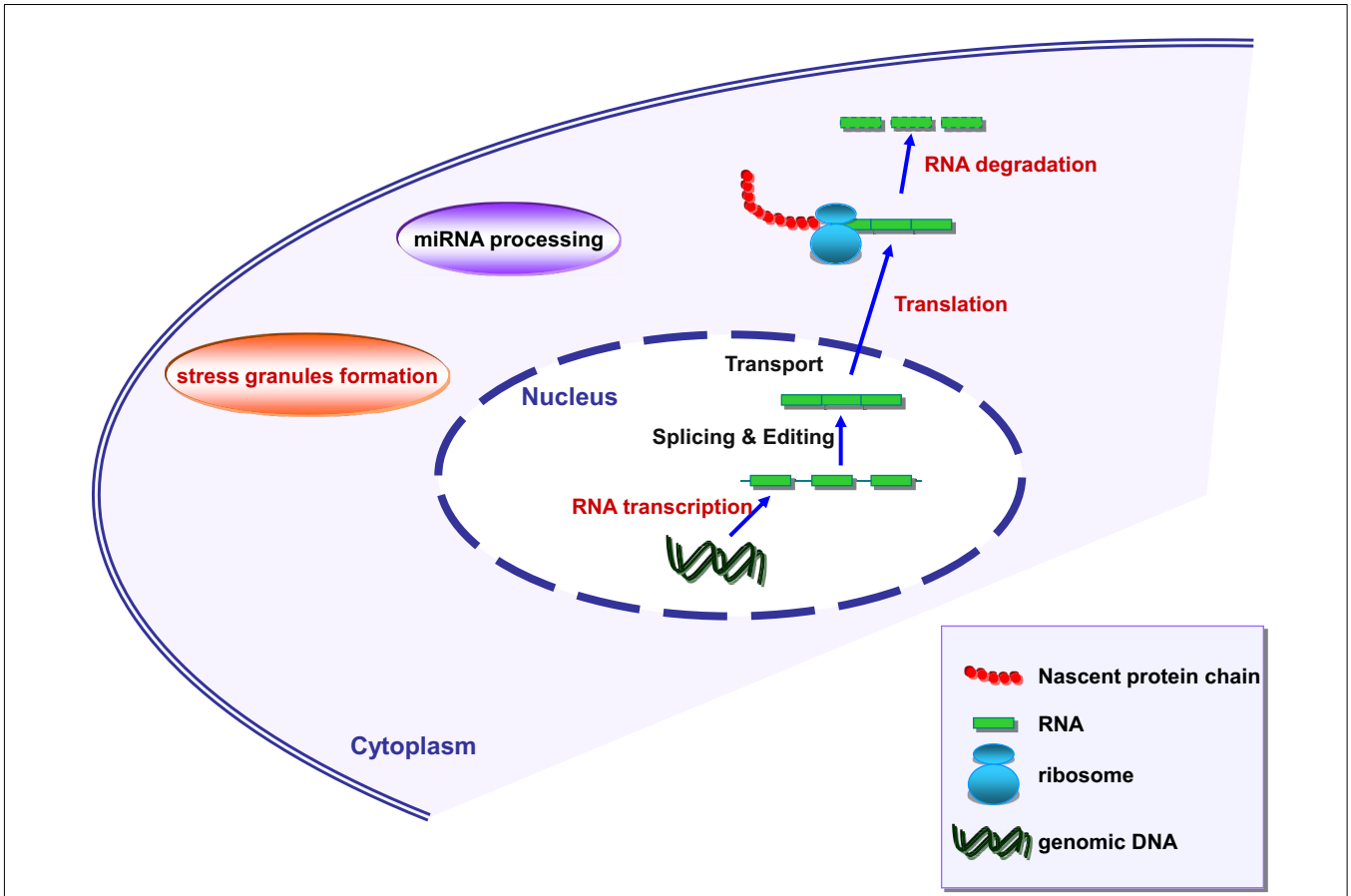

FIGURE 2 | Schematic representation of RNA metabolism in mammalian cells. Marked in red are the processes where angiogenin has been shown to be involved.

death signals and up-regulating survival signals cannot be ruled out (**Figure 1**).

Supporting the first scenario, evidence for a pathological role of glia on motoneurons death in ALS has been clearly established, and activated microglia, astrogliosis, and infiltrating lymphocytes coincide with motoneuron injury in ALS spinal cord (Appel et al., 2011). In addition, the toxic effect of astrocytes derived from ALS patients or mouse models on motoneurons has been recently reported (Diaz-Amarilla et al., 2011; Haidet-Phillips et al., 2011). Further studies are therefore required to explore whether angiogenin alters the secretome of astroglia.

CONCLUSION

It is not yet clear whether the pathological role of astrocyte and/or other glial cells on ALS disease progression is simply that of increased toxicity, or instead of failure to provide adequate protection against stress signals – internal and/or external. One appealing possibility is a model where an initial stress signal ("hit") would trigger neurotoxicity. In this scenario, angiogenin (or other ALS-associated proteins) could function as a "rescue message" to astrocytes. Loss-of-function mutations in the *ANG* gene could dramatically increase the susceptibility of motoneurons to stress-induced injury. From a therapeutic perspective, angiogenin delivery may be a viable approach for the treatment of ALS or other neurodegenerative disorders.

Of note, a first "hit" could also be the presence of another ALS-related mutation, such as TDP-43, FUS, or SOD1 mutant proteins (the latter known to exercise its pathological effect through a toxic gain-of-function profile). Cases of ALS patients with mutations in more than one gene have been observed (see **Table 1** for reference), as well as the observation of angiogenin mutations, previously linked to ALS, in healthy control subjects (Corrado et al., 2007). This so-called "double hit" hypothesis could be one possible explanation for an ALS scenario where many minor insults or individually harmless genetic polymorphisms put together or acting synergistically, could cause the disease phenotype. Corroborating this hypothesis, a recent study has demonstrated that the frequency of families with multiple mutations is higher than one might expect on the basis of chance ($P = 1.57 \times 10^{-7}$; van Blitterswijk et al., 2012).

Notwithstanding the cumulative evidence gathered thus far, the role of angiogenin in the physiology and pathophysiology

of the nervous system, in particular ALS and Parkinson's disease, requires further investigation. It is tempting to speculate that the neuroprotective role of angiogenin occurs via a double action both on motoneurons and astrocytes through the reprogramming of protein synthesis. Therefore, the identification of angiogenin substrates and products, together with the understanding of their physiological roles during the context of neurodegeneration may pave the way to new exciting therapeutic possibilities.

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Cause or effect: misregulation of microRNA pathways in neurodegeneration

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During normal aging or neurodegenerative diseases, neuronal survival and function depend on protein homeostasis, which is regulated by multiple mechanisms, including the microRNA (miRNA) pathway. In different cells types, the absence of Dicer, a key miRNA processing enzyme, leads to neurodegeneration through cell-autonomous and non-cell-autonomous mechanisms. Loss of certain miRNAs also causes neurodegeneration in some model organisms. On the other hand, miRNA expression is misregulated in patients with different neurodegenerative diseases. Thus, the miRNA pathway appears to be essential in the pathogenesis of several age-dependent neurodegenerative conditions; however, our understanding of the underlying mechanism remains rudimentary. The precise causal relationships between specific miRNAs and neurodegeneration in humans need to be further investigated.

Keywords: Alzheimer's disease, ALS, FTD, microRNAs, neurodegeneration, C9ORF72, CHMP2B, TDP-43

INTRODUCTION

Alzheimer's disease (AD), frontotemporal dementia (FTD), Parkinson's disease (PD), and other neurodegenerative disorders are a major health problem in both developed and developing countries (Hampel et al., 2011; Reitz et al., 2011; Wittchen et al., 2011). Since no effective treatments are available, it is unlikely that the adverse societal effects of these disorders will be substantially alleviated in the near future. These disorders are characterized by progressive neuronal dysfunction that initially affects selected groups of neurons in specialized neuronal circuits. A number of cellular and molecular mechanisms lead to neuronal demise. Among them, neurotoxicity induced by misfolding, mislocalization, or abnormally elevated concentrations of particular protein species seems to be a common theme (Jucker and Walker, 2011; Lee et al., 2012; Selkoe et al., 2012). Mutations in several genes that are apparently functionally unrelated can cause the same neurodegenerative disease. Multiple environmental factors (e.g., viral infections and exposure to certain toxins) might also contribute to the development of a neurodegenerative disease (Ahmed and Wicklund, 2011; Gao and Hong, 2011). Much remained to be learned about how the complex interactions of environmental and genetic factors initially lead to the misregulation of protein homeostasis and subsequently to neuronal dysfunction.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression by degrading their target mRNAs or repressing their translation (Ambros et al., 2003; Bartel, 2004; Ghildiyal and Zamore, 2009). Initially discovered in *Caenorhabditis elegans* (Lee et al., 1993), miRNAs have been found in plants, invertebrates, mammals, and humans (Bartel, 2009). Two major features of miRNAs indicate their potential contributions to neurodegenerative diseases. First, miRNAs can simultaneously regulate many target transcripts, and up to 50% of all coding genes may be regulated by miRNAs (Krol et al., 2010). Thus, miRNAs are central regulators of genetic networks. Second, miRNAs ensure stable protein levels under variable conditions and are therefore essential for the robustness of biological processes (Herranz and Cohen, 2010). Given the importance of protein homeostasis and the diversity of cellular pathways potentially leading to neurodegeneration, it has been hypothesized that miRNAs might contribute to neurodegenerative diseases (Eacker et al., 2009; Lau and de Strooper, 2010; Sonntag, 2010).

In this review, we briefly describe the biogenesis of miRNAs and their potential involvement in the evolution of the human brain. Then we will discuss accumulating evidence that miRNAs are important contributors to neurodegenerative diseases. Indeed, some observations suggest that miRNA alterations can disrupt protein homeostasis and may be at the root of neurodegenerative processes (**Figure 2**). Conversely, other data strongly suggest that altered miRNA networks are a consequence of abnormal neuronal physiology (**Figure 3**). Examples in specific neurodegenerative diseases will be presented.

miRNA BIOGENESIS

Although several alternative mechanisms also exist (Yang and Lai, 2011), the canonical pathway for miRNA biogenesis involves a primary transcript generated by RNA polymerase II (Lee et al., 2004). The primary miRNA is cleaved by a nuclear complex containing Drosha and DGCR8, giving rise to a hairpin precursor molecule (pre-miRNA) of 70–100 nt (Lee et al., 2003; Han et al., 2004). This pre-miRNA is exported to the cytoplasm, where Dicer, a RNA-III nuclease, catalyzes the final cleavage in the maturation process, resulting in an imperfect RNA duplex (Hutvagner et al., 2001). One strand (guide strand) is loaded into an RNA-induced silencing complex (RISC) to bind the target mRNA; the other strand (passenger strand) is usually destroyed (Chendrimada et al., 2005; Bartel, 2009). miRNAs control gene expression at

the post-transcriptional level through imperfect base pairing with specific sequences, located mostly in the 3′UTRs of mRNAs. After recognition, miRNA–target interactions often result in mRNA degradation or inhibition of mRNA translation (Krol et al., 2010; **Figure 1**).

ROLE OF miRNA IN BRAIN EVOLUTION

Neurodegenerative diseases are considered devastating disorders because they often impair cognitive and executive functions. Understanding how the human brain acquired such functions is a challenging task that might also provide important insights into the mechanisms of neurodegeneration. It was noted four decades ago that genetic differences among species do not account for brain divergence and that brain evolution could have been driven by changes in gene expression levels (King and Wilson, 1975). Early comparative transcriptome analyses confirmed this hypothesis and revealed more human-specific than chimpanzee-specific expression changes in the prefrontal cortex (PFC); no such changes were observed in blood, liver, or other tissues (Enard et al., 2002; Caceres et al., 2003). These findings supported the idea that brain-specific changes in gene expression levels shaped the evolution of the nervous system.

To determine whether miRNAs participated in this process, miRNA expression profiles in human and chimpanzee brains were compared (Berezikov et al., 2006). Many of the newly identified brain miRNAs were expressed only in humans, and many were restricted to primates. Another study reported that 10–35% of miRNAs were expressed in the human brain but not in chimpanzee or macaque brains (Hu et al., 2011). More importantly, developmental profiles of brain miRNAs and their target genes showed the fastest rates of human-specific evolutionary change (Somel et al., 2011). Although experimental evidence is still lacking, it is an attractive hypothesis that rapidly evolving miRNAs in the human brain are essential for neuronal function and maintenance.

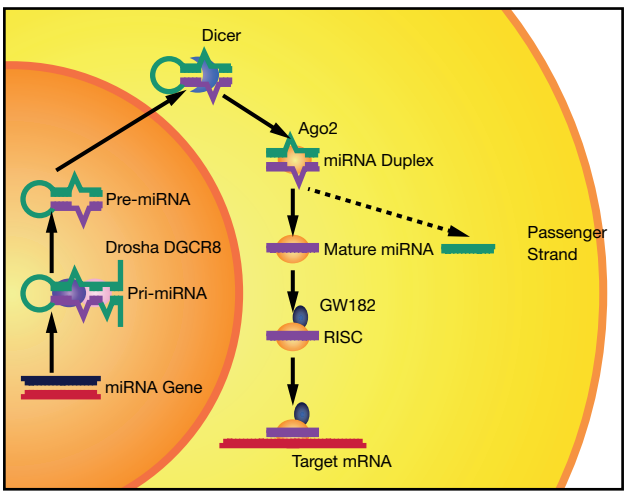

FIGURE 1 | The canonical miRNA biogenesis pathway. miRNAs are produced from long Pol II transcripts (pri-miRNA). A nuclear complex containing Drosha (purple oval) and DGCR8 (pink oval) cleaves the primary transcript and generates a precursor miRNA (pre-miRNA). After nuclear export, pre-miRNA is further processed by Dicer (blue croissant). Then, Ago2 (yellow oval) binds to the complex formed by miRNA duplex and Dicer. Ago2 induces Dicer dissociation and the release of the passenger strand from the complex. Finally, other proteins, such as GW182 (dark blue), associate with Ago2 and form a RISC complex that recognizes and then silences (by mRNA degradation and/or translation inhibition) a target mRNA.

GLOBAL LOSS OF miRNAs CAUSES NEURODEGENERATION

Genetic disruption of miRNAs biogenesis pathways has been used to probe the potential link between miRNAs and neurodegeneration. In mouse cerebellar Purkinje cells, conditional knockout of Dicer leads to age-dependent cerebellar degeneration and ataxia (Schaefer et al., 2007). Cell-type specific deletion of Dicer in striatal, retinal, spinal, and cortical neurons produced similar results (Cuellar et al., 2008; Damiani et al., 2008; Davis et al., 2008; Haramati et al., 2010). Dicer deletion also altered the phosphorylation pattern of tau before neuronal cell loss (Hebert et al., 2010), indicating that some mechanisms of neurodegeneration might be controlled through miRNAs. Of note, neurodegeneration in the absence of Dicer could also result from the toxic accumulation of pre-miRNAs or from the loss of other Dicer functions unrelated to miRNAs biogenesis. However, reduced production of a small proportion of miRNAs as a result of DGCR8 haploinsufficiency also leads to neuronal dysfunction (Stark et al., 2008; Fenelon et al., 2011; Schofield et al., 2011), supporting the notion that neurodegeneration could indeed arise from loss of miRNAs.

Glial cell defects may also profoundly influence neuronal survival (Ilieva et al., 2009; Prinz et al., 2011). Indeed, neurodegeneration ensues after targeted deletion of Dicer in astrocytes (Tao et al., 2011), oligodendrocytes (Shin et al., 2009), and Schwann cells (Pereira et al., 2010; Verrier et al., 2010; Wu et al., 2012). Thus, alteration of miRNA networks has the potential to disrupt neuronal function in a cell-autonomous or non-cell-autonomous manner and lead to neurodegenerative phenotypes.

LOSS OF INDIVIDUAL miRNAs LEADS TO NEURODEGENERATION

Genetic analyses have revealed essential roles for specific miRNAs in long-term neuronal survival, for example in mice lacking *miR-124-1* (Sanuki et al., 2011), one of the most well studied miRNAs in neuronal development (Gao, 2010). The mouse genome has three *miR-124* loci (*miR-124-1*, *-2*, and *-3*). Deletion of *miR-124-1*, the dominant source of this miRNA, increases apoptosis in the hippocampus and retina, causing a significant decrease in brain size. Furthermore, in the tail-suspension test, adult mutant mice exhibit a front and hind limb clasping response, a common phenotype in mouse models of neurodegenerative disorders. This effect seems to be mediated by regulation of the transcription factor Lhx2 (Sanuki et al., 2011). It is unclear whether the neurodegeneration also reflects the absence of miR-124's well-documented developmental functions (Cao et al., 2007; Visvanathan et al., 2007; De Pietri Tonelli et al., 2008; Cheng et al., 2009; Maiorano and Mallamaci, 2009). This question could be answered by using a conditional knockout approach.

Another miRNA that might be involved in neuronal survival is miR-8 (Karres et al., 2007), which is not brain-specific and has a complex pattern of expression. Mutant flies lacking *miR-8*

have limb and wing defects and increased apoptosis in the central nervous system. Moreover, concomitant decrease of the transcriptional repressor atrophin in *miR-8* mutant flies partially rescued the phenotypes (Karres et al., 2007). It remains to be determined whether miR-8 contributes to neurodegeneration in mammals.

miRNAs IN NEURODEGENERATIVE DISEASES

Microarray studies have shown that the brain expresses a wide range of miRNAs, suggesting that these small RNA molecules participate in nervous system physiology (Lagos-Quintana et al., 2002; Miska et al., 2004; Lim et al., 2005; Manakov et al., 2009). More importantly, profiling studies revealed profound changes in several miRNAs (i.e., miR-9, miR-29 cluster, miR-107, miR-125b, and miR-128) in patient brains such as that of AD patients (Kim et al., 2007; Lukiw, 2007; Hebert et al., 2008; Johnson et al., 2008; Wang et al., 2008, 2011; Nunez-Iglesias et al., 2010). Although these global changes in miRNA expression under pathological conditions should be interpreted cautiously, they support the notion that dysregulation of miRNAs networks is a common theme in neurodegenerative diseases.

ALZHEIMER'S DISEASE

A pathological hallmark of AD, the most prevalent neurodegenerative disease, is the accumulation of plaques formed by short β-amyloid (Aβ) peptides, commencing in the hippocampus, and spreading progressively throughout the brain (Ballard et al., 2011; Selkoe et al., 2012). It is likely the accumulation is caused by both increased production and impaired clearance of Aβ. Aβ peptides exert toxic effects and elicit an inflammatory response. Both may contribute to disrupt neuronal homeostasis and alter the integrity of neuronal networks involved in learning, memory, and other cognitive functions. Aβ peptides are generated through proteolytic cleavage of the amyloid precursor protein (APP) by γ-secretase and β-site APP-cleaving enzyme 1 (BACE1; O'Brien and Wong, 2011).

Amyloid precursor protein and BACE1 each contain several miRNA target sites in their 3′UTRs. Several miRNAs have been reported to repress APP expression, including miR-16, miR-101, miR-106a, and miR-520c (Patel et al., 2008; Liu et al., 2010; Long and Lahiri, 2011). miR-137 and miR-181c regulate serine palmitoyltransferase, which modulates the Aβ level (Geekiyanage and Chan, 2011). Interestingly, polymorphisms in miRNA binding sites in the 3′UTR of APP gene could influence the binding efficiency of these miRNAs. Thus, miRNAs might fine tune APP expression, which may enhance or limit the risk of AD (Delay et al., 2011).

A significant decrease in neuronal miR-107 expression and a parallel increase in BACE1 have been observed in AD patients (even those at the earliest stages of AD; Wang et al., 2008, 2011; Nelson and Wang, 2010). The 3′UTR of BACE1 mRNA has functional binding sites for miR-29 (Hebert et al., 2008), miR-107 (Wang et al., 2008), and miR-124 (Fang et al., 2012). Interestingly, miR-107 also controls the expression of other proteins relevant to AD pathology, such as cofilin (Yao et al., 2010), an actin-binding protein that accumulates in cytoplasmic inclusions known as Hirano bodies (Hirano, 1994). Thus, a single miRNA deregulation could activate multiple potentially pathogenic cascades upstream of Aβ accumulation.

Another possibility is that alterations in miRNAs in AD brain are a consequence of amyloid deposits. For instance, miR-106b is aberrantly expressed in APPswe/PSE9 mice (Wang et al., 2010a), and miR-146a levels are increased in AD brains and in several mouse models of AD (Li et al., 2011). *In vitro* exposure of hippocampal neurons to Aβ peptides preferentially decreases the levels of mature miRNAs (only a small fraction of miRNAs were upregulated; Schonrock et al., 2010). miRNAs were similarly deregulated in the hippocampus of APP23 mice at the onset of plaque formation. Overall, these studies suggest that miRNA deregulation is an essential pathogenic mechanism that is induced by Aβ aggregation and contributes to the progression and severity of AD.

POLYGLUTAMINE DISEASES

Polyglutamine (polyQ) diseases are a group of nine neurodegenerative disorders caused by an unstable CAG expansion in the coding region of their respective associated genes (Orr and Zoghbi, 2007). Apart from this common feature, polyQ diseases have distinct clinical presentations, and the proteins involved in these diseases have no structural/functional homology (**Table 1**). Huntington disease (HD), the most frequent polyQ disease, is characterized by the progressive loss of striatal neurons and motor impairment (typically resulting in the involuntary writhing movements called chorea) and is often associated with cognitive and behavioral deficits (Shoulson and Young, 2011).

The causal mutation in HD is an expanded repetition of the CAG trinucleotide in the first exon of the gene encoding huntingtin (HTT; Gilliam et al., 1987), a large protein (3300 amino acids) whose functions remains mostly unknown. HTT associates with Ago2 in P-bodies, and HTT depletion impairs miRNA-mediated gene silencing (Savas et al., 2008). Expanded HTT may sequester RNA processing factors in the cytoplasm (Jiang et al., 2011).

miRNAs were implicated in HD pathogenesis by two lines of evidence. First, the levels of repressor element 1 silencing transcription (REST) factor, a major pathogenic pathway in HD (Buckley et al., 2010), is elevated in HD neurons, resulting in repression of hundreds of key neuronal genes (Zuccato et al., 2003, 2007; Johnson et al., 2010b). Canonical and non-canonical REST binding motifs have been mapped in close proximity to 22 miRNA sites in the human genome, including several miRNAs that are abundant in neurons (Bruce et al., 2004; Jothi et al., 2008; Yu et al., 2011). Could abnormal REST deregulate miRNAs network in HD patients? REST and its cofactor coREST possess functional target sites for miR-9 and miR-9*, respectively (Packer et al., 2008), and miR-9 and miR-9* (together with miR-7, miR-124, miR-132, and other miRNAs) are downregulated in HD patients (Johnson et al., 2008; Packer et al., 2008; Marti et al., 2010). miRNA deregulation in HD was confirmed by profiling studies in animal models, although there was a high degree of variability among the models (Lee et al., 2011). These observations strongly suggest that altered miRNA transcription is a major event in HD pathogenesis.

Besides HD, there are eight other polyQ diseases: dentatorubral–pallidoluysian atrophy, spinal and bulbar muscular atrophy, and spinocerebellar ataxia (SCA) 1, 2, 3, 6, 7, and 17 (Gatchel and Zoghbi, 2005). In fly models of SCA3, reduction of

Table 1 | List of miRNAs and the neurodegenerative diseases to which they have been associated.

miRNA	Disease	Type of evidence	Mechanism	Reference
miR-7	PD	*In vitro* reporter assay Overexpression *in vitro*	Regulation of α-synuclein	Doxakis (2010), Junn et al. (2009)
miR-8		miR-8 flies	Upregulation of atrophin	Karres et al. (2007)
miR-9/9*	HD	Profiling studies *In vitro* reporter assays	REST–coREST	Marti et al. (2010), Packer et al. (2008)
miR-9/9*	ALS	Profiling in mouse mode	Neurofilament expression	Haramati et al. (2010)
miR-16	AD	Profiling in mouse model Overexpression *in vitro*/*in vivo*	Regulation of APP levels	Liu et al. (2010)
miR-19	SCA1	*In vitro* reporter assays *In vitro* gain of function	Regulation of ataxin-1	Lee et al. (2008)
miR-29	AD	Profiling in patients *In vitro* reporter assay Overexpression/blocking *in vitro*	Regulation of BACE1 levels	Hebert et al. (2008)
miR-29b	FTD	*In vitro* reporter assay Overexpression *in vitro*	Regulation of progranulin	Jiao et al. (2010)
miR-34		SCA3 overexpression in fly	Protective role	Liu et al. (2012)
miR-101	AD	*In vitro* reporter assays Overexpression/blocking *in vitro*	Regulation of APP levels	Long and Lahiri (2011)
miR-101	SCA1	*In vitro* reporter assays *In vitro* gain of function	Regulation of ataxin-1	Lee et al. (2008)
miR-106a	AD	*In vitro* reporter assays Overexpression *in vitro*	Regulation of APP levels	Patel et al. (2008)
miR-106b	AD	Expression in mouse model	TGF-β	Wang et al. (2010a)
miR-107	AD	Profiling in patients *In vitro* reporter assay *In situ* hybridization in patients	Regulation of BACE1 levels	Wang et al. (2008)
miR-107	AD	*In vitro* reporter assay Levels in mouse models	Regulation of cofilin	Yao et al. (2010)
miR-107	FTD	*In vitro* overexpression	Regulation of progranulin	Wang et al. (2010b)
miR-124		miR-124-1 knockout mouse	Altered expression of Lhx2	Sanuki et al. (2011)
miR-124	AD	Overexpression *in vitro*/*in vivo*	Regulation of BACE1 levels	Fang et al. (2012)
miR-130	SCA1	*In vitro* reporter assays *In vitro* gain of function	Regulation of ataxin-1	Lee et al. (2008)
miR-133b	PD	Profiling in patients	???	Kim et al. (2007)
miR-137	AD	Profiling in patients *In vitro* reporter assay Blocking *in vitro*	Regulation of Aβ levels	Geekiyanage and Chan (2011)
miR-144	SCA1	Profiling in patients *In vitro* reporter assay	Regulation of ataxin-1	Persengiev et al. (2011)
miR-146a	AD	Profiling in patients Expression in cell lines and mouse models	Downstream of Aβ	Li et al. (2011)
miR-153	PD	*In vitro* reporter assay Overexpression *in vitro*	Regulation of α-synuclein	Doxakis (2010)
miR-181c	AD	Profiling in patients *In vitro* reporter assay Blocking *in vitro*	Regulation of Aβ levels	Geekiyanage and Chan (2011)
miR-520c	AD	*In vitro* reporter assays Overexpression *in vitro*	Regulation of APP levels	Patel et al. (2008)
miR-659	FTD–ALS	Human polymorphism	Regulation of progranulin	Rademakers et al. (2008)

miRNA processing after knockout of Dicer1 markedly enhances the toxicity induced by mutant ataxin-3 (Bilen et al., 2006). In parallel genetic screens, a single miRNA, *bantam*, was identified as a potent downstream modulator of both polyQ and tau toxicity in

flies (Bilen et al., 2006). In a recent paper, miR-34 was shown to be protective against expanded SCA3 (Liu et al., 2012). Subsequent work suggested that miR-19, miR-101, and miR-130 are important for the post-translational regulation of ataxin-1 (Lee et al., 2008). Inhibition of those miRNAs enhanced the cytotoxicity of polyQ-expanded ataxin-1 in human cells. Moreover, miR-144, a highly conserved miRNA, also regulated ataxin-1 expression and appeared to be associated with aging. Ataxin-1 levels are higher in the cerebellum and cortex of SCA1 patients than in healthy aged brains (Persengiev et al., 2011). On the other hand, ataxin-2 might be required for miRNA function (McCann et al., 2011), further supporting the intimate association between miRNAs and polyQ diseases.

PARKINSON'S DISEASE

Parkinson's disease is a neurodegenerative disorder that primarily affects movement. Clinical symptoms include bradykinesia (decreased ability to start and continue movements), resting tremor, and rigidity. These symptoms are due to the relatively selective loss of dopaminergic neurons in the substantia nigra (Dauer and Przedborski, 2003) and reflect the impairment of neuronal networks important for regulating motor function. The past two decades have witnessed significant advances in the identification of distinct genetic loci at which pathogenic mutations are associated with parkinsonism (for review, see Lesage and Brice, 2009; Zimprich, 2011). Most research is focused on genes that have been conclusively linked to PD pathogenesis, including those encoding α-synuclein, leucine-rich repeat kinase 2, PTEN-induced putative kinase 1, parkin, and DJ-1.

Profiling studies of PD brains have revealed abnormalities in miRNA content (Kim et al., 2007; Minones-Moyano et al., 2011). One of the miRNAs found to be downregulated in these studies, miR-133b, plays a major role in the development of midbrain dopaminergic neurons by regulating the transcription factor Pitx3 (Kim et al., 2007). miR-7 and miR-153 control the expression of α-synuclein (Junn et al., 2009; Doxakis, 2010). Since intracellular levels of this protein appear to be critical in mediating its toxicity, deregulation of those miRNAs might lead to increased toxic levels of α-synuclein.

AMYOTROPHIC LATERAL SCLEROSIS AND FRONTOTEMPORAL DEMENTIA

Amyotrophic lateral sclerosis (ALS) is a progressive, lethal, degenerative disorder characterized by the selective death of motor neurons in the brain and spinal cord (Pasinelli and Brown, 2006; Ferraiuolo et al., 2011). ALS shares many clinical, pathological, and molecular features with FTD, the second most common early-onset dementia (Ferrari et al., 2011). Clinically, FTD progresses from an insidious onset of behavioral changes, impaired frontal executive functions, and language deficits to more severe cognitive defects and, finally, to generalized dementia (Boxer and Miller, 2005). In familial FTD cases, the mutated locus has been identified in the genes encoding tau (Hong et al., 1998; Hutton et al., 1998), VCP (Watts et al., 2004), CHMP2B (Skibinski et al., 2005), progranulin (Baker et al., 2006; Cruts et al., 2006), and C9ORF72 (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Among them, CHMP2B (Parkinson et al., 2006; Cox et al., 2010), VCP (Johnson et al., 2010a), and C9ORF72 (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Stewart et al., 2012) have also been implicated in ALS. Moreover, the RNA-binding proteins TDP-43 and FUS have been strongly implicated in both FTD and ALS (Arai et al., 2006; Neumann et al., 2006, 2009; Sreedharan et al., 2008; Vance et al., 2009).

As described above, Dicer deletion in spinal motor neurons mimics most of the clinical (e.g., progressive paralysis) and pathological (e.g., astrocytosis and signs of axonopathy) features of ALS (Haramati et al., 2010). Whether the miRNA pathway is involved in the molecular pathogenesis of FTD/ALS caused by *C9ORF72* repeat expansion is unknown. However, a potential involvement for the miRNA pathway in other forms of FTD or ALS comes from limited studies on TDP-43. TDP-43 is mutated in a subset of ALS patients (Sreedharan et al., 2008), and the expression levels of some miRNAs are affected in *TDP-43* mutant flies (Buratti et al., 2010). Biochemical interactions between TDP-43 and Drosha, a key miRNA processing enzyme (Han et al., 2004), have been observed (Gregory et al., 2004; Ling et al., 2010). These findings raise the possibility that TDP-43 may play a role in miRNA processing; however, the mechanism remains to be elucidated and whether endogenous Drosha and TDP-43 physically interact needs to be demonstrated.

Some miRNAs are emerging as important contributors to ALS pathogenesis. The muscle-specific miR-206 is upregulated upon nerve injury and is required for regeneration of neuromuscular synapses. Moreover, miR-206 deficiency accelerates disease progression in a mouse model of ALS (Williams et al., 2009). miR-9/9*, an evolutionarily conserved and multifunctional miRNA (Yuva-Aydemir et al., 2011), is also potentially involved in ALS. Profiling of miRNA expression in motor neurons harboring an *SMN1mut* allele found in pediatric spinal motor atrophy revealed decreases of more than 90% in miR-9 and miR-9* levels (Haramati et al., 2010). More importantly, changes in the expression levels of the neurofilament subunits likely contribute to the disease, and miR-9 is an upstream regulator of the neurofilament mRNAs.

Several other miRNAs might be linked to FTD–ALS through different mechanisms. For example, miR-29b and miR-107 regulate progranulin levels (Jiao et al., 2010; Wang et al., 2010b). Since progranulin haploinsufficiency can cause FTD[114,115], excessive levels of those miRNAs might decrease progranulin levels and be a risk factor for the disease. Consistent with these observations, a genetic polymorphism in the 3′UTR of the progranulin gene is associated with a higher risk of FTD–ALS, and multiple miRNAs are misregulated in FTD with TDP-43 pathology (Rademakers et al., 2008; Kocerha et al., 2011). This genetic variant (rs5848) affects the miR-659 binding site, resulting in more efficient binding and, consequently, decreased progranulin levels. It is not known whether translational regulation by miRNAs is a common mechanism in FTD caused by progranulin deficiency or in other neurodegenerative diseases (Rollinson et al., 2011).

SUMMARY

Although much progress has been made in our understanding of how miRNAs control gene expression at the post-transcriptional level during development, their contributions

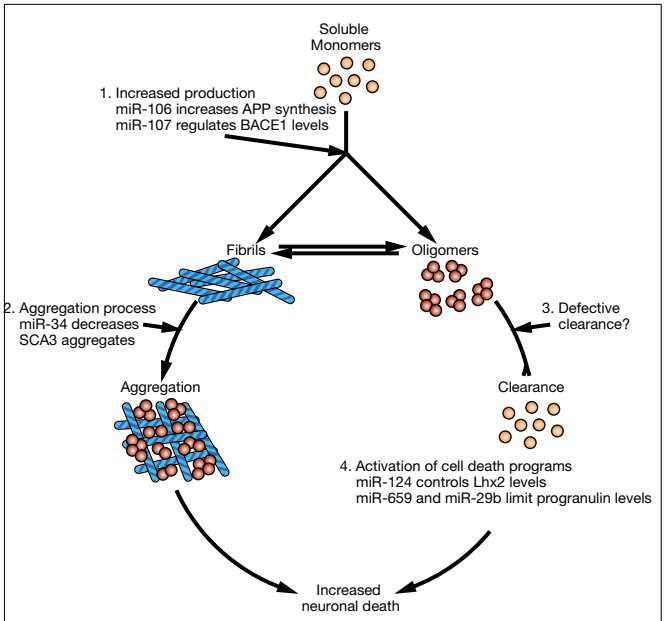

FIGURE 2 | Potential mechanisms of neurodegeneration induced by altered miRNA networks. Schematic representation of cellular pathways that could be affected downstream of miRNAs. (1) Defects in miRNAs could increase the levels of aggregation-prone proteins either directly (i.e., miR-106a and APP in AD) or indirectly (i.e., miR-107 acting through BACE1 or miR-137 acting through serine palmitoyltransferase in AD). (2) miRNAs could control the expression of proteins involved in proper folding or quality control, increasing the risk of protein aggregation. (3) miRNAs could impair the removal of aggregated proteins and therefore increase their levels and toxicity. (4) Finally, altered miRNAs might result in neuronal cell death due to increased levels of certain transcription factors (i.e., miR-124 controls neuronal survival by limiting the expression of Lhx2) or the imbalance between pro-survival and pro-apoptotic signals (i.e., in FTD, polymorphism rs5848 results in more efficient binding of miR-659 and decreased levels of the pro-survival factor progranulin).

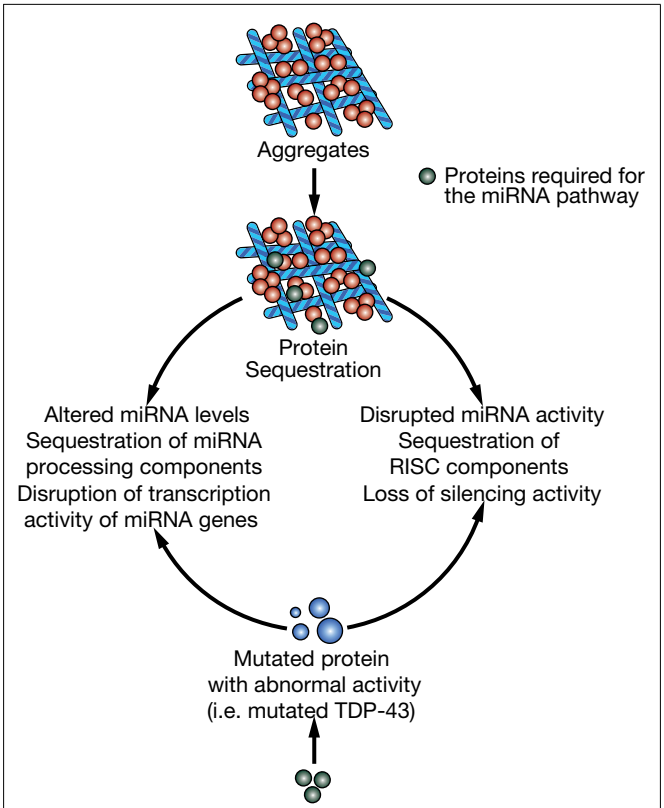

FIGURE 3 | Potential mechanisms of miRNA deregulation downstream of protein aggregation/dysfunction. Two basic mechanisms could affect miRNA regulatory networks: (1) alteration of miRNA levels through deregulation of transcription (i.e., miR-9 downstream of HTT and REST) or processing (i.e., TDP-43 altering Drosha function) and (2) interference with RISC activity (i.e., ataxin-2 seems to be required for optimal miRNA silencing).

to neurodegenerative disease remain poorly understood. Many fundamental questions need to be addressed. Is miRNA pathway disruption a downstream consequence or a cause of neurodegeneration (**Figure 2** vs. **Figure 3**)? Are miRNAs essential for the proper regulation of aggregation-prone proteins or do they control additional pathogenic pathways? Are individual miRNAs especially important in particular neurodegenerative diseases? Which miRNA target or targets are relevant for the disease? To address these questions, it will be essential to generate novel experimental models, such as conditional knockouts in which developmental defects can be circumvented, allowing assessment of the functions of specific miRNAs in the adult brain. We expect many exciting findings will be made in years to come.

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