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"Mnk1/2 KINASES REGULATE MEMORY AND AUTISM-RELATED BEHAVIOURS VIA Syngap1"

Master's Thesis

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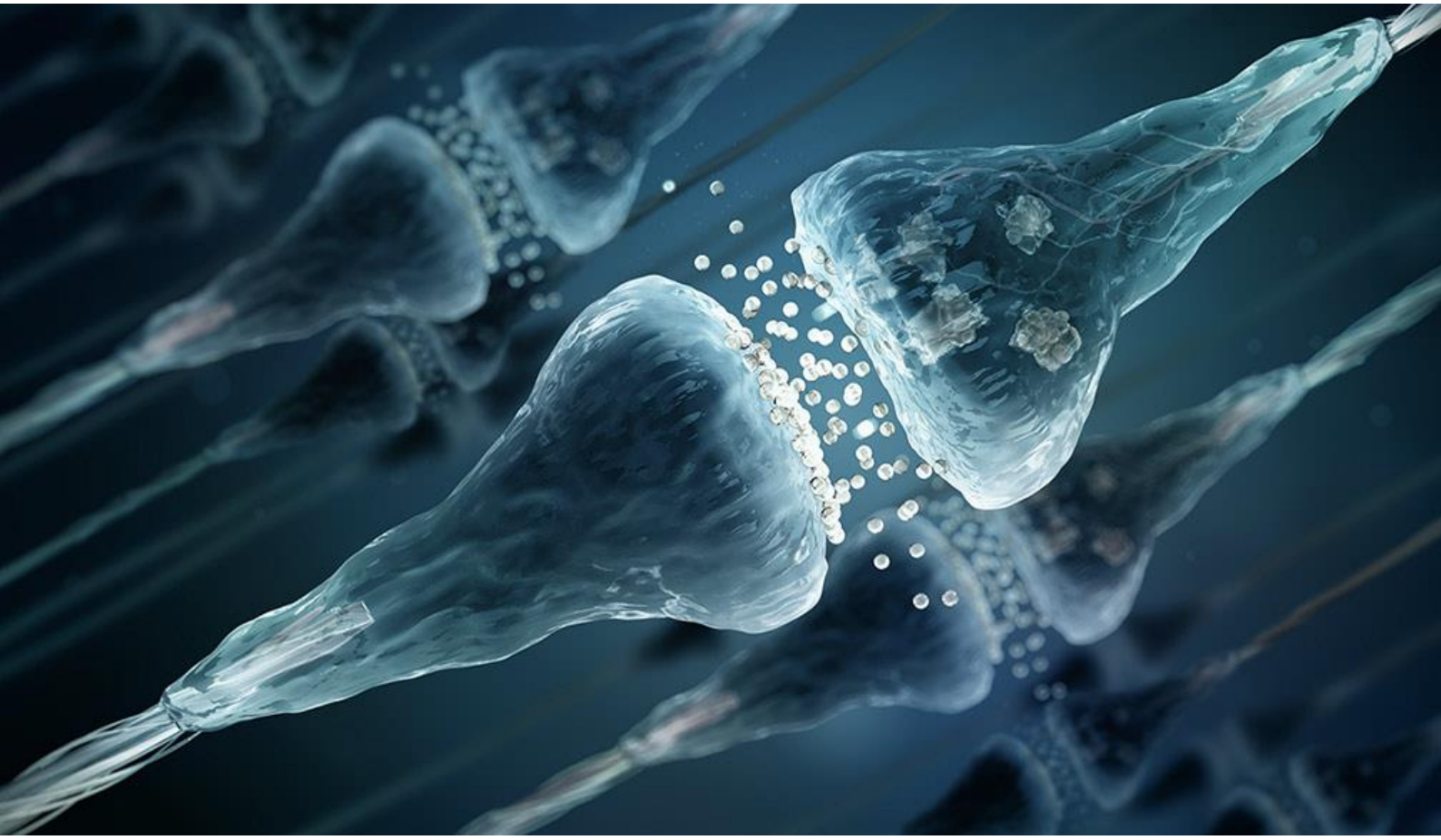

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Περίληψη

Οι πρωτεϊνικές κινάσες 1 και 2 (Mnk1/2) που αλληλεπιδρούν με τις μιτογόνο-ενεργοποιούμενες πρωτεϊνικές κινάσες (MAPK) ρυθμίζουν μια πληθώρα λειτουργιών στα κύτταρα, κυρίως μέσω φωσφορυλίωσης του καλύτερα χαρακτηρισμένου υποστρώματος τους, του ευκαρυωτικού παράγοντα έναρξης της μετάφρασης 4E (eIF4E) στο αμινοξικό κατάλοιπο Ser209. Αυτή η μεταπτυχιακή εργασία βασίζεται σε πρωτεομικά και φωσφοπρωτεομικά δεδομένα από την έρευνα των Chalkiadaki et al., (2022), στον εγκέφαλο διαγονιδιακών ποντικών όπου έχει γίνει απαλοιφή των γονιδίων *Mnk*1/2 (Mnk DKO) τα οποία καταδεικνύουν ότι οι Mnk1/2 διαθέτουν πρόσθετους καθοδικούς τελεστές στον εγκέφαλο, πέραν του eIF4E. Μέσω ανοσοκατακρήμνισης εντοπίστηκε η συναπτική Ras GTPase ενεργοποιούμενη πρωτεΐνη 1 (Syngap1) που κωδικοποιείται από ένα συνδρομικό γονίδιο της Διαταραχής Αυτιστικού Φάσματος (ΔΑΦ), ως καθοδικός στόχος της Mnk1 ενώ ταυτόχρονα αποδείχθηκε μειωμένη φωσφορυλίωση (S788) σε Mnk DKO ποντίκια. Παράλληλα, ανάλυση του μονοπατιού της κινάσης mTOR έδειξε μειωμένη ενεργότητα σε *Mnk1*$^{+/-}$*/Mnk2*$^{+/-}$ συναπτοσώματα εγκεφάλου ποντικών. Επιπρόσθετα, η αναστολή έκφρασης της Syngap1 στον εγκέφαλο Mnk DKO ποντικών μέσω αδενο-συσχετιζόμενου ιού- διαμεσολαβούμενης αφαίρεσης ανέστρεψε την απορρύθμιση της σηματοδότησης mTOR και της ολικής πρωτεϊνοσύνθεσης. Η φαρμακολογική αναστολή των Mnks μέσω ενός αναστολέα της κινάσης (eFT508) ανέστρεψε επίσης την απορρύθμιση της σηματοδότησης και της ολικής πρωτεϊνοσύνθεσης. Τα δεδομένα αυτά σε συνδυασμό με τα συμπεριφορικά αποτελέσματα της μελέτης των Chalkiadaki et al., (2022), που δείχνουν πλήρη αναστροφή φαινοτύπων που σχετίζονται με ΔΑΦ, παρέχουν ισχυρές ενδείξεις ότι η Syngap1 ρυθμίζει τη σηματοδότηση mTORC1 και τη σύνθεση πρωτεϊνών και ότι η ρύθμιση μέσω του Mnk-Syngap1 άξονα είναι ζωτικής σημασίας για συμπεριφορές στην ΔΑΦ όπως η κοινωνική αλληλεπίδραση, η μάθηση και η μνήμη.

Abstract

Mnk1/2, the MAPK (mitogen-activated protein kinase) interacting protein kinases 1 and 2 modulate several functions in cells, mainly via phosphorylation of their best characterised substrate, eukaryotic translation initiation factor 4E (eIF4E) on Ser209. This MSc thesis is based on 'omics data from Chalkiadaki et al., (2022) which suggest that Mnk1/2 posses additional downstream effectors in the brain. We identified the synaptic Ras GTPase activating protein 1 (Syngap1), encoded by a syndromic Autism Spectrum Disorder (ASD) as a downstream target of Mnk1 since Syngap1 immunoprecipitated with Mnk1 and showed reduced phosphorylation (S788) in double knockout for *Mnk* (Mnk DKO mice). Syngap1 inhibition in Mnk DKO mouse brain via Adeno-associated virus (AAV)-mediated knockdown reversed dysregulation of signalling and total protein synthesis. Pharmacological inhibition of Mnks using a kinase inhibitor (eFT508) also rescued the dysregulation of signalling and total protein synthesis. These data taken together with the behavioural results of Chalkiadaki et al., (2022) which show rescued ASD related phenotypes, provide strong evidence that the genetic ASD risk factor Syngap1 regulates mTORC1 signalling and protein synthesis and that the Mnk-Syngap1 axis is crucial for ASD linked behaviours such as social interaction, learning, and memory.

Chapter 1: Introduction

1.1 MAP kinases

Mitogen-activated protein (MAP) kinases are part of a family of ubiquitous, evolutionarily conserved, proline-directed protein kinases that phosphorylate amino acids serine and threonine (Pearson et al., 2001). MAP kinases play a pivotal role in signal transduction pathways while also regulating intracellular events including acute responses to hormones, cell proliferation and survival, gene expression and apoptosis (Pearson et al., 2001). A ''domino'' of phosphorylations occur upon activation of the MAPK cascade. Following an intrinsic or extrinsic stimulus, an upstream MAPK phosphorylates the next MAPK. For instance, MAP3K activates MAP2K which then, in turn, activates MAPK. MAPK protein phosphatases (MKPs) dephosphorylate both phospho-threonine and phospho-tyrosine residues on MAPKs, leading to inactivation of phosphorylation events (Soares-Silva et al., 2016). The sequences of the first MAP kinases became available between 1989 and 1991. These kinases were Kss1p and Fus3p in the pheromone response pathway of the budding yeast and extracellular signal-regulated kinases 1, 2 and 3 (ERK1, ERK2 and ERK3) of mammals (Avruch, 2007). At that time, two substrates, myelin basic protein (MBP) and microtubule-associated protein-2 (MAP2) were used to measure the activity of ERK1 and ERK2. The kinases were named MBP and MAP2 kinases (Avruch, 2007). To this day, the MAP acronym is retained. However, it now has a different meaning, as the name "mitogen-activated protein kinase was assigned to these enzymes to acknowledge the fact that they had first been detected as mitogen-stimulated tyrosine phosphoproteins in the early 1980s (Avruch, 2007). The most well-known MAPK pathways in mammalian cells are the ERK1/2, the c-JUN N-terminal kinase 1,2 and 3 (JNK1/2/3) and lastly, the p38 MAPK α, β, γ and δ pathways. Growth factors, hormones and proinflammatory stimuli are needed in order to activate ERK1/2, while JNK1/2/3 and p38 can be activated by cellular and environmental stress, in addition to proinflammatory stimuli (Owens & Keyse, 2007; Soares-Silva et al., 2016).

1.2 p38 pathway

Cellular stress is the main factor that activates the mammalian p38 MAPKs. Stress can include ultraviolet (UV) irradiation, heat shock, high osmotic stress, lipopolysaccharide, protein synthesis inhibitors, proinflammatory cytokines (such as IL-1 and TNF-α) and certain mitogens (Zhang & Liu, 2002). Four isoforms of p38 are known, p38 α, β, γ and δ (Zhang & Liu, 2002). While other Mitogen Activated Protein kinase kinases (MAPKKs) can phosphorylate only some p38 isoforms, like MKK3 which phosphorylates p38 α,γ and δ, all isoforms can by phosphorylated by the MAPK kinase MKK6 (Zhang & Liu, 2002). P38 MAPK appears to play a major role in various cellular processes. The most important ones are apoptosis, proliferation, differentiation and survival of cells, inflammation and other stress responses. p38 activity is required in Cdc42-induced cell cycle arrest at G1/S (Takenaka et al., 1998). Inhibition of cyclin-D1 expression can regulate this inhibitory role. Activated p38 can cause mitotic arrest in somatic cell cycles at the spindle assembly check point (Takenaka et al., 1998). p38 plays another pivotal role in various vertebrate cell differentiation processes such as adipocytes, cardiomyocytes, chondroblasts, erythroblasts, myoblasts and neurons (Nebreda & Porras, 2000; Zhang & Liu, 2002).

1.3 ERK1/2 pathway

ERKs have been the best characterized MAPKs and the Raf-MEK-ERK pathway represents one of the best characterized MAPK signaling pathway. The classical ERK family (p42/44 MAPK) is known to be an intracellular checkpoint for cellular mitogenesis (Zhang & Liu, 2002). Other processes that are regulated by the ERK cascade are cell adhesion, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription (Zhang & Liu, 2002). The stimulation of tyrosine kinase receptors (RTKs) provokes the activation of MAPKs in a multistep process. The essential linkers from epidermal growth factor receptors to MAP kinase are an adaptor protein named Grb2, Sos, which is a guanine nucleotide exchange protein, p21ras, a small GTP binding protein, a cascade of protein kinases defined sequentially as (Mitogen Activated Protein (MAP) kinase kinase kinase) MAPKKK Raf, and MAPKK such as MEK1 and MEK2. MEK1/2 catalyze the phosphorylation of

ERK1/2 at Tyr204/187 and then Thr202/185. These phosphorylations at these sites are required for enzyme activation (Roskoski, 2012). While MEK families and the Raf kinase have narrow substrate specificity, ERK1/2 have the ability to catalyze the phosphorylation of hundreds of cytoplasmic and nuclear substrates including regulatory molecules and transcription factors (Roskoski, 2012). The activated ERKs translocate to the nucleus and transactivate transcription factors. This leads to changing gene expression to promote growth, differentiation or mitosis (Roskoski, 2012). G protein-coupled receptors (GPCRs) can also lead to activation of MAPKs mediated by stimulation of a large number of complex cascades (Blaukat et al., 2000). One novel mechanism is that GPCRs stimulation can lead to tyrosine phosphorylation of RTK, such as the (Epidermal growth factor receptor) EGFR, which ultimately results in ERK activation (Zhang & Liu, 2002).

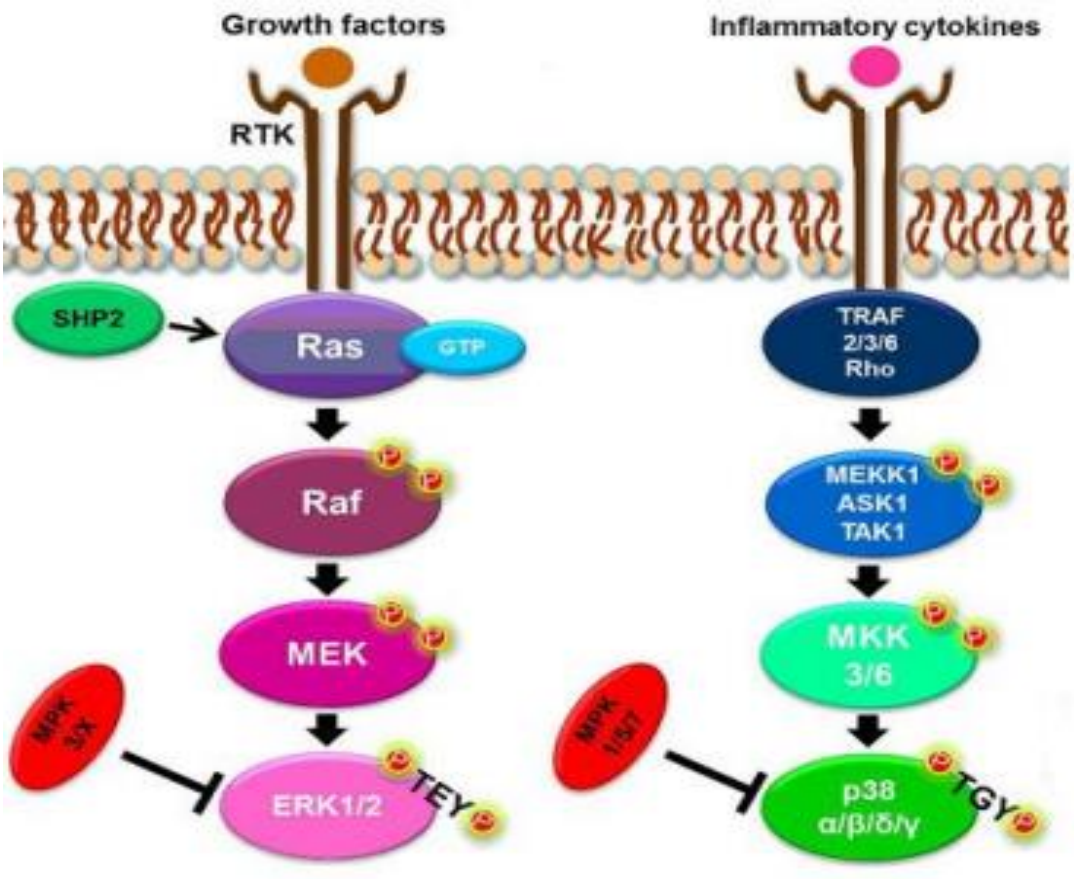


Figure 1. Schematic illustrations of the ERK1/2, p38 signaling pathways. Adapted from Soares-Silva et al., 2016.

2.1 Mnk *kinases*

Erk and p38 phosphorylate the MAPK (mitogen-activated protein kinase) interacting protein kinases 1 and 2 (Mnk1/2) which can exert multiple biological functions due to their ability to respond to a wide range of external stimuli such as mitogens as well as stress inducers (Roux & Blenis, 2004). Mnk1 and Mnk2 are serine/threonine kinases, both contain a carboxyl terminus in which a biding domain for MAPK kinases does exist. The N-terminal basic amino acid rich region of all isoforms contain a polybasic sequence that binds (eukaryotic translation initiation factor 4 G) eIF4G and importin and can mediate their localization in the nucleus (Parra-Palau et al., 2003). Although Mnk1 and Mnk2 appear to have a substantial similarity in their coding sequences and motifs each gene of these proteins gives rise to two transcripts via alternative splicing which differ towards the 3'end of their coding regions. In the human brain, MNK1b/2b, the short forms, have an independent activity from MAPK kinases because they lack the MAPK binding C-terminal. These isoforms also lack a nuclear export sequence while still retaining the nuclear localization signal and therefore both MNK1b and MNK2b are preferentially localized to the nucleus and in PML bodies which also contain eukaryotic translation initiation factor 4E (eIF4E). There is a need for a better understanding of these kinases because their regulation is not well known (O'Loghlen et al., 2004; Scheper et al., 2003). There are some important differences between MNK1a and MNK2a isoforms. The polybasic sequence that binds eIF4G and importin can interact with ERK1/2 and certain 'stress-activated' p38 MAPKs and tightly regulate MNK1/1a which is cytoplasmic, whereas MNK2/2a which is largely nuclear only interacts with ERK resulting in a high basal activity (Buxade et al., 2008; Waskiewicz et al., 1997). Different stimuli is needed to activate MNK1 or MNk2. Mitogens and stress inducing agents such as anisomycin or sorbitol as well as by cytokines such as type Ⅰ and type Ⅱ interferons (IFNs), tumor necrosis factor (TNF)-α, interleukin (IL)-1b, growth factors, and ultraviolet (UV) radiation, are needed to activate MNK1 (Joshi et al., 2009) whereas MNK2 exhibits high basal activity that is more resistant to the inhibition of Erk and p38 (Parra-Palau et al., 2005; X. Wang et al., 1998).

2.2 Upstream regulation of Mnks

The p21 activated kinase 2 (PAK2/γ-Pak) is a critical effector that link Rho GTPases to cytoskeleton reorganization and nuclear signaling can negatively regulate its kinase activity (Orton et al., 2004) by phosphorylating Mnk1 on Thr22/Ser27 resulting in decreased affinity for eIF4G and potentially interferes with Mnk1 mediated phosphorylation of eIF4E (Joshi & Platanias, 2014). Additionally, Pak2 also phosphorylates eIF4G and so inhibiting it's interaction with eIF4E. Another regulator that has a negative impact on Mnk's activity is the protein phosphatase 2A (PP2A) (Joshi and Platanias 2014).The serine/threonine phosphatase activity of PP2A has broad substrate specificity and diverse cellular functions. Among the targets of PP2A are proteins of oncogenic signaling cascades, such as Raf, MEK, and AKT, where PP2A may act as a tumor suppressor (McConnell & Wadzinski, 2009). As seen from the study of Li et al., 2010, the phosphorylation of eIF4E which is a target of Mnk1 was increased when small interfering RNA mediated knockdown of PP2A or pharmacological inhibition of PP2A was done. As mentioned above Mnk kinases can be regulated by p38 and Erk1/2 mitogen-activated protein kinases since they phosphorylate them on Thr 197/202. Therefore Mnks can exert multiple biological functions due to their ability to respond to a wide range of external stimuli such as mitogens as well as stress inducers and phosphorylate downstream effectors.

2.3 Downstream regulation of Mnks

Mnk kinases are known to have a number of downstream effectors such as the eIF4E, eIF4G, hnRNPA1, Sprouty, PSF (polypyrimidine tract-binding protein) and Cytosolic phospholipase A2.

2.3.1 Sprouty 2 acts as a negative regulator of multiple receptor tyrosine kinase pathways by negatively controlling the Erk MAPK pathway (Cabrita & Christofori, 2008). Although more work needed to be done in order for relevance of Mnk mediated phosphorylation of Spry2 to be understood, it appears that Mnk kinases

negatively or positively regulate Spry2 expression in a cell-specific manner, depending on the presence of additional regulatory cellular signals (DaSilva et al., 2006; Edwin et al., 2010).

2.3.2 hnRNPA1 and ***PSF*** play an important role in mediating the translation of AU rich elements containing mRNAs such as the TNF-α mRNA. Buxadé et al., (2005) showed that TNF-a production reduced when the activity/ expression of Mnk was inhibited though regulating TNF-α via its effector hnRNPA1, while in 2008 Buxade and colleagues showed that Mnk mediated phosphorylation of PSF was found to enhance its binding to the TNF-α mRNA containing AREs (AU-rich elements). Notably, PSF phosphorylation by Mnk did not affect the stability or the nuclear cytoplasmic localization of PSF or the bound TNF-α mRNA, but its effects on TNF-α mRNA translation need more clarification.

2.3.3 Cytosolic phospholipase A2 (cPLA2) also is mediated by Mnk activity. cPLA2 catalyzes the release of arachidonate acid from glycerophospholipids to provide the precursor of the eicosanoids and it is activated by increased cytosolic calcium. It is found cPLA2 is phosphorylated by Mnk on Ser 727 resulting in the enhancement of its enzymatic activity (Hefner et al., 2000).

2.3.4 eIF4E, the cap binding protein, is a major and well characterized target of Mnk kinases. Mnks phosphorylate eIF4E on Ser 209 in response to mitogens and cellular stress, through activation of either ERK or p38 pathway (Knauf et al., 2001; Ueda et al., 2004). The biochemical consequence of eIF4E and the role in regulating mRNA translation need to be more determined. Many studies proposed that phosphorylation of eIF4E by Mnk after the formation of the pre-initiation translation complex reduces its affinity for the 5' m7G cap, leading to the release of eIF4E and thereby enabling it to be available for another round of initiation of mRNA translation (Scheper & Proud, 2002). Mnk1 mediated phosphorylation of eIF4E regulates its release from eIF4G. eIF4G along with its binding partners and the small ribosomal subunits are important components of the 48S initiation complex required for translation initiation (Scheper & Proud, 2002). Studies suggest that Mnk1 is unable to interact with eIF4E in the absence of eIF4G and a mutant eIF4E lacking the

ability to bind eIF4G is not a good Mnk1 substrate (Pyronnet et al., 1999; Rhoads, 1999).

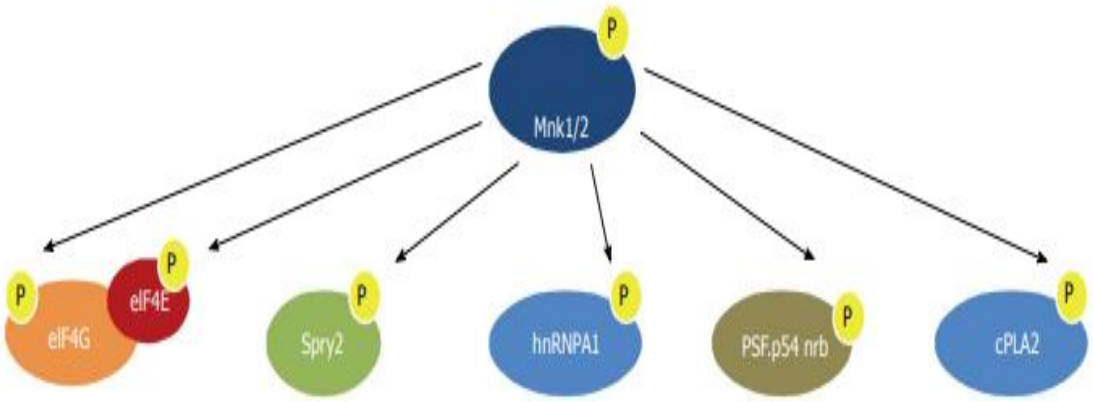


Figure 2. Schematic illustration of the Mnk downstream effectors. Adapted from (Joshi & Platanias, 2014).

3. Regulation of mRNA translation via eIf4E phosphorylation

A lot of studies suggest that eIF4E is not necessary for survival, development and reproduction since deletion of *Mnk1* and/or *Mnk2* in mice and therefore the eIF4E mediated phosphorylation did not affect these areas. Notably, cells from *Mnk1* and *Mnk2* deficient mice did not exhibit any defects in cap dependent translation or general protein synthesis, indicating that Mnk mediated phosphorylation of eIF4E is not critical under basal conditions but may be important during their activation with external stimuli (Ueda et al., 2004). This view is reinforced with other studies in which knock-in mice expressed a mutant eIF4E ($eIF4E^{S209Ala}$) which cannot be phosphorylated and they did not exhibit any developmental or viability defects (Furic et al., 2010). Phosphorylation of eIF4E is important for the translation of mRNAs containing 5' untranslated terminal regions (UTRs) with extensive secondary structure (Gong et al., 2020). All these date suggest that phosphorylation of eIF4E might not be critical for general mRNA translation, but it might be important for the translation of specific mRNAs, induced by specific stimuli (Koromilas et al., 1992).

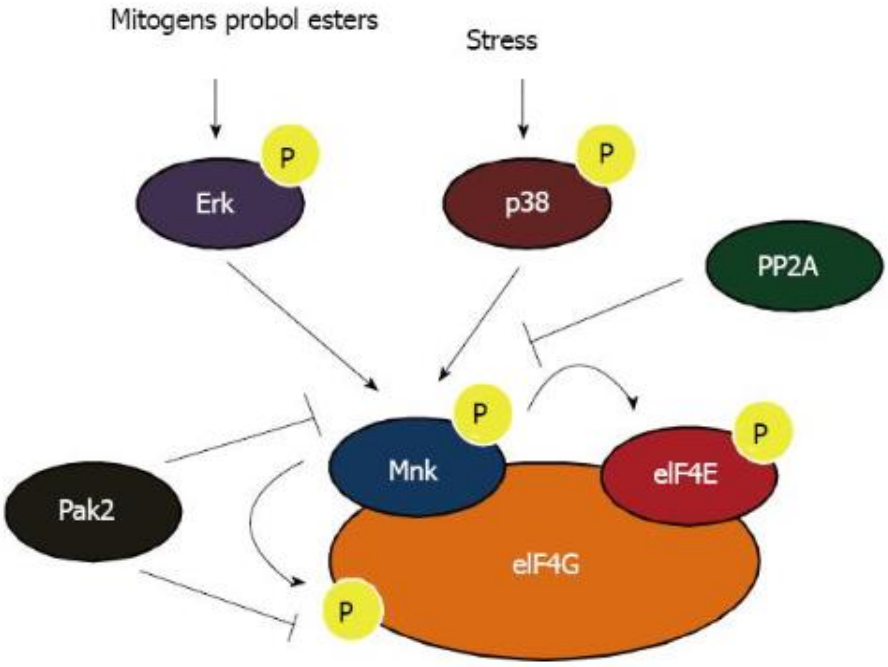


Figure 3. Schematic illustration of the Mnk mediated regulation of cap-dependent translation. Adapted from (Joshi & Platanias, 2014).

4. Translational regulation of specific mRNA transcripts via Mnk

Preferential translation of subsets of mRNAs is critical for synaptic function, plasticity and ultimately behaviors linked to neurological disorders such as ASD. In the brain, Mnk kinases participate in the regulation of a translational complex containing eIF4E, CYFIP1 (cytoplasmic FMR1 interacting protein 1), and FMR1 (fragile X messenger ribonucleoprotein 1) (Joshi & Platanias, 2014). The formation of this translation initiation complex is being blocked by the interaction of CYFIP1 with eIF4E, resulting in the blocking of eIF4E–eIF4G binding (Napoli et al., 2008). Translation could also be controlled by Mnk via regulation of the repressor CYFIP1/FMR1 complex. Stimuli can trigger the dissociation of the CYFIP1/FMR1 complex from the mRNA cap structure, allowing eIF4E-eiF4G interactions and thus promoting translation of FMR1 target mRNAs (Napoli et al., 2008; Panja et al., 2014). Proteins regulated by FMR1, are involved in neurotransmission and synaptic plasticity and were shown to overlap with proteins controlled by Mnk1 in mouse cortical neurons in culture, in response to Brain-Derived Neurotrophic Factor treatment (BDNF) (Genheden et al., 2015). Interestingly, in a mouse model of FXS (Fragile X Syndrome) core significant FXS-

correlated behaviors (social, repetitive/stereotypic) were rescued when Mnks where inhibited genetically or pharmacologically (Gkogkas et al., 2014).

5. Fragile X syndrome

FMR1 is encoded by the FMR1 gene in humans and mutations in this gene are the cause of FXS which is characterized by intellectual disability, hyper arousal and anxiety, repetitive behaviors, and morphological abnormalities (Saldarriaga et al., 2014). The precise mechanism by which FMR1 regulates translation is still unclear. However, one plausible mechanistic explanation is that FMR1 binds to several ASD-linked mRNAs and represses translation at the elongation stage through a mechanism involving direct binding to the 60S ribosome (Ascano et al., 2012; Darnell et al., 2011). More recent studies suggest that FMR1 preferentially stimulates the translation of long mRNAs, leading to excessive translation of ribosomal protein coding mRNAs (Seo et al., 2022), or that that FMR1 activates the translation initiation of large proteins in Drosophila oocytes, without affecting elongation (Flanagan et al., 2022). Together, these recent findings further complicate our understanding of FMR1 translational control mechanisms. Two very important pathways, Phosphoinositide 3-kinase (PI3K)- Protein kinase B (Akt)- mechanistic Target of Rapamycin (mTOR) (Sharma et al., 2010) and the Ras- ERK-Mnk (Osterweil et al., 2010) regulate the translational inhibitory activity of FMR1. These cascades play an important role in phosphorylating translation initiation factors and therefore activate cap dependent translation

6. The contribution of mTOR in translation

mTOR is a serine/threonine protein kinase, conserved during evolution that belongs in the PI3K-related kinase (PIKK) family (Laplante & Sabatini, 2009). mTOR forms two distinct protein complexes, known as mTOR Complex 1 (mTORC1) and 2 (mTORC2). mTORC1 has five components, which are mTOR, the catalytic subunit of the complex, regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8 or GβL), proline-rich AKT substrate 40kDa (PRAS40) and DEP-domain containing mTOR-interacting protein (Deptor) (Laplante & Sabatini, 2009). mTORC1 is partially inhibited by rapamycin. It has the ability to unify multiple signals

that indicate the availability of growth factors, nutrients and energy in order to promote cellular growth and catabolic processes during stress (Zarogoulidis et al., 2014). It is activated through growth factors and hormones, such as insulin, which use Akt to signal the protein complex, inactivating tuberous sclerosis complex 2 to prevent inhibition of mTORC1. After activation, mTORC1 phosphorylates other downstream elements such as 4E-BP1 and p70 S6 kinase, suppresses autophagy through Atg13 and ULK1, and activates transcription that leads to increased mitochondrial activity or adipogenesis (Zarogoulidis et al., 2014). mTORC2, on the other hand, consists of 6 proteins, several of which are common to mTORC1. These are mTOR, rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor-1 (Protor-1), mLST8 and Deptor, an endogenous inhibitor of mTORC1 (Laplante & Sabatini, 2009). It uses Akt and promotes cell survival through its activation while also mediating cytoskeletal dynamics and ion transport and growth by activating PKCα and phosphorylating SGK1, respectively (Laplante & Sabatini, 2009). The mammalian target of rapamycin (mTOR) signaling pathway integrates both intracellular and extracellular signals and is responsible for regulating many vital cellular processes. These include cell metabolism, growth, proliferation and survival (Yoon, 2017). The first upstream element of the pathway is PI3K. PI3Ks can be divided into three subtypes, Class I, Class II and Class III according to their structure and substrate specificity. The most studied among these is class I which can be activated directly by cell surface receptors like tyrosine kinase receptors or growth factors (Liu et al., 2009). Activation of class I PI3K by these receptors leads to phosphorylation of phosphatidylinositol 4,5-bisphosphate, generating phosphatidylinositol-3,4,5-trisphosphate (PIP3). As a second messenger, PIP3 in turn activates Akt through phosphorylation as well. Akt then targets and phosphorylates many proteins, including Glycogen synthase kinase-3 (GSK-3) and mTOR (Xu et al., 2020). As mentioned before the signaling pathway of mTOR contributes in the initiation of translation in a cap-dependent manner this is the result of mTOR-mediated phosphorylation of eukaryotic Initiation Factor 4E-Binding Proteins (4EBPs), which are inhibitors of eIF4E and S6Ks (ribosomal protein S6 Kinases) to promote translation initiation (Hay & Sonenberg, 2004). There are three 4E-BP

paralogs (1, 2 and 3) and 4E-BP2 is enriched in the mammalian brain, especially in the hippocampus. Non-phosphorylated 4E-BPs bind with high affinity to eIF4E, thus preventing eIF4F complex formation and consequently inhibit translation. Upon phosphorylation by mTORC1, 4E-BPs dissociate from eIF4E, allowing eIF4F complex formation and relief of translation inhibition. 4E-BPs have been implicated in cancer progression innate immunity circadian rhythms, learning and memory and ASD (Banko et al., 2005, 2006; Cao et al., 2013; Colina et al., 2008; Gkogkas et al., 2013). Genetic ablation of 4E-BP2 (Gkogkas et al., 2013) or overexpression of eIF4E (Santini et al., 2013) cause autism-like behaviors and synaptic plasticity deficits in mice. Information from Simons Foundation Autism Research Initiative (SFARI) gene database (https://gene.sfari.org/) indicates implicate that several mTOR pathway genes are strongly associated with several PI3K/mTOR pathway-related genes are ASD-risk genes (Vasic et al., 2021).

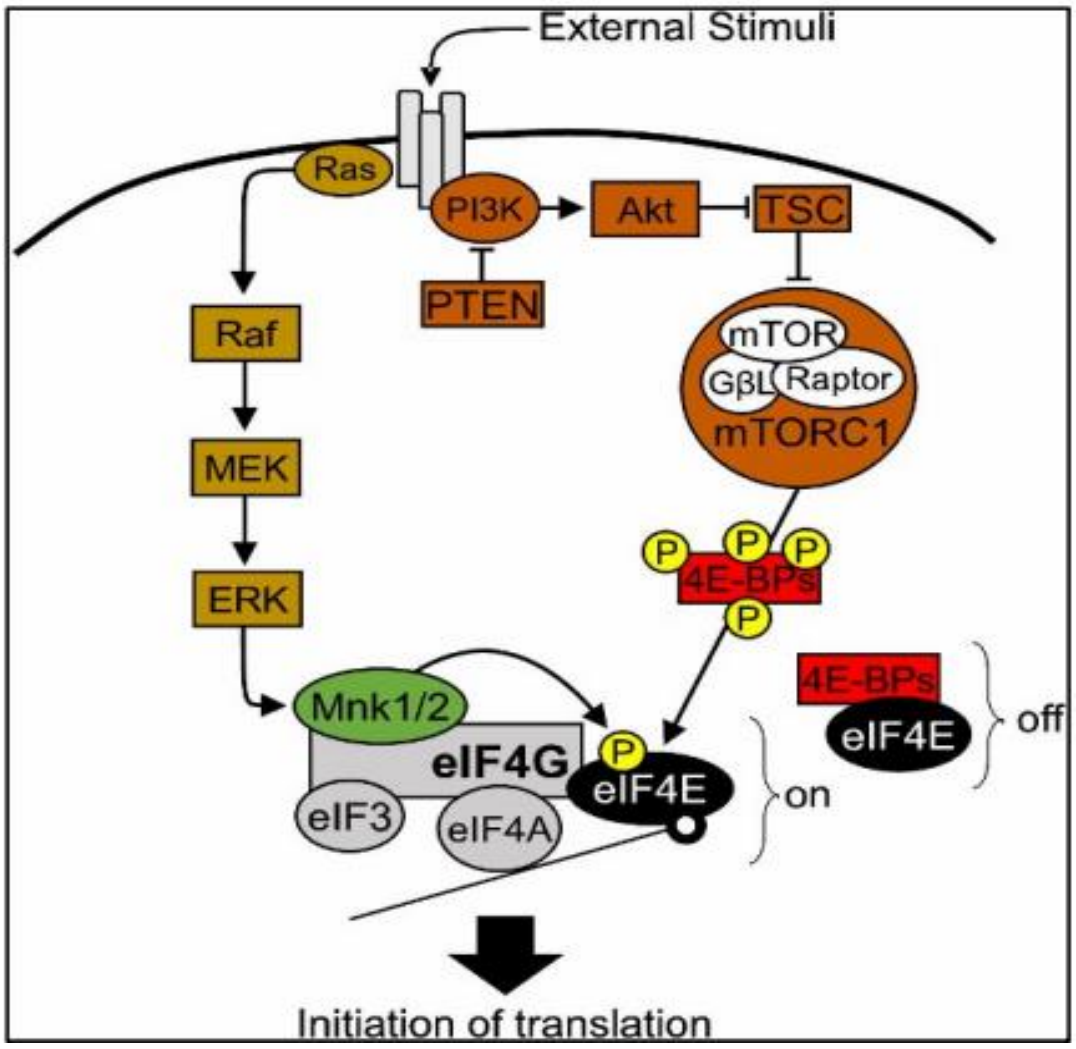


Figure 4. Translational Control via mTORC1. Signaling pathways upstream of translation initiation containing the mTORC1 pathway (orange) and the parallel MAPK/ERK/Mnk pathway (brown). Both pathways converge on initiation of protein synthesis by the cap-binding protein eIF4E.

7. Autism Spectrum Disorder

ASD is a complex neurodevelopmental disorder caused by a combination of genetic, environmental and epigenetic factors (Martens & van Loo, 2009). ASD is characterized by impaired social interactions altered verbal and nonverbal communication and social reciprocity, as well as repetitive behaviors and restricted interests (Montes & Halterman, 2006). In most cases the precise etiology is unknown, however is now established that ASD is to a very large extent a polygenic disorder (Elsabbagh, 2012). According to the Autism and Developmental Disabilities Monitoring Network's ASD prevalence estimates in 2016, one in fifty-four children

aged 8 years was diagnosed with ASD (Maenner et al., 2016). It is now estimated that ∼1% of the human population is diagnosed with ASD. There is a strong genetic basis for autism, but the risk architecture is highly heterogeneous, and a large number of genes have been implicated, two of them are TSC complex subunit 1, 2 (TSC1, TSC2) which are downstream regulators of mTORC1 (Abrahams & Geschwind, 2008; Sato, 2016). Syndromic forms of ASD or ASD-related syndromes commonly involve disruptions of the mTOR (mechanistic Target of Rapamycin, mTORopathies) and the RAS-RAF-MEK-ERK pathways (RASopathies) (Vasic et al., 2021).

Hörnberg and colleagues showed in 2020 that pharmacological inhibition of Mnk restores mRNA translation, oxytocin signaling and social novelty in a mouse model of the syndromic ASD gene Neuroligin 3 (Ngln3) indicating a relationship between ASD and MNKs. Chalkiadaki et al. in 2022 revealed a novel link between Mnk kinases and the ASD risk gene encoding synaptic Ras GTPase activating protein 1 (Syngap1).

8. Syngap1

Notably, Syngap1 is one of the most frequently mutated and ASD implicated *RAS* genes (Berryer et al., 2013). Syngap1 is a RAS GTPase-activating protein, ∼140 kDa which is encoded by *Syngap 1* gene (Chen et al., 1998). RAS has a slow intrinsic GTPase activity and Syngap1 negatively regulate RAS by enhancing the hydrolysis of GTP to GDP (Ligeti et al., 2012). Syngap is part of N-methyl-d-aspartate receptor (NMDAR) multiprotein complex which is established in the glutamate synapses, and is activated by $Ca2^{+}$/calmodulin-dependent protein kinase II (CaMKII) (Fan et al., 2014). RAS has been shown to play a fundamental role in a form of synaptic plasticity called long-term potentiation (LTP), in part by modulating actin dynamics and AMPARs (α-amino-3-hydroxy-5-methylisoxazole propionate-receptors) insertion into the postsynaptic membrane during LTP (Qin et al., 2005). The insertion of AMPARs happens when the MEK– ERK pathway is activated by Ras. It is next so, *Syngap1*$^{+/-}$ mice to show increased activation of the MEK–ERK pathway, increased insertion of AMPAR at the synaptic membrane, and impaired learning and memory a view that was confirmed by Carlisle et al., 2008; Komiyama et al., 2002; Rumbaugh et al., 2006. It is also known that phosphorylation of Syngap1 by cyclin-dependent

kinase 5 (CDK5) activates Rap1 that increases endocytosis of AMPAR however, the relationship between Syngap1 and RAP in the context of synaptic plasticity remains unclear (Krapivinsky et al., 2004; Pena et al., 2008).

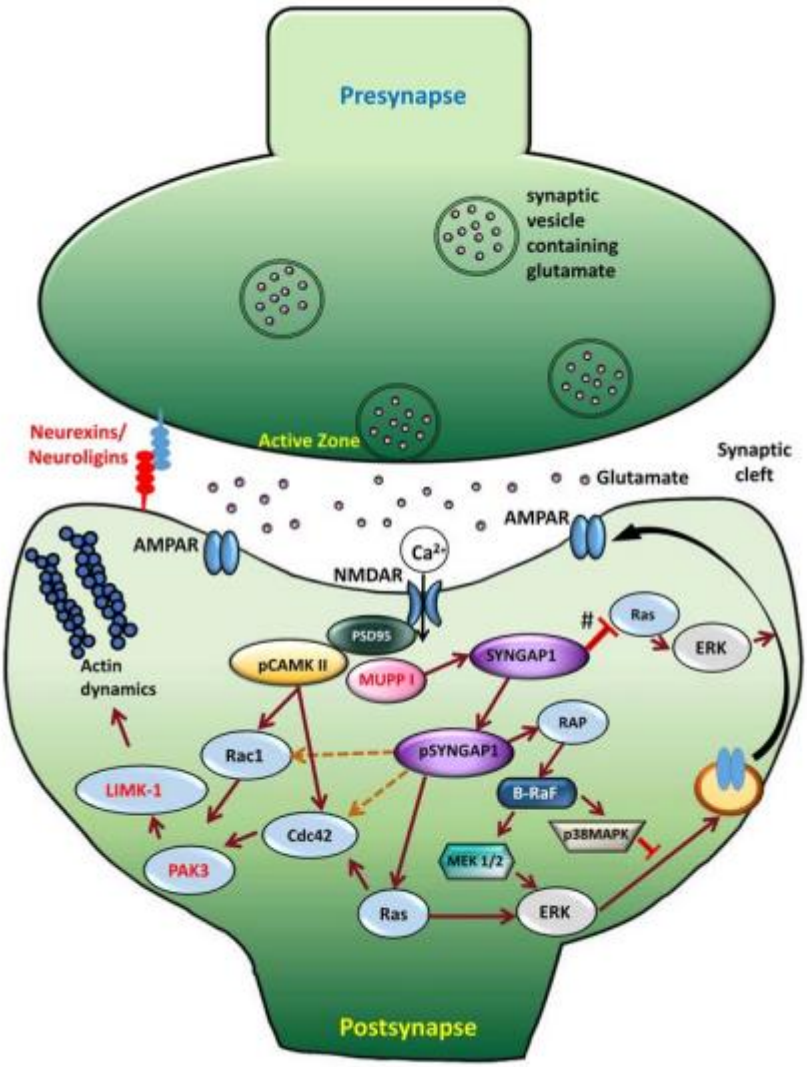


Figure 5. Schematic illustration of the signaling pathways recruited phosphorylation of SYNGAP1 (Jeyabalan & Clement, 2016).

9. Hypothesis and Aims

The master thesis was based on initial findings by Chalkiadaki et al., which are summarized here:

1. Mnk1/2 knockout mice display impaired synaptic plasticity (hippocampal long-term potentiation), learning and memory (Morris water maze and contextual fear conditioning).
2. Mnk1/2 knockout in mouse brain alters brain translatome (assessed with ribosome profiling) and phospho-proteome (assessed wish phospho-proteomics)

eIF4E (P-Ser209) is one of the best studied substrates of MNKs in the brain (and other tissues). However, it was shown that phospho-mutant Ser 209 (*Eif4e*^{Ser209Ala}) mice displayed intact synaptic plasticity, learning and memory (Amorim et al., 2018), while Mnk DKO (double knock-out) mice exhibited impaired hippocampal synaptic plasticity, learning and memory (Chalkiadaki et al., 2022). Furthermore, comparison of Mnk DKO whole brain translatome to *Eif4e*^{Ser209Ala} revealed only a small overlap (Chalkiadaki et al., 2022). Thus, we hypothesized that there are other yet unidentified Mnk1/2 downstream targets underlying Mnk1/2-mediated translational regulation in the brain and more broadly synaptic plasticity, learning and memory.

To test this hypothesis, we set the following Aims:

1. To identify and characterise binding partners of Mnk in the brain by performing immunoprecipitation.
2. To compare Mnk direct binding partners with the list of phospho-substrates (phospho-proteomics) in Mnk DKO mouse brain (from Chalkiadaki et al., 2022).
3. To characterise upstream signalling and measure protein synthesis in Mnk DKO brain.

The mechanistic work we performed helped established a link between Mnks and Syngap1, which was further elaborated in Chalkiadaki et al., by showing that:

1. Syngap1 inhibition in Mnk DKO mouse brain via intrahippocampal stereotaxic injection of AAV9 expressing shRNA specific to Syngap1 rescued learning and memory deficits and reversed dysregulation of signalling and total protein synthesis.
2. Pharmacological inhibition of Mnks using a kinase inhibitor (eFT508) rescued autism-related behavioural deficits and dysregulation of signalling and total protein synthesis.

Chapter 2: Material and Methods

Behavioural experiments, Quantitative Proteomics and Phospho-Proteomics, Ribosome Profiling and Bioinformatics Analysis were performed at University of Edinburgh (Chalkiadaki et al., 2022). I will only describe methodologies, which I performed as part of my MSc project results.

Co-Immunoprecipitation

Whole brain of *Mnk1*$^{-/-}$*Mnk2*$^{-/-}$, *Mnk1*$^{+/+}$*Mnk2*$^{+/+}$, *Syngap1*$^{+/+}$ *and Syngap1*$^{+/-}$ mice were homogenised by K. Chalkiadaki at the University of Edinburgh and processed at FORTH. All animal procedures were in accordance with UK Home Office and Canadian Council on Animal Care regulations and were approved by the University of Edinburgh and University of Ioannina, ANILAB and Prefecture of Epirus (C. Gkogkas project licence holder). Fresh lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1% CHAPS + protease inhibitor + phosphatase inhibitor) and Dounce glass homogenizer were used in order to homogenize the whole mouse brain samples on ice. After the homogenization, the samples were incubated for 20 min at 4^{o}C with rotation, and then they were centrifugated at 16,000 x *g* for 10 min at 4^{o}C. The supernatants of the homogenates were precleared with 50 µl of pre-washed protein A agarose slurry beads on rotator for 30 min at 4^{o}C and then, they were centrifugated at 3,500 rpm for 1 min at 4^{o}C to collect the precleared supernatants. After that, incubation of the precleared took place with either 3 µg of normal rabbit IgG (CST #2729S) or 3 µg of Mnk1 antibody (CST #2195S) for 30 min at 4^{o}C, followed by incubation with 50 µl of pre-washed protein A agarose slurry beads overnight at 4^{o}C. The mixtures were centrifuged at 3,500 rpm for 1 min at 4^{o}C to remove the supernatant as unbound fraction. Beads were washed 3 times with lysis buffer for 10 min at 4^{o}C and the bound proteins were eluted in 2x SDS sample buffer.

Synaptosomal preparation

Phosphoserine IP

The Synaptosomal fraction of one hemisphere from wild-type mice (*Mnk1*$^{+/+}$*Mnk2*$^{+/+}$) was homogenised in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1% CHAPS +

protease inhibitor + phosphatase inhibitor) on ice using Dounce glass homogenizer. The homogenates (whole brain or synaptosome) were incubated for 20 min at 4°C on rotator followed by centrifugation at 16,000 x *g* for 10 min at 4°C, and then the supernatants were collected and precleared with 50 µl of protein A agarose slurry beads for 30 min at 4°C. The mixtures were centrifuged at 1,500 x *g* for 1 min at 4°C and the supernatants were collected. The precleared supernatants (1.8 mg of whole brains, 600 µg of synaptosome fractions) were incubated with phosphoserine antibody (Millipore, AB1603) for 30 min at 4°C, were incubated with 50 µl of protein A agarose slurry beads at 4°C, overnight followed by a centrifugation at 1,500 x *g* for 1 min at 4°C. The first supernatants were collected as unbound fractions. Beads were washed with lysis buffer 3 x 10 min, at 4°C and the bound proteins were eluted in 2x SDS sample buffer.

Plasmid generation (mutant Syngap1)

Mouse Syngap1 coding sequence (CDS; wild-type or S788A or S788D) were synthesised using Custom Gene Synthesis (Biomatik, Canada) and were subcloned to the pBSK(+) vector and subsequently transferred using blunt cloning to a pcDNA3.1(+) backbone (Addgene) to generate the final plasmids. Syngap1 sequence was confirmed with Sanger sequencing.

Mammalian cell culture and transfection

Human Embryonic Kidney cells (HEK-293H ATCC® CRL-1573) were cultured (37°C, 5% CO2) in Dulbecco's modified Eagle's medium (DMEM, 11995065) supplemented with 10% fetal bovine serum (10500064) and 1% Pen/Strep (15140148). Transient transfection was performed with Lipofectamine 3000 (L3000008) in Opti-MEM (31985070) following the manufacturer's protocol for 48 h. All cell culture and transfection reagents were from ThermoFisher Scientific.

SUrface SEnsing of Translation (SUnSET) assay

After 48h of transfection, cells were pulsed with 5 µg/mL puromycin hydrochloride for 1 hr in culture medium. Cells were washed three times with ice cold PBS and lysed in RIPA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-

100, 0.1% Sodium Deoxycholate, 0.1% SDS, 140 mM NaCl; all from Sigma, supplemented with phosphatase and protease inhibitors; Roche) for immunoblotting with anti-puromycin antibody (MABE343; 12D10 Sigma). Puromycin incorporation was quantified with ImageJ (immunoblot signal intensity) and was normalised to control (no puromycin or no antibody) and membrane background signals.

Syngap1 immunoprecipitation

500 µg of transfected HEK-293H lysate or mouse brain synaptosomes resuspended in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin and 1mM PMSF) and were incubated with 20 uL of protein G beads (Invitrogen, 10003D) preincubated with 1 µg rabbit IgG (Sigma, 12-370) or Syngap1 antibody (ThermoFisher Scientific, rabbit polyclonal, PA1-046) for 1h at 4 ^{o}C. Samples were washed thrice with RIPA buffer and either processed with immunoblotting or stored at -80 ^{0}C.

Active Rheb (Rheb-GTP) immunoprecipitation assay

Rheb-GTP was detected with immunoblotting using the Rheb Pull-Down Activation Assay Kit (New East Biosciences, 81201). Transfected HEK293-H cells were washed thrice with PBS and lysed in a glass Dounce homogenisers. Lysates were left on ice for 15 min with occasional vortexing and then centrifuged at 4oC at 16,000 x *g* for 20 min. A total of 1mg of the supernatant was used to immunoprecipitate active-Rheb, to which 1 µg anti-Rheb-GTP antibody (New East Biosciences, Cat. # 26910) and 20 µL of A/G Agarose bead slurry (New East Biosciences, Cat. # 30301) were added. Samples were incubated for 1 hour at 4oC with gentle agitation. Beads were collected through centrifugation at 5,000 x g for 1 min, washed thrice with Assay/Lysis Buffer (New East Biosciences) and resuspended in SDS-PAGE buffer for immunoblotting.

Immunoblotting

Dissected brain tissue, HEK293-H lysates, immunoprecipitates, or synaptosomes were homogenized in RIPA (see above) or buffer B (50 mM MOPS/KOH pH 7.4, 100

mM NaCl, 50 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% NP-40, 7 mM β-mercaptoethanol), both supplemented with protease and phosphatase inhibitors (Roche), using a glass Dounce homogenizer (~30 strokes). Samples were incubated on ice for 15 min, with occasional vortexing, and cleared by centrifugation for 20 min at 16,000 *g* at 4°C. The supernatant was used for western blotting after the protein concentration of each sample was determined by measuring A280 Absorbance on a NanoDrop (ThermoFisher Scientific). 50 µg of protein per lane were prepared in SDS Sample Buffer (50mM Tris pH 6.8, 100 mM DTT, 2% SDS, 10% Glycerol, 0.1% bromophenol blue), heated to 98°C for 5 min and resolved on 10-16% polyacrylamide gels. Proteins were transferred to 0.2 µm nitrocellulose membranes (Bio-Rad), blocked in 5% BSA in TBS-T (10 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 1 h at RT, incubated with primary antibodies overnight at 4°C and with secondary antibodies for 1 h at RT. Primary antibodies were diluted in 1% BSA in TBS-T containing 0.02% Na azide, and between incubations membranes were washed extensively in TBS-T. Blots were imaged using Azure Biosystems 600. For quantitative Western Blotting, the intensity of each protein band was measured in triplicate to minimize measuring variability. HSC70 or β-actin were used as a loading control. Data are shown as arbitrary units (AU) as a proxy for protein expression, after normalization to control (for protein phosphorylation: phospho-protein values were measured with Image J or Image Studio™ Lite and divided to total protein and to loading control: HSC70, *β*-actin or GAPDH band intensity values, after subtracting immunoblot background intensity).

eFT508 treatment

eFT508 (MCE, New Jersey, USA), which was dissolved as concentration stocks (3.22 mg/mL) in Tween 80 (20% v/v), DMSO (32% v/v) and PBS (48% v/v) were freshly diluted in PBS to appropriate working concentrations (1 mg/mL). Intraperitoneal injections (1 mg/kg) were carried out daily for 5 days prior to behavioural tests and throughout the experiment. Behavioural tests were performed 24 h after the last injection at the University of Edinburgh. I processed tissue for the experiments described herein.

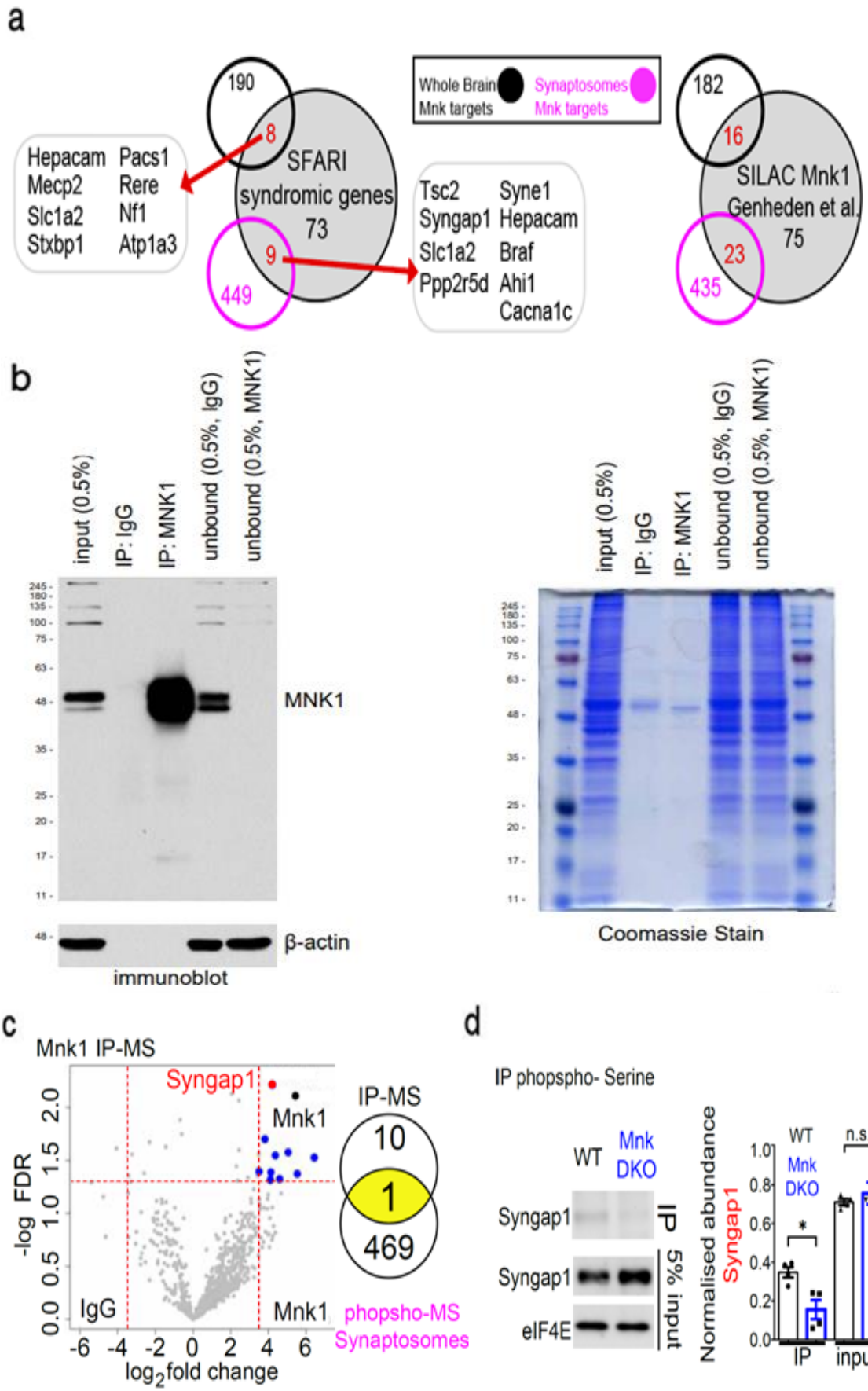
Chapter 3: Results

Identification of Mnk direct binding partners as a means to identify cardinal downstream effectors on protein synthesis.

To study the molecular substrates of Mnk1/2 kinases in the brain, Chalkiadaki et al., performed label-phospho-proteomics mass-spec analysis of whole brain and synaptosomal lysates from wild-type and Mnk DKO mice. This analysis revealed pervasive changes in the whole brain and synaptic phosphoproteome. Given the spatial memory phenotypes in Mnk DKO mice and the emerging role of Mnks in ASD, we compared the phosphoproteomics datasets from Chalkiadaki et al., to the SFARI syndromic genes and identified several common targets while there is also a significant overlap between them and a previous SILAC proteomics datasets from Mnk1 mouse knockout neurons **(Fig. 6a)**. To identify targets that may directly bind to and are phosphorylated by Mnks, we performed an immunoprecipitation assay using the Mnk1 antibody in whole brain lysates. In immunoprecipitates we detected a single band of ~48 kDa corresponding to Mnk1, evident by Coomassie blue stain and confirmed by immunoblotting with Mnk1-specific antisera, which was absent from Immunoglobulin G (IgG) control **(Fig. 6b).** Quantitative mass-spectrometric analysis performed by Chalkiadaki et al., at the University of Edinburgh detected Mnk1 along with 10 other proteins as the top statistically significant targets (± 3.5 $\log_2$ fold-change and False Discovery Rate <0.05) among the immunoprecipitated proteins **(Fig. 6c).** Interestingly, the only common target between the Mnk1 interactors and the differentially phosphorylated proteins in the Mnk DKO mice was Syngap1 **(Fig. 6c)**, which is linked to intellectual disability and is a syndromic ASD gene (Gamache et al., 2020).

To further confirm the Mnk-regulated phosphorylation of Syngap1 we performed immunoprecipitation with anti-phosphoserine antibody in wild-type and Mnk DKO brain lysates and immunoblotting with the Syngap1 antibody, which revealed significantly reduced levels of phosphorylated Syngap1 in Mnk DKO compared with WT brain lysates (~65% decrease; **Fig. 6d** IP-phosphoserine). Notably this was a modest effect, which could be explained if the Mnk-Syngap1 interaction is transient or other proteins are required as part of a multi-protein complex. From the synaptic

phosphoproteome analysis in Chalkiadaki et al., a highly enriched consensus motif ("SPEAK") for Mnks was identified. Because this motif is similar to motifs of other kinases (Glycogen synthase kinase-3 (GSK3) (Beurel et al., 2015), CDK5 (Sang et al., 2019) and ERK (Carlon, 2013), it is plausible that Mnks may work in synergy with other kinases to phosphorylate a given subset of proteins, and in particular Syngap1.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190
8
SFARI
syndromic genes
73
9
449
Hepacam Pacs1
Mecp2 Rere
Slc1a2 Nf1
Stxbp1 Atp1a3
Whole Brain
Mnk targets
Synaptosomes
Mnk targets
Tsc2 Syne1
Syngap1 Hepacam
Slc1a2 Braf
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16
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435
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Syngap1
Mnk1
IgG
Mnk1
-log FDR
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phopsho-MS
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IP phopspho- Serine
WT
Mnk DKO
Syngap1
Syngap1
eIF4E
IP
5% input
Normalised abundance
Syngap1
WT
Mnk DKO
n.s.
IP
input

Figure 6. Comparison of MNK1 DKO phosphoproteomics dataset with previous published datasets

a. Overlap of Mnk DKO differentially phosphorylated targets with SFARI syndromic ASD genes (https://gene.sfari.org/) and studies identifying FMR1-targets (Darnell et al., 2011) and (Ascano et al., 2012). Overlap between Mnk DKO differentially phosphorylated targets and SILAC proteomics from (Genheden et al., 2015). **b.** Representative immunoblot of different fractions of IgG and Mnk1 antibody immunoprecipitation (IP) and total lysates from mouse brain probed with antisera against the indicated proteins; β-actin is the loading control and Coomasie-stained gel. **c.** Identification of Mnk1 protein interactome in whole brain by co-immunoprecipitation-mass-spectrometry (IP-MS). Volcano plot shows proteins co-enriched with Mnk1 using anti-Mnk1 antibody (over IgG). A cut-off of ± 3.5 $\log_2$ fold-change (dashed vertical lines) and *FDR<0.05* (dashed horizontal line) was applied. Ven diagram demonstrates the overlap between proteins co-enriched with Mnk1 and the proteins with reduced phosphosites in Mnk DKO synaptosomes (Chalkiadaki et al., 2022). **d.** Analysis of Syngap1 phosphorylation in WT (wild-type) and Mnk DKO whole brains. Proteins with phospho-Ser residues were immunoprecipitated by specific anti-phospho-Serine antibody and the presence of Syngap1 was measured by western blot. The bar graph represents the relative enrichment of Syngap1 protein in each genotype in the input and phospho-Ser-enriched fractions. n = 4 for each genotype; Students *t*-test, $*p < 0.05$.

Analysis of signalling pathways in Mnk DKO brain synaptosomes, upstream of protein synthesis

From the phosphoproteomics dataset, two phosphorylation sites on Syngap1 were identified. The first one is S788, which is downregulated in Mnk DKO and the second one is S1165 which is upregulated in Mnk DKO, both sites are highly conserved between mouse, rat and human. Intriguingly, these sites are proximal to known CDK5 and CamKIIα phosphorylation sites on rat Syngap1 (Walkup et al., 2015).

Recent analysis of several human Syngap1 variants (Meili et al., 2021) showed that CDK5 phosphorylates Syngap1 at S788. Taking into consideration previous research linking Syngap1 to regulation of protein synthesis (Barnes et al., 2015; C. C. Wang et al., 2013) we hypothesised that phosphorylated Syngap1 S788 could be a newly detected effector of Mnk1 in brain, contributing in the regulation of translation and ultimately synaptic plasticity, memory, and autism-like behaviours. To further uncover the link between Mnk1 and Syngap1, and because Syngap1 is known to regulate signalling pathways upstream of translation (*e.g.,* upregulation of mTORC1 signalling in *Syngap1*$^{+/-}$ mouse brain, Wang et al., 2013), we examined three key pathways linked to translational control (mTORC1, Akt and MAPK) in synaptosomes isolated from Mnk DKO and wild-type mice by immunoblotting for phospho-ribosomal protein S6 (rpS6 240/244 and 235/236 sites), phospho-Akt(S473) and phospho-ERK (T202/Y204), respectively **(Fig. 7a,b)**. We did not detect any significant changes in Akt or MAPK signalling **(Fig. 7b)**, however a significant decreased phosphorylation of rpS6 on both sites (240/244, 235/236) in Mnk DKO compared with wild-type was detected thus indicating decreased mTORC1 activity **(Fig.7a)**.

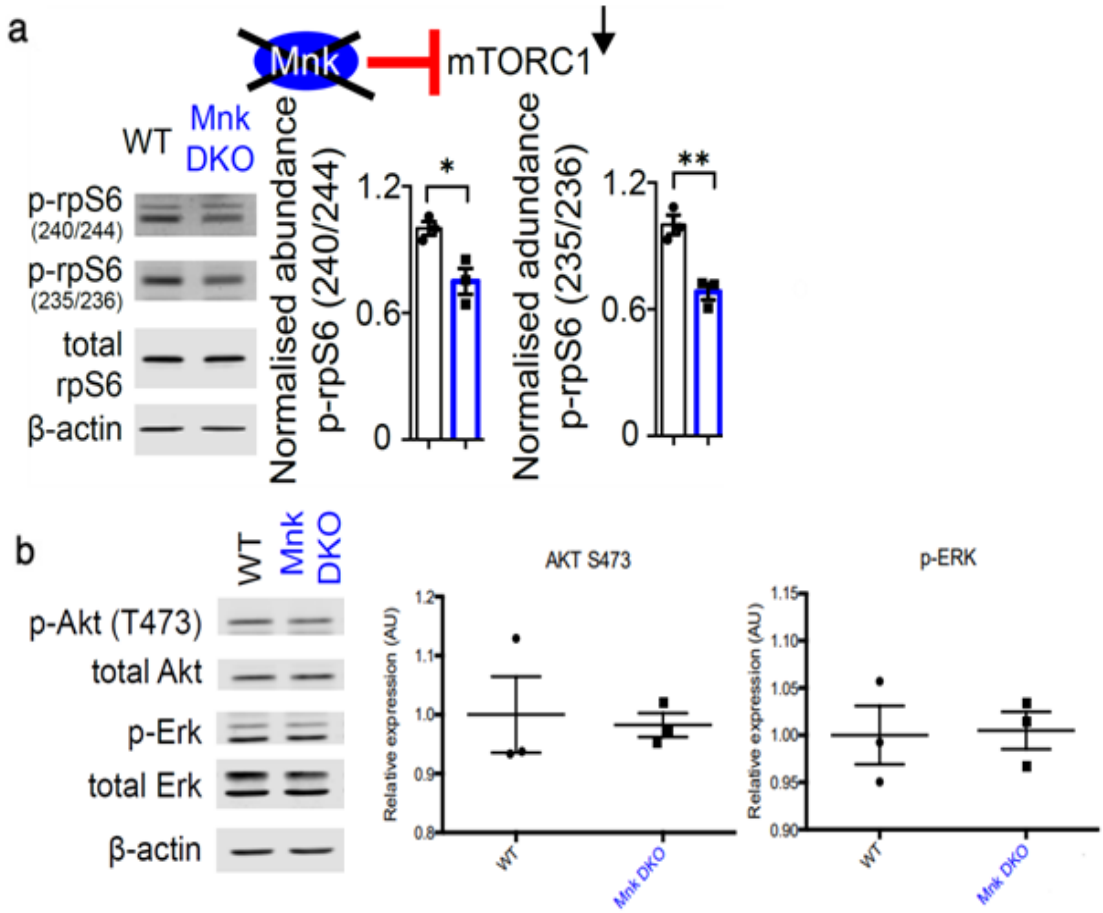

Figure 7. Signaling pathways in MNK DKO brain synaptosomes

a.mTORC1 activity in synaptosomes from Mnk DKO mouse brain. (Left) Representative images from immunoblotting of synaptosome lysates probed with antisera against the indicated proteins; β-actin is the loading control. (Right) Quantification of relative protein expression in immunoblotting experiment. Normalised expression of two phosphosites on rpS6 (240/244, 235/236) is depicted; n = 3 for each genotype, Unpaired *t*-test with Welch's correction. **b.** Representative immunoblots for signaling pathways upstream of translational control in synaptosomes probed with antisera against the indicated proteins; *β*-actin is the loading control and quantification of protein expression in immunoblots using arbitrary units (AU). n=3 for each genotype; Students t-test.

Based on these findings, we formed a new *Hypothesis*: Mnk-mediated phosphorylation of Syngap1 promotes mTORC1 through Rheb GTPase

As it is known from the study of Walkup et al., 2015, Akt and Erk are downstream to Ras GTPase, which is regulated by Syngap1 GAP activity. However, we did not detect any significant changes in the Akt or Erk signalling in immunoblotting for phospho-Akt (S473) and phospho-ERK (T202/Y204) comparing wild-type and Mnk DKO mice. In contrast, we observed significantly decreased phosphorylation of rpS6 on both sites (240/244, 235/236) in Mnk DKO compared with wild-type. Although it seems to be paradoxical that Syngap1 regulates mTORC1 without affecting Erk or Akt, we hypothesized that Syngap1 S788 phosphorylation may regulate mTORC1 via increased GAP-activity for the Rheb GTPase**,** in a parallel signalling pathway that bypasses Ras **(Fig. 8).** This hypothesis is also supported by previous research indicating that Rheb which activates mTORC1 is regulated by Syngap1 (C. C. Wang et al., 2013).

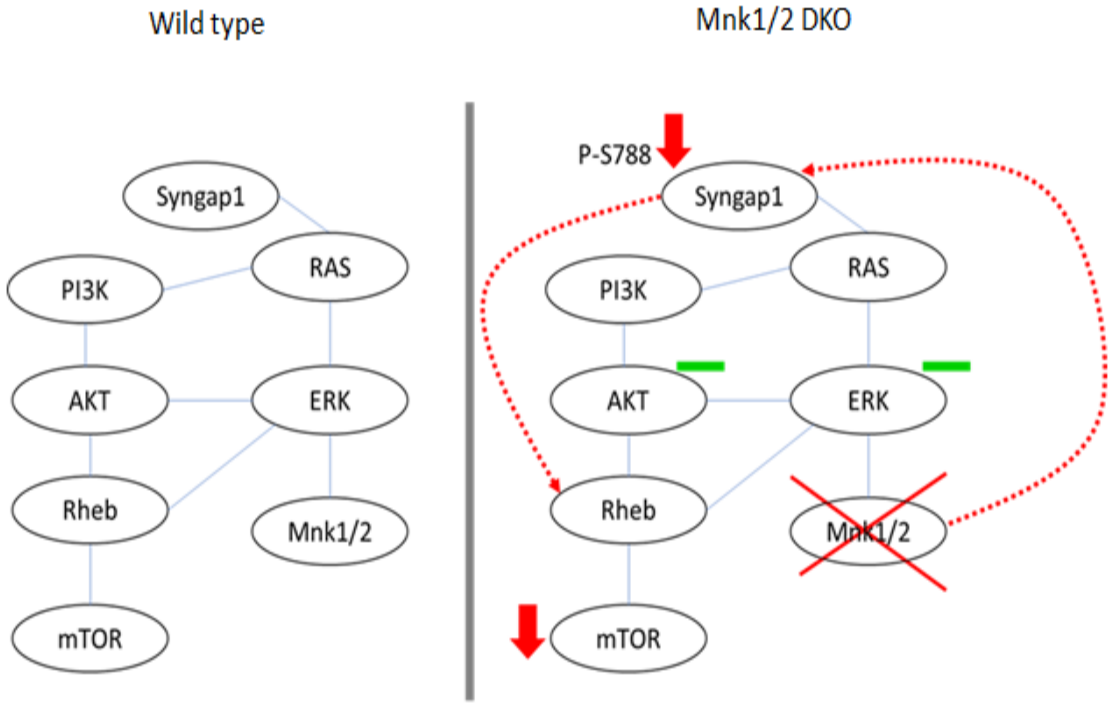

Figure 8. Schematic illustration of the hypothesis that Syngap1 S788 phosphorylation may regulate mTORC1 via increased GAP-activity for the Rheb GTPase.

Mnk-mediated phosphorylation of Syngap1 promotes mTORC1 activity and protein synthesis

We generated Syngap1 wild-type and phospho-mutant (Syngap1^{S788A}) or phospho-mimetic (Syngap1^{S788D}) expression constructs to examine if S788 phosphorylation alters Syngap1 GAP activity towards Rheb GTPase. And so we performed active-Rheb (Rheb-GTP) immunoprecipitation from HEK293 cells transfected with the different mutants of Syngap1 (Fig. 9). Wild-type and S788A expression led to reduced active Rheb (Rheb-GTP) recovered in the IP **(Fig. 9)**. These results were have been further confirmed by using immunoprecipitated (IP) Syngap1 from HEK-293H cells transfected with the different mutants of Syngap1, using a recombinant Rheb GTPase assay based on luminescence (summarized in Chalkiadaki et al.,).

Thus, Mnk-mediated phosphorylation of Syngap1 on S788 promotes mTORC1 activity and thereby protein synthesis, possibly via reduced Syngap1 GAP-activity for Rheb-GTPase independent of p-ERK or p-Akt.

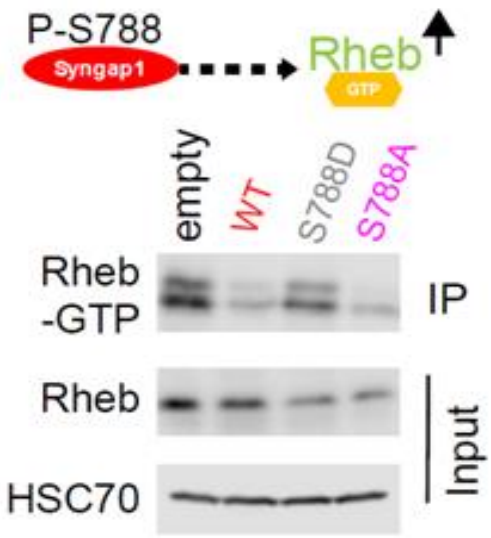

Figure 9. Active Rheb (Rheb-GTP) immunoprecipitation-based assay.

Syngap1 wild-type, phospho-mutants or empty vector groups were probed with antisera against the indicated proteins using IP and total lysates; HSC70: loading control (the experiment was replicated 2 times).

Syngap1 phosphorylation on S788 increases global protein synthesis

To assess the role of the Syngap1 S788 phospho-site in regulation of global protein synthesis, we used Syngap1 wild-type and phospho-mutant (*Syngap1*^{S788A}; phospho-mutant or *Syngap1*^{S788D} phospho-mimetic) expression constructs and performed transient transfection in HEK-293H cells. We then pulsed HEK-293H cells with puromycin and detected its incorporation into nascent peptides with immunoblotting (SUnSET method) **(Fig. 10a)**. Expression of wild-type Syngap1 led to a ~30% decrease in puromycin incorporation (a proxy for global protein synthesis), compared with empty vector Phospho-mimetic *Syngap1*^{S788D} expression displayed loss of function in inhibiting protein synthesis, while phospho-mutant *Syngap1*^{S788A} expression inhibited protein synthesis akin to wild-type **(Fig. 10b)**. These data, in conjunction with the reduction in Syngap1 S788 phosphorylation in Mnk DKO brain (Chalkiadaki et al., 2022) suggest that Mnk phosphorylation of Syngap1 on S788 promotes protein synthesis.

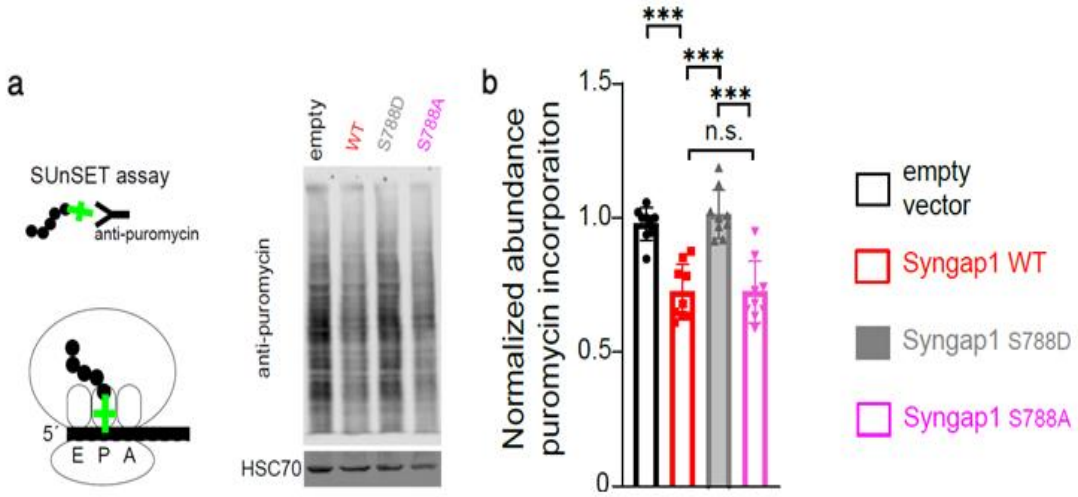

Figure 10. Puromycin incorporation assay in HEK-293H cells transfected with wild-type, phospho-mutant (S788A) or phospho-mimetic (S788D) Syngap1

a. Puromycin incorporation assay in HEK-293H cells transfected with wild-type or phospho-mutant (S788A) or phospho-mimetic (S788D) Syngap1. **b**. Representative immunoblot analysis of lysates probed with antisera against the indicated proteins;

HSC70 is the loading control. One-way ANOVA with Tukey's post-hoc, *$p < 0.05$. Empty vector Syngap1 WT (9) Syngap1 S788D (9) Syngap1 S788A (9).

AAV9-shRNA-mediated Syngap1 inhibition and chronic eFT508 treatment normalised mTORC1 signalling in Mnk DKO and Syngap1$^{+/-}$ mice.

Based on the newly found Mnk-Syngap1 interplay via mTORC1, we further hypothesised that: a) Syngap1 inhibition in Mnk DKO and b) pharmacological inhibition of Mnk in Syngap$^{+/-}$ mice would normalise mTORC1 signalling.

To this end, adeno-associated virus 9 (AAV9) expressing short-hairpin RNAs (shRNAs) against mouse *Syngap1* mRNA, driven by the U6 promoter (AAV9-Syngap1-shRNA, were prepared by Vector Biolabs. AAV9-Syngap1-shRNA or an AAV9 expressing scrambled sequence was injected into the hippocampus of wild-type and Mnk DKO mice. The hippocampus of the AAV9-Syngap1-shRNA was then lysated to study the mTORC1 signalling. In Mnk DKO mice, shRNA against Syngap1 but not scrambled-shRNA restored reduced phosphorylation of rpS6 (240/244), suggesting that mTORC1 activity was normalised to wild-type levels **(Fig. 11b)**. These data are consistent with the results of the behavioural experiments which indicate that increased Syngap1 phosphorylation and thus activity downstream of Mnk, underlies memory deficits in Mnk DKO mice (Chalkiadaki et al., 2022).

Tomivosertib (eFT508) is a brain-permeable, which is highly specific inhibitor of Mnks (Reich et al., 2018). Pharmacological modulation of Mnks emerged as a promising therapeutic avenue in autism spectrum disorders (Gkogkas et al., 2014; Hörnberg et al., 2020). Since Mnk-mediated phosphorylation suppresses Syngap1 activity, we reasoned that inhibition of Mnk might increase Syngap1 activity and restore mTORC1 signalling. Indeed Mnk inhibition via chronic eFT508 treatment reduced enhanced phosphorylation of rpS6 (240/244) to wild-type levels **(Fig. 11a).** We chose to examine only the 240/244 site, as it is the major output of mTORC1, while 235/236 can be phosphorylated by other kinases (Hutchinson et al., 2011). These data are consistent with the results of the behavioral rescue experiments in Chalkiadaki et al., which indicate that pharmacological inhibition of Mnk by eFT508

corrects behavioural phenotypes in Syngap1$^{+/-}$ mice. Altogether, pharmacological inhibition of Mnk kinase activity in Syngap1$^{+/-}$ mice with eFT508 normalized exaggerated mTORC1 signaling in Syngap1$^{+/-}$ mice, and reversed autism-related behaviours.

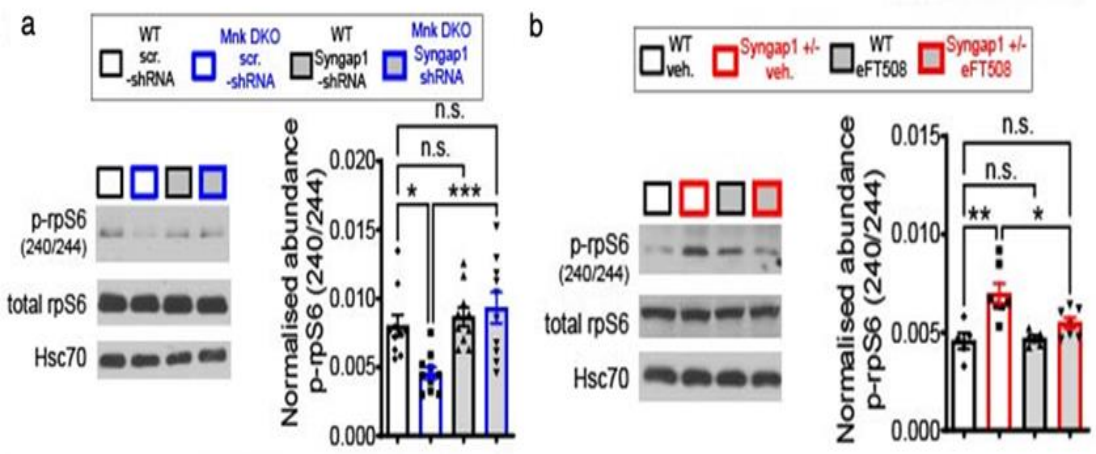

Figure 11. AAV9-shRNA-mediated Syngap1 inhibition and chronic eFT508 treatment normalised mTORC1 signalling in Mnk DO mice and Syngap1$^{+/-}$ mice.

a. Immunoblot analysis and quantification of hippocampal tissue lysates isolated from animals analysed in Chalkiadaki et al., 2022 study. One-way ANOVA; Bonferroni's post-hoc. WT scr (10) Mnk DKO scr (10) WT shRNA (10) Mnk DKO shRNA (10) **b.** Immunoblot analysis and quantification from hippocampal tissue lysates isolated from animals analysed in Chalkiadaki et al., 2022 study. One-way ANOVA; Bonferroni's post-hoc. WT veh (5) WT eFT508 (7) Syngap1 veh (5) Syngap1 eFT508 (7). Lysates were probed with antisera against the indicated proteins. Hsc70; loading control. All data are shown as mean ± S.E.M. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Mnk DKO: *Mnk1$^{-/-}$Mnk2$^{-/-}$*, Syngap1+/-: *Syngap1$^{+/-}$*, WT: *Mnk1$^{+/+}$Mnk2$^{+/+}$* or *Syngap1$^{+/+}$*.

Based on my work and other findings in Chalkiadaki et al., the following mechanism could be proposed **(Fig. 12).** Mnk1 phosphorylates Syngap1 downstream of MAPK resulting in the regulation of memory and autism-related behaviours. Syngap1 is an inhibitor of mTORC1, controlling protein synthesis, memory and autism-related behaviours. Mnks phosphorylate Syngap1 on S788 to reduce its GAP activity for Rheb

and thus promote mTORC1 activity and protein synthesis, which are required for memory and autism-related behaviours **(Fig. 12 left)**. Depletion of Mnks reduces Syngap1 S788 phosphorylation, increasing Syngap1 inhibitory GAP activity, reducing active Rheb and thus decreasing mTORC1 activity and protein synthesis, leading to memory impairment and autism-related behaviours. **(Fig. 12 right).**

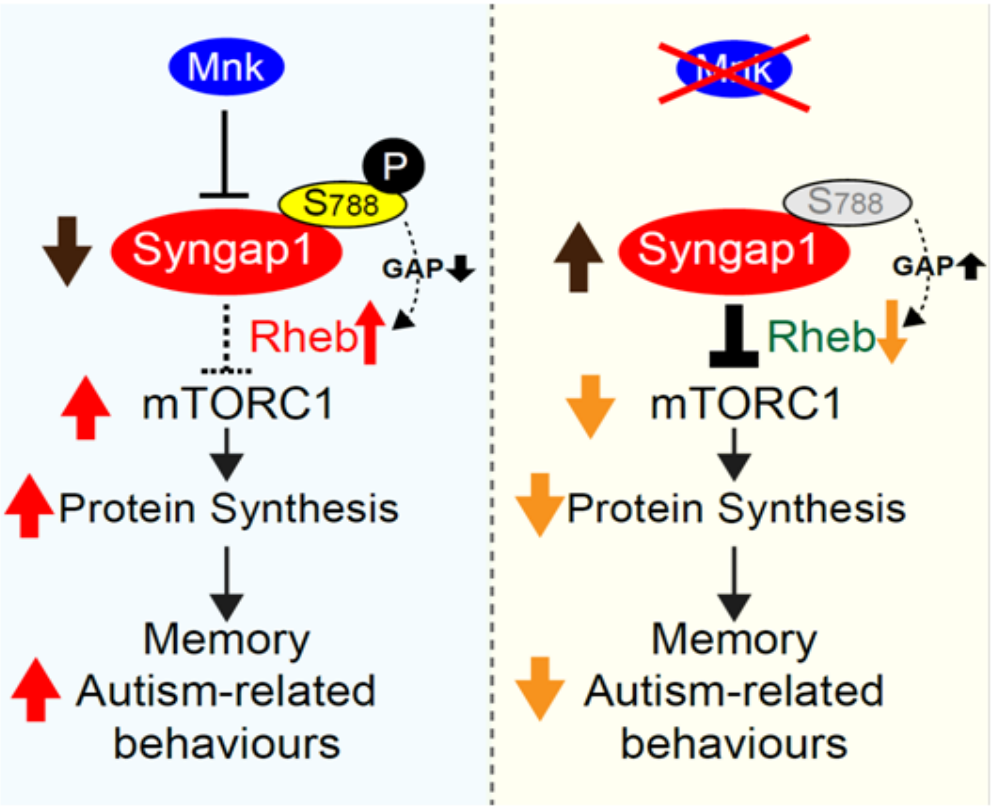

Figure 12. Schematic illustration of the proposed mechanisms for the Mnk-Syngap1 interplay.

Chapter 4: Discussion

This MSc thesis based on 'omics data from Chalkiadaki et al., 2022, provides strong evidence in support of a previously unknown link between Mnk kinases and the genetic ASD risk factor Syngap1 in regulating mTORC1 signalling and protein synthesis. Pharmacological and AAV-mediated knockdown experiments by Chalkiadaki et al., further revealed that the Mnk-Syngap1 axis is crucial for ASD-linked behaviours such as social interaction, learning, and memory.

While it is widely known that Mnk kinases contribute to the regulation of phosphorylation and activity of proteins involved in many different cellular functions including development, apoptosis, autophagy, oncogenesis, inflammation etc., (Roux & Blenis, 2004) only a few substrates of Mnks have been identified in the brain and importantly their roles remain elusive. eIF4E one of the well-known substrates of Mnks can partially account for the plethora of Mnks-regulated functions in the brain. It appears that Ser 209 phosphorylation of eIF4E may be required for the translation of mRNAs coding for ECM proteins (Amorim et al., 2018). This is in line with previous literature showing translational control of ECM proteins by phospho eIF4E in cancer (Furic et al., 2010; Preston et al., 2022; Robichaud et al., 2015) and in fragile X syndrome (Gantois et al., 2017; Gkogkas et al., 2014). More specifically it has been shown that eIF4E phosphorylation promotes the translation of mRNAs encoding the matrix metalloproteinases (MMPs) MMP3 and MMP9, which are overexpressed in PC3 prostate cancer cells and promote invasion and metastasis by rearrangement of the ECM resulting in tumorigenesis and prostate cancer progression (Furic et al., 2010). Robichaud et al., showed in 2015 phosphorylation of eIF4E promotes EMT and metastasis via translational control of SNAIL and MMP-3 while it has been also shown that phosphorylation of eIF4E in the stroma drives the production and spatial organization of collagen type I in the mammary gland (Preston et al., 2022). As for the FXS, it is known that pharmacogenetic inhibition of eIF4E-Dependent Mmp9 mRNA translation reverses fragile X syndrome-like phenotypes (Gkogkas et al., 2014). Metformin, the most widely used drug for type 2 diabetes, rescues core

phenotypes in Fmr1$^{-/y}$ mice and selectively normalizes ERK signaling, eIF4E phosphorylation and the expression of MMP-9 (Gantois et al., 2017).

Unlike Eif4e^{Ser209Ala} mice, Mnk DKO mice exhibited impaired hippocampal synaptic plasticity, learning and memory (Chalkiadaki et al., 2022). When comparing Mnk DKO whole brain translatome (ribosome profiling) to Eif4e^{Ser209Ala} only a small overlap between Mnk DKO and Eif4e^{Ser209Ala} translatomes was revealed, chiefly comprising of ECM-related genes. While ECM regulation was linked to synaptic function, plausibly Syngap1 may account for several functions that are not regulated by eIF4E. Mnks, which are part of MAPK signalling, play a crucial role in regulating synaptic plasticity, learning and memory (Kelleher et al., 2004). Long-term Potentiation; LTP (a cellular process in hippocampal and other neurons which underlies synaptic plasticity, learning and memory), is mediated by sustained BDNF signalling to Mnk and Mnk-dependent regulation of translation in two functionally and mechanistically distinct stages (Panja et al., 2014). Syngap1 also regulates plasticity in the brain (Araki et al., 2020), thus plausibly the newly identified Mnk-Syngap1 axis can be pivotal in regulating LTP. Apart from plasticity and memory, many of the mRNAs that are regulated by MNKs in the MAPK pathway are upregulated in patients with syndromic ASD (Stivaros et al., 2018). Recently, Hornberg and colleagues showed that pharmacological inhibition of Mnk restores mRNA translation, oxytocin signaling and social novelty in a mouse model of the syndromic ASD gene Ngln3 indicating a relationship between ASD and MNKs. Given the spatial memory phenotypes in Mnk DKO mice and the emerging role of Mnks in ASD, we compared the phosphoproteomics datasets to SFARI syndromic genes and identified several common targets, while there is also a significant overlap between the datasets based on Chalkiadaki et al., 2022 and a previous SILAC proteomics dataset from Mnk1 mouse knockout neurons. Recently it was shown that phospho-eIF4E (Amorim et al., 2018) and Mnk kinases (Aguilar-Valles et al., 2018) were linked to Major Depressive Disorder (MDD), using mouse models. Syngap1 mutations engender a complex pathology which also involves depressive phenotypes (Wright et al., 2022), thus the Mnk-Syngap1 link may also be relevant to MDD. The work performed in this thesis and in Chalkiadaki et al., revealed novel aspects of Mnk kinase biology. The

consensus motif ("SPEAK") for Mnks which is similar to motifs of other kinases (ERK, CDK5) thus we reasoned that it is plausible Mnks work in synergy with other kinases to phosphorylate a given subset of proteins. Such candidate kinases include CamKIIα and Cdk5, which are known to phosphorylate Syngap1 (Walkup et al., 2015). Consequently, Mnks could phosphorylate Syngap1 directly or in complex with CamKIIα/Cdk5, recruiting and phosphorylating Syngap1 on S788 (CamKIIα site) and S1165 (Cdk5 site). This is reminiscent of the mechanism by which Mnks phosphorylate eIF4E on Ser209, requiring first binding to eIF4G (Waskiewicz et al., 1997). Mnk1 binding to Syngap1 at synaptic sites could lead directly or indirectly (via Cdk5/CamKIIα) to S788 phosphorylation, thus regulating local translation. We observed that mTORC1 is downregulated in Mnk DKO mice, while Akt and Erk phosphorylation was not altered. Syngap1 displays GAP activity for several small GTPases upstream of Akt, Erk and mTOR (e.g. Ras). In S788 phosphomutant lysates (but not phosphomimetic) and in Mnk DKO synaptosomes (where S788 phosphorylation was reduced) I measured increased Syngap1 GAP activity for Rheb, leading to inhibition of active Rheb. As Rheb is an upstream mTORC1 activator (Bai et al., 2007) collectively these findings may explain reduced mTORC1 signalling in Mnk DKO, concomitant with reduced Syngap1 S788 phosphorylation. p-Akt and p-ERK could remain unaltered in Mnk DKO due to homeostatic regulation of different phosphosites on Syngap1. Furthermore, expression of phospho-mimetic Syngap1^{S788D} displays loss of function in inhibiting protein synthesis compared with wild-type Syngap1. This suggests that Syngap1 S788 phosphorylation by Mnks is required to stimulate protein synthesis. It would be interesting to study more the interplay between Syngap1 and Mnk and uncover if any other proteins participate in this signalling pathway. Furthermore, we reveal that in addition to eIF4E, Mnks have additional downstream effectors, which regulate mRNA translation and maybe there are other pathways that are unidentified. Plausibly, in synaptic sites, Mnk interaction with Syngap1 may exert local translational control via Syngap1/Rheb/mTORC1 pathway. Whereas in other sites Mnk1-mediated phosphorylation of eIF4E on Ser209 could have a more prominent, yet mTORC1-independent effect on the translatome. Moreover, Mnk1 via eIF4E, Syngap1, or other unknown mediators may have divergent effects on mRNA translation in different brain regions and thus

regulate different behaviours (e.g. memory, ASD-related or affective behaviours). In conclusion, the work performed in this thesis highlighted and further elaborated the mechanistic link between Mnk and the syndromic autism risk protein-coding gene Syngap1 **(Fig. 12)**. This constitutes a previously unidentified mechanism of translational control in brain downstream of MAPK, with wider implications for synaptic plasticity, learning, memory, and autism-related behaviours and other neurological disorders.

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