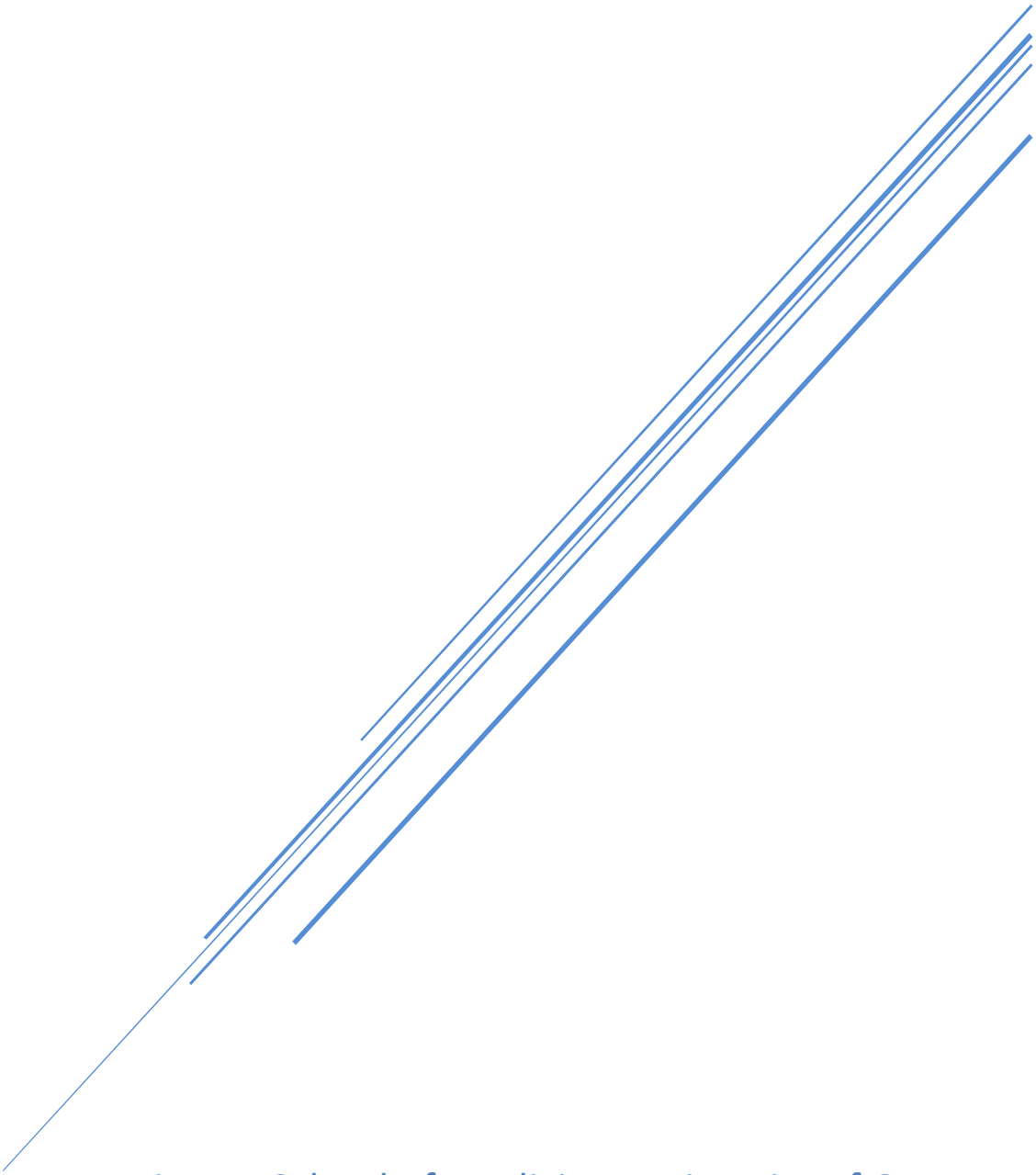
REGULATION OF PROTEIN SYNTHESIS IN THE BRAIN

And implications of its dysregulation in the development of neuropsychiatric disorders



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Abstract

mRNA translation is a fundamental cellular function that is tightly regulated by intracellular signaling pathways. Translation initiation is a crucial step of this process in eukaryotic cells and is, thus, under the most extensive regulation. Dysregulation of protein synthesis in the brain has been implicated in various neuropsychiatric disorders, among which autism spectrum disorder (ASD) is the focus of the present study. Mutations of the contactin-associated protein-like 2 (*CNTNAP2*) gene are associated with development of ASD and related disorders in humans and lead to ASD-like phenotypes in mice. However, a molecular mechanism to explain these observations is lacking. Here we show that knockout of CNTNAP2 in mice leads to excessive mRNA translation, potentially mediated by the hyperactivation of the mammalian target of rapamycin complex 1 (mTORC1) that we observed. In the second part of this thesis, we make use of a previously described technique to isolate and study adult neural stem cells (NSCs) in culture.

Περίληψη

Η μετάφραση του mRNA είναι μια θεμελιώδης κυτταρική λειτουργία που βρίσκεται κάτω από αυστηρό έλεγχο μέσω των ενδοκυτταρικών σηματοδοτικών μονοπατιών. Η έναρξη της μετάφρασης είναι το στάδιο το οποίο υπόκειται στο μεγαλύτερο μέρος της ρύθμισης. Αποδιοργάνωση αυτής της διαδικασίας εμπλέκεται σε διάφορες νευροψυχιατρικές διαταραχές, από τις οποίες η διαταραχή φάσματος του αυτισμού είναι αντικείμενο της παρούσας έρευνας. Μεταλλάξεις στο γονίδιο contactin-associated protein-like 2 (*CNTNAP2*) έχουν συσχετιστεί με την εμφάνιση του αυτισμού και συγγενών διαταραχών σε ανθρώπους και προκαλούν παρόμοιους φαινότυπους σε πειραματόζωα. Παρόλα αυτά, ο μοριακός μηχανισμός που ευθύνεται για αυτά τα φαινόμενα παραμένει άγνωστος. Η παρούσα μελέτη δείχνει πως απενεργοποίηση του γονιδίου αυτού σε μύες οδηγεί σε αύξηση της πρωτεϊνοσύνθεσης, για την οποία ευθύνεται πιθανώς η παρατηρούμενη υπερ-ενεργοποίηση του mammalian target of rapamycin complex 1 (mTORC1). Στο δεύτερο κομμάτι της εργασίας, παρουσιάζεται η χρήση μιας εδραιωμένης τεχνικής για την απομόνωση και τη μελέτη νευρικών βλαστοκυττάρων από ενήλικους μύες.

1. INTRODUCTION

1.1 Autism Spectrum Disorder

1.1.1 General description and clinical aspects

Autism Spectrum Disorder (ASD) includes a range of conditions comprised by autism, Asperger's disorder, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified. These disorders are considered to be of neurodevelopmental origin and share common phenotypical characteristics including deficits in various aspects of social interaction, limited interests, stereotyped/repetitive behaviors, and language impairment. ASDs are further characterised by an early age of onset (1 to 3 years), yet the observed symptoms are of varying severity, hence the definition of a "spectrum"[1].

ASD affects approximately 1% of the population, with a male-to-female prevalence of 4 to 1. Moreover, it is often associated with the presence of intellectual disability (~30% of ASD individuals) and epilepsy (20-25% of individuals). Other comorbid disorders include Attention-Deficit/Hyperactivity Disorder (ADHD), anxiety disorders, depression, sleep disorders, and gastrointestinal disorders.[1,2]

1.1.2 Genetics

Despite the high heritability of ASD (70-90% concordance in studies of monozygotic twins), there is no single gene responsible for its development. Instead, the disorder has a genetically complex basis, with the "rare variant – common disease" hypothesis of etiology currently being the most well suited to explain the findings of the numerous genetic and genomic studies. This model is supported by the observation of monogenic forms of ASD in about 10-15% of the affected individuals, caused by mutations of a single gene such as: Fragile-X syndrome (FXS, *FMR1*), Rett syndrome (*MECP2*), Tuberous Sclerosis Complex (TSC, *TSC1/2*), PTEN hamartoma tumor

syndrome (PHTS, *PTEN*), Angelman syndrome (*UBE3A*), Neurofibromatosis type I (NF-1, *NF1*) and familial ASD (*NLGN3/4*, *NRXN1*, *SHANK3*). [2,3]

Most of the gene products responsible for the disorders mentioned above impinge in some way on the regulation of protein synthesis in the brain. Dysregulation of this fundamental cellular process has been implicated both in human and animal studies as a critical factor leading to the development of ASD. Thus, below follows a brief overview of the mechanisms of cap-dependent mRNA translation, which is the prevalent mode of mRNA translation in most eukaryotic cells.[4]

1.2 Cap-dependent mRNA translation

1.2.1 Basic Principles

Proteins are the fundamental building blocks of all living cells and, thus, protein homeostasis (proteostasis) – including the processes of synthesis, folding, transport and degradation of proteins – is a tightly regulated aspect of cellular function.

Protein synthesis, or mRNA translation, is believed to account for approximately 20% of the cell's energy consumption, making it the most demanding of cellular processes. As such, it is subject to extensive regulation by both extracellular (e.g. nutrients, growth factors) and intracellular (e.g. amino acid availability, energy status) signals. In comparison to transcriptional control, translational modulation permits higher temporal precision in the proteome changes.[4–6]

Eukaryotic mRNA translation can be divided into different phases, namely initiation, elongation, termination and ribosome recycling. Although all of these stages are under regulatory control, the rate-limiting step of the process is thought to be translation initiation. Consequently, it is no surprise that most of the translational regulation is present at this point. Prominent in this process are the eukaryotic initiation factors (eIFs) and their complexes, some of which are going to be described below.[7]

Translation initiation itself is subdivided into four steps **(Figure 1)**:

i. Ternary complex assembly and 43S preinitiation complex formation. eIF2, GTP and Met-tRNA$_i$ assemble to form the ternary complex (TC), which subsequently associates with the 40S ribosomal subunit to create the 43S preinitiation complex (43S PIC). This is promoted by the factors eIF1, eIF1A and eIF3.

ii. Binding of the 43S PIC to the mRNA to form the 48S initiation complex. The 43S PIC attaches to the capped 5'-end of the mRNA in a process that is facilitated by the eIF4F complex. eIF4F is a heterotrimer comprised by eIF4E (which binds the m^{7}G cap), eIF4A (which is an ATP-dependent RNA helicase, responsible for unwinding of the secondary structure of the mRNA) and eIF4GI/II (a scaffolding protein mediating the formation of the eIF4F complex and the joining of the 43S PIC, mainly through its interaction with eIF3). Another important factor in this process is the poly(A)-binding protein (PABP). PABP binds the poly(A) tail at the 3'-end of the mRNA and, by interacting with eIF4G, mediates the circularization of the mRNA, which is thought to facilitate the formation of the 48S complex.

iii. Recognition of the AUG start codon. Once the 48S complex is formed, it starts "scanning" downstream (towards the 3'-end of the mRNA) until it encounters an AUG start codon. Complementary base pairing of the start codon with the anticodon of the Met-tRNA$_i$ leads to hydrolysis of the TC-bound GTP to GDP by eIF5 and arrest of the scanning mechanism.

iv. Joining of the 60S ribosomal subunit and formation of the 80S ribosome. After the hydrolysis of GTP, eIF2-GDP (along with the factors eIF1, eIF1A and eIF3) dissociate from the surface of the 40S subunit, an event that then permits the 60S ribosomal subunit to interact with the 40S and form the functional 80S ribosome. Subsequently, the ribosome proceeds to the stage of elongation, the elaboration of which is beyond the scope of this introduction.[4,6-8]

Apart from the translation initiation mechanism described above, a small group of eukaryotic mRNAs are translated via a cap-independent mechanism. This mechanism makes use of the internal ribosome entry sites (IRES), allowing the recruitment of the preinitiation complex directly to the start codon, similar to what occurs in bacteria. The IRES-dependent mode of translation initiation is often seen with viral mRNAs, helping viruses avoid some of the host defenses.[4,9]

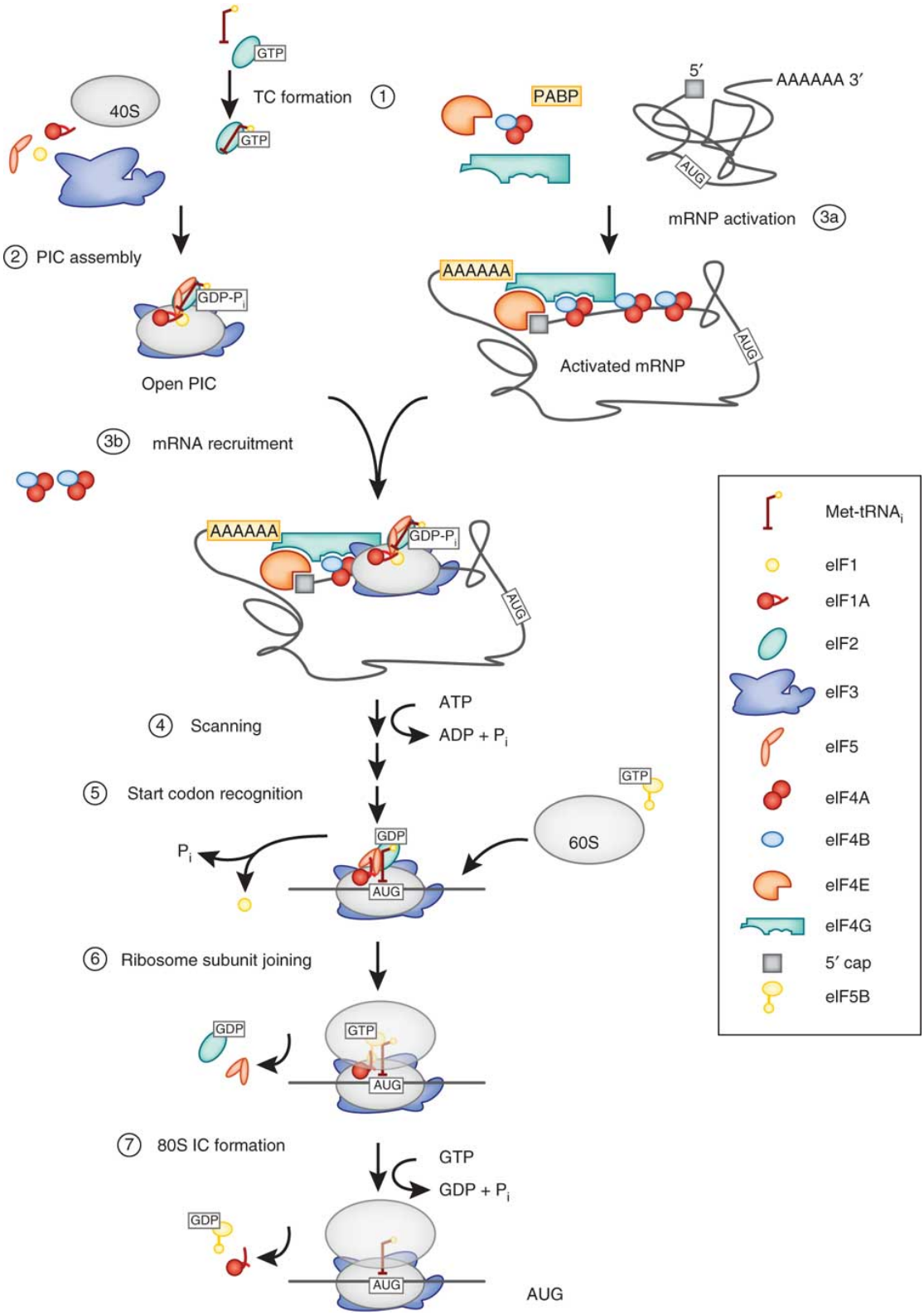

Figure 1. The mechanism of translation initiation in eukaryotes. The main processes that take place during translation initiation in eukaryotic cells are presented in this figure. Adapted from [7] .

1.2.2 Signaling pathways regulating cap-dependent translation

eIF2α phosphorylation

A major regulatory point in the process of translation initiation is the phosphorylation of eIF2α, which inhibits the recycling of eIF2 to the ternary complex and, consequently, leads to a reduction in the general translation rate (although the translation of a subset of mRNAs is upregulated instead). The phosphorylation takes place on Ser51 and is controlled by four protein kinases: (i) protein kinase R (PKR), activated by the presence of double-stranded RNA, (ii) PKR-like endoplasmic reticulum kinase (PERK), activated by misfolded or unfolded proteins in the endoplasmic reticulum, (iii) heme-regulated eIF2α kinase (HRI), activated in case of heme deficiency and (iv) general control nonderepressible 2 kinase (GCN2), activated by amino acid insufficiency and UV-irradiation. As is evident, all of the above kinases participate in the cellular response to different stressors and decrease general translation in order to preserve energy and increase the cell's chances of survival.[6,10,11]

4E-BPs and mTOR

Another crucial point where much of the translational regulation is exerted is the formation of the eIF4F complex and its interaction with the m^7G mRNA cap. eIF4E-binding proteins (4E-BPs) compete with eIF4G for binding to eIF4E (the cap-binding protein) inhibiting eIF4F formation and, thus, leading to a decrease in the translation rate of a specific subset of "eIF4E-sensitive" mRNAs[12–14]. There are three known isoforms of 4E-BP (1, 2 and 3), of which 4E-BP2 is the main isoform found in the brain.[15]

Phosphorylation of 4E-BPs by the mammalian target of rapamycin (mTOR) kinase leads to their dissociation from eIF4E and, thus, disinhibition of mRNA translation. Phosphorylation takes place in a hierarchical manner, first on the residues Thr37/Thr46 and then on Ser65/Thr70.[5,14,16]

mTOR is a central integration point for various cues, both extracellular (e.g. insulin, growth factors, hormones) and intracellular (e.g. amino acids, glucose, energy status), and regulates cellular functions as diverse as growth, proliferation, differentiation and autophagy.[17–19] The mTOR kinase forms two distinct complexes, mTORC1 and mTORC2, that have different components and downstream targets, are activated by different mechanisms and exhibit dissimilar sensitivity to drugs. mTORC1 is the more extensively studied of the two and it is responsible for the phosphorylation of the 4E-BPs. The distinctive component of mTORC1 is the scaffolding protein Raptor (replaced by Rictor in mTORC2), which in large part confers the substrate specificity

to the complex. Moreover, mTORC1 is sensitive to inhibition by rapamycin, whereas mTORC2 is largely rapamycin-insensitive, unless a longer period of treatment is applied.[20]

mTORC1 lies downstream of the phosphatidylinositol 3-OH kinase (PI3K)/Akt (known also as PKB) pathway which is activated by extracellular stimuli such as growth factors and insulin. Sequential activation of PI3K, PI3K-dependent kinase 1 (PDK1) and Akt leads to phosphorylation and inactivation of the tuberous sclerosis complex (TSC, consisting of TSC1 and TSC2 proteins). This, in turn, releases a small G-protein, Ras-homolog enriched in brain (Rheb), from inhibition by TSC1/2. Rheb is then free to activate mTORC1.[21] Conversely, mTORC1 activity is suppressed by phosphatase and tensin homolog (PTEN), which is a phosphatase that catalyzes the conversion of phosphatidylinositol-3,4,5-triphosphate (PIP_3) to the biphosphate product (PIP_2). Thus, it reverses the reaction carried out by PI3K, downregulating the whole downstream pathway. Another negative regulator of mTORC1 is 5' AMP-activated protein kinase or AMPK. AMPK is activated under conditions of cellular energy deficiency and inhibits mTORC1 by activating TSC2 and phosphorylating Raptor, which leads to its sequestration. This serves to suppress protein synthesis and inhibit energy-demanding processes such as growth and proliferation.[5]

Apart from 4E-BPs, mTORC1 also influences mRNA translation through the phosphorylation and activation of the ribosomal protein S6 kinases (S6Ks, 1 and 2). Substrates of the S6Ks include the ribosomal protein S6 (rpS6), eIF4B (phosphorylation of which promotes the recruitment of the 43S PIC to the mRNA), programmed cell death protein 4 (Pdcd4, which inhibits eIF4A activity and phosphorylation of which targets it for degradation) and eukaryotic elongation factor 2 kinase (eEF2K, phosphorylation and deactivation of which promotes the elongation stage of translation).[4,5,22]

Other pathways

Another pathway that modulates cap-dependent translation is the mitogen-activated protein kinase (MAPK) pathway. In response to various mitogens the MAPK cascade is activated, leading to sequential phosphorylation and activation of Ras, Raf, MEK and ERK. Apart from the other diverse effects on cellular function, this pathway results in the activation of the MAPK-interacting kinases 1 and 2 (Mnk1/2). The Mnks, in turn, phosphorylate eIF4E on Ser209, an event that is thought to stimulate the translation of at least a subset of mRNAs.[23–25]

Another important regulator of cap-dependent translation is the Fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein that represses translation through its interaction with the cytoplasmic FMRP-interacting protein 1 (CYFIP1). CYFIP1 disrupts the assembly of the eIF4F complex in a manner analogous to the 4E-BPs, as it binds to eIF4E and competitively inhibits its binding to eIF4G.[4,26]

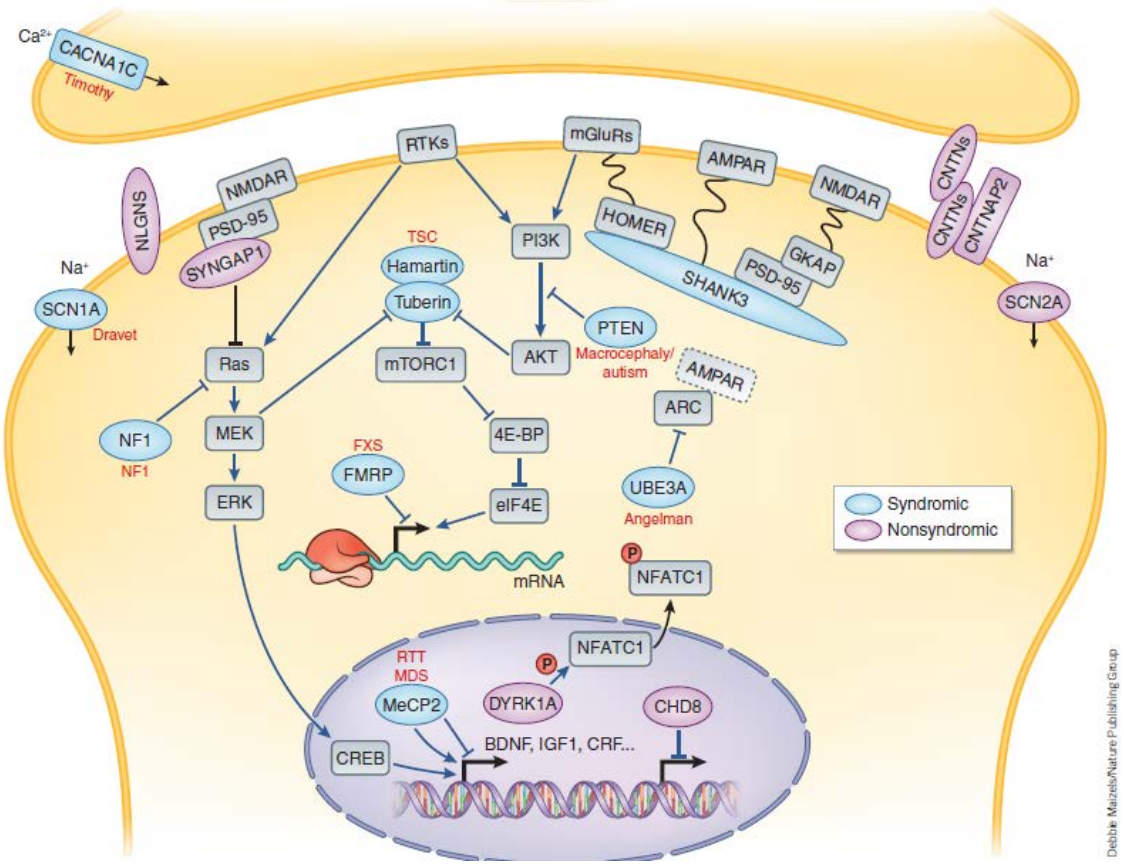

Figure 2. Intracellular signaling pathways regulating mRNA translation. Some of the pathways discussed in the main text above, such as the PI3K/Akt/mTOR and Ras/MAPK pathways, are depicted here. Also, some of the synaptic proteins implicated in ASD (NLGNs, SHANK3, CNTNAP2, SYNGAP1) that are described in section 1.3 are also presented in the figure. Adapted from [27]

1.2.3 Dysregulation of mRNA translation in disease

The significance of the precise regulation of cap-dependent mRNA translation for correct physiological function is evidenced by the numerous disorders that arise when it is aberrant. Apart from its repeated implication in various forms of cancer, there are many examples of neuropsychiatric conditions that result from dysregulation of protein synthesis.[4,23,24,28] Some of these are briefly described below.

Fragile X Syndrome (FXS) and Tuberous Sclerosis Complex (TSC) are both syndromic forms of autism accompanied by intellectual disability. FXS is the leading ge-

netic cause of autism and one of the most prevalent causes of intellectual disability. Patients exhibit behavioural, cognitive and physical abnormalities.[29] FXS is caused by mutations in the *FMR1* gene, located on the X chromosome, that lead to its silencing.[30] Additionally, loss of FMRP in a mouse knock-out (KO) model leads to excessive protein synthesis in hippocampus, thalamus and hypothalamus.[3,31]

TSC can be caused by mutations in either of the two components of the protein complex, TSC1 (hamartin) or TSC2 (tuberin). There is a high prevalence of ASD in patients with TSC and other neuropsychiatric disorders associated with TSC include intellectual disability, ADHD, depression, anxiety and epilepsy. Loss-of-function mutations in TSC1/2 lead to hyperactivation of the mTORC1 pathway and, consequently, exaggerated mRNA translation. This has been shown to give rise to diverse abnormalities in brain function, ranging from developmental defects in neuronal growth and migration to impairments in synaptic function and intracellular signaling.[3,32,33]

Two additional conditions associated with ASD that result from inactivation of negative regulators of protein synthesis are neurofibromatosis type 1 (NF1) and PTEN hamartoma tumor syndrome (PHTS). Although the proteins involved are part of different upstream pathways (NF1 inhibits the MAPK pathway, whereas PTEN suppresses the PI3K/Akt pathway), the effects of their inactivation in mice are similar, including deficits in learning and memory, as well as impairment in long term potentiation (LTP).[33–36]

Finally, the role of eIF4E and the 4E-BPs (4E-BP2 specifically) in the etiology of pathophysiological and behavioural changes associated with ASD has been extensively investigated. Early studies showed that in mice lacking 4E-BP2 LTP was enhanced after one train of high-frequency stimulation compared to wild-type (WT) controls, whereas there was an impairment of LTP induced by a stronger stimulus. Moreover, these mice performed poorly in the Morris water maze (MWM) test that assesses spatial memory as well as in tests of motor coordination and balance. Working memory was also found to be affected in these mice.[15,37] A later study identified impairments in social behaviour and increased stereotyped/repetitive behaviours in 4E-BP2 KO mice, results that parallel some of the behavioural abnormalities seen in ASD patients. The same study discovered an alteration of the excitation to inhibition ratio in the hippocampus. Although both excitatory and inhibitory activity were found to be increased, their relative change favored excitation.[38] Lastly, in a mouse model where eIF4E was overexpressed (essentially producing the same effect as deleting 4E-BP2) there were similar findings with respect to social interactions and repetitive behaviours. Alterations were also seen in both the basal electrophysiological properties in the medial prefrontal cortex (mPFC) of these mice and the long term depression (LTD) in striatum and hippocampus.[39]

1.3 Synaptic dysfunction in ASD

1.3.1 Excitation/inhibition imbalance in ASD

A recurrent finding in mouse models of ASD has been an increased ratio of excitation to inhibition.[40–43] Additionally, there is a high comorbidity of ASD with epilepsy.[1] Taken together, these findings led to the initial hypothesis that ASD arises due to excessive excitatory drive onto certain brain networks involved in the observed behavioural aberrations.[44] However, this view might be too simplistic, as more recent studies have provided evidence that there are also cases of elevated inhibitory activity and/or decreased excitation.[45–48] These findings suggest that deviation from the normal balance of activity in the brain, irrespective of its direction, may produce the physiological and behavioural abnormalities observed in ASD and related disorders. It has been recently proposed that the eventual effects are similar in both cases due to the organism's attempt to maintain network homeostasis.[49]

1.3.2 Protein homeostasis and synaptic dysfunction

A conclusion that can be drawn from the aforementioned studies is that regardless of the gross network effects that may arise (excitation/inhibition balance), synaptic dysfunction appears to be at the core of ASD pathophysiology. The synapse is the major point of communication in the nervous system and it is not difficult to imagine how widespread the effects of synaptic transmission aberrations are.

This dysfunction might be caused by abnormalities in the homeostasis of proteins involved in the normal synaptic function, for example due to changes in their translation or degradation rate. Indeed, it has been shown that proteins associated with ASD have an important effect on synaptic function. FMRP regulates the translation of many synaptic mRNAs – including metabotropic glutamate receptor type 5 (mGluR5) and N-methyl-D-aspartate receptor (NMDAR) subunits, as well as many structural proteins of both the pre- and postsynapse.[50] On the other hand, ubiquitin-protein ligase E3A (UBE3A), whose loss of function gives rise to Angelman syndrome (a characteristic of which is a high prevalence of autism), has been shown to be responsible

for the ubiquitination of the immediate early gene Arc. Among other functions, Arc regulates the trafficking of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate type glutamate receptors (AMPARs). Thus, a loss of UBE3A leads to an increase of Arc and a consequent decrease of AMPARs in the postsynaptic membrane.[51]

1.3.3 Synaptic proteins implicated

Apart from these examples, however, genetic studies have also implicated genes whose products are directly involved in the structure and function of synapses. Ion channels, neurotransmitter receptors, scaffolding proteins and cell adhesion molecules (CAMs) have all been associated in some way with the etiology of ASD. Some examples are discussed below.

Neuroligins and neurexins

Neuroligins (NLGNs) are a family of cell adhesion proteins that are localized postsynaptically and form a trans-synaptic complex with their presynaptic receptors, neurexins (NRXNs, which have been also found postsynaptically). The NLGN-NRXN complex has been shown to be important for proper synaptic function, but not for initial formation of synaptic contacts *in vivo*.

NLGNs and NRXNs accomplish their role of organizing the pre- and postsynaptic compartments by interacting with scaffolding proteins, such as postsynaptic density protein 95 (PSD95, found in excitatory synapses), gephyrin (inhibitory synapses), and others. NLGN1 and NLGN2 are associated with excitatory and inhibitory synapses respectively, while NLGN3 and NRXNs are found in both types of synapses.

Various mutations in the *NLGN3* and *NLGN4* genes have been repeatedly identified in patients with conditions ranging from ASD and intellectual disability to ADHD and Tourette's syndrome. Animal studies with mice carrying mutations in one of the *Nlgn1-3* genes have discovered social interaction deficits and memory impairments, as well as changes in the excitation/inhibition balance, which are to be expected considering the function of these proteins. Additionally, mutations in the *NRXN* genes, particularly *NRXN1*, have been associated mainly with schizophrenia, but also with ASD and intellectual disability.[52–56]

SHANKs

SH3 and Ankyrin-domain-containing proteins (Shanks) are large scaffolding proteins localized at the postsynaptic density (PSD) of excitatory synapses. All members of the family (Shank1-3) are highly expressed in the brain and are critical for the for-

mation and maturation of dendritic spines. They organize the PSD by forming huge protein complexes with other synaptic proteins. Interactors of Shanks include AMPA, NMDA and metabotropic glutamate receptors, cytoskeletal proteins, as well as other scaffolding proteins.

Mutations in the *SHANK3* gene are responsible for the Phelan-McDermid syndrome (PMS), which shares some behavioural phenotypes with ASD. Moreover, mutations in all three *SHANK* genes have been implicated in the etiology of various conditions, including ASD, intellectual disability, ADHD and schizophrenia. *SHANK3* is the gene most heavily associated with the occurrence of ASD.

Loss of Shank3 function leads to various ASD-like phenotypes, including social interaction deficits, increases in repetitive/stereotyped behaviours, and impairments in communication and cognitive function. Also, loss of excitation/inhibition balance is a regular finding, along with changes in the expression of glutamate receptors and impairments in LTP.[55–57]

SYNGAP1

Synaptic Ras-GTPase-activating protein (SYNGAP1) is expressed exclusively in the brain, predominantly in synapses of excitatory neurons. It interacts with proteins of the postsynaptic density and participates in the same multi-protein complex as NMDARs. SYNGAP1 activates the GTPase activity of Ras and Rap leading to their inactivation, modulating thus their downstream pathways. These regulate the MAPK pathway, which has been discussed previously, the trafficking of AMPARs and the organization of the actin network in dendritic spines. Consequently, SYNGAP1 activity is critical for the maturation and general function of dendritic spines.

Several mutations have been identified in the human *SYNGAP1* gene, most of which are implicated in the etiology of intellectual disability. However, its involvement has been also found in ASD, schizophrenia and epileptic disorders.

Mice homozygous for the deletion of the gene do not survive long after birth, but heterozygous mice (*SYNGAP*$^{+/-}$) exhibit deficits reminiscent of those seen in human patients. Abnormal social interactions, increased repetitive/stereotyped behaviours, impairments of spatial and working memory, reduced prepulse inhibition (PPI), and increased susceptibility to seizures have all been observed in this mouse model. At the same time, *SYNGAP*$^{+/-}$ mice present alterations in the rate of spine maturation, LTP impairments and a general imbalance in the excitation/inhibition ratio.[58,59]

1.3.4 CNTNAP2

Contactin-associated protein-like 2 (CNTNAP2 or Caspr2) is a large (~150 kDa) transmembrane protein, part of a group of contactin-associated proteins (CNTNAP1-5) that are structurally related to the neurexin group of CAMs. Caspr2 has a well-established role in the neuron-glia interactions that are critical for the correct organization of the nodes of Ranvier in myelinated axons. Myelination is a very important process that allows the saltatory conduction of action potentials along axonal processes, a function which requires properly organized nodes of Ranvier. Specifically, Caspr2 interacts with contactin-2 (also known as TAG-1) and this interaction is responsible for the clustering of potassium channels at the juxtaparanodal domain of the nodes of Ranvier.[60–62] However, apart from its role in myelination, which takes place postnatally, CNTNAP2 expression during embryonic development suggests that it has additional functions in the brain.[63]

CNTNAP2 is the largest gene in the human genome (spanning 2.3 Mb) and is highly expressed in a circuit including the prefrontal cortex, the striatum and the thalamus, a network known to be involved in cognitive functions.[63] Mutations in this gene were first associated with Tourette's syndrome and obsessive compulsive disorder (OCD).[64] Subsequently, mutations were identified in patients with a condition called cortical dysplasia-focal epilepsy (CDFE). CDFE is considered to be a syndromic form of ASD and is characterized by epileptic seizures, language impairments, intellectual disability, hyperactivity, and a high prevalence of ASD. Additionally, aberrations in neuronal migration were observed in these patients.[65] Subsequently, a large number of genetic studies have associated mutations in *CNTNAP2* with an array of neuropsychiatric disorders, including various language disorders, ASD, epilepsy, intellectual disability, schizophrenia, depression, and ADHD.[66–68] Notably, the effects of CNTNAP2 on normal language development seem to be particularly pronounced, a finding made more intriguing by the fact that *CNTNAP2*'s transcription is regulated by the factor forkhead box P2 (FOXP2), whose role in human language evolution has been extensively studied.[69]

Animal models of *CNTNAP2* disruption have identified many physiological and behavioural phenotypes that parallel the abnormalities seen in human patients. Homozygous knock-out of the gene in mice resulted in epileptic seizures, abnormalities in neuronal migration and reduction of γ-amino butyric acid (GABA)-ergic interneurons in various areas of the brain. Network abnormalities were also observed, with the synchronicity of neuronal firing being decreased. Moreover, these mice exhibited deficits in social interactions and communication, as well as increased repetitive/stereotyped behaviours.[70] CNTNAP2 and its binding partner contactin-2 have been found to be present in the synaptic fraction of brain lysates.[71] Accordingly, neu-

ronal culture and *in vivo* studies have identified a role for CNTNAP2 in dendritic spine development and stability, along with dendritic arborisation.[72,73] These functions are consistent with the synaptic pathology observed in previously described models of ASD and point to a common pathophysiology accounting for the effects of mutations in most genes associated with the disorder.

The *CNTNAP2* knock-out model might also be useful in the discovery of novel therapeutic approaches to ASD. Already, risperidone, which is being used for the treatment of ASD, has been shown to reduce the repetitive behaviours observed in the mouse model, in accordance with what is seen in humans.[70] Furthermore, oxytocin, a neuropeptide with a known involvement in various aspects of social behaviour, appears able to rescue some of the social interaction deficits seen in *CNTNAP2* mutants.[74,75]

1.4 Hypotheses – Aims

1.4.1 CNTNAP2

Despite the numerous genetic studies and the more recent studies involving *CNTNAP2*-KO mice, the precise function of CNTNAP2, apart from the one in myelination, remains elusive. Furthermore, the mechanism which leads to the physiological and behavioural aberrations observed in both human patients and animal models remains largely unknown.

It is reasonable to assume that the defects in neuronal migration and dendritic spine stability observed in *CNTNAP2* mutants might lead to the identified alterations in the excitation/inhibition balance.[70,72] This, in turn, could lead to the increased appearance of epileptic seizures in these animals.[70] As was previously described, shifts in the excitation/inhibition ratio arise frequently due to mutations in genes that encode proteins involved in synaptic function and the regulation of protein synthesis.

Thus, we hypothesize that *CNTNAP2* mutations might result in synaptic dysfunction and dysregulation of mRNA translation, which have been repeatedly associated with ASD and related neuropsychiatric disorders. Since these cellular functions are

tightly connected and regulated, it is easy to imagine how a small initial aberration in either one might precipitate a vicious circle eventually leading to the observed phenotypes. However, it would be more challenging to establish which of these is a primary effect of the loss of CNTNAP2 and which a secondary consequence.

In the present study, we aimed to identify whether homozygous knock-out of *CNTNAP2* in mice leads to alterations in the global rate of protein synthesis, similarly to what is seen in other models of ASD. Additionally, we examined whether the cap-dependent regulation of translation, specifically the formation of the eIF4F complex, was affected in these mutants. Finally, we investigated the potential molecular mechanisms responsible for the manifestation of the abnormalities described above.

1.4.2 4E-BP2 deamidation

4E-BP2 is the major isoform of the eIF4E-binding proteins expressed in the mammalian brain. However, phosphorylation of 4E-BP2 could not be detected in the adult mouse brain. Instead, deamidation of two asparagine residues (Asn99 and Asn102) to aspartates was identified.

Deamidation is the spontaneous conversion of glutamine and asparagine residues to glutamate and aspartate, respectively, by the removal of an amide group. Deamidation has been shown to occur in multiple proteins with rates depending on the amino acid sequence of the protein, the pH of the environment and the temperature. It has been proposed that deamidation functions as a “molecular clock”, regulating the lifespan of proteins and other biological events.[76]

It was discovered that the deamidated form of 4E-BP2 shows a greater association with Raptor and that this interaction is responsible for reduced binding of the deamidated 4E-BP2 to eIF4E. Furthermore, in cell culture experiments, it was shown that deamidation diminishes the ability of 4E-BP2 to repress translation of eIF4E-sensitive mRNAs. Finally, the same study discovered that the processes of phosphorylation and deamidation of 4E-BP2 exhibit opposite trajectories in the postnatal brain (the first decreasing and the latter becoming more evident with time) with approximately the same time course. Notably, these changes parallel the decline seen in the activation of the PI3K/Akt pathway during the same period.[77]

Significance for adult neurogenesis

Among the many questions that arise, one that we found interesting concerns the role of 4E-BP2 deamidation in adult neurogenesis. It has been well established that neurogenesis persists in certain regions of the adult mammalian brain, the most

prominent of which are the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus (DG), which is part of the hippocampal formation.[78]

There has been sufficient evidence that the PI3K/mTOR/4E-BP pathway is directly involved in the control of proliferation versus differentiation of neural stem cells. However, the reports seem to be conflicting in some cases. For example, it has been shown that increasing the expression of Akt (which induces the activity of mTORC1) leads to a robust increase of adult hippocampal neural progenitor cell (NPC) proliferation at the expense of differentiation into neurons or glia.[79] Another study found that activation of Akt is decreased with neuronal differentiation, which is consistent with the previous study.[80] On the other hand, various studies show that mTORC1 activation induces differentiation of the NSCs into an intermediate cell type, transient amplifying cells (TACs), which have a high but finite proliferative capacity before terminally differentiating. Apparently, this happens at the cost of NSC self-renewal and leads to depletion of their population.[19,81] Moreover, it was demonstrated that mTOR regulates cell cycle dynamics and that this ability is dependent on the 4E-BPs (as opposed to mTOR's effects on cell size for example, which are mediated through the S6 kinases).[18,82] Combined with the finding that the cell cycle length – particularly the length of the G1 phase – heavily influences cell fate (as in NSC self-renewal versus neurogenesis), the above data suggest a direct role for mTOR and 4E-BP2 in the regulation of adult neurogenesis.[83]

Taking into account the postnatal "switch" of 4E-BP2 phosphorylation to deamidation in the brain, it is intriguing to investigate whether this alteration might have a functional role in the control of adult neurogenesis. Finally, there is already some evidence linking neurogenesis to the etiology of ASD.[84] Additionally, it seems safe to assume that there is some ongoing pathology during adulthood, since treatment during this period can rescue some of the aberrations seen at least in animal models of ASD.[85] Taking these together, discovering the involvement of 4E-BP2 deamidation in the process of adult neurogenesis, and the implication of the latter in ASD pathophysiology, carries promise regarding potential future treatments for the disorder.

2. MATERIALS AND METHODS

2.1 Animals

All procedures involving mice were carried out in accordance with UK Home Office guidelines set out in the Animals (Scientific Procedures) Act 1986. Wild-type (*Cntnap2*$^{+/+}$, WT) and knock-out (*Cntnap2*$^{-/-}$, KO) mice from the *B6.129(Cg)-Cntnap2*^{tm1Pele}*/J* strain (The Jackson Laboratory, [60]) were used for the CNTNAP2 experiments. WT C57BL/6J mice were used for the neurosphere culture generation. Food and water were available ad libitum and mice were kept on a 12-h light/dark cycle.

2.2 Reagents

Chemicals and drugs used in the present work were purchased from Sigma-Aldrich, unless otherwise indicated.

2.3 SUnSET assay

The surface sensing of translation (SUnSET) method which allows the assessment of general protein synthesis has been previously described[86]. It takes advantage of puromycin incorporation into newly synthesized proteins (when present in low concentrations) to quantify the rate of their production. Briefly, acute hippocampal slices were obtained in ice-cold cutting artificial cerebrospinal fluid (cCSF: 86mM NaCl, 1.2mM NaH_2PO_4, 2.5mM KCl, 25mM $NaHCO_3$, 25mM glucose, 75mM sucrose, 0.5mM$CaCl_2$, 7mM $MgCl_2$) using a Leica VT 1200S vibratome and allowed to recover for 45min at 32^{o}C in an incubation chamber which contained normal aCSF (124mM NaCl, 1.2 mM NaH_2PO_4, 2.5mM KCl, 25mM $NaHCO_3$, 20mM glucose, 2mM $CaCl_2$, 1mM $MgCl_2$) and was equilibrated with 95% O_2/ 5% CO_2. Afterwards, half of the slices from each animal were incubated in a 5µg/ml puromycin dihydrochloride in aCSF solution for another 45min, while the rest were kept in normal aCSF. Finally, at the end of the incubation period, the slices were recovered and snap frozen in liquid nitrogen.

2.4 Immunoblotting

2.4.1 Lysis – homogenization. Tissues were lysed and homogenized in **RIPA Buffer** (10mM Tris pH 8.0, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS, 140 mM NaCl) plus protease and phosphatase inhibitors (Roche), except for the cap-pull down assay (described below). Lysates were left on ice for ~15min, occasionally vortexed gently, before centrifugation at 16.000xg for 20min at 4°C. Afterwards, the supernatant from each sample was transferred to a new tube and kept at -80°C prior to use.

2.4.2 Protein concentration. The concentration of the lysates was measured using the Bradford assay (Bio-Rad), so that equal amounts of each sample could be loaded in each well of the gel.

2.4.3 Running. The samples were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in **1x SDS-PAGE Loading Buffer** (50mM Tris pH 6.8, 100mM DTT, 2% SDS, 10% glycerol, 0.1% bromophenol blue) and run in a 10, 12 or 16% acrylamide gel, depending on the size of the target proteins. The run was carried out at 90V in **1x Running Buffer** (25mM Tris, 190mM glycine, 0.1% SDS)

2.4.4 Transfer. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Bio-Rad) in **1x Transfer Buffer** (25mM Tris, 190mM glycine, 20% methanol) at 30V overnight at 4°C. The quality of the transfer was checked with Ponceau S solution staining.

2.4.5 Blocking. The membrane was incubated for 1h in **blocking solution**, 5% milk in **TBS-T solution** (10mM Tris pH 7.5, 150mM NaCl, 0.1% Tween 20), at room temperature.

2.4.6 Primary antibodies. The membrane was washed three times with TBS-T solution before being incubated with the primary antibody at 4°C overnight. The primary antibody was diluted 1:1000, unless otherwise specified, in 1% bovine serum albumin (BSA, Cell Signaling) in TBS-T solution. Primary antibodies against the following proteins were used: puromycin and Caspr2 (Merck Millipore); 4E-BP2, phospho-4E-BP (Thr37/46), phospho-eIF4E, phospho-S6 (Ser240/244), p44/42 MAPK and phospho-MAPK (Thr202/204) (Cell Signaling); HSC70, eIF4E, and S6 (Santa Cruz).

2.4.7 Secondary antibodies. After three more washes, the membrane was incubated with the secondary antibody, which was anti-rabbit or anti-mouse IgG, horseradish peroxidase (HRP)-linked (Cell Signaling), for 1h at room temperature. The secondary antibody was diluted 1:5000 in blocking solution.

2.4.8 Detection. The membrane was washed three times and was then incubated either with Pierce ECL (Thermo Scientific) or Clarity ECL (Bio-Rad) substrate for enhanced chemiluminescence. The membrane was then ex-

posed to a CL-XPosure film (Thermo Scientific) for an appropriate amount of time and the film was developed.

2.5 m^{7}GTP pull-down assay

The tissue was lysed in **Buffer A** (50mM MOPS/KOH pH 7.4, 100mM NaCl, 50 mM NaF, 2mM EDTA, 2mM EGTA, 1% NP40, 1% sodium deoxycholate, 7 mM BME + protease and phosphatase inhibitors (Roche)) using a 2ml glass homogenizer. The lysate was left on ice for 15min with occasional vortexing and then centrifuged at 16.000xg for 15min at 4^{o}C. The supernatant was transferred to a new tube and the protein concentration was measured using a NANODROP 2000 spectrophotometer (Thermo Scientific). A sample of the lysate was kept at -80^{o}C for SDS-PAGE (**Whole lysate)**.

The m^{7}GTP agarose beads (Jena Biosciences) were washed three times with **Buffer B** (50mM MOPS/KOH pH 7.4, 100mM NaCl, 50 mM NaF, 0.5mM EDTA, 0.5 mM EGTA, 7 mM BME, 0.5 mM PMSF (Thermo Scientific), 1mM Na_3VO_4 and 0.1mM GTP) and collected.

For each sample, 500µg of measured protein was added to 50µl of washed m^{7}GTP beads and the total volume was brought to 1ml with Buffer B. The mixture was incubated for 1.5h at 4^{o}C on a rotating wheel. After centrifugation, the supernatant (not containing beads) was kept at -80^{o}C **(Not bound fraction)**.

The beads were washed three times with Buffer B and, after the final wash, the supernatant is discarded. An equal volume of 2x SDS-PAGE loading buffer was added to each tube and the sample was boiled to achieve elution of the m^{7}GTP-bound proteins. Finally, the samples were kept at -20^{o}C prior to immunoblotting **(Cap-bound fraction)**.

2.6 Neurosphere cultures

Neurospheres are the clusters, consisting of NSCs and their progeny, which form when neurogenic areas of the mammalian brain are dissociated and cultured in a serum-free and growth factor-supplemented environment. Neurosphere cultures are one of the two ways – the other being adherent monolayer cultures – to investigate NSC function (e.g. proliferation/differentiation) *in vitro*. They were first described in 1992[87] and are now considered to represent a reasonably accurate model of the *in vivo* neurogenic niche of the NSCs.[88]

We generated neurosphere cultures according to the protocol described in [89] Briefly the process is as follows:

i. SVZ/DG microdissection. The animal was killed by cervical dislocation and the brain was removed. The target areas (SVZ and DG) were dissected under a stereoscope. The whole process was done in ice-cold 1x phosphate buffer saline (PBS).

ii. SVZ dissociation and culture. The tissue was minced with a scalpel blade for ~1min and then incubated in 1ml of 0.05% Trypsin-EDTA (Thermo Scientific) for 7min at 37°C. Then, 1ml of 0.125 mg/ml trypsin inhibitor containing 0.01 mg/ml DNaseI (in NEUROBASAL medium) was added to stop the reaction. Afterwards, the suspension was centrifuged at 300xg for 5min and the resulting pellet was resuspended in 1ml of **culture medium** (see below for composition). The cells were dissociated by gentle pipetting and culture medium was added to a total volume of 5ml. Then, the suspension was passed through a 40μm cell strainer to clear away undissociated tissue and centrifuged at 300xg for another 5min. Finally, the resulting pellet was resuspended in 200μl of culture medium, diluted to a total volume of 20ml with culture medium and plated across a 96-well plate (200μl/well). The cells were cultured at 37°C with 5% CO_2.

iii. DG dissociation and culture. The dissected DG was minced in the same way and incubated in 1ml of prewarmed **PDD enzyme mix** (see below) for 20min at 37°C, with regular mixing. The tissue was dissociated by gentle pipetting and incubated for another 10min at 37°C. The dissociating step was repeated and the suspension was centrifuged at 200xg for 5min. After resuspending the pellet in 1ml of **buffer solution** (described below), the total volume was brought up to 10ml with the same solution and centrifugation at 200xg for 5min followed. The resulting pellet was resuspended in 5ml of 20% Percoll (GE Healthcare) and re-centrifuged at 500xg for 15min. After resuspension of the pellet in 10ml of buffer solution, another 5min centrifugation at 200xg followed. Finally, the pellet was resuspended in 200μl culture medium, diluted to 20ml and cultured as described for the SVZ culture.

iv. Solutions.

- **Culture Medium:** NEUROBASAL Medium, 2% B-27, 1x GlutaMAX (Thermo Scientific), 2μg/ml heparin (Stemcell Technologies), 50 units/ml Penicillin/Streptomycin (Thermo Scientific), 20 ng/ml purified mouse receptor-grade epidermal growth factor (EGF), and 20 ng/ml recombinant bovine fibroblast growth factor (FGF-2) (PeproTech).
- **PDD enzyme mix:** Papain 2.5 U/ml, DNaseI 250 U/ml (Sigma-Aldrich) and Dispase 1 U/ml (Stemcell Technologies) in NEUROBASAL medium.

- **Buffer solution:** 1x HBSS, 2 mM HEPES (pH 7.4) (Thermo Scientific), 30 mM Glucose and 26 mM NaHCO3 (Sigma-Aldrich).

2.7 Image analysis

Images were analyzed using ImageJ (NIH).

3. RESULTS

3.1 *CNTNAP2* knock-out increases general mRNA translation

To assess global mRNA translation rate in hippocampal slices from 6 to 8-week-old WT and *CNTNAP2*$^{-/-}$ mice, we used the SUnSET assay as a measure of de novo protein synthesis, as previously described.[86] Puromycin incorporation was higher in KO animals compared to WT controls, without reaching the statistical significance level we defined as α=0.05, although a clear trend was observed **(Figure 3)**. These results suggest that *CNTNAP2* knock out leads to an increase in global protein synthesis in these mice. However, a larger sample size is required to reach a definitive conclusion.

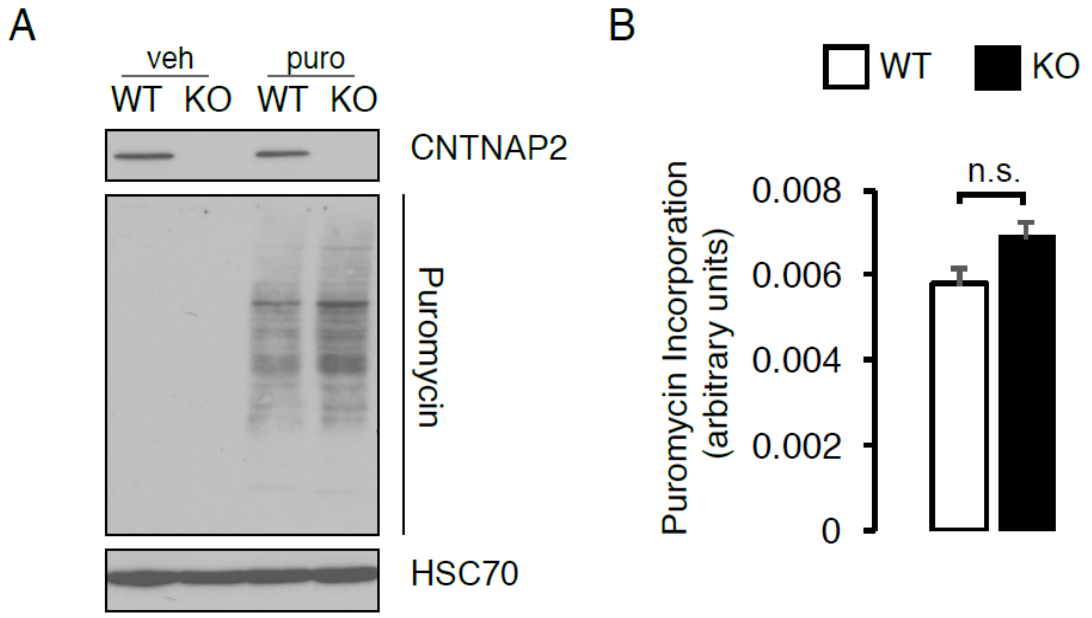

Figure 3. A) Western Blot analysis of hippocampal slice lysates from WT and *CNTNAP2*-KO mice, treated with vehicle (veh) or puromycin (puro). Representative immunoblots, probed with antibodies against Cntnap2 and puromycin, are shown. Heat shock cognate 70 kDa (Hsc70) was used as a loading control. **B)** Quantification of immunoblots from **A** for puromycin incorporation, normalized to the loading control. n=8 for WT and n=10 for KO, two-tailed t-test with equal variances assumed returned a p-value of 0.0533. The bars represent mean ± SEM.

3.2 mRNA cap complex formation

To further investigate whether the observed changes in global translation were due to alterations in cap-dependent mRNA translation regulation we examined eIF4F complex formation. We performed a pull-down assay with immobilized γ-Aminophenyl-m^{7}GTP agarose beads on lysates from cortex and cerebellum of adult (6-8 weeks) WT and *CNTNAP2*$^{-/-}$ mice. The whole lysates (**Input**) and the cap-bound fractions (**m^{7}GTP-agarose pulldown**) were analyzed by Western Blotting and subsequently probed for components of the eIF4F complex as shown in **Figure 4**. However, HSC70, which is not known to interact with the mRNA cap or any eIF4F components, was also detected in the m^{7}GTP-bound fraction. This finding indicates that the results of the present experiment are not reliable and the procedure requires optimization to ensure the purity of the m^{7}GTP-bound fraction.

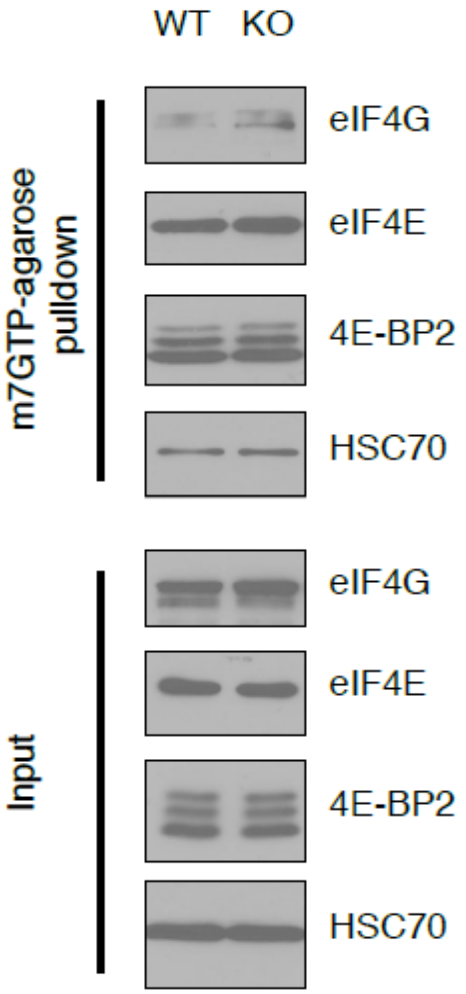

Figure 4. Immunoblotting analysis of cap-bound **(m^{7}GTP-agarose pulldown)** and whole lysates **(Input)** from cortices of WT and KO animals. Representative images, in which the membranes were probed with antibodies against the specified proteins, are shown. Roughly the same results were observed with cerebellum tissue lysates (data not shown).

3.3 Increased activation of mTORC1 targets, but not the MAPK pathway in *CNTNAP2*$^{-/-}$ mice

To elucidate the molecular mechanisms responsible for the effects of *CNTNAP2* mutations on mRNA translation, we focused initially on two major signaling pathways known to modulate cap-dependent mRNA translation, the MAPK and the PI3K/mTORC1 pathways.

The MAPK pathway, through its downstream effectors Mnk1/2, leads to phosphorylation of eIF4E on Ser209. This leads to an increase in translation of a subset of mRNAs.[23] However, Western Blotting analysis of lysates from WT and KO cortices did not reveal significant differences in phosphorylation of either p44/42 MAPK on Thr202/204, or eIF4E on Ser209 **(Figure 5,** bottom graphs**)**. These results suggest that the MAPK is not activated in the absence of CNTNAP2.

mTORC1, which lies downstream of the PI3K/Akt pathway, is considered a "master regulator" of mRNA translation. This role is achieved mainly through phosphorylation of the 4E-BPs, an event that leads to de-repression of mRNA translation.[13] mTORC1 activation also leads to phosphorylation of the ribosomal protein S6 (rpS6). Levels of phosphorylated rpS6 are considered as a good measure of mTORC1 activation and they are found consistently elevated in neurodevelopmental disorders such as FXS and TSC. However, the precise role of rpS6 in mRNA translation regulation remains unknown.[90] Here we show that phosphorylation of both 4E-BP2 (on Thr37/46) and rpS6 (on Ser240/244) is elevated in *CNTNAP2*$^{-/-}$ mice compared to control animals. **(Figure 5,** top graphs**)**

Taken together, these results suggest that in *CNTNAP2*$^{-/-}$ animals mTORC1 is hyperactivated, while the MAPK pathway remains apparently unaffected.

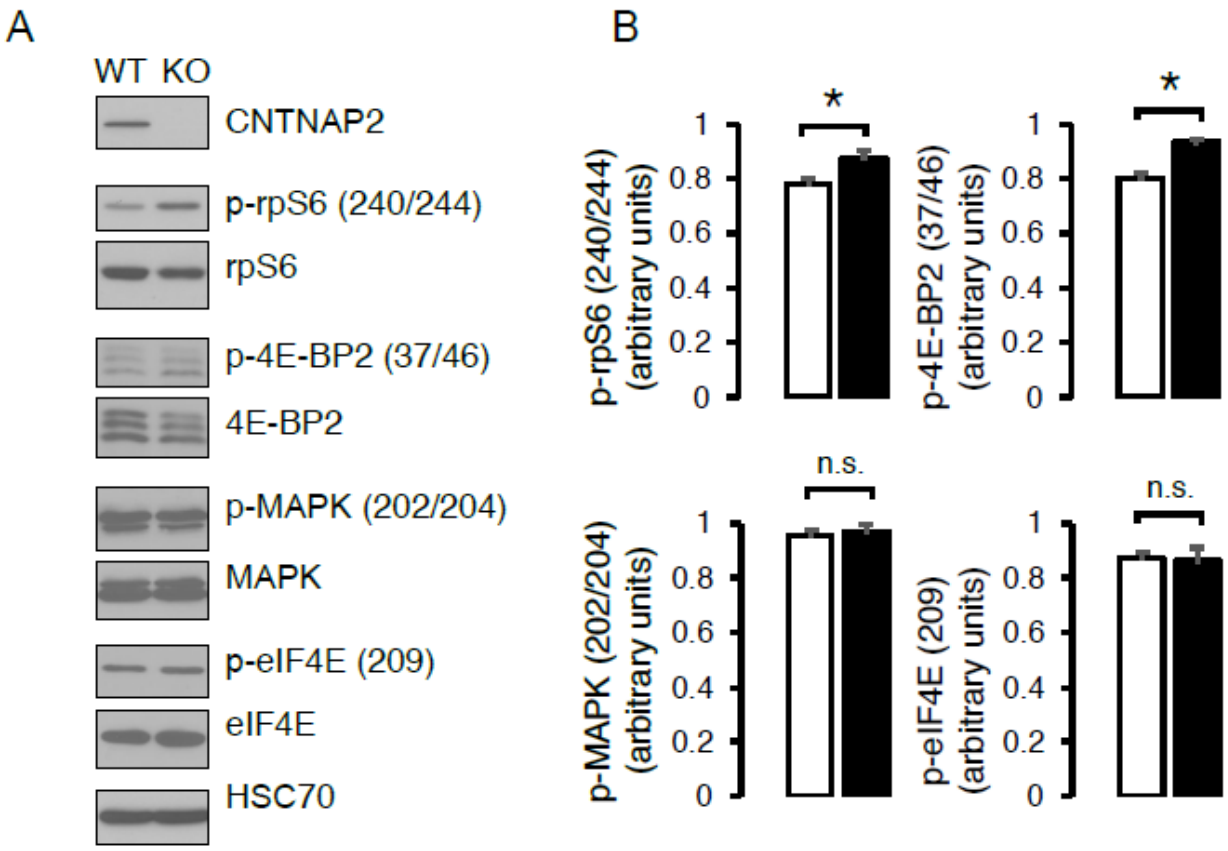

Figure 5. A) Representative immunoblot images from WT and KO animals, probed for the proteins indicated. HSC70 was used as a loading control. **B)** Quantification of immunoblots from **A**, with phosphorylated proteins normalized against their respective total ones (e.g. p-rpS6/rpS6). n=5 for phospho- and total rpS6, n=3 for phospho- and total MAPK and eIF4E, and n=2 for phospho- and total 4E-BP2. Two-tailed t-test assuming equal variances for p-rpS6 returned a p-value of 0.0322, while two-tailed t-test assuming unequal variances for p-4E-BP2 returned a p-value of 0.0329. The bars represent mean ± SEM.

3.4 Pilot neurosphere cultures

Two adult WT mice were used to carry out the pilot experiment. Neurospheres were successfully generated in considerable numbers from the SVZ of both animals **(Figure 6)**. DG-derived neurospheres were much rarer and were not consistently seen in the different wells of the plates.

After 10-12 days in culture, the neurospheres grew to approximately 150µm diameter (data not shown) and were passaged in order to maintain the culture, as described in [89]

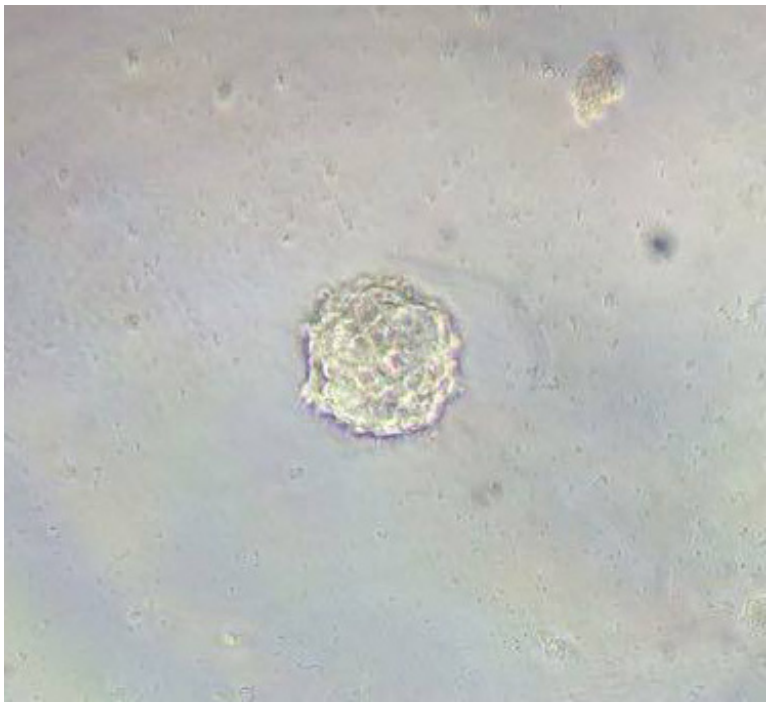

Figure 6. A representative image of an SVZ-derived neurosphere after 7 days in culture.

4. DISCUSSION

In this study, we examined the molecular mechanisms that might be responsible for the abnormalities associated with mutations in the *CNTNAP2* gene. In humans, *CNTNAP2* mutations have been implicated in various neuropsychiatric disorders, including ASD, epilepsy and schizophrenia. The behavioural and physiological abnormalities identified in animal models closely parallel the patient phenotypes.[66]

Most ASD-related disorders share common pathophysiological mechanisms, among which dysregulation of cap-dependent mRNA translation and dysfunction of synaptic activity are prominent.[33,55] Given the similarity of phenotypes observed in *CNTNAP2*-mutant animals[70,72,73] with other models of ASD[38,41], we assumed a shared physiological substrate. Consequently, we examined protein synthesis and its regulation by intracellular signaling pathways in a *CNTNAP2*$^{-/-}$ mouse model.

In our model, there is a trend for increased global protein synthesis in the hippocampus of KO mice compared to WT controls. However, statistical significance was not reached at this point due to sample size limitations. Additional experiments will increase the statistical power of the study and substantiate the present results. Based on power calculations, we would need 2 additional animals per group to reach statistical significance.

Formation of the eIF4F complex is a crucial step in mRNA translation initiation.[4] Thus, we aimed to discover whether alterations in its formation might account for the observed changes in mRNA translation in our model. However, we were unable to draw any definitive conclusions, due to technical complications that affected the outcome of the m^{7}GTP-pulldown assay. Once these are addressed, we will be able to provide answers that will help in the characterization of CNTNAP2 function and dysfunction in the nervous system.

Regarding the regulation of mRNA translation, we focused on two major signaling pathways, the PI3K/mTORC1 and the MAPK pathways. Activation of both pathways has been shown to stimulate mRNA translation through different mechanisms. mTORC1-mediated phosphorylation of 4E-BPs leads to their dissociation from eIF4E and subsequent de-repression of translation.[5] On the other hand, activation of the Ras/MAPK pathway by mitogens leads to phosphorylation of eIF4E by Mnk1/2, which also enhances mRNA translation.[23]

Here we show that genetic inactivation of *CNTNAP2* leads to excessive activation of mTORC1. 4E-BP2 and rpS6, both downstream targets of the kinase, exhibit in-

creased phosphorylation in mutant animals compared to controls. This finding is consistent with the hyperactivation of the PI3K/mTORC1 pathway that is regularly observed in syndromic forms of ASD, including TSC and PTHS.[3] Given the localization of both CNTNAP2 and PI3K on the synaptic membrane[71], it is not unreasonable to assume that there might be a functional interaction between the two. This interaction might be direct, or alternatively mediated through cytoskeletal and scaffolding proteins. Absence of CNTNAP2 could lead to reorganization of the protein networks present at the postsynaptic membrane and, in turn, to constitutive activation of PI3K and its downstream signaling pathway. However, to confirm this assumption, additional experiments are required. Firstly, it has to be established that components upstream of mTORC1 (for example Akt) are indeed activated in *CNTNAP2*-KO animals. Furthermore, immunoprecipitation assays could help identify potential interactors of CNTNAP2 at the synapse. Alternatively, a technique called BioID, which has already been used by our group, takes advantage of a bacterial biotin ligase to identify proteins interacting with a protein of interest.[91] Interactions of contactin-2, the binding partner of CNTNAP2, should also be taken into consideration.

On the other hand, components of the MAPK pathway do not appear to be affected in mutant animals. Specifically, p44/42 MAPK and eIF4E phosphorylation levels were found to be similar in both wild-type and *CNTNAP2*-KO mice.

Collectively, these findings suggest that *CNTNAP2* inactivation potentially increases mRNA translation through selective activation of the PI3K/mTORC1, but not the MAPK, pathway. However, as discussed above, the mechanism behind this selectivity has to be examined further.

Elucidation of the molecular mechanisms of CNTNAP2 function would aid in the discovery of new and more effective treatments for ASD and related conditions. If the regulation of mRNA translation is indeed affected, targeting the mTOR pathway, which is one of the “master” regulators of protein synthesis, might be beneficial. Indeed, mTORC1 inhibitors such as rapamycin have already been used in some models of ASD with promising results.[85,92,93]

Finally, we successfully generated neurosphere cultures from adult mice, following the protocol from[89] This system is ideal for the study of NSC properties, such as proliferation and differentiation potential. Given the role of 4E-BP2 in NSC self-renewal[19], and its developmental regulation[77], we intend to explore its effects on adult neurogenesis using this established technique.

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