Translational control of neuronal mRNAs

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Abstract

The processes of learning and memory require accurate translational control of neuronal mRNAs. This control can be carried out through the regulation of the general translation machinery and upstream factors, as well as through the recognition and selective translation of specific mRNAs. In this work we explore two molecular mechanisms of translational control in neurons. First, we investigate the roles of RNA helicases eIF4A1 and DHX29 in controlling the translation of synaptically-localized mRNAs. We demonstrate the preferential translation of mRNAs with extensively structured 5'UTR, underlining the importance of this region in translational control. We also pinpoint the post-synaptic localization of DHX29 and propose a technique for monitoring the rate of 5'UTR-dependent translation *in vivo* with the use of a photoconvertible fluorescent reporter protein. Second, we study the role of cap-dependent translation in autism spectrum disorders (ASD) by examining translationally controlled mRNAs. We show that knockout of eIF4E binding protein 2 (4E-BP2, eIF4E repressor downstream of mTOR) or overexpression of eIF4E in mice lead to increased translation of neuroligin mRNAs, which can explain the autistic-like behaviours in these mice. Moreover, we design short hairpin RNAs packaged in lentiviral particles to modulate the expression of neuroligins *in vivo*. We use this technology to validate the mechanism of dysregulated neuroligin mRNA translation as the causal factor for the autism-like behaviour in the 4E-BP2 knockout mice. Finally, we propose a mechanism for the exaggerated translation of neuroligin mRNAs through their 5'UTRs. In conclusion, in this work we provide evidence that translational control of specific brain mRNAs is crucial for synaptic function and behaviour.

Résumé

Les processus d'apprentissage et de mémoire requièrent un contrôle précis de la traduction des ARNm présents dans les neurones. Ce contrôle peut être exercé au niveau de la machinerie traductionnelle et des facteurs en amont de celle-ci, mais aussi au niveau des ARNm, en reconnaissant et en traduisant les molécules messagères de façon sélective. Dans cet ouvrage nous étudions deux mécanismes de régulation traductionnelle dans les cellules nerveuses. En premier lieu, nous explorons les rôles des hélicases eIF4A1 et DHX29 dans la régulation de la traduction des ARNm localisés aux synapses. Nous confirmons que la traduction des ARNm contenant une région 5' non traduite (5'UTR) complexe est favorisée, soulignant de ce fait l'importance de cette région dans la régulation traductionnelle. Nous démontrons que DHX29 se trouve surtout dans l'élément postsynaptique et proposons une méthode de surveillance du taux de traduction *in vivo* selon la complexité de la 5'UTR à l'aide d'un gène rapporteur codant une protéine fluorescente photoconvertible. En second lieu, nous étudions le rôle de traduction dans le cas des troubles du spectre autistique (TSA) en examinant la régulation traductionnelle de certains ARNm. Nous démontrons que l'invalidation (knock-out) de 4E-BP2 (eIF4E-binding protein 2) ou la surexpression de eIF4E dans des souris mène à la traduction accrue des ARNm des neuroligines, ce qui pourrait expliquer le comportement autistique de ces souris. De plus, nous concevons des lentivirus remplis de petits ARN en épingle à cheveux (shRNA) destinés à moduler l'expression des neuroligines *in vivo*. Nous utilisons cette technologie afin de prouver que la dysrégulation de la traduction des ARNm des neuroligines est une cause majeure du comportement autistique parmi les souris knock-out pour 4E-BP2. Enfin, nous proposons une explication de la traduction exagérée des ARNm des neuroligines en examinant leur 5'UTR. Pour conclure, dans cet ouvrage nous fournissons la preuve de l'importance de la régulation traductionnelle des ARNm pour le propre fonctionnement du système nerveux.

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Preface and contribution of authors

The work presented in this thesis is composed of two parts. The first chapter deals with RNA helicases and their involvement in translation initiation and in regulation of a specific subset of mRNAs with complex 5'UTRs. The results presented here have never been published or presented outside of this lab. In the second chapter, I present the work and experiments that I have performed for the recently published paper concerning autism-related deficits in mice with dysregulated translational control (Gkogkas et al., 2013). All the work presented in this thesis is my own, with the exception of metabolic labelling experiments, performed by Christos Gkogkas. Results of behavioural and electrophysiology experiments performed by Christos Gkogkas and Arkady Khoutorsky are mentioned for the purpose of presenting a complete picture, but are not credited as my work. For the list of contributions to the paper by other authors, please see the "Author Contributions" section of the paper.

Introduction

The central dogma of molecular biology and the principles of translational control

In 1958, Francis Crick first proposed a universal scheme describing the flow of genetic material in a living organism. He called it the "central dogma" of molecular biology. According to Crick, genetically encoded information is passed from one biopolymer to the next in a highly precise and unidirectional manner: from DNA to RNA to protein, but never from protein to nucleic acid or from protein to protein (Crick, 1958, 1970).

Crick's "central dogma" has since then become an important pillar of molecular biology, one that has been reaffirmed, but also revised and completed as the scientific community made new discoveries. The core of it, however, has remained unchanged to this day and is forever at the heart of molecular research. In a process termed transcription, genetic information is "transcribed" from the double-stranded DNA molecule to RNA, known as messenger RNA (mRNA). This information is then converted from the nucleic acid code to the entirely different amino acid code as a protein is synthesised according to the mRNA template. This process is known as translation. There are instances where an RNA molecule can be reverse-transcribed to DNA and a DNA molecule can be replicated into another DNA molecule, but a protein molecule is the final and irreversible product of this cellular production line.

Proteins constitute the workforce of the cell. Not only do they act as important structural components, proteins play a crucial role as enzymes, responsible for catalyzing biochemical reactions and for synthesizing all biopolymers including polysaccharides, other proteins, and nucleic acids. Moreover, proteins confer to the organism the phenotype specified by the genetic

material. Crick wrote that "the main function of the genetic material is to control [...] the synthesis of proteins" (Crick, 1958). Considering that all our body functions down to the workings of our brain are controlled by enzymes, that dysregulation in protein function is often the main cause of disease, and that peptides are the first and foremost targets in drug development, it is not difficult to see the reasoning behind Crick's bold hypothesis. It is also clear that studies of the processes involved in the control of protein synthesis, vital to life as we know it, hold the key to understanding various diseases, including cancer, metabolic diseases, and neurodegenerative disorders.

Translation is an intricate process involving a large number of molecular players and a substantial portion of a cell's energy (Mathews, Sonenberg, & Hershey, 2007). It is essential that such a biologically important and energetically costly process be tightly regulated. Although gene expression is also regulated at the level of transcription, translational regulation allows for rapid changes in protein levels via the translational control of existing mRNAs (Sonenberg & Hinnebusch, 2009). It is now known that the cellular abundance of proteins is primarily controlled at the level of translation (Schwanhausser et al., 2011). The regulation of translation is of particular importance in situations requiring an immediate response: cellular response to stress and apoptosis (Holcik & Sonenberg, 2005), regulation of cell growth and division (Jorgensen & Tyers, 2004), and during differentiation and development (Kuersten & Goodwin, 2003).

The process of translation is generally divided into four steps: initiation, elongation, termination, and ribosome recycling. Among these, translation initiation is the most highly regulated and rate-limiting step, since the "cost" of terminating the translational process at this step by dealing with the mRNA transcript is far less than the energy expenditure that would be entailed by dealing

with the multiple protein copies downstream of translation (Mathews et al., 2007). The following work concentrates solely on the process of translational regulation at the step of initiation.

The mRNA transcript

To convert the genetic code into a functional protein, the translation machinery acts on the mRNA transcript of a gene. Therefore, it is important to understand the basic structure of an mRNA before discussing the specific means of translational control. Multiple factors determine the translational success of an mRNA, such as its inherent stability and the presence of factors influencing global and transcript-specific translation (Dever, 2002; Gebauer & Hentze, 2004). These factors include changes in the phosphorylation state of translational machinery components, ribosomal abundance, and presence of structures "earmarking" specific mRNAs for translational control (Mata, Marguerat, & Bähler, 2005).

The 5'UTR and the cap structure

The 5 prime untranslated region (5'UTR) of an mRNA transcript is the region located directly upstream of the protein-coding region. Because the transcript is "read" by the translational machinery in a 5' to 3' direction, the 5'UTR is located at the "beginning" of the transcript. As its name suggests, the 5'UTR does not code for a part of the protein, but is instead necessary for controlling gene expression. A number of studies report translation regulation of specific mRNAs while global translation remains unaffected. These mRNAs are characterized by long 5'UTRs with complex secondary structures and AUG codons (van der Velden & Thomas, 1999). These features impede the movement of the translational machinery along the mRNA strand and

hinder translation initiation; in fact, a structure with a free energy of -50 kcal/mol is enough to severely retard translation initiation (Pelletier & Sonenberg, 1985). Interestingly, such transcripts often encode proteins involved in developmental processes, including proto-oncogenes, growth factors, and transcription factors (Kozak, 1987; van der Velden & Thomas, 1999).

The 5' cap structure is located directly upstream of the 5'UTR and is present in most eukaryotic mRNAs, distinguishing them from prokaryotic transcripts (Shatkin, 1976). It is involved in adequate splicing of the mRNA precursor (Konarska, Padgett, & Sharp, 1984), in the export of the mRNA from the nucleus (Hamm & Mattaj, 1990), and in preventing degradation of the transcript by 5' exonucleases while promoting mRNA stability (Ross, 1995). Most importantly, the 5' cap structure promotes translation by facilitating the formation of the translation initiation complex (Shatkin, 1976; Topisirovic, Svitkin, Sonenberg, & Shatkin, 2011). The binding of the eIF4E cap-binding protein to the cap structure is considered to be the rate-limiting step of translation initiation (Niedzwiecka et al., 2002). The cap is important, but not essential for translation initiation: initiation and scanning can occur in its absence (Gunnery, Mäivali, & Mathews, 1997). However, the presence of the cap in combination with a poly(A) tail (discussed next) dramatically enhances initiation on an mRNA strand.

The cap is added co-transcriptionally to the 5' end of the nascent RNA strand in a series of enzymatic steps. It consists of a methylated guanosine residue (m^7G, 7-methylguanosine) linked to the first nucleotide (N) of the mRNA transcript via an inverted 5' to 5' triphosphate linkage (normally, nucleotides are linked to each other 5' to 3'): m^7G(5')ppp(5')N (Topisirovic et al., 2011). This unusual link is designed to "confuse" 5' exonucleases, thus protecting the mRNA from degradation.

The 3'UTR and the poly-A tail

The 3' untranslated region (3'UTR) is found immediately after the coding region. It contains sequences necessary for controlling mRNA stability, in particular sequences recognized by microRNAs (K. Chen et al., 2008; von Roretz & Gallouzi, 2008) and sequences promoting sub-cellular localization, crucial during development (Gavis & Lehmann, 1994; Kuersten & Goodwin, 2003). It also contains one or multiple polyadenylation signals, recognised on the newly-made pre-mRNA right after transcription. Enzymes in the nucleus cleave the 3' end and append a poly(A) tail onto the future mRNA molecule; the poly(A) tail is a sequence of several adenine bases at the very end of the mRNA transcript, necessary for nuclear export, stability, and translation of the mRNA. Because multiple polyadenylation sites may be present in certain genes, polyadenylation can produce more than one transcript from a single gene (Proudfoot, Furger, & Dye, 2002). The poly(A) tail is the binding site for the poly(A)-binding protein (PABP), a protein which also associates with eIF4G, which is bound by eIF4E (both in the eIF4F complex), which binds to the 5' m^7G cap, thus efficiently circularizing the mRNA molecule (Sonenberg & Hinnebusch, 2009). This "closed-loop" formation is believed to promote re-initiation of translation by the translation machinery, increasing translational efficiency. In fact, under normal cellular conditions the 5' cap and the poly(A) tail work in synergy to enhance the rate of translation (Jacobson, 1996). This synergy is achieved through the interaction of eIF4F and PABP. Disruption of the interaction between these factors dramatically reduces translational efficiency (Michel, Poncet, Piron, Kean, & Borman, 2000).

Thus, both the 5'UTR and the 3'UTR contain sequences or structural features designed to affect the rate of translation. These are known as *cis*-acting elements. They influence translation by interacting with *trans*-acting elements, usually proteins, which carry out the processes affecting

translation. The major *trans*-acting elements involved in the process of translation initiation will be discussed in the context of cap-dependent translation initiation.

Cap-dependent translation initiation: mechanisms and molecular players

In eukaryotes, translation initiation is a highly regulated rate-limiting step involving a large host of molecular players. The goal of translation initiation is the identification of the AUG start codon by the initiator methionyl tRNA (Met-tRNAi) in the ribosomal peptidyl (P) site. Unlike in bacteria, where ribosome recruitment depends on the Shine-Dalgarno sequence (Shine & Dalgarno, 1975), translation initiation in eukaryotes is mostly cap-dependent (see Figure 1 for a diagram), meaning that a 5' cap and associated initiation factors are necessary for efficient translation initiation. In fact, while translational control in bacteria is effected thought the modulation of accessibility to the Shine-Dalgarno sequence, eukaryotes employ a scanning mechanism where the small 40S ribosomal subunit is loaded with Met-tRNAi in a pre-initiation complex (PIC) and scans the 5'UTR in search of the start codon (Sonenberg & Hinnebusch, 2009).

Translation initiation requires a pool of separated small (40S) and large (60S) ribosomal subunits, at least 12 eukaryotic initiation factors (eIFs), as well as hydrolysis of ATP and GTP (Mathews et al., 2007). Because translation is a cyclical process, these subunits and factors are constantly assembled and recycled (Jackson, Hellen, & Pestova, 2010). As previously mentioned, in the canonical cap-dependent translation paradigm, the m^7G cap structure at the 5' end of the mRNA and the 3' poly(A) tail is recognised by specific eIFs that "circularize" the

mRNA strand and provide a platform for PIC binding. This step is known as mRNA activation and requires energy in the form of ATP hydrolysis.

The 43S PIC is composed of the small 40S ribosomal subunit, the Met-tRNAi, and the initiation factors 1, 1A, 2, 3, and 5. The Met-tRNAi and a GTP-bound eIF2 are first assembled into what is known as the ternary complex before joining the other components of 43S. The preassembled PIC is recruited to the 5' end of the mRNA by the cap-binding factor eIF4E which, along with eIF4A (an RNA helicase) and eIF4G (a scaffold protein), is part of the eIF4F complex. Factors eIF4B and eIF4H aid the positioning process by assisting eIF4A in unwinding the 5' cap-proximal region and preparing it for PIC binding (Jackson et al., 2010). It has been demonstrated that 43S complexes are intrinsically capable of 5' end-dependent attachment to and scanning of mRNAs with unstructured 5'UTRs (Pestova & Kolupaeva, 2002). However, almost all eukaryotic 5'UTRs have some degree of secondary structure, which means that initiation factors are necessary to disrupt these structures and prepare the cap-proximal are for binding by the 43S complex.

Once properly positioned near the cap, the 43S PIC scans the 5'UTR in a 5' to 3' direction, a process which also requires ATP hydrolysis. The complex progresses along the mRNA strand, resolving secondary structures, until the AUG start codon is recognised through perfect complementarity with the anticodon of the initiator tRNA. Once the start codon is recognised, scanning terminates and the GTPase-activating protein eIF5 forces the eIF2 GTPase to irreversibly hydrolyse the GTP molecule bound to it. The release of eIF2-GTP and of other initiation factors and the binding of the 60S large ribosomal subunit result in the formation of the 80S complex, ready to proceed to the elongation step of translation (Mathews et al., 2007).

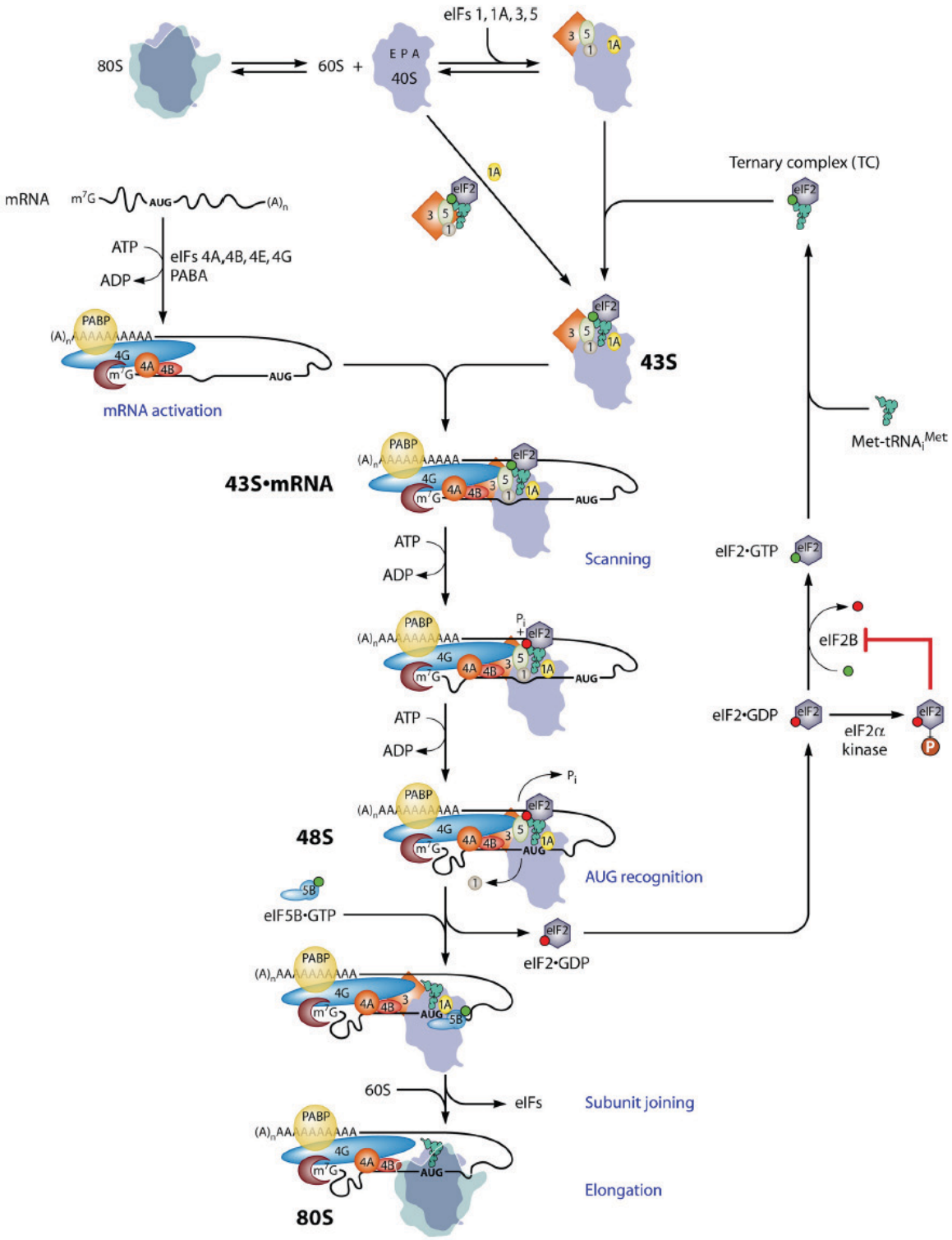


Figure 1. The mechanism of cap-dependent translation initiation in eukaryotes. See text for a brief summary. Figure adapted from (Hinnebusch, 2011).

The cap-binding protein eIF4E and the 4E-BPs

Of particular interest in the process of translation initiation and regulation is the cap-binding factor eIF4E. It was first identified as a polypeptide that bound strongly and specifically to the 5' cap structure (Sonenberg, Rupprecht, Hecht, & Aaron, 1979) and is one of the two major cap-binding proteins described to date (Topisirovic et al., 2011). As previously discussed, eIF4E binding to the cap is one of the most important rate-limiting steps in translation initiation. Indeed, because eIF4E is the least abundant initiation factor, formation of the eIF4F complex is dependent on its availability (Raught & Gingras, 1999). This leads to believe that eIF4E is a crucially important target for regulating translation initiation and multiple studies have proven this to be the case.

Structurally, eIF4E resembles a cupped hand "holding" the cap structure in its palm (Marcotrigiano, Gingras, Sonenberg, & Burley, 1997). The guanine base itself is "sandwiched" between the Trp-56 and Trp-102 residues of eIF4E, while interactions with other residues also strengthens eIF4E's grip on the cap (Marcotrigiano et al., 1997). The affinity of eIF4E for the cap is further enhanced through its binding to eIF4G (Gross et al., 2003).

One of the most common ways of regulation of protein function is through phosphorylation. The protein switches between a phosphorylated and an unphosphorylated state, one of which is active while the other is not. Phosphorylation of mammalian eIF4E mostly occurs on a single residue, Ser-209 (Joshi et al., 1995). This phosphorylation is carried out by the kinases Mnk1 and Mnk2 which physically interact with eIF4G and are advantageously placed to carry out their function (Pyronnet et al., 1999). In its phosphorylated state, eIF4E has a greater affinity for the cap. Although it has been found that eIF4E phosphorylation has an insignificant effect on the translation rates in vitro, it has been shown that under nutrient-limiting conditions in *Drosophila*,

decrease in eIF4E phosphorylation might limit organism growth (Reiling, Doepfner, Hafen, & Stocker, 2005). Thus, eIF4E phosphorylation might not be vital under normal conditions, but may play a critical role in response to environmental stress.

A much stronger control over eIF4F complex formation is exerted by the eukaryotic initiation factor 4E binding protein (4E-BP) family. 4E-BPs are translational repressors that compete with eIF4G for an overlapping binding site on eIF4E: binding of one of these factors to eIF4E excludes binding of the other factor. Thus, a 4E-BP (in its hypophosphorylated form) binding to eIF4E disrupts the formation of the eIF4F complex and effectively inhibits cap-dependent translation initiation (Mathews et al., 2007).

Mammals possess three 4E-BPs: 4E-BP1, 4E-BP2, and 4E-BP3. 4E-BP1 is the main form in adipocytes, while 4E-BP2 is predominant in brain tissue (Tsukiyama-Kohara et al., 2001). In accordance with this distribution, 4E-BP1 knockout mice are leaner than their wild-type counterparts (Tsukiyama-Kohara et al., 2001) and 4E-BP2 knockout mice display learning and memory deficiencies (Banko et al., 2005). As for 4E-BP3, it has been described to interact with eIF4E in the nucleus and cytosol of culture cells (Kleijn, Scheper, Wilson, Tee, & Proud, 2002). It appears that it is involved in regulating eIF4E-mediated nuclear export of mRNAs (C.-C. Chen, Lee, & Chang, 2012). However, it has not been as extensively studied as the other two isoforms and its mechanism remains unknown.

The mTOR signalling pathway

Because translation is a highly demanding process in terms of energy and amino acid availability, a stringent regulatory mechanism must be set up in order to make appropriate decisions when a request for translation is received by the cell. Phosphorylation of translation

factors is the most common way to regulate their activity and this phosphorylation is carried out by intracellular signalling pathways. One such pathway – or signalling cascade – is the mTOR pathway.

The target of rapamycin (TOR) proteins are evolutionally conserved, high molecular weight kinases that play a key role in growth, proliferation, and survival. The mammalian TOR kinase (mTOR) processes signals from hormones and growth factors as well as from nutrient sensors in the cell and integrates this information in order to accordingly direct translation initiation (Hay & Sonenberg, 2004).

mTOR is found in the cell in two multiprotein complexes: mTORC1 and mTORC2, each with a distinct signalling function. mTORC1 contains the Raptor protein and is sensitive to the antibiotic, immunosuppressant, and anticancer drug rapamycin. This complex directly phosphorylates 4E-BP and S6K proteins downstream of itself and indirectly regulates the phosphorylation of other translation factors. On the other hand, mTORC2 is insensitive to rapamycin, but it regulates proteins upstream of the mTOR pathway and signals to the actin cytoskeleton.

Here is a brief overview of the mTOR signalling cascade (see Figure 2). Various growth factor and hormone receptors, upon receiving a signal from outside of the cell, trigger the activation of phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) by phosphoinositide 3-kinase (PI3K). PIP3 then activates PDK1 (phospholipid-dependent kinase 1) and protein kinase Akt. Activated Akt represses the tumour suppressor TSC2 and by doing so contributes to the increase of Rheb-GTP levels which leads to mTORC1 activation. mTOR phosphorylates downstream factors, regulating translation initiation (Mathews et al., 2007). However, although it is known that multiple factors

appear to be phosphorylated as a result of signalling upstream of mTOR, it is still not completely clear how signalling downstream of mTOR is carried out.

In reality, signalling leading to translation regulation is much more complex than described here. There appears to be extensive crosstalk between the mTOR and the Ras-MAP kinase pathways, and likely between other pathways as well. However, because of the limited scope of this work, discussion will be based on the pathway mechanism described above. Next are described two factors involved in the mTOR signalling cascade that are of importance to this work.

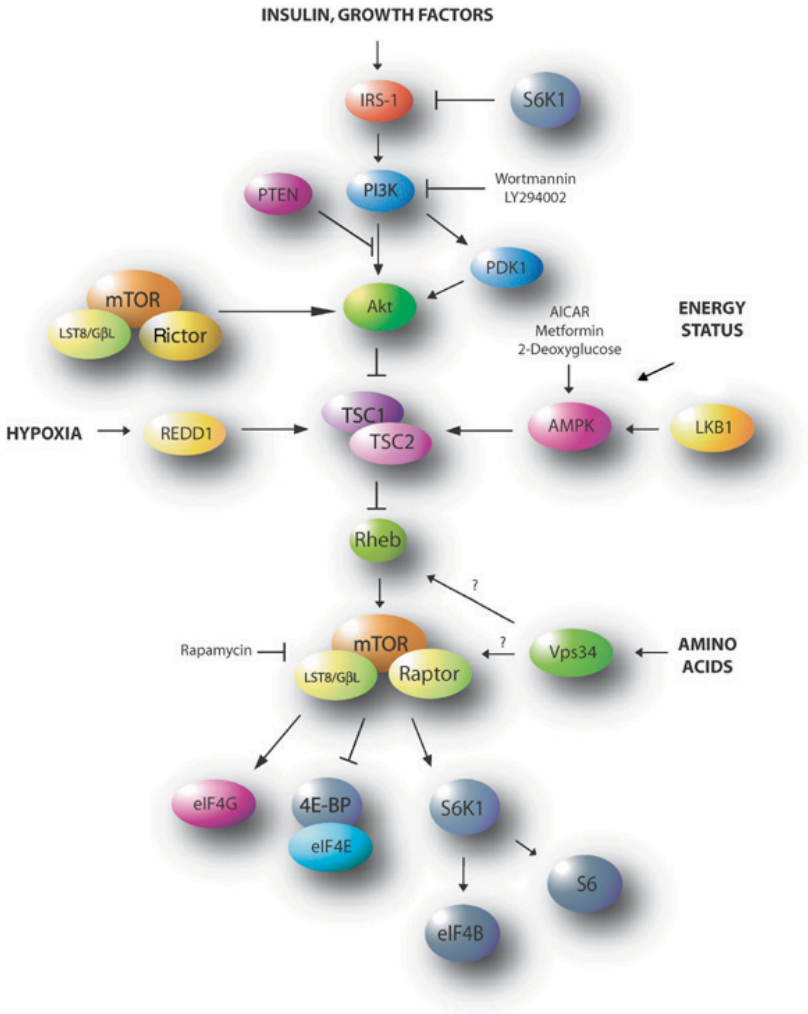


Figure 2. The mTOR signalling pathway. See text for a brief summary. Figure adapted from (Mamane, Petroulakis, LeBacquer, & Sonenberg, 2006)

PTEN

The phosphatase and tensin homolog (PTEN) is a tumour suppressor known to be mutated in a large number of cancers (Maehama & Dixon, 1999). It acts at the very top of the mTOR signalling cascade by dephosphorylating and depleting the levels of PIP3 (Bader, Kang, Zhao, & Vogt, 2005). As previously discussed, PIP3 is responsible for activating the mTOR pathway. Therefore, mutations in PTEN lead to increased levels of PIP3 and to a constitutively active mTOR signalling cascade, causing problematic increases in translation.

Although cancer is the most obvious disease related to PTEN dysregulation, because PTEN is one of the most commonly mutated tumour suppressors, it is not the only problem associated with aberrant PTEN function. Other diseases include those characterized by the development of noncancerous tumours known as hamartomas, such as the Cowden syndrome, among others. More recently, PTEN dysfunction has also been associated with autism spectrum disorders (Kelleher & Bear, 2008; Napoli et al., 2012).

TSC2

Tuberous sclerosis protein 2 (TSC2) is part of the TSC1/TSC2 tumour suppressor complex. In the mTOR pathway discussed above, TSC2 integrates signals triggered by hormones/growth factors and energy and stress levels and regulates the presence of Rheb-GTP in the cell by converting it to Rheb-GDP. Mutations in TSC2 (or in TSC1) result in a decreased control of cell growth and may lead to tuberous sclerosis, a diseases causing benign tumour growth in the brain and in other vital organs. Moreover, because of the role played by the TSCs in translational regulation in neurons, among other cells, tuberous sclerosis patients often also display intellectual disability and autism spectrum disorders (Auerbach, Osterweil, & Bear, 2011).

The role of mRNA helicases in ribosomal scanning

Scanning along the 5'UTR is not a straightforward process. The PIC often encounters extensive secondary mRNA structures or bound proteins in its path. To overcome these obstacles, energy in the form of ATP is used by helicases: enzymes that "unwind" – directly or indirectly – the structured 5'UTRs of mRNAs. This elaborate mechanism is conserved in eukaryotes from yeast to mammals (Altmann & Linder, 2010). The best-characterized mRNA helicase to date is the eukaryotic initiation factor 4A (eIF4A), also known as DDX2, believed to play a key role in unwinding highly structured 5'UTRs. However, it has been proposed that other mRNA helicases may have non-redundant and often complementary roles in translation initiation (Merrick, 2010). These include, but are not limited to, Ded1/DDX3, DHX29, RHA, and VAS (Parsyan et al., 2011). Below are outlined the roles of some mRNA helicases of interest to this work.

eIF4A

The canonical translational helicase eIF4A is an abundant DEAD-box protein whose sequence analysis gave birth to the DEAD-box protein family (Linder et al., 1989). Part of the cap-binding eIF4F complex, eIF4A exhibits RNA-dependent ATPase activity and has long been believed to be the most active helicase responsible for unwinding mRNA 5'UTR secondary structures during translation initiation (Linder, 2006). The helicase activity of yeast and mammal eIF4A is weak and is often enhanced by eIF4G and eIF4B (or eIF4H) (Marintchev et al., 2009; Rogers, Richter, Lima, & Merrick, 2001; Schütz et al., 2008). Moreover, as previously mentioned, 43S complexes possess the intrinsic ability to bind to unstructured 5'UTRs and scan the mRNA to the initiation codon (Pestova & Kolupaeva, 2002). This leads to suggest that the requirement of eIF4A helicase activity is dependent on the degree of secondary structure in the 5'UTR (Svitkin et al.,

2001). It has been suggested and argued that eIF4A acts by "pulling" on the mRNA strand to pass it through the mRNA-binding cleft of 40S instead of unwinding the strand at the leading edge of the small subunit. Such a mechanism would possibly require an additional RNA helicase to unwind the mRNA at the leading edge, a task probably partly accomplished by the intrinsic RNA-unwinding ability of the ribosome.

Ded1 and DDX3

The DEAD-box RNA helicase Ded1 in *Saccharomyces cerevisiae* is required for translation initiation and is essential for viability (Iost, Dreyfus, & Linder, 1999). It has been suggested several times that Ded1 is a more potent helicase than eIF4A when it comes to scanning long 5'UTRs (Berthelot, Muldoon, Rajkowitsch, Hughes, & McCarthy, 2004; Chuang, Weaver, Liu, & Chang, 1997; Marsden, Nardelli, Linder, & McCarthy, 2006). Interestingly, transcription intermediary factor 1 (Tif1) and Tif2 (yeast homologues of mammalian eIF4A) and Ded1 are non-redundant and the ability to scan long 5'UTRs seems to rely more on Ded1 than on eIF4A activity (Berthelot et al., 2004). Moreover, the DED1 gene in yeast can be functionally replaced by its mouse homologue, PL10, suggesting an evolutionary conservation of Ded1 function in translation (Chuang et al., 1997). Another mammalian homologue of Ded1 is DDX3. There are contradictory data concerning the function of DDX3 in translation. The combined evidence of studies in yeast and mammals seems to favour the role of DDX3 in translation of mRNAs with long or structured 5'UTRs, but further characterization is required for a better understanding of its function (Parsyan et al., 2011).

DHX29

Unlike eIF4A and Ded1/DDX3, the recently characterised helicase DHX29 is a DEAH-box protein (Pisareva, Pisarev, Komar, Hellen, & Pestova, 2008). *In vitro* (Pisareva et al., 2008) and *in vivo* (Parsyan et al., 2009) studies suggest that, similarly to Ded1, DHX29 is required to promote translation of mRNAs with structured 5'UTRs. However, it has been demonstrated that Ded1 and DHX29 have different mechanisms of action (Abaeva, Marintchev, Pisareva, Hellen, & Pestova, 2011), leading to the question of whether yeast has a DHX29 orthologue. Although BLAST searches identified YLR419w as the protein most closely related to DHX29 in *S. cerevisiae*, reverse searches suggested that YLR419w is closer to mammalian DHX57 and DHX36 than to DHX29 (Abaeva et al., 2011). Moreover, YLR419 is a nonessential gene and its protein product has no known binding partners related to the translation machinery (Stevenson & McCarthy, 2008). Thus, YLR419 does not seem to be a functional DHX29 orthologue. There is evidence to believe that DHX29 acts by remodelling the 40S subunit of the ribosome (Pisareva et al., 2008) rather than by "unwinding" the mRNA strand, although the canonical helicase function cannot be completely excluded (Abaeva et al., 2011). The current hypothesis is that DHX29 causes conformational changes in the 40S subunit through its NTPase activity (Parsyan et al., 2011). It has also been suggested that DHX29 has the ability to monitor mRNA entry into the 43S complex by opening and closing the 40S subunit (Abaeva et al., 2011). Determining the structure of the 43S complex associated with NTP- and NDP-bound forms of DHX29 will further elucidate the mechanism of its action.

Translational control of synaptic plasticity in learning and memory

The nervous system is a highly sophisticated and complex mechanism that perceives outside signals, integrates and analyses the information, and coordinates the body's response to its environment. Even a minor flaw in the execution of these tasks has tremendous repercussions on the body's ability to function adequately. It is not surprising that defects of the nervous system, whether inherent or acquired, lead to debilitating neurodegenerative diseases and severely affect an individual's quality of life.

Most multicellular animals possess a nervous system, although varying in complexity. In most animals it consists of two components: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS contains the brain and the spinal cord, while the PNS consists of nerves – long fibres branching off the CNS and connecting it to all other parts of the body. The most basic unit and building block carrying out the function of the nervous system is the neuron, also known as the "nerve cell". This essential cell will be described in greater detail below. Aside from neurons, the nervous system also includes glial cells – or glia – which do not directly participate in signal transmission, but provide support and nutrition, maintain homeostasis, produce myelin (an insulating material crucial for neuronal function), and aid signal transmission between neurons. Glia are essential for proper function of the nervous system and virtually every aspect of nervous system function involves a partnership between neurons and glia (Barres, 2008). It is suspected that glial malfunction is an important factor in multiple neurodegenerative diseases (Miller, 2005).

The neuron and the synapse: an overview

The nervous system is defined by the existence of the neuron. The most fundamental property of neurons is their ability to communicate with each other and with specific target cells by sending "point-to-point" signals. Unlike the relatively slow signals that certain cells send through the blood stream (hormones, growth factors, etc.), neuronal signals achieve high levels of rapidity and specificity.

A typical neuron is a polarized cell. The neuronal body – or the soma – is the central part of the cell, containing the nucleus. Leading to the soma in terms of the chronology of signal transmission is the dendritic tree – a mass of cellular extensions each sporting many branches which receive the majority of input signals from other neurons. At the other pole of the cell is the axon – a cable-like projection often extending several times (or hundreds of times) the diameter of the soma in length. Signals propagate along the axon in the form of electrochemical waves termed "action potentials". The axon has several processes along its length and also branches out at its terminal to form connections with the dendrites or soma of the next neuron or with other target cells.

The point at which the axon of one neuron connects with the dendrite of another neuron in order to propagate the information is known as the synapse. Synapses can be of two kinds: fast-responding electrical synapses simply propagate the electric signal down to the next neuron, while chemical synapses – more common and more functionally diverse – use neurotransmitters to transmit information. Synapses can be either excitatory or inhibitory, meaning that they either increase or decrease the activity of the next neuron in line.

In a chemical synapse, the cell that sends the signal is called presynaptic, while the cell that receives the signal is called postsynaptic. The presynaptic area at the very tip of the axon

contains neurotransmitter-filled vesicles that release their content into the space between the presynaptic and postsynaptic cells (the synaptic cleft) when triggered by the oncoming action potential. The neurotransmitter molecules bind to the receptors located on the postsynaptic membrane, eliciting a corresponding response (excitatory, inhibitory, or otherwise modulatory) in the target cell. There exist hundreds of different synapses and neurotransmitter, not to mention a vast array of receptors. The signal transduction possibilities are thus highly specific and diverse.

Synaptic plasticity and protein synthesis

Synaptic plasticity is defined as a series of changes in the strength of synaptic connections as a result of their use or disuse. Long-term potentiation (LTP) and long-term depression (LTD) occurring at glutamate excitatory synapses and responsible for learning-based synapse reinforcement or weakening, respectively, are the most extensively studied mechanisms of synaptic plasticity (Malenka & Bear, 2004). Studies of the rodent hippocampus – a brain structure critical for processing information about space, time, and relationship between objects – have provided the bulk of information about these processes.

It has been shown almost 30 years ago that LTP required protein synthesis in vivo (Krug, Lössner, & Ott, 1984). Subsequent studies differentiated two phases of LTP – E-LTP (early) and L-LTP (late) – and showed that L-LTP is sensitive to both transcription and translation inhibitors, while E-LTP is not sensitive to either, meaning that out of the two only L-LTP requires new gene expression (Huang & Kandel, 1994; Nguyen, Abel, & Kandel, 1994). Moreover, L-LTP itself consists of an early translation-dependent and transcription-independent phase, indicating a need for immediate protein synthesis. Several transmitter receptors have been

shown to couple to translation regulation, for example β-adrenergic receptors promoting eIF4E activation (Gelinas et al., 2007) It is now known that LTD also requires protein synthesis (Huber, Kayser, & Bear, 2000).

The strength of the synapse is affected through a variety of mechanisms, mostly based in the post-synaptic area. It can be regulated by the number of ion channels or neurotransmitter receptors in the post-synaptic membrane or by the amount of connections between the cells and is highly dependent on protein synthesis in the so-called post-synaptic density (PSD): a cluster of receptors, scaffolding proteins, and signalling molecules, anchored in the post-synaptic membrane and working together to concentrate and position the neurotransmitter receptors in the synaptic cleft. This molecular lattice appears to form the basis for neural adaptations (Holtmaat & Svoboda, 2009). Moreover, dysfunctional PSD components have been reported to be responsible for several neuropsychiatric diseases, particularly for those displaying cognitive deficits, such as schizophrenia and Alzheimer's disease (Iasevoli, Tomasetti, & Bartolomeis, 2013). Thus, for proper synapse function there must be a complete and functional set of PSD components the presence of which is of course regulated through local translation.

A single neuron may form as many as 10^4 synapses, each of which can be regulated independently to achieve synaptic plasticity. This means that precise mechanisms are required to bring newly synthesised proteins to specific synapses. Although a large amount of protein synthesis critical for neuronal function occurs at the soma, to achieve a tighter spacial and temporal control of signal-induced gene expression it is much more advantageous for the cell to have a local translation system in place. It has been known for a while now that dendrites and dendritic spines contain polyribosomes, translation factors, and mRNAs, creating the necessary environment for protein synthesis (Iacoangeli & Tiedge, 2013).

The importance of 4E-BP phosphorylation for eIF4F complex formation and for translation initiation has been previously discussed. Not surprisingly, it has been found that enhanced 4E-BP phosphorylation is present in both LTP and mGluR-LTD (Banko et al., 2005; Kelleher, Govindarajan, Jung, Kang, & Tonegawa, 2004). 4E-BP phosphorylation is known to be regulated ERK, PI3K, and mTOR pathways and it is known that mTOR directly phosphorylates 4E-BP. Recent studies have demonstrated that mTOR is required for several protein synthesis-dependent forms of synaptic plasticity, making mTOR-dependent 4E-BP phosphorylation an important part of learning and memory (Mathews et al., 2007). Moreover, it has been demonstrated that mice lacking 4E-BP2 – the predominant 4E-BP form in the mouse hippocampus (Banko et al., 2005) – display various abnormalities when subjected to memory and behaviour tests (Banko et al., 2007), making 4E-BP2 an interesting subject of future research.

Autism spectrum disorders and translational control

Autism spectrum disorders (ASDs) encompass neurodevelopmental conditions characterized by specific behaviours: abnormal social interactions, deficits in communication, and repetitive or restricted interests or behaviours (Lord, Cook, Leventhal, & Amaral, 2000). On a larger scale, ASDs are part of the pervasive developmental disorders (PDD) category, but only three of the disorders listed in this group are considered to be ASDs. These are autistic disorder, Asperger syndrome, and pervasive developmental disorder not otherwise specified (PDD-NOS) (Johnson, Myers, & Disabilities, 2007; Tchaconas & Adesman, 2013). ASDs are one of the most prevalent

neurodevelopmental disorders in children today. In 2008, 1 in 88 children was affected with an ASD, the prevalence in males being four times higher than in females (Tchaconas & Adesman, 2013). Because of increased media coverage and rapidly expanding scientific knowledge of autistic disorders, both the physicians and the general public have gained awareness of ASDs in the past two decades (Johnson et al., 2007).

Autistic traits usually become apparent in infancy and are confirmed in the first three years of a child's life. The difficulty in diagnosis resides in the fact that autism is a heterogeneous condition, which is to say that no two children or adults present the same characteristics. However, the universal autistic traits are easily recognised and are usually consistent from one individual to another, despite the developmental differences and specific behaviours (Lord et al., 2000).

ASDs are complex heritable disorders, involving multiple genes and displaying great phenotypic variation (Johnson et al., 2007; Persico & Bourgeron, 2006). Many candidate genes have been proposed, including those playing a role in brain development and neurotransmission, as well as genes involved in neurogenetic syndromes that are associated with ASDs, such as genes causing the fragile X syndrome or tuberous sclerosis (Johnson et al., 2007). An extra layer of complexity is added when one considers the environmental effects believed to modulate phenotypic expression. The results of the California Autism Twin Study – the largest ever ASD twin study – published in 2011 indicate that ASDs are 55% attributable to environmental factors shared by twins, a percentage much higher than those predicted by earlier studies (Hallmayer J & et al., 2011). The environmental effects potentially leading to autism are now gaining increasing attention among researchers. Factors of interest include parental age, maternal infections during pregnancy, multiple births, and low birth weight (Tchaconas & Adesman, 2013).

Educational therapies focusing on behaviour, communication, and social responsiveness form the core of ASD treatment. There exist various approaches, often making it difficult for physicians to select an optimal therapy for a patient (Tchaconas & Adesman, 2013). Although there are no medications available to treat the core symptoms of ASDs, drugs are often used to target associated symptoms such as hyperactivity, impulsiveness, inattention, aggression, irritability, anxiety, and withdrawal (Tchaconas & Adesman, 2013). Advances in biochemical research have led to the discovery of potential genetic targets and to the development of target-specific treatments that may lead to future therapies, but additional studies are necessary to determine whether these medications are appropriate for use in patients.

Autism spectrum disorders and translation

As previously mentioned, autism is one of the most heritable neurodevelopmental disorders with a highly complex genetic basis. However, several single-gene disorders are also associated with ASDs, indicating an increased risk of autism conferred by the mutation. Conversely, a significant percentage of patients affected with ASDs present mutations in one of these genes. Such disorders collectively account for only 10-15% of autism cases, but their molecular bases point to common pathogenic pathways shared by ASDs (Kelleher & Bear, 2008).

The identification of mutations in neuroligins as the underlying genetic cause of certain autism cases has provided crucial information about the defects in synaptic function closely linked to ASD pathogenesis (Persico & Bourgeron, 2006; Zoghbi, 2003). Mouse models of mutations that cause ASDs in humans point to disrupted synaptic function: excessive or diminished excitatory synaptic connectivity (Chao, Zoghbi, & Rosenmund, 2007; Hanson & Madison, 2007) and alterations in the excitation/inhibition (E/I) ratio (Dani et al., 2005; Tabuchi et al., 2007). It

appears that these changes are caused by a dysregulation of synaptic protein synthesis. Hyperconnectivity of neuronal circuits due to increased synaptic protein synthesis is believed to be a cause of ASDs (Gkogkas et al., 2013; Levinson & El-Husseini, 2005). The gene products mutated in single-gene autism-associated disorders act as negative regulators of protein synthesis, leading to altered synaptic plasticity and autistic phenotypes. The most studied of these gene products are the previously described FMRP, TSC1/2, and PTEN (Kelleher & Bear, 2008).

The fragile X mental retardation protein (FMRP) binds to over 400 distinct mRNAs to repress their translation. Loss of FMRP expression causes translational derepression in these target mRNAs, an effect observed in the *Fmr1* knockout mouse where the modelled loss of FMRP function results in a cerebral protein synthesis increase of about 20% (Qin, Kang, Burlin, Jiang, & Smith, 2005). Similarly, inactivation of TSC1/2 and loss of PTEN function in neurons upregulates mTORC1 activity, resulting in enhanced neuronal translation. *Pten* knockout mice exhibit deficits in cognition and social interaction (Kwon et al., 2006). Mice lacking *Tsc1* as well as $Tsc2^{+/-}$ mice display synaptic plasticity and memory deficits and present autism-like phenotypes (Auerbach et al., 2011; Ehninger et al., 2008; Tsai et al., 2012). All of these deficits are rescued by rapamycin. The association of mutations in FMRP, PTEN, and TSC1/2 with ASDs suggests that translational control is an important mechanism leading to autistic phenotypes.

Results

Chapter 1: The role of helicases in the control of synaptic local translation

As has been previously mentioned, translational control at the synapses plays an important role in regulating synaptic plasticity. However, the role of mRNA helicases in the control of synaptic plasticity, learning and memory has not been previously studied. The following experiments and results are the beginning of an attempt at understanding the mechanisms by which helicases are involved in translational control at the synapse. Two mRNA helicases – eIF4A1 and DHX29 – were studied for their ability to distinguish between mRNAs with structured and unstructured 5'UTRs. Biochemical results showed that both helicases regulate the same transcripts, but behavioural analyses in mice indicated that these helicases might be recruited in different situations and at different times (unpublished data). An assay for monitoring the rate of synaptic translation was proposed and preliminary tests were carried out. The data presented lays the groundwork for future investigation.

DHX29 is predominantly localized post-synaptically in neurons

To determine the distribution and localization of DHX29 in neurons, we isolated synaptosomes from neurons and verified for their protein content by immunoblotting. Some of the proteins probed for along with DHX29 were GFAP (glial fibrillary acidic protein, an intermediate filament protein), Synapsin I (a pre-synaptically found protein associated with synaptic vesicles), PSD95 (a post-synaptic density protein), αCaMKII (a protein known to localize at synapses and

to play a major role in long-term potentiation), Arc/Arg3.1 (synaptic plasticity marker), and Pol II (DNA polymerase II, necessary for DNA repair). As expected, Synapsin I, PSD95, αCaMKII, and Arc protein levels were enriched in synaptosomal fractions, while GFAP (mostly associated with mitosis and predominantly expressed in astrocytes) was less present at synapses. Pol II, usually confined to the nucleus, was entirely absent from the synaptosomal fractions. DHX29 was also found to localize at the synapses (Figure 3a). Neurons stained for endogenous DHX29 also showed the protein clustering at synaptic puncta along the dendrites (Figure 3b).

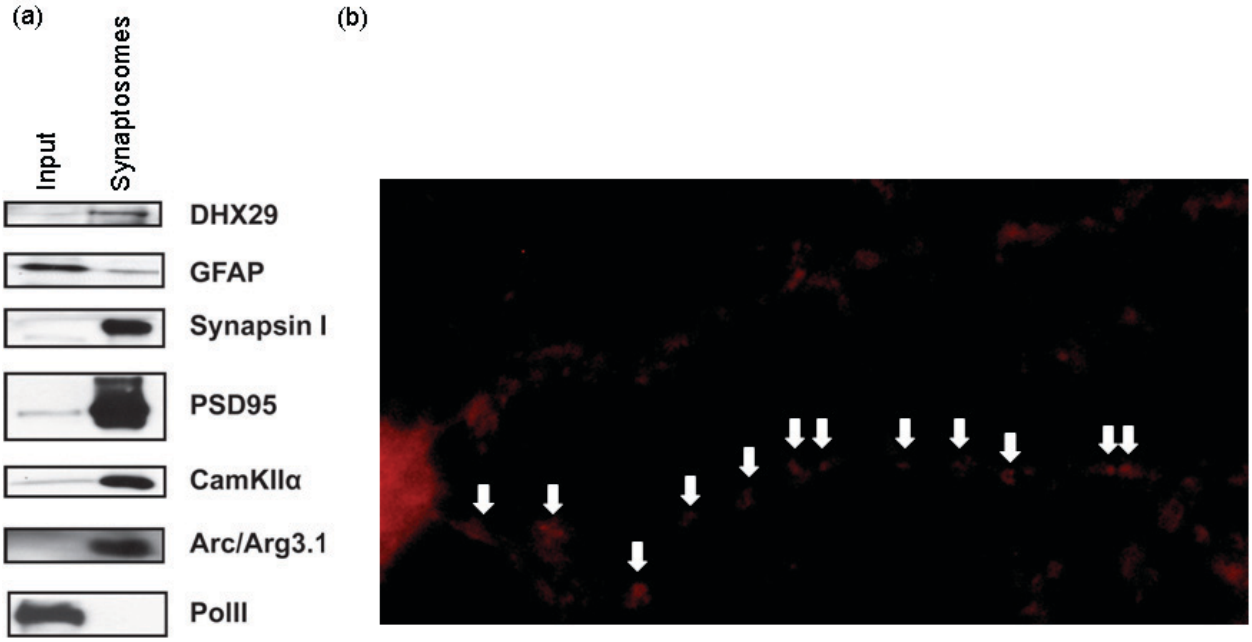


Figure 3. (a) Proteins enriched in synaptosomal fractions detected by immunoblotting. **(b)** Neuron stained with immunofluorescence for endogenous DHX29, labelling the puncta at which the helicase is concentrated.

To further determine whether DHX29 localized pre- or post-synaptically, neurons expressing a DHX29-GFP fusion protein were probed with fluorescently tagged Synapsin I and PSD95 antibodies (Figure 4). A merged view of the stained images showed a better co-localization of

DHX29 with PSD95 than with Synapsin I, indicating that DHX29 is more readily localized post-synaptically.

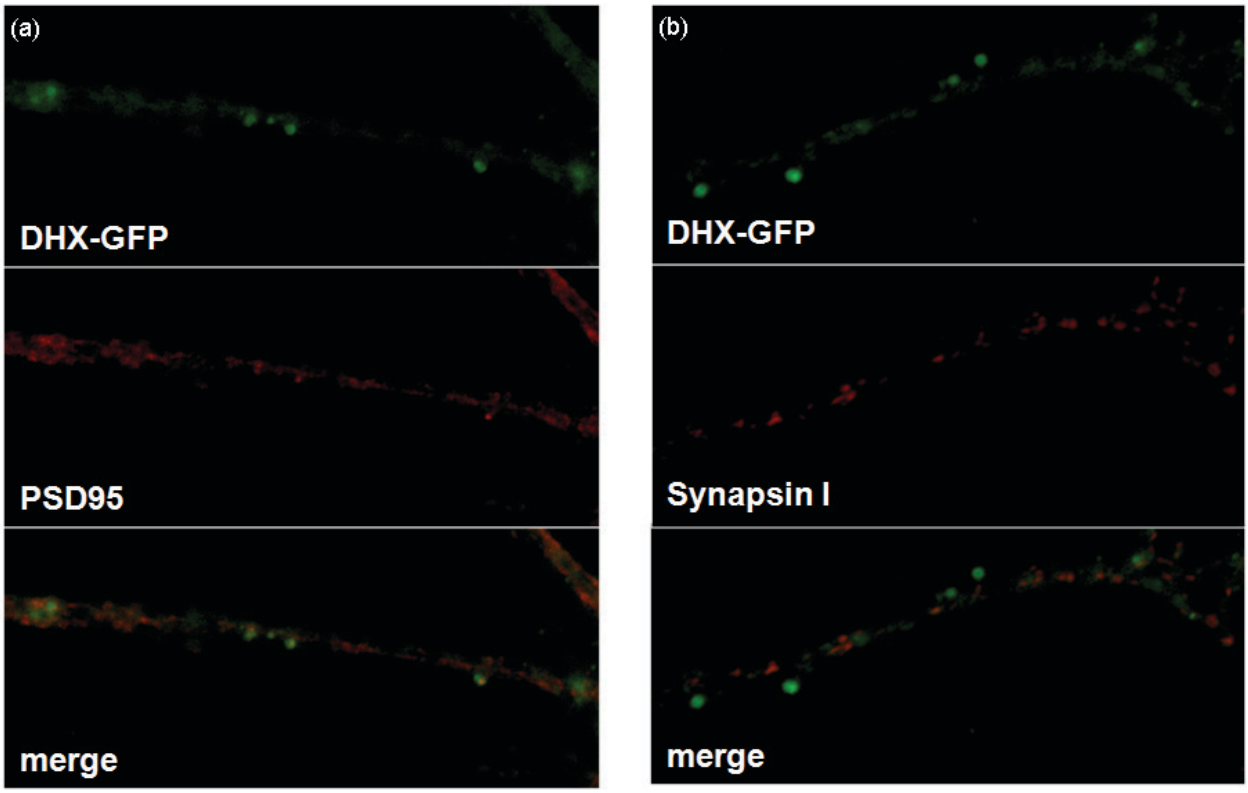


Figure 4. Immunofluorescent view of DHX29-GFP co-localization with PSD95 **(a)** and with Synapsin I **(b)** in neurons.

Polysomal profiles of *DHX29* and *eIF4A1* knockdowns in neurons

To assess the global role played by the helicases eIF4A1 and DHX29 in translation, we performed knockdowns using shRNAs against *DHX29*, *eIF4A1*, and scrambled mRNAs in cortical neurons. The resulting polysome profiles showed that only knockdown of *eIF4A1* affected the overall polysome trace as is demonstrated by a shift to lighter polysomes and by the

increase in the 80S peak, while knockdown of *DHX29* did not affect the polysome profile (Figure 5a). Knockdown efficiency was assessed by immunoblotting (Figure 5b).

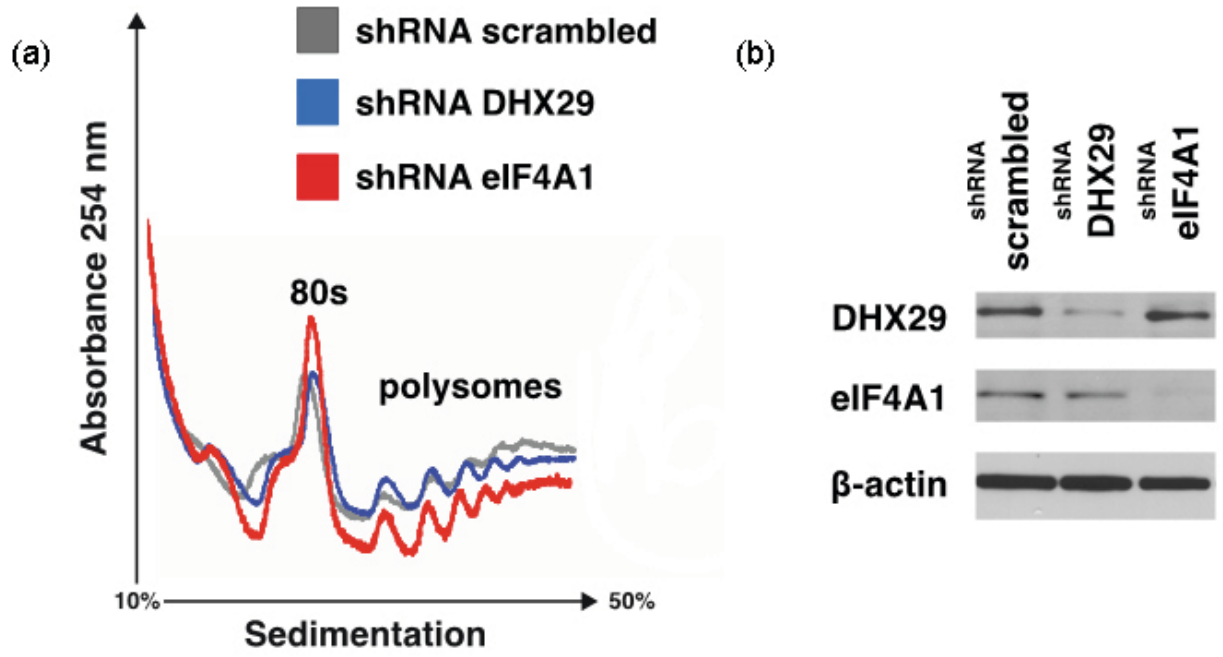
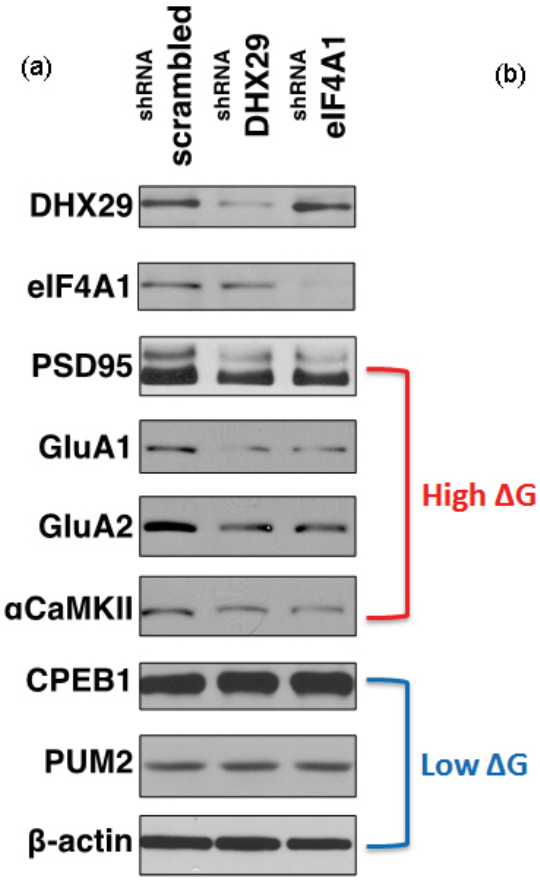


Figure 5. (a) Polysome profile of cortical neurons infected with lentiviruses expressing shRNA against scrambled, DHX29, and eIF4A1 mRNA. **(b)** Assessment of the knockdown by immunoblotting. The shRNAs are specific for the genes targeted and contribute to the decrease in abundance of the relevant proteins.

mRNA translation based on 5'UTR complexity

We divided mRNA transcripts that have previously been found to be present at synapses (Kye et al., 2007) as well as other transcripts of interest to us into two categories based on the complexity of the secondary structure of their 5'UTRs (Figure 6b). Since helicase activity is in large part based on the complexity of the 5'UTR of a transcript, we predicted that knockdown of helicases would predominantly affect the translation of messages with a highly structured 5'UTR. Indeed,

as visualized by immunoblotting, in *DHX29* and *eIF4A1* knockdowns, levels of protein coded by mRNAs with highly structured 5'UTRs are decreased in comparison to wild-type, while the knockdown of helicases does not have the same effect on proteins encoded by mRNAs with low complexity 5'UTRs (Figure 6a).



(b)

Gene (protein)	ΔG (kcal/mol)
Mtap2 (MAP2)	-125.7
Gria1 (GLUA1)	-106.0
Gria2 (GLUA2)	-123.3
Dlg4 (PSD95)	-131.80
Grin1 (NR1)	-121.1
Actb (β-actin)	-16.9
Cpeb (CPEB)	-6.4
Pum2 (PUM2)	-11.9

Figure 6. (a) Effect of the knockdowns of *DHX29* and *eIF4A1* on levels of protein coded for by mRNAs with structured (high ΔG) and unstructured (low ΔG) 5'UTRs. (**b**) Gibbs free energy (ΔG) values of selected mRNAs with high- and low-complexity 5'UTRs. Larger negative values (red) indicate a highly structured 5'UTR.

To visualize the difference in translational control of these transcripts, we collected polysomal fractions from *DHX29* and *eIF4A1* knockdown neurons as well as from wild-type (scrambled

shRNA) neurons. The RNA from these fractions was extracted, reverse transcribed, and analyzed by qRT-PCR using specific primers for genes of interest (Figure 8). From these results, we also observed that upon *DHX29* and *eIF4A1* knockdown, there is a shift to lighter polysomes in the translation of mRNAs with highly structured 5'UTRs, while no such shift occurs in the case of mRNAs with lighter 5'UTRs (with the exception of actin, to be discussed).

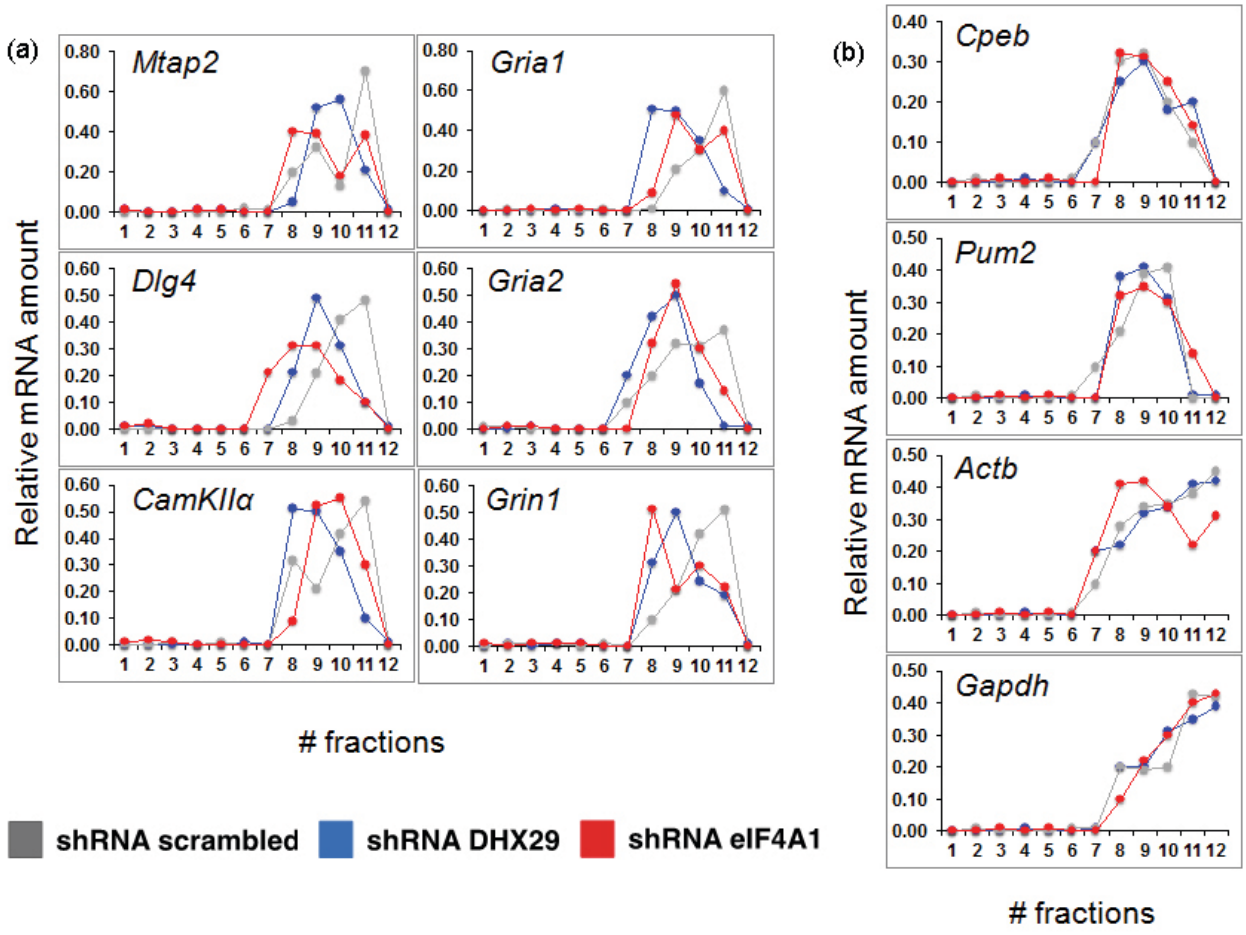


Figure 7. qRT-PCRs preformed on RNA fractions extracted from *DHX29*, *eIF4A1*, and scrambled KD polysomes isolated from dissociated cortical neuronal cultures. Results shown for mRNAs with structured (**a**) and unstructured (**b**) 5'UTRs.

Translation rate can be monitored with the use of a photonvertible reporter

To study the effects of helicases on local synaptic translation, it is important to monitor the rate of translation at synapses. One of the assays widely used is photoconvertible translation reporter, Kaede (Ando, Hama, Yamamoto-Hino, Mizuno, & Miyawaki, 2002; Banerjee, Neveu, & Kosik, 2009; Dittrich, Schäfer, & Schwille, 2005). Kaede is a green fluorescent protein which upon synthesis fluoresces green, much like GFP. Its green chromophore can be photoconverted by UV light (λ = 350-410) to the red chromophore form (Figure 8). This conversion is irreversible. One can photoconvert all of the tagged protein in a certain area to fluoresce red and then simply track the rate of appearance of new green fluorescence in stimulated neurons, be they wild-type or knockouts of genes of interest.

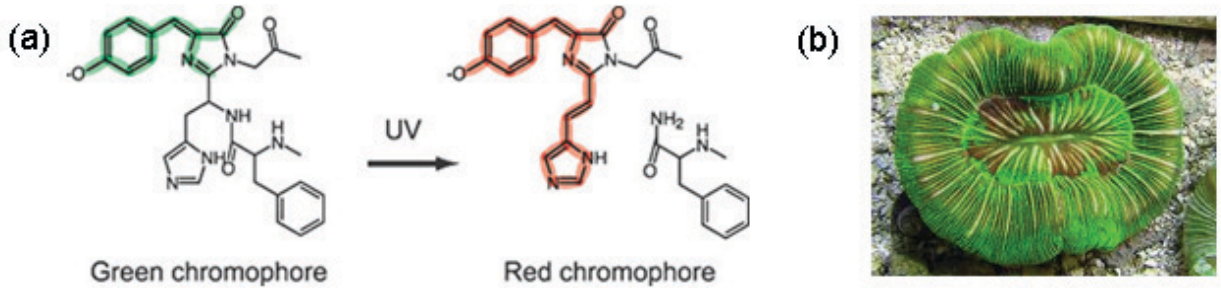


Figure 8. (a) The irreversible photoconversion of the chromophore molecule (image taken from http://www.brain.riken.jp/bsi-news/bsinews25/no25/research1e.html). **(b)** *Trachyphyllia geoffroyi*, the stony coral from which the Kaede protein is extracted (image taken from http://www.vissenforum.nl/infohoek/Artikel/1206).

Since helicase activity depends on the 5'UTR structure of a transcript, we decided to create constructs containing, in tandem, the 5'UTR of an mRNA transcript of interest, the Kaede reporter, and the 3'UTR of αCaMKII (for post-synaptic localization). These constructs were then packaged into lentiviruses which were used to infect neuronal cultures (Figure 9).

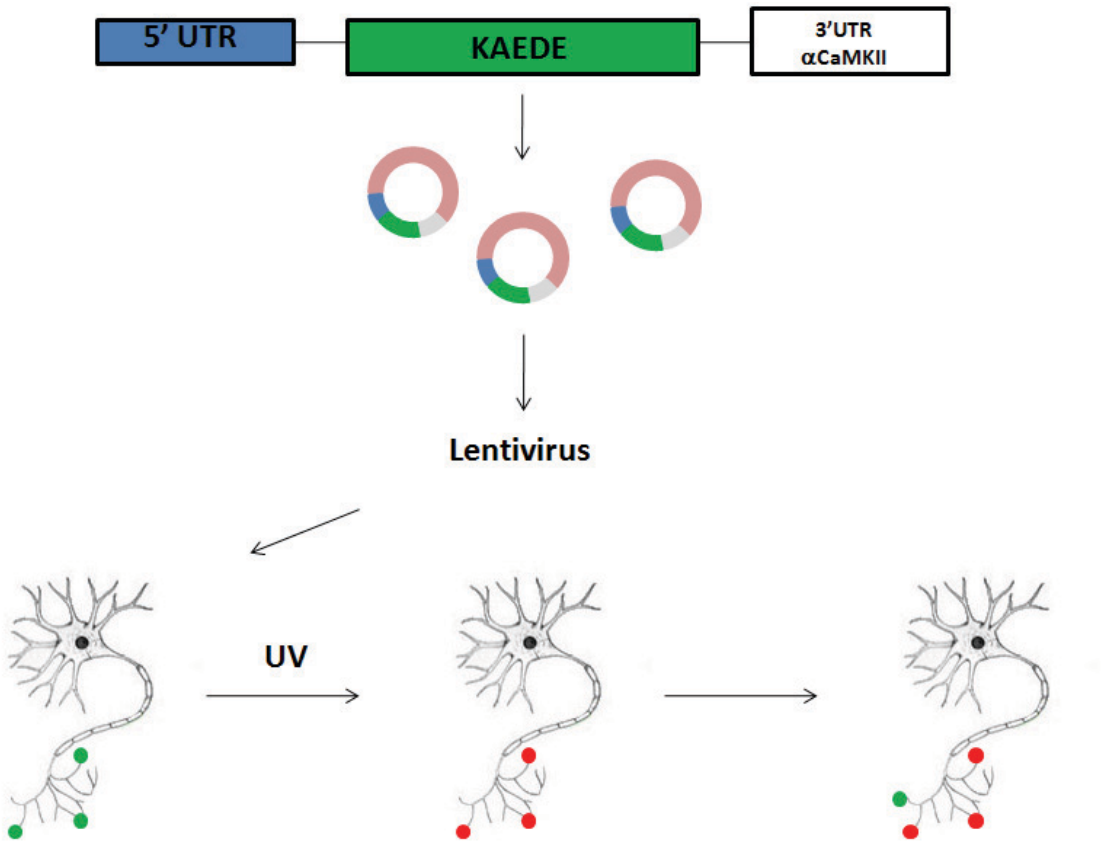


Figure 9. Proposed scheme for monitoring the translation rate of mRNAs with structured and unstructured 5'UTRs at the synapses.

Seven constructs were prepared containing the 5'UTRs of mouse *Gria1*, *Dlg4*, *Grin1*, *Pum2*, *Cpeb1*, *αCaMKII*, and *Actb*, as well as a construct containing no insert. One of the constructs (with the *αCaMKII* 5'UTR) was used to assess the efficacy of photoconversion. As a test in HEK293T cells, all infected cells were first visible in the green channel, but not in the red channel. After a ten-second UV photoconversion period, green fluorescence was significantly reduced, but red fluorescence appeared (Figure 10). New proteins synthesized should be apparent in the green channel.

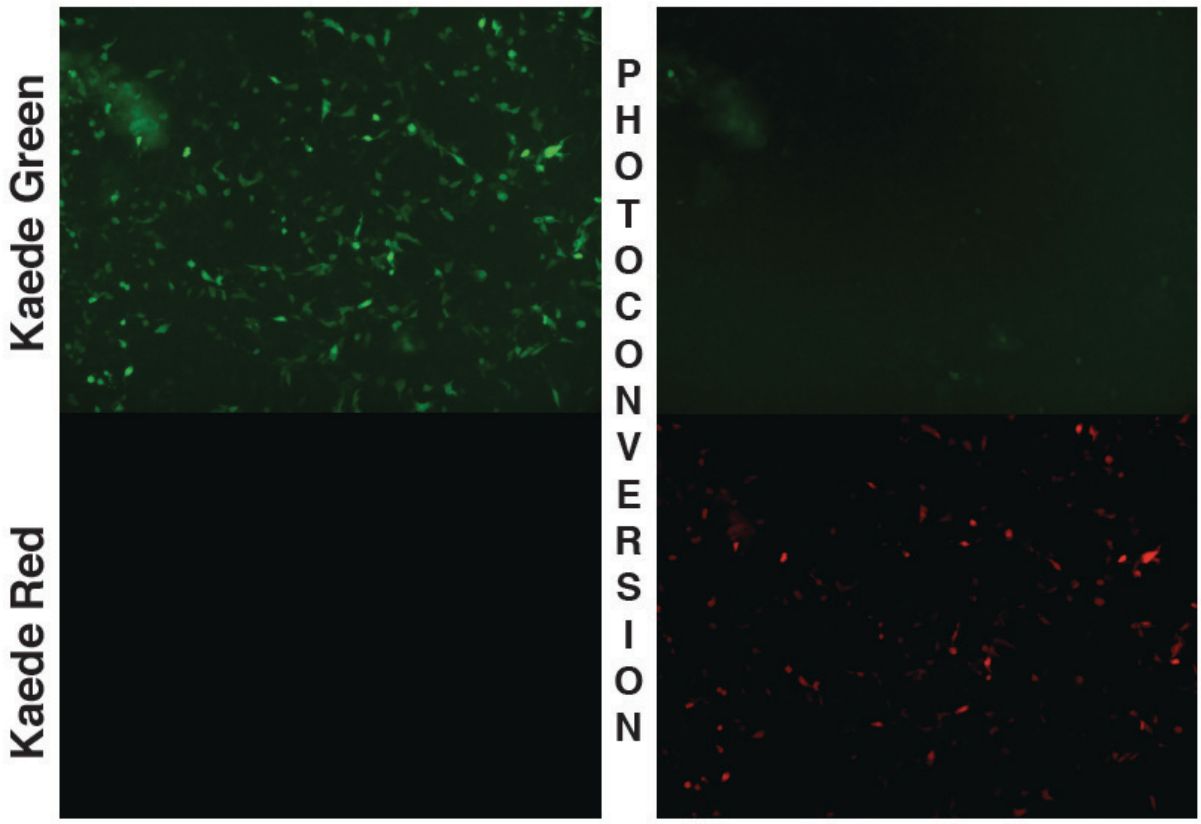


Figure 10. Representative example of photoconversion: the αCaMKII 5'UTR-Kaede- αCaMKII 3'UTR construct undergoes irreversible photoconversion from green to red when irradiated with UV light for ten seconds. Both forms were observed at the same intensity and contrast settings for both channels.

Chapter 2: Autism-related deficits via dysregulated eIF4E-dependent translational control

It has been previously discussed that increased synaptic protein synthesis leads to hyperconnectivity of neuronal circuits, believed to cause ASDs and that mTOR plays an important role in ASD development through upstream signalling. However, regulatory mechanisms downstream of mTOR are still unclear. This work shows that knockout of the eukaryotic translation initiation factor 4E-binding protein 2 (*Eif4ebp2*) – a gene coding for 4E-BP2, the eIF4E repressor downstream of mTOR – or eIF4E overexpression leads to an increased translation of neuroligins (postsynaptic adhesion protein previously linked to ASDs). *Eif4ebp2* knockout mice were shown to exhibit autistic behaviours (abnormal social interactions, deficits in communication, and repetitive or restricted interests or behaviours) as well as an increased excitation to inhibition (E/I) ratio. Pharmacological inhibition of eIF4E activity and normalization of NLGN1 (but not NLGN2) protein levels were found effective in rescuing ASD-like phenotypes and in restoring the normal E/I ratio (Gkogkas et al., 2013).

Neuroligins and eIF4E-dependent translation

We hypothesized that ASD-like phenotypes in *Eif4ebp2* knockout mice arise as a result of altered translation of a subset of mRNAs, the initiation of which is controlled by eIF4E activity. To test this hypothesis, we assessed the translation initiation rates of hippocampal lysates from *Eif4ebp2* knockout, eIF4E-overexpressing (βT-*Eif4e*), and wild-type mice by polysome profiling. In both cases, the polysome profiles in *Eif4ebp2* knockout and βT-*Eif4e* mice were not significantly altered as compared to wild-type littermates (Figure 11). These results were in

agreement with previous reports as well as with the [^{35}S]Met/Cys incorporation test performed on wild-type and *Eif4ebp2* knockout mice (Figure 11).

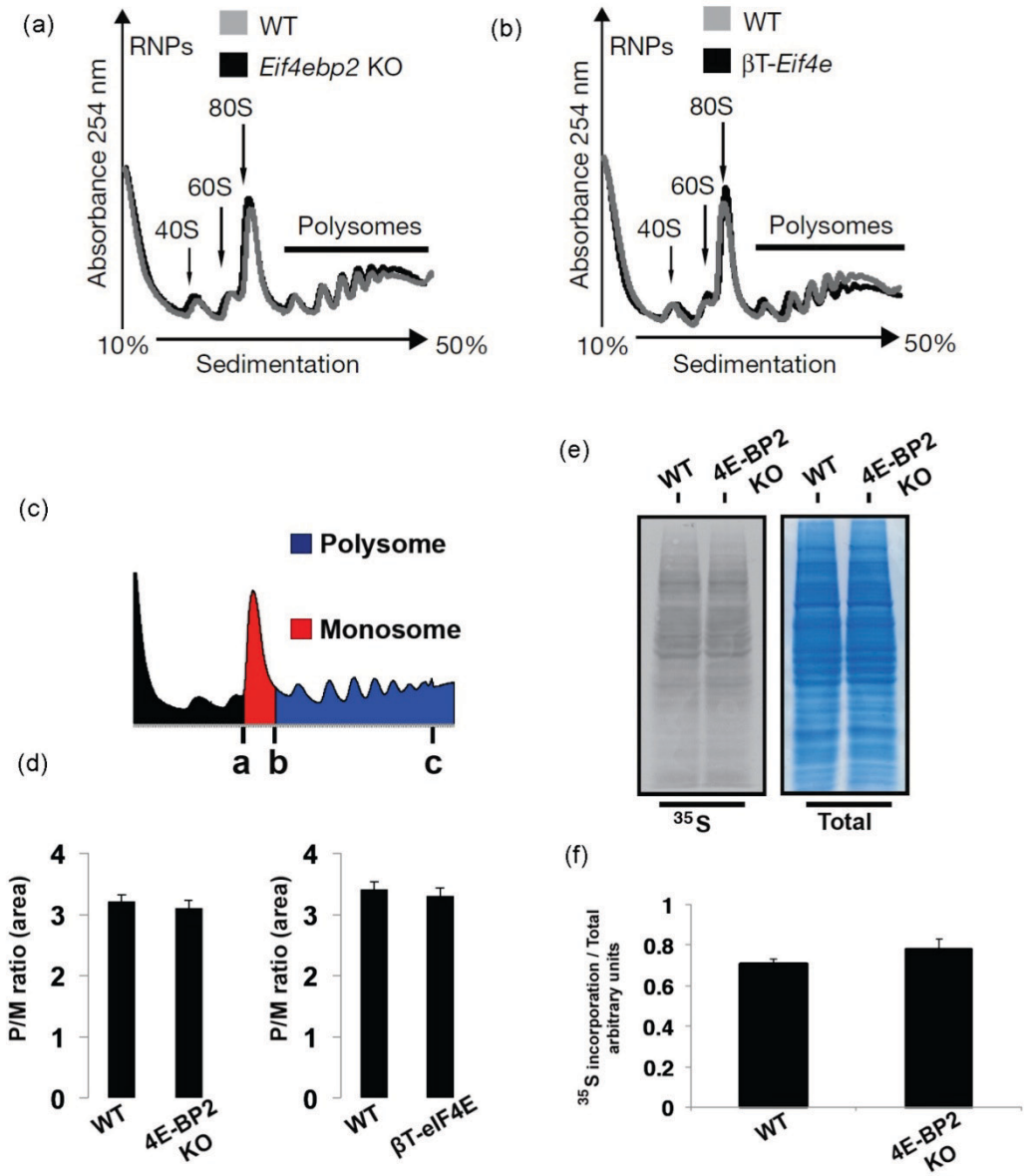


Figure 11. Polysomal profiles from hippocampal lysates of wild-type and *Eif4ebp2* knockout mice (**a**) and of wild-type and βT-*Eif4e* mice (**b**). Positions of 40S, 60S, and 80S ribosome peaks and polysomes are indicated. (**c**) Graphic depiction of the calculation of the polysome to monosome ratio (P/M) using the definite integral for the A_{254} absorbance

function. (**d**) Quantification of P/M ratio from polysome profiles generated from hippocampal *Eif4ebp2* KO, βT-*eIF4E* or WT lysates. No changes in the P/M ratio are observed between the examined genotypes (p=0.092, p=0.112 respectively); (n=4), Student's *t*-test. (**e**) Representative images of SDS-PAGE gel transferred to nitrocellulose measuring ^{35}S-Methionine incorporation from acute hippocampal slices (left) and GelBlue-stained SDS-PAGE gel of total protein loaded on gel (right) from *Eif4ebp2* KO or WT littermate mice. (**f**) Quantification of ^{35}S-Methionine incorporation normalized to total protein amount from (a). No changes in global translation are observed between *Eif4ebp2* KO and WT mice (p=0.214), n=3; Student's *t*-test.

Next, we selected 24 mRNAs of interest coding for proteins known to be associated with ASD (Table 1). We examined their polysome distribution in Eif4ebp2 knockout, eIF4E-overexpressing (βT-Eif4e), and wild-type polysome fractions. Out of the 24 mRNAs examined, only neuroligin (Nlgn1, Nlgn2, Nlgn3, and Nlgn4) mRNA profiles were shifted toward heavier polysome fractions, indicating an increased translation of these mRNAs (Figure 12a,b). Moreover, protein amounts of all four neuroligins were increased in crude and synaptosomal extracts from Eif4ebp2 knockout hippocampi and in the synaptosomal fractions of βT-Eif4e mice (Figure 12c,d). The mRNA levels were found to be the same between mutant and wild-type mice, indicating that these changes were indeed due to translation and not transcription (Figure 13). No translational changes were observed for other mRNAs coding for adhesion and scaffolding proteins such as neurexins, PSD95, gephyrin, SHANK2, SHANK3, and SAPAP3. From these findings we concluded that relief of translational suppression, either by loss of *Eif4ebp2* or by eIF4E overexpression selectively enhances the synthesis of neuroligins, causing an imbalance between adhesion and scaffolding proteins.

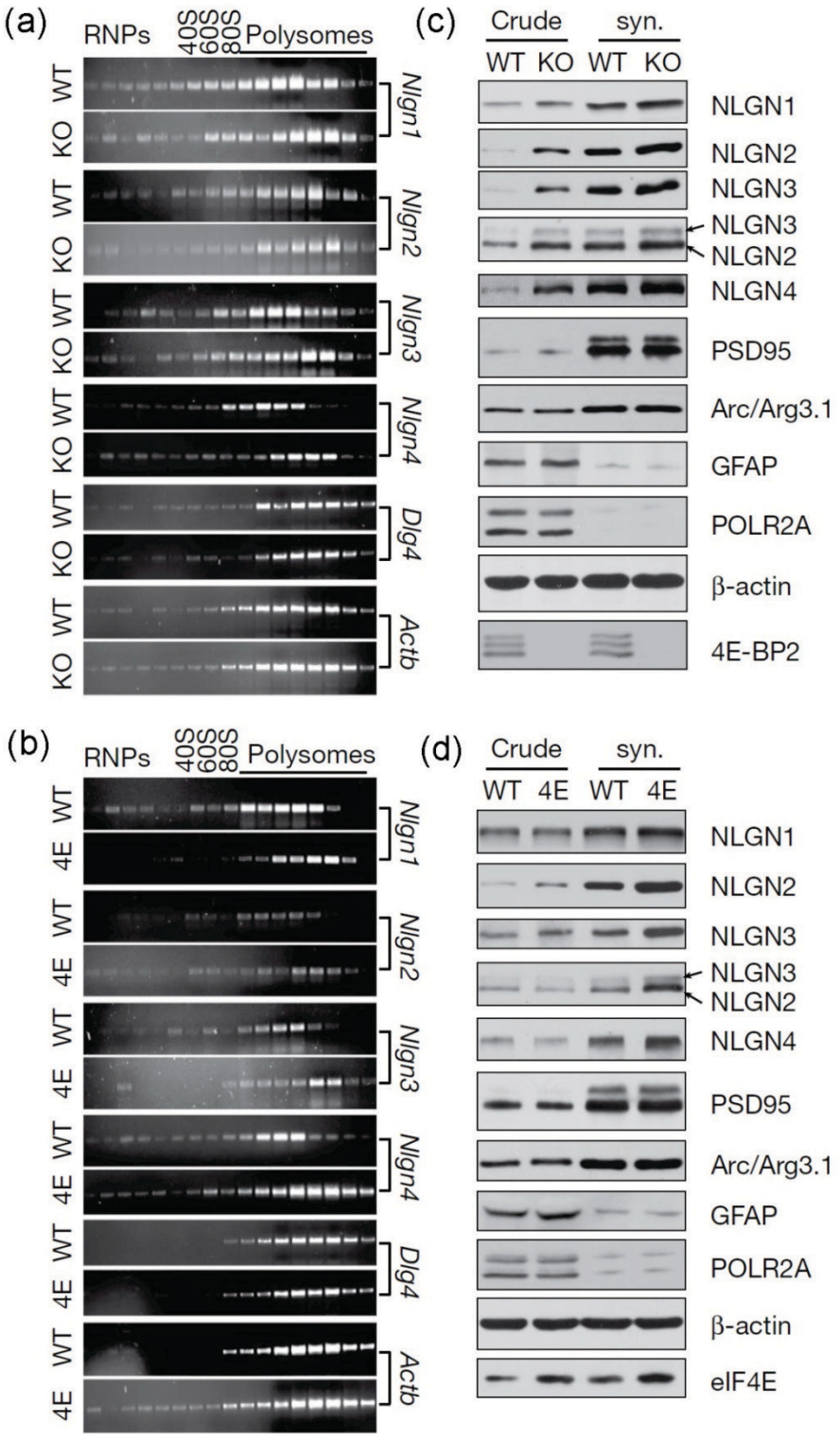


Figure 12. (a) (b) RT-PCR of RNA extracted from polysome fractions from WT and *Eif4ebp2* KO hippocampi (a) and from βT-*Eif4e* hippocampi (b). Representative gel images are shown; n=4 per group. **(c) (d)** Representative immunoblots of crude and synaptosomal (syn.) fractions from hippocampal lysates of WT and *Eif4ebp2* KO mice (c) or from WT and βT-*Eif4e* mice (d).

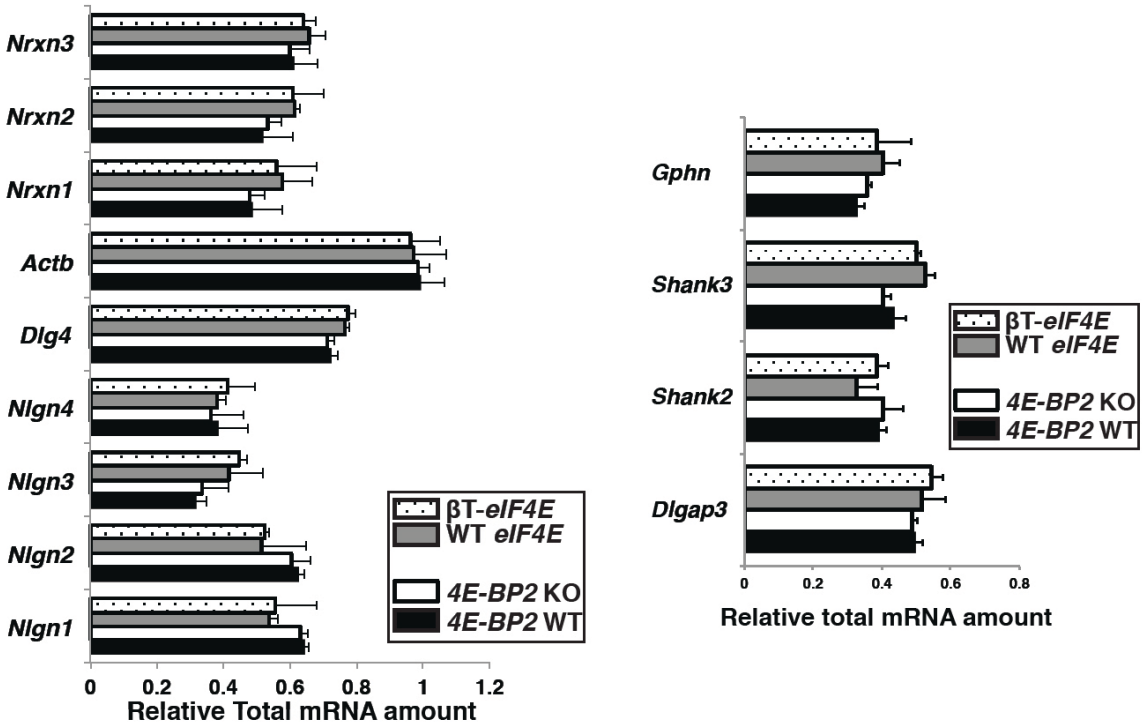


Figure 13. qRT-PCR analysis of total RNA in *Eif4ebp2* KO and WT and in βT-*eIF4e* and WT mice. No change is observed in the amounts of *Nlgn1*, *2*, *3*, and *4*, *Dlg4*, *Actb*, *Nrxn1*, *2*, and *3*, *Dlgap3*, *Shank2*, *Shank3*, and *Gphn* mRNAs (n=4 for *Eif4ebp2* KO and WT, βT-*eIF4e* and WT mice).

Table 1. Translational profiling of ASD-related mRNAs and controls in *Eif4ebp2* knockout and βT-*Eif4e* mice.

Gene	Shifts in *Eif4Ebp2* knockout	Shifts in βT-*Eif4e*
Nlgn1	yes	yes
Nlgn2	yes	yes
Nlgn3	yes	yes
Nlgn4	yes	yes
Nrxn1	no	no
Nrxn2	no	no
Nrxn3	no	no
Dlg4	no	no
Dlgap3	no	no
Shank2	no	no
Shank3	no	no
Gphn	no	no
Cdh9	no	no
Cdh10	no	no
Gabrb3	no	no
Itgb3	no	no
En2	no	no
MeCP2	no	no
A2bp1	no	no
Gapdh	no	no
Gfap	no	no
Actb	no	no
Ctnna3	no	no

Nlgn1 knockdown in *Eif4ebp2* mice

The adhesion protein NLGN1 is present exclusively at excitatory synapses and is known to promote excitatory synaptic transmission (Chubykin et al., 2007; Dahlhaus et al., 2010). It has been established that in *Eif4ebp2* knockout mice neuroligin mRNA translation is enhanced and that these mice are characterized by an increased E/I ratio. Given this information, we hypothesized that decreasing levels of the NLGN1 protein in particular would restore the E/I balance and reverse the ASD-like behaviours in *Eif4ebp2* knockout mice. To this effect, we

produced lentiviruses containing *Nlgn1* and *Nlgn2* shRNA. We observed a decrease in NLGN1 and NLGN2 protein levels, but not of other neuroligins, in mice injected with these lentiviruses (Figure 3a). The knockdowns were performed with two different shRNAs for each *Nlgn1* and *Nlgn2*, yielding similar results. Lentiviral titer was determined by a colony formation assay (Figure 14b,c). Knockdown of NLGN1, but not of NLGN2 helped restore the E/I ratio and to rectify the ASD-like behaviour in the mice treated with the lentiviruses (results not shown)

eIF4E sensitivity of neuroligins is confined to their 5'UTR

It has been previously demonstrated that increased mTOR signalling to its downstream effectors preferentially promotes the translation of eIF4E-sensitive mRNAs with extensive secondary structures in their 5'UTRs (Koromilas, Lazaris-Karatzas, & Sonenberg, 1992). To test whether neuroligin transcripts relied on their 5'UTR for selective translation, we expressed reporter mRNAs containing the full length 5'UTRs of *Nlgn1* and *Nlgn2* as well as those of *Dlg4* (PSD95) and *Actb* fused to luciferase in cells with elevated mTORC1 signalling (*Pten*+/-, *Tsc2* KO) or enhanced cap-dependent translation (*Eif4ebp2* KO, eIF4E overexpression) (Figure 15). We observed that neuroligin 5'UTRs are better translated in these cells than in wild-type cells, while the translation levels of the transcripts coding for scaffolding proteins remain unchanged. These results reinforce the idea that neuroligin mRNAs are specifically targeted by the translation machinery in pathways with dysregulated translation and that this specificity is conferred by their 5'UTRs.

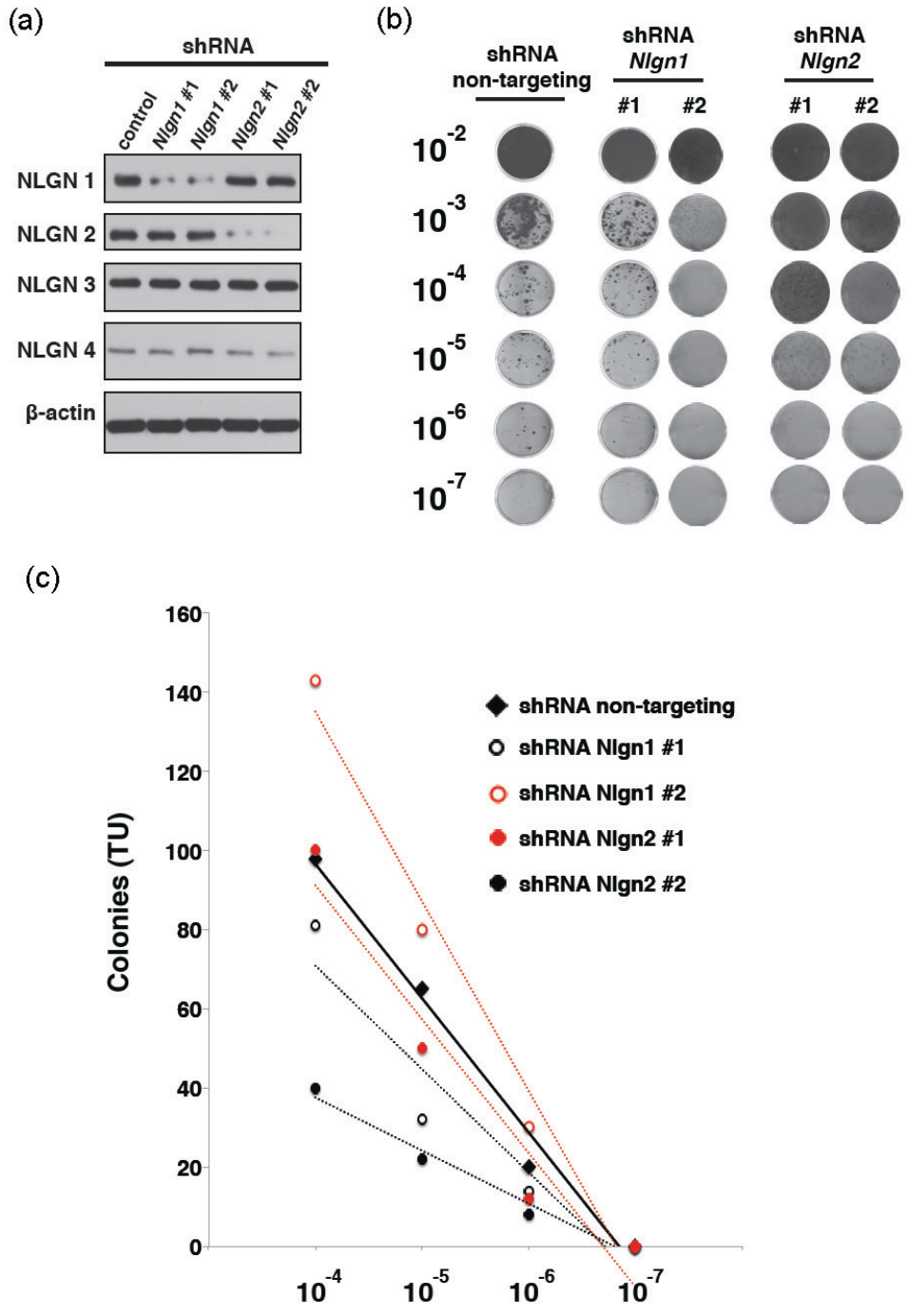


Figure 14. (a) Immunoblot analysis of lysates from N2A cells infected with lentiviruses expressing different shRNAs against *Nlgn1*, *Nlgn2*, or a non-targeting sequence. Reduced expression of NLGN1 of NLGN2 is observed in cells infected with relevant shRNAs. **(b)** Colony formation to determine lentiviral titer. Representative images from N2A cells infected with different dilutions of lentiviruses encoding for shRNA #1 and #2 against *Nlgn1*, and with shRNA #1 and #2 against *Nlgn2* or with non-targeting shRNA, stained with crystal violet. **(c)** Quantification of transducing units per ml (TU/ml) of the lentiviruses in (b).

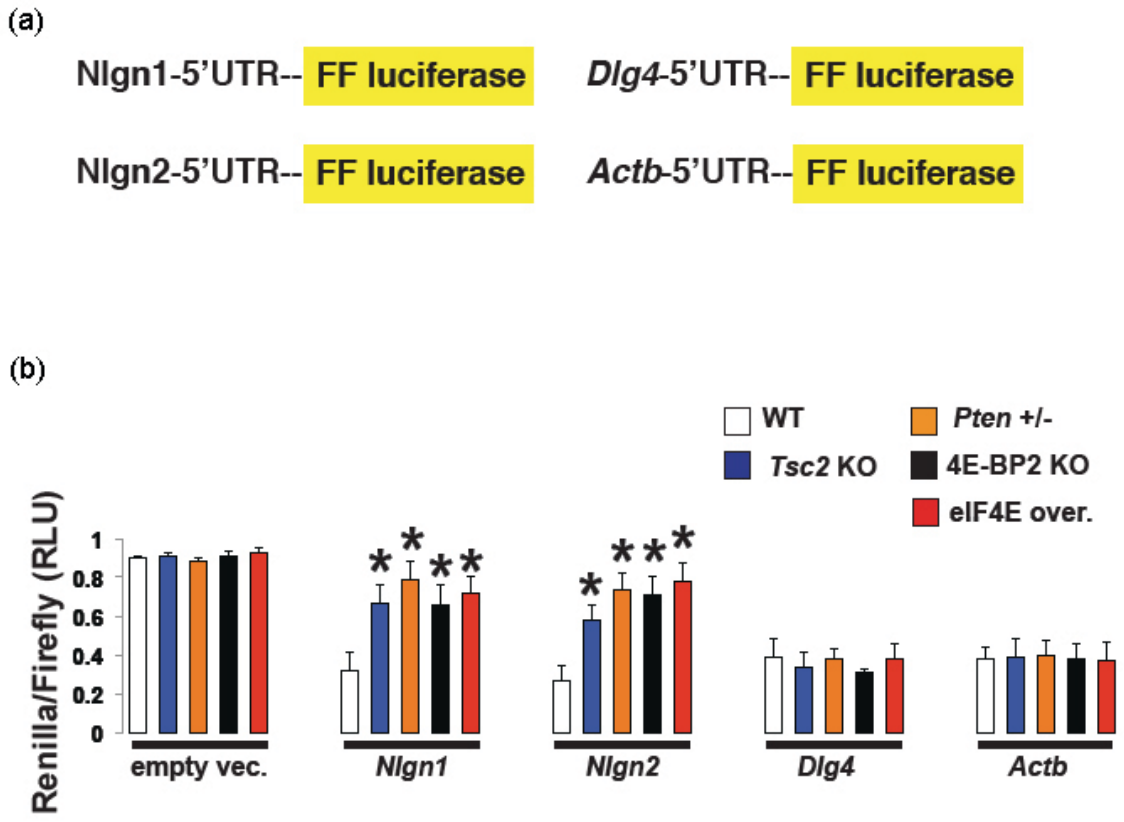


Figure 15. (a) Reporter luciferase vectors with 5'UTRs of *Nlgn1, 2, Dlg4, Actb* cloned upstream of the firefly luciferase genes. **(b)** Luminescence of firefly luciferase expressed as relative light units (normalized to renilla luciferase luminescence) for the depicted MEF lines. *Nlgn* 5'UTRs are translated more in cell lines with enhanced mTORC2 signalling or increased eIF4E-dependent translation; one-way ANOVA; Bonferroni's post-hoc *p<0.001 (n=4). All data are presented as mean ±SEM.

Discussion

The role of helicases in the control of synaptic local translation

The role of RNA helicases in synaptic plasticity, learning, and memory has never been investigated. Even less is known of DHX29, a recently discovered helicase whose exact role and mechanism are still being elucidated (Marintchev, 2013). In this work, we have shown that DHX29 localizes in a predominantly post-synaptic fashion. This suggests that translation through DHX29 is important for translational control of post-synaptic proteins which are important for synaptic and brain function.

We have observed no effect on the polysome profile by knocking down DHX29 in cortical neurons. However, a similar knockdown in HeLa cells previously performed in our lab yields profiles with an increased 80S peak and reduced polysome peaks (Parsyan et al., 2009). A possible explanation for this discrepancy is the fact that neurons are post-mitotic cells. Fully differentiated neurons are usually permanently quiescent while continuing to perform their functions (Herrup & Yang, 2007). This results in a lower level of basal translation in non-stimulated neurons. We hypothesized that DHX29 knockdown does not have a profound effect on overall translation because this helicase is involved in translation of specific transcripts. eIF4A1, while also regulating these transcripts, is responsible for general translation: knockdown of eIF4A1 has a significant effect on the polysome profile. Moreover, the effects of other RNA helicases have not been considered in this work. It would be interesting to perform a knockdown

of the helicases eIF4A2 and DDX3 and observe their effects on the polysome profile and mRNA transcripts.

The RNA helicases examined here promoted translation of transcripts with highly structured 5'UTRs over those with relatively unstructured 5'UTRs. qRT-PCR analyses performed on cDNA reverse-transcribed from RNA extracted from polysome fractions showed a shift to lighter polysomes for mRNAs with structured 5'UTRs, but no shift for mRNAs with unstructured 5'UTRs. However, in eIF4A1 knockdown cells, *Actb* (*β*-actin) displays a shift to lighter polysomes. The importance of *β*-actin in several neuronal mechanisms is a possible explanation to these results. It has previously been shown that *β*-actin mRNA localizes more frequently to neuronal processes and growth cones, providing the growth cone with autonomous control of its structure (Bassell et al., 1998). This is also necessary for changes in the shape and size of dendritic spines, which are correlated with the strength of excitatory synaptic transmission and depend on remodelling of the actin cytoskeleton. Since it is believed that learning and memory depend on the strengthening and weakening of synaptic connections, actin should play an important role in these processes. It has been demonstrated that the actin cytoskeleton does not only contribute to the structure of the synapses, but also takes part in synaptic activities such as organizing the postsynaptic density and localizing the translation machinery. Moreover, it has been shown to be deficient in various memory disorders (Hotulainen & Hoogenraad, 2010). Finally, it has previously been demonstrated that expression of housekeeping genes such as β-actin and GAPDH cannot always be assumed to be constant (Glare, Divjak, Bailey, & Walters, 2002). In view of these data, it is possible that our knockdown of eIF4A1 severely affects β-actin translation, regardless of its 5'UTR.

We proposed the use of the photoconvertible Kaede reporter to track protein synthesis at the synapse. While this technique has previously been successfully used in developing embryos (Hatta, Tsujii, & Omura, 2006) and, more importantly, in stimulated neurons (Banerjee et al., 2009), our experimental procedure has yet to be refined. We have confirmed that our constructs can be successfully photoconverted by UV light, but have yet to test them in neurons. Moreover, because basal translation in neurons is low, the rate of protein synthesis in unstimulated neurons may be difficult to track. Proper stimulation conditions have yet to be determined for performing this experiment and for quantifying the results.

Autism-related deficits via dysregulated eIF4E-dependent translational control

We showed that neuroligin mRNAs are translated more efficiently in two models of enhanced eIF4E activity: in *Eif4ebp2* knockout mice and in βT-*Eif4e* eIF4E-overexpressing mice. These results highlight the important role played by 4E-BP2 and eIF4E in translational control of postsynaptic mRNAs. Although there appears to be no effect on general translation in the mice models we used, enhanced translation machinery activation favours the translation of specific transcripts. As previously reported, increased mTOR signalling to its downstream effectors promotes the preferential translation of a subset of mRNAs, knows as "eIF4E-sensitive", that possess extensively structured 5'UTRs (Koromilas et al., 1992).

We have also shown that the 5'UTR is the main element that distinguishes neuroligin mRNAs from the pool of other mRNAs to be translated. Reporter mRNAs containing the full-length 5'UTRs of *Nlgn1*, *Nlgn2* fused to the luciferase reporter are better translated in cells with

elevated mTORC1 signalling and/or enhanced cap-dependent translation than those containing the 5'UTRs of *Dlg4* and *Actb*, as compared to parental cells. Moreover, preliminary data indicates that the 5'UTRs of neuroligin mRNAs possess a repeated structural element absent from other mRNA 5'UTRs (data not shown). The discovery of these elements (similar in structure, but not in sequence), may explain the preferential increase in neuroligin mRNA translation in response to increased eIF4F activity.

Knockdown of neuroligin mRNAs yielded interesting results (not shown here): only neuroligin 1, but not neuroligin 2 contributed to restoring E/I balance and normal behaviour in mice. These results contribute to the understanding of the link between the translational control of neuroligins through eIF4E, E/I balance, and ASD-like phenotypes.

Conclusions

This work focused on investigating the mechanisms that regulate the translation of neuronal mRNAs. We demonstrated that RNA helicases eIF4A1 and DHX29 preferentially regulate mRNAs with highly structured 5'UTRs at neuronal synapses. We also determined that DHX29 localized post-synaptically, making it an important factor in learning and memory control. Similarly, we showed that eIF4E overexpression selectively promotes translation of neuroligin mRNAs and that this specificity resides in the 5'UTR of the transcripts. Taken together these results lead us to highlight the significance of the 5'UTR region of mRNAs and to prove the importance of translational control at synapses for regulating learning, memory, and behaviour.

Materials and methods

Immunoblotting

The reagents and solutions discussed in this section were prepared using the recipes listed in the Lab FAQs booklet by Roche. It may be found online at http://www.roche-applied-science.com/PROD_INF/MANUALS/labfaqs/lab_faqs.pdf.

SDS-PAGE gels were prepared as described in the Roche manual, depending on the desired density: water, acryl-bis-acrylamide mix (29:1, 30%, BioShop), Tris (1.5M, pH 8.8 or 0.5M, pH 6.8), SDS (10%), ammonium persulfate (10%), and TEMED (BioShop). Protein lysate concentration was balanced using a Bradford assay (Bio-Rad). The lysates to be separated by electrophoresis were mixed with 2X SDS-PAGE loading dye (0.15M Tris, pH 6.8; 1.2% SDS; 30% glycerol; 15% β-mercaptoethanol; bromophenol blue) and boiled for 5 minutes at 100°C. The lysates were loaded onto the cast gels alongside size markers (Fermentas) and run at constant amperage of 15 mA per gel in running buffer until the desired separation was reached. The proteins from the SDS-PAGE gels were transferred onto 0.2 μm nitrocellulose membranes using the electroblotting technique in transfer buffer. The transfer was performed either overnight at constant voltage of 30 V at 4°C or over the period of 1.5 hours at constant voltage of 100°C. Following transfer, the nitrocellulose membranes were blocked in 5% BSA (in TBS-T buffer) for 20 minutes, with gentle rocking at 4°C. They were then cut into strips according to the size of the protein of interest and incubated in primary antibody solution overnight. After incubation with primary antibody, the membranes were briefly washed three times in TBS-T and then incubated in secondary antibody for 1 hour with constant rocking at room temperature. Finally, the membranes were once again washed three times in TBS-T. Following the last wash,

protein bands were visualized using chemiluminescence (Western Lightning-ECL, PerkinElmer) and autoradiography film (Kodak).

Primary antibodies were stored in 5% BSA in TBS-T at 4°C. 0.01% sodium azide was added to maintain antibody integrity during storage. Secondary antibodies used were either ECL anti-mouse or anti-rabbit IgG (GE Healthcare), diluted 1:5000 in TBS-T and discarded after use. Antibodies against indicated proteins were: GFAP, PSD95, αCaMKII, β-actin, eIF4E-BP2, eIF4E (Cell Signaling); Synapsin I (BD Biosciences); Arc/Arg3.1, POLR2A, Pol II (Santa Cruz); DHX29 (Novus); NLGN4, eIF4A1, CPEB1, GluA1 (Abcam); PUM2, GluA2 (Millipore); NLGN1, NLGN2/3, NLGN2, NLGN3 (Synaptic Systems). Antibodies were used at dilutions specified by the manufacturer.

Cell culture

Unless otherwise specified, all cell lines were grown on BD Falcon tissue culture dishes in DMEM (Wisent) supplemented with 10% FBS (Wisent) and 1% penicillin/streptomycin (Wisent) at 37°C, 5% CO_2.

Dissociated neuronal culture

Intact cortices were isolated from E16-E18 C57BL/6 embryos in HBSS (Invitrogen). Meninges were removed and cortical neurons were separated by mechanical dissociation and mild trypsinization. Cells were plated on poly-L-lysine-coated (1 mg/ml, Sigma-Aldrich) glass-bottom chamber slides (BD Falcon) in Neurobasal medium (Gibco) supplemented with 1% B-27 (Gibco), 1% Glutamax (Wisent), and 1% penicillin- streptomycin (Wisent). After 48 h, neurons

were treated with 1 μM Cytosine β-D-arabinofuranoside (Sigma-Aldrich) for another 48 h to inhibit non-neuronal cell growth.

Synaptosome preparation

Intact cortices from wild-type mice were dissected on ice-cold PBS and homogenized in 320 mM sucrose, 1 mM EDTA, 5 mM Tris-HCl (pH 7.4), and 25 μM DTT. Synaptosomes were isolated on a discontinued (3, 10, 15, 23%) Percoll (GE Healthcare) gradient. The fraction between 15 and 23% Percoll was isolated and re-suspended in 2X SDS-PAGE sample buffer.

Immunofluorescence

Primary cortical neurons grown on culture slides were infected with a lentivirus carrying the DHX29-GFP DNA cloned into the pLenti6/V5-DEST Gateway vector (Invitrogen) by Alexey Karetnikov in our lab. Once the cells were expressing the GFP-tagged DHX29 protein, they were fixed with 4% PFA and incubated with primary antibodies for one hour: either with PSD95 (1:200 in PBS, Cell Signaling) or with Synapsin I (1:200 in PBS, BD Transduction Laboratories). After another PBS wash, the neurons were incubated with fluorescently-tagged secondary antibodies: Alexa Fluor 594 goat anti-mouse and goat anti-rabbit (1:400 in PBS, Invitrogen). Fluorescence was observed using a Zeiss Axio Scope microscope equipped with GFP and TRITC filters.

Staining for neuronal DHX29 was performed in a similar fashion on primary cortical neurons, using a DHX29 (Novus) primary antibody.

Photoconversion

The Zeiss Axio Scope microscope was used to detect the fluorescence and for the photoconversion of the Kaede chromophore. Green fluorescence was detected using the GFP filter, photoconversion was carried out for ten seconds using the DAPI filter, and red fluorescence was detected with the TRITC filter.

Plasmid preparation

Plasmid DNA was transformed into chemically competent bacteria (OneShot Stbl3 *E. coli*, Invitrogen) and grown overnight on ampicillin selection plates (100 μg/ml). To this effect, at least 10 ng of DNA were added to bacteria in a microcentrifuge tube, incubated on ice for 30 minutes, heat-shocked at 42°C for 30 seconds, and incubated on ice for two more minutes. 250 μl of LB culture (antibiotic-free) were added to the tube which was then incubated for 1 hour with shaking at 37°C. The bacteria was then plated onto selection plates and left to grow overnight in a 37°C incubator (Isotemp Incubator, Fisher Scientific). The next day, isolated colonies were picked to be grown in 500 ml LB culture with ampicillin selection (100 μg/ml) at 37°C with shaking, overnight. In the case where glycerol stocks of the plasmid-containing bacteria were available, the transformation and selection steps were omitted: 300 μl of the glycerol stock were used to inoculate the 500 ml overnight cultures.

For all plasmid purification, the QIAGEN Plasmid Maxi Kit was used. The purification procedure may be found online at http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/Plasmid-DNA/QIAGEN-Plasmid-Maxi-Kit#resources. 800 μl of the culture were removed to prepare 10% glycerol stocks kept at -80°C (800 μl bacteria + 200 μl 50% glycerol). The DNA pellet obtained was dissolved in 500 μl water

and the concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Cloning

For the luciferase constructs, full-length 5'UTRs of mouse *Nlgn1* (NCBI reference NM_001163387.1), *Nlgn2* (NM_198862.2), *Actb* (β-actin; NM_007393.3), and *Dlg4* (PSD95; NM_0078643) were amplified from mouse tail genomic DNA, using specially designed primers and cloned into pGL4.13 firefly luciferase vector (Promega), upstream of the firefly luciferase gene. Restriction sites for restriction enzymes HindIII (AAGCTT) and for NheI (GCTAGC) were included in the primers. The random sequences CGATCGAT and ATCGATCG were appended before the restriction sites to allow for efficient attachment of the restriction enzyme. The online restriction map tool was used to select appropriate restriction enzyme sites (http://www.vivo.colostate.edu/molkit/mapper/).

Primers used for amplification:

Nlgn1 F: 5'-CGATCGATAAGCTTTGAGGGATATAG-3'

R: 5'-ATCGATCGGCTAGCTGTCTGGTACAG-3'

Nlgn2 F: 5'-CGATCGATAAGCTTTCAAGCCACTAA-3'

R: 5'-ATCGATCGGCTAGCGGGAAAGGAAG-3'

Actb F: 5'-ATCGATCGAAGCTTCTGTCGAGTC-3'

R: 5'-CGATCGATGCTAGCGGCGAACTGG-3'

Dlg4 F: 5'-ATCGATCGAAGCTTCCAGCTCATG-3'

R: 5'-CGATCGATGCTAGCGTTGGGGGGC-3'

The primers were used to amplify the genes of interest from mouse tail genomic DNA, resulting in DNA segments flanked by restriction enzyme sites. The PCR program used to amplify the segments was as follows: (1) 95°C for 5 minutes, (2) 95°C for 30 seconds, (3) 65°C for 30 seconds, (4) 72°C for 30 seconds, repeat steps 2-4 25 times, 72°C for 5 minutes. 2 µl of genomic DNA were used for the amplification. The polymerase used was Pfu recombinant DNA polymerase (Fermentas). The amplified DNA was separated by electrophoresis on an ethidium bromide agarose gel of a density appropriate to the size of the segments, excised, and purified using the QIAquick Gel Extraction Kit (QIAGEN). The protocol used may be found online at http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/DNA-Cleanup/QIAquick-Gel-Extraction-Kit#resources. The purified DNA segments as well as the pGL4.13 plasmid were sequentially digested with HindIII and NheI restriction enzymes. For the DNA segments, all of the purified DNA (30 µl) was mixed with 2 µl NheI enzyme (Thermo Scientific), 4 µl 1X Tango buffer (supplied with the enzyme), and 4 µl water. For the plasmid, 1 µl of DNA (about 1500 ng) was mixed with 1 µl NheI enzyme, 2 µl 1X Tango buffer, and 16 µl water. 300 ng of plasmid DNA are sufficient for one ligation, therefore the amount was scaled for the number of ligations to be performed. The digestion reactions were carried out at 37°C over the period of three hours. The digested segments were purified with the QIAquick gel extraction kit (omitting the steps for the gel extraction) and the next restriction reaction was set up. Both for the inserts and for the plasmid, all of the DNA purified (30 µl) was mixed with 2 µl HindIII enzyme (Thermo Scientific), 4 µl 1X buffer R (supplied with the enzyme), and 4 µl water. The digestion reactions were carried out at 37°C over the period of three hours. Once digestion was complete, the DNA segments and the plasmid were separated on an agarose gel, excised, and purified using the Silica Bead DNA Gel Extraction Kit (Thermo

Scientific). The protocol used may be found online at http://www.thermoscientificbio.com/uploadedFiles/Resources/k0513-product-information.pdf. The ligation step was performed as follows: 1/5 of the total extracted plasmid DNA was mixed with each of the DNA segments (entire volume extracted), 1 µl T4 DNA ligase (Thermo Scientific), 2 µl 10X T4 DNA ligase buffer (supplied with the enzyme), and enough water to bring the reaction volume to 20 µl. Four reactions containing the digested plasmid and each of the four inserts plus one reaction containing no insert were prepared. The ligation took place at room temperature over a two-hour period. Following ligation, the constructs were cloned into chemically competent bacteria (Stbl3 *E. coli*, Invitrogen) and purified as previously described.

The plasmid constructs used to create the Kaede reporters were prepared in a similar fashion. 5'UTRs of interest were amplified from mouse tail genomic DNA, using specifically designed primers (see below). They were cloned into a pWPXL plasmid already containing the Kaede reporter fused to the 3'UTR of αCaMKII (obtained from the Kosik lab) (Banerjee et al., 2009) using the PacI (TTAATTAA) and SwaI (ATTTAAAT) restriction enzymes (Thermo Scientific). The restriction reactions were performed at 37°C in 2X buffer PacI (Thermo Scientific) for PacI and at 30°C in 1X buffer O (thermo Scientific) for SwaI. The ligation and cloning steps were performed as described above.

Primers used for amplification:

Gria1 F: ATCGATCGATTTAAATCGGGAGGGTGAGAGAGGCTG

R: CGATCGATTTAATTAAATTCCTTTTTACATTGGCGAAAAAG

Dlg4 F: ATCGATCGATTTAAATCCAGCTCATGCCCCAGCCCCAG

R: CGATCGATTTAATTAAGTTGGGGGGCCTGGCCGCGG

Grin1 F: ATCGATCGATTTAAATAGAACGCGTAGGTCCCGCTC

R: CGATCGATTTAATTAAGAGCCTCGGGCACAGCGGGC

Pum2 F: ATCGATCGATTTAAATGTTGTTGTGAGTCTCTGTGC

R: CGATCGATTTAATTAATTCATCCACAGATCAAACAG

Cpeb1 F: ATCGATCGATTTAAATGCTGCTCGCTGCAAAAATAG

R: CGATCGATTTAATTAAGGGGTCCGGCGTGGCCCTAAG

Actb F: ATCGATCGATTTAAATCTGTCGAGTCGCGTCCACCC

R: CGATCGATTTAATTAAGGCGAACTGGTGGCGGGTGTG

Metabolic labelling

Acute hippocampal slices were prepared from wild-type or *Eif4ebp2* knockout or *β*T-*Eif4e* mice as described earlier (Bidinosti et al., 2010) and 30 mCi/ml [^{35}S]Met/Cys was added for 3 hours. Slices were homogenized in 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol, and lysates were centrifuged at 16,000 x *g*. Total extracts were resolved by SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes, which were then exposed onto autoradiography film (KODAK) for 1 week and developed. For total protein calculations, lysates were analysed by SDS-PAGE electrophoresis and the gels were stained with GelCode Blue Stain Reagent (Pierce), according to the manufacturer's protocol. Signals were quantified using ImageJ (NHE).

Polysome profile analysis

Intact mouse hippocampi were washed with ice-cold PBS containing 100 µg/ml cycloheximide, homogenized, and lysed in a hypotonic lysis buffer (5mM Tris-HCl, pH 7.5; 2.5 mM $MgCl_2$; 1.5

mM KCl; 100 µg/ml cycloheximide; 2 mM DTT; 0.5% Triton X-100; 0.5% sodium deoxycholate). Lysate concentration was balanced using a Bradford assay (Bio-Rad) and by measuring total RNA concentration using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Balanced lysates were loaded onto 10-50% sucrose density gradients (20 mM HEPES-KOH, pH 7.6; 100 mM KCl; 5 mM $MgCl_2$) and centrifuged at 35,000 r.p.m. for 2 h at 4°C in the Optima L-80 XP ultracentrifuge (Beckman Coulter). After the spin, gradients were fractionated and the absorbance at 254 nm wavelength was continuously recorded using the BR-188 density gradient fractionation system (Brandel and Teledyne ISCO). The chase solution used was 60% sucrose (w/v) with addition of methylene blue. The data was collected using the TracerDAQ software (Measurement Computing). Polysome-to-monosome ratio was calculated as the area under the A_{254} absorbance curve, using the function describing the absorbance values, processed with the definite integral command in MATLAB (MathWorks).

RNA extraction

Total and fractionated RNA was isolated using Trizol (Invitrogen). The protocol can be found online at http://tools.invitrogen.com/content/sfs/manuals/trizol_reagent.pdf. 1 ml of Trizol reagent was added to each fraction and the samples were incubated for 5 minutes at room temperature before proceeding with the extraction. 200 µl of room-temperature chloroform were then added to the samples, the tubes were shaken vigorously for 15 seconds, and then incubated at room temperature for 3 minutes. The samples were centrifuged at 12,000 x *g* for 15 minutes in an Eppendorf microcentrifuge (model 5424) placed at 4°C. This centrifuge was used for all centrifugation steps. Following centrifugation, the upper phase containing the RNA was transferred to a fresh tube and mixed with 500 µl of room-temperature isopropyl alcohol. The

samples were incubated at -80°C overnight to allow for RNA precipitation. The next day, the samples were centrifuged at 12,000 x *g* for 10 minutes at 4°C. After the centrifugation, the supernatant was removed and the resulting pellet was washed with 1 ml of 75% ethanol at room temperature and centrifuged at 7,500 x *g* for 5 minutes. The wash was removed as much as possible and the RNA pellet was air-dried for 10-15 minutes. The dry pellet was resuspended in 20 µl RNase-free distilled water (Invitrogen) and the RNA concentration was measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific).

RT-PCR

The RNA extracted from polysomal fractions was reverse-transcribed using the SuperScript III Reverse Transcriptase kit (Invitrogen) and random hexamers from Roche. A protocol may be found online at http://tools.invitrogen.com/content/sfs/manuals/superscriptIII_man.pdf. 400 ng of RNA were mixed with 1 µl random hexamers (50 µM), 1 µl dNTP mix (10 mM), and water to bring the total up to 13 µl. The mix was heated at 65°C for 5 minutes (no hotlid), cooled at 4°C for 5 minutes, placed on ice for 5 minutes, briefly centrifuged, and placed on ice. The heating and cooling steps were carried out on the C1000 Thermal Cycler (Bio-Rad). Next, the following reagents were added to the contents of each tube and mixed by gently pipetting up and down: 4 µl of the 5X first-strand buffer supplied with the kit (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$), 1 µl DTT (100 mM, also included in the kit), 1 µl recombinant RNasin ribonuclease inhibitor (Promega), and 1 µl SuperScript III reverse transcriptase, for a total volume of 20 µl. The mix was incubated at 25°C for 5 minutes, then at 50°C for 60 minutes, and at 70°C for 15 minutes. The tubes were cooled to 22°C and the cDNA was kept at -20°C. For

RT-PCR results depicted on agarose gels, products were amplified only 20-25 cycles to remain within the range of the qRT-PCR reaction.

qRT-PCR

Total and polysomal fraction reverse-transcribed RNA (cDNA) was analysed using the iQ SYBR Green Supermix reagent (Bio-Rad) and the LightCycler 480 real-type PCR system (Roche). LighCycler 480 96-well plates and sealing foil were used. For each polysomal fraction collected, the reaction mix was as follows: 1 µl cDNA (from RT-PCR), 0.5 µl primer mix (10 µM), 5 µl iQ SYBR Green Supermix reagent, and 3.5 µl water, for a total of 10 µl per well. The amplification reaction was carried out at the following conditions: (1) 95°C for 2 minutes, (2) 95°C for 15 seconds, (3) 55°C for 15 seconds, (4) 68°C for 20 seconds, with steps 2-4 repeated 45 times. The melting curve analysis for each product was performed as follows: 95°C for 15 seconds, 60°C for 15 seconds, 20-minute hold, 95°C for 15 seconds. Results are presented in arbitrary units as relative amounts, using serial dilutions (1, 1:10, 1:100, 1:1000, 1:10000) of cortical or hippocampal RNA as qRT-PCR concentration standards. The primers used are summarized below (Table 2).

Lentiviral transduction and titration

Lentiviruses were produced by co-transfection of HEK293T cells with the transfer vector pLKO.1, the packaging plasmid psPAX2, and the envelope plasmid pMD2.G along with Lipofectamine 2000 transfection reagent (Invitrogen) and Opti-MEM serum-free medium (Gibco). The virus was concentrated over a sucrose cushion (20% sucrose in HBSS) by centrifugation at 20,000 RPM for 2 hours at 4°C (Optima L-80 XP Ultracentrifuge, Beckman

Coulter). For mouse *Nlgn1* knockdown, shRNA1 was TRCN0000032022 and shRNA2 was TRCN0000032020. For mouse *Nlgn2* knockdown, shRNA1 was TRCN0000180497 and shRNA2 was TRCN0000184441. The non-targeting shRNA was SHC002. All constructs were obtained from MISSION (Sigma-Aldrich).

N2A cells used for validation of shRNAs and siRNAs and MEFs used for titration of the lentiviruses were maintained in DMEM (Gibco) supplemented with 10% FBS (company) and 1% penicillin/streptomycin (Gibco) at 37°C, 5% CO_2. Viral titer (TU/ml) was calculated using puromycin selection of MEFs infected with serial viral particle dilutions and stained with crystal violet solution (0.2% crystal violet; 20% methanol). Colonies were counted using the CellCount plug-in in ImageJ (NIH). Viral titres were adjusted to 1.4 x 10^7 TU/ml for *in vivo* infections.

Table 2. Primers used for qRT-PCR analyses.

Primer name	Primer sequence (5'-3')	NCBI accession #
Fw-Mtap2	GGTTCCTCAGCTTGTCTCTAAC	NM_001039934.1
Rev-Mtap2	TTCTCTGGGCTCTTGCTTATTC	
Fw-Grin1	CCAGATGTCCACCAGACTAAAG	NM_001177656.1
Rev-Grin1	GTTCACCTTAAATCGGCCAAAG	
Fw-Cpeb	GAGCAGCACACAGTCAGTATTA	NM_001252525.1
Rev-Cpeb	GGAGTCTAGCCTCTCTCTCTATG	
Fw-Pum2	CAGCGTCCTATTACTCCAAGTC	NM_001160219.1
Rev-Pum2	GCCAGGTCCATGAGAGAATAAA	
Fw-Gria1	ACCACTACATCCTCGCCAAC	NM_001113325.2
Rev-Gria1	TCACTTGTCCTCCACTGCTG	
Fw-Gria2	AACGGCGTGTAATCCTTGAC	NM_001083806.1
Rev-Gria2	CACCAGGGAGTCGTCGTAGT	
Fw-Camk2a	TCTGAGAGCACCAACACCAC	NM_177407.4
Rev-Camk2a	CGATTGCTTATGGCTTCGAT	
Fw-Nlgn1	ACAGGAGAACATCGTTTCCAGCCT	NM_138666.3
Rev-Nlgn1	ATACAGGAGCAAACTGAGTGGCGT	
Fw-Nlgn2	ACTATCTTTGGGTCTGGTGC	NM_198862.2
Rev-Nlgn2	ATGAGCATGTCGTAGTTGAGG	
Fw-Nlgn3	GTGAAATCCTGGGTCCTGTG	NM_172932.3
Rev-Nlgn3	GTCTTCATCTTCATCCCCGTC	
Fw-Nlgn4	AGGACGCGCACGTGATCTCTTAAT	EU350930
Rev-Nlgn4	TTTCTGAGGCAGTGGGATGACTGT	
Fw-Dlg4	ATCGGTGACGACCCATCCATCTTT	NM_007864.3
Rev-Dlg4	TCCCGGACATCCACTTCATTGACA	
Fw-Dlg2	TAAAGCAGTGGAAGCCCTCAAGGA	NM_011807.3
Rev-Dlg2	ACAGTCTCCAATATGGGTCGCCTT	
Fw-Dlgap3	TGGATGGACAGTCAGTCAAGCGAA	NM_198618.4
Rev-Dlgap3	AGTGATAAGTCCTGGCTTTGGCCT	
Fw-Shank2	AGAAGAGGACACGGATGGCTTTGT	NM_001081370.2
Rev-Shank2	ATGACATTTGCCTTTGGGCCTGAG	
Fw-Shank3	TAGCCTTCAAGACGCGCTCAACTA	NM_021423.3
Rev-Shank3	TCTGGGCATAAACTCTCCGCTTGT	
Fw-Gphn	ATGATCCTCACCAACCACGACCAT	NM_145965.2
Rev-Gphn	TGCCGATATAGTCCCACCCAACAA	
Fw-Nrxn1	GCAGTCGCCTTATCCTTAGAC	NM_020252.3
Rev-Nrxn1	GGCTGATTCGCTTTATGTTTAGG	

Fw-Nrxn2	CAATGGGTTGTTGCTCTTCAGCCA	NM_001205234.1
Rev-Nrxn2	ATTCACCATCATTGACCTTGCGGC	
Fw-Nrxn3	ATGGTGCGGTCTCCTTGGTCATTA	NM_001198587.1
Rev-Nrxn3	TGCCGAAGATTGCGTGTCACTTTG	
Fw-Cdh9	ACGAAAGACCTGTACACAGCCAGT	NM_009869.1
Rev-Cdh9	ATTATGCCTGATTCCGGGTCCACT	
Fw-Cdh10	GATGGAGATGGCACGGATATG	NM_009865.2
Rev-Cdh10	GAGGATCGACTGAAAACAGGAG	
Fw-Gabrb3	CTCCCACAGTTCTCCATTGTAG	NM_008071.3
Rev-Gabrb3	GGATTGAGGGCATATACGTCTG	
Fw-Itgb3	AAGAACGAGGATGACTGTGTC	NM_016780.2
Rev-Itgb3	ATATTGGTGAAGGTGGAGGTG	
Fw-En2	CGCTTGGGTCTACTGCAC	NM_010134.3
Rev-En2	CCCGTGGCTTTCTTGATTTTG	
Fw-MeCP2	CAGGCAAAGCAGAAACATCAG	NM_001081979.1
Rev-MeCP2	GTCAAAATCATTAGGGTCCAAGG	
Fw-A2bp1	ACACAGAAAGCAAGTCCCAG	BC059002.1
Rev-A2bp1	CAATTTCTCCCTCGCCCTATC	
Fw-Gapdh	TCAACAGCAACTCCCACTCTTCCA	NM_008084.2
Rev-Gapdh	ACCCTGTTGCTGTAGCCGTATTCA	
Fw-Gfap	GGAAATTGCTGGAGGGCGAAGAAA	NM_001131020.1
Rev-Gfap	TGGTGAGCCTGTATTGGGACAACT	
Fw-Actb	TGTGATGGTGGGAATGGGTCAGAA	NM_007393.3
Rev-Actb	TGTGGTGCCAGATCTTCTCCATGT	
Fw-Ctnna3	GCGCAGGTTTCTCAGGAG	NM_001164376.1
Rev-Ctnna3	CACAGTGAACGTTTGGATCTG	
Fw-Grik2	GTTCCTCACATACAGACCCG	NM_001111268.1
Rev-Grik2	GCCCCTCTTCATCTCTTTCAG	
Fw-Cntnap2	CAGATCAGTGCCATTGCAACCCAA	NM_001004357.2
Rev-Cntnap2	AGGGTTTCCAGTTTCTCCCTGTGT	
Fw-Cacna1c	AATGATTCGGGCCTTTGTTCAGCC	NM_009781.3
Rev-Cacna1c	TACCACCTTGCCCTTGAACTTCCT	
Fw-Bc1	TTTAGCTCAGTGGTAGAGCGCTTG	NR_038088
Rev-Bc1	GGTTGTGTGTGCCAGTTACCTTGT	

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