THE ROLE OF 4E-BPs IN SYNAPTIC PLASTICITY IN THE DEVELOPING NERVOUS SYSTEM AND IN THE MATURE CEREBELLUM

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ABSTRACT

At many excitatory synapses across the nervous system, synaptic plasticity requires acute protein synthesis. Recent data shows that in the hippocampus, 4E-BP2 and cap-dependent translation critically regulate both the early and late forms of long term potentiation at excitatory synapses. Yet, it is not known whether 4E-BPs and cap-dependent translation are directly involved in synaptic plasticity outside of the hippocampus. Moreover, it has been established that structural changes are involved in the long-term maintenance of memories and involve the PI3K-AKT-mTOR pathway. However, it remains unclear how the mTOR pathway affects the structural changes necessary for the long-term maintenance of plasticity, and if structural plasticity requires 4E-BPs and cap-dependent translation. To study these two issues, I examined long term depression at the parallel fiber-Purkinje cell synapse (PF-PC LTD) in 4E-BP2 knockout mice (4E-BP2 KO) and I investigated the effects of genetically removing 4E-BP1 on synaptic refinement at synaptically silent cholinergic synapses in sympathetic ganglia (α3/4E-BP-DKO mice). I asked (1) what is the effect of 4E-BP2 genetic deletion on PF-PC LTD and on motor learning?; (2) does genetic deletion of 4E-BP2 affect the molecular signaling pathways that regulate LTD?; (3) what is the role of 4E-BP in synaptic elimination? To address these questions, I used electrophysiological, molecular biology and imaging techniques.

I found that genetic deletion of 4E-BP2 had an unexpected effect on cerebellar plasticity, converting PF-PC LTD into LTP. This effect was not due to changes in morphology of the cerebellum or in excitatory synaptic innervation of PCs, nor was it caused by defects in calcium signaling in the PC. This raised the question: how does removing 4E-BP2 convert PF-PC LTD to an LTP? To test this, I used pharmacological inhibitors to block key molecules whose role in PF-PC synaptic plasticity were previously established and found that inhibition of PP2A was the only pharmacological intervention that rescued PF-PC LTD. PP2A is a complex formed by catalytic, structural and regulatory subunits, which are

themselves regulated by activators and inhibitors. To test whether genetic deletion of 4E-BP2 alters PP2A protein levels, I performed a western blot analysis and found that at baseline, expression of the PP2A activator PTPA is enhanced and that the inhibitor PPME1 is decreased. Moreover, I found that 4E-BP2-KO mice have defects in VOR gain adaptation when exposed to gain-down stimuli, consistent with the notion that disruptions in PF-PC LTD correlated with aberrant motor learning.

Next, I tested the role of 4E-BP1 on the reorganization of cholinergic inputs innervating developing sympathetic neurons in the Superior Cervical Ganglion (SCG). I showed that while SCG neurons from WTs are innervated by 7-8 axons at birth, they remain innervated by only 1-3 inputs by post-natal day 30 (P30). To test the effect of removing rapid synaptic transmission on refinement, I used mice in which the α3-nicotinic receptor subunit was genetically knocked out (α3-KO). To count inputs in these mice, I first had to unsilence the synapses; to this end, I restored synaptic activity by infecting mice with an α3-containing adenoviral construct expressed for 48 hours. I showed that presynaptic inputs innervating α3-KO SCG fail to refine, and P30 α3-KO SCG neurons remain innervated by 7-8 axons. Surprisingly, I found that genetically removing 4E-BP1 in α3-KO SCG was sufficient to restore synaptic elimination.

My results (1) establish that 4E-BP2 is critical for PF-PC LTD and VOR gain adaptation; (2) demonstrate that genetic deletion of 4E-BP2 enhances PP2A activation which converts PF-PC LTD to LTP; and (3) reveal that knocking out 4E-BP1 enables synaptic elimination to occur in the absence of synaptic activity. Taken together, my results suggest that 4E-BP is a critical regulator of plasticity; 4E-BP2 is essential for PF-PC LTD, whereas activity-dependent inactivation of 4E-BP1 is necessary for structural plasticity at cholinergic synapses.

RÉSUMÉ

La plasticité synaptique de plusieurs synapses excitatrices à travers le système nerveux nécessite une synthèse protéique aiguë. Des données récentes montrent que dans l'hippocampe, 4E-BP2 et la traduction dépendante de la coiffe, m^7GTP, régulent de manière critique les formes précoces et tardives de potentialisation à long terme. Pourtant, on ne sait pas si les 4E-BPs et la traduction dépendante de la coiffe sont directement impliqués dans la plasticité synaptique à l'extérieur de l'hippocampe. De plus, il a été établi que des changements structurels au niveau des synapses sont impliqués dans le maintien à long terme des mémoires et impliquent la voie de signalisation PI3K-AKT-mTOR. Cependant, on ne sait pas encore comment la voie de signalisation mTOR affecte les changements structurels nécessaires pour maintenir la plasticité synaptique à long terme, et si la plasticité structurelle nécessite 4E-BP et la traduction dépendante de la coiffe. Pour étudier ces deux questions, j'ai examiné la dépression synaptique à long terme au niveau de la synapse entre les fibres parallèles et les cellules de Purkinje (FP-CP DLT) chez des souris qui possèdent une délétion du gène 4E-BP2 (4E-BP2 KO) et j'ai étudié les effets de l'élimination génétique du gène 4E-BP1 sur le développement des terminaisons nerveuses cholinergiques dans les circuits autonomes, un phénomène qui requiert la plasticité structurelle, dans lesquels l'activité post-synaptique est absente (souris α3 / 4E-BP-DKO). J'ai demandé (1) quel est l'effet de la délétion génétique de 4E-BP2 sur la FP-CP DLT et sur l'apprentissage moteur ?; (2) l'élimination génétique de 4E-BP2 affecte-t-elle les voies de signalisation moléculaire qui régulent la DLT ?; (3) quel est le rôle de 4E-BP dans le développement des terminaisons nerveuses? Pour répondre à ces questions, j'ai utilisé des techniques d'électrophysiologie, de biologie moléculaire et d'imagerie optique.

J'ai découvert que la délétion génétique de 4E-BP2 avait un effet inattendu sur la plasticité dans le cervelet, convertissant FP-CP DLT en PLT. Cet effet n'est pas dû à des changements dans la morphologie du cervelet ou dans

les contacts synaptiques excitateurs reçus par les CP, ni à des défauts de signalisation du calcium dans la CP. Hors, comment la délétion génétique de 4E-BP2 convertit-elle la FP-CP DLT en PLT? Pour tester cela, j'ai utilisé des inhibiteurs pharmacologiques pour bloquer des molécules clés avec un rôle établi dans la plasticité synaptique des synapses FP-CP. J'ai trouvé que l'inhibition pharmacologique de PP2A était la seule intervention qui a rétabli la FP-CP DLT. PP2A est un complexe formé de sous-unités catalytiques, structurelles et régulatrices, elles-mêmes régulées par des activateurs et des inhibiteurs. Pour tester si l'élimination génétique de 4E-BP2 modifie les niveaux de protéine PP2A, j'ai effectué une analyse Western Blot. J'ai constaté que dans le cervelet des souris avec un délétion de 4E-BP2, l'expression de l'activateur de PP2A, PTPA, est élevé et que celle de l'inhibiteur de PP2A, PPME1, est diminué comparé au souris contrôles. De plus, j'ai trouvé que les souris sans 4E-BP2 ont des défauts dans l'adaptation du gain du réflexe oculaire vestibulo en réponse à différentes fréquences de mouvement, ce qui est cohérent avec l'idée que les perturbations de la FP-CP DLT soient corrélées avec des problèmes d'apprentissage moteur.

Ensuite, j'ai testé le rôle de 4E-BP1 sur la réorganisation des terminaisons nerveuses cholinergiques en contact avec les neurones sympathiques dans le ganglion cervical supérieur (GCS). J'ai montré qu'à la naissance, les neurones GCS des souris sont innervés par 7 à 8 neurones, alors qu'ils restent innervés par seulement 1 à 3 neurones rendu au jour 30 post partum (P30). Pour tester le rôle de transmission synaptique rapide sur l'élimination des axones présynaptiques, j'ai examiné la conséquence d'éliminer activité synaptique rapide. Pour ce faire, j'ai utilisé le GCS de souris dans lequel la sous-unité du récepteur α3-nicotinique a été génétiquement éliminée (α3-KO). Pour compter le nombre d'axones qui innerve une neurone sans transmission synaptique dans le GSC, j'ai d'abord dû restauré l'activité synaptique; pour ce faire, j'ai infecté des souris avec un vecteur adénoviral contenant α3 exprimée pendant 48 heures. J'ai montré que sans activité synaptique rapide, le nombre d'axones présynaptiques innervant la neurone GCS dans les α3-KO reste comparable à

celui des nouveau né, puisque les neurones P30 α3-KO GCS sont reste innervés par 7 à 8 axones. Étonnamment, j'ai trouvé que l'élimination génétique de 4E-BP1 dans le GCS des α3-KO était suffisante pour restaurer l'élimination synaptique.

Mes résultats (1) établissent que 4E-BP2 est critique pour la DLT et l'adaptation du gain du réflexe oculaire vestibulo; (2) démontrent que la délétion génétique de 4E-BP2 augmente l'activation de PP2A, ce qui convertit FC-CP DLT en PLT; et (3) révèlent que l'élimination génétique de 4E-BP1 permet l'élimination synaptique en l'absence d'activité synaptique. Ensemble, mes résultats suggèrent que 4E-BP est un régulateur critique de la plasticité; 4E-BP2 est essentiel pour FC-CP DLT, tandis que l'inactivation de 4E-BP1, dépendant de l'activité synaptique rapide, est nécessaire pour la plasticité structurelle au niveau des synapses cholinergiques.

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I have many people to thank for making my years in the lab productive and a rich learning experience. Pejmun, thank you for taking me as your student years ago. Talking about science with you is always thrilling, making the world feel full of exciting possibilities and discoveries. Thank you, Brigitte, for being a constant source of positivity, I leave every interaction with you feeling uplifted. Merci pour tous les cafés et les croissants aux amandes! Yumaine, thank you for pushing me to always be better and for making all those late nights at the lab so much more fun. Dr. Cooper, the first lesson I learnt from you was to learn to think for myself and it's perhaps the one most impactful lesson that was reinforced throughout my time at the lab. I learnt so much from you about being a good scientist, writing and speaking well (still working on that!) and about working hard and with passion, and I am extremely grateful for your mentorship over the years. Importantly, thank you for supporting my decision to move away from academia and making the writing of this thesis as smooth as possible.

I'm fortunate to have been surrounded by many other peers and friends that also impacted my time at the lab. I apologize in advance to all the people I am forgetting to mention, but hopefully you're not reading this anyway! Thanks to Nancy for all the help making our experiments run more smoothly and for being such a lovely person. Bowie lab, thanks for all the discussions, advice with troubleshooting and borrowed reagents. Derek and Reza, I appreciate all your thoughtful questions and guidance during my committee meetings. Thanks to the Sonenberg Lab for supplying me with mice and Peng for help with the cerebellum western blot analysis. Thanks to Vanessa from the Cullen lab for help with the behavioral experiments. Evan, Ryan and Felix thank you for your feedback on my thesis, you guys are the best in every way.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

My findings reveal a novel role for 4E-BPs in regulating two forms of activity-dependent plasticity: one at PF-PC synapses and the other at cholinergic synapses in the autonomic nervous system. In chapter 3, I demonstrate that in the absence of 4E-BP2, PF-PC LTD gets converted into an LTP. Moreover, I show 4E-BP2-KOs have defects in motor learning, specifically in the vestibular ocular reflex gain-down adaptation. This data supports previous findings that alterations in PF-PC LTD affect motor learning. In addition, although the importance of acute translation of PF-PC LTD was previously established, my work is the first to show that the cap-dependent translation initiation factor, eIF4F, is required.

In chapter 4, I follow up on my observation that PF-PC LTD is converted into an LTP in 4E-BP2-KOs. My work illustrates a novel role for 4E-BP2 in the regulation of PP2A activity. I found that in 4E-BP2-KOs, the level of the activator of PP2A PTPA (protein phosphatase 2A phosphatase activator) is upregulated whereas the level of the PP2A inhibitor PPME1 (Protein phosphatase methylesterase-1) is decreased. Pharmacologically blocking PP2A activity during LTD induction restores PF-PC LTD in 4E-BP2-KOs. Taken together, these findings show that 4E-BP2 is necessary for the regulation of PP2A activity and to regulate the phosphatase/kinase balance involved in the induction of synaptic plasticity at PF-PC synapses.

In chapter 5, I discover a novel role for 4E-BP in synaptic refinement. My work shows that postsynaptic activity is critical for refinement to occur at cholinergic, superior cervical ganglion synapses. Mice in which the nicotinic receptor α3 subunit is genetically deleted (α3-KOs) have no rapid synaptic transmission and this completely prevents synaptic refinement from occurring. Immunostaining experiments show that 4E-BP1 activity is enhanced in α3-KOs compared to WTs, and proteomic analysis revealed that the translation of several proteins is altered in α3-KOs compared to WTs. This work also shows that genetically deleting 4E-BP in α3-KOs (α3/4E-BP-DKO), largely restored the

proteome. Fascinatingly, this study is amongst the first to show that synaptic refinement can occur in the absence of synaptic activity since α3/4E-BP-DKOs undergo synaptic refinement. In addition, my work shows that removing 4E-BP2 in otherwise WT animals has little effect on synaptic elimination.

CONTRIBUTIONS OF AUTHOR

4E-BP1/2 DKO mice were a gift from the Sonenberg lab (McGill University, Department of Biochemistry), and α3/4E-BP-DKO mice were generated by my labmate Y. Chong.

In chapter 3, I prepared cerebellar slices and performed all of the electrophysiological experiments. My labmate Y. Chong performed the immunohistochemistry experiments, assisted in the calcium imaging experiments, and analyzed the imaging data. V. Chang of the Cullen lab (McGill University, Department of Physiology) performed the gain-down VOR experiments and analyzed the VOR data. I analyzed all the other data, performed the statistical analysis and created the figures.

In chapter 4, I performed all the electrophysiological experiments, analyzed the data and created the figures. The western blot analysis was performed by Dr. P. Wang from the Sonenberg lab (McGill University, Department of Biochemistry).

In chapter 5, I performed all of the electrophysiological recordings and data analysis. My colleagues B. Pie and N. Grenier generated the adenoviruses, and N. Grenier and Y. Chong performed the adenoviral injections. Y. Chong performed immunohistochemistry experiments and analysis. J.Penney performed the western blot analysis. Figures were originally put together by Y. Chong and I for our manuscript (Chong et al., 2018), and I put together original versions for my thesis.

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION AND RATIONALE

During early development, neurons extend their axons to form synapses with designated target neurons as they establish neural circuits. Initially, these axonal projections are somewhat diffuse, and the functions of these early circuits are relatively imprecise. As development proceeds, however, neuronal activity improves the precision of these circuits by causing the synaptic connections to refine through a process that involves strengthening some inputs and eliminating others. This rearrangement of synaptic connections, or synaptic plasticity, persists throughout the life of the animal and provides the organism with the ability to adapt neural circuits to changes in its environment and to altering neuronal activity.

Considerable progress has been made in unraveling mechanisms that enable neurons to respond to changes in synaptic activity. These mechanisms involve a myriad of signaling mechanisms, usually culminating in new gene expression. Neurons are highly polarized cells with hundreds of synapses that can be located along dendrites several hundreds of microns from the cell body, and axonal nerve terminals are located even further. This raises the issue: how do the products of newly expressed genes in the nucleus mediate synaptic changes hundreds of microns from the cell body? In part, the answer is that the dendrites and nerve terminals contain messenger RNA encoding key proteins essential for synaptic plasticity, as well as ribosomes to translate these mRNA into proteins. And, recent evidence over the past 10-15 years has demonstrated that local mRNA translation and protein synthesis at active synapses is essential to induce and maintain functional or structural synaptic plasticity of these synapses.

For my thesis, I have focused on mechanisms involved in synaptic plasticity. Specifically, I have concentrated on the regulation of mRNA translation and how it underlies changes in the function and structure of synapses. My research addressed the question: how does disrupting the regulation of mRNA translation affect activity-dependent synaptic plasticity?

To provide context for my experiments, I begin with a brief review of our current understanding of how mRNA translation is regulated in neurons. Then, I discuss how regulation of mRNA translation affects plasticity at excitatory synapses, focusing on synapses in the hippocampus, among the best-studied synapses in the CNS, and ones involved in spatial learning. To learn if the results obtained from hippocampal synapses are applicable to other synapses in the CNS, I investigated excitatory synapses in the cerebellar cortex, synapses that are known to be involved in motor learning. Specifically, for my doctoral research, I investigated the consequence of dysregulating mRNA translation on the plasticity of excitatory synapses made by parallel fibers, the axons that emanate from Golgi neurons, onto the distal dendrites of Purkinje neurons. In addition, given that structural plasticity supports long term changes in synaptic strength, I have also investigated how the regulation of mRNA translation affects structural plasticity. For this, I examined the synaptic refinement of excitatory synapses during postnatal development.

1.2 THE REGULATION OF mRNA TRANSLATION IN NEURONS

1.2.1 Synaptic Activity Activates mTOR Through the PI3K Pathway and the ERK Pathway

Several experiments conducted over 15 years ago provided the first indications that synaptic transmission initiates mRNA translation in postsynaptic neurons (Kang and Schuman, 1996; Weiler et al., 1997; Bagni et al., 2000; Aakalu et al., 2001; Yin et al., 2002; Ju et al., 2004). Experiments using a variety of model systems ranging from isolated synaptosomes, neurons in culture and intact brain tissue showed that glutamate, acting on postsynaptic ionotropic and metabotropic glutamate receptors, and growth factors, such as neurotrophins and insulin-like growth factor 1 (IGF-1) acting on tyrosine kinase receptors (Trk), initiate the phosphatidylinositol 3-kinases (PI3K)/protein Kinase B (known as Akt) pathway and the extracellular signal-regulated kinase (ERK) pathway (Kang and Schuman, 1996; Bagni et al., 2000; Yin et al., 2002; Thomas and Huganir, 2004). The PI3K and ERK pathways converge on two complexes that contain the target of rapamycin (mTOR), a serine/threonine kinase (Kim et al., 2002; Sarbassov et al., 2004), mTORC1 and mTORC2. While the functions of mTORC2 are less clear, mTORC1 plays a critical role in mRNA translation initiation (reviewed by (Klann and Dever, 2004)). Below, I review how the PI3K and ERK pathways activate mTORC1.

1.2.1.1 mTORC1 Regulation by the PI3K Pathway

Insulin-like growth factor 1 (IGF-1) and neurotrophin 3 (NT-3) are among the main growth factors that stimulate mRNA translation in neurons. Insulin-like growth factor 1 (IGF-1) binds to IGF-1 receptors and stimulates insulin substrate-1 (IRS-1) leading to PI3K activation, whereas NT-3 binds with high affinity to TrkC and activates PI3K through the intermediary molecules, Shc, Grb2, and Gab -1 or -2 (Segal, 2003). PI3K, in turn, phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2), a membrane-bound phospholipid, to activate Akt which then activates mTORC1 indirectly by phosphorylating and inhibiting tuberin

(TSC2), a major subunit of the tuberous sclerosis complex (TSC) (Sabatini, 2006).

TSC is a heterodimer consisting of TSC1 (hamartin) and TSC2 (tuberin). TSC2 is a GTPase-activating protein (GAP) that hydrolyzes GTP bound to the small G protein Ras-homolog enriched in brain (Rheb). Phosphorylation of TSC2 decreases GAP activity, promoting Rheb and mTORC1 activation (Hay and Sonenberg, 2004) (Figure 1). Interestingly, in humans, TSC1/2 mutations are linked to autism spectrum disorder (Smalley, 1998), indicating the importance of this pathway for proper neural function.

1.2.1.2 mTORC1 regulation by the ERK pathway

The ERK pathway is activated by excitatory neurotransmission (reviewed in (Thomas and Huganir, 2004), figure 2). Glutamate released from presynaptic nerve terminals acts on ionotropic AMPA receptors (AMPA-R) and NMDA receptors (NMDA-R), as well as metabotropic glutamate receptors (mGluR1-5) leading to an increase in intracellular calcium, either through direct permeation through AMPA-Rs and NMDA-Rs, through membrane depolarization and activation of voltage-gated calcium channels (VGCC), or in the case of mGluRs, through activation of phospholipase C (PLC) and hydrolyzation of phosphoinositide phospholipids ($PIP_2 \rightarrow PIP_3$) to produce inositol 1,4,5-trisphosphate (IP_3) which binds to receptors on the endoplasmic reticulum to release calcium.

Elevations in cytosolic calcium activate ERK through a pathway that involves Ras–GTP (Ras: Rat sarcoma viral oncogene small G-protein), activation of Raf (Raf: Rapid Accelerated Fibrosarcoma), and the mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) (Figure 2). How calcium activates Ras signaling is not fully understood, but likely involves Ras–guanyl nucleotide exchange factors (GEFs) and Ras–GTPase (GAPs) (Rosen et al., 1994; Yun et al., 1999). Active ERK phosphorylates and activates p90S6K (RSK), which in turn phosphorylates and activates PDK1 (Frodin et al., 2000), leading to Akt

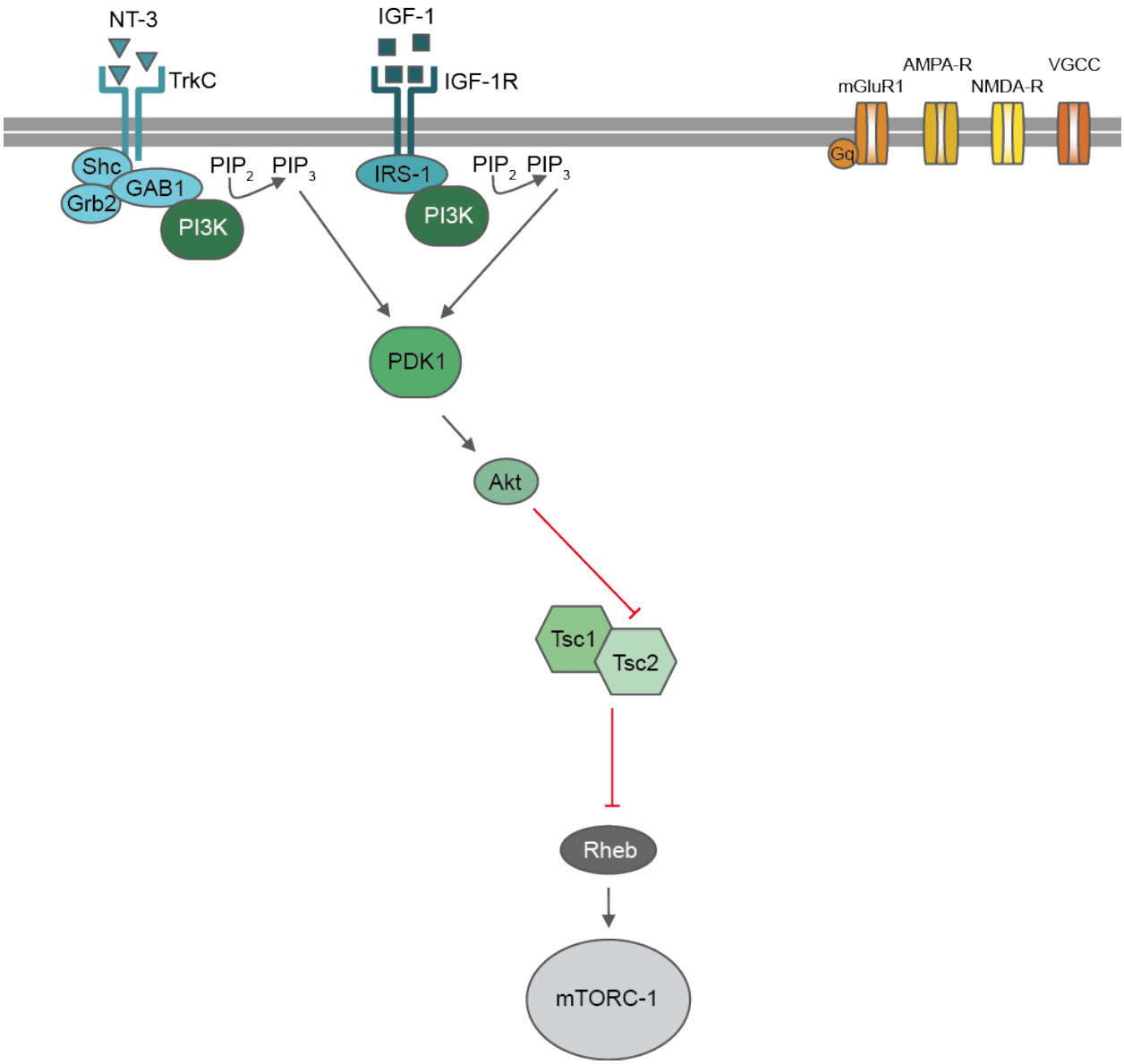

Figure 1 Growth factors, through the PI3K pathway, regulate mTORC1. At the postsynaptic membrane, growth factors such as NT-3 and IGF-1 activate TrkC and IGF-1 receptors, respectively. This leads to PI3K activation; active PI3K phosphorylates membrane-bound phospholipid PIP_2 to form PIP_3. PIP_3 recruits Akt and PDK1 to the plasma membrane, enabling the PDK1 to phosphorylate Akt, which activates Akt. Active Akt inhibits TSC2, which in turn disinhibits Rheb, activating mTORC1.

activation, TSC2 inhibition and downstream activation of mTORC1 through the same molecular pathway described in section 1.2.2.1. In addition, active ERK and active RSK can both impact mTORC1 function by phosphorylating TSC2 directly (Figure 2) (Roux and Blenis, 2004; Ma et al., 2005).

Although many of the signaling molecules and pathways upstream of mTORC1 had initially been discovered in non-neuronal cells (Hay and Sonenberg, 2004), it is now clear that these signaling pathways are also initiated in neurons by excitatory synaptic transmission.

1.2.2 mTORC1 and cap-dependent mRNA translation

Translation occurs in three steps: initiation, elongation and termination. In eukaryotes, initiation is generally the rate-limiting step and begins when a ribosome binds to the start codon (AUG) of the mRNA. The secondary structures of non-coding nucleotides adjacent to the AUG (the 5' untranslated region (5' UTR)) can hinder ribosome binding to the start codon (Figure 3A), and therefore the 5'UTR must be unwound and stabilized, a process that requires several initiation factors and the assembly of the eukaryotic initiation complex (referred to as "eukaryotic initiation factor 4F" or eIF4F). eIF4F comprises 12 proteins, including an RNA helicase, eIF4A, to disrupt the secondary structures, a scaffolding protein, eIF4G, and eIF4E which binds to the 5' end of mRNA (Figure 3B).

The assembly of eIF4F is essential to recruit ribosomes to AUG. A crucial first step is the binding of eIF4E to the 5' end of the mRNA, or cap structure containing a m7G(5')ppp(5')N sequence (Figure 3B) (Gingras et al., 1999). In eukaryotes, essentially all mRNA translation requires the binding of eIF4E to the cap structure and therefore mRNA translation is referred to as cap-dependent translation.

The availability of eIF4E for cap-binding is regulated by the eIF4E binding proteins (4E-BPs), proteins that sequester eIF4E and prevent it from interacting

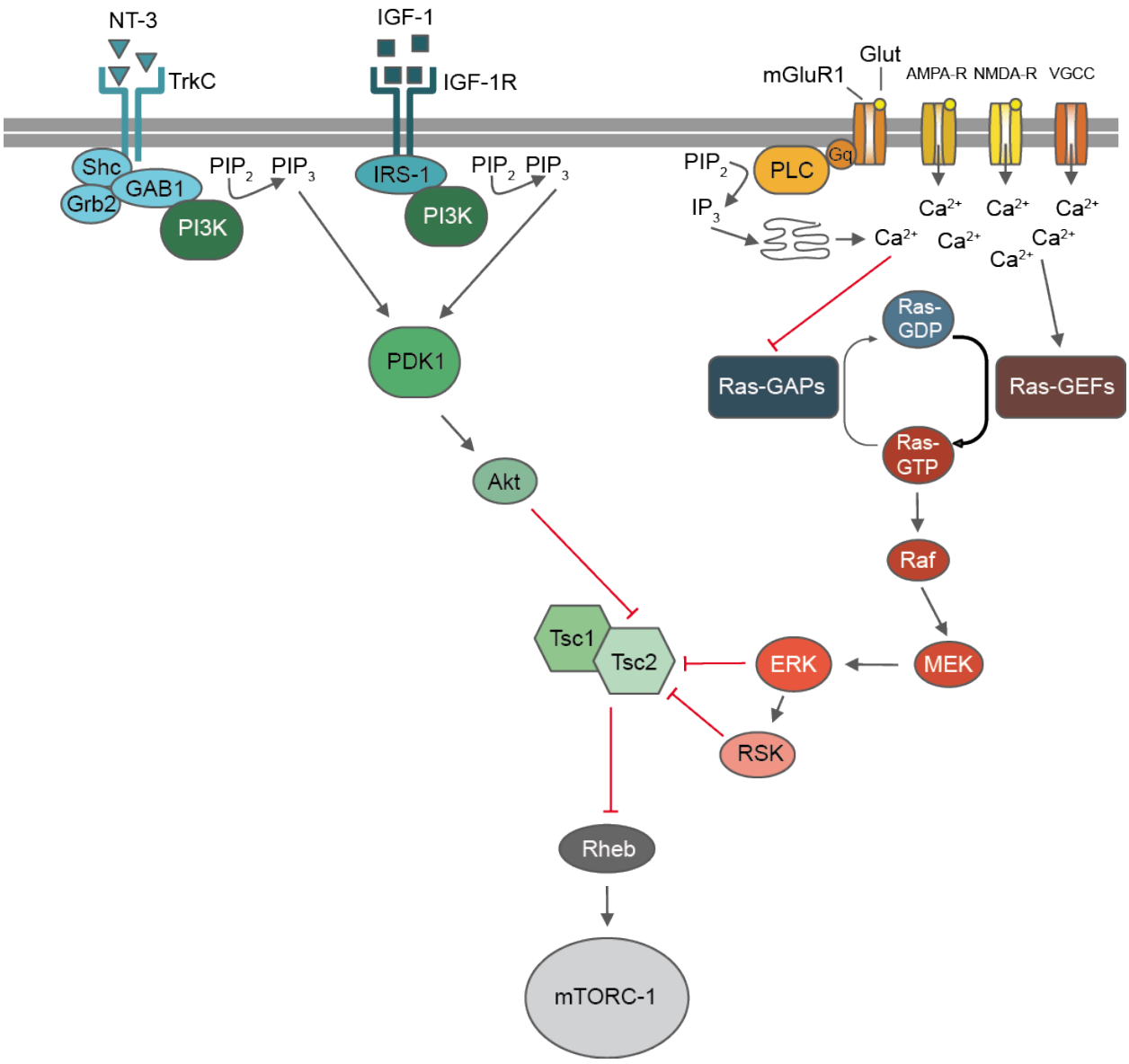

Figure 2 Glutamate activates the ERK pathway to regulate mTORC1. Glutamate binds to postsynaptic AMPARs and mGluRs, which depolarizes the postsynaptic neuron leading to elevated cytosolic calcium. Elevated calcium converts Ras-GDP to Ras-GTP. Ras-GTP activates Raf, which activates MEK, leading to ERK activation. ERK inactivates TSC2 directly, leading to mTORC1 activation. ERK also indirectly inhibits TSC by activating RSK. RSK inhibits TSC2 directly, or upstream by activating PDK1; this, in turn, leads to mTORC1 activation.

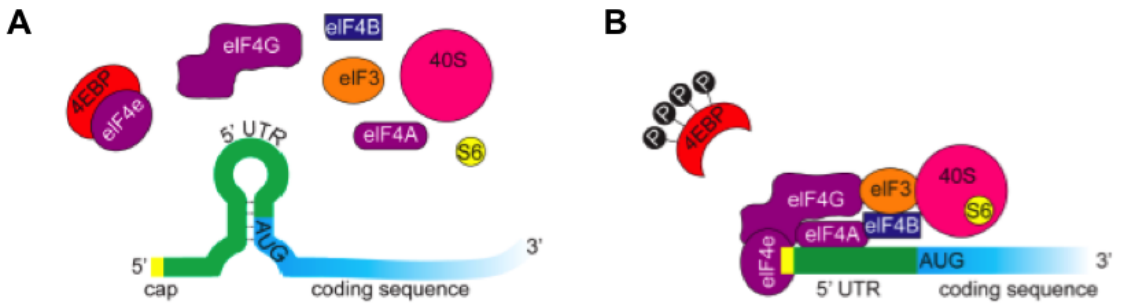

Figure 3 4E-BPs regulate cap-dependent translation initiation by modulating eIF4E availability. **A** Hypophosphorylated 4E-BPs bind and sequester eIF4E, preventing it from binding the 5' cap of mRNA and blocking eIF4F assembly. Secondary structures in the 5' UTR of mRNA prevent access of ribosomes of the AUG of mRNA. **B** Hyperphosphorylated 4E-BPs release eIF4E, enabling it to bind to the 5' end of mRNA and assemble with the other components of eIF4F, including the helicase eIF4A and the scaffolding protein eIF4G. eIF4F unwinds and stabilizes the secondary structures in the 5' UTR of the mRNA and facilitates the recruitment of ribosomes to the AUG of mRNA to initiate translation.

with mRNA (Figure 3A). There are 3 4E-BP genes: 4E-BP1, 4E-BP2 and 4E-BP3 (Poulin et al., 1998). 4E-BP2 is the predominant variant in the central nervous system, whereas 4E-BP1 is mainly expressed in the peripheral neurons.

The affinity of 4E-BPs for eIF4E depends on their phosphorylation state (Figure 3B). 4E-BP has 4 phosphorylation sites: Thr37, Thr46, Thr70 and Ser65 (Gingras et al., 2001), and when hypophosphorylated, 4E-BPs bind and sequester eIF4E in the cytoplasm. When these 4 residues are phosphorylated, 4E-BPs release eIF4E, allowing eIF4E to bind to the 5' cap and initiate mRNA translation (Figure 3B) (Pause et al., 1994; Haghighat et al., 1995; Mader et al., 1995; Marcotrigiano et al., 1999).

1.2.3 the TOR pathway regulates homeostatic synaptic plasticity in *Drosophila*

4E-BP's phosphorylation status is directly controlled by mTORC1 (Gingras et al., 2001) and excitatory synaptic transmission indirectly leads to the initiation of cap-dependent mRNA translation by activating mTORC1 (Hay and Sonenberg, 2004; Thomas and Huganir, 2004). A clear demonstration of this link is the work done at the neuromuscular junction (NMJ) in *Drosophila Melanogaster*.

1.2.3.1 Homeostatic regulation of synaptic strength in *Drosophila*

Drosophila Melanogaster is an excellent model to investigate the link between synaptic plasticity and mRNA translation regulation, in part, because of the ease with which one can delete or overexpress specific genes in a cell-type-specific manner (Sobels, 1974). Using this approach, Penney, Haghighi, and co-workers demonstrated that the mTOR pathway is critical for maintaining synaptic function at the *NMJ* (Penney et al., 2012).

Briefly, neurotransmission at the *D. Melanogaster* NMJ is mediated by glutamate. The postsynaptic glutamate receptors are composed of GluRIIC,

GluRIID, and GluRIIE subunits that form a tetramer with either GluRIIA or GluRIIB (Han et al., 2015); GluRIIB containing receptors have a lower mean open time than GluRIIA containing receptors (DiAntonio et al., 1999). Under basal conditions, presynaptic nerve terminals spontaneously release single vesicles of neurotransmitter, or quantum, and the postsynaptic current evoked by a quantum is referred to as a mini excitatory junction current (mEJC). When the action potential (AP) arrives at the presynaptic nerve terminal, tens of quanta are released simultaneously, giving rise to evoked synaptic transmission, known as the excitatory junction current (EJC). The number of quanta released by the AP, calculated by dividing the EJC by the mEJC (EJC/mEJC), is known as the quantal content (QC).

Genetically deleting GluRIIA (GluRIIA -/-) leaves only GluRIIB containing receptors, and consequently, a single vesicle of neurotransmitter produces a smaller mEJC; unexpectedly, however, the EJC in GluRIIA -/- is the same size as in controls, indicating that the number of quanta released by the presynaptic terminal increases in a homeostatic manner to maintain the size of the EJC (Petersen et al., 1997). This work was among the first to show that homeostatic plasticity regulates synaptic strength at neuronal synapses.

1.2.3.2 TOR regulates homeostatic plasticity at the *Drosophila* NMJ

Considering the accumulating evidence that TOR activity and mRNA translation influence synaptic growth, function, and plasticity in neurons (Tang et al., 2002; Ehninger et al., 2008; Swiech et al., 2008; Buckmaster et al., 2009; Hoeffer and Klann, 2010; Sharma et al., 2010), Penney et al. (2012) extended these studies to understand whether the mTOR (TOR in Drosophila) pathway is involved in homeostatic plasticity. Specifically, they deleted the *Tor* gene and asked if the compensatory increase in quantal content at the NMJ of GluRIIA -/- larvae still occurred.

Genetic deletion of both copies of *Tor* is lethal, however, genetically removing one copy of *Tor* decreases TOR levels by half, therefore Penney and

colleagues created GluRIIA-*Tor* double mutants (GluRIIA -/-; *Tor* +/-). They also examined larvae with a hypomorphic *Tor* mutation - that is, a *Tor* mutation that greatly reduces TOR activity (GluRIIA -/-; *Tor* -/-). Interestingly, removing one copy of *Tor* or hypomorphic TOR completely prevented the compensatory increase in QC in GluRIIA -/- larvae. Importantly, these *Tor* manipulations had no effect on basal neurotransmission in control GluRIIA +/+ larvae. In addition, Penney et al. (2012) blocked TOR pharmacologically with rapamycin and found that blockade of TOR prevented the compensatory increase in QC in GluRIIA -/- mutants. The results from this work established that TOR is required for the compensatory increase in QC in GluRIIA -/- mutants.

Reduced TOR activity, by genetic deletion or pharmacological inhibition, should increase hypophosphorylated 4E-BPs and greatly decrease eIF4E availability to initiate cap-dependent translation (see Figure 3). However, it was unclear from the above experiments whether this homeostatic compensation at the NMJ depended on eIF4E and cap-dependent translation. To test this, Penney et al. (2012) genetically deleted eIF4E in GluRIIA -/- mutants. Again, removing both eIF4E genes is lethal, but removing a single gene is not. Therefore, they genetically removed one copy of eIF4E in GluRIIA -/- larvae (GluRIIA -/-; eIF4E +/-) and found that it completely prevented the homeostatic plasticity in GluRIIA mutants. These results indicate that homeostatic regulation of synaptic strength at the NMJ critically acts through eIF4E.

The homeostatic compensation at the *Drosophila* NMJ is presynaptic in nature and raises the question: does TOR regulate the compensatory mechanism in the presynaptic neuron, postsynaptic cell, or both? To address this question, Penney et al. (2012) rescued *Tor* in GluRIIA -/-; Tor -/- mutants in a cell-specific manner. They found that only rescuing *Tor* in muscle and not in the presynaptic neuron restored the size of the EJC in GluRIIA -/-; Tor -/- mutants. Similarly, knocking down *Tor* using RNAi only postsynaptically prevented the homeostatic increase in QC in GluRIIA -/- mutants. Therefore, TOR acts

postsynaptically and the increase in presynaptic quantal release occurs in a retrograde manner.

1.2.3.3 TOR activity regulates the strength of synaptic connections at the *Drosophila* NMJ

These results clearly show that the mTOR pathway is required for homeostatic plasticity when synaptic activity is perturbed in GluRIIA -/- mutants. However, does mTOR activation also play the same role under normal conditions? Genetically reducing TOR function has no effect in otherwise wild-type larvae - but what about increasing TOR function? To address this, Penney et al. (2012) overexpressed the *Tor* transgene in postsynaptic muscles (Tor OE). Interestingly, they found that overexpressing TOR significantly increased EJCs due to increased QC as the quantum size was normal. Moreover, this effect could be blocked by genetically removing one copy of eIF4E (Tor OE; eIF4E +/-).

To demonstrate that the enhanced EJC was due to enhanced mRNA translation, Penney et al. (2012) prevented the increase in QC and EJC in Tor OE mutants by blocking protein synthesis pharmacologically using cycloheximide. These results indicate that the TOR pathway directly regulates synaptic strength by increasing availability eIF4E and enhancing cap-dependent translation.

Given that TOR regulates homeostatic plasticity through eIF4E, one might predict that 4E-BP is also involved and that removing 4E-BP would free-up eIF4E to initiate translation. *D. Melanogaster* have only one 4E-BP, encoded by the *Thor* gene. *Thor* can be knocked out with mutagenesis in one of two ways; the Thor1 mutation completely eliminates the *Thor* gene, and Thor2 introduces a mutation that prevents 4E-BP mRNA translation. Kauwe et al. found significant increases in the nerve-evoked EJC and QC in both Thor1 and Thor2 mutants (Kauwe et al., 2016). Consistent with the Tor OE findings, restoring 4E-BP only in the muscle restored EJCs to wild-type levels, indicating a retrograde effect. These data demonstrated that one of the main effects of mTOR activation at the

drosophila NMJ is the phosphorylation and inhibition of 4E-BP, which frees eIF4E and enhances cap-dependent translation.

Taken together, this work at the Drosophila NMJ reveals a novel mode of action for TOR activity in regulating and maintaining the strength of synaptic connections. Recent work, reviewed below, indicate that the mTORC1 pathway also plays a critical role in strengthening synaptic connections in the rodent hippocampus (Tang et al., 2002; Banko et al., 2005; Gkogkas et al., 2013).

1.3 SYNAPTIC PLASTICITY IN THE MAMMALIAN HIPPOCAMPUS

Excitatory synapses in mammals can change their strength, either potentiate or depress. This plasticity of functional connections referred to as long term potentiation (LTP) or long term depression (LTD), can persist for many days and is widely believed to be the cellular substrate for learning and memory.

1.3.1 Excitatory synapses in the hippocampus undergo long term potentiation.

The first speculation that changes in synaptic strength underlie learning and memory was made in 1911 (Cajal, 1909). As a possible mechanism, Hebb postulated over 70 years ago that: “When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased” (Hebb, 1949). It took another 20 years, however, for such a mechanism to be demonstrated experimentally. In a ground-breaking series of experiments, Bliss and Lomo showed that brief, high-frequency stimulation of hippocampal excitatory synapses in anesthetized rabbits produced a rapid and long-lasting increase in strength that could persist for many days (Lømo, 1966; Bliss and Gardner-Medwin, 1973). It is now clear that LTP is widespread at excitatory glutamatergic synapses in the nervous system.

In the decade that followed the initial discovery of LTP, researchers found that LTP could be reliably induced in acute hippocampal brain slices, opening the way for a plethora of cell and molecular experiments. Importantly, it was shown that LTP in the CA1 region of the hippocampus critically depends on the activation of postsynaptic NMDA receptors (NMDAR-dependent LTP) (Collingridge et al., 1983) and appears to be involved in associative, episodic-like, and spatial memory (Malenka and Bear, 2004).

1.3.1.1 Induction of NMDAR-dependent long-term potentiation in the CA1 region of the hippocampus

In early experiments, NMDA-dependent LTP was shown to be induced by stimulating the presynaptic input with tetani consisting of a train of 50-100 stimuli at 100 Hz or more (Bliss and Collingridge, 1993). But overtime, more physiological stimuli were also shown to be effective, including 'primed-burst stimulation', which consists of a single pulse followed at 200 ms by a single burst of 4 shocks at 100 Hz (Rose, 1988), and 'theta-burst stimulation' (4 shocks at 100 Hz delivered at an interburst interval of 200 ms) (Larson et al., 1986), a pattern that mimics action potential firing commonly seen in hippocampal pyramidal neurons (Ranck, 1973) and that corresponds to the theta frequency found in the EEG of exploring rats (Vanderwolf, 1969). Moreover, it was shown that primed-burst stimulation evokes the early phase of LTP, E-LTP, that last about 60 minutes, whereas longer-lasting theta-burst stimulation gives rise to the "late" phase of LTP, L-LTP, which lasts over 3 hours *in vitro* and may last for several days in freely moving animals (Bliss and Collingridge, 1993). To convert E-LTP to L-LTP requires new protein synthesis and gene transcription.

1.3.1.2 Long term depression is NMDAR-dependent in the CA1 region of the hippocampus

In addition to synaptic potentiation, mechanisms exist to depress synaptic strength. Nearly 20 years after the discovery of LTP, Dudek and Bear discovered its counterpart, long term depression (LTD) which depresses potentiated synapses or reduces synaptic strength of non-potentiated synapses below baseline. In slices of the CA1 region of the hippocampus, LTD can be induced by low-frequency stimulation (LFS: 0.5–3 Hz, 900 repetitions), and interestingly, it also requires activation of NMDARs and calcium influx (Dudek and Bear, 1992; Mulkey and Malenka, 1992). Moreover, LTD requires protein synthesis for stable expression; however, in contrast to L-LTP, stable expression of LTD is impaired only by inhibiting mRNA translation, but not gene transcription.

1.3.2 Molecular mechanisms underlying NMDAR-dependent LTP and LTD in the hippocampus

Whether the synaptic stimulation of NMDARs induces LTP or LTD depends on the downstream activation of kinases (LTP) or phosphatases (LTD) (Figure 4) (Lisman, 1989, 1994). Calcineurin (also known as protein phosphatase 2B, PP2B) has a much greater affinity for Ca^{2+} than Ca^{2+}/calmodulin kinase II (CaMKII) and protein kinase C (PKC). Consequently, the low-frequency LTD-inducing stimuli produce a small increase in postsynaptic calcium that preferentially activates PP2B but not CaMKII (Figure 4, left side) and causes the dephosphorylation of different substrates to induce LTD. Conversely, high-frequency, LTP-inducing stimuli produce sufficient calcium influx to activate CaMKII and PKC (Figure 4, right side), as well as activating adenylate cyclase and protein kinase-dependent cAMP (PKA) that act as an inhibitory feedback mechanism by phosphorylating inhibitor 1 (I-1) to inhibits phosphatase 1 (PP1) and prevent inactivation of CaMKII. In addition to increasing CaMKII kinase activity (De Koninck and Schulman, 1998), LTP-inducing stimuli recruits CaMKII to sites adjacent to activated synapses (Hudmon et al., 2005; Flynn et al., 2012; Lemieux et al., 2012).

1.3.2.1 mGluR-dependent LTD at excitatory synapses in the hippocampus

Shortly after the discovery of NMDAR-dependent LTD, researchers discovered a mechanistically distinct form of LTD (mGluR-dependent LTD) that requires activation of Gq coupled, metabotropic glutamate receptors (Gp1 mGluRs) (Huber et al., 2000). mGluR-dependent LTD can be induced by low-frequency stimulation (paired pulses at 1 Hz; PP-LFS), or pharmacologically with the selective mGluR1 agonist, DHPG [(RS)-3,5-dihydroxyphenylglycine] (Snyder et al., 2001; Xiao et al., 2001).

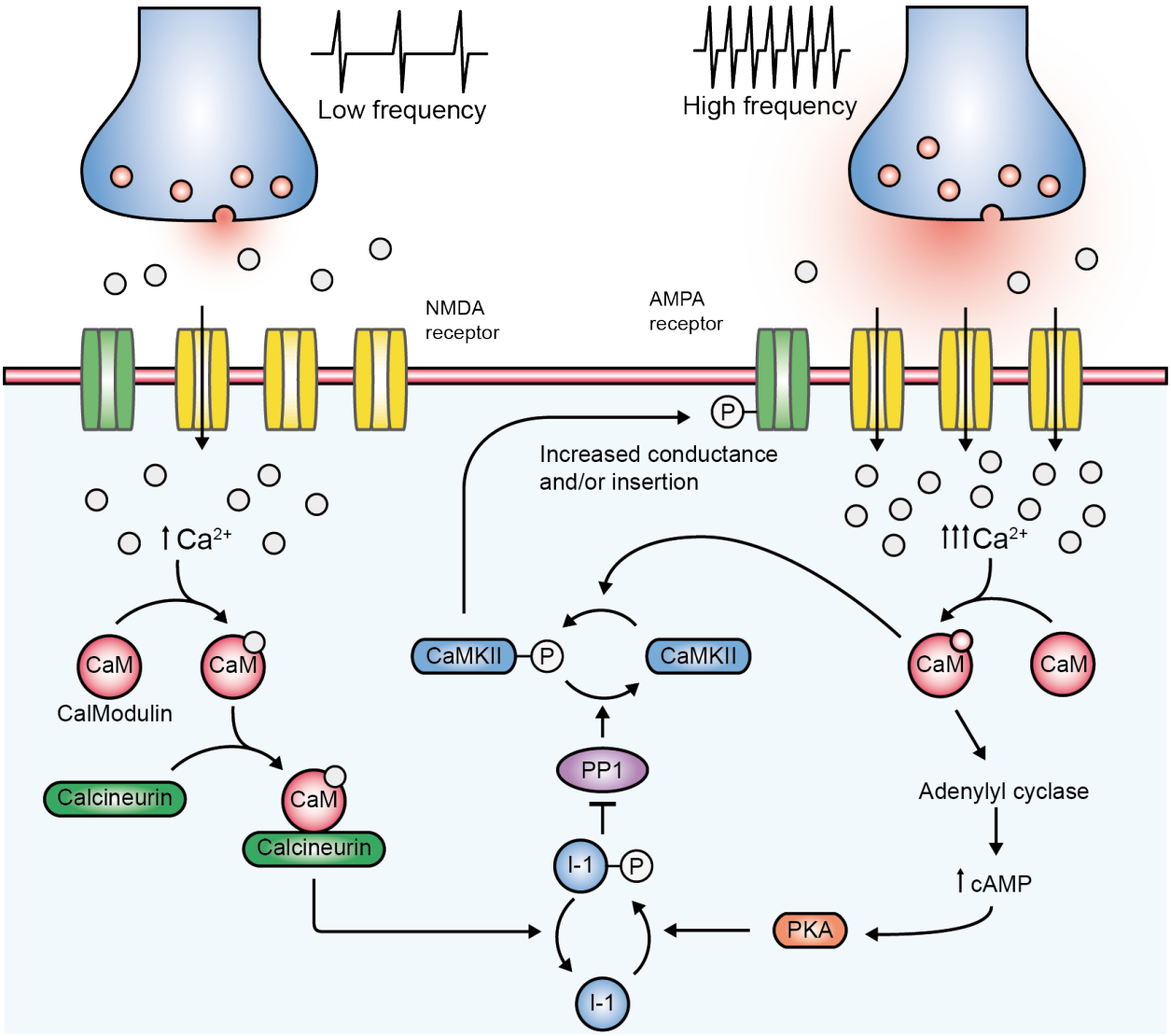

Figure 4 Molecular pathways underlying long term depression (left) and long term potentiation (right) at excitatory synapses in the hippocampus. **Left:** Low-frequency presynaptic stimulation (LFS: 0.5–3 Hz, 900 repetitions) leads to long term depression at the postsynaptic neuron. Presynaptic nerve stimulation induces a slight calcium elevation in the postsynaptic neuron, which activates calmodulin (CaM) and Calcineurin (PP2B) leading to the dephosphorylation and inactivation of inhibitor-1 (I-1). This, in turn, disinhibits protein phosphatase 1 (PP1), which dephosphorylates and deactivates Ca^{2+}/calmodulin kinase II (CaMKII), leading to the dephosphorylation and internalization of postsynaptic AMPARs. **Right:** High-frequency stimulation greatly increases postsynaptic calcium levels, activating CaM, Protein Kinase A (PKA) and CaMKII. Active CaMKII phosphorylates AMPARs, leading to their membrane insertion, whereas active PKA phosphorylates and activates I-1, inhibiting PP1, which prevents CaMKII inactivation.

1.3.2.2 LTP and LTD signaling pathways influence AMPAR membrane dynamics

NMDA-dependent LTP and LTD, and mGluR-dependent LTD are manifested by an increase (LTP) or decrease (LTD) in the density of AMPARs in the postsynaptic membrane. Synapses on CA1 neurons are composed of GluR1 and GluR2 AMPAR subunits. Phosphorylation of GluR1 regulates the biophysical properties of AMPARs, whereas phosphorylation of GluR2 changes its affinity for PDZ domain-containing proteins and affects the dynamics of AMPARs in the postsynaptic membrane. These PDZ-containing proteins include glutamate receptor interacting protein (GRIP1) and GRIP-associated protein 1 (GRASP1), which promote AMPAR membrane insertion (Ye et al., 2000; Seidenman et al., 2003; Hoogenraad et al., 2010; Pfennig et al., 2017), and protein interacting with C-kinase 1 (PICK1), which promotes AMPAR endocytosis (Moretto and Passafaro, 2018).

GRIP1 interacts with the S880 and T876 residues of the GluR2 subunit in a phosphorylation-dependent manner and facilitates new AMPAR insertion into the postsynaptic membrane (Shin et al., 2003; Anggono and Huganir, 2012; Pfennig et al., 2017). Point mutations that mimic S880 phosphorylation disrupt GluR2-GRIP1 interaction and depress synaptic transmission, whereas mutations that prevent S880 phosphorylation preserve the GRIP1-GluR2 interaction and potentiate synapses (Seidenman et al., 2003). GRASP1 also promotes AMPAR exocytosis by associating with GRIP1, facilitating AMPAR exocytosis from recycling endosomes (Ye et al., 2000; Hoogenraad et al., 2010).

Conversely, PICK1 functions as a counterpart to GRIP1 and promotes AMPAR internalization (Moretto and Passafaro, 2018). Phosphorylation of the S880 and T876 residues of GluR2 disrupts the GRIP1-GluR2 interaction, enabling GluR2 to interact with PICK1, promoting AMPAR endocytosis by interacting with AP2 complex and initiating the assembly of the endocytic machinery (Fiuza et al., 2017).

AMPAR membrane dynamics play an important role in synaptic plasticity at various excitatory synapses (Chung et al., 2000). For example, in the cerebellar cortex, parallel fiber synapses on Purkinje neurons contain AMPARs composed of GluR2/GluR3 subunits, but in contrast to synapses on CA1 hippocampal neurons, phosphorylation of GluR2-Ser880 by PKC induces LTD (Xia et al., 2000; Chung et al., 2003) (see Figure 6). In Purkinje neurons, kinases and phosphatases play an inverse role in altering synaptic strength, however, the roles of GRIP1 and PICK1 are preserved. Accordingly, Schonewille et al. found that mutations that disrupt the interaction of GluR2 with PICK1, such as PICK1 KO, GluR2Δ7 or GluR2K882A block AMPAR internalization and prevent parallel fiber synaptic LTD (Schonewille et al., 2011).

1.3.2.3 Long-term changes in synaptic strength require *de novo* protein synthesis

There is abundant evidence that *de novo* protein synthesis is also required for synaptic plasticity in the hippocampus. Pharmacological inhibition of mRNA translation with anisomycin or emetine blocks NMDA-dependent LTP (Frey et al., 1988; Huang et al., 1996; Bradshaw et al., 2003), as well as NMDA-dependent LTD (Kauderer and Kandel, 2000; Manahan-Vaughan et al., 2000; Sajikumar and Frey, 2003) both *in vivo* and in hippocampal slices. Likewise, pharmacological inhibitors of mRNA translation prevent the induction of mGluR-dependent LTD in hippocampal slices and cultured hippocampal neurons (Huber et al., 2000; Snyder et al., 2001; Hou and Klann, 2004).

1.3.3 The role of the mTORC1 pathway in synaptic plasticity, learning, and memory

As discussed previously (see sections 1.2.2.1 and 1.2.2.2), excitatory synaptic transmission activates mTORC1 through the PI3K and ERK pathways. mTOR hyperphosphorylates 4E-BPs, which in turn frees up eIF4E to initiate cap-dependent translation. Translation plays a critical role in long-term changes of synaptic strength, particularly for L-LTP and mGluR-dependent LTD.

1.3.3.1 The role of mTORC1 in hippocampal plasticity

The first study to suggest that the mTOR pathway is involved in hippocampal synaptic plasticity blocked mTORC1 pharmacologically with the inhibitor rapamycin and showed that it prevented the induction of L-LTP but not E-LTP. This is consistent with the finding that L-LTP but not E-LTP requires mRNA translation (Tang et al., 2002). To determine whether this effect on L-LTP occurred through the mTORC1 target 4E-BP, Banko et al. generated a mouse with a deletion in 4E-BP2, the isoform present in the CNS (4E-BP2 KO) (Banko et al., 2005). Interestingly, deleting 4E-BP2 enhances E-LTP and converts it to L-LTP, an effect that can be prevented pharmacologically with anisomycin. These experiments suggest that 4E-BP inactivation converts E-LTP to L-LTP in a translation-dependent manner, however, they did not directly address the roles of eIF4E or eIF4F.

In a follow-up study, Gkogkas et al. showed that the pharmacological inhibitor 4E-GI1, a molecule that disrupts eIF4E/eIF4G association by binding to eIF4E, restored normal E-LTP in 4E-BP2-KO mice, indicating that enhanced eIF4F formation and cap-dependent translation initiation is directly involved in converting E-LTP to L-LTP (Gkogkas et al., 2013).

Given these results, it would be reasonable to expect that even stronger stimuli that give rise to L-LTP would enhance L-LTP beyond WT levels. Surprisingly, however, Banko et al. (2005) found that L-LTP does not occur in 4E-BP2-KOs. One possibility is that excessive initiation complex formation obstructs translation rather than enhances it. Nevertheless, this work clearly illustrates that 4E-BP2 is critical for LTP in the hippocampus, and it appears to act by regulating the rate of cap-dependent translation initiation.

1.3.3.2 Genetic deletion of 4E-BP2 affects learning and memory

Banko et al. (2005) used the Morris water maze and conditioned fear memory test to show that mice with a deletion in 4E-BP2 exhibited impaired

spatial learning and memory as well as deficits in conditioned fear-associative memory, indicating the importance of 4E-BP2-dependent mRNA translation for learning and memory. One question is whether these findings on the effects that cap-dependent mRNA translation has on synaptic plasticity can be generalized beyond the hippocampus.

One hint comes from a follow-up study by Banko et al. who subjected 4E-BP2-KO mice to a battery of learning and memory tests that are known to require synaptic plasticity outside of the hippocampus, including the rotarod motor learning test, a task that involves the cerebellar cortex (Banko et al., 2007). Unlike their wild type counterparts,4E-BP2-KOs fail to improve at the rotarod test, suggesting that motor learning is impaired and implying that plasticity of excitatory synapses in the cerebellar cortex may also require 4E-BP2. My interest is to understand how disrupting the regulation of mRNA translation affects activity-dependent synaptic plasticity in the cerebellar cortex. In the following sections, I provide a brief review of plasticity at excitatory synapses on Purkinje cells in the cerebellar cortex to provide context for my experiments.

1.4 CEREBELLAR PLASTICITY

1.4.1 The anatomy of the cerebellar cortex

Anatomically, the cerebellar cortex has a relatively simple and stereotyped structure with easily identifiable cell types. In transverse slices of cerebellar tissue, one can clearly distinguish 3 cell layers (Figure 5). The middle layer, or Purkinje cell (PC) layer, consists of a row of Purkinje cell bodies whose axons are the sole output of the cortex and form inhibitory, GABAergic connections with neurons in the deep cerebellar nuclei. The dendritic arbor of a Purkinje cell is shaped like a flat fan that measures about 250 μm across and extends 250 μm to the top of the molecular layer (Eccles, 1967).

PC dendrites receive 2 excitatory, non-overlapping inputs. One input is from the axons of the granule cells. Granule cells are densely packed into the innermost layer, the granule cell layer, and receive excitatory connections from mossy fibers from the vestibular nerve; their axons, called parallel fibers (PFs), form synapses onto the distal dendrites of Purkinje cells. A single PF axon forms only 1-2 synapses onto a single PC; however, each PC receives synapses from thousands of PFs axons. Therefore, in experimental settings, increasing stimulation to PF axons give rise to a graded PF-EPSC response (Eccles et al., 1966b; Eccles et al., 1966a; Eccles, 1967). At least 16 PFs must be simultaneously activated for the postsynaptic PC to reach its action potential threshold (Delaney and Jahr, 2002), even though PCs are innervated by thousands of PFs.

The other excitatory input onto PCs is from climbing fibers (CFs). CFs are afferents from inferior olive neurons whose cell bodies are located in the deep cerebellar nucleus and receive inputs from the visual system. A single CF axon synapses onto the proximal dendrites of a PC and forms hundreds of synaptic connections (Eccles, 1967). CF activation provides a strong, all-or-nothing depolarizing stimulus to Purkinje cells (Eccles et al., 1966b; Eccles et al., 1966a; Eccles, 1967), causing the PC to fire complex spikes,

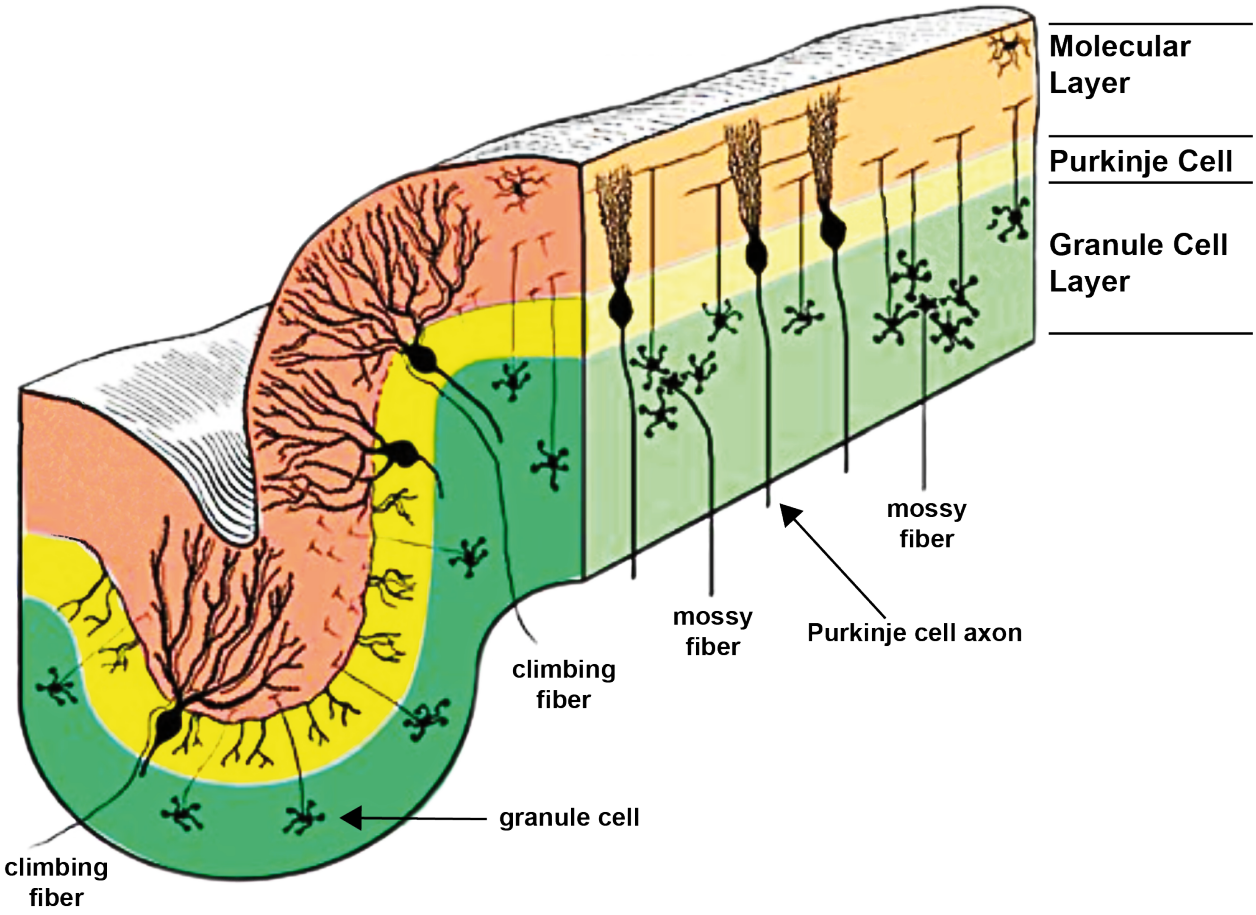

Figure 5 Cartoon of the transverse layers of the cerebellar cortex. The innermost layer is the granule cell layer, which contains densely packed granule cells, the cell bodies of the parallel fibers. The middle layer is the Purkinje cell layer and contains a single cell row of Purkinje cell bodies. The outer layer is the molecular layer into which the Purkinje cell dendrites fan out and where PF and CF synaptic connections onto the Purkinje cell are formed.

characterized by an initial prolonged large-amplitude spike followed by a high-frequency burst of smaller-amplitude action potentials (Granit and Phillips, 1956; Thach, 1967). These complex spikes occur at 1-3 Hz. In contrast, PC activation by PFs causes it to fire simple spikes that occur at a rate of 17-150Hz (Raman and Bean, 1999). PCs are also innervated by several classes of inhibitory neurons, but I will not review them here as my focus primarily is on excitatory synapses.

1.4.2 Cerebellar plasticity and cerebellar-dependent motor learning

A benchmark test for rapid motor learning is the vestibulo-ocular reflex (VOR). The function of the VOR is to maintain the stability of the retinal image when the head turns in one direction by eliciting a counterbalancing eye movement in the opposite direction. The reflex consists of vestibular afferent activation of cells in the vestibular nuclei, which in turn activates the oculomotor neurons. As mentioned in section 1.4.1, vestibular afferents also drive Purkinje cells through activation of the mossy fiber (MF)-PF pathway. PCs provide inhibitory input to the vestibular nuclei and are thus able to modulate the VOR gain. The "gain" of the VOR is defined as the ratio of the peak velocity of the eye movement divided by the peak velocity of the eye movement during the head turn. When the image is perfectly stabilized on the retina, the gain of the rotational VOR is 1.0. The gain of the VOR needs to be adjusted when the image is moving in the same direction as the head (gain down) or opposite direction as the head (gain up). In these cases, retinal slips are initially higher until the VOR adjusts. Retinal slips activate the CF input to PCs, providing an error signal (Simpson and Alley, 1974). Over trials, the gain of the VOR is adjusted and fewer errors occur, which is considered a form of motor learning.

Based on the structure and electrophysiological activity of the cerebellar cortex (Eccles, 1967), together with strong evidence for the cerebellum's involvement in motor learning (Brindley, 1964), Marr and Albus provided the first model for how the cerebellum contributes to motor learning: when there is a difference between the actual movement and the expected movement, CFs fire

to convey error signals and initiate a correction in the firing frequency of the PC by depressing PF synapses, and that that depression underlies motor learning (Marr, 1969; Albus, 1971).

1.4.2.1 Long-term depression at the PF-PC synapse

In an elegant series of experiments, Ito et al. tested the Marr-Albus hypothesis by pairing PF and CF stimulation. PF-PC evoked responses can be generated by electrically stimulating the vestibular nerve to activate granule cells which cause the PF-PC synapses to fire and evoke action potentials in the PCs (simple spikes) (Ito and Kano, 1982). Likewise, electrical stimulation of CF afferents drives PCs to evoke complex spikes, consisting of several large EPSPs and bursts of APs. Co-stimulating the vestibular nerve (PFs) and climbing fiber afferents (CFs) at 4/sec for 25 sec per trial caused a long-term depression of the PF-PC synapses, and this LTD persisted for at least one hour. This finding provided evidence that CFs can act as an error signal and provided physiological evidence for the Marr-Albus hypothesis.

1.4.2.2 The role of PF-LTD in motor learning

While PF-LTD provides a compelling mechanism for motor learning, the evidence linking LTD to motor learning was only indirect. Severing the inferior olive or CF axons completely disrupts the VOR (Itō, 1984), confirming that the CF input is essential. However, whether PF-LTD was directly responsible for motor learning had not yet been examined. To draw such a link, one would need to disrupt PF-LTD and examine the effect on motor learning. Using this approach in cerebellar slices, researchers found that pharmacological inhibitors, and in particular, genetic manipulations could disrupt long-term depression of PF-EPSCs induced by co-stimulation of PFs and CFs. Then, using these mutant mice, these groups tested motor learning and, in most cases, found that mutants that had impaired LTD also had impaired motor learning. This provided persuasive evidence in support of the Marr-Albus hypothesis that PF-PC LTD is directly involved in motor learning.

However, there is not always a correlation between disruptions in LTD and with the absence of motor learning. As reviewed above (section 1.3.2.2), LTD in the hippocampus and in the cerebellum is mediated by AMPA receptor endocytosis. Schonewille et al. blocked AMPAR endocytosis by mutating GluR2 to prevent S880 phosphorylation by PKC (GluR2K882A KI), or to stop PICK1-GluR2 interaction (PICK1 KO and GluR2Δ7 KI). These manipulations abolished PF–LTD, but contrary to the experiments that strongly support the Marr-Albus hypothesis referred to above, these mutant mice did not have impairment in motor learning (Schonewille et al., 2011). Possibly, compensatory mechanisms altered cerebellar circuitry in these mutants that allowed for motor learning in the absence of PF-PC LTD. Nevertheless, these findings led researchers to consider that alternative mechanisms may underlie motor learning. One such possibility is that PF-PC LTP plays a role in motor learning (Schonewille et al., 2010).

1.4.2.3 Cerebellar LTP

The first evidence for post-synaptically expressed LTP at PF-PC synapses came two decades after the discovery of PF-PC LTD (Lev-Ram et al., 2002). Briefly, while paired PF and CF stimulation at 1Hz for 300 repetitions leads to LTD at PF-PC synapses, PF stimulation alone leads to LTP (Lev-Ram et al., 2002). In other words, the key difference between whether LTP or LTD is induced is the coincident activation of the CFs.

1.4.3 Molecular mechanisms involved in cerebellar plasticity

Like excitatory synapses in the hippocampus, elevation in intracellular calcium is necessary for the induction of plasticity at PF-PC synapses, and the levels of intracellular calcium determine whether synapses are strengthened or weakened. Stimulation of CFs causes large depolarization and complex spikes in PCs, which leads to a major influx of calcium through P/Q voltage-gated calcium channels (VGCC) (Llinas and Sugimori, 1980; Stockle and Ten Bruggencate, 1980; Ekerot and Oscarsson, 1981). This CF mediated postsynaptic calcium influx is critical for LTD; preventing an increase in intracellular calcium

concentration by using calcium chelators or VGCC inhibitors prevents PF-PC LTD (Konnerth et al., 1990; Coesmans et al., 2004). Moreover, using calcium uncaging techniques to artificially increase the calcium concentration in the PC during LTP induction results in PF-PC LTD instead of LTP (Coesmans et al., 2004). Interestingly, high concentrations of calcium chelators (30mM BAPTA) can also prevent PF-LTP, which shows that despite having a lower threshold, PF-LTP also requires calcium influx (Coesmans et al., 2004). The levels of intracellular calcium strengthen or weaken synapses by activating different second messenger pathways; in contrast to the hippocampus (reviewed in section 1.3.2), high calcium activates the kinase pathway, whereas low calcium levels activate phosphatases (Figure 6). In addition, CF stimulation can be mimicked by large depolarizing voltage steps to increase postsynaptic calcium influx through VGCC (Crepel and Jaillard, 1991; Blond et al., 1997). Therefore, in experimental settings, one can induce LTD with either paired PF and CF stimulation or by pairing PF stimulation with PC depolarization.

1.4.4 The role of translation in cerebellar LTD

It is clear that mRNA translation is necessary for LTP and LTD at synapses in the hippocampus. However, to date, there are only two studies that have examined the role of acute translation in cerebellar LTD. The first study used cultures of isolated Purkinje cells (PCs) from embryonic mice (Linden, 1996). In this model, there are no other cell types present and Purkinje cells are devoid of synaptic connections. Therefore, to mimic nerve transmitter release, glutamate is briefly applied from a pipette and the resulting glutamate evoked inward current (IGlut) is recorded by performing perforated-patch voltage-clamp experiments onto the PCs. In this set-up, depression of IGlut is induced by six repetitive glutamate test pulses paired with a 3 s long depolarization step to 0 mV, which depresses IGlut to ~50% of baseline. When the cultured cells were treated with the translation inhibitor anisomycin immediately after the pairing protocol, IGlut depressed transiently and then increased to 83% ± 7.9% of

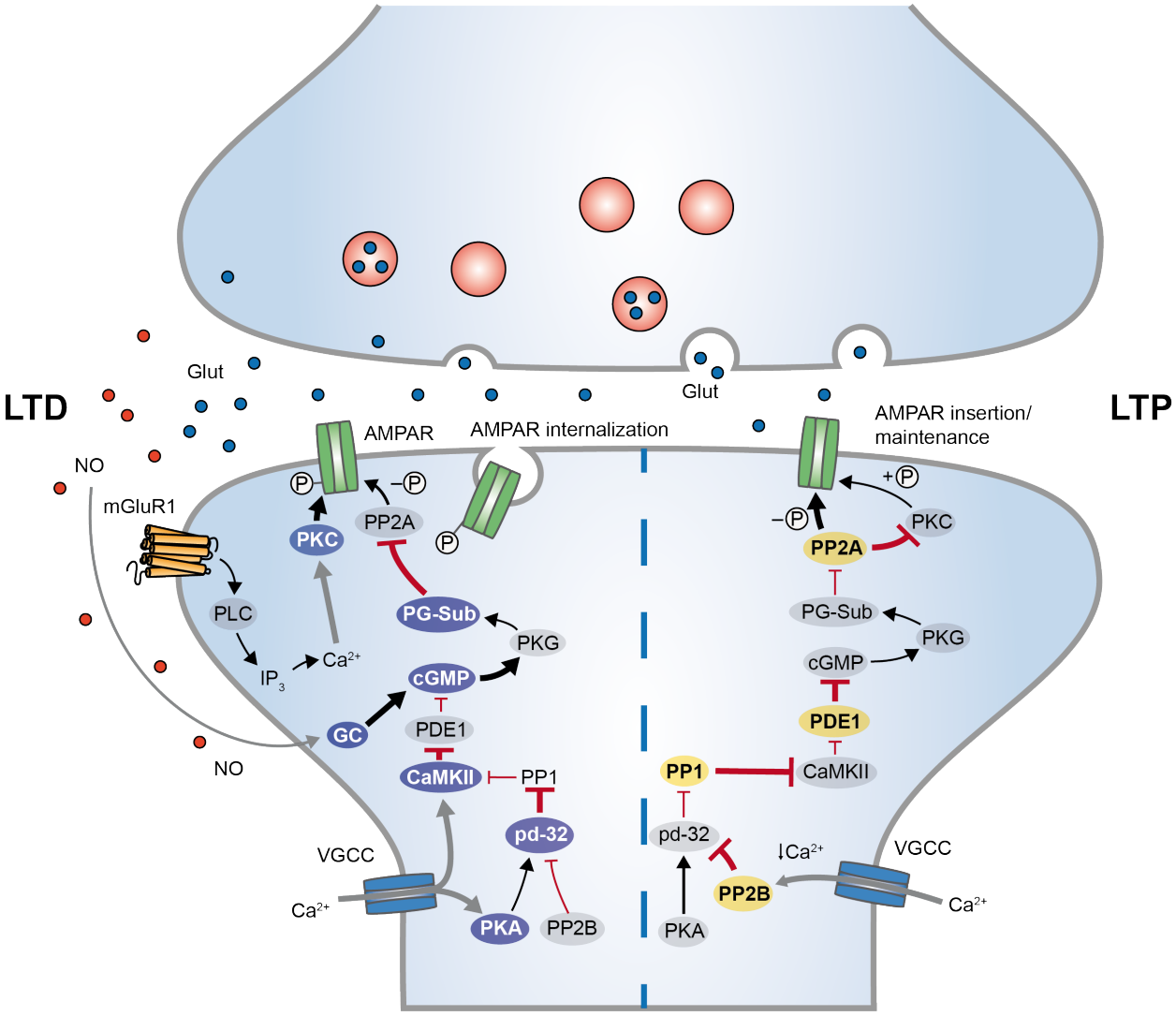

Figure 6 Molecular mechanisms underlying cerebellar LTD (left) and LTP (right). **Left:** LTD induction requires elevation of postsynaptic calcium through activation of P/Q voltage-gated calcium channels and release IP_3 calcium stores via mGluR1 activation. This leads to PKC activation, which phosphorylates GluR2-Ser880, leading to AMPAR internalization. The elevated calcium also activates PKA and CaMKII, which act in concert to inhibit phosphatases and prevent GluR2-Ser880 dephosphorylation. **Right:** In lower calcium conditions, phosphatase activity is favored, leading to LTP. PP2B gets activated, which inhibits pd-32, disinhibiting PP1. PP1 then inhibits CaMKII, which disinhibits PDE-1, leading to PP2A disinhibition. Active PP2A dephosphorylates GluR2-Ser880, leading to its insertion and maintenance on the postsynaptic membrane. Likewise, PP2A inhibits PKC, preventing AMPAR phosphorylation and internalization.

baseline. Meanwhile, anisomycin application had no effect on the basal IGlut. These data indicate that acute protein synthesis is necessary for the depression of IGlut. If these results from isolated cells reflect what happens at postsynaptic AMPARs when pairing CF and PF stimuli, then they suggest that acute protein synthesis is necessary for maintenance of LTD at PF-PC synapses. However, the molecular mechanisms in isolated PCs extracted from embryonic mice may differ from those in mature PCs in the intact cerebellum given the lack of synaptic connectivity and the absence of glia.

The second study used acute slices from adult rats to examine the role of acute translation in cerebellar LTD (Karachot et al., 2001). This study showed that LTD can be completely blocked by inhibiting translation; furthermore, the blockade is most complete when the inhibition is during LTD-induction, or immediately preceding or following it, and is less effective as the time between LTD-induction and the translation inhibition increases.

These two studies strongly suggest a role for acute translation in cerebellar LTD. Consistent with these findings, removing 4E-BP2 affects motor learning (Banko et al., 2007). In addition, Karachot et al. (2001) completely blocked PF-PC LTD with the mRNA cap analog (7-methyl GTP) (Karachot et al., 2001), an experiment that suggests that eIF4E and 4E-BP are involved in PF-PC LTD. The experiments in my thesis obtained direct evidence that 4E-BP plays a direct role in PF-LTD.

1.5 STRUCTURAL PLASTICITY

In addition to undergoing changes in synaptic strength, synapses can also have considerable structural modifications. This structural plasticity entails rewiring synaptic connections, either by selectively adding and stabilizing certain connections and/or removing others (Bernardinelli et al., 2014). This structural plasticity, in parallel to functional plasticity, is thought to have a critical role in learning and memory (Lamprecht and LeDoux, 2004; Johansen-Berg, 2007; Bernardinelli et al., 2014).

Structural plasticity in adults has many features in common with the rewiring of connections during early postnatal life, a process referred to as synaptic refinement (Purves and Lichtman, 1980; Lichtman and Colman, 2000; Lamprecht and LeDoux, 2004; Johansen-Berg, 2007; Bernardinelli et al., 2014). It is clear that structural plasticity requires new protein synthesis, but little is known about molecular mechanisms involved. The molecular mechanisms involved in rearranging connections during development are also unclear, but several decades of experiments lay the groundwork for further experimentation. Therefore, I investigated whether the regulation of mRNA translational regulation has a role in the synaptic refinement during early postnatal development.

1.5.1 Synaptic refinement

In utero, neurons establish diffuse connections that refine during the first few weeks following birth by eliminating redundant synapses while stabilizing and strengthening the remaining connections to improve performance (reviewed in (Purves and Lichtman, 1980; Lichtman and Colman, 2000; Kano and Hashimoto, 2009)). Synaptic refinement occurs widely in the developing nervous system (Purves and Lichtman, 1980; Schlaggar et al., 1993; Sanes and Lichtman, 1999; Buffelli et al., 2002; Katz and Crowley, 2002; Zou et al., 2004; Bleckert and Wong, 2011; Hong and Chen, 2011; Yasuda et al., 2011; Takeuchi et al., 2014), and has been extensively studied at the neuromuscular junction (NMJ) (Purves and Lichtman, 1980; Sanes and Lichtman, 1999).

Briefly, at birth, as many as 6-8 motor axons converge on a single muscle cell, as measured electrophysiologically by counting the jumps in postsynaptic potentials in response to increasing motor nerve stimulation. By 2 weeks, however, muscle cells are innervated by only 1 axon, indicating that redundant axons are gradually eliminated over the first few postnatal weeks (Redfern, 1970; Brown et al., 1976). Anatomical studies revealed that the elimination of synaptic connections during this developmental period is accompanied by the addition of synaptic contacts from the remaining motor axon as the input strengthens (Lichtman et al., 1987). A major question is which axons are eliminated and which one is maintained. The results from several experiments done at the NMJ and elsewhere in the nervous system suggest that afferent axons compete with one another to innervate target cells (Katz and Shatz, 1996; Lichtman and Colman, 2000; Zhang and Poo, 2001; Cohen-Cory, 2002; Kano and Hashimoto, 2009).

1.5.1.1 Activity-dependent synapse elimination

Experimental findings at the NMJ have demonstrated that blocking postsynaptic activity, either by surgically removing a piece of tendon to prevent muscle contraction (Benoit and Changeux, 1975; Riley, 1978), or by paralyzing the muscle cells pharmacologically by blocking the postsynaptic nicotinic receptors with curare (Srihari and Vrbova, 1978) or alpha bungarotoxin (Brown et al., 1981; Brown et al., 1982; Balice-Gordon and Lichtman, 1994), delays or prevents synaptic elimination; conversely, electrically stimulating the motor nerve hastens input elimination (O'Brien et al., 1978; Zelena, 1979; Thompson, 1983). These experiments clearly show that synaptic activity plays an important role in synaptic elimination, however, how is activity involved in intrasynaptic competition and elimination?

In an elegant series of experiments, Balice-Gordon and Lichtman showed that pharmacological blockade of neurotransmission throughout a junction prevents synapse elimination; however, focal application alpha-bungarotoxin to

block neurotransmission at a small region of the NMJ causes synapse elimination at the site where the receptors were blocked (Balice-Gordon and Lichtman, 1994). This study suggested the active synaptic sites “win” by destabilizing inactive synapses in their vicinity.

1.5.1.2 Model from the NMJ

Based on experiments such as these, Lichtman and colleagues put forward a model suggesting that post-synaptic activation of a cell creates a homosynaptic protective signal, or “good factor” that prevents the active presynaptic input from becoming destabilized (Lichtman and Colman, 2000). This mechanism enables the postsynaptic neuron to identify and preserve synapses belonging to the same axon and therefore fire synchronously. The active inputs in turn produce an intersynaptic “punishing signal” to destabilize inputs that are not active at the same time. This mechanism enables the postsynaptic neuron to identify and preserve synapses from the “winning” axon while destabilizing and eliminating the other axons that are not synchronously activated. Although there is good experimental support for this model, the molecular identity of the factors involved still remains elusive.

1.5.1.3 Synaptic elimination in cerebellar cortex

Like motor axons at the NMJ, climbing fibers innervating Purkinje cells undergo significant remodeling over the first postnatal weeks. At birth, several CFs innervate the Purkinje cell and are restricted to the Purkinje cell body. In the weeks that follow, as the Purkinje cell grows, all but one CF axon are eliminated from the cell body while the remaining axons establish hundreds of new CF synapses on the PC’s proximal dendrites. In an elegant study, Bosman et al. used electrophysiological and imaging methods to show that this refinement involves activity-dependent competition (Bosman et al., 2008). They found that at birth, all CFs are equal in strength, but over the first postnatal week one CF strengthens. Electrophysiological analysis revealed that the synapses of that “winning” CF undergo LTP, while synapses from the other CFs undergo LTD and

are subsequently eliminated. After elimination takes place, the “winning” CF axon extends along the proximal PC dendrites where it establishes several tens of new synapses (Bosman et al., 2008). This study and several others indicate that both elimination and remodeling of CF-PC inputs require an increase in postsynaptic calcium (Miyazaki et al., 2004; Mikuni et al., 2013).

While CF-PC refinement is a valuable model to study structural plasticity in the CNS, the heterogeneity of synaptic inputs to PCs adds complexity. For instance, synaptic activity from PFs, the other excitatory input onto PCs, is also critical for proper CF elimination (Crepel et al., 1976; Hashimoto et al., 2001). Moreover, PCs receive inhibitor inputs from GABAergic neurons and their role in refinement is not fully understood. Therefore, to examine the role of cap-dependent translation in structural plasticity, I opted for a simpler model: the sympathetic nervous system.

1.5.1.4 Synaptic elimination in the sympathetic nervous system

Synaptic elimination in the sympathetic nervous system has been well established (Lichtman and Purves, 1980; Lichtman et al., 1980; Purves and Lichtman, 1980), particularly in the superior cervical ganglion (SCG) (Lichtman, 1977; Lichtman and Purves, 1980; Purves and Lichtman, 1985). The SCG neurons receive sole afferent innervation from the axons of cholinergic preganglionic neurons in the intermediate horn of the spinal cord (Moller and Baeres, 2002; Glebova and Ginty, 2005). At birth, SCG neurons receive 7-11 inputs, but are innervated by only 1-3 inputs in adulthood (Purves and Lichtman, 1978).

SCG neurons receive no other excitatory inputs nor do they receive inhibitory inputs; therefore, refinement is not confounded by other inputs to postsynaptic neurons in the circuit. Furthermore, fast synaptic transmission in the SCG is exclusively mediated by presynaptic acetylcholine released onto one class of postsynaptic receptors: α3-containing nicotinic acetylcholine receptors (nAChRs) (Xu et al., 1999; Rassadi et al., 2005). Importantly, fast synaptic

transmission can be genetically abolished in the mouse SCG by disrupting the α3 gene (referred to as α3-KO) without compromising the survival of the animal. In addition, synaptic activity can rapidly be restored to these silent synapses in α3-KO mice by infecting postsynaptic neurons with α3-expressing adenoviruses (Krishnaswamy and Cooper, 2009). Moreover, in the absence of postsynaptic activity, the presynaptic terminals in α3-KO mice persist and appear ultrastructurally normal (Rassadi et al., 2005; Krishnaswamy and Cooper, 2009). This raises an intriguing question - what happens to synaptic refinement in α3-KO mice?

1.5.2 Role for mRNA translational in synaptic refinement

The role of mRNA translational in synaptic refinement is largely unknown. McCann et al. showed that pharmacologically blocking protein synthesis in individual muscle cells causes the presynaptic nerve to withdraw without affecting muscle cell function (McCann et al., 2007). These findings suggest that post-synaptic protein synthesis is involved in the retrograde signaling of a “good factor” required for presynaptic input maintenance (McCann et al., 2007). However, this experiment did not directly examine the role of translation regulation. Therefore, it remains to be seen whether regulating mRNA translation has any role in synaptic refinement.

My interest was to determine whether activity-dependent refinement was disrupted by dysregulating mRNA translation at mammalian neuron-neuron synapses. For my experiments, I chose to examine the refinement of preganglionic axons that innervate postsynaptic sympathetic neurons, in part because of its relative simplicity, but equally relevant, I had access to valuable mouse models, including α3-KO mice and 4E-BP-KO mice, that I could exploit in my investigations on how cap-dependent translation influences activity-dependent synaptic refinement.

1.6 THESIS QUESTIONS

For my thesis, I addressed the following questions:

- Is Cerebellar LTD impacted by cap-dependent translation regulation?
- How is PF-PC LTD affected in 4E-BP2-KO mice?
- Does genetic deletion of 4E-BP2 affect the molecular signaling pathways that regulate LTD? Are the effects of removing 4E-BP2 acute and/or are they present at baseline?
- Do 4E-BP2-KO mice have defects in motor learning tasks such as VOR?
- What happens to synaptic elimination in α3-KO mice?
- Given that the mTOR pathway is activated by synaptic activity, how is it affected in α3-KO?
- What is the role of 4E-BP in synaptic elimination?

CHAPTER 2

METHODS

2.1 Mice

A colony of C57BL/6 4E-BP1/2 KO mice (gift from Dr. N. Sonenberg) were maintained by breeding homozygous animals (Tsukiyama-Kohara et al., 2001; Banko et al., 2005, referred to as 4E-BP2-KO). Mice with a deletion in the alpha 3 nicotinic subunit gene (Xu et al., 1999; referred to as α3-KO) were maintained on an outcrossed background (Krishnaswamy and Cooper, 2009). α3+/- mice were crossed with 4EBP1/2-/- mice (Tsukiyama-Kohara et al., 2001; Banko et al., 2005) to generate 4EBP1/2-/-; α3-/-mice (referred to as α3/4E-BP-DKO). Genotypes were determined by PCR. All procedures for animal handling were carried out according to the guidelines of the Canadian Council on Animal Care.

2.2 Tissue preparation

2.2.1 Cerebellar Slices

P60 mice were killed with CO2 and transcardially perfused with 20mL of room temperature (23-25°C) NMDG ACSF solution containing (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 MgSO4, 0.5 CaCl2, adjusted to pH 7.3, and bubbled for at least 30 minutes prior to use with 95% O2/ 5% CO2. The cerebella were dissected out into NMDG ACSF (23-25°C), glued to the vibratome cutting platform (Compresstome VF-200, Precisionary Instruments) with cyanoacrylate adhesive and embedded in 2% agar (Sigma). Sagittal slices (250 μm thick) were cut from the cerebellum vermis using a zirconium ceramic blade (EF-INZ10, Cadence) and incubated for 10 minutes in NMDG ACSF at 32-34°C. After, the slices recovered for 1h at room temperature in HEPES ACSF consisting of (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 MgSO4, 0.5CaCl2 adjusted to pH 7.4 with NaOH or HCl, and bubbled with 95% O2/ 5% CO2. Slices were stored at room temperature in oxygenated standard ACSF

consisting of (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH2PO4, 24 NaHCO3, 5 HEPES, 12.5 glucose, 2MgSO4, 2CaCl2 (pH adjusted to 7.4 with NaOH or HCl) until required for recording.

Slices from P21 were prepared by transcardially perfusing mice with 20mL of ice-cold (0°C) HEPES ACSF solution. The cerebellum was dissected and sliced into HEPES ACSF (0°C). Slices were transferred into oxygenated standard ACSF for recovery and storage until ready for use. All other steps were identical to those used to prepare cerebellar slices from P60 mice.

All solutions were oxygenated for at least 30 minutes prior to use, and recipes were obtained from https://www.brainslicemethods.com/

2.2.2 SCG ganglia

SCGs were acutely dissected in oxygenated Tyrode's solution and continuously perfused (3-4 mL/min) with oxygenated solution at 33–34°C. For the dissection, mice under the age of P7 were stunned and decapitated and older mice were anesthetized in a cage filled with carbon dioxide and then decapitated. The head, ventral side up, was placed and secured with needles in a dissection dish and perfused with oxygenated Tyrode's solution. Using fine-tipped scissors and forceps, the skin, muscle layer, and trachea were removed, and the SCG, found near the carotid bifurcation, was located. The SCGs, with both the pre- and postganglionic nerves intact, were dissected from surrounding tissue in the neck and placed in a small Sylgard-coated recording dish containing 1mL of oxygenated Tyrodes's solution. The connected tissue of the carotid artery, on which the ganglion sits, was used to pin the preparation down with minutien pins, avoiding damage to the ganglion. The tissue around the nerves was carefully removed to ensure a good seal for suction electrodes in the recording bath. The recording dish was mounted on the stage of a dissecting microscope (Nikon, Tokyo, Japan) and perfused with oxygenated Tyrodes's solution maintained at 35-37°C at 3mL/min.

2.3 Electrophysiology

2.3.1 Electrophysiological Recordings in the Cerebellum

Whole-cell recordings were obtained from visually identified PCs under a confocal microscope (FV-1000, Olympus) using a 40X water-immersion objective and IR900nm DIC optics. To patch PCs, I used 2.5-4 MΩ glass electrodes filled with intracellular solution containing (in mM): 60 CsCl, 40 Cs D-gluconate, 20 TEA-Cl, 1 EGTA, 4 MgCl2, 4 ATP, 0.4 GTP, and 30 HEPES, adjusted to pH 7.3 with CsOH. In the recording chamber, the slice was perfused with standard ACSF (4 ml min-1) at room temperature. Bicuculline (10 μM, Tocris) was always added to block IPSPs. To stimulate PFs and CFs, we used a glass electrode (3-6 μm tip diameter) filled with standard ACSF. Focal stimulation of PFs was achieved by applying a square-wave pulses (0.1ms in duration, 100V in amplitude) using a stimulator (World Precision Instruments) in the molecular layer one-third from the pial surface and focal stimulation of CFs was achieved by applying a square pulses (0.1ms in duration, 100V in amplitude) in the granule layer. I recorded ionic currents from PCs with a VE-2 amplifier (Alembic Software, Montreal, Quebec, Canada). I compensated for series resistance (~40-60%) and monitored changes in series resistance and input resistance by delivering a 50ms long, -5 mV hyperpolarizing voltage step. If series resistance or input resistance changed more than 20%, data was discarded. Stimulation and acquisition were performed with IGOR Pro (WaveMetrics, Lake Oswego, OR), and the data were analyzed offline. The signals were filtered at 3 Hz and digitized at 20 kHz. PF-EPSCs were recorded at a holding potential of -80 mV, and CF-EPSCs at a holding potential of -20 mV. Paired pulse to CFs and PFs were delivered at a 50ms interval. The intrinsic firing frequency of PCs was recorded in a cell-attached configuration.

2.3.1.1 LTD Protocols

LTD was induced using one of two stimulation protocols. The first, by conjunctive stimulation (CJS) (PF stimuli concomitant with 30 ms depolarization of the PC to -30 mV at 1Hz, 300s) after the acquisition of a stable baseline response for 20 minutes at 0.1 Hz (Uemura et al., 2007). Following the CJS, I recorded the PF-EPSC at a rate of 0.1 Hz for up to 40 minutes. The amplitude of the baseline PF-EPSCs and the PF-EPSCs following CJS were averaged every 60 seconds, and the PF-EPSCs following CJS were normalized to the baseline PF-EPSCs for each PC.

The second LTD inducing protocol consists of parallel fibers trains of five pulses at 100 Hz accompanied by 100-ms-long depolarization of the Purkinje cell to 0 mV repeated 30 times at 2-s intervals (Shin and Linden, 2005).

2.3.1.2 LTP Protocol

LTP was induced by stimulating PFs at 1Hz for 300s after the acquisition of a stable baseline response for 20 minutes at 0.1 Hz. Following the stimulation, I recorded the PF-EPSC at a rate of 0.1 Hz for up to 40 minutes (Lev-Ram et al., 2002).

2.3.1.3 Pharmacological inhibitors

To assess the role of individual molecules in the LTD and/or LTP pathway, specific pharmacological inhibitors were either perfused in the extracellular solution or included in the pipette saline.

To block cap-dependent translation initiation during LTD induction, the translation inhibitor 4EGI-1 (50 μm, Calbiochem), which selectively blocks the eIF4E-eIF4G interaction, was perfused in the recording chamber 10 minutes prior to starting the LTD stimulation until 10 minutes after the LTD stimulation ended (Gkogkas et al., 2013).

Several pharmacological inhibitors were used to understand the molecular mechanisms involved in PF-PC LTD in the cerebellum on 4E-BP2-KOs. To block mGluR1 signaling, I perfused recording solution containing the mGluR antagonist CPCCOEt (100 μM, Tocris Bioscience, Ellisville, MO, USA) into the recording chamber for 10 minutes prior to starting the LTD stimulation until 10 minutes after the LTD stimulation ended (Mitsumura et al., 2011).

To block phosphatase activity in cerebellar slices, the general phosphatase inhibitor Okadaic Acid (1uM, Sigma, St. Louis, MO) was added to the extracellular solution and perfused into the recording chamber 30 minutes prior to inducing LTD until the end of the recording. To block PP1, inhibitory peptide inhibitor-2 (I-2; 100 nM, New England BioLabs, Beverly, MA) was added to the pipette solution and allowed to perfuse into the PC for 20 minutes after whole-cell configuration was achieved before recording was started. PP2B was blocked using Cyclosporin A (100uM, Tocris, Ellisville, MO) added to the extracellular solution and perfused into the recording chamber 30 minutes prior to inducing LTD until the end of the recording. PP2A was inhibited using Fostriecin (50 nm) added to the pipette saline (Belmeguenai and Hansel, 2005).

2.3.2 Electrophysiological Recordings of the SCG

2.3.2.1 Extracellular recordings

To record the nerve-evoked postganglionic compound action potential (CAP), the preganglionic nerve was connected to a stimulator (365R Isolator, World Precision Instruments, Sarasota, FL) with a suction electrode and the postganglionic trunk to a differential amplifier (DP-301, Warner Instrument Corp, Hamden, CT). To measure compound action potentials in SCGs, the preganglionic nerve was stimulated and the output from the recording electrode amplified (by 1000x) and then digitized by an input/output NI-PCI-6259 data acquisition card (National Instruments, Austin, TX) and filtered between 10Hz and 3kHz, recorded, displayed and analyzed off-line on a Pentium II-based

computer using Igor (Wavemetrics, Lake Oswego, OR) (Krishnaswamy and Cooper, 2009).

2.3.2.2 Intracellular recordings

To count the number of inputs to SCG cells, the preganglionic nerve of the dissected SCG preparation was held in a suction electrode. The electrode was connected to the stimulator (World Precision Instruments) and the preganglionic nerve was stimulated with depolarizing current at 0.8-1 Hz through an input/output data acquisition card (NI-PCI-6259, National Instruments) and Igor software (Wavemetrics). To record from ganglion cells, I used 80–120 mΩ glass microelectrodes (G150F-4, Warner Instruments) made with a DMZ universal puller (Zeitz Instruments, Munich, Germany). Stable intracellular recordings were achieved with a high inertial precision microdrive (Inchworm 8200; EXFO, Vanier, Quebec, Canada) attached to a micromanipulator (SM11; Narshige, Tokyo, Japan) that drove the electrode through the ganglion. The recording electrode was filled with 1M KAc and connected by a thin silver chlorided wire to the head stage of an Axoclamp 2A amplifier (Axon Instruments, Union City, CA) used in current-clamp mode; hyperpolarizing constant current pulses were applied through the recording electrode. Membrane potential was digitized with a data acquisition card (National Instruments), sampled at 10 kHz, recorded and analyzed offline on a Pentium II-based computer with Igor (Wavemetrics) (Krishnaswamy and Cooper, 2009). Measurements in neonatal animals were challenging because of the small size of SCG neurons; to record from these cells, very fine intracellular electrodes with resistances of 100-130 MΩ were used. Only neurons with resting membrane potentials under -40 mV were included in my study.

2.3.2.3 Analysis of disparity between inputs

To measure the convergence of preganglionic axons innervating a sympathetic neuron, the preganglionic nerve was stimulated with voltages of increasing strength while holding the neuron at ~ -90 mV to prevent EPSPs from

triggering action potentials. If the resting membrane potential of the neuron was above -90 mV, hyperpolarizing current was injected in the cell. In some experiments, I also included QX314 in the recording electrode to prevent action potentials. At maximal stimulus strength, the EPSP max is the sum of the EPSPs evoked by each of the axons innervating that neuron (Eq. 1). Increasing the strength of the stimulus to the preganglionic nerve activates axons of different thresholds which results in discrete jumps in the amplitude of the EPSPs. I used these discrete jumps as a measure of the number of axons innervating the neuron. To isolate the EPSP evoked by individual axons, I averaged at least 10 traces for each discrete jump and subtracted the average EPSP evoked by that axon and all axons of lower thresholds from the average EPSP evoked only by axons of lower threshold (Eq. 2).

For N axons innervating a neuron,

Eq. 1

$$\mathrm{EPSP}_{max} = \sum_{n=1}^{N} \mathrm{EPSP}_n$$

To isolate the EPSP n evoked by axon n,

Eq. 2

$$\mathrm{EPSP}_n = \sum_{n=1}^{n} \mathrm{EPSP}_n - \sum_{n=1}^{n-1} \mathrm{EPSP}_{n-1}$$

To calculate the disparity index, DI, for each neuron, I divided the standard deviation, SD, of the EPSPs by the mean EPSP (Hashimoto and Kano, 2003). (Eq. 3.)

Eq. 3

$$DI = SD/M$$

$$SD = \sqrt{\frac{\sum_{n=1}^{N} (EPSP_n - M)^2}{N-1}}$$

$$M = \mathrm{EPSP}_{max}/N$$

2.4 Calcium imaging

Calcium imaging of Purkinje cells was performed on an upright confocal microscope. The intracellular solution contained (in mM) 135 Cs-methanesulfonate, 10 CsCl, 10 HEPES, 4 Na2ATP, 0.4 Na3GTP, and 0.15 Oregon Green BAPTA-1 hexapotassium salt and 0.1 Alexa Fluor 568 (pH 7.25). I started imaging 20 min after the establishment of whole-cell configuration to allow for dendritic perfusion of the intracellular solution. Using the Alexa Fluor 568 signal, a region of interest approximately half-way between the cell body and the pial surface was selected. Using a 488 laser, the region of interest was sampled at 10Hz while we delivered a depolarizing voltage step to 0mV for 100ms to the PC cell body at 1Hz. The data was acquired with FluoView and the image data were analyzed with ImageJ (Kim et al., 2008).

2.5 Western blot analysis.

For SCG samples, SCGs were rapidly dissected and flash-frozen in liquid nitrogen. Western blot analysis was performed according to manufacturer's protocols using the following primary antibodies: anti-phopho-4E-BP1 (T37/46), anti-nonphospho-4E-BP1 (t 46) (Cell Signaling).

For the cerebellum, mice were killed using CO2, transcardially perfused with standard aCSF, and the cerebella rapidly removed and flash-frozen in liquid nitrogen. Western blot analysis was performed according to manufacturer's protocols using the following primary antibodies against: PPP2B4 (PTPA), PPME1, regulatory subunit B56ε, Regulatory subunit B55β, Structure subunit Aα/β, Catalytic subunit Cα/β, 4E-BP1, 4E-BP2, Regulatory subunit B56δ, Regulatory subunit B55α.

Protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The gel images were scanned and band intensities were quantified using MetaMorph software (Molecular Devices).

2.6 VOR

2.6.1 Head-post implantation and craniotomy

Mice were anesthetized with an intramuscular injection of a mixture of atropine (5 × 10-4 mg/g), ketamine (10-1 mg/g), acepromazine maleate (2 × 5.10-2 mg/g), xylazine (10-1 mg/g), and sterile saline. Animals were then secured in a stereotaxic frame. A custom-built head holder was then cemented to the skull. Afterwards, animals were kept in isolated cages and closely monitored during the first 48 h. In addition, care was provided to avoid hypothermia and dehydration (Beraneck and Cullen, 2007).

2.6.2 Recording sessions

During the experiment, animals were placed in a custom-built Plexiglas tube at the center of a turntable surrounded by a striped drum (vertical black and

white stripes, visual angle width of 5°). The animal's head was fixed 35° nose down to align the horizontal semicircular canals with the horizontal plane (Vidal et al., 2004; Calabrese and Hullar, 2006). The turntable was used to apply passive vestibular rotational stimuli (Medrea and Cullen, 2013).

Eye movements were monitored using the video-oculography method previously described by (Stahl et al., 2000). Turntable velocity was measured with an angular velocity sensor (Watson Industries). Head and body position were measured with magnetic search coils. Data acquisition was controlled by a QNX-based real-time data-acquisition system (REX). Eye- and head-position signals were low-pass filtered at 250 Hz by an 8 pole Bessel filter and sampled at 1 kHz. The sampled signals were digitally filtered at 125 Hz. During each experiment eye, head, body and table movements were recorded on digital audio tape for later playback and off-line analysis.

2.6.3 Behavioral protocol

The VOR gain was measured by delivering 0.2, 0.4, 0.8, 1, 2 or 3 Hz ±10°/s peak velocity sinusoidal turntable rotations in the dark. Measurements were taken in 30 s blocks. Any cycle containing a saccade or motion artifact was deleted from the analysis. Head and eye velocity traces were aligned on the zero crossings of head velocity and then averaged. Fourier analysis was then used to extract amplitude and phase from the averaged traces (Boyden and Raymond, 2003). The VOR gain was calculated to be the ratio of the eye velocity amplitude to the head velocity amplitude, and the VOR phase was calculated to be the eye velocity phase minus the head velocity phase, minus 180°. Thus, a perfectly compensatory VOR would have a phase of zero.

Motor learning was induced in the VOR by pairing head rotation with rotation of the optokinetic drum. For the gain-down stimulus, the illuminated drum was held stationary relative to the mouse, while ±10°/s sinusoidal turntable rotation was delivered. The frequency of the sinusoidal rotation for gain-down stimuli was 0.2, 0.4, 0.8, 1, 2 or 3 Hz. Habituation experiments involved ±10°/s

sinusoidal turntable rotation in the dark at either 0.5 or 1 Hz. For each training session, mice were trained in three 10 min periods. After each 10 min period, the VOR was measured during two 30 s blocks of turntable rotation in the dark. In between the two blocks, a bright light lasting approximately 1/6 s was flashed to maintain animal alertness, followed by an 8 s pause before beginning the second block of eye movement measurement (Boyden and Raymond, 2003).

2.7 Adenovirus

Full-length α3 neuronal nAChR subunit cDNA was ligated into pAdTrack-synapsin 1 (Ad-α3/Syn), and replication-deficient viral vectors were made according to He et al. (1998), as described previously (Krishnaswamy and Cooper, 2009). The synapsin promoter was only active in SCG neurons for ~2 weeks; to overexpress α3 for longer times, we used the human ubiquitin C promoter (Schorpp et al., 1996). P28 α3-KO or α3/4E-BP-DKO and P60 α3-KO mice were infected with either Ad-α3/Syn or Ad-α3/Ubi adenovirus by intravenous tail vein injections at a concentration of ~10 7 pfu/mL in 200-300 μL 1X PBS; pups younger than P7 were infected by intraperitoneal injection.

2.8 Immunohistochemistry

2.8.1 Calbindin staining in the Cerebellum

For calbindin immunostaining, mice (P21-P30) were deeply anesthetized by CO_2, cervically dislocated and intracardially perfused with ice-cold HEPES ACSF followed by 4% paraformaldehyde. Brains were postfixed for 2–16 h and equilibrated with 30% sucrose overnight. Sagittal sections (50 μm) were permeabilized in PBS with Triton-X 0.25% (Tx-PBS) and treated as follows in Tx-PBS based solutions: they were preincubated with 10% normal donkey serum solution (Jackson Immunoresearch USA, West Grove, PA, USA) for 1 h at room temperature (RT) and incubated with mouse anti-Cb (1:1,000; Swant, Marly, Switzerland; code 300) overnight at 4 °C, then incubated with secondary antibody (Alexa 488-conjugated anti-mouse, 1:200; Jackson Immunoresearch) for 2 h at room temperature and rinsed.

For the quantification of Purkinje cell density, images were acquired with a confocal microscope (FV-1000, Olympus) with a 60X, NA 1.42 PlanApo N oil-immersion objective. The data was acquired with FluoView and the image data was analyzed with ImageJ. Calbindin-positive Purkinje cells were counted in random fields (5–10/animal), and their number was normalized by the length of the Purkinje cell PC layer section that was analyzed and averaged within each animal.

2.8.2 P-4E-BP1, 4E-BP1 and MAP-1A staining in SCG slices

SCGs were fixed and sliced into 100 μm sections. Sections were incubated in primary antibodies (P-4E-BP1 or 4E-BP1 with MAP-1A) for 48 hours, then in secondary antibodies for 1 hour, and mounted for imaging. WT and α3-KO samples were processed in parallel. Primary antibodies: Rabbit anti-P-4E-BP1 (1:600; Cell Signaling, Danvers, MA), rabbit anti- 4EBP1 (1:600; Cell Signaling), goat anti-MAP-1A (1:360; Santa Cruz Biotechnology, Dallas, TX); Secondary antibodies: TRITC donkey anti-rabbit (1:500; Jackson ImmunoResearch Laboratories) and Alexa Fluor 647 donkey anti-goat (1:500; Life Technologies).

2.9 Statistical analysis

To test for statistical differences between two samples, I used unpaired two-tailed t-tests assuming equal variance.

To test for statistical differences between three or more samples, I used a one-way ANOVA to determine if one or more samples were significantly different. If the p-value calculated from the F-statistic was less than 0.05, we used a post hoc Tukey HSD test to identify which pairs of samples were significantly different from each other.

CHAPTER 3

THE ROLE FOR THE TRANSLATION REGULATOR 4E-BP2 IN SYNAPTIC PLASTICITY OF PARALLEL FIBER-PURKINJE CELL SYNAPSES

INTRODUCTION

Recent evidence indicates that genetic deletion in mice of 4E-BP2, a critical regulator of mRNA translation (4E-BP2 KO), alters synaptic plasticity in the hippocampus and hippocampal-dependent learning and memory (Banko et al., 2005; Banko et al., 2007; Gkogkas et al., 2013). Moreover,4E-BP2-KO mice have impaired motor learning (Banko et al., 2007), implying that 4E-BP may also affect synaptic plasticity in the cerebellar cortex. It has long been held that motor learning involves LTD at synapses between parallel fibers and Purkinje cells (Ito and Kano, 1982; Yuzaki, 2013). Yet, recent studies showed that in calcineurin (PP2B) knockout mice, motor learning is impaired but PF-PC LTD is intact (Schonewille et al., 2010). Further studies revealed that genetic mutations that disrupt AMPAR endocytosis prevent PF-PC LTD but have no effect on motor learning (Schonewille et al., 2011). These results have led researchers to suggest that mechanisms in addition to PF-PC LTD may be involved in motor learning.

Cerebellar PF-PC LTD involves many of the key molecules that play a role in hippocampal LTP (Belmeguenai and Hansel, 2005). In addition, like synaptic plasticity in the hippocampus, cerebellar PF-PC LTD requires mRNA translation and protein synthesis (Linden, 1996; Karachot et al., 2001), and genetically removing the translation regulator 4E-BP2 (4E-BP2 KO) disrupts LTP in the hippocampus. Moreover, 4E-BP2 has been shown to play an important role in motor learning (Banko et al., 2007). Yet, whether 4E-BP has a role in the plasticity of PF-PC synapses has not been investigated. My experiments in this chapter address this question.

METHODS (For complete description, see Chapter 2)

Mice

Electrophysiological experiments used P21-P30 4E-BP1/2 -/- mice (referred to as 4E-BP2-KO). VOR experiments used mice between 2-4 months of age.

Cerebellar Slices

For electrophysiological experiments and calcium imaging, 250uM transverse slices were acutely prepared following the protocol outlined in Section 2.2.1

Electrophysiology

LTD was induced using one of two protocols. The first protocol consists of a depolarizing voltage step to -30mV for 30ms applied to the PC paired with a single PF stimulation, repeated at 1Hz for 5 minutes. The second protocol consists of parallel fibers trains of five pulses at 100 Hz accompanied by 100-ms-long depolarization of the Purkinje cell to 0 mV repeated 30 times at 2-s intervals. To inhibit cap-dependent translation during LTD induction, the regular standard aCSF was substituted with standard ACSF containing 4EGI-1 (50uM, Calbiochem), perfused at 3-4ml/minute for 10 minutes before LTD induction until 10 minutes after the stimulation ended. LTP was induced by a single PF stimulation, repeated at 1Hz for 5 minutes.

Immunohistochemistry

We immunostained cerebella for calbindin 28K and examined overall cerebellar structure as well as the number and density of Purkinje cells (see Section 2.8.1. for details).

Calcium Imaging

Purkinje cells were patched with an electrode containing 0.1mM Alexa Fluor 568, used to visualize the dendrites and select a region of interest, and 0.15 mM Oregon Green BAPTA-1 hexapotassium salt. Using a 488 laser, the

region of interest was sampled at 10Hz while I delivered a 100ms depolarizing voltage step to 0mV the PC cell body at 1Hz (for the complete protocol, see section 2.4).

VOR

The VOR protocol used is described in detail in Section 2.6. Briefly, animals were placed in a custom-built Plexiglas tube at the center of a turntable surrounded by a striped drum. The VOR gain was measured by delivering 0.2, 0.4, 0.8, 1, 2 or 3 Hz ±10°/s peak velocity sinusoidal turntable rotations in the dark; measurements were taken in 30 s blocks. Motor learning was induced in the VOR by pairing head rotation with a rotation of the optokinetic drum. Mice were trained in three 10 min periods and the gain-down stimulus was delivered by holding the illuminated drum stationary relative to the mouse, while a ±10°/s sinusoidal turntable rotation was delivered.

RESULTS

Cerebellar PF-PC LTD requires cap-dependent translation

In acute cerebellar slices from P21-30 wildtype mice (WTs), pairing PF-stimulation with a 30 ms step depolarization of the postsynaptic Purkinje cells to -30 mV repeatedly at 1Hz for 5 minutes depressed PF-EPSCs to 80% of baseline (Figure 1A). To address whether cap-dependent mRNA translation is necessary for PF-PC LTD, I prevented eIF4F formation by applying the pharmacological inhibitor, 4EGI-1, which out-competes eIF4E for the binding site on eIF4G, thereby blocking the initiation of mRNA translation. Treating cerebellar slices with 4EGI-1 (50 μM) for 30 minutes prior to LTD induction completely blocked LTD (Figure 1B). This shows that the interaction of eIF4E and 4G, presumably leading to the formation of eIF4F and cap-dependent mRNA translation, is directly involved in cerebellar LTD.

4E-BP2-KO mice show reversed polarity in the plasticity in PF synapses

Given that 4E-BP2 regulates eIF4E availability, I asked whether removing 4E-BP alters PF-PC LTD. Previous work on hippocampal synapses showed that deleting 4E-BP2 increases E-LTP yet prevents L-LTP (Banko et al., 2005). Using cerebellar slices from 4E-BP2-KO mice at P21-30, I found, unexpectedly, that the usual LTD induction protocol (paired PF-stimulation at 1Hz for 5 minutes with a 30 ms depolarizing voltage step of the PC to -30mV) potentiated PF-PC synapses to approximately 120% of baseline (Figure 2). These results indicate that in the absence of 4E-BP2, LTD inducing stimuli give rise to LTP.

4E-BP2 KO mice have normal PF-PC LTP

PF-stimulation alone, without conjunctive depolarization, gives rise to PF-PC LTP (Lev-Ram et al., 2002). To determine whether the absence of 4E-BP2 alters PF-PC LTP, I stimulated PFs at 1Hz for 5 minutes without the paired depolarization of the PC. In cerebella from wild-type mice, PF-EPSCs potentiated to 120% of baseline (Figure 3A). Likewise, in cerebella from 4E-BP2-KO mice, PF-stimulation alone potentiated PF-EPSCs to 120% of baseline (Figure 3B). These results indicate that the removal of 4E-BP does not affect the induction of LTP at PF-PC synapses.

Cerebellar morphology, Purkinje Cell density, Purkinje Cells soma size and Purkinje Cells intrinsic firing frequency are not affected in 4E-BP2-KO mice

To determine whether the cerebellum of 4E-BP2-KO mice have any anatomical defects that might possibly affect the plasticity of PF synapses, cerebella were immunostained for calbindin 28K to examine the overall cerebellar structure as well as the number and density of Purkinje cells. The gross anatomical structure of cerebella in 4E-BP2-KO mice was similar to those in wild-type mice (Figure 4A), and there were no statistical differences between WT and 4E-BP2-KO cerebella in PC cell density (Figure 4B), or PC soma size (Figure 4C). Moreover, I did not detect any significant difference in the intrinsic

firing frequency of PCs in acute slices from 4E-BP-KOs compared to WTs (Figure 5A).

Deleting 4E-BP does not affect the developmental elimination of Climbing Fiber axons or synaptic transmission at PF or CF synapses

At birth, PCs are innervated by multiple CFs, but due to input elimination, only one CF remains after the 3rd postnatal week (Hashimoto and Kano, 2003). To examine whether removing 4E-BP affects CF elimination, I counted the number of innervating CFs physiologically by recording the jumps in the EPSC with increasing stimulus strength to the CF axons in the molecular layer. At P21, in both WTs and 4E-BP2-KOs, the PCs were innervated by a single CF (Figure 5B), suggesting that CF elimination proceeds normally in 4E-BP2-KO mice.

In addition, I examined whether the removal of 4E-BP affected basal synaptic transmission at climbing fiber synapses. I found no significant difference in the amplitude of CF-EPSCs between WTs and 4E-BP2-KOs (Figure 5C). Moreover, I examined the CF release probability by measuring the CF paired-pulse ratio and found no significant difference between WTs and 4E-BP2-KOs (Figure 5D). Taken together, these data suggest that CF innervation is normal in 4E-BP2-KOs.

Furthermore, I examined the short-term plasticity of PF synapses and found no statistical difference in PF paired-pulse ratio between WTs and 4E-BP2-KOs (Figure 5E). These data suggest that PF release probability has not changed in 4E-BP2-KO mice.

A presynaptic form of LTP can also be induced in PF nerve terminals which manifests as an increase release probability (Salin et al., 1996). To test whether the LTD protocol changes PF release probability in 4E-BP2-KOs, I compared the PF paired-pulse ratio before and after applying the LTD inducing protocol. I found no significant difference in the release probability between WT and 4E-BP2-KO cerebella (Figure 5F).

Taken together, these data suggest that neither PC innervation nor release probability are altered by 4E-BP2-KO mice and therefore cannot explain why stimuli that induce LTD at PF-PC synapses in wild-type mice induce LTP in 4E-BP2-KO mice.

4E-BP2 KO mice have normal calcium transients

Induction of PF-PC LTD requires a large calcium elevation in Purkinje cells, and conversely, PF-PC LTP is induced in low levels of intracellular calcium (Coesmans et al., 2004). This raises the possibility that if calcium influx is impaired in 4E-BP2-KOs during the LTD stimulation protocol, then PF-PC may undergo LTP instead of LTD.

To test this possibility, I measured depolarization evoked changes in intracellular calcium concentrations with the calcium indicator, Oregon-Green-BAPTA-1. Purkinje cells in WT and 4E-BP2-KO mice showed no significant difference in calcium influx in response to depolarizing voltage steps to 0mV for 100ms, indicating that deletion of 4E-BP2 does not affect calcium influx through voltage-gated calcium channels (Figure 6 A, B).

Cerebella in 4E-BP2-KO mice have specific defects in PF-PC LTD

In cerebella from both WT and 4E-BP-KO, stimulating parallel fibers without conjunctive CF stimulation or depolarization induces LTP at PF-PC synapses. On the other hand, stimulating parallel fibers with conjunctive CF stimulation or depolarization induces LTD in WT cerebella, but LTP in cerebella from 4E-BP-KO mice. Therefore, if the conjunctive depolarizing stimuli were ineffective in 4E-BP-KO cerebella, the result would be PF-PC LTP. To rule out this possibility, I used an LTD-inducing protocol that does not induce LTP in the absence of the paired conjunctive stimulation (Shin and Linden, 2005). This protocol consists of parallel fibers trains of five pulses at 100 Hz accompanied by 100-ms-long depolarization of the Purkinje cell to 0 mV repeated 30 times at 2-s intervals. In WT cerebella, this protocol depressed PF-EPSCs to 80% of baseline (Figure 7A). In contrast, in 4E-BP2-KOs, this protocol potentiated PF-EPSCs to

120% of baseline (Figure 7B). Importantly, in both WT and 4E-BP-KOs, leaving out the conjunctive depolarization resulted in no significant change from baseline (Figure 7C, D). These results indicate that protocols that induce PF-PC LTD in cerebella from WT mice induce PF-PC LTP in cerebella from 4E-BP-KO cerebella.

4E-BP2 KO mice change in gain requires acute cap-dependent translation

Deleting 4E-BP dysregulates cap-dependent mRNA translation by freeing up eIF4E to interact with eIF4G. Therefore, I investigated whether the change from LTD to LTP in cerebella from 4E-BP2-KO mice could be prevented by blocking acute mRNA translation with the inhibitor, 4EGI-1. Applying 4EGI-1 to cerebellar slices from WT mice before inducing PF-PC LTD with the burst stimulation and conjunctive depolarization protocol described in Figure 7 prevented LTD in WTs (Figure 8A). Importantly, in slices from 4E-BP2-KO cerebella, 4EGI-1 prevented the LTD-inducing stimuli from inducing PF-PC LTP (Figure 8B). These results demonstrate that PF stimulation with conjunctive depolarization requires acute mRNA translation to triggers PF synaptic plasticity. In WT cerebella, this acute mRNA translation leads to LTD at PF-PC synapses; whereas, in cerebella where mRNA initiation is dysregulated by removing 4E-BP, this LTD inducing stimulation leads to LTP.

4E-BP2 KO mice show defects in adaptation of the vestibulo-ocular reflex

Mutations that disrupt PF-PC LTD can disrupt motor learning (but see (Schonewille et al. 2010, 2011). Therefore, I examined whether motor learning was affected in 4E-BP2-KOs. Specifically, I tested the vestibular ocular reflex (VOR), a reflex that moves the eye to stabilize images on the retina in response to head movement. At baseline, the VOR was comparable between WTs and 4E-BP2-KOs (Figure 9A). When exposed to gain-down stimuli, over repeated trials, WTs showed appropriate motor learning with decreased gain of their eye movement (Figure 9B). 4E-BP2-KO mice also had a decrease in gain during gain-down experiments compared to baseline (Figure 9C); however, this motor

learning was less efficient than WTs when the rotation frequency exceeds 1 Hz (Figure 9D). This suggests that motor learning impaired in 4E-BP2-KOs compared to WTs and correlates with the altered synaptic plasticity. (For technical reasons, gain-up VOR experiments could not be examined.)

DISCUSSION

Taken together, my results show that 4E-BP2 is necessary for PF-PC synapses to undergo LTD. In 4E-BP2-KO mice, stimuli that normally give rise LTD of PF-PC synapses induce LTP instead. This change in gain correlates with impaired motor learning;4E-BP2-KO mice show impairments in the VOR gain-down compared to wild type controls. These results are consistent with previous studies suggesting that LTD is involved in motor learning (Yuzaki, 2013). Some recent studies imply that LTP or PC intrinsic firing frequency, rather than LTD, are responsible for motor learning (Belmeguenai et al., 2010; Titley and Hansel, 2016). However, I did not detect a change in LTP or intrinsic PC firing in cerebella from 4E-BP2-KO mice.

Previous work has shown that acute mRNA translation is necessary for PF-PC LTD to occur (Linden, 1996; Karachot et al., 2001). My results show that cap-dependent mRNA translation is specifically involved in LTD. Inhibiting acute translation in cerebellar slices from 4E-BP2-KO mice prevents PF-PC LTD, suggesting that in the absence of 4E-BPs, plasticity still depends on acute mRNA translation. However, more work needs to be done to determine why removing 4E-BP2 produces a change in gain, from LTD to LTP. We know that LTD requires kinase activity, leading to AMPAR phosphorylation and internalization (Chung et al., 2000) and that LTP induction requires phosphatase activity (Belmeguenai and Hansel, 2005; Schonewille et al., 2010), AMPAR dephosphorylation, and exocytosis (Chung et al., 2000); one possibility is that the kinase/phosphatase balance may be shifted, such that LTP is favored. Alternatively, it’s possible that receptor internalization is affected in 4E-BP2-KOs, and that is why LTD cannot occur.

Relevantly, using a mouse model with a global genetic deletion poses certain caveats, as there may be compensatory developmental effects. While my results show that no major differences in the morphology of the cerebellum and in the excitatory innervation, it is possible that subtle changes went undetected. For example, PCs also receive inhibitory innervation, and the balance between excitatory and inhibitory inputs may have been altered (Belmeguenai et al., 2010). This would inevitably disrupt the PCs' firing activity and affect motor learning. More work would need to be done to address this issue. One way to rule out any compensatory developmental effects would be to use an animal model where the 4E-BP2 genetic knockout is specific to PCs and could be temporarily controlled. Likewise, such conditional KO mice would also rule out the possibility that the changes are caused by the absence of 4E-BP2 in the presynaptic inputs.

Interestingly, 4E-BP2 gene deletion mimics a mutation in humans that causes autism (Gkogkas et al., 2013). Therefore, my observations on the effects of removing 4E-BP2 on PF-PC synaptic plasticity in mice may have relevance to the pathology underlying this disease in humans. In humans, mutations in upstream regulators of mTOR, including TSC (Tuberous Sclerosis) and PTEN are risk factors for ASD (Zhou and Parada, 2012; Jeste, 2009), and animal models with either TSC or PTEN mutations show autism-like deficits and abnormal PC morphology and innervation (Tsai et al., 2012; Cupolillo et al., 2016). I showed that PCs in 4E-BP2-KO mice, however, do not appear to have any anatomical defects. Since PTEN and TSC are further upstream the mTOR pathway, it is possible that they affect additional signaling processes that account for the anatomical abnormalities.

It's unclear whether PTEN and TSC mutant mice have defects in PF-PC LTD, as PF-PC has yet to be studied in most animal models of autism. One of the few studies that looked at PF-PC LTD in an animal model for ASD, the mouse model (patDp/+) for the human 15q11-13 duplication, showed that PF-PC synapses also potentiate with LTD inducing protocols (Piochon et al., 2014).

Taken together, my work and that of Piochon et al. suggest that altered PF-PC plasticity may be common in autistic patients and possibly underlie some of the behavioral deficits. Consistent with this idea, both autistic animal models (Kloth et al., 2015) and humans with autism (Hansel, 2019), show deficits in delay eyeblink conditioning, a form of associative sensory learning requiring cerebellar plasticity. Studying PF-PC LTD in ASD animal models will clarify if my findings and those of Piochon et al. are more broadly applicable to animal models and patients with ASD, and if defects in PF-PC plasticity contribute to ASD pathology.

CONCLUSION

Taken together, my results show that 4E-BP2 is critical for PF-PC LTD. Genetic deletion of 4E-BP2 appears to affect the LTD signaling pathway downstream of the induction step since calcium influx is not affected. Moreover, LTD in 4E-BP2-KOs still requires acute translation since inhibiting cap-dependent translation with 4EGI-1 blocks LTD. More work is needed to understand the molecular mechanisms underlying the change in gain from LTD to LTP occurs in 4E-BP2-KOs, and this question is addressed in Chapter 4.

I also demonstrated that genetic deletion of 4E-BP2 impairs motor learning in gain-down VOR. This correlation supports the notion that impaired PF-PC LTD affects motor learning. A more detailed understanding of how 4E-BP2 affects the LTD molecular signaling pathway will broaden our understanding of motor learning and may also illuminate a component of the pathology in autistic patients.

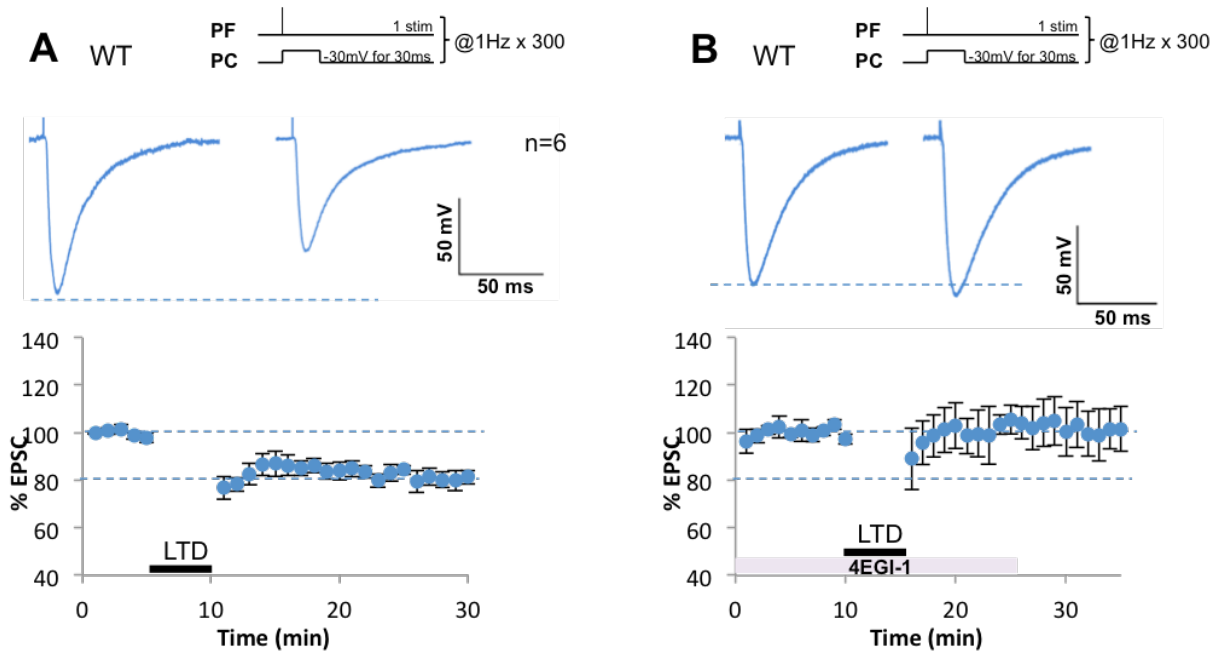

Figure 1 PF-PC LTD requires cap-dependent translation. **A** In WTs, pairing PF-stimulation at 1Hz for 5 minutes with a depolarizing voltage step to the Purkinje cell body to -30 mV for 30ms depresses PF-EPSCs to 80% of baseline (n=6). **B** Treating cerebellar slices with a pharmacological inhibitor that blocks the initiation of cap-dependent mRNA translation, 4EGI-1 (50 μM), for 30 minutes prior to LTD induction completely blocks LTD (n=6).

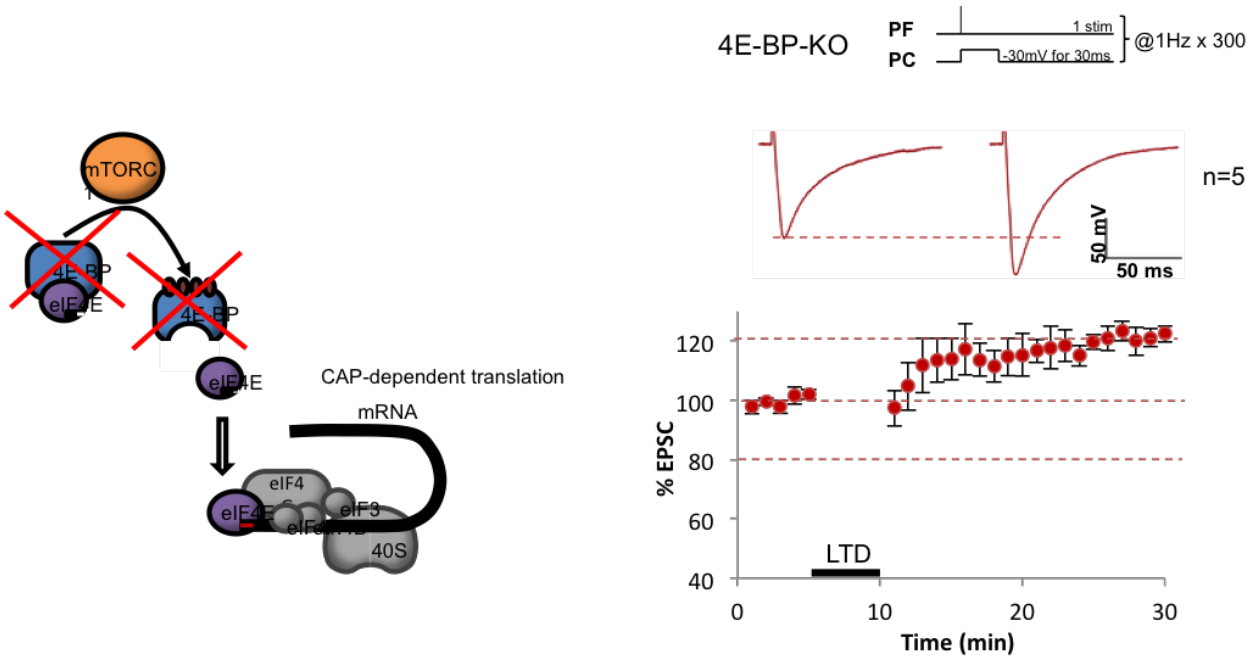

Figure 2 Genetic deletion of 4E-BP2 reverses the polarity in the plasticity in PF synapses. **Left:** Cartoon of the regulatory pathways of the cap-dependent translation initiation when 4E-BP2 is genetically deleted. In the absence of 4E-BPs, eIF4E is free to assemble into the translation initiation complex (eIF4F) and translation is dysregulated. **Right:** In acute cerebellar slices from 4E-BP2-KO mice, the LTD induction protocol (paired PF-stimulation at 1Hz for 5 minutes with a depolarizing voltage step of the PC to -30mV for 30ms) potentiates PF-PC synapses to approximately 120% of baseline (n=5).

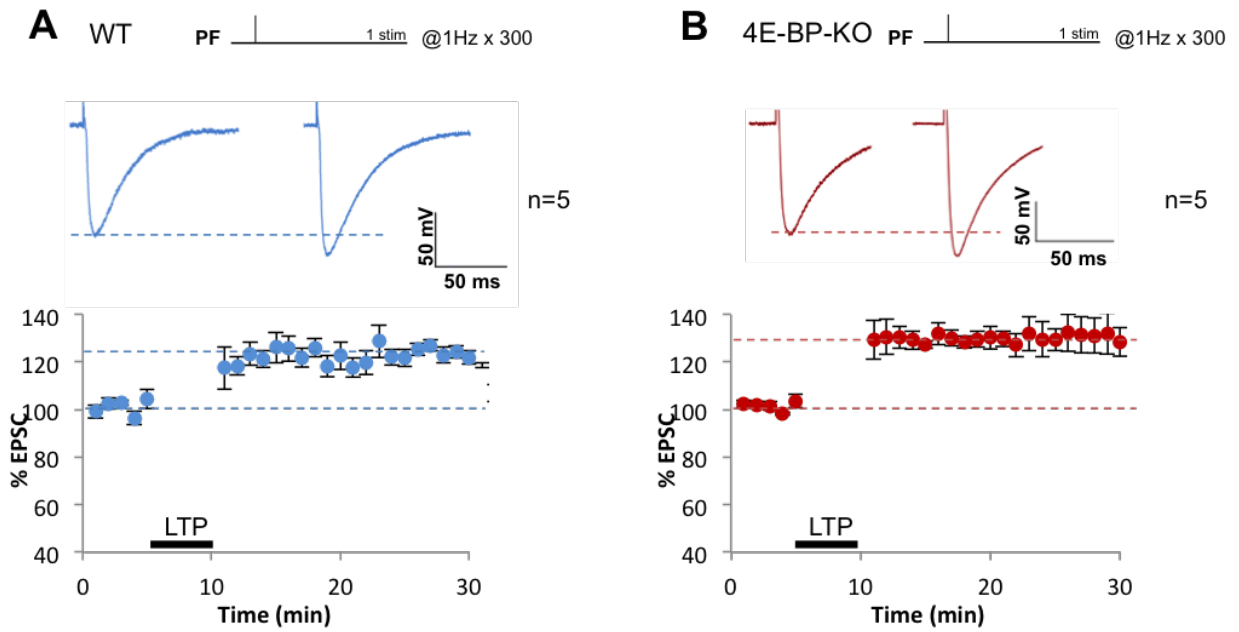

Figure 3 PF-PC LTP is normal in 4E-BP2-KO mice. **A** In WTs, PF-stimulation alone at 1Hz for 5 minutes, without conjunctive depolarization, potentiates PF-EPSCs to 120% of baseline (n=5). **B** In cerebella from 4E-BP2-KO mice, PF-stimulation alone potentiated PF-EPSCs to 120% of baseline (n=5).

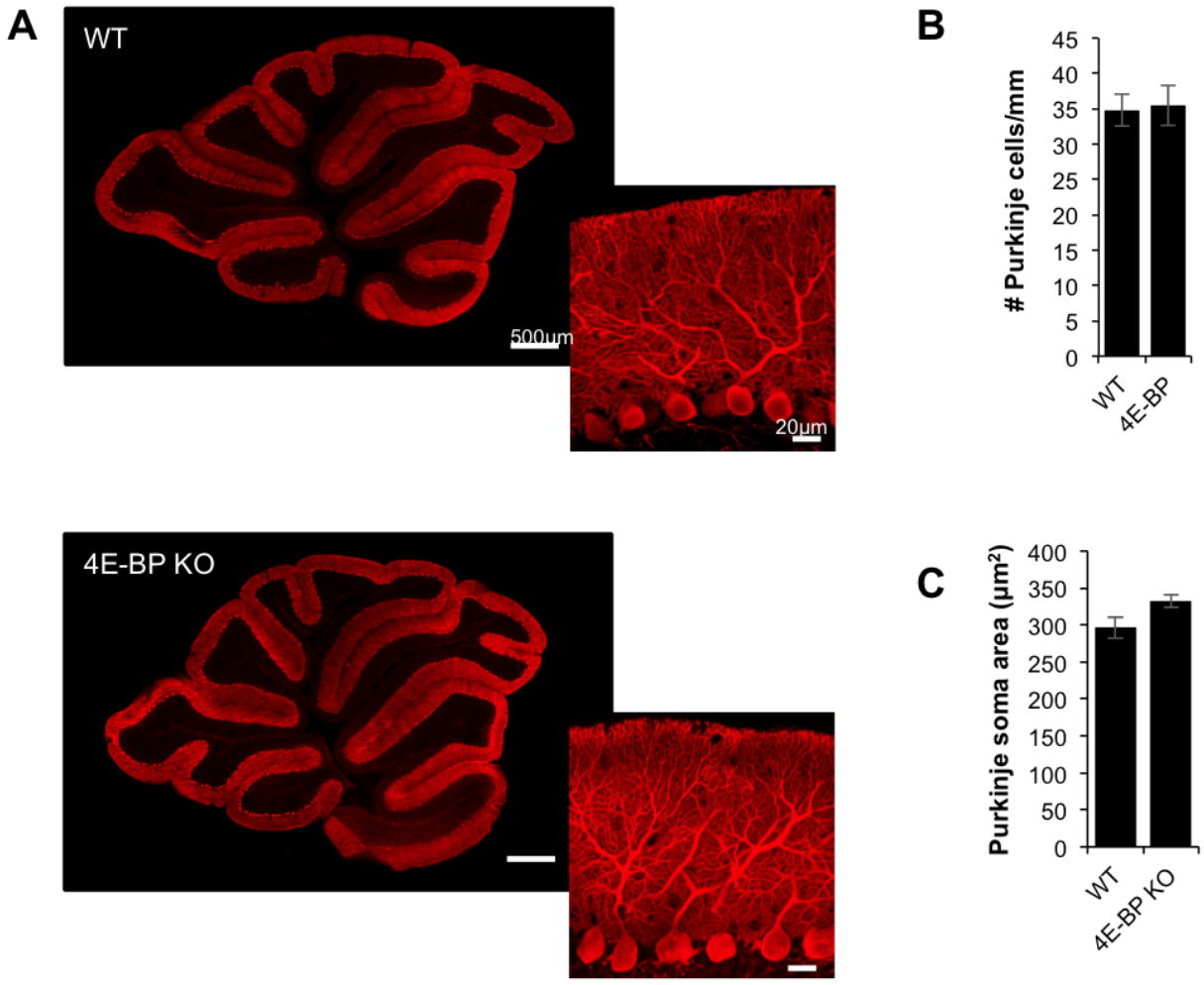

Figure 4 The cerebellum of 4E-BP2-KO mice is anatomically normal. **A** The gross anatomical structure of cerebella, assessed by staining transverse slices for calbindin 28K (red), is comparable between wild-type mice (top) and in 4E-BP2-KO mice (bottom). Scale bar: 500µM, 20µM. **B** There were no statistical differences between WT and 4E-BP2-KO cerebella in PC cell density. **C** PCs from WT and 4E-BP2-KO cerebella had comparable soma size.

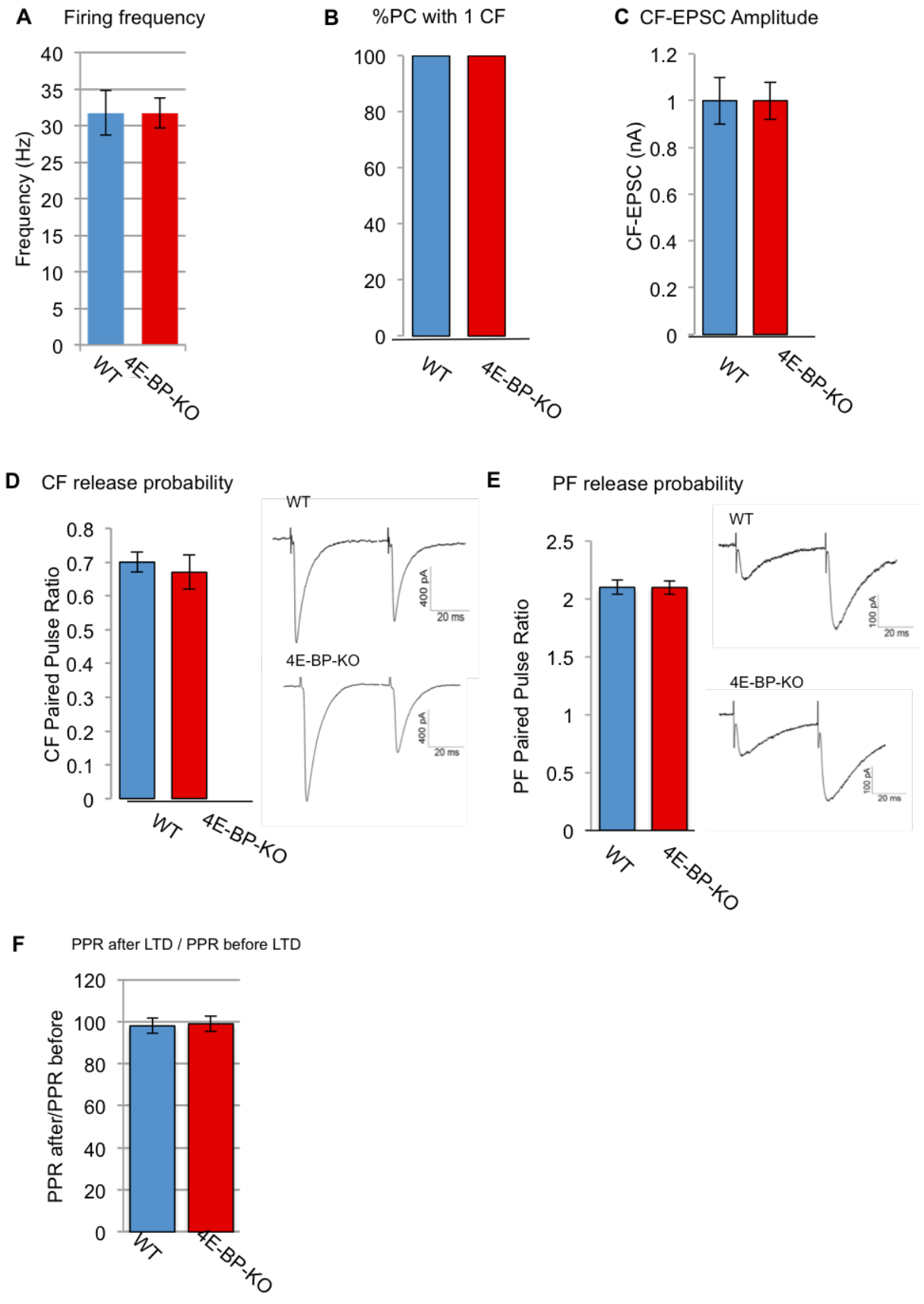
A
Firing frequency
Frequency (Hz)
40
35
30
25
20
15
10
5
0
WT
4E-BP-KO
B
%PC with 1 CF
100
80
60
40
20
0
WT
4E-BP-KO
C
CF-EPSC Amplitude
CF-EPSC (nA)
1.2
1
0.8
0.6
0.4
0.2
0
WT
4E-BP-KO
D
CF release probability
CF Paired Pulse Ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
4E-BP-KO
WT
400 pA
20 ms
4E-BP-KO
400 pA
20 ms
E
PF release probability
PF Paired Pulse Ratio
2.5
2
1.5
1
0.5
0
WT
4E-BP-KO
WT
100 pA
20 ms
4E-BP-KO
100 pA
20 ms
F
PPR after LTD / PPR before LTD
PPR after/PPR before
120
100
80
60
40
20
0
WT
4E-BP-KO

Figure 5 Genetic deletion of 4E-BP2 does not affect synaptic transmission at excitatory synapses onto PCs. **A** Intrinsic firing frequency of PCs in acute slices from 4E-BP-KOs (n=5) compared to WTs (n=4) (p=0.28). **B** At P21, PCs in both WTs (n=9) and 4E-BP2-KOs (n=10) are innervated by a single CF. **C** The amplitude of CF-EPSCs is comparable between WTs (n= 9) and 4E-BP2-KOs (n=10) (p=0.18). **D** CF paired-pulse ratio, measured by giving two pulses 50 ms appart, is not statistically different between WTs (n= 9) and 4E-BP2-KOs (n= 9) (p=0.10). **E** Paired pulse facilitation, measured by giving two pulses 50 ms apart, is comparable between WTs (n= 18) and 4E-BP2-KOs (n= 17) (p=0.46). **F** PF paired-pulse ratio before and after applying the LTD inducing protocol is not statistically different in neither WTs (n= 5) nor 4E-BP2-KO cerebella (n= 5) (p=0.42).

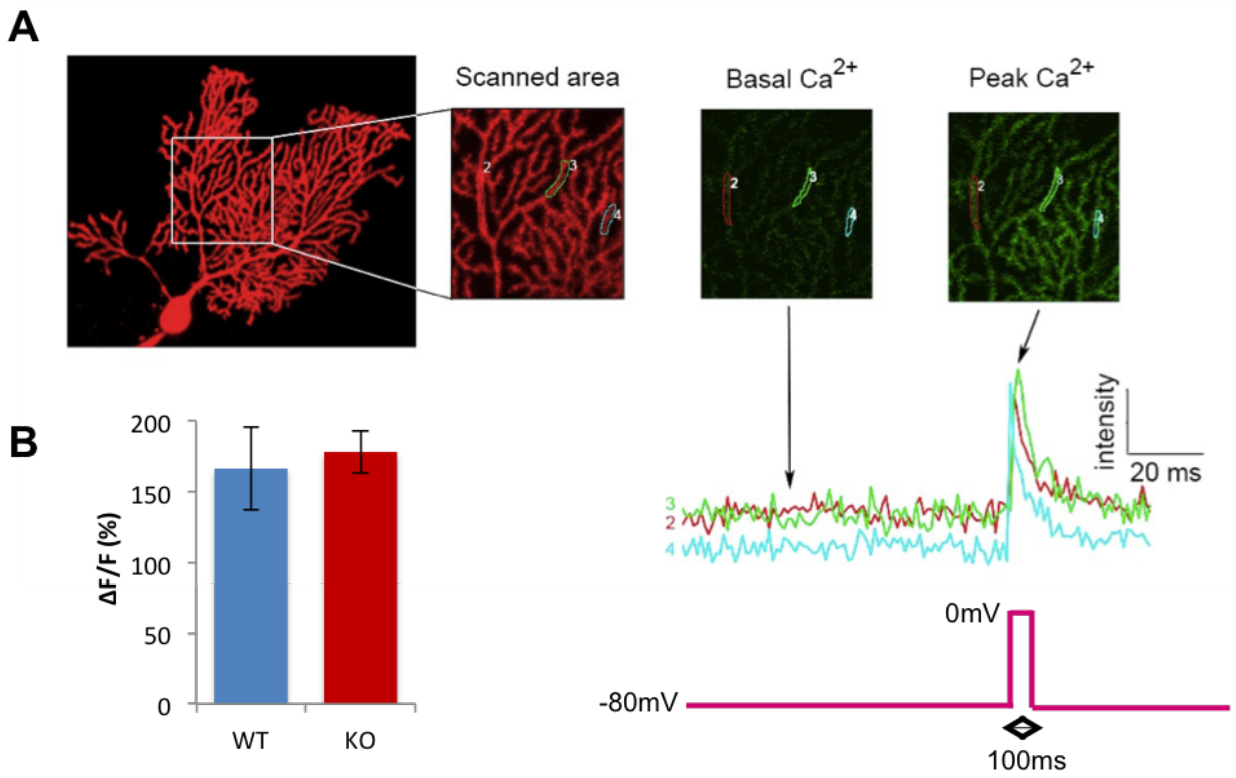

Figure 6 4E-BP2-KO mice have normal calcium transients. **A** Left to right: Image of a PC filled with Alexa Fluor 568 (red), the corresponding OGB-1 signal (green), and the change in fluorescent intensity response to a depolarizing voltage step. Scanned areas were selected using the Alexa Fluor 568 signal; example areas are labeled in the first inset as 2, 3, and 4. Calcium levels were measured in these areas of interest over time by measuring the OGB-1 fluorescent signal. To induce calcium influx, a 100ms depolarizing voltage step was delivered to the PC cell body through a patch electrode. **B** The change in fluorescence was comparable between WT and 4E-BP2-KO mice.

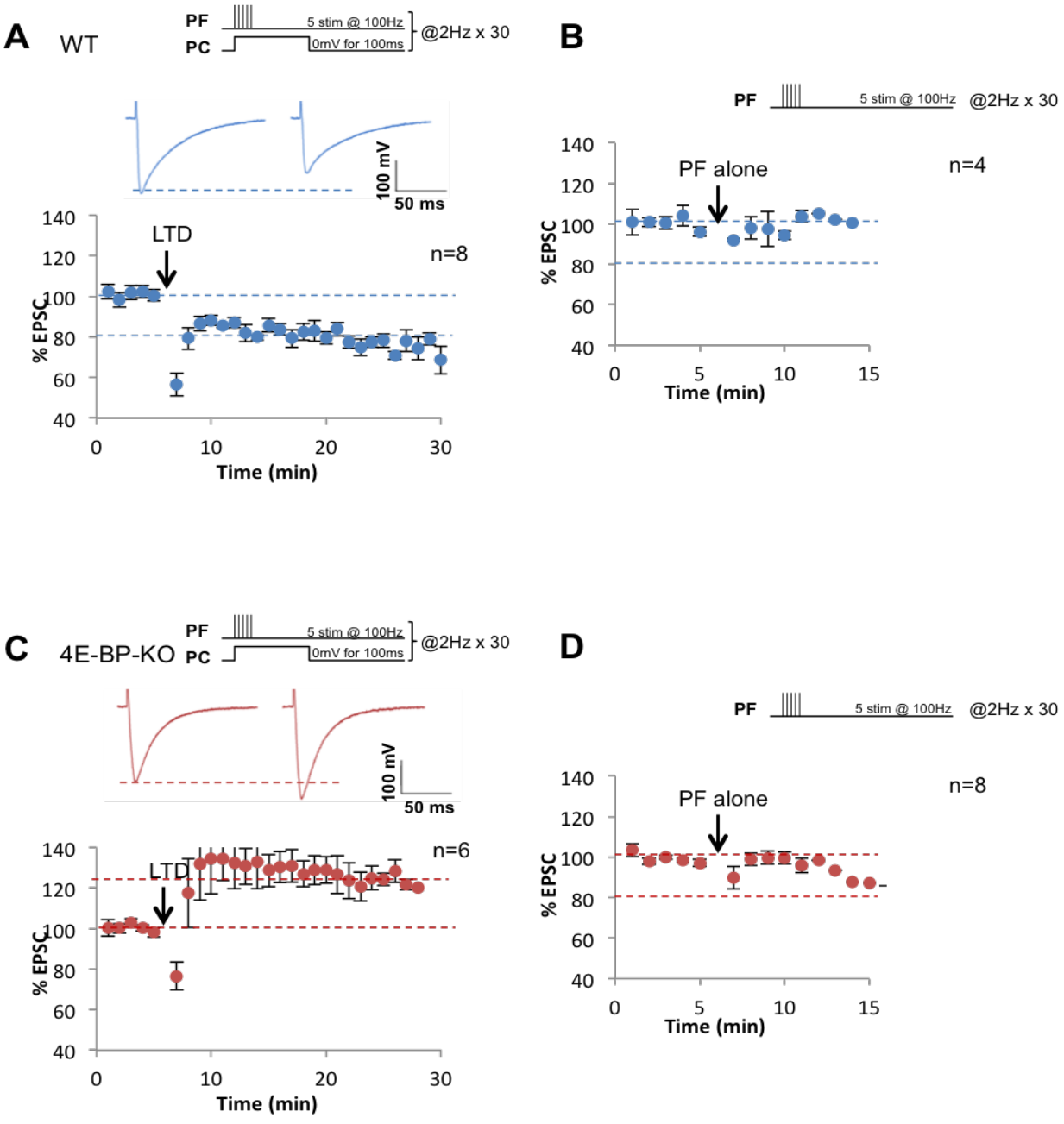
A
WT
PF
PC
5 stim @ 100Hz
0mV for 100ms
@2Hz x 30
100 mV
50 ms
LTD
n=8
% EPSC
Time (min)
B
PF
5 stim @ 100Hz
@2Hz x 30
PF alone
n=4
% EPSC
Time (min)
C
4E-BP-KO
PF
PC
5 stim @ 100Hz
0mV for 100ms
@2Hz x 30
100 mV
50 ms
LTD
n=6
% EPSC
Time (min)
D
PF
5 stim @ 100Hz
@2Hz x 30
PF alone
n=8
% EPSC
Time (min)

Figure 7 An LTD protocol consisting of paired PF burst stimulation and PC depolarization also alters PF-PC LTD in 4E-BP2-KO mice. **A** In WTs, an alternate LTD protocol consisting of parallel fibers trains of five pulses at 100 Hz accompanied by 100-ms-long depolarization of the Purkinje cell to 0 mV repeated 30 times at 2-s intervals depresses PF-EPSCs to 80% of baseline (n= 8). **B** in 4E-BP2-KOs, this protocol potentiates PF-EPSCs to 120% of baseline (n = 8). **C** In WTs, delivering parallel fibers trains of five pulses at 100 Hz only, leaving out the conjunctive depolarization, resulted in no significant change from baseline (n= 4). **D** in 4E-BP2-KOs, delivering parallel fibers trains of five pulses at 100 Hz accompanied without conjunctive depolarization, resulted in no significant change from baseline (n= 8).

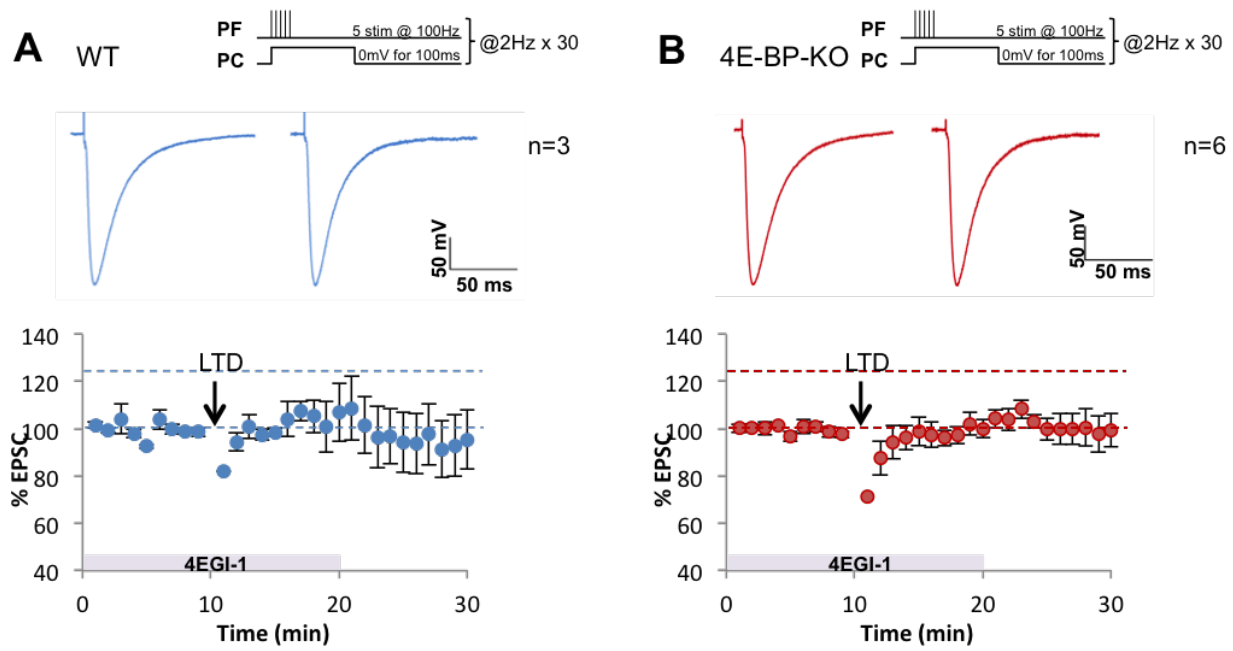

Figure 8 The change in gain in 4E-BP2-KO mice requires acute cap-dependent translation. **A** In WTs, applying 4EGI-1 (50 μM) to cerebellar slices before inducing PF-PC LTD with the burst stimulation and conjunctive depolarization protocol blocks LTD (n= 3). **B** In slices from 4E-BP2-KO cerebella, 4EGI-1 prevented the LTD-inducing stimuli from producing a change from baseline (n= 6).

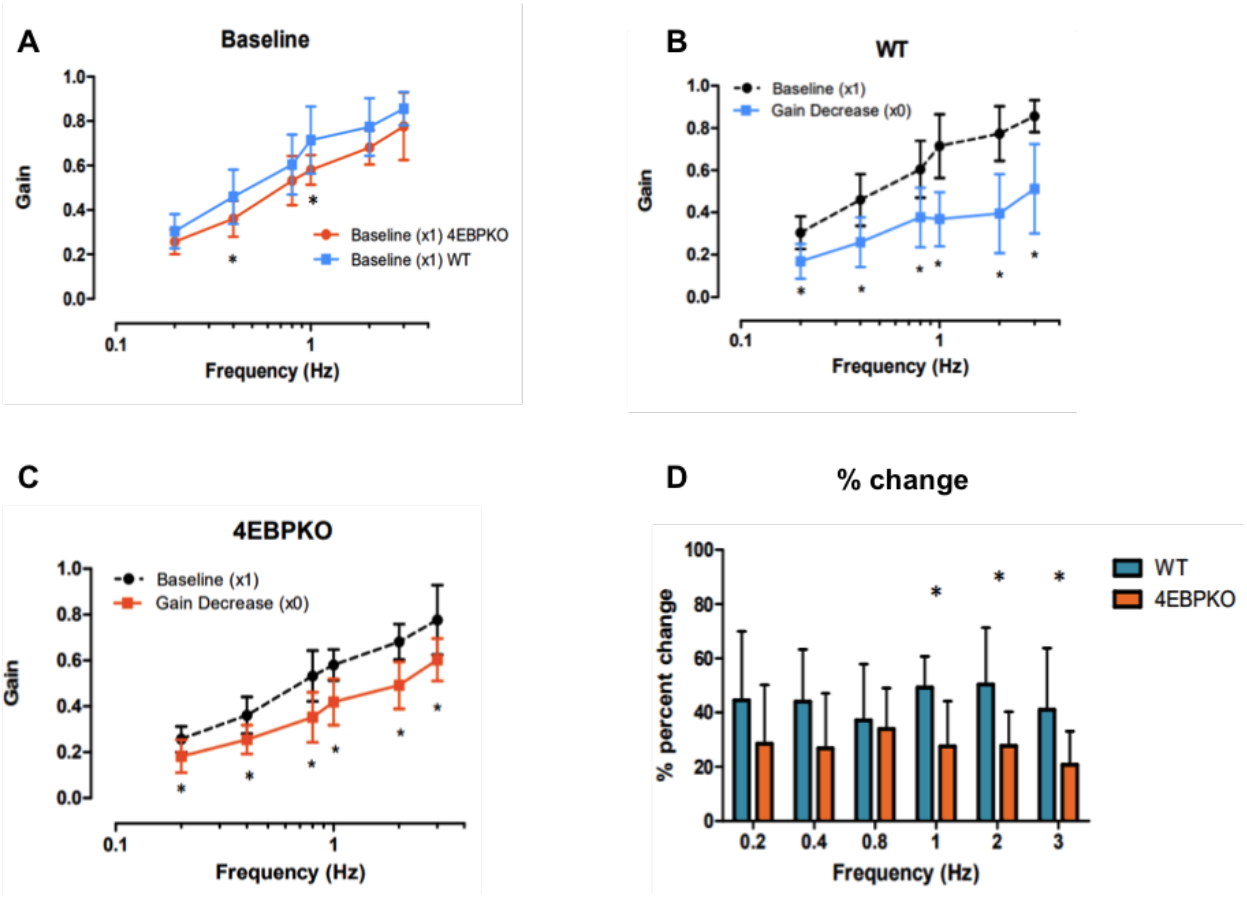

Figure 9 4E-BP2-KO mice have deficits in the adaptation of the vestibulo-ocular reflex. **A** At baseline, the VOR is comparable between WTs (n=8 mice) and 4E-BP2-Kos (n=8 mice). **B** When exposed to gain-down stimuli, over repeated trials, WTs have appropriate motor learning which decreases the gain of their eye movement (gain down: blue trace). **C** 4E-BP2-KO mice also have a decrease in gain during gain-down experiments compared to baseline (gain down: orange trace). **D** The percent change between the gain after gain decrease stimuli versus baseline is plotted against rotational frequencies. Motor learning in 4E-BP2-KOs is less efficient than WTs when the rotation frequency exceeds 1 Hz.

CHAPTER 4

4E-BP REGULATES CEREBELLAR PF-PC LTD VIA PROTEIN PHOSPHATASE 2A

INTRODUCTION

In the previous chapter, I showed that deregulating cap-dependent translation by deleting eIF4E binding proteins, 4E-BPs, converts LTD at parallel fibres-Purkinje cell synapses into LTP. In wildtypes, stimulating PFs alone produces LTP at PF-PC synapses, whereas stimulating PFs together with conjunctive depolarization, or climbing fiber stimulation, produces LTD. I showed that after deleting 4E-BPs, PF stimulation still gives rise to LTP at PF-PC synapses, however, paired conjunctive depolarization and PF stimulation leads to LTP instead of LTD. In this chapter, I investigate underlying molecular mechanisms involved in the conversion LTD to LTP at PF-PC synapses in 4E-BP-KO mice.

At PF-PC synapses, the postsynaptic AMPA receptors are composed of GluR2/GluR3 subunits. Phosphorylation of Ser-880 on the GluR2 subunit leads to receptor internalization, resulting in LTD; on the other hand, dephosphorylation of Ser-880 leads to receptor maintenance and/or insertion on the membrane, resulting in LTP (Xia et al., 2000; Chung et al., 2003; Schonewille et al., 2011). This plasticity at PF-PC synapses depends on how postsynaptic Ca^{2+} levels activate the relevant kinase and phosphatase pathways (Konnerth et al., 1990; Coesmans et al., 2004). The major kinase pathways activate CaMKII and PKC; whereas a major phosphatase pathway activates protein phosphatase 2A (PP2A); moreover, the relevant molecules in these kinase and phosphatase pathways interact (Kawaguchi et al., 2013). Briefly, calcineurin (PP2B) has a greater affinity for Ca^{2+} than CaMKII and PKC and when PFs are stimulated alone, they evoke a small increase in postsynaptic calcium that preferentially activates PP2B. PP2B activation leads to the inhibition of CamKII and downstream activation of PP2A (Lisman, 1989; Lisman 1994). PP2A

simultaneously inhibits PKC and dephosphorylates Ser-880 on the GluR2 subunit, leading to LTP (Chung et al., 2000). Conversely, when PFs are stimulated with conjunctive CF stimulation or depolarization, there is a large influx of calcium that activates PKC and CaMKII signaling cascades. These two pathways act cooperatively culminating in LTD (Kawaguchi et al., 2013). Whereas PKC directly phosphorylates AMPARs, leading to their internalization (Chung et al., 2000), the role of CaMKII is primarily to inhibit phosphatase activity (Kawaguchi et al., 2013). LTD induction also involves a secondary, inhibitory feedback mechanism; elevated calcium activates adenylate cyclase, which activates the protein kinase-dependent cAMP (PKA). PKA phosphorylates inhibitor 1 (I-1) which inhibits phosphatase 1 (PP1) and prevents inactivation of CaMKII (see chapter 1, figure 6 for an illustration of the molecular mechanisms).

Interestingly, the genetic deletion of PP2B completely prevents LTP and instead leads to LTD (Schonewille et al., 2010). Conversely, perfusing constitutively active PP2B into the PC prior to inducing LTD shifts it to an LTP (Belmeguenai and Hansel, 2005). This is due to a change in the balance between kinase and phosphatase activity, which impacts AMPAR membrane insertion and removal.

The question is, in 4E-BP2-KOs, which part of the LTD pathway is affected? The problem could be at the level of the kinase/phosphatase balance and secondary messenger signaling, or it could occur at the level of induction, or directly affect AMPAR internalization. My previous work (see chapter 3) showed that calcium signaling is not altered in 4E-BP2-KOs and therefore is unlikely to cause the conversion of LTD to LTP. However, LTD induction also requires mGluR1 signaling, and deleting 4E-BP may impair this pathway, or it may impair a component of the downstream kinase and/or phosphatase signaling pathways. In this chapter, I have investigated these various possibilities.

METHODS

Mice

All experiments in chapter 4 used P21-P30 4E-BP1/2 -/- mice (referred to as 4E-BP2-KO) and age-matched wildtype controls.

Cerebellar Slices

For electrophysiological experiments, 250uM transverse slices were acutely prepared following the protocol outlined in section 2.2.1.

Electrophysiology

LTD was induced by delivering parallel fibers trains of five pulses at 100 Hz accompanied by 100-ms-long depolarization of the Purkinje cell to 0 mV repeated 30 times at 2-s intervals. The solutions, setup and other stimulation protocols used are described in detail in section 2.3.1.

Pharmacological inhibitors

The mGluR antagonist CPCCOEt (100 μM, Tocris Bioscience, Ellisville, MO, USA) was used to block mGluR1 signaling (Mitsumura et al., 2011). General phosphatase activity was inhibited using the general phosphatase inhibitor Okadaic Acid (1uM, Sigma, St. Louis, MO). To block PP1, inhibitory peptide inhibitor-2 (I-2; 100 nM, New England BioLabs, Beverly, MA) was used. PP2B was blocked using Cyclosporin A (100uM, Tocris, Ellisville, MO) and PP2A was inhibited using Fostriecin (50 nm) (Belmeguenai and Hansel, 2005). The detailed protocol is described earlier in section 2.3.1.3.

Western Blot Analysis

Western blot analysis was performed according to manufacturer's protocols using the following primary antibodies against PPP2K4 (PTPA), PPME1, regulatory subunit B56ε, regulatory subunit B55β, structure subunit Aα/β, catalytic subunit Cα/β, 4E-BP1, 4E-BP2, regulatory subunit B56δ and regulatory subunit B55α. A detailed protocol can be found in section 2.5

RESULTS

mGluR1 signaling is not affected in 4E-BP2-KOs

PF-PC LTD induction requires mGluR1 activation and downstream calcium release from IP_3 stores. Blocking mGluR1 pharmacologically prevents PF-PC LTD (Aiba et al., 1994; Mitsumura et al., 2011). Therefore, I asked whether inactivating mGluR1 on cerebellar Purkinje cells affects LTD induction in 4E-BP2-KO mice.

To test this possibility, I continuously perfused cerebellar slices from WT and 4E-BP2-KO mice with the mGluR1 inhibitor, CPCCOEt (100 μM), starting 10 minutes before LTD induction. Blocking mGluR1 did not affect the baseline amplitude of PF-EPSCs (see baseline of Figure 1, A and B). On the other hand, blocking mGluR1 prevented the LTD induced in wildtype slices by conjunctive PF stimulation and depolarization, consistent with previous work (Figure 1A). Likewise, in cerebella from 4E-BP2-KO mice, blocking mGluR1 prevented any change in PF-EPSPs from baseline after conjunctive PF stimulation and depolarization (Figure 1B). These results suggest that the change from LTD to LTP at PF-PC synapses in 4E-BP2-KOs is not a result of altered mGluR activation.

General phosphatase inhibition restores LTD in 4E-BP2-KOs

Next, I investigated downstream second messenger signaling. When the kinase/phosphatase balance favors kinase activity, AMPARs become phosphorylated and internalized, leading to LTD. When the kinase/phosphatase balance shifts towards phosphatase activity, on the other hand, the postsynaptic receptors become dephosphorylated, inserted and stabilized on the membrane and LTP occurs. In acute slices from wildtype mice, blocking phosphatases prevents PF-LTP (Belmeguenai and Hansel, 2005). Therefore, it is possible that in cerebella from 4E-BP2-KO mice, LTD induction leads to enhanced phosphatase activity instead of kinase activity, thereby producing LTP instead of LTD. If so, inhibiting phosphatase activity should prevent LTP.

To test this, I blocked phosphatase activity in acute slices using the general phosphatase inhibitor Okadaic Acid (1uM). In wildtypes, PF stimulation at 1Hz for 5 minutes potentiated synapses to 120% of baseline (see chapter 3, Figure 3A), but in the presence of Okadaic Acid, LTP was blocked and became an LTD (Figure 2A). In slices from 4E-BP2-KOs, LTD induction (PF stimulation with conjunctive depolarization) gave rise to LTP (see chapter 3, Figure 7C). In the presence of Okadaic Acid, however, LTD induction in 4E-BP2-KO slices depressed PF-PC synapses to ~85% of baseline (Figure 2B). This strongly suggests that in 4E-BP2-KOs, the induction of LTP instead of LTD with conjunctive stimulation is the result of increased phosphatase activity. Three phosphatases are known to be involved in LTP at PF-PC synapses: protein phosphatase 1 (PP1), PP2A and Calcineurin (PP2B) (Belmeguenai and Hansel, 2005). Therefore, investigated each one individually.

PP1 inhibition does not rescue LTD in 4E-BP2-KOs

First, I examined the role of PP1 using the PP1 inhibitory peptide inhibitor-2 (I-2) (100 nM). In acute slices from WTs, I-2 added to the pipette saline blocked PF-LTP induction (Figure 3A). Likewise, LTP induction in 4E-BP2-KOs was blocked in the presence of I-2 (Figure 3C). However, stimulating PF with conjunctive depolarization in the presence of I-2 had little effect on the potentiation of PF-PC synapses (Figure 3B). These data indicate that enhanced phosphatase activity occurs downstream of PP1.

PP2B inhibition does not rescue LTD in 4E-BP2-KOs

Next, I blocked PP2B pharmacologically by perfusing acute cerebellar slices with the PP2B-selective inhibitor cyclosporin A (100 μm). PP2B inhibition blocks LTP in WTs (Figure 4A) and in 4E-BP2-KOs (Figure 4C). However, when I induced LTD in 4E-BP2-KOs in the presence of cyclosporin A, the PF-PC synapses still potentiated (Figure 4B). Therefore, the enhanced phosphatase activation is not caused by PP2B and occurs downstream.

PP2A inhibition restores LTD in 4E-BP2-KOs

Finally, I tested the effect of inhibiting PP2A by adding the PP2A inhibitor fostriecin (50nM) to the pipette saline. PP2A is directly responsible for dephosphorylating Ser-880 on the GluR2 subunit and also inhibits PKC, blocking AMPAR phosphorylation and endocytosis. In WTs, inducing LTP in the presence of the fostriecin prevented LTP (Figure 5A). Similarly, blocking PP2A in 4E-BP2-KOs during LTD induction also blocked LTP (Figure 5C). Interestingly, blocking PP2A during LTD induction restored LTD in 4E-BP2-KOs, and PF-PC synapses depressed to ~85% of baseline (Figure 5B). This suggests that in 4E-BP2-KOs, PP2A activation is enhanced and converts LTD to LTP; when PP2A activity is inhibited, LTD is restored.

Cerebella of 4E-BP2-KO mice have higher levels of the PP2A activator PTPA and lower levels of the inhibitory protein PPME1

Another caveat is that PP2A is regulated by many molecules and is itself composed of multiple subunits (Kaur and Westermarck, 2016). It is possible that in 4E-BP2-KO mice, PP2A levels are altered, or that its activity is enhanced due to lower levels of PP2A inhibitors, or higher expression of PP2A activators. To this end western blot analysis of PP2A subunits and their regulators were performed using cerebella from 4E-BP2-KOs and WT controls. Although we found no changes in the expression of the structural, regulatory, or catalytic subunit of PP2A, preliminary evidence suggests that in 4E-BP2-KO mice there was less PP2A inhibitory protein, PPME1 (protein phosphatase methylesterase-1) and slightly more of the PP2A activator, PTPA (protein phosphatase 2A phosphatase activator) (Figure 6). This suggests that PP2A activation may be altered at baseline, which could shift the kinase/phosphatase balance, favoring phosphatase activity and contributing to the change in gain from LTD to LTP in 4E-BP2-KO mice.

DISCUSSION

These results strongly suggest that PP2A regulation is critical for PF-PC LTD and is under the control of cap-dependent translation regulation. In 4E-BP2-KOs, PP2A activity is enhanced which converts LTD to LTP; blocking PP2A pharmacologically in acute slices is sufficient to restore LTD.

While PP2A is involved in shifting LTD to LTP in 4E-BP2-KOs, the mechanism of action remains unclear. One possibility is that at baseline, PP2A activity in Purkinje cells is already enhanced, since we detected more of the PP2A activator, PTPA, and less of the inhibitor, PPME1, in the cerebella of 4E-BP2-KOs compared to control. While this change does not appear to affect PF-PC synapses under basal conditions, it could affect the kinase/phosphatase balance after LTD induction, leading to enhanced phosphatase activity instead of kinase activity, and giving rise to LTP instead of LTD. However, our western blot study used the whole cerebellar vermis; whether the levels of PTPA and PPME1 are altered specifically in PCs remains to be confirmed. Additionally, PP2A regulation is complex (Kaur and Westermarck, 2016), and there could be additional proteins involved that we did not examine. A comprehensive proteomic analysis of 4E-BP2-KO cerebella would further illuminate how PP2A activation is affected in 4E-BP2-KOs.

Another possibility is that in 4E-BP2-KOs, the translation of PP2A, or of its regulators, is acutely enhanced during LTD induction, leading to enhanced PP2A activity and LTP. In chapter 3, I showed that acute blockade of cap-dependent translation initiation blocks LTD in wildtypes and also prevents any change from baseline in the 4E-BP2-KOs. However, it remains unclear which mRNAs are acutely translated during LTD induction in WTs, and whether the same mRNA (or different mRNA such as that of PP2A) are being acutely translated in 4E-BP2-KOs. It’s possible that acute translation plays a permissive role without affecting the sign of the change in gain. A better understanding of the target mRNA that is acutely translated during PF-PC LTD will clarify this issue.

An intriguing finding from Chapter 3 is that genetic deletion of 4E-BPs has behavioral consequences; 4E-BP-KOs have impaired motor learning in VOR gain-down adaptation. Given that blocking PP2A restores normal LTD in 4E-BP-KOs, it would be interesting to test whether the pharmacological blockade of PP2A in the cerebellum would rescue motor learning. This data would help contribute to our understanding of the role of LTD in motor learning.

Finally, as I mentioned in the discussion of Chapter 3, 4E-BP2 gene deletion mimics a mutation in humans that causes autism, and 4E-BP2-KO mice are an animal model for autism spectrum disorder (ASD) (Gkogkas et al., 2013). My results in Chapter 3 are consistent with the idea that defects in cerebellar function may contribute to ASD pathology, and this follow-up work points to a role for enhanced PP2A activity. Although Piochon et al. (2014) found a similar shift from LTD to LTP in the mouse model for the human 15q11-13 duplication (patDp/+), an animal model for ASD, they did not examine the molecular mechanisms involved. It would be interesting to see if altered PP2A activity contributes to the change in gain in patDp/+ mutants as well. This data would further illuminate the link between cap-dependent translation regulation, cerebellar function, and ASD.

CONCLUSION

Taken together, my results suggest that PP2A activity is enhanced in 4E-BP2-KOs and converts LTD to LTP. However, the mechanism of action by which PP2A activity is altered remains to be examined. One possibility is that in the absence of 4E-BP2, basal levels of PP2A regulators are altered which leads to enhanced PP2A activity during LTD induction, shifting LTD to an LTP. Additionally, there may be acute translational effects on PP2A or its regulators which lead to a change in gain, since blocking cap-dependent translation prevents plasticity in 4E-BP2-KOs. More work will be necessary to clarify how 4E-BP2 affects PP2A levels and activity.

Given that 4E-BP2-KO mice are an animal model for ASD, it would be interesting to explore whether the findings from 4E-BP2-KO mice are more broadly applicable in animal models of ASD and if PP2A is also affected in those models.

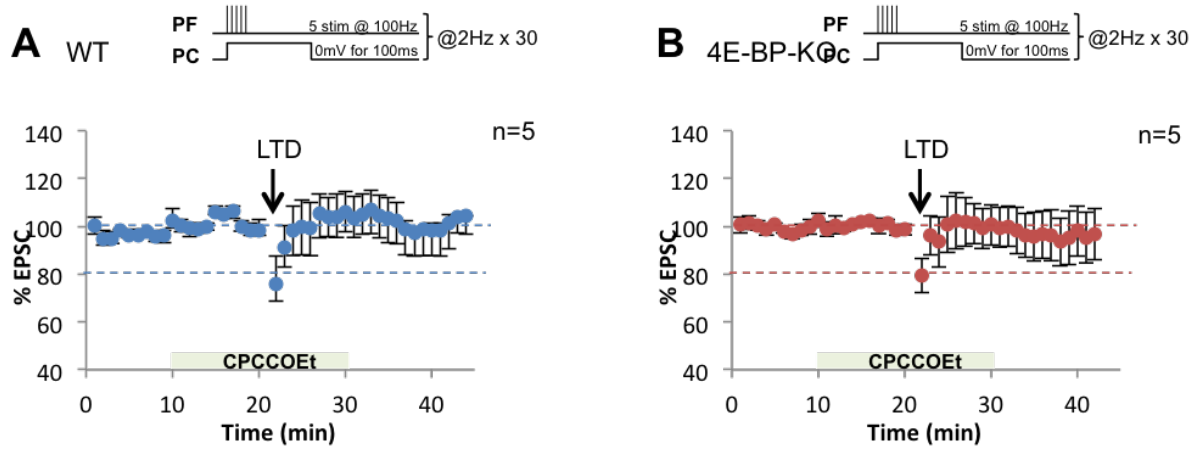

Figure 1 mGluR1 signaling is not affected in 4E-BP2-KOs. **A** Blocking mGluR1 with the pharmacological inhibitor CPCCOEt (100 μM) prevents PF-PC LTD in WTs (n=5). **B** Pharmacological inhibition of mGluR1 in 4E-BP2-KOs prevents any change from baseline (n=5).

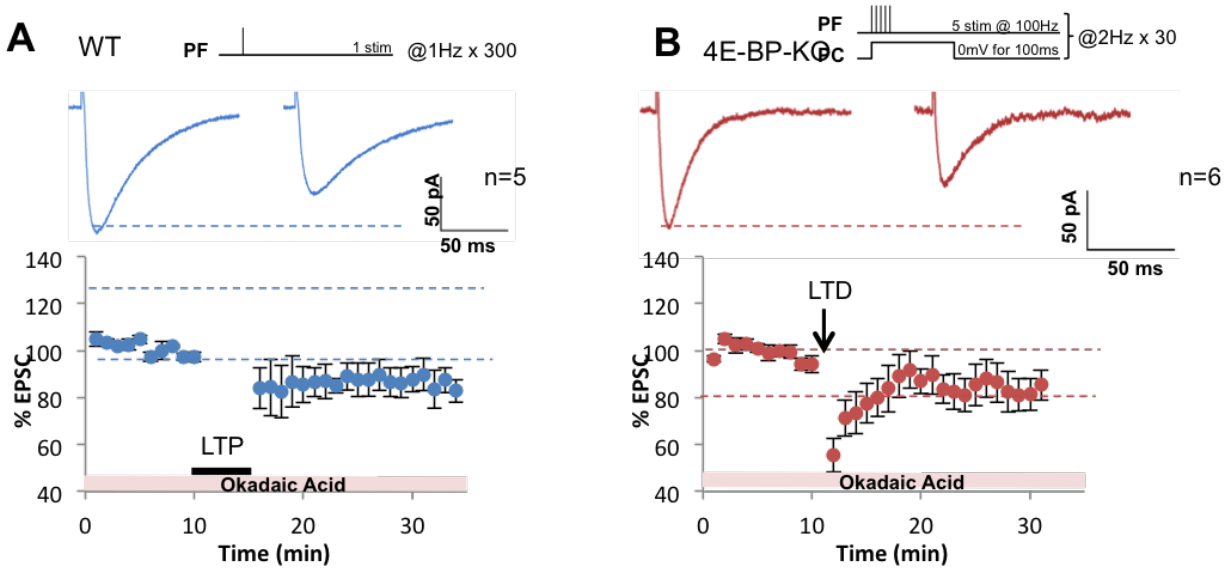

Figure 2 General phosphatase inhibition using the phosphatase inhibitor Okadaic Acid restores LTD in 4E-BP2-KOs. **A** In WTs, pharmacological inhibition of phosphatase activity with the antagonist Okadaic Acid (1uM) blocks LTP induction (n=5). **B** in 4E-BP2-KOs, inducing LTD in the presence of Okadaic Acid restores LTD, depressing PF-EPSCs to ~85% of baseline (n=6).

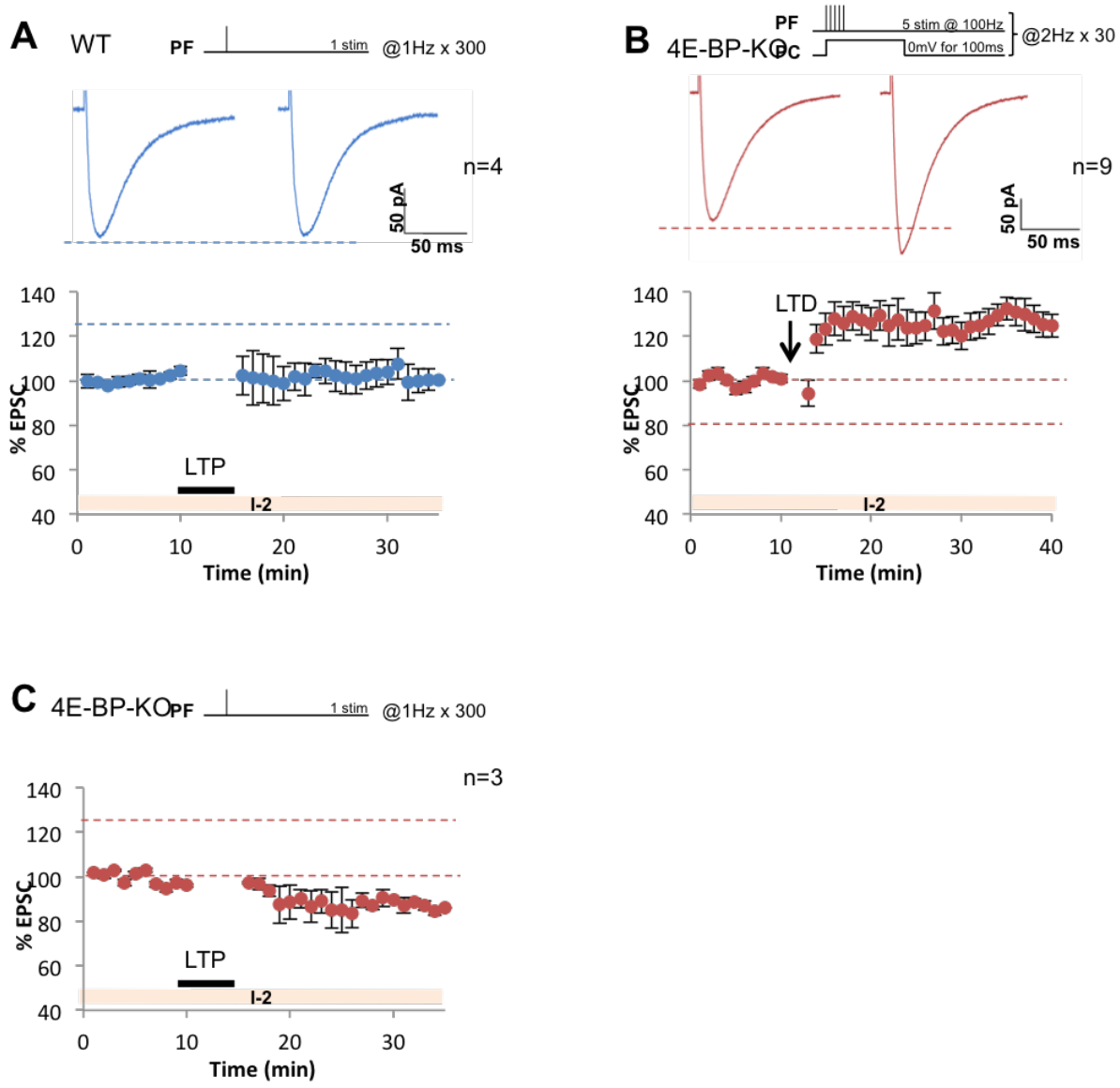

Figure 3 PP1 pharmacological inhibition does not restore LTD in 4E-BP2-KOs. **A** In WTs, pharmacological inhibition of PP1 with I-2 (100 nM) blocks LTP induction (n=4). **B** in 4E-BP2-KOs, PP1 inhibition has no effect on the LTP induced with the LTD protocol (n=9). **C** PP1 inhibition effectively prevents LTP in 4E-BP2-KOs (n=3).

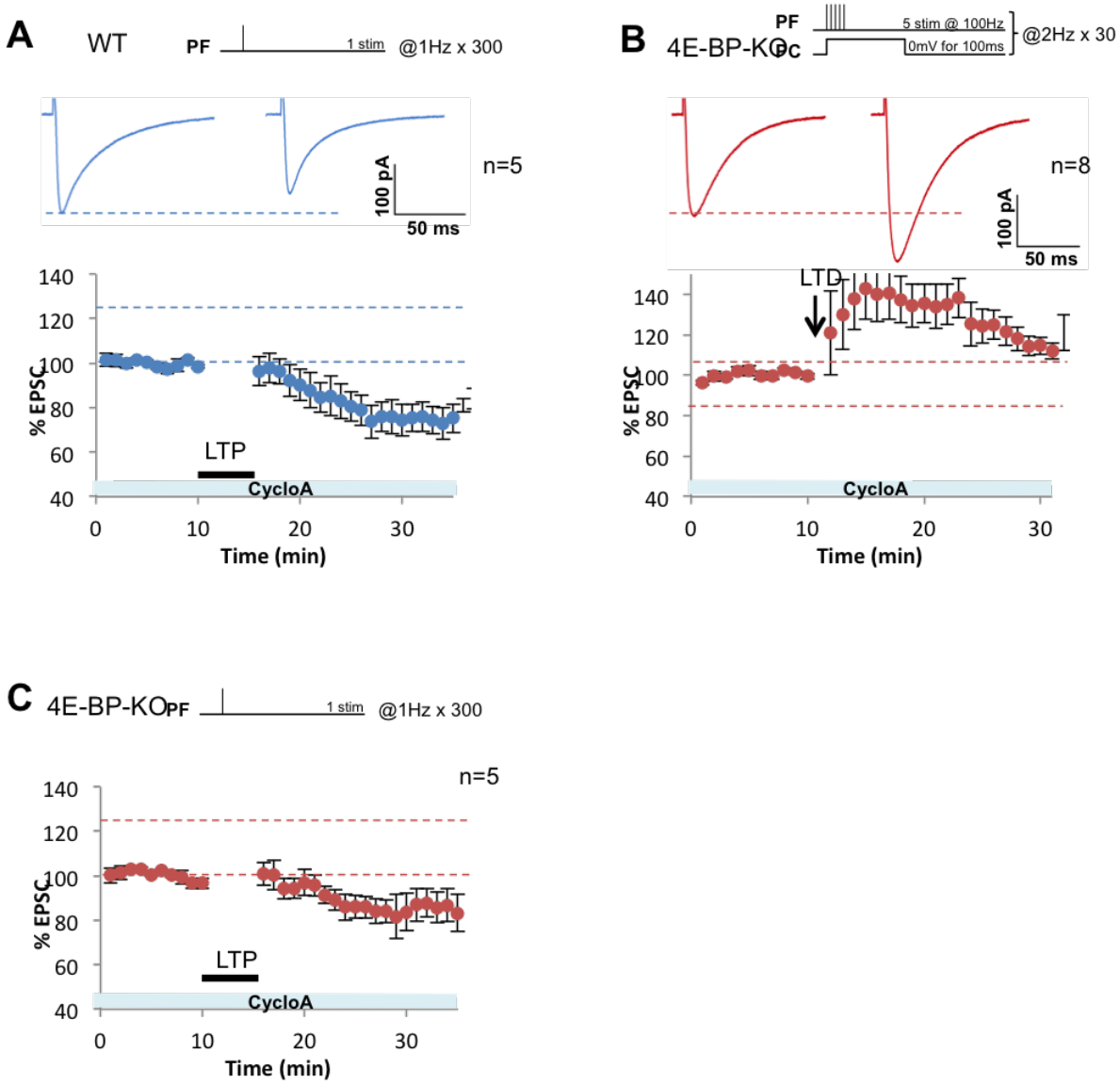

Figure 4 Blocking PP2B with Cyclosporin A has no effect on the change in gain in 4E-BP2-KOs. **A** Pharmacological inhibition of PP2B with the antagonist Cyclosporin A (100uM) completely blocks LTP in WTs and instead induces LTD (n=5). **B** in 4E-BP2-KOs, blocking PP2B has no effect on the LTP induced by the LTD protocol (n=8). **C** Blocking PP2B in 4E-BP2-KOs during LTP induction prevents potentiation (n=5).

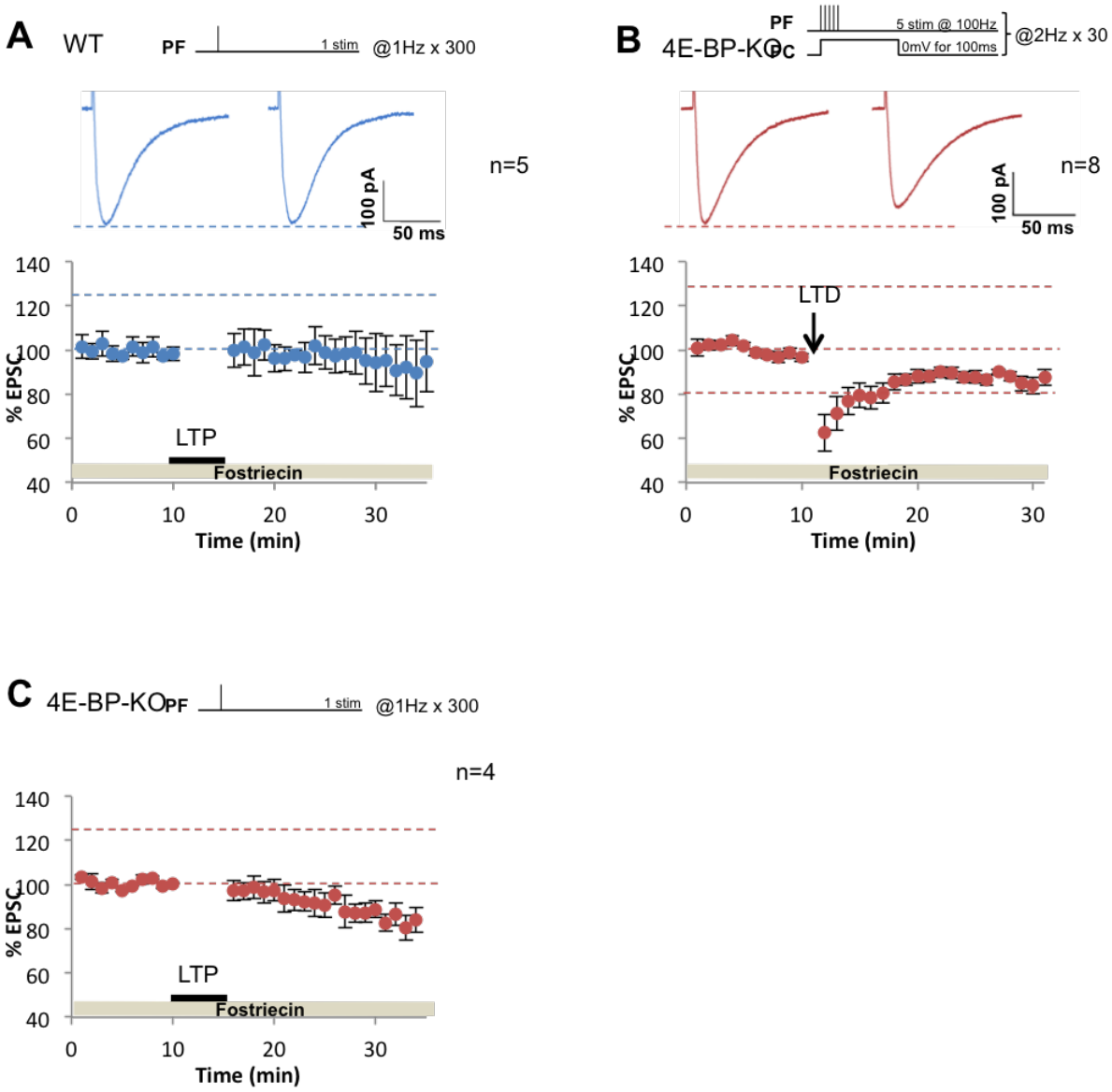

Figure 5 PP2A pharmacological inhibition restores LTD in 4E-BP2-KOs. **A** In WTs, PP2A inhibition using the pharmacological inhibitor Fostriecin (50 nm) blocks LTP (n=5). **B** in 4E-BP2-KOs, blocking PP2A during LTD induction depresses synapses to ~85% of baseline (n=8). **C** in 4E-BP2-KOs, blocking PP2A during LTP induction prevents potentiation (n=4).

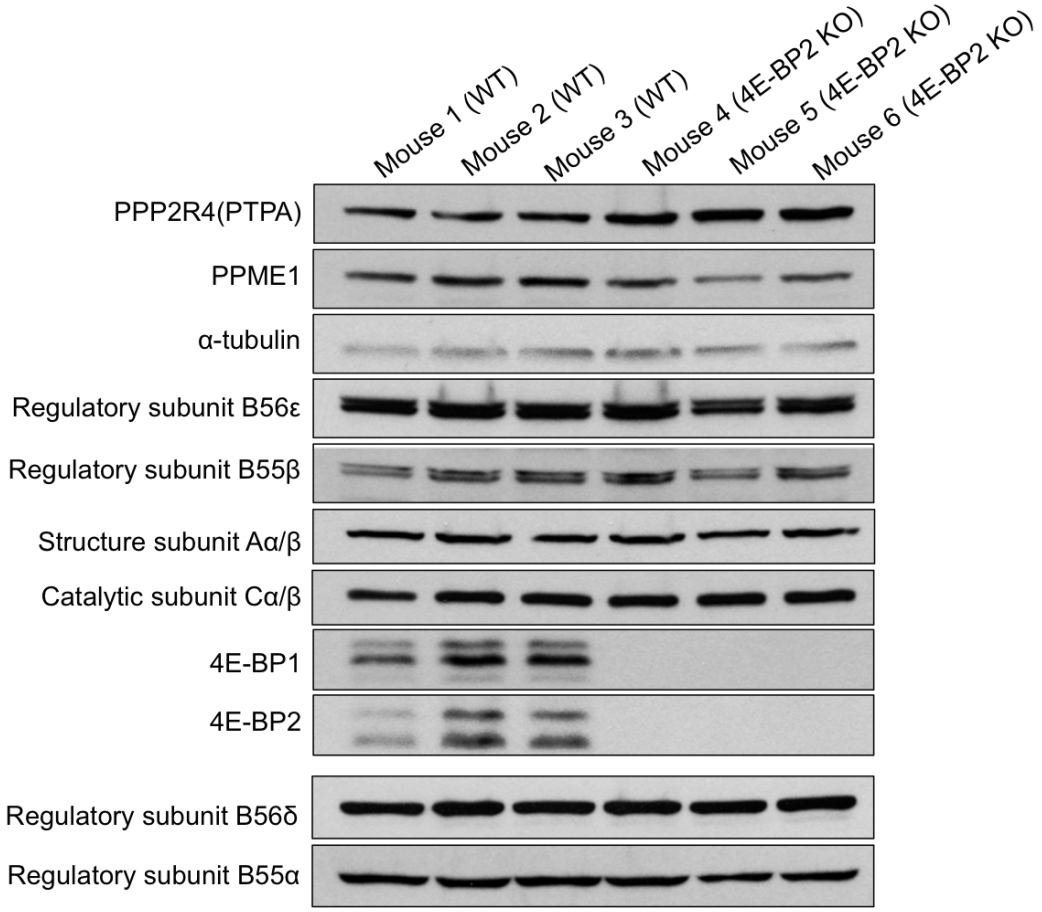

Figure 6 Western blot of 4E-BP2-KO and WT cerebella, staining for PP2A and its regulators. In 4E-BP2-KOs the PP2A activator PTPA is expressed in higher levels and the PP2A negative regulator PPME1 is expressed in lower levels compared to WT controls. The levels of PP2A subunits, including the structure subunits, catalytic subunits, and regulatory subunits are comparable between WT mice and 4E-BP2-KOs.

CHAPTER 5

REMOVING 4E-BP ENABLES SYNAPSES TO REFINE WITHOUT POSTSYNAPTIC ACTIVITY

INTRODUCTION

In the previous chapters, I showed that cap-dependent translation and 4E-BP2 are necessary for functional plasticity at PF-PC synapses. Specifically, genetic deletion of 4E-BP2 converts LTD into LTP. This suggests that regulation of cap-dependent translation by 4E-BPs is necessary for synapses to appropriately strengthen or weaken in response to environmental stimuli.

More broadly, my interest is to understand the link between synaptic activity and synaptic plasticity. Recent studies show that structural plasticity, particularly the re-arrangement of synaptic connections, supports long term changes in synaptic strength and learning and memory (Lamprecht and LeDoux, 2004; Johansen-Berg, 2007; Bernardinelli et al., 2014). Therefore, I wondered whether cap-dependent translation has a role in this process? While the role for structural plasticity in learning and memory is novel, its role in the rearrangement of synaptic connections in the developing nervous system as neural circuits refine their connection is well established (Purves and Lichtman, 1980; Lichtman and Colman, 2000; Kano and Hashimoto, 2009). Therefore, I investigated the role of synaptic activity and cap-dependent translation on the rearrangement of synaptic connections during early postnatal development.

My work in chapters 3 and 4 focused on Purkinje cells in the cerebellar cortex. Therefore, the refinement of climbing fibers innervating Purkinje cells might seem like an appropriate model. However, given that Purkinje cells also receive excitatory innervation from parallel fibers and inhibitory innervation, the link between post-synaptic activation, cap-dependent translation and structural changes is not clear-cut and may be confounded by these other inputs. To address the role of activity and translation in structural plasticity, I investigated a

simpler model: preganglionic innervation of sympathetic neurons in the Superior Cervical Ganglion (SCG).

The SCG is a useful model to address these questions since SCG neurons are innervated exclusively by a homogeneous group of excitatory cholinergic preganglionic axons; therefore, refinement of preganglionic axons is not confounded by other inputs to the postsynaptic neurons. At birth, SCG neurons are innervated by 7-8 preganglionic axons, and over the next 2-3 postnatal weeks, the peripheral autonomic circuits are refined by eliminating redundant inputs and strengthening others (reviewed in (Purves and Lichtman, 1980; Lichtman and Colman, 2000)). Furthermore, SCG neurons express only 1 class of postsynaptic receptors: α3-containing nicotinic acetylcholine receptors (nAChRs) (Xu et al., 1999; Rassadi et al., 2005). Genetic deletion of the α3 nAChR subunit gene from mice (α3-KO) completely eliminates rapid synaptic transmission at SCG synapses (Rassadi et al., 2005; Krishnaswamy and Cooper, 2009). Nevertheless, ultrastructurally normal synapses persist on α3-KO SCG neurons (Krishnaswamy and Cooper, 2009). In addition, synaptic activity can be restored at synapses by infecting postsynaptic neurons with α3-expressing adenoviruses (α3-Ad) (Krishnaswamy and Cooper, 2009).

This process of synaptic refinement is widespread in the developing nervous system (Purves and Lichtman, 1980; Schlaggar et al., 1993; Sanes and Lichtman, 1999; Buffelli et al., 2002; Katz and Crowley, 2002; Zou et al., 2004; Bleckert and Wong, 2011; Hong and Chen, 2011; Yasuda et al., 2011; Takeuchi et al., 2014). The current model is that synaptic refinement occurs by activity-dependent competition (Katz and Shatz, 1996; Lichtman and Colman, 2000; Zhang and Poo, 2001; Cohen-Cory, 2002; Kano and Hashimoto, 2009). This model suggests that synapses that most effectively depolarize the postsynaptic cell “win” and strengthen, whereas the less effective synapses are destabilized and pruned. However, many of the molecular details remain unclear. Using the mouse SCG as a model, I investigated the refinement of preganglionic axons in the absence of postsynaptic activity. In addition, I investigated whether cap-

dependent mRNA translation plays a role in the refinement of preganglionic axons.

METHODS

Mice

These experiments used α3-KO and α3/4E-BP-DKO mice at birth (P1-3), one month (P28-30) and two months (P54-60), as well as age-matched WT and 4E-BP-KO as controls (see section 2.1)

Tissue preparation

Acutely dissected ganglia were dissected in oxygenated Tyrode's solution, pinned in a sylgard coated dish with minutia pin and mounted on the stage of a dissection microscope. See section 2.2.2 for the detailed protocol.

Electrophysiological recordings

To record nerve evoked compound action potentials, the preganglionic nerve was suctioned into an electrode connected to a stimulator (365R Isolator, World Precision Instruments, Sarasota, FL) and the postsynaptic nerve was suctioned into a recording electrode connected to an amplifier. See section 2.3.2.1 for more information.

I performed intracellular recording to count the number of inputs innervating an SCG neuron, and the complete protocol is outlined in section 2.3.2.2. Briefly, the postsynaptic neuron was penetrated by a sharp microelectrode connected to an amplifier used in current-clamp mode, and the presynaptic nerve was tightly suctioned in a stimulating electrode. The stimulus strength started at zero and was slowly increased until the first EPSP was recorded. The stimulus was increased until there were not more additional stepwise increases in EPSP size ($EPSP_{max}$), indicating that all innervating neurons has reached their threshold. To ensure that all EPSPs remained

subthreshold, I either injected current in the SCG neuron to hold the cell at -90 mV, or added QX314 in the recording electrode solution.

All data were recorded and analyzed offline on a Pentium II-based computer with Igor (Wavemetrics), and only cells with a resting membrane potential under -40 mV were used in my study.

Analysis of disparity

To measure the number of inputs innervating a cell, I measured the number of discrete jumps in the EPSP size between the first EPSP, evoked at the lowest stimulus strength, and EPSP max. To determine the size of each individual EPSP, I averaged 10 or more traces for each discrete jump and subtracted the average EPSP evoked by that axon and all axons of lower thresholds from the average EPSP evoked only by axons of lower threshold (see Eq. 2, section 2.3.2.3). To calculate the disparity between neurons, I divided the standard deviation, SD, of the EPSPs by the mean EPSP (see Eq. 3, section 2.3.2.3)

Adenovirus

For experiments where synaptic activity was restored for 48 hrs, an Ad-α3/Syn adenovirus was used. Rescue experiments, which restored activity for 30 days, used an Ad-α3/Ubi adenovirus (see section 2.7 for the full protocol).

Immunohistochemistry

For the detailed protocol, see section 2.8.2. Briefly, WT and α3-KO SCGs were sliced into 100 μm sections and immunostained with primary antibodies against P-4E-BP1 or 4E-BP1.

RESULTS

Disparity between preganglionic inputs increases over the first postnatal month

To determine the number of preganglionic axons innervating a single SCG neuron, I recorded the evoked EPSPs intracellularly while gradually increasing the stimulus strength to the preganglionic nerve. As each preganglionic axon reaches its threshold, it produces a discrete jump in the EPSP, therefore, as the stimulus to the preganglionic nerve increased and more axons reached their threshold, the size of the EPSPs increased in a stepwise manner. By counting the number of discrete jumps in EPSP size, I quantified the number of axonal inputs innervating a given SCG neuron. In WT mice at postnatal day 1-3 (P1-3), SCG neurons were innervated by 7-8 preganglionic axons (Figure 1A). By P8-9, SCG neurons were innervated by 5-6 axons and by one month (P28-30), SCG neurons were innervated by only 1-3 axons (Figure 1B; 1D). Given that the EPSPs increase in a stepwise manner, the EPSP produced by each individual axon can be measured by subtracting the compound EPSP by the next largest EPSP (Figures 1A, B middle panels).

During the first postnatal month, when some preganglionic axons were eliminated, 1 or 2 of the remaining inputs increased in strength. At P1-3, the EPSPs produced by individual axons were similar in size; however, at P28, as some inputs strengthened, the EPSPs produced by individual axons were more variable. I quantified the disparity in the strength of the EPSPs on each neuron in two ways. In one, I calculated the disparity index as the ratio of the standard deviation (SD) of the EPSPs evoked by each axon divided by the mean (SD/M) (Hashimoto and Kano, 2003). The disparity index was low at P1-3, suggesting that the EPSPs are nearly equal in size, but increased significantly by P28-30, reflecting the increase in strength of 1 or more inputs. The other method involved computing the difference in strength between the strongest and second strongest input expressed as a percentage of the maximum compound EPSP. At P1–P3, the difference between the strongest and the next strongest input was <5%,

whereas at P28–P30 the difference was 35% (Figure 1C). These data are consistent with previous findings on rat SCG (Purves and Lichtman, 1984) and show that SCG neurons undergo considerable elimination during the first postnatal month while one or more of the persisting inputs increases in strength.

Preganglionic axons do not refine in the absence of rapid synaptic transmission

To determine whether preganglionic axon refinement requires postsynaptic activity, I examined the innervation of SCG neurons in α3-KO mice. In α3-KO, SCG neurons are synaptically silent (Figure 2A). Therefore, to count the number of preganglionic inputs, I needed to restore synaptic activity at these silent synapses. To do this, I infected α3-KO mice with adenoviruses expressing the α3 cDNA under the control of the human synapsin (syn) promoter and recorded from SCG neurons 24-48 hrs later. SCG neurons from α3-KO mice, infected with Ad-$\alpha3_{syn}$ were innervated by 7-8 preganglionic axons at in P1-3 (Figure 2B), not statistically different from the number on P1-3 SCG neurons from WT mice (Figure 1A). In contrast, α3-KO SCG neurons at P28-30 remain innervated by 7-8 preganglionic axons (Figure 2C), unlike the number innervating WT SCG at P28-30 (Figure 1B). Interestingly, the number of preganglionic axons innervating α3-KO SCG neurons at P28-30 is not different from the number innervating SCG neurons in WT at P1-3 WT and P1-3 KO mice (Figure 2E), and the EPSPs produced by single axons were similar in size and showed little disparity (Figure 2D). These findings suggest that in the first postnatal month, synaptic activity is necessary for the elimination of redundant synapses and for the strengthening of those that persist.

In addition, P28-30 α3-KO mice have considerable morphological differences compared to their WT counterparts. P30 WT SCG neurons have 5 primary dendrites and presynaptic axons are primarily targeted to dendrites, with some synapses on the cell body. In contrast, P30 α3-KO SCG neurons have only 3 primary dendrites and significantly less dendritic growth than their WT counterparts, and innervation is largely concentrated on the SCG cell body

(Chong et al., 2018). These data suggest that postsynaptic activity is also critical for dendritic growth and presynaptic axon targeting.

Preganglionic axons refine when synaptic activity is restored in α3-KO SCG

Given that synaptic activity during the first postnatal month is required for synaptic elimination, I investigated whether restoring synaptic activity in α3-KO mice for one month could rescue refinement. To this end, I infected P30 α3-KO mice with Ad-α3 and quantified the number of preganglionic axons innervating SCG neurons at P56-64. I found that one month after infection, α3-KO SCG neurons were innervated by only 2–3 preganglionic axons (Figure 3A), similar to that of WT neurons at P60 (Figure 3B), whereas non-infected control α3-KO SCG neurons at P60 continued to be innervated by 7–8 axons (Figure 3C; 3D). This suggests that one month of synaptic activity is sufficient to induce the preganglionic axons to refine.

Hypophosphorylated 4E-BP1 is increased in α3-KO mice

Next, I investigated whether cap-dependent translation has a role in activity-dependent refinement. Synaptic activity stimulates mTOR, which in turn phosphorylates its target, 4E-BPs (Thomas and Huganir, 2004) (Figure 4A). To determine whether synaptic activity phosphorylates 4E-BP in SCG neurons, we immunostained SCG slices with antibodies against phosphorylated 4E-BP1 and total 4E-BP1, the dominant isoform of 4E-BP in the PNS. At birth, there was no significant difference in the levels of phosphorylated 4E-BP1 in α3-KO SCG neurons when compared to age-matched WT neurons (see (Chong et al., 2018)). At 1 month, however, phosphorylated 4E-BP1 in α3-KO SCG was 25%–30% less than in WT SCG (Figure 4C), while total 4E-BP1 was comparable (Figures 4B). This suggests that 4E-BPs are more active in α3-KOs compared to WTs, and that the rate of mRNA translation initiation is likely decreased.

Synaptic elimination is not significantly affected by 4E-BP1 genetic deletion

To address the role of 4E-BP1 in synaptic elimination, I investigated the effects of genetically deleting 4E-BP1 (4E-BP-KO) on synaptic refinement. Genetic deletion of 4E-BP frees up eIF4E to bind the 5' cap of mRNA and deregulates the initiation of cap-dependent translation. A western blot analysis confirmed that there is no 4E-BP protein in the SCG of 4E-BP-KO mice (Figure 5A), yet this does not affect synaptic activity in SCG ganglia (Figure 5B). At P1-3, SCG neurons in 4E-BP-KO mice are innervated by 7-9 preganglionic axons, not significantly different from the number innervating SCG neurons in WT pups (Figure 5C). By P8-9, SCG neurons in 4E-BP-KO mice are innervated by ~4 presynaptic axons, suggesting that synaptic elimination is accelerated in 4E-BP-KO mice compared to WTs that receive 5-6 inputs (Figure 5D). By P28-30, 4E-BP-KO mice are innervated by 2-3 inputs (Figure 5E). At P28-30 the number of innervating axons and the disparity index of 4E-BP-KO mice was not statistically different from that in WTs, suggesting that 4E-BP1 genetic deletion does not significantly affect synaptic refinement.

Genetic deletion of 4E-BP1 enables synaptic elimination in α3-KO SCG neurons

Next, I investigated the effects of removing 4E-BP1 in α3-KO mice (α3/4E-BP-DKO). First, I tested whether α3/4E-BP-DKO mice have rapid synaptic transmission; I recorded the CAP and found that there was no change from baseline, suggesting that α3/4E-BP-DKOs have no rapid synaptic transmission (Figure 6A). To determine whether removing 4E-BP1 has an effect on synaptic refinement in the absence of synaptic activity, I recorded from SCG neurons in P28-30 α3/4E-BP-DKO mice that were infected with Ad-α3 24-48 hrs earlier. Unexpectedly, I found that SCG neurons from P28-30 α3/4E-BP-DKO mice were innervated by 1-3 presynaptic axons (Figure 6B), not different from the number of preganglionic axons innervating SCG neurons in aged-matched WT mice (Figure 6B, 6C). This surprising result suggests that in the absence of 4E-BPs, synaptic

refinement can take place independently of synaptic activity. These findings strongly suggest that under normal circumstances, synaptic-dependent competition acts through 4E-BP1. Interestingly, there does not appear to be much disparity between the EPSPs on α3/4E-BP-DKO SCG neurons (Figure 6D), suggesting that in the absence of synaptic activity, there are no dominant axons. This indicates that while removing 4E-BP1 has little effect on elimination in WTs, it is sufficient to drive elimination in the absence of synaptic activity.

DISCUSSION

Taken together, my data shows that postsynaptic activity is critical for synaptic refinement to occur. In α3-KO mice, presynaptic axons fail to refine and accumulate on the cell body. Restoring synaptic activity is sufficient to restore synaptic elimination and corrects axon targeting. This work clearly demonstrates that synaptic activity is necessary and sufficient for synaptic refinement in the mouse SCG. In order to understand if there is a critical period after which SCG synapses can no longer refine, we would need to repeat the rescue experiments at different ages; based on preliminary data, refinement still occurred in mice up to 8 months of age (data not shown).

A key finding from this study is that in the absence of synaptic activity, refinement can still occur when 4E-BP1 is inactivated (or genetically removed). However, contrary to WTs, α3/4E-BP-DKOs do not exhibit the differential strengthening of one input. This finding suggests that synaptic activity acts through 4E-BP1 and cap-dependent translation to induce synaptic elimination, but other activity-dependent mechanisms must be involved in the selective strengthening of synaptic inputs during development. Moreover, these findings challenge the notion that the selective strengthening of one presynaptic input, through activity-dependent competition, is the impetus for the elimination of the other, weaker synapses (Katz and Shatz, 1996; Kano and Hashimoto, 2009).

The fact that α3/4E-BP-DKOs undergo seemingly normal refinement raises a perplexing question: are the resulting circuits functional? If one were to

restore synaptic activity, do these circuits support physiological functions? α3-KO mice have disrupted autonomic functions, including gastrointestinal dysmotility, dilated and poorly responsive pupils, decreased lacrimation, reduced heart rate variability, dilated bladder, reduced levels of plasma catecholamines, and hypotension (Vernino et al., 2004; Vernino et al., 2008). We could answer this question by restoring synaptic activity in α3/4E-BP-DKO mice later on in life, and testing whether their autonomic function is restored. The answer to this question would reveal whether synaptic activity is necessary to build functional circuits that support physiological functions.

Our immunostaining experiments strongly support a model where during development synaptic activity leads to the hyperphosphorylation and inactivation of 4E-BP1 which enhances cap-dependent translation of key mRNA necessary for synaptic refinement. In synaptically silent α3-KO SCGs, the levels of active, hypophosphorylated 4E-BP1 increased, suggesting that eIF4E is inhibited and cap-dependent translation is decreased.

While these data imply that activity-dependent inactivation of 4E-BP1 is critical for elimination to occur, my work does not directly test this. One way to confirm this theory would be to understand the impact of increasing 4E-BP1 activity at active synapses, either by overexpressing a constitutively active form of 4E-BP1 or by blocking mTOR with rapamycin. If inactivating 4E-BP1 is a critical step, then increasing its activity should adversely affect synaptic elimination in otherwise functional synapses. More work is necessary to directly test the role of 4E-BP1 at active SCG synapses. Likewise, the key molecules involved in refinement remain largely unknown, and more research will be necessary to elucidate their identities.

Finally, one cannot ignore the fact that SCG neurons are part of a circuit receiving afferent innervation from the axons of preganglionic neurons in the intermediate horn of the spinal cord and synapsing onto their targets, which include arterial smooth muscles and sweat glands in the face and deep targets such as the iris muscle and pineal gland in the skull (Moller and Baeres, 2002;

Glebova and Ginty, 2005). Targets of SCG neurons do not receive any other excitatory innervation; therefore, they are also synaptically silent. Previous work shows that activity-dependent retrograde signals are involved in regulating presynaptic SCG inputs (Krishnaswamy and Cooper, 2009). Given that the SCG-target synapses are also silent, SCG neurons themselves are not receiving activity-dependent retrograde signals. It would be relevant to understand how silencing the targets of the SCG neurons impacts SCG neuronal development. One way to test this would be to electrically or optogenetically stimulate the downstream targets of α3-KO SCG neurons and see how activity in the targets, and examine how it affects refinement and morphology of α3-KO SCG neurons.

CONCLUSION

In summary, this study shows that 4E-BP-regulated mechanisms are involved in the growth of dendrites, the targeting of synapses, and the refinement of preganglionic axons in the absence of rapid synaptic transmission. Our results suggest that, in the absence of synaptic activity, where synaptic refinement is curtailed, genetic removal of 4E-BP activates a molecular program that can compensate for the lack of activity-dependent mechanisms that normally direct synaptic reorganization and refinement of presynaptic inputs.

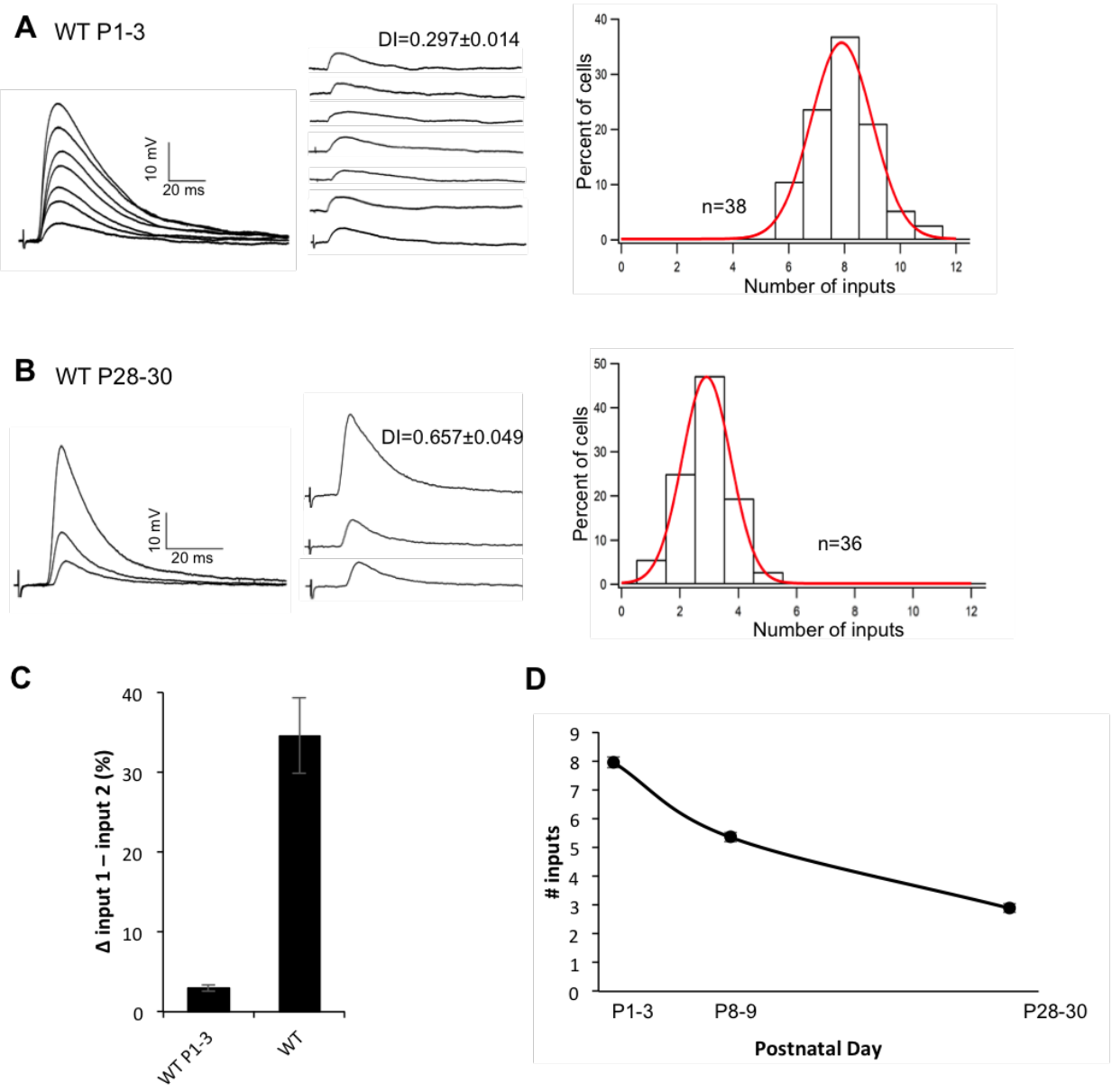

Figure 1 Presynaptic inputs refine over the first postnatal month. **A** Left: Sample graded EPSPs recorded from an SCG neuron from P1-3 WT mice. Middle: Example traces of the individual EPSPs from each input innervating a P1-3 SCG neuron; the disparity index (DI) is 0.297±0.014. Right: Gaussian distribution of the number of inputs innervating a P1-3 SCG neuron. On average, P1-3 SCG neurons are innervated by 7-8 preganglionic axons (n=38). **B** Left: Sample graded EPSPs recorded from an SCG neuron from P28-30 WT mice. Middle: Disparity between inputs increases by P28-30 (DI=0.657±0.049). Right: P28-30 SCG neurons are innervated by 1-3 inputs (n=36) **C** The difference between the strongest input and the second strongest input is <5% at P1–P3 and increases to 35% by P28–P30. **D** The average number of inputs innervating a WT SCG neuron decreases overtime; at P1-3 they are innervated by 7-8 inputs, at P8-9, 5-6 inputs (n=37) and at P28-30, 1-3 axons.

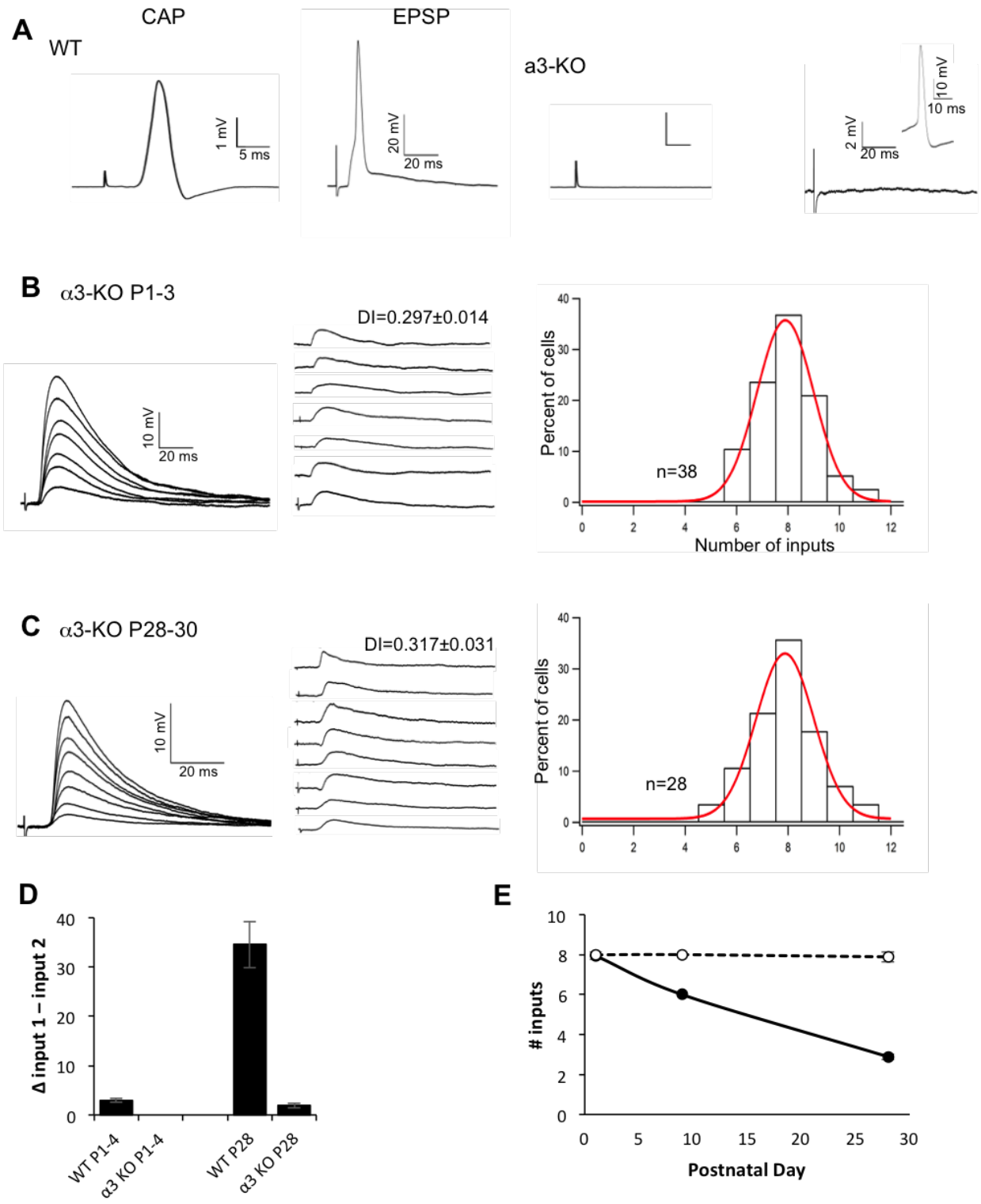
A
CAP
EPSP
WT
1 mV
5 ms
20 mV
20 ms
a3-KO
10 mV
10 ms
2 mV
20 ms
B
α3-KO P1-3
10 mV
20 ms
DI=0.297±0.014
Percent of cells
n=38
Number of inputs
C
α3-KO P28-30
10 mV
20 ms
DI=0.317±0.031
Percent of cells
n=28
D
Δ input 1 – input 2
WT P1-4
α3 KO P1-4
WT P28
α3 KO P28
E
inputs
Postnatal Day

Figure 2 Presynaptic axons in α3-KO SCGs fail to refine in the absence of synaptic activity. **A** Compound action potential (CAP) and evoked postsynaptic potential (EPSP) for a WT (left) and an α3-KO mouse (right). **B** Left: Sample graded EPSPs recorded from an SCG neuron from a P1-3 α3-KO mouse in which activity was restored for 24-48hrs with Ad-α3. Middle: Disparity between inputs increases by P28-30 (DI=0.297±0.014). Right: P28-30 are innervated by 1-3 inputs (n=38). **C** Left: Graded EPSPs from the SCG of a P28-30 α3-KO mouse in which activity was restored for 24-48hrs. Middle: Disparity between inputs increases by P28-30 (DI=0.317±0.031). Right: P28-30 are innervated by 1-3 inputs (n=28). **D** The disparity between inputs is comparable between P28-30 α3-KOs and P1-3 WTs and is significantly lower than in P28-30 WTs. **E** In α3-KOs, the number of inputs innervating SCG neurons does not change between P1-3, P7-8, and P28-30.

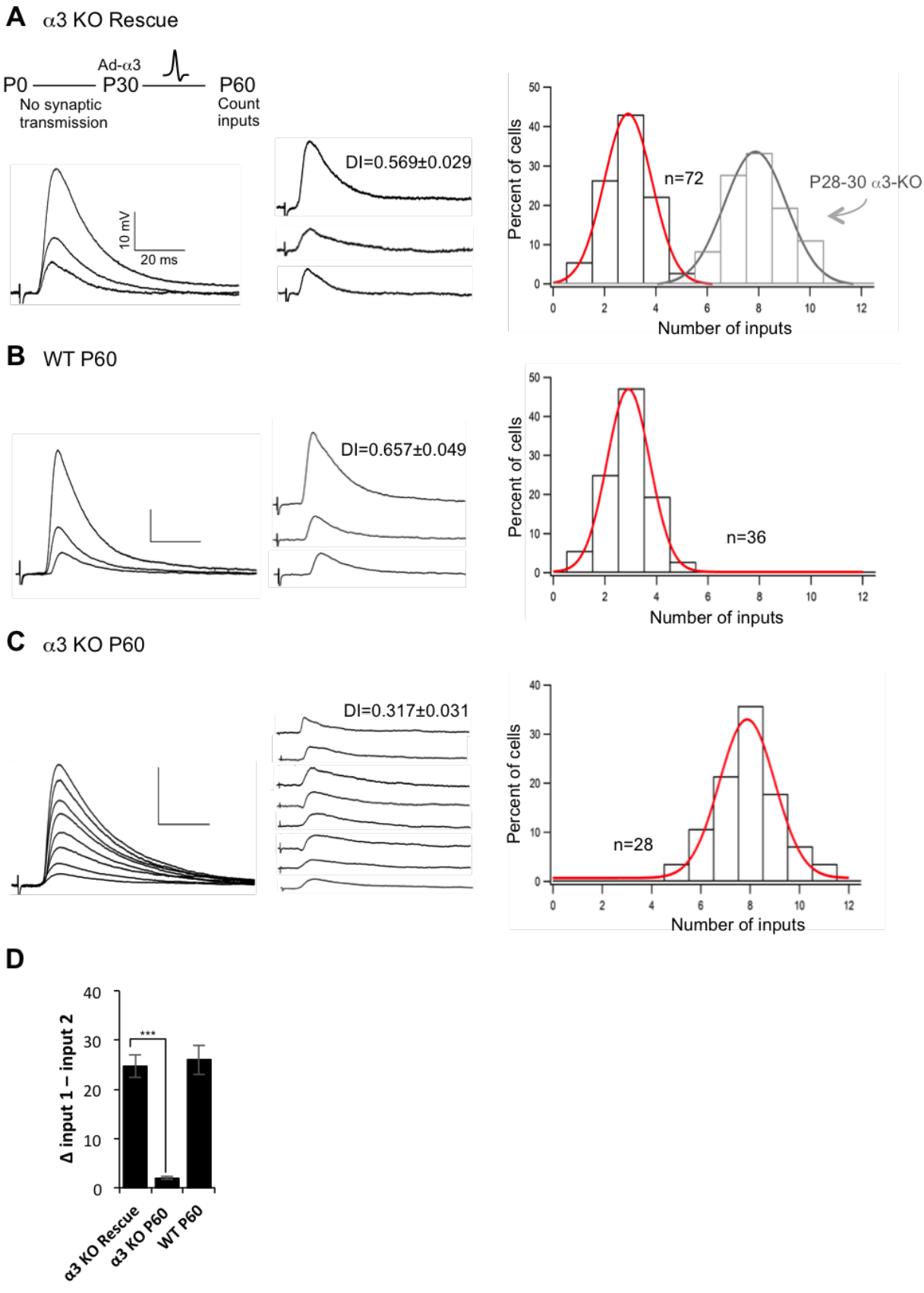
A α3 KO Rescue
Ad-α3
P0
P30
P60
No synaptic transmission
Count inputs
10 mV
20 ms
DI=0.569±0.029
Percent of cells
n=72
P28-30 α3-KO
Number of inputs
B WT P60
DI=0.657±0.049
Percent of cells
n=36
Number of inputs
C α3 KO P60
DI=0.317±0.031
Percent of cells
n=28
Number of inputs
D
Δ input 1 – input 2
***
α3 KO Rescue
α3 KO P60
WT P60

Figure 3 Restoring synaptic activity in α3-KO SCG allows preganglionic axons to refine. **A** Left: Graded EPSPs from the SCG of a P54-60 α3-KO mouse in which activity was restored for one month. Middle: Samples EPSPs from individual inputs; one month of activity increases the disparity between inputs (DI=0.569±0.029). Right: P28-30 α3-KO SCG with activity restored for one month are innervated by 1-3 inputs (n=72, red trace), significantly fewer than before activity was restored (grey trace). **B** Left: Graded EPSPs from the SCG of a P54-60 WT mouse. Middle: At P54-60, the disparity between inputs is comparable to P54-60 α3-KO SCG in which activity was restored for one month (DI=0.657±0.049). Right: P54-60 SCG neurons are innervated by 1-3 inputs (n=36). **C** Left: Sample graded EPSPs recorded from an SCG neuron from a P54-60 α3-KO mouse in which activity was restored for 24-48hrs with Ad-α3. Non-infected control α3-KO SCG neurons at P54-60 have a disparity comparable to P1-3 WTs (DI=0317±0.031; middle) and continue to be innervated by 7–8 axons (n=28; right). **D** The difference between the largest EPSP and the second-largest EPSP is comparable in α3-KO with synaptic activity restored for one month and P60 WT mice, and is significantly larger than P60 α3-KO mice in which activity was not restored.

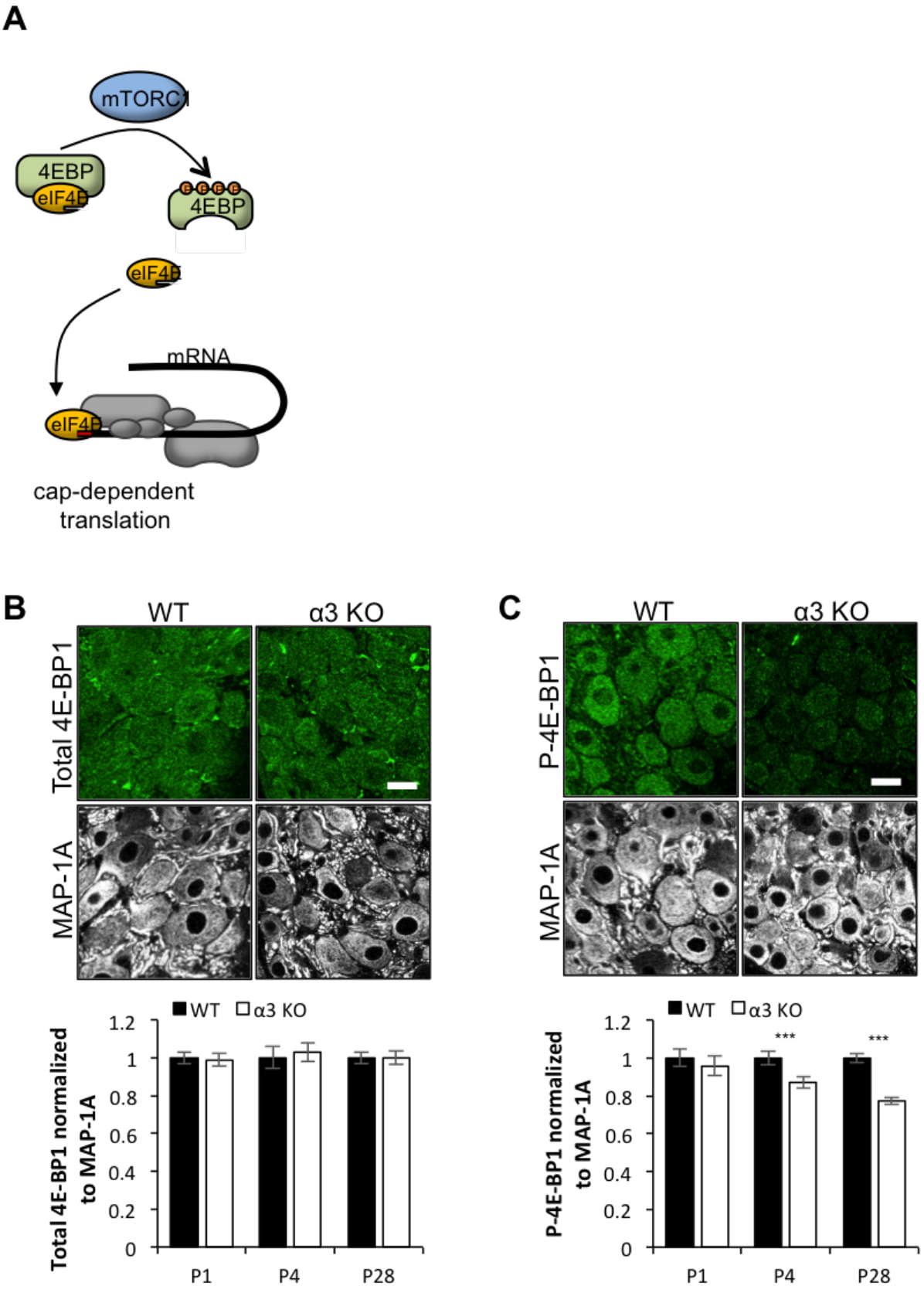
A
mTORC1
4EBP
eIF4E
4EBP
eIF4E
mRNA
eIF4E
cap-dependent
translation
B
WT
α3 KO
Total 4E-BP1
MAP-1A
WT
α3 KO
Total 4E-BP1 normalized to MAP-1A
1.2
1
0.8
0.6
0.4
0.2
0
P1
P4
P28
C
WT
α3 KO
P-4E-BP1
MAP-1A
WT
α3 KO
P-4E-BP1 normalized to MAP-1A
***
***
1.2
1
0.8
0.6
0.4
0.2
0
P1
P4
P28

Figure 4 Hypophosphorylated 4E-BP1 is increased in α3-KO mice. **A** Cartoon of the mTOR pathway; synaptic activity stimulates mTOR, which in turn phosphorylates its target, 4E-BPs. **B** At P28-30 months, total 4E-BP1 is at comparable levels between WT and α3-KO SCG. The bar graph shows levels of total 4E-BP1 normalized to MAP-1A at P1, P4, and P30. **C** Phosphorylated 4E-BP1 in α3-KO SCG is 25%–30% less than in WT SCG. The bar graph shows levels of phospho-4E-BP1 normalized to MAP-1A at P1, P4, and P30.

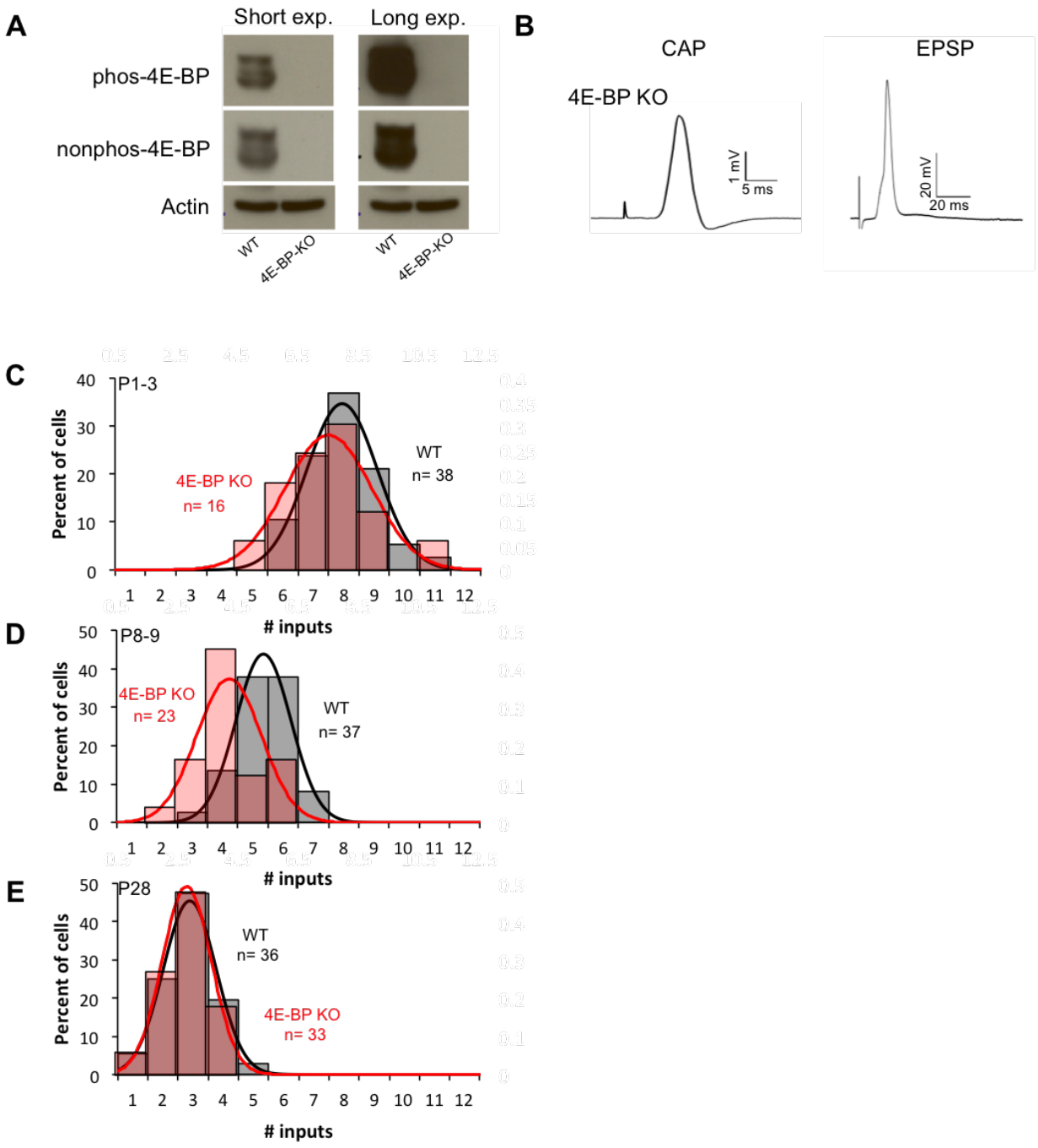

Figure 5 4E-BP1 KOs have relatively normal synaptic elimination. **A** A western blot analysis shows 4E-BP protein in the SCG WTs but not in SCG 4E-BP-KO mice. **B** Sample traces of CAP and EPSPs in 4E-BP-KOs. **C** At P1-3, SCG neurons in 4E-BP-KO mice are innervated by 7-9 preganglionic axons (n=16; red), similar to age-matched WTs (grey). **D** By P8-9, SCG neurons in 4E-BP-KO mice are innervated by ~4 presynaptic axons (n=23; red) compared to WTs that receive 5-6 inputs (grey). **E** By P28-30, 4E-BP-KO mice are innervated by 2-3 inputs (n=33 ;red), similar to age-matched WTs (grey).

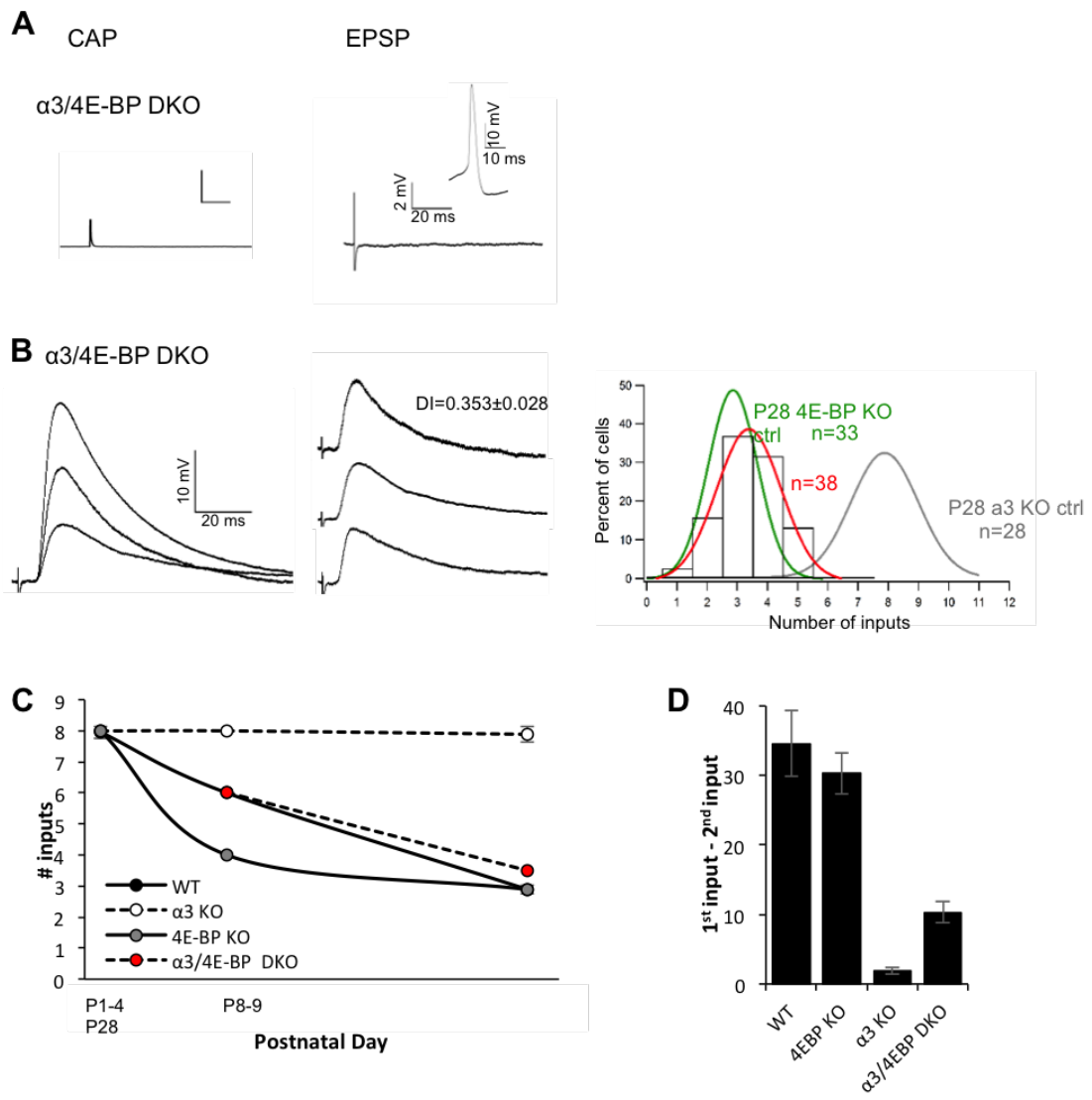

Figure 6 Knocking out 4E-BP1 enables synaptic elimination in α3-KO SCG neurons. **A** Sample traces of CAP and EPSPs in α3/4E-BP-DKO mice. **B** Sample graded EPSPs in an α3/4E-BP-DKO neuron with synaptic activity restored for 48hrs (left). Individual EPSPs from presynaptic inputs show little disparity (DI=0.353±0.028) (middle). SCG neurons from P28-30 α3/4E-BP-DKO mice are innervated by 1-3 presynaptic axons (n=38, red trace), comparable to that in P28 4E-BP-KOs (green trace) and significantly different from P28 α3-KOs (grey trace) (right). **C** The number of inputs innervating α3/4E-BP-DKO is comparable to WTs at P1-3, P8-9, and P28-30. **D** There is little disparity between the EPSPs on P28-30 α3/4E-BP-DKO SCG neurons compared to age-matched WTs and 4E-BP-KOs.

CHAPTER 6

DISCUSSION

4E-BP2 is critical for PF-PC LTD

I show that in 4E-BP2-KO mice, stimuli that normally induce LTD of PF-PC synapses instead produce LTP. This result demonstrates that 4E-BP2 is critical for PF-PC synapses to undergo LTD. The question is, how does 4E-BP2 regulate LTD? Since I used a mouse model with a global 4E-BP2 genetic deletion, one possibility is that compensatory developmental effects lead to the change in gain. I examined the cerebellar and PC morphology, and found no significant difference between WTs and 4E-BP2-KOs. Likewise, PCs in 4E-BP2-KOs received normal excitatory innervation and their intrinsic firing frequency was comparable to WTs. I did not examine inhibitory innervation of PCs, but I pharmacologically blocked GABAergic synaptic transmission in both WTs and KOs during my LTD experiments. Therefore, it is unlikely that gross structural changes occur in 4E-BP2 KO mice that could account for the change in gain that I detected. It is possible, however, that subtle difference caused by a global genetic deletion 4E-BP went undetected in my experiments. Therefore, in future experiments it would be preferable to use an animal model in which 4E-BP2 genetic deletion is temporally controlled and cell-specific.

One possibility is that in 4E-BP2-KOs, calcium signaling is decreased and does not reach the threshold necessary to induce LTD and that in low calcium, PF stimulation instead induces LTP (Coesman et al., 2004). To examine this possibility, I performed calcium imaging experiments while delivering depolarizing voltage steps to PCs and found no difference between WTs and 4E-BP2-KOs. This indicates that calcium influx through voltage-gated calcium channels during my depolarizing voltage steps is unchanged. Nevertheless, I opted for a second LTD inducing protocol, one in which the unpaired-PF stimulation did not induce LTP. Using this protocol, I showed that 4E-BP2-KOs still undergo LTP instead of

LTD. Thus, the change in gain is not likely to be caused by defects in calcium signaling.

It's been previously established that PF-PC LTD requires acute translation (Linden 1996; Karachot et al., 2001), however it was never directly examined whether cap-dependent translation regulation was involved. Moreover, given that genetically removing 4E-BP2 dysregulates translation (Gkogkas et al., 2013), I wondered whether PF-PC LTD in 4E-BP2-KOs still depends on acute mRNA translation. To test these questions, I blocked cap-dependent translation initiation using an inhibitor that disrupts the formation of the cap-initiation complex, and found that in both WT controls and 4E-BP2-KOs, blocking cap-dependent translation initiation prevents any change in PF-PC synaptic strength induced by the LTD protocol. This indicates that PF-PC LTD requires acute cap-dependent translation regulation, and that synaptic plasticity at the PF-PC synapses require acute translation even in the absence of 4E-BP2. Additional experiments are required to identify the acutely translated protein(s) involved in the plasticity of PF synapses.

Increased PP2A activity leads to the change in gain from LTD to LTP in 4E-BP2-KOs

My pharmacological experiments indicate that in 4E-BP2-KOs, the change in gain from LTD to LTP involves enhanced PP2A activity. This suggests that under normal conditions, inhibition of PP2A is a critical step for LTD to occur and is consistent with previous work that showed that shifts in the kinase/phosphatase balance in PCs can alter the gain of the plasticity (Hansel et al., 2006; Schonewille et al., 2010).

My work also suggests a novel role for cap-dependent translation regulation in the modulation of PP2A activity, however the link between cap-dependent translation regulation and PP2A activity is unclear. Relevantly, PP2A is a complex composed of catalytic, regulatory and structural subunits that are each regulated by activators and inhibitors (Kaur and Westermarck, 2016). Given

that in 4E-BP2-KOs, the change in gain requires acute cap-dependent translation, LTD induction may acutely increase the concentration of one of the PP2A subunits or of one of it's activators, resulting in enhanced PP2A activity. Acutely blocking the translation of PP2A mRNA or that of it's regulatory proteins, using mRNA interference for example, would be one way to test whether their acute translation is involved in the change from LTD to LTP. An alternative possibility is that under baseline conditions, protein levels in 4E-BP mutants are already altered and primed to activate PP2A. Preliminary western blot analysis supports this idea: we detected more of the PP2A activator, PTPA, and less of the inhibitor PPME1 in the cerebella of 4E-BP2-KOs compared to controls. While these changes do not appear to affect PF-PC synapses under basal conditions, they could affect the kinase/phosphatase balance during LTD induction, giving rise to LTP instead of LTD.

While the exact mechanism by which genetic deletion of 4E-BP2 enhances PP2A activity is unclear, it appears that removing 4E-BP2 increases PP2A activation. This in turn shifts the kinase/phosphatase balance, such that kinase activity is suppressed and the dephosphorylation of AMPARs is favored, leading to their maintenance and expression on the postsynaptic membrane, producing LTP. Whether PP2A activity is enhanced through acute translation or is already enhanced at baseline remains to be tested.

LTD in motor learning

Several studies from the past two decades reinforced the hypothesis that PF-PC LTD underlies motor learning by showing that pharmacological treatments or genetic modifications in mice that disrupted PF-PC LTD were associated with impairments in VOR and/or eyeblink conditioning (Yuzaki, 2013), however there are some contradictory findings (Schonewille et al., 2011).

The most convincing correlations between PF-PC LTD and motor learning come from mice in which the gene deletions were restricted to Purkinje cells, achieved using the PC-specific promoter L7. Examples include the PC-specific

mGluR1 deletion, which prevents LTD and causes motor coordination deficits (Ichise et al., 2000), as well as conditional knockout mice with a PC-specific ablation of cGMP-dependent kinase I (PKGI), which show reduced LTD and an impairment of VOR gain adaptation (Feil et al., 2003). Consistent with these findings, mutations in GluRδ2, the orphan glutamate receptor that is predominantly expressed in PCs, inhibit LTD (Kashiwabuchi et al., 1995) and impair eyeblink conditioning (Kishimoto et al., 2001). Both LTD and eyeblink conditioning are completely restored by Purkinje-cell-specific expression of the wild-type GluRδ2 transgene (Kakegawa et al., 2008). Taken together, these data strongly suggest that PF-PC LTD underlies motor learning. My data are consistent with these findings; I found that in the absence of the negative regulation of cap-dependent translation by 4E-BP2, PC-PF LTD and VOR were both impaired. Moreover, my results showed that preventing PP2A with the pharmacological inhibitor Fostriecin was sufficient to restore LTD in 4E-BP2-KOs. It would be interesting to test whether VOR is restored in animals treated with a PP2A inhibitor.

Controversy over the role of PF–LTD in motor learning arose from recent reports that have examined several lines of genetically modified mice (Steinberg et al., 2006; Schonewille et al., 2010; Schonewille et al., 2011). Mutations that affect AMPA receptor endocytosis prevent PF-PC LTD but have no effect on motor learning (Steinberg et al., 2006). Conversely, in mice with a Purkinje cell-specific deletion of calcineurin (PP2B-KO), PF–LTD in cerebellar slices is normal but eyeblink conditioning and VOR adaptation are impaired (Schonewille et al., 2010). These findings suggest that PF–LTD is not required for cerebellar motor learning (Schonewille et al., 2011). Since Schonewille et al. (2010) found that PF-PC LTP was disrupted in PP2B-KO mice, it is likely that PF-PC LTP is involved in motor learning. Studying whether PF-PC LTP is affected in other mouse models with disrupted motor learning would clarify this issue. On the other hand, my data show that in 4E-BP2-KO mice, the VOR is impaired but PF-PC LTP is intact.

It's important to acknowledge the caveat that genetic deletion of a gene since birth may give rise to compensatory mechanisms that confuse the link between PF-PC LTD and motor learning. For example, cerebellar slices from PP2B-KO mice have intact PF-PC LTD, in contrast, acute blockade of PP2B by including calcineurin inhibitory peptides in the patch pipette completely blocks LTD induction in wild-type cerebellar slices (Fujiwara et al., 2007; Nomura et al., 2012). Since PP2B activity is involved in regulating clathrin–dependent endocytosis (Biou et al., 2008), the LTD observed in PP2B-KOs may use other compensatory mechanisms. This example illustrates the importance of using temporally specific ablation of genes to clarify the role of PF-PC LTD and motor learning. Along these lines, it may be relevant to test the role of 4E-BP2 in adult cerebellar plasticity and motor learning using conditional 4E-BP2 knockout mice, as this would rule out any developmental compensatory mechanisms. Given that 4E-BP2-KO mice are expected to show enhanced mRNA translation, temporal genetic deletion would also circumvent the potential caveat that a change in the baseline levels of protein affects secondary signaling in Purkinje cells. While my results support a link between PF-PC LTD and motor learning, more work is needed to understand fully the link between secondary signaling, PF-PC plasticity and motor learning.

How does acute mRNA translation give rise selectively to LTP or LTD at PF-PC synapses?

Based on my results, PF-PC LTD (see Chapter 3) and PF-PC LTP (preliminary data) require acute cap-dependent translation. The question is, how can LTP and LTD both require acute mRNA translation, yet give rise to opposite changes in synaptic strength? Do LTP and LTD require the translation of distinct mRNAs? If so, how are the correct mRNA selectively translated during LTP or LTD induction, respectively? Or, is translation simply permissive for plasticity to occur: could it be that translation is necessary for plasticity to take place but does not in and of itself affect the direction of the change in gain? If distinct mRNA are translated to give rise to LTP and LTD, then 5'UTR of mRNA or one of the many

regulators of translation could be involved in the selective translation of the correct mRNA.

5' UTRs

mRNAs generally contain secondary structures in their 5' untranslated region (5'UTR) that may be involved in conferring specificity to translational regulation. Excessive secondary structure in the 5'UTR of mRNAs can block access to the initiation site (AUG) and negatively influence translation. Previous results showed that mTOR activity influences translation of specific genes based on the complexity of their 5'UTR and that mRNA with the most complex 5'UTRs are most sensitive to changes in mTOR activity (Penney et al., 2012). This effect likely involves the cap-binding complex eIF4F, which unwinds and stabilizes the 5'UTR (Sonenberg, 1994; Ma and Blenis, 2009).

In the cerebellum, LTD entails higher levels of synaptic activation (PF and CF co-stimulation) and higher intracellular calcium compared to LTP (PF stimulation alone). As reviewed in Chapter 1.2.1, synaptic activity is linked to mTOR activation and cap-dependent translation initiation. If LTP and LTD differentially activate mTOR, then this may also affect which mRNA, based on their 5'UTR structures, get translated.

Other regulators of translation

In addition to 4E-BP, there are several regulators of translation that could be involved in selectively translating target mRNA at PF-PC synapses during synaptic plasticity. These include eIF2α, elongation factors, and FMRP, which have also been shown to critically regulate synaptic plasticity in the hippocampus (Huber et al., 2002; Costa-Mattioli et al., 2005; Hou et al., 2006; Nosyreva and Huber, 2006; Park et al., 2008).

Briefly, eIF2α influences which start codon and open reading frame is read by the translation machinery. Phosphorylation of eIF2α impairs general translation, but it paradoxically results in upregulation of translation of a subset of

mRNAs that contain upstream open reading frames (Chesnokova et al., 2017). Given that eIF2α dephosphorylation is activity-dependent (Takei et al., 2001; Costa-Mattioli et al., 2005), it could bias which target mRNA is/are translated during LTP or LTD.

Similarly, the phosphorylation of eEF2 by the eEF2 kinase is a key regulatory step in translation elongation and occurs in an activity-dependent manner (Piccoli et al., 2007). While phosphorylation of eEF2 decreases general translation, it promotes the translation of specific, poorly translated mRNAs (Brendler et al., 1981; Godefroy-Colburn and Thach, 1981; Walden et al., 1981; Scheetz et al., 2000; Chotiner et al., 2003; Park et al., 2008). In eEF2 kinase knockout mice, where eEF2 is constitutively active and promotes elongation, mRNA translation is altered and mGluR-LTD is impaired (Park et al., 2008). These findings demonstrate that elongation factors can be an effective mechanism to control translation of specific mRNAs, ultimately influencing synaptic plasticity. However, their involvement in PF-PC synaptic plasticity remains to be tested.

Finally, FMRP inhibits the translation of a subset of mRNAs that encode synapse-specific genes (Darnell et al., 2001). Genetic deletion of the FMRP gene (Fmr1-KO) enhances the expression of synapse-specific genes (Brown et al., 2001; Garber et al., 2006) and enhances mGluR-LTD (Huber et al., 2002; Nosyreva and Huber, 2006; Banko et al., 2007). This suggests that FMRP actively represses the translation of select mRNA and provides an alternate molecular mechanism by which select pools of mRNA may be selected in response to LTP or LTD inducing stimuli.

While the involvement of eIF2α, eEF2 and FMRP remains to be tested in the cerebellum, they provide alternate molecular mechanisms by which LTD and LTP could involve the differential regulation of target mRNA in response to the respective induction protocols.

Potential link to Autism Spectrum Disorder

Recent evidence suggests a role for the cerebellum (Hansel, 2019) and for cap-dependent translation regulation (Gkogkas et al., 2013; Gkogkas and Sonenberg, 2013) in Autism Spectrum Disorder (ASD). My results may have relevant implications for the role of 4E-BPs and the cerebellum in ASD. Briefly, ASD is a neurodevelopmental disorder characterized by difficulties with social interaction and communication, and by restricted and repetitive behavior (Fombonne, 2009). While ASD is believed to have a genetic basis, the specific molecular mechanisms remain largely unclear and there are no reliable tools for diagnosis or intervention. Therefore, a better understanding of ASD is paramount for designing effective therapies.

Autistic-like behaviors in 4E-BP2-KO mice

Interestingly, mice with a deletion in the 4E-BP2 gene (4E-BP2-KO) exhibit autistic-like behaviors (Gkogkas et al., 2013). Pharmacologically blocking cap-dependent translation initiation in 4E-BP2-KOs *in vivo* completely reversed the autistic-like behaviors in 4E-BP2-KO mice (Gkogkas et al., 2013). These data provide compelling evidence that dysregulation of cap-dependent translation is an underlying cause of austistic-like behavioural abnormalities in 4E-BP2-KO mice.

Moreover, Gkogkas and colleagues found that hippocampal neurons in 4E-BP2-KO mice receive proportionally more synaptic excitation than inhibition but that pharmacologically blocking cap-dependent translation initiation with 4EGI-1 restores normal excitation/inhibition (E/I) balance and rescues behavior. These findings support the hypothesis that changes the E/I balance results in aberrant information processing and may ultimately lead to ASD (Rubenstein and Merzenich, 2003; Uhlhaas and Singer, 2012). One caveat is that the excitatory and inhibitory innervation of 4E-BP2-KO neurons was tested in hippocampal slice cultures and the excitatory/inhibitory ratio was measured by analyzing spontaneous miniature postsynaptic potentials. Therefore, it is unclear if these

findings reflect evoked activity in the intact hippocampus. Moreover, it was previously shown that hippocampal E-LTP is enhanced and that L-LTP is abolished in 4E-BP2-KOs (Banko et al., 2005), and it is unclear if alterations in long term plasticity are linked to behavioral abnormalities.

The study by Gkogkas et al. links dysfunction in hippocampus with ASD-like behaviors in 4E-BP2-KOs. However, one cannot rule out the possibility that ASD-like behavioral deficits in 4E-BP2-KOs are, at least in part, due to abnormalities outside of the hippocampus. My work clearly demonstrates that 4E-BP2-KOs have disrupted PF-PC LTD and suggests that alterations in PF-PC synaptic plasticity may be involved in ASD pathology. My work also supports previous findings that suggest that cerebellar dysfunctions are involved in ASD, which I discuss in more detail below. In order to better understand the involvement of the cerebellum in ASD, however, it would be useful to generate a mouse model in which 4E-BP2 genetic deletion is restricted to PCs and test these mutants for ASD-related behaviors. Likewise, it would be interesting to examine how rescuing LTD of PF-PC synapses, for example via PP2A pharmacological inhibition, affect ASD-like behaviors in 4E-BP2-KO mice.

Evidence for a role for the cerebellum in ASD

ASD is often associated with abnormalities in cerebellar morphology (Fatemi et al., 2012), and functional magnetic resonance imaging has revealed differences in the functional connectivity and the activation of cerebellar circuits in patients with ASD (Mostofsky et al., 2009). Mutations that give rise to ASD in humans also lead to autistic-like behaviors in mouse models, and these have been leveraged to gain a better understanding of the role of the cerebellum in ASD. One compelling study used mice in which tuberous sclerosis 1 (Tsc1) is deleted only in Purkinje cells (Tsc1-L7-KO). In humans, a mutation in Tsc1 is associated with autism comorbidity. Tsc1-L7-KO mice exhibit autism-resembling social deficits (Tsai et al., 2012). This study strongly supports the idea that the cerebellum is involved in ASD, since the Tsc1 genetic deletion was restricted to PCs. Interestingly, Tsc1-L7-KO mice have deficits in motor learning (Tsai et al.,

2012), suggesting that PF-PC LTD may also be affected, however this was not tested.

Interestingly, Tsc1 is an upstream negative regulator of mTORC1 (see Chapter 1.2.1) suggesting that in PCs of Tsc1-L7-KO mice, the level of phosphorylated, inactive 4E-BP is increased. Additionally, genetic mutations in other regulators of mTORC1, such as AKT serine/threonine kinase (AKT), phosphatase and tensin homolog (PTEN), and Raf-1 proto-oncogene, serine/threonine kinase (Raf) (see Chapter 1.2.2, Figure 2), are linked with ASD in humans. Taken together with my results, these data hint at the possibility that altered 4E-BP activity and cap-dependent translation regulation are more broadly involved in ASD. However, more work will be necessary to test this theory, and examine whether 4E-BP and cap-dependent translation in PCs is directly involved.

Given that the role of the cerebellum in autism is relatively novel, we know very little about how the synaptic organization, wiring and plasticity of the cerebellum is affected in animal models of ASD (Napoli et al., 2008; Baudouin et al., 2012; Grant, 2012; O'Roak et al., 2012; Zhou and Parada, 2012; Ha et al., 2016; Peter et al., 2016). However, previous work suggests that defects in cerebellar plasticity correlate with ASD-related behavioural defects (Koekkoek et al., 2005; Baudouin et al., 2012; Piochon et al., 2014). In a mouse model for the human 15q11-13 duplication (patDp/+), one of the most frequently observed genetic aberrations in autism, PF-PC LTD is occluded and delayed eyeblink conditioning is impaired. patDp/+ mice also exhibit incomplete developmental elimination of CFs and innervation from multiple CFs persists in adults. These findings point to deficits in synaptic plasticity and pruning as potential causes for motor problems and abnormal circuit development in autism (Piochon et al., 2014).

Similar results were observed in a mouse model of Fragile X syndrome. Genetic mutations of the Fragile X mental retardation 1 gene, Fmr1, causes Fragile X syndrome in humans and has high comorbidity with ASD. Mice with a

Purkinje cell-specific deletion of the Fragile X mental retardation 1 gene Fmr1 (L7-Fmr1) not only exhibit autistic-like behavioral impairments, but also have impaired delay eyeblink conditioning, and enhanced LTD (Koekkoek et al., 2005). No other structural changes or changes in excitatory innervation were found in L7-Fmr1 mice. These results strongly suggest that impaired cerebellar plasticity correlates with ASD.

My findings are consistent with results from patDp/+ and L7-Fmr1 mutants; in 4E-BP2-KO mice, both PF-PC LTD and motor learning are impaired. Can these cerebellar abnormalities explain specific ASD symptoms? It has been suggested that the cerebellum not only controls movement, but is also involved in cognitive functions and might play a role in higher-order behaviours (Schmahmann, 1991, 2004; Buckner, 2013). My results, taken together with those from the patDp/+ and the L7-Fmr1 studies suggest that cerebellar dysfunction may contribute to non-motor symptoms of ASD as well. In order to better understand the role of the cerebellum in autism, we need to test cerebellar plasticity and connectivity in all mouse models of ASD.

Eyeblink conditioning as a diagnostic tool for ASD

Although motor problems are not a core issue in autism, there are important advantages to studying motor behaviour in animal models of ASD. Whereas social behaviours are very different between humans and mice, motor learning, particularly eyeblink conditioning, is evolutionarily conserved (Fanselow and Poulos, 2005), allowing for direct comparisons between ASD-related behavioural symptoms in human patients and animal models.

Most patients with ASD show alterations to delay eyeblink conditioning (Sears et al., 1994; Oristaglio et al., 2013), suggesting that it may be a useful, early biomarker for ASD. To test whether these findings hold in animal models of ASD, eyeblink conditioning was tested in five mouse models that have previously shown face validity for autism (Silverman et al., 2010), with alterations in social behavior, ultrasonic vocalization, and repetitive behaviors. All 5 mouse models

performed poorly in eyeblink conditioning, although in different ways depending on which cell types of the cerebellum were affected by the genetic mutations. Some mice blinked too soon or too late after the light appeared; others blinked weakly or less frequently; and some did not blink at all. This suggests that autism can affect the processing of sensory information in the cerebellum in different ways (Kloth et al., 2015). These findings are important because they demonstrate that autism-related genetic mutations disrupt cerebellar learning and identify the cerebellum as an important anatomical target for future diagnosis and intervention.

Genetic deletion of 4E-BP1 enables synaptic refinement to occur at synaptically silent synapses

Chapter 5 of my thesis looked at the role of cap-dependent translation regulation in plasticity from another perspective; while it's involvement in synaptic plasticity had been previously established (Banko et al., 2005; Gkogkas et al., 2013), its role in structural plasticity has not been directly examined. Consistent with previous work (Benoit and Changeux, 1975; Riley, 1978; Srihari and Vrbova, 1978; Brown et al., 1981; Brown et al., 1982; Balice-Gordon and Lichtman, 1994), my results show that synaptic refinement does not occur at silent SCG synapses, but surprisingly, genetically removing 4E-BP1 is sufficient to restore normal synaptic elimination. This data suggests that in WT mice, synaptic activity leads to mTOR activation, which hyperphosphorylates and inactivates 4E-BPs, leading to the translation of key proteins involved in refinement and presynaptic elimination of redundant inputs; in the absence of synaptic activity, 4E-BP1 is hypophosphorylated, blocking translation and refinement. This model is supported by my western blot and proteomic analysis (Chong et al., 2018) that show that synaptically silent neurons have elevated levels of active, hypophosphorylated 4E-BP1 and altered postsynaptic protein levels.

One caveat is that my work does not directly address the role of 4E-BPs at active synapses. Presumably, increasing 4E-BP activation, for example with a

constitutively active 4E-BP or by pharmacologically inactivating mTOR with rapamycin, would interfere with refinement, but this remains to be investigated.

Are the effects of translation regulation on synapse elimination are presynaptic or postsynaptic in nature? The α3-KO mutation only affects the postsynaptic neurons, and silencing SCG neurons enhances active 4E-BP1 in the SCG. Moreover, rescuing synaptic activity in the postsynaptic neurons is sufficient to drive input elimination, even after one month of synaptic silence. Therefore, it seems likely that activity-dependent translation in the postsynaptic neuron is critical for proper elimination, and acts on presynaptic inputs in a retrograde manner. However, using a conditional knockout of 4E-BP where it is only genetically removed for SCG neurons would help clarify this issue.

Unresolved issues in synaptic refinement

In spite of decades of research into the reorganization of developing circuits, the underlying molecular mechanisms remain largely unknown. My data suggests that activity-dependent regulation of mRNA translation is critically involved, demonstrating that deleting 4E-BP1 by-passes the need for synaptic activity in refinement. Interestingly, however, in the absence of synaptic activity, genetic deletion of 4E-BP1 does not enable the selective strengthening of 1-2 of the persisting presynaptic inputs. Therefore, differential strengthening must occur downstream of another activity-dependent secondary signaling pathway. Our findings (Chong et al., 2018) reveal that in the absence of synaptic activity, the proteome is changed significantly. Of the 2100 genes screened, 83 were significantly different between wild type SCG and synaptically silent SCG neurons (α3-KO). Fascinatingly, genetic deletion of 4E-BP1 in the synaptically silent SCG (α3/4E-BP-DKO) restored the protein levels for 60% (51/83) of these genes. This implies that genetic deletion of 4E-BP1 restores the translation of the proteins involved in presynaptic elimination, however, the molecules involved in differential strengthening of inputs must be amongst the 40% that are not rescued by 4E-BP1 genetic deletion. One question is, what are the molecular identities of these mRNA targets?

Over the last two decades, a few molecules involved in synaptic refinement have been identified. These appear to vary across synapses (Nguyen and Lichtman, 1996); Lichtman and Colman, 2000; Nguyen et al., 1998; Gonzalez et al., 1999), adding to the complexity of understanding the molecular pathways that are implicated. At the SCG, brain derived neurotrophic factor (BDNF) appears to be important for synapse formation (Causing et al., 1997). The number of presynaptic puncta dramatically increases on sympathetic neurons that overexpress BDNF, whereas sympathetic neurons that lack BDNF receive significantly fewer synapses (Causing et al., 1997). NGF also promotes maintenance of pre-synaptic inputs onto SCG neurons; exogenous NGF prevents the loss of synaptic contacts on ganglion cells after axotomy, whereas antiserum to NGF causes synapse loss (Nja and Purves, 1978). These results suggest that target-derived BDNF and NGF are involved in the formation and maintenance of preganglionic nerves. Whether these molecules have a role in the refinement of presynaptic inputs is less clear.

CHAPTER 7

CONCLUSION

In conclusion, my work shows a novel role for 4E-BPs in regulating two forms of activity-dependent plasticity, one at PF-PC synapses and the other at cholinergic synapses in the autonomic nervous system.

First, I conclude that 4E-BP2 is critical for PF-PC LTD. Genetic deletion of 4E-BP2 converts PF-PC LTD at PF-PC synapses to LTP. Moreover, I conclude that this change in plasticity involves PP2A. My findings lead to a model where 4E-BP2 tightly regulates the translation of molecules that affect PP2A activity. In so doing, it affects the balance between the kinase or phosphatase pathways, converting LTD responses into LTP responses. I conclude that in the absence of 4E-BP2, key mRNA are differentially translated such that PP2A activity is enhanced. As a consequence, the kinase/phosphatase balance is shifted toward phosphatase activity, receptor dephosphorylation and expression on the membrane, giving rise to LTP. In addition, I conclude that 4E-BP2 is required for motor learning in VOR gain-down adaptation through its effects on PF-PC LTD.

In addition, I conclude that 4E-BP-dependent mechanisms are involved in the growth of dendrites, the targeting of synapses, and the refinement of preganglionic axons during postnatal synaptic reorganization. In the absence of synaptic activity, synaptic refinement is curtailed, but genetic removal of 4E-BP activates a molecular program that can compensate for the lack of activity-dependent mechanisms that normally direct synaptic reorganization and refinement of presynaptic inputs. My model of this process is as follows: during development, synaptic activity inactivates 4E-BP1, leading to the synthesis of key proteins involved in refinement, that act on the presynaptic terminals in a retrograde manner. In the absence of synaptic activity, 4E-BP1 activity is enhanced, prevents the translation of key proteins and blocks refinement. Removing 4E-BP1, however, upregulates translation of the target mRNA, enabling refinement to take place even in the absence of synaptic activity.

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