RNP granule remodelling in response to neuronal activity

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Remodelage des granules ARN en réponse à l'activité neuronale

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Thesis Summary

One of the most fascinating – and still open – questions in neuroscience is how neuronal cells can form, store and then recall memories. Previous work has shown that Long-term memory (LTM) formation requires *de novo* protein synthesis, involving not only translation of newly transcribed RNAs, but also local, experience-induced translation of quiescent mRNAs carried and stored at synapses. For their transport and translational control, mRNAs are packaged with regulatory RNA binding proteins (RBPs), mainly translational repressors, into ribonucleoprotein (RNP) granules. To date, how neuronal RNP granules are remodelled in response to neuronal activity to relieve translation repression of mRNAs is unclear. Furthermore, the functional impact of such a remodelling in the establishment of long-term memories remains to be demonstrated *in vivo*. The objective of my PhD was to 1) investigate the *in vivo* mechanisms underlying activity-dependent remodelling of neuronal RNP granules; 2) test the hypothesis that RNPs could be involved in LTM-underlying mechanisms by regulating gene expression. To this end, I used as paradigm RNPs containing the conserved RBP Imp in *Drosophila*. First, I studied the impact of neuronal activity on Imp RNP properties by treating *Drosophila* brain explants with either KCl or the tyramine neuropeptide. In both cases, a disassembly of Imp RNPs was observed, characterized by a loss of both Imp and other RNP-component granular patterns, and a de-clustering of RNP-associated mRNA molecules. RNP disassembly could be reverted upon Tyramine withdrawal and was not observed in hyperpolarized neurons. Furthermore, my data suggest that RNP-disassembly is linked to increased translation of associated mRNAs, consistent with a model in which activity-induced RNP remodelling would lead to translational de-repression. Second, I investigated the mechanisms controlling RNP remodelling. A candidate regulator was CamkII, a conserved Ca^{2+} -activated kinase identified as a partner of Imp in an IP-Mass Spectrometry analysis. During my PhD, I could validate the Imp-CamkII interaction and showed that it is not mediated by RNA but depends on CamkII activity. Furthermore, I showed that inactivating CamkII function prevents the disassembly of Imp RNPs observed upon neuronal activation of brain explants, suggesting that CamkII may be involved in the activity-dependent remodelling of Imp RNP granules. These results are particularly interesting in the context of establishment of LTM, as CamkII has long been recognized as essential for LTM. Moreover, we recently showed in *Drosophila* that interfering with Imp function in a population of CNS neurons involved in learning and memory – the Mushroom Body γ neurons -, dramatically impairs LTM and that this effect relies on Imp C-terminal Prion-like domain, a domain known to be involved in RNP homeostasis. Altogether, my thesis work suggests a model where CamkII-dependent remodelling of Imp RNPs in response to neuronal activation might underlie LTM formation *in vivo*.

Keywords: *Drosophila,* RNA binding proteins, neuronal RNP granules, neuronal activity, LTM

Résumé de la thèse

Une des questions les plus fascinantes – et les plus ouvertes – en neuroscience est de comprendre comment les cellules neuronales contribuent à la formation, le maintien puis le rappel des souvenirs. Des travaux antérieurs ont montré que la formation de la mémoire à long-terme (MLT) requiert la synthèse *de novo* de protéines, impliquant non seulement la traduction d'ARNs nouvellement transcrits, mais aussi la traduction locale, induite par l'expérience, d'ARNms latents transportés et stockés dans les synapses. En vue de leur transport et du contrôle de leur traduction, les ARNms sont empaquetés avec des protéines de liaison aux ARNs (RBP), qui sont majoritairement des répresseurs de traduction, dans des granules ribonucléoprotéiques (RNP). La manière dont les granules RNP neuronaux sont remodelés en réponse à l'activité neuronale pour lever la répression traductionelle des ARNms est pour l'instant peu claire. En outre, l'impact fonctionnel d'un tel remodelage sur l'établissement de la MLT reste à démontrer *in vivo*. L'objectif de mon doctorat était 1) d'étudier les mécanismes *in vivo* qui sous-tendent le remodelage des granules RNP neuronaux ; 2) de tester l'hypothèse que les granules RNP pourraient être impliqués dans les mécanismes de renforcement de la MLT en régulant l'expression génétique.

Dans cette optique, j'ai utilisé comme modèle des granules RNP contenant la RBP conservée Imp chez la drosophile. Tout d'abord, j'ai étudié l'impact de l'activité neuronale sur les propriétés des granules RNP Imp, en traitant des explants de cerveau soit avec du KCl, soit avec le neuromodulateur Tyramine. Dans les deux cas, un désassemblage des granules RNP Imp - caractérisé par une dé-granulation à la fois de Imp et d'autres composants – est observé. Le désassemblage des granules RNP est réversible après retrait de la tyramine, et n'a pas été observé dans les neurones hyperpolarisés. Il ne dépend pas strictement du domaine de type prion qui se trouve à l'extrémité carboxy-terminale de Imp, un domaine connu pour être impliqué dans l'homéostasie des granules RNP. De plus, mes données suggèrent que ce désassemblage soit lié à une augmentation de la traduction des ARNms associés, ce qui est cohérent avec un modèle dans lequel le remodelage des granules RNP induit par l'activité des neurones induit une dé-répression de la traduction.

Ensuite, j'ai recherché les mécanismes contrôlant le remodelage des granules RNP. Un candidat pour cette régulation était CamkII, une kinase conservée activée par le calcium, et identifiée comme partenaire de Imp dans une analyse d'immunoprécipitation-spectrométrie de masse. Au cours de mon doctorat, j'ai pu valider l'intéraction Imp-CamkII et montrer qu'elle n'est pas médiée par l'ARN, mais dépend de l'activité de CamkII. De plus, j'ai montré qu'inhiber l'activité de CamkII empêche le désassemblage des granules RNP Imp observé lors de l'activation

neuronale, suggérant que CamkII pourrait être impliquée dans le remodelage des granules RNP Imp induit par l'activité neuronale. Ces résutats sont particulièrement intéressants dans le contexte de l'établissement de la MLT, car CamkII est depuis longtemps reconnue comme y étant essentielle. Plus encore, nous avons récemment démontré chez la drosophile qu'inactiver la fonction de Imp dans une population de neurones du cerveau central impliquée dans l'apprentissage et la mémoire – les neurones du Mushroom Body – altère radicalement la MLT. En conclusion, mes résultats sont cohérents avec un modèle où le remodelage des granules RNP Imp en réponse à l'activation neuronale dépend de CamkII, et pourrait contribuer à la formation de la MLT *in vivo*.

Mots clés : *Drosophile,* protéines de liason à l'ARN, granules RNP neuronaux, activité neuronale, MLT

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List of abbreviations

ACh: Acetylcholine

AD: Alzheimer Disease

ALE: Alternative Last Exon

ALS: Amyotrophic Lateral Sclerosis

AMPAR: α-Amino-3-hydroxy-5-Methyl-4-isoxazolepropionic Acid Receptor

APA: Alternative PolyAdenilation

Arc: Activity-regulated cytoskeleton-associated protein

ATP: Adenosin Tri-Phosphate

BDNF: Brain-Derived Neurotrophic Factor

BONCAT: Bio-Orthogonal Non-Canonical Aminoacid Tagging

CBs: Cajal Bodies

CPEs: Cytoplasmic Polyadenylation Elements

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DANs: Dopaminergic Neurons

DRF: Diaphanous-Related Formins

dsRBM: double-stranded RNA Binding Motif

DTEs: Dendritic Targeting Elements

eCLIP: Enhanced Crosslinking and Immunoprecipitation

EJC: Exon Junction Complex

EM: Electron Microscopy

FMRP: Fragile X Mental Retardation Protein

FP: Fluorescent Protein

FRAP: Fluorescence Recovery After Photobleaching

GO: Gene Ontology

HEK: Human Embryonic Kidney

ISH: *in situ* Hybridization

KH: K-Homology

KIF5: Kinesin5

LCRs: Low Complexity Regions

LLPS: Liquid-Liquid Phase Separation

lncRNA: long noncoding RNA

LTD: Long-Term Depression

LTF: Long-Term Facilitation

LTM: Long-Term Memory

LTP: Long-Term Potentiation

MAP2: Microtubule-Associated Protein 2

MB: Mushroom Body

MBONs: Mushroom Body Output Neurons

MCIDs: Multiple Copies of Interaction Domains

mESC: murine Embryonic Stem Cell

miRNA: micro RNA

mRNA: messenger RNA

MT: Microtubule

NMDA: N-Methyl-D-Aspartate

NMDAR: NMDA Receptor

NSC: Neural Stem Cell

NTEs: Neurite Targeting Elements

PABP: Poly(A)-Binding Protein

PAMs: Protocerebral Anterior Medial neurons

PAR-CLIP: Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation

P-bodies: Processing bodies

PLDs: Prion-Like Domains

PLM: Posterior Lateral Microtubule

PTM: Post-Translational Modification

RBP: RNA Binding Protein

RGC: Retinal Ganglion Cell

RGC: Retinal Ganglion Cell

RNA: RiboNucleic Acid

RNP: RiboNucleoProtein

RRM: RNA Recognition Motif

rRNA: ribosomal RNA

SCA-2: Spinocerebellar Ataxia

SG: Stress Granule

Shh: Sonic Hedgehog

SMTI: Single Molecule Translation Imaging

snoRNPs: small nucleolar RNPs

snRNA: small nuclear RNA

snRNPs: small nuclear RNPs

STM: Short-Term Memory

TRAP: Translating Ribosome Affinity Purification

tRNA: transfer RNA

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I – Introduction

mRNA localization is a powerful means to control gene expression in space and time. As a consequence of this process, protein synthesis is restricted to specific cellular compartments, thus achieving an impressive level of signalling cascade refinement. This fascinating process was observed for the first time more than 30 years ago by William R. Jefferis in the ascidia *Styela plicata*. Since then, localized transcripts were observed in virtually all species and mRNA segregation is nowadays recognized as a prevalent mechanism used by cell types ranging from fibroblasts to oocytes, from eggs to embryos and mature adults.

mRNA localization is particularly prevalent in neurons, that have a very challenging morphology. These cells are highly compartmentalized, consisting of a nucleus-containing cell body from which neuronal processes (dendrites and axons) extend. Not only these processes have a variable length that can reach up to hundreds of centimetres, but they also need to promptly respond to different stimuli, hence requiring a constant protein supply. As defined by Christine Holt and Erin Schuman as the “decentralized dogma”, this need can be fulfilled by mRNAs subcellularly localized in neurites, locally translated *on-site* and rapidly available for response to external cues.

mRNA localization in neurons relies on the assembly of macromolecular complexes composed of RNA Binding Proteins (RBPs) and target mRNAs, called “RNP granules”. These assemblies are membraneless organelles that form through liquid-liquid phase separation in a highly regulated fashion. All the steps of their journey are tightly controlled: the recognition of target mRNA sequences by their respective RBPs, their transport *via* cytoskeletal tracks, their anchoring, the final release of the transcripts and subsequent translation.

The local control of the neural proteome in neurites has been proposed as a key mechanism impacting several neural aspects: neural architecture, synaptic plasticity and - very importantly - learning and memory processes. Although *in vivo* evidence is scarce, RNA localization and local translation are believed to be crucial events for the establishment of local changes at synapses, ultimately leading to learning and memory formation.

In the first part of the introduction, I will discuss the principles of mRNA localization (§ 1) and briefly provide a non-neuronal example of mRNA segregation in fibroblasts (§1.1). The second part will focus on how neurons localize (§2) and locally translate (§3) mRNAs, with particular attention to the role of local translation in memory formation (§4). The third part will be dedicated to RNP

granule composition, assembly and function as sensors of external stimuli (§5). Finally, I will describe the role of the IMP RBP family – and mainly of its *Drosophila* member dImp – (§6) and conclude with the specific objectives of my thesis (§7).

1. mRNA localization: overview and advantages

Asymmetrical distribution of organelles and molecules within the cells generates cell polarity, the establishment of which is crucial for cellular function and for the development of an organism. Cell polarity, indeed, has been shown to regulate various development-related key processes such as cell motility, stem cell division and cell differentiation. Consistent with a major and ancestral function of cell polarity, protein polarity complexes such as the Crumbs, Scribble and PAR complexes are well conserved throughout the animal kingdom. If affected, these so-called polarity determinants function cause partitioning defects and loss of polarisation (Bulgakova and Knust 2009; Su et al. 2012; Thompson 2013; Lang and Munro 2017).

mRNAs, not only proteins, are found to be asymmetrically distributed within cells. The first studies highlighting polarized mRNA distribution date back to the 80s and showed RNA localization in different cell types, ranging from eggs and embryos (Jeffery et al. 1983) to more specialized cells such as neurons (Garner et al. 1988).

With the discovery of mRNAs asymmetrically localized in a vast number of cytoplasmic territories of oocytes and embryos in different species, the crucial role for development and the widespread presence of localized transcripts became more evident (Martin and Ephrussi 2009; Medioni et al. 2012; Cody et al. 2013). For instance, in the *Drosophila* oocyte, an elegant control of the specification of body axes is exerted by localized mRNAs: the differential distribution of *gurken, bicoid, nanos* and *oskar* mRNAs guarantees the establishment of the correct patterning along the embryonic axes (Lasko 2012).

The advent of genome-wide approaches confirmed the importance of mRNA segregation in various tissues, revealing not only that asymmetrically distributed mRNAs have a role in cellular architecture and function, but also that this mechanism is prevalent throughout cell types and species and concerns a huge number of transcripts (Tomancak et al. 2002; Moccia et al. 2003; Zhong et al. 2006; Blower et al. 2007; Lecuyer et al. 2007; Mili et al. 2008; Jambor et al. 2015; Misra et al. 2016; Zappulo et al. 2017). Global analysis of mRNA localization in *Drosophila* embryogenesis, for example, established that 71% of mRNAs are subcellularly localized, showing in most cases exclusion from certain regions of the embryo (Lecuyer et al. 2007). This percentage is 7-times higher than what had been previously estimated (Tomancak et al. 2002). To date, subcellular targeting of mRNAs to nearly all cellular compartments was shown, and new-

generation techniques have permitted the study of localized mRNAs in organelles classically difficult to isolate, such as peroxisomes, or mitochondrial outer membranes (Yarmishyn et al. 2016; Fazal et al. 2019).

The link between mRNA segregation and cell polarity relies on the translation of localized transcripts *in situ*, a process that eventually establishes protein asymmetry. This hypothesis is further supported by the close spatial correlation between localized mRNAs and their corresponding protein products (Lecuyer et al. 2007; Zappulo et al. 2017), inferring that mRNAs are localized and can then be translated at their final destination.

What are the advantages of transporting mRNAs rather than proteins? Different hypotheses were proposed (Holt and Bullock 2009): if on one hand this strategy has an obvious energy-saving gain for the cell as one single mRNA can be translated multiple times, on the other hand, spatially restricting protein production has different functional advantageous implications. irst, mRNAs expression can be spatially controlled, avoiding detrimental ectopic expression of active proteins in the wrong compartment and inappropriate protein-protein interactions; second, local protein accumulation can be achieved promptly in response to compartment-specific stimuli; and third, newly synthetized proteins show minimal post-translational modifications, thus exhibiting specific signalling properties.

1.1 Cell migration in fibroblasts as a case study: a tale of localized mRNAs

Fibroblasts are connective tissue cells with pleiotropic functions in organ morphogenesis, immune response, maintenance of tissue integrity and wound healing. These cells can move on 2D surfaces by forming extensions called lamellipodia at the leading edge of the cells and, when polarized, actively migrate toward the polarizing stimulus. Lamellipodia are actin-rich structures whose protrusion is tightly linked to actin polymerization and has been shown to depend – among others - on the Arp2/3 complex and Dia1 (Goulimari et al. 2005; Sixt 2012; Suraneni et al. 2012). ARP2/3 is a nucleator complex promoting the branching of actin networks (Mullins et al. 1998) and Dia1 is part of the DRFs family (Diaphanous-Related Formins) involved in the nucleation of actin polymerization (Pollard 2007; Chesarone and Goode 2009).

Interestingly, polarized migrating fibroblasts exhibit asymmetric distribution of different cytoskeletal mRNAs, suggesting that localization of these transcripts could extensively contribute to the mechanisms underlying fibroblast polarization and cell motility (Liao et al, 2015). Furthermore, the observation that mRNAs coding for distinct cytoskeleton-related proteins or for different protein isoforms are found differently located within the cell strongly suggests a tightly regulated orchestrated mechanism. Among the localized mRNAs found within fibroblasts, ***β-actin*** mRNA is

probably the most studied example of localized transcript: Lawrence and Singer first showed that it accumulates at the leading extremities of chicken embryonic fibroblasts more than thirty years ago (Lawrence and Singer, 1986) (Figure 1). Consistent with a crucial role of mRNA segregation in fibroblast migration, misslocalization of *β-actin* mRNA was reported to decrease cell motility (Condeelis and Singer, 2005). Interestingly, α- and *γ-actin* mRNAs have a different fate (Hill and Gunning, 1993; Kislauskis et al., 1993; Bulinski, 2006), suggesting an isoform-specific mechanism controlling *β-actin* distribution. This may be linked to the different function of actin isoforms, as α- and γ-actin proteins are required in the perinuclear area and in stress fibres (Hill and Gunning, 1993; Kislauskis et al., 1993), while β-actin polymerization is crucial for the formation of lamellipodia and thus for the migrating behaviour of this cell type (Hofer et al, 1997; Shestakova et al, 2001) (Figure 1C).

β-actin mRNA is localized through a 54 nt-long *cis*-acting element called zipcode, present in its 3'UTR region and essential for its localization (Kislauskis et al., 1994). This sequence is recognized by the RBP and translational repressor Zipcode Binding Protein 1 (ZBP1/IMP1/IGF2BP1), *via* two out of its four K-Homology RNA-recognition domains (Ross et al, 1997; Chao et al., 2010). In fibroblasts, ZBP1 binds *β-actin* mRNA perinuclearly and transports it in a repressed state towards the leading edge (Wu et al. 2015), where the mRNA is released (Figure 1C). *β-actin* mRNA is then anchored at the leading edge with the help of proteins such as EF1-alpha (Liu et al, 2002). The discharged mRNA is then bound by ribosomes and translated (Rodriguez et al, 2006; Wu et al. 2015) *in loco* (Figure 1C).

As previously mentioned, fibroblasts show several localized mRNAs coding for proteins required for fibroblast migration. Interestingly, the mRNAs encoding the ARP 2/3 complex and Dia1, for instance, are localized in different cellular compartments (Figure 1C): while *ARP2/3* mRNAs are found – as *β-actin* – at the fibroblast leading edge (Mingle et al, 2005) together with their corresponding proteins (Machesky et al, 1997), *Dia1* mRNA is on the contrary observed in the perinuclear region (Liao et al, 2011) (Figure 1C).

Although a whole picture of the underlying molecular mechanisms is still missing, these data support the idea that Actin and the ARP2/3 complex might be locally translated at the leading edge, thus promoting actin polymerization and consequent cell motility. In support of this hypothesis, all the seven mRNAs encoding the subunits of the ARP2/3 complex are localized at the leading edge, possibly leading to quick and coordinated complex assembly during or after translation (Liao et al, 2015).

However, although this example describes a fine and tightly regulated one-to-one protein-mRNA mechanism, the control of mRNA localization is way more complex and involves a large network of interactors. ZBP1 itself is able to bind and likely control more than 100 mRNAs other than *β-actin* (Patel et al, 2012; Hafner et al, 2010; Jønson et al, 2007). Besides, various proteins have been

found to control *β-actin* mRNA segregation and trafficking (Wächter et al, 2013; Ceci et al, 2012; Pan et al, 2007; Itoh et al. 2002; Rackham and Brown, 2004; Dormoy-Raclet et al, 2007; Ascano Jr et al, 2012; Mukherjee et al, 2019), as for example FUBP3/MARTA2, which is directing *β-actin* to the fibroblast leading edge independently of ZBP-1 (Mukherjee et al, 2019).

In conclusion, asymmetrical localization of mRNAs represents a key regulatory step, crucial for proper cellular function. Molecularly, it depends on sequences present within localized mRNAs and recognized by RBPs, which in turn mediate mRNA transport toward specific compartments. Differentially localized proteins participate to compartment-specific pathways, leading to sophisticated and locally-regulated cellular mechanisms.

2. mRNA localization in neurons

Neurons are highly specialized polarized cells, structurally composed of a cell body (that contains the nucleus) and emanating neuronal processes. Neuronal processes – dendrites and axons – account for up to 99% of the cytoplasm volume. Neuronal morphology represents a unique challenge in terms of spatial control of gene expression for at least two reasons: first, neurons extend their processes far from the cell body, generating a peculiar need for fine-tuning gene expression in space and time; second, dendrites and axons can be very long, measuring up to several millimetres and hundreds of centimetres respectively; third, neurons form numerous synapses whose activity and protein content need to be dynamically and individually regulated.

In this framework, mRNA targeting plays a pivotal role in concentrating corresponding proteins in specific compartments, participating in the regulation of neuronal polarity and function (Campbell and Holt 2001; Leung et al. 2006; Welshhans and Bassell 2011; Medioni et al. 2012; Medioni et al. 2014; Glock et al. 2017; Wong et al. 2017; Terenzio et al. 2018; Biever et al. 2019). For instance, mRNA localization is required *in vitro* and *in vivo* for axon steering, branching, growth and remodelling in different systems (Campbell and Holt 2001; Donnelly et al. 2013; Wong et al. 2017; Lee et al. 2018; Sahoo et al. 2018), and it is also crucial for synaptic plasticity and related cognitive functions (Koekkoek et al. 2005; Bramham and Wells 2007; McEvoy et al. 2007; McFleder et al. 2017; Nakayama et al. 2017; Sahoo et al. 2018).

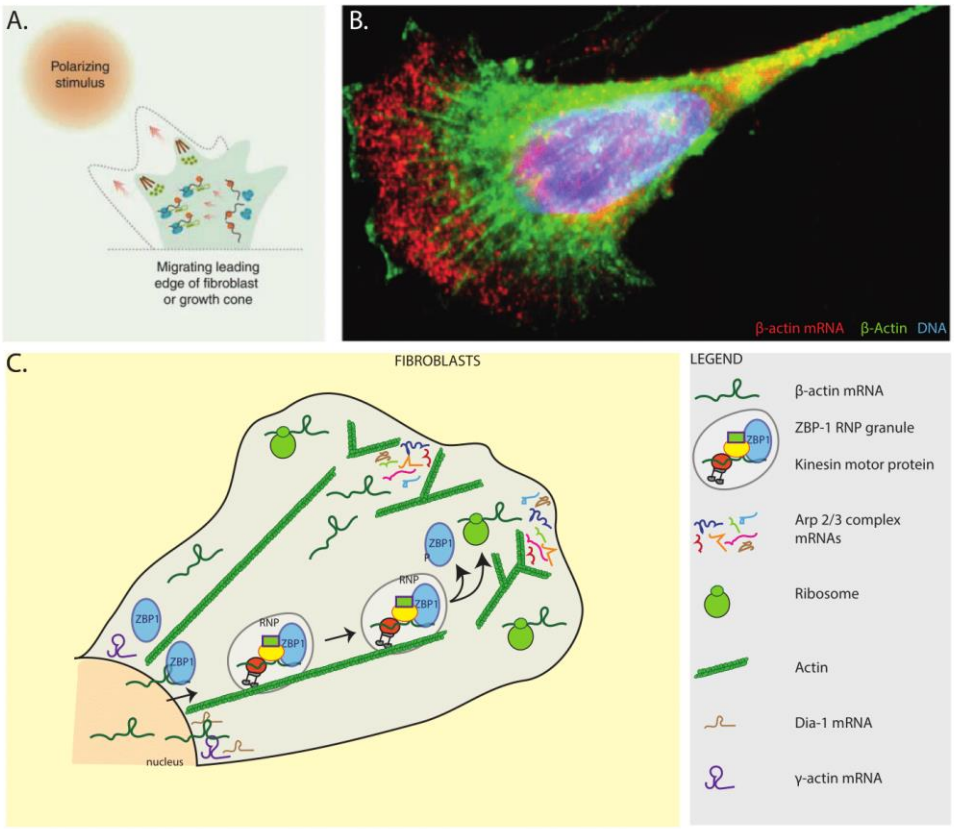

Figure 1. mRNA localization in migrating fibroblasts.
A) scheme of a fibroblast growth cone showing the leading edge migration towards the polarizing stimulus. B) Image of a migrating fibroblast with β-actin mRNA labeled in red and actin protein in green. Figure of A. Wells. C) scheme of β-actin mRNA localization in the fibroblast leading edge: *β-actin* mRNA reaches the leading edge either by diffusion or by direct transport. In this latter case, β-actin transcript is bound by ZBP-1 in the perinuclear area and transported in ZBP1 RNP granules towards the leading edge, where it is released. Other mRNAs involved in actin polimerization are differentially localized, such as Dia1 and γ-actin in the perinuclear area, and the seven Arp2/3 complex mRNAs in the leading edge.
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A) Holt, C. E. and S. L. Bullock (2009). Science 326(5957): 1212-1216.
B) Dahm, R. and M. Kiebler (2005). Nature 438(7067): 432-435.
C) inspired by Shav-Tal, Y. and R. H. Singer (2005). J Cell Sci 118(Pt 18): 4077-4081.

2.1 mRNA localization in neurites: Brief history

The first evidence of targeted mRNAs localizing in dendrites arose in the 80s, when Davis and colleagues reported mRNAs in rat hippocampal neurons (Davis et al, 1987). In the following 15 years, many mRNAs were observed in dendrites using *in situ* hybridization (ISH) (Figure 2A and 2B) including: the microtubule-associated protein 2 (*MAP2*) (Figure 2A) (Garner et al, 1988), the α-subunit of the Ca^{2+}/calmodulin-dependent kinase II (*CamkII-α*) (Miyashiro et al, 1994) (Figure 2B), the activity-regulated cytoskeleton-associated protein (*Arc*) (Lyford et al, 1995) (Figure 2B) and the NMDA receptor (*NMDAR*) NR1 subunit (reviewed in Steward and Schuman, 2003).

Similarly to dendrites, mRNA segregation was also observed in axons. The presence of *kinesin* mRNA in squid giant axons (Gioio et al, 1994; Chun et al. 1996) represented the first step towards the understanding of the long-distance transport of RNA molecules from the soma.

The use of compartmentalized "Campenot" chambers first and of microfluidic devices later enabled the isolation of axonal and somatodendritic compartments and the identification of compartment-specific mRNAs (Campenot 1977; Taylor et al. 2005; Park et al. 2006; Taylor et al. 2009). Using this approach on primary cultures of rat cortical and hippocampal neurons (Taylor et al. 2005; Park et al. 2006; Taylor et al. 2009), more than 300 axonally-localized mRNAs were identified, showing an enrichment in cytoskeletal components, transport, protein synthesis machinery and mitochondrial maintenance (Taylor et al. 2009).

At the same time, evidence arose about the order of magnitude of this phenomenon: hundreds of mRNAs localised in neuronal processes were found in microdissected Aplysia neurons via RNA isolation (Moccia et al, 2003; Moroz et al, 2006) whereas microarrays and RNA-seq studies established that thousands of RNAs are localized in hippocampal dendrites (Poon et al, 2006;).

With the advent of transcriptomic approaches, hundreds of mRNAs were further localized in dendrites and axons, confirming that RNA content is highly regulated (Miyashiro et al, 1994; Moccia et al, 2003; Sung et al, 2004; Taliaferro et al, 2014; Zivraj et al, 2010; Andreassi et al, 2010; Gumy et al, 2011, Tushev et al, 2018; Hafner et al, 2019, Cajigas et al, 2012). This pool – much enriched in cytoskeletal proteins, translation regulators and neurite-related proteins (Moccia et al, 2003; Tushev et al, 2018; Hafner et al, 2019) - is very dynamically regulated in response to external cues, such as activity. For example, mRNAs such as *Arc* are transcribed in response to activity and then rapidly transported to dendrites (Link et al, 1995; Lyford et al, 1995) where translation starts (Steward et al, 1998; Steward and Worley, 2001). Although *Arc* shows the most dramatical fold increase upon synaptic stimulation (de Solis et al, 2017), this is not an isolated case: *CamkII-α* and *β-actin* mRNAs localize at dendrites upon neuronal stimulation (Rook et al, 2000; Tiruchinapalli et al, 2003), where *BDNF* also is increasing upon stimulation (de Solis et al, 2017).

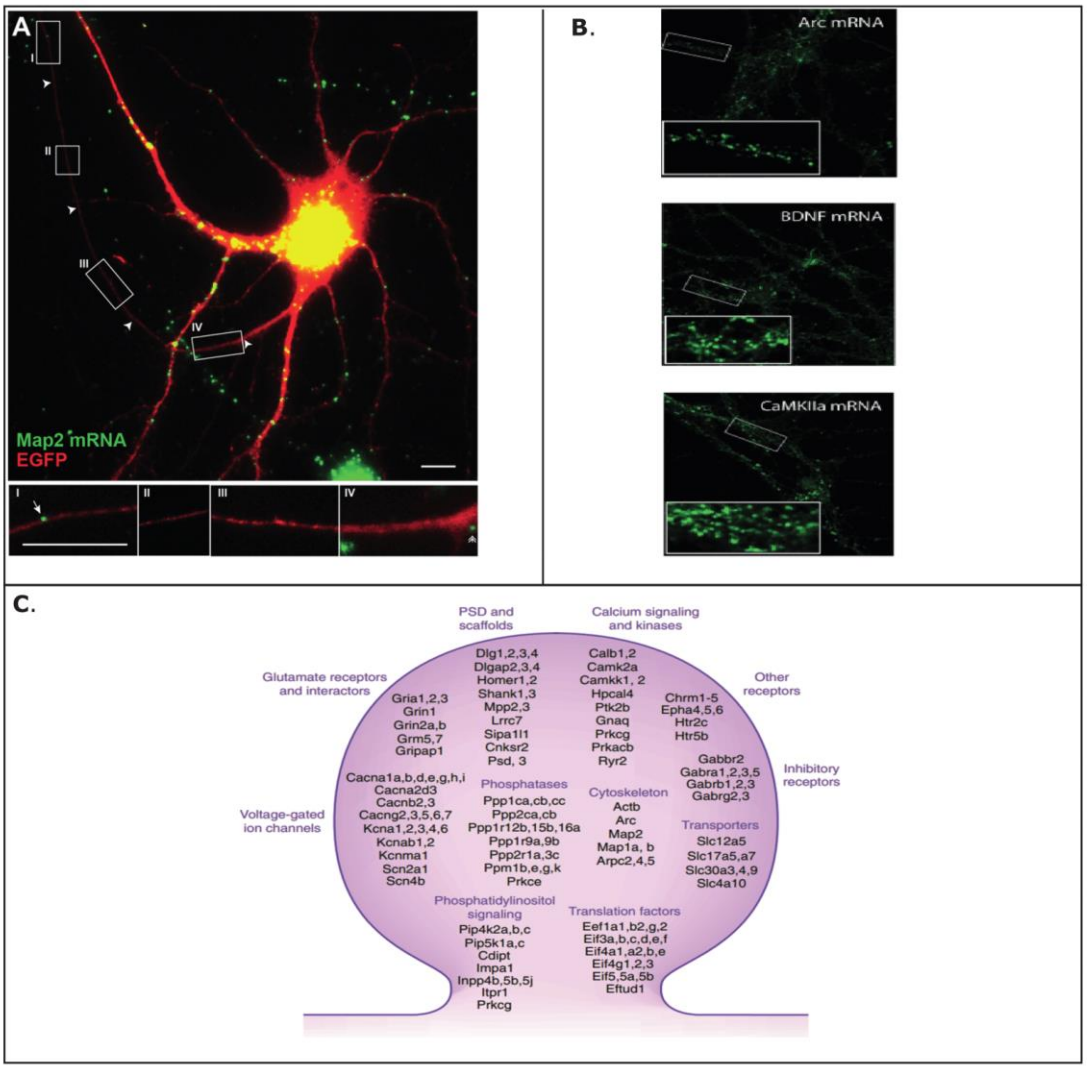

Figure 2. mRNAs localized in neurites.
A) *Map2* mRNA (in green) labeled by fluorescent *in situ* hybridization in cortical neuron dendrites expressing EGFP (red in the figure). The four insets I to IV in the bottom panel correspond to magnified views of the boxed regions portrayed in the upper image. Scale bar : 10 µm.
B) *Arc, BDNF* and *CamkII* mRNA in hippocampal neuron dendrites revealed by immuno-FISH, the longest part of the onset corresponds to approximately 20 µm. The magnification of the boxed area is fivefold. C) Scheme of a post-synaptic density indicating mRNA families identified therein by RNA-seq. These transcripts code for synaptically-relevant proteins and have been found in the neuropil of hippocampal area CA1.

References:
A) Balasanyan, V. and D. B. Arnold (2014). PLoS One 9(3): e92349.
B) Raju, C. S., N. Fukuda, C. Lopez-Iglesias, C. Goritz, N. Visa and P. Percipalle (2011). Mol Biol Cell 22(11): 1864-1877.
C) Holt, C. E., K. C. Martin and E. M. Schuman (2019). Nat Struct Mol Biol 26(7): 557-566

2.2 Mechanisms of RNA targeting

2.2.1. *cis*-acting elements

RNA localization relies on the interplay between *cis*-acting elements within the mRNA sequence and proteins able to recognize them, termed *trans*-acting factors (discussed in § 2.2.3).

Cis-acting elements have been identified in numerous localized mRNAs targeted to different compartments in different species. These localization signals reside in the RNA sequence and are necessary and sufficient for the correct targeting of mRNAs.

To date, a body of evidence shows that localization to dendrites relies on the presence of *cis*-acting elements initially called Dendritic Targeting Elements (DTEs). The term DTEs can refer not only to sequences found within the mRNAs, but also to particular RNA secondary structures. However, this nomenclature tends to be misleading since to date numerous *cis*-acting elements have been shown not only to direct mRNAs to or along dendrites, but also to target them to axons. Hence these sequences can be referred to as Neurite Targeting Elements (NTE) instead. Structural RNA motifs can address mRNAs to specific locations as well. For instance, G-quartet RNA structures are found in high proportion in dendritic mRNAs and have been shown to be necessary and sufficient to target mRNAs to neurites (Subramanian et al, 2011). Furthermore, other *cis*-acting elements firstly described in cells other than neurons have been shown to address mRNAs to neural processes, as in the case of the AC-rich zipcodes and the UUUUAU sequences called Cytoplasmic Polyadenylation elements (CPEs) (Huang et al. 2003).

Regarding the described NTEs, they are very heterogeneous. They can be found anywhere along the transcript, but also show a very variable length. In fact, although several groups have identified targeting elements in the 3'UTRs of mRNAs such as *Map2*, *Arc*, *β-actin, Shank1, BDNF, Rgs4, CamkII-α* or *Tau* (Mayford et al. 1996; Blichenberg et al. 1999; Aronov et al. 2001; Bockers et al. 2004; Kobayashi et al. 2005; Meer et al. 2012; Klein et al. 2016; Bauer et al. 2019), evidence shows that NTEs can be also found elsewhere. In the case of *neuritin,* localization elements are located in both 5' and 3'UTRs (Merianda et al, 2013) whereas *vasopressin* mRNA contains a DTE partially present in the coding region. *PKMζ* mRNA also presents two DTEs: one partly covering the 5'UTR and the coding region and the other – a RNA stem-loop motif – residing in the 3'UTR (Prakash et al, 1997; Muslimov et al, 2004). Interestingly, *CamkII-α* presents *at least* two localization elements in its 3'UTR that extensively differ in terms of size: while one is 20 nt-long (Mori et al, 2000), the second one accounts for 1200 nt (Blichenberg et al, 2001).

The heterogeneity and multiplicity of NTEs indicate that targeting can be mediated by different mechanisms, perhaps redundant. Remarkably, different localizing sequences can be found within

the same mRNAs, probably as a part of complex regulation where they can – cooperatively or not – dictate complex targeting patterns. For instance, *CamkII-α* mRNA also contains a CPE and a G-quartet structure in its 3'UTR, facilitating translocation (Huang et al, 2003; Subramanian et al, 2011).

However, not all localized mRNAs contain targeting elements, showing that there might be alternative ways to achieve correct neurite localization, as illustrated by targeting sequences found in retained introns (Buckley et al. 2011; Khaladkar et al. 2013).

2.2.2. the 3'UTR code

3'UTRs are enriched in *cis*-acting elements dictating mRNA sub-localization. In addition, elements controlling stability and translational efficiency are commonly encoded within 3'UTRs (Tian and Manley, 2017). Because of their pleiotropic role in controlling mRNA fate and their recently discovered diversity, 3'UTRs have been suggested to serve as a molecular code that helps establishing mRNA fate. Their length and properties are regulated by alternative polyadenylation (APA), a crucial post-transcriptional mechanism that affects more than 70% of mammalian transcripts (Derti et al. 2012; Hoque et al. 2013).

3'UTR heterogeneity is regulated in a development- and tissue-specific fashion and has been connected to differential RNA regulation (Ulitsky et al. 2012; Lianoglou et al. 2013; Mayya and Duchaine 2019). Interestingly, a body of evidence is emerging, suggesting that the neuronal transcriptome is characterized by specific 3'UTR signatures and that alternative 3'UTRs might regulate the differential localization of isoforms in neuronal compartments. For example, studies of the 3'UTR repertoire have demonstrated that in neuronal tissues transcripts have extended 3'UTRs compared to other tissues (Miura et al. 2013; Guvenek and Tian 2018). A recent mapping of alternative 3'UTRs performed in murine embryonic stem cell (mESC)-derived neurons established that hundreds of genes present differentially-localized 3'UTR isoforms (Ciolli Mattioli et al. 2019). *Cdc42* and *BDNF* mRNAs, for instance, can exhibit short or long 3'UTRs found respectively in somata or neuritis (An et al. 2008; Ciolli Mattioli et al. 2019). These two compartments are enriched in different transcripts and can also be characterized by the presence of different isoforms of the same gene, generally correlating with alternative last exons (ALEs) (Taliaferro et al. 2016). Moreover, Tushev and colleagues analyzed RNA samples derived from either the somatic or the neuropil layer of microdissected rat brain slices and found that, although both compartments were enriched in neurite- and synapse-specific transcripts, further relevant differences could be observed (Tushev et al. 2018). Neuropile transcripts possess 3'UTRs with (i) a higher content in GC and predicted secondary structures, (ii) increased length, (iii) a higher tendency to be composed of repeated elements and (iv) they also exhibit longer half-lives (Tushev et al. 2018). These peculiarities are likely connected to functions or requirements for their localization. For

example, the longer half-life suggests that transcripts localized in neurites may need to be more stable, perhaps to survive their long journey to distal neurite compartment, or to allow several local translation rounds. Moreover, the increased length and enrichment in RNA motifs likely reflects the fact that they contain several *cis*-acting elements and serve as platforms for the binding of *trans*-acting factors and subsequent efficient transport to neurites.

2.2.3. Trans-acting factors in transport

Localization of mRNAs depends not only on *cis*-acting determinants present within the RNA sequence, but also on the action of *trans*-acting factors, proteins that recognize and bind these determinants.

ZBP-1, for instance, recognizes the 54-nt long zipcode (Chao et al, 2010; Patel et al, 2012), whereas the Fragile X Mental Retardation Protein 1 (FMRp) is reported to bind several diverse NTEs, including G-quadruplex RNA structures (DeMarco et al, 2019; McAninch et al, 2017). To date, numerous *trans*-acting factors have been identified and described using biochemical and computational approaches. Most of these key players in mRNA trafficking are RBPs organized in different modules, enabling concomitant binding to mRNAs and other regulators. Several RNA binding domains have been described so far, depending on the protein fold and on the affinity with RNA. Examples are the RNA Recognition Motifs (RRMs), the K homology (KH) domains or the double-stranded RNA binding motif (dsRBM), found for instance in known *trans*-acting factors such as Fmrp, ZBP-1 and Staufen respectively (Di Liegro et al, 2014). An intriguing property of RBPs is that they can act as bridges, connecting the mRNAs to molecular motors to ensure mRNA transport. Staufen for instance has been shown to bind mRNAs, to colocalize with kinesin5-(KIF5) associated granules (Kanai et al, 2004) and to mediate transport of mRNAs toward neurites (Sharangdhar et al, 2017; Bauer et al, 2019). In addition, kinesins are reported to interact with RBPs such as FMRp and ZBP1 together with their target mRNAs: in both cases an increased interaction between these proteins and kinesins correlated with active transport to dendrites (Dictenberg et al, 2008; Urbanska et al, 2017).

2.3 Dynamic mRNA transport to neurites

Subcellular targeting of mRNAs in neurons depends on active transport along cytoskeletal tracks. To date, the velocity measured for mRNA transport remains heterogeneous within the µm/s and mRNAs have been reported to travel in both anterograde and retrograde manners, sometimes with a bias for anterograde transport. Interestingly, changes in velocity and directionality have been associated to neurite maturation state (Knowles, 1996) or to activity levels (Bauer et al, 2019). In

particular, Bauer and colleagues observed an anterograde bias in the transport of *Rgs4* mRNA to dendrites in hippocampal neurons that is dependent on neuronal activity (Bauer et al, 2019). Other examples of mRNAs whose transport responds to activity bursts have been mentioned in § 2.2.

Several studies have proven that for mRNA transport to happen the MT cytoskeleton has to be intact (Knowles, 1996; Kohrmann et al, 1999; Kanai et al, 2004; Medioni et al, 2014). Notably, the transport of various RNA/RBP cargoes have been reported along Microtubules (MTs), involving stable or transient interactions with proteins belonging to the kinesin family or dynein (Das et al, 2019; Kanai et al, 2004; Davidovic et al, 2007; Jeong et al, 2007; Xu et al, 2013; Bianco et al, 2010; Urbanska et al, 2017; Aronov et al, 2002). While dynein mediates minus end-directed retrograde movements (van Niekerk et al, 2007), Kinesin transport is generally directed toward the plus-end of microtubules. Due to the unified polarity of microtubules in axons and partly in dendrites (Rolls 2011), kinesins are particularly well-suited for anterograde transport. Different members of the kinesin family have been shown to mediate mRNA transport: KIF5 has been extensively linked to transport of mRNAs such as *CamkII-α* and *Arc* to dendrites (Kanai et al, 2004), whereas KIF3A helps *Tau* trafficking to axons (Aronov et al, 2002). In addition, kinesins are reported to interact with RBPs such as FMRp and ZBP1 together with their target mRNAs: in both cases an increased interaction between these proteins and kinesins correlated with active transport to dendrites (Dictenberg et al, 2008; Urbanska et al, 2017). Consistent with kinesin function in RNA transport, in the case of pathological conditions such as ALS, FUS inclusions form and sequester kinesin-1, thus impeding a correct RNA localization in axons, a common described outcome of this disease (Yasuda et al, 2017; Khalil et al, 2018).

However, MT-based mechanisms are not the only ones controlling mRNA localization. In fact, mRNAs can be targeted in an actin- and rather myosin- dependent fashion (Balasanyan and Arnold, 2014). This alternative mechanism can be achieved through either direct or indirect binding of Myosin motor proteins. Myosin family members can be plus-end directed (Myosin Va) or minus-end directed (Myosin VI) and they are likely not involved in long-distance transport. Nevertheless, they have been shown to direct mRNA cargo movements in two different ways: (i) they can mediate short-range transport via microfilaments (Mallardo et al. 2003; Canclini et al. 2014; Kalinski et al. 2015; Yoon et al. 2016) or (ii) they can rather bind mRNA thus decreasing its availability for MT-dependent transport (Nalavadi et al. 2012; Calliari et al. 2014) as it has been proven in hippocampal neurons, where Myosin Va inhibits ZBP1 transport to axons and restricts its anterograde movement (Nalavadi et al. 2012).

2.4 mRNA anchoring in neurites: sushi tonight?

Once mRNAs reach neurites, finely-tuned mechanisms must be in place to anchor the mRNAs for

subsequent translation. Although this step is crucial for mRNA localization and further regulation to occur, evidence is scarce regarding its molecular triggers. If on one hand, the endocytic pathway has been linked to mRNA anchoring in *Drosophila* oocyte and embryo (Pratt and Mowry 2013); on the other hand, actin and actin-binding proteins have been proposed to participate in mRNA docking (Doyle and Kiebler 2011; Medioni et al. 2012). In neurites, very few data exist elucidating mRNA docking mechanisms. Recent studies provided evidence that *β-actin* mRNA docking depends on F-actin in both mouse hippocampal dendrites and *Xenopus* retinal ganglion cell (RGC) axons (Yoon et al, 2016; Turner-Bridger et al, 2018). Notably, in the latter work, disrupting actin cytoskeleton did not affect *β-actin* directed transport but increased diffusive mobility of the mRNA (Turner-Bridger et al, 2018). Very interestingly, this same effect of increased diffusion was observed upon puromycin treatment, suggesting that ribosomes might be active players in *β-actin* anchoring in axons (Turner-Bridger et al, 2018).

Interestingly, mRNAs seem to experience cycles of pausing/moving and dock at specific sites, probably in response to external stimuli (Doyle and Kiebler 2011; Yoon et al. 2016; Wong et al. 2017; Bauer et al. 2019). Wong and colleagues, for instance, observed mRNA docking at random sites just in 16% of the cases, whereas mRNAs were found specifically at axonal protrusion sites in the remaining 84% of the cases (Wong et al. 2017). Insights into the dynamics of mRNA anchoring further revealed that transcripts show continuous movements – which are bi-directional – and notably are stationary for periods of time in the range of minutes and then are briefly motile (this time in the range of seconds) (Yoon et al. 2016; Wong et al. 2017).

These data hence suggest that mRNAs continuously redistribute along neurites, as further demonstrated by the fact that transcripts traffick along entire neurites, also in regions that were previously unoccupied by mRNA (Kim and Martin 2015; Yoon et al. 2016).

Remarkably, Yoon and colleagues observed an increased recruitment of mRNAs upon glutamate uncaging, with stimulated regions exhibiting a higher mRNA density compared to neighbouring regions, suggesting that the transcripts previously localized in these latter regions probably contribute to the mRNA increase at activated loci (Yoon et al. 2016).

These findings challenged the vision that mRNAs could act as on-hold outposts and wait for regulatory cues while being anchored (Park et al. 2014) and rather confirmed that mRNAs patrol synapses, circulating throughout neurites via bi-directional transport, and are promptly recruited to activated synapses (Doyle and Kiebler 2011).

Since this mechanism might resemble what happens in japanese restaurants, where customers take food from the continuously active sushi belt according to their hunger, a mechanism called “sushi-belt model” was proposed for mRNA docking at synapses (**Figure 3**) (Doyle and Kiebler, 2011). According to this model, mRNAs would be transported within neurites via bi-directional movements, transiently remaining at some synapses, and then being transported toward others,. A

dynamic sorting process induced by synaptic activity and characterized by a series of activity-regulated docking and undocking events was recently described for *Rgs4* mRNA in rat hippocampal dendrites, supporting the sushi-belt model (Bauer et al, 2019). In the proposed model, active synapses would trap mRNAs for local translation, thus inducing and/or supporting synapse-specific long-lasting changes. This phenomenon may provide the molecular bases underlying the “synaptic tagging” hypothesis (Frey and Morris, 1997), and may be integrated in a framework where local translation occurs acutely in response to stimuli at synapses, where mRNAs are captured and anchored. Consistent with this model, research carried out in *Aplysia* sensory neurons has demonstrated that although mRNAs can be indeed delivered throughout the whole neuron, translation of these transcripts locally increases at specific active synapses (Kim and Martin 2015).

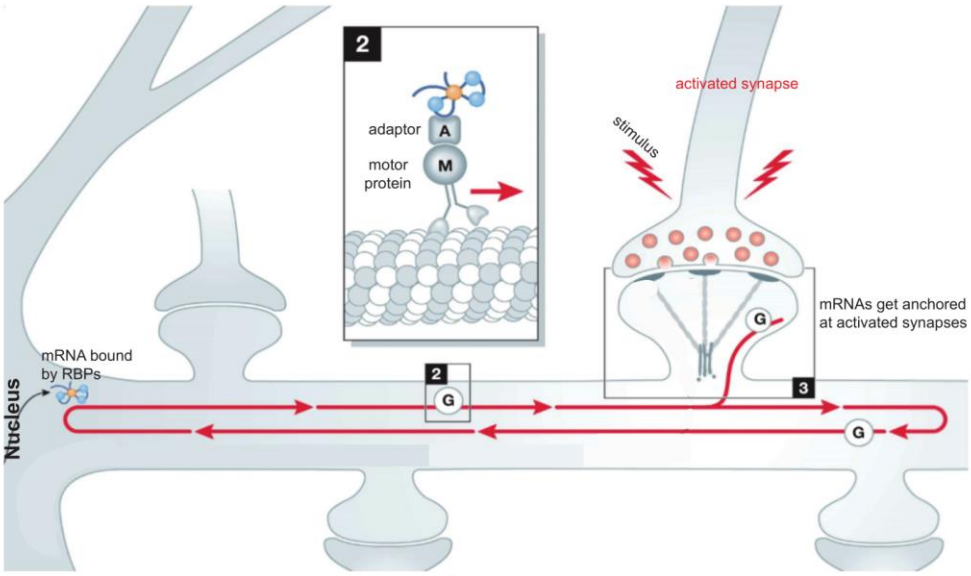

Figure 3. sushi belt model for mRNA trafficking and anchoring at activated synapses
The sushi belt model proposes that mRNAs (R) - bound to RBPs (A) and motor proteins (M) - constantly traffick along neurites and patrol synapses. In response to stimulation, specific synapses get activated. mRNAs are thus captured and anchored at these activated synapses, inducing changes therein, possibly via local translation.
References:
Adapted from Doyle M, Kiebler MA. 2011. EMBO J 30: 3540-3552

3. local translation in neurons

Although an obvious outcome of mRNA localization is local translation, demonstrating that local translation of mRNAs is actually happening in axonal and dendritic processes is very challenging and has required the development of advanced technologies.

Historically speaking, polyribosomes had been observed at synapses by Steward and Levy already in 1982 (Steward and Levy 1982) and then by Kiebler and DesGroseillers in 2000 (Kiebler and DesGroseillers 2000). However, while polysomes had been clearly observed in dendrites, EM analyses had not been as conclusive in axons, shedding doubts about the actual presence of the translational machinery therein. Axons were however later shown to be able to locally adjust their proteome. In particular, radioactively-labeled somaless cultures clearly demonstrated the occurrence of on-site protein synthesis in *Xenopus* RGC axons (Campbell and Holt 2001). Further evidence suggested that the peculiar distribution of the translational machinery in axons could explain its previous poor detection in EM studies. In fact, in rabbit and rat root nerve fibers, small restricted ribosomal domains were found scattered in the periphery of axons, whereas in hippocampal neurons the translational machinery was found to be associated with the membranous receptor DCC (Koenig et al. 2000; Tcherkezian et al. 2010). Another explanation for the underestimation of the axonal translation machinery relies on the possibility that axons use monosomes rather than polysomes for synthetizing proteins (Klein et al. 2016). Monosomes would indeed be undistinguishable from other dark stained particles in EM studies because of their small size (Harris and Weinberg 2012). In accordance with this hypothesis, a surprising number of transcripts localized at neuronal processes appear to be tightly associated with monosomes and are enriched in synaptic proteins (Biever et al. 2019).

Our knowledge regarding mRNAs and other molecules important for protein synthesis localizing in neurites has now deeply increased. For instance, it was recently demonstrated that in the adult rodent brains, 75% of both pre- and post-synaptic compartments do contain the main requirements for translation, *i.e*. rRNAs, ribosomes and polyadenylated mRNAs (Hafner et al. 2019). Furthermore, protein synthesis is carried out at synapses physiologically, even in the absence of exogenous stimulation: when labelling active translating ribosomes, Hafner and colleagues observed that 40% of presynaptic terminals (both inhibitory and excitatory) and 60% of dendritic spines were translationally-active at rest (Hafner et al. 2019). The nature of the transcripts stored at synapses revealed by transcriptomic profiling of mature mice synaptosomes showed an enrichment in genes involved in transport, signalling and metabolism, translation, formation/scaffolding of synaptic vesicles and active zone architecture (Hafner et al. 2019).

3.1 the neural translatome

High throughput techniques have been adopted in the recent years to study the translatome (mRNAs being actively translated and thus bound by ribosomes) of both dendritic and axonal compartments. This body of work has shown that both synaptic compartments display active translation, throughout development and in mature neurons, under basal conditions and in response to activity.

In murine mixed glial and neuronal cultures, quantitative analysis of nucleotide levels in response to KCl depolarization revealed that thousands of transcripts are significantly altered upon neuronal stimulation and that, among them, hundreds are altered just at the level of translation (Dalal et al. 2017). Analysis of newly translated proteins can be achieved with the BONCAT (bio-orthogonal non-canonical amino acid tagging) technique that was applied in rat hippocampal neurons (Schanzenbacher et al. 2016). This method – which permits to label and thus identify newly synthetized proteins - together with pharmacological manipulation of neuronal activation (obtained by inhibiting and reactivating neurons cyclically) unravelled hundreds of differentially regulated nascent proteins (Schanzenbacher et al. 2016). Intriguingly, these proteins do mainly participate in neuronal functions (neurite outgrowth and guidance, synapse functioning, filopodia formation) (Schanzenbacher et al. 2016).

To study the translatome in entire organisms, *in vivo* techniques were developed relying on cell-specific Ribosome tagging (Sanz et al. 2009). These techniques aim at extracting ribosome-associated mRNAs upon immunoprecipitation of HA-tagged Rpl22 proteins (Sanz et al. 2009). A version implemented for axons, the axon-TRAP (Translating Ribosome Affinity Purification), was further applied to the axonal projections of Retinal Ganglion Cells (RGCs) in mouse (Shigeoka et al. 2016). Using this approach, the authors showed that both immature and mature axons can synthetize proteins and gave important hints about how the translatome is regulated compartment-wise during development (Shigeoka et al. 2016). Gene Ontology (GO) analysis of the translated mRNAs, for example, revealed that while growing axons mainly translate mRNAs involved in neuron morphogenesis and axon elongation, mature axons rather translate mRNAs regulating synaptic transmission and neurotransmission (Shigeoka et al. 2016). Strikingly, translational regulators as mTORC1 have expression peaks during axonal maturation that are coherently overlapping with a steep increase in the translation of target mRNAs (Shigeoka et al. 2016), suggesting a fine-tuned stage-specific translational programme.

This programme is likely regulated also depending on the neural cell type. Different types of neurons do indeed show diverse local translatomes probably linked to cell type-specific post-transcriptional control, as shown in mouse by synap-TRAP (Ouwenga et al. 2018).

3.2 mechanisms underlying local translation

During their travel, mRNAs are bound by regulatory *trans*-acting factors. Analysis of RBPs and proteins associated to mRNAs in neurons have highlighted a huge number of translational repressors, suggesting that mRNAs travel in a translationally-inactive state throughout neurites (El Fatimy et al. 2016; Elvira et al. 2006; Fritzsche et al. 2013). Furthermore, mRNAs to be localized – together with their bound proteins – seem to associate with stalled ribosomes (Krichevsky and Kosik 2001; Mallardo et al. 2003; El Fatimy et al. 2016; Das et al. 2019). Further details have been elucidated through the years, leading to the description of several elegant processes of transport of mRNAs in a repressed state to neurites, where the consequent mRNA translation occurs (Iocoangeli and Tiedge 2013; Fernandez-Moya et al. 2014). One of the best-studied examples is given by FMRp (Figure 4). This RBP associates with several mRNAs (such as *CamkII, cofilin* or *psd95)* in neurons and mediate their transport in a repressed state to dendrites (Iocoangeli and Tiedge 2013). Consistently, inactivation of FMRp leads to increased translational levels of target mRNAs, as shown for *psd95* or *cofilin* for instance (Ifrim et al. 2015; Feuge et al. 2019). This translational repression is possibly achieved by an action of FRMp at the level of translational elongation: notably, FMRp induces ribosome stalling, thus impeding its translocation (Khandjian et al. 2004; Udagawa et al. 2013; Das et al. 2019). Numerous other mRNAs and proteins have been characterized (some of which are depicted in Figure 4). One transcript example is ***β-actin*** mRNA, which has been shown to be largely (although not completely) translationally inactive during transport (Wu et al. 2016). Moreover, its *trans*-acting factor, ZBP-1, inhibits translation of its bound target mRNAs by preventing 80S ribosome formation to then release the repression in response to phosphorylation at the final location (Hüttelmaier et al, 2005). Indeed, ***β-actin*** repression is relieved upon dissociation of ZBP-1 from ***β-actin***, following a Src-mediated ZBP-1 phosphorylation (Huttelmaier et al. 2005; LePellettier et al. 2017) (Figure 4).

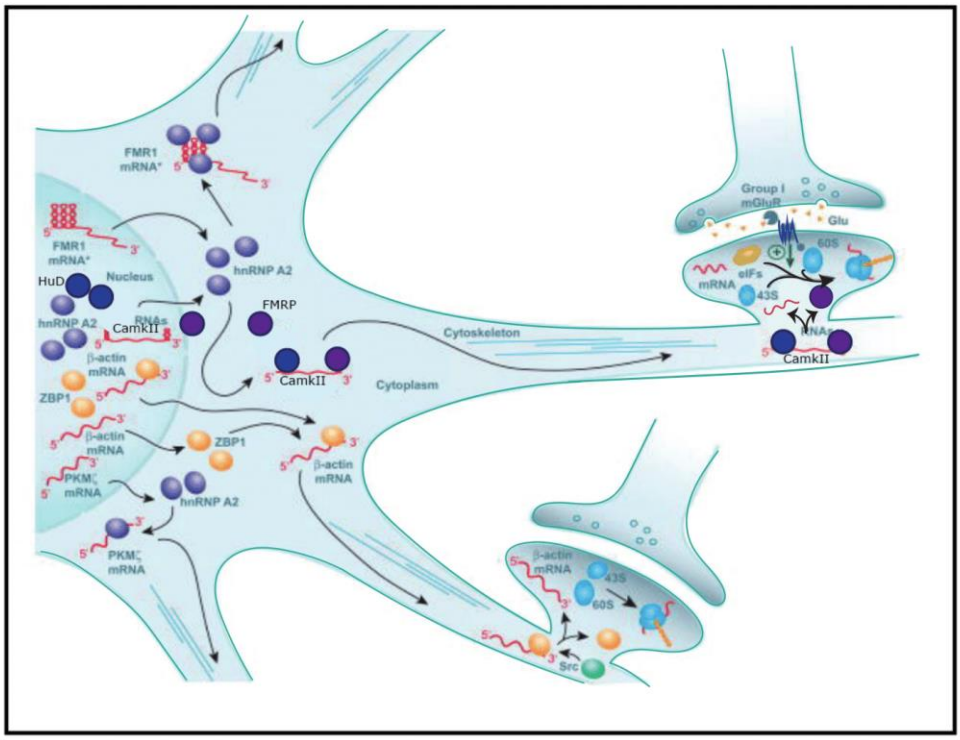

Figure 4. mRNA localization and translation in dendrites.
Specific mRNA:protein interactions are established in the perinuclear area. The assembled RNP granule is transported along cytoskeletal tracks to the synapses where local translation occurs in response to stimuli. Here few examples are described: hnRNP A2 protein recognizing *PKMζ* and *Fmrp* mRNAs, ZBP-1 binding *β-actin* mRNA and releasing it at synapses upon phosphorylation by the Src protein, *CamkII-α* mRNA bound either by FMRP or HuD (here drawn together) and translated in response to stimuli in the post-synaptic density.
References:
Iacoangeli A, Tiedge H. 2013. Trends Biochem Sci 38: 47-55 (adapted)

3.3 dynamics of local translation: achieving "space" and "time" resolutions

Local translation in neurons needs to be dynamically regulated, in order to respond promptly to the cell needs and specific stimuli. Visualization of local protein synthesis with high spatial and temporal resolution has recently been made possible thanks to single molecule real-time imaging. These approaches permit not only to spatially monitor where mRNA molecules are translated, but also to determine if local translation occurs in a stochastic fashion or in "hot spots", in response to external signals.

The Single Molecule Translation Imaging (SMTI) technique, for example, exploits the fast-folding and fast-bleaching fluorescent protein (FP) Venus which is inserted between the 5'UTR and the coding region of the mRNA of interest (Strohl et al. 2017). Imaging of reporter constructs expressed in animal models is composed of cycles of imaging-bleaching, allowing the visualization of single translation events lasting for hundreds of milliseconds before the signal is bleached again. When this system was used to visualize local *β-actin* synthesis in Xenopus retinal axons, sporadic translation events were registered, mainly occurring at the same location, suggesting a monosome-based protein synthesis (Strohl et al. 2017). However, the application of the same technique on hippocampal dendrites, showed that for *Arc* and *FMRP* mRNAs, translation was rather stochastic or bursty, and not occurring in the same spots, suggesting that translation activation might not follow the same mechanism (Tatavarty et al. 2012).

To date, the SunTag method is the most adapted to visualize *in vivo* translation events of single mRNAs. It is based on two different tag arrays: one that marks the nascent peptide and the other that labels the messenger RNA (Figure 5A) (Tanenbaum et al. 2014; Morisaki et al. 2016; Wang et al. 2016; Wu et al. 2016; Yan et al. 2016). Tandem copies of the peptide tag are visualized *via* a co-expressed GCN4 antibody fused with GFP that recognizes and binds its cognate peptide with high affinity, leading to the formation of such bright and localized GFP foci, that the system was called SUperNova (SunTag) (Tanenbaum et al. 2014). Repeated mRNA tags (MS2 or PP7 hairpin motifs) are recognized with high affinity by co-expressed M2 or PP7 bacteriophage coat proteins fused to a different fluorescent protein (Figure 5A) (Coller and Wickens 2007; Chao et al. 2008). The Suntag approach has been used so far to answer to general questions, such as the spatial distribution of protein synthesis in neurons, or to track individual translational events in both dendrites and axons (Wang et al. 2016; Wu et al. 2016; Cioni et al. 2019). Live imaging experiments in hippocampal neurons exploiting this technique gave remarkable insights into local translation dynamics: translation in dendrites was shown to be bursty and switching between "on" and "off" states, whereas mRNAs were observed moving after translation had already started, suggesting that active transport and translation of mRNAs are not mutually exclusive (Wang et al. 2016; Wu et al. 2016)

Very recently, the SunTag itself was implemented with the orthogonal MoonTag system that allows visualization of multiple single mRNAs and thus follow more complex translational events (Figure 5B/D) (Boersma et al. 2019). Furthermore, other genetically-encoded probes with the same purposes have been developed, such as the HA-frankenbody which can light up multiple proteins tagged with hemagglutinine in all cell compartments (Figure 5C) (Zhao et al. 2019) and can be used together with other reporters such as the SunTag to visualize different mRNAs.

These powerful tools will be critical to monitor local translation of several mRNAs at the same time and to address numerous open questions, such as: how fast mRNAs are translated in response to external cues? And where? Are different compartments simultaneously yet differently responding to stimuli? The high spatio-temporal resolution achievable using these approaches makes them particularly suitable to tackle these questions.

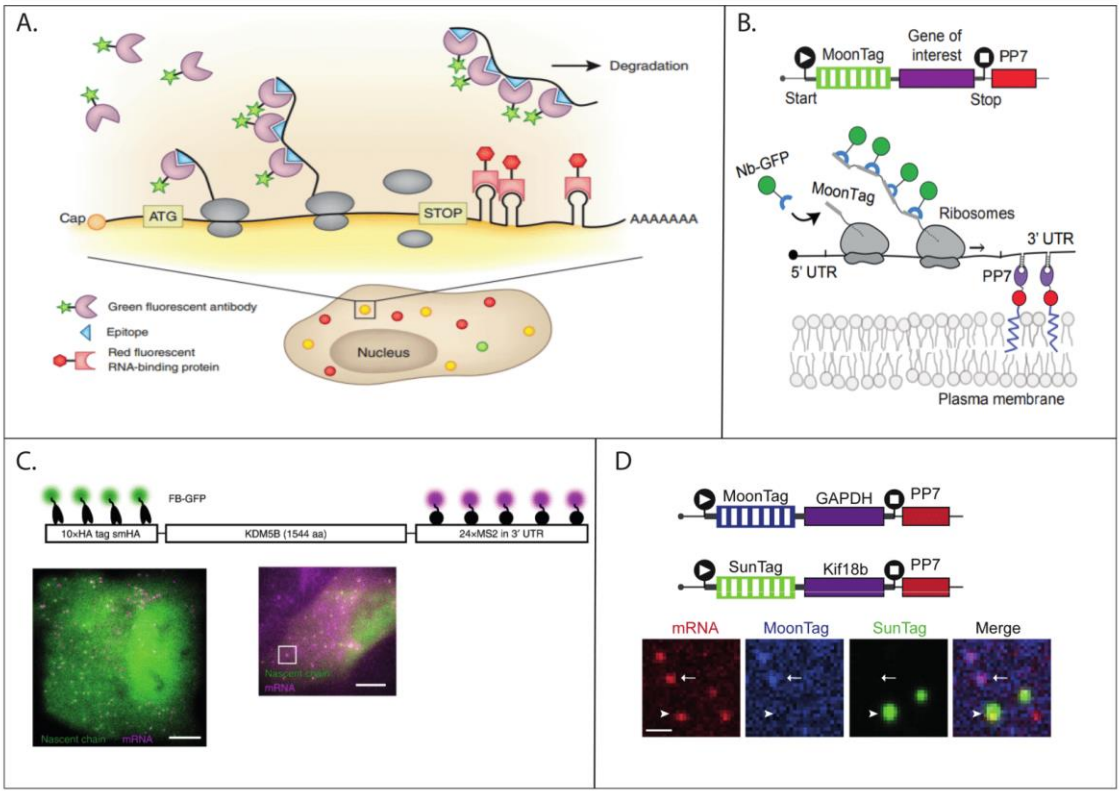

Figure 5. Dual reporters to visualize in vivo translation events.
A) The SunTag approach allows the visualization of both the mRNA and the newly synthetized peptide thanks to different co-expressed fluorescent molecules. B) and C) the Moontag and the HA-frankenbody are two other systems that allow the visualization of mRNAs and their corresponding nascent polypetides. These systems are based on the same principle of the SunTag, but exploit other tags to recognize the translated proteins. Examples of detection of KDM5B in U2OS cells . Scale bar: 10 µm D) The different systems can be used in combination to detect distinct translational events and thus simultaneously follow different mRNAs, as shown here for GADPH and Kif18b. Scale bar: 1µm
References:
A) Wu, J. and S. R. Jaffrey (2016). Nat Biotechnol 34(7): 723-724
B, D) Boersma, S., D. Khuperkar, B. M. P. Verhagen, S. Sonneveld, J. B. Grimm, L. D. Lavis and M. E. Tanenbaum (2019). Cell 178(2): 458-472 e419
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4. local translation and memory formation: a long-lasting story

4.1- Local translation is required for Long-Term Memory

Long-Term Memory (LTM) formation relies on long-lasting changes at the synapses and long-range activation of signalling cascades. Molecularly speaking, while Short-Term Memory (STM) is established *via* modifications of already produced proteins, LTM requires *de novo protein* synthesis. This requirement can be fulfilled not only by translating newly transcribed mRNAs in the cell body, but also by the experience-induced translation of mRNAs already localized at synapses. For this reason, local translation at synapses and LTM formation are thought to be tightly related.

Long-lasting changes in synaptic strength induced by repeated stimulations within a neuronal pathway are thought to underlie LTM. These changes can be manifested by a strengthening or a weakening of synapses, respectively called Long-Term Potentiation (LTP) and Long-Term Depression (LTD) (Malenka and Bear 2004; Buffington et al. 2014). Both processes were shown to require local translation (Sutton and Schuman 2006). For example, pioneer work performed by Kang and Schuman (Kang and Schuman 1996) showed that neurothropin-induced plasticity in the hippocampus leads to an increase in synaptic transmission. This increase is rapid, yet long-lasting. Moreover, while local translation is not required right away for the strengthening of the synapses, it is essential for the later synaptic potentiation (Kang and Schuman 1996). As in this study the authors demonstrated that the translational source was not the neuronal soma, dendrites (and later on axons) were indicated as possible reservoirs of translatable mRNAs (Kang and Schuman 1996).

In addition, another form of synaptic plasticity called Long-Term Facilitation (LTF) is observed upon administration of a noxious stimulus. Upon repeated stimulation with serotonin, *Aplysia* stimulated sensory neurons display facilitation selectively at the stimulated locus, whereas no changes occur where no stimulation is applied (Martin et al. 1997). This site-specific LTF actively required local translation in both *Aplysia* and mouse and was impacted by pharmacological inhibition of protein synthesis (Frey and Morris 1997; Martin et al. 1997; Huber et al. 2000; Kandel 2001; Costa-Mattioli et al. 2009; Luscher and Huber 2010).

4.2. LTM and dendritic mRNA localization are intimately connected

Dendritically localized mRNAs represent the substrate of local translation mechanisms. Perturbing their localization was shown in different *in vivo* contexts to alter memory formation. *CamkII-α* mRNA - particularly enriched at synapses - codes for an essential player in memory formation (see 4.3). Disrupting the dendritic localization of *CamkII-α* by restricting it to somata dramatically impacts synaptic plasticity and causes memory impairments in mouse (Miller et al. 2002). Moreover, mice carrying RNG105/caprin1 mutations disrupting the dendritic subcellular localization of target mRNAs exhibit dramatic LTM deficits (Nakayama et al. 2017). Further consistent with a tight *in vivo* link between local translation and memory formation, a nearly complete reduction in mRNA translation is observed in brains of patients affected by one of the major diseases impacting memory, Alzheimer Disease (AD) (Langstrom et al. 1989).

Because of their role in regulating the fate of associated transcripts and restricting translation in space and time, RBPs represent strong candidates for memory regulators. Indeed, evidence has arisen about their involvement in memory formation (Sudhakaran and Ramaswami 2017; Mirisis and Carew 2019). To date, however, the mechanisms by which these proteins could exert their function in such processes is far from elucidated. Furthermore, the fact that investigating their local function is particularly challenging has represented a clear obstacle to their study.

4.3. Achieving perduring molecular memory

Localization of mRNA and consequent on-site translation allow individual synapses to respond to specific stimuli and change their properties accordingly. However, formation of long-term memories raises two main molecular questions: first, how is local translation *actually* triggered? And second, how are the induced structural changes self-maintained for long periods of time, thus allowing memories that outlast the normal protein turnover?

Different models were proposed over the past years to explain the perdurance of molecular and structural changes linked to memory traces (Asok et al. 2019), among which two are discussed below. The first one relies on RBPs capable of forming self-propagating amyloid-like aggregates *via* structural changes in response to activity (see § 4.3.1). The second one relies on a locally translated self-activating kinase induced by activity (see § 4.3.2).

4.3.1. The CPEB protein family in the persistence of memory

The CPEB protein family has raised an increasing interest in this context for different reasons. First, CPEBs are involved in mRNA recognition and translational regulation (Richter 2007; Richter and Klann 2009; Lu et al. 2017). Second, CPEBs are essential for memory formation (Richter and

Klann 2009; Lu et al. 2017). Third, they can exist not only in a monomeric form, but also in a self-propagating prion-like conformation *in vitro* (Sudhakaran and Ramaswami 2017). In mammals, CPEB1 recognizes 3'UTR-contained CPEs (U-rich sequences) and it is involved in a translational regulation pathway together with its other partners: Gld2, a poly(A) polymerase; PARN, a deadenylase and a translation inhibitor called Ngd (Richter and Klann 2009). Once activity bursts in dendrites, CPEB1 is phosphorylated and its conformational change induces the release of PARN from the original RNA complex. Associated mRNAs thus undergo poly(A)-tail elongation, promoting the binding of the Poly(A)-Binding Protein (PABP) and the recruitment of the translational machinery (Udagawa et al. 2012). mRNAs abundantly localized in dendrites such as *CamkII* are regulated by this pathway (Huang et al. 2002). In mice, CPEB knock-out leads to memory and synaptic plasticity defects in Schaffer collateral-CA1 or hippocampal neurons which correlate to altered mRNA expression (Alarcon et al. 2004; Berger-Sweeney et al. 2006; Udagawa et al. 2012).

In *Aplysia*, Si and colleagues studied a neuron-specific isoform of CPEB whose amount at synapses is increased by serotonin (Si et al. 2003) and discovered that ApCPEB protein can switch from a monomeric form to an activity-dependent self-sustaining multimeric one that may represent a mark for active synapses (Si et al. 2010). The discovery that the ApCPEB multimers resulted from the formation of ApCPEB homotypic interactions and the amyloid features of such multimers led the authors to propose that ApCPEB may act as a prion-like protein. Because of their unique properties of self-propagating and inducing structural changes in other proteins, prion proteins represent suitable players in memory maintenance. In fact, they are capable of forming stable aggregates, which likely allows the molecular persistence of memories by sustaining changes at specific active synapses (Si and Kandel 2016). Consistently, neuronal activity would be the trigger of the oligomerization of prion-like protein, as happens for ApCPEB in response to serotonin (Si et al. 2010), leading to local and persistent synaptic plasticity (Sudhakaran and Ramaswami 2017).

The discovery that the *Drosophila* CPEB Orb2 also exists in the two described forms (a monomer and a self-sustaining prion-like oligomer) was beneficial to the understanding of the molecular mechanisms behind this persistent synaptic plasticity, Orb2 forms are differentially distributed within the neurons: while the monomeric form was detected in the neuronal cell body, the oligomer was found enriched in the synaptic region (Majumdar et al. 2012). Furthermore, the transition between the two Orb2 forms depends on neuronal activity: stimulation induced by the neuromodulator Tyramine, for instance, was reported to increase the proportion of the Orb2 oligomeric form (Majumdar et al. 2012). The function of CPEB proteins in memory was also recapitulated in *Drosophila*: Orb2 was shown to be essential for LTM formation, but not for STM establishment (Keleman et al. 2007; Majumdar et al. 2012). To further show that this memory phenotype is tightly linked to the formation of the self-sustaining Orb2 oligomers, Majumdar and

colleagues made use of an Orb2 mutation that specifically inhibits the oligomer formation whitout affecting the monomeric form nor the overall protein level (Majumdar et al. 2012). Flies carrying such a mutation were defective in forming long-lasting memory, resulting in the formation of memories persisting for no more than 48h (Majumdar et al. 2012).

But what is the molecular and functional impact of Orb2 oligomerization? The two structural states of Orb2 were shown to differentially impact mRNA translation (Khan et al. 2015) (Figure 6): Orb2 monomer inhibits translation whereas Orb2 oligomer acts as a translational activator (Khan et al. 2015) (Figure 6C). This may be linked to the differential recruitment of functional partners, as the deadenylase CG13928 and the poly(A)-Binding protein CG4612 were among others shown to bind specifically to the monomer and the oligomer respectively (Khan et al. 2015) (Figure 6C). These results strongly suggest that Orb2 transitioning into functional aggregates controls LTM formation by regulating translation at synapses, a result further supported by the mRNA interactome of Orb2, composed of both known and novel players in LTM (Stepien et al. 2016) (Figure 6D). Orb2 was also shown to regulate the expression of a CamkII reporter in an activity-dependent fashion in the *Drosophila* Mushroom Body (MB), the fly functional ortholog of the hippocampus (Kruttner et al. 2015).

Altogether, studies that focused on the role of Orb2 in the persistence of memory showed that 1) activity-dependent structural changes are responsible for long-term memory consolidation; 2) transition into higher-order complexes promotes mRNA translation and 3) mRNAs involved in memory are de-repressed following repeated activity spikes likely at synapses.

The mechanism by which CPEB/Orb2 transitions between two states represents a clear bridge between local translation and LTM. However, the multiplicity of RBPs existing and recognizing distinct subsets of mRNAs could vouch for the hypotheses that diverse parallel mechanisms exist and are still undescribed.

Self-sustained post-translational protein modifications, indeed, are also good candidates that can account for long-lasting changes underlying memory formation (see § 5.3.3).

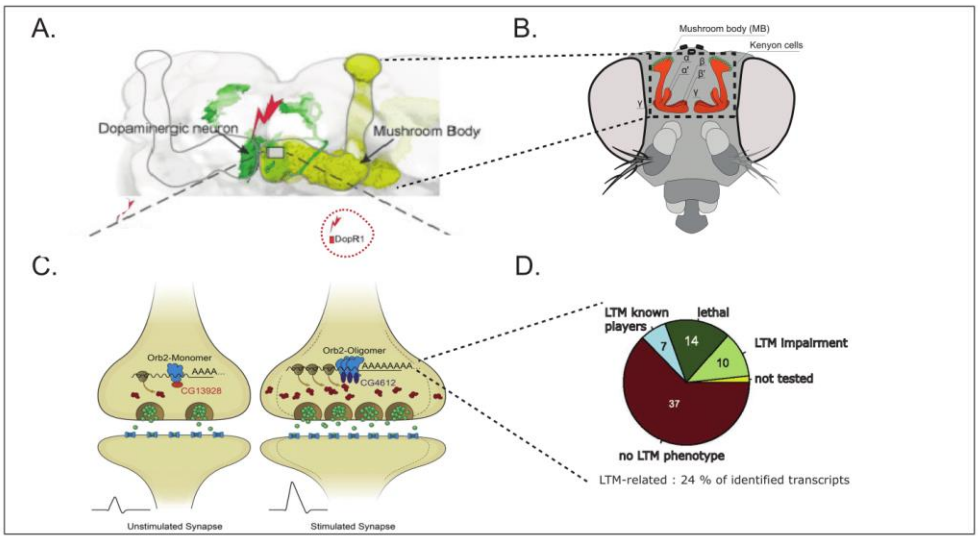

Figure 6. Orb2-mediated mechanism in LTM formation in *Drosophila*.
The main structures responsible for memory formation in *Drosophila melanogaster* are the Mushroom Bodies (A, B). A) In courtship conditioning, LTM relies on dopaminergic neurons (black arrow) projecting into the distal part of the MB γ lobe. C) Upon neuronal stimulation, Orb2 transition from a monomeric state inhibiting translation to an oligomeric form associated to an active translation. This functional switch is linked to Orb2 interaction with different proteins in the two conditions: the deanylase CG13928 interacts with the monomer, whereas the poly(A)- Binding Protein CG4612 is associated with the oligomer. D) The mRNAs bound by Orb2 show an enrichment in LTM-related transcripts.
References:
A) Kruttner S, Traunmuller L, Dag U, Jandrasits K, Stepien B, Iyer N, Fradkin LG, Noordermeer JN, Mensh BD, Keleman K. 2015. Cell Rep 11: 1953-1965
B) Felipe Delestro online portfolio
C) Khan MR, Li L, Perez-Sanchez C, Saraf A, Florens L, Slaughter BD, Unruh JR, Si K. 2015. Cell 163: 1468-1483
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4.3.2. CamkII: a long-term player in memory

CamkII is the most abundant protein present at the post-synaptic sites and represents 2% of the total hippocampal protein content (Erondu and Kennedy 1985; Hudmon and Schulman 2002). In mammals, CamkII has been shown to modify signalling cascades required for LTP (Nicoll 2017) and to be essential for hippocampal learning and memory (Giese et al. 1998; Yamagata et al. 2009; Zalcman et al. 2019). Strikingly, the molecular mechanism underlying CamkII activation can be seen as a molecular memory process that includes a first activity-induced acute activation followed by an autonomous and persistent activation loop (Figure 7). In fact, CamkII activation requires activity bursts in the form of calcium spikes (Ca^{2+} bound to Calmodulin) to perform a first auto-phosphorylation on threonine 286, but then remains autonomously active for a certain amount of time (Zalcman et al. 2018) (Figure 7C). The auto-phosphorylation step is essential for two reasons: first, it impedes the kinase to revert to its previous inactive conformation; second, it decreases the affinity for the Ca^{2+}/CaM complex, permitting a Ca^{2+}-independent activation of CamkII (Hudmon and Schulman 2002; Zalcman et al. 2018).

CamkII holoenzyme is a dodecamer composed of two rings of six subunits each. Subunits can in mammals be encoded by one of several CamkII genes and end up being all phosphorylated and active following the first stimulus (Srinivasan et al. 1994; Brocke et al. 1999; Gaertner et al. 2004) (Figure 7B). Each subunit is composed of a N-terminal catalytic domain (responsible for the enzymatic activity of CamkII) followed by a regulatory domain (with important regulatory residues, such as the threonine 286, and the calmodulin-binding site), a linker and the association domain (which mediates the multimerization).

CamkII functions have been extensively studied at different levels in neurons. CamkII regulates several activity and plasticity related pathways, acting through diverse mechanisms: as a locally-activated kinase but also as a scaffold (Joiner and Griffith 1999; Nesler et al. 2016). Regulation of the Eag potassium channel by CamkII illustrates the first function. These two proteins can activate each other: Eag is phosphorylated by CamkII but at the same time can locally activate it (Wang et al. 2002; Sun et al. 2004), creating an interplay that likely modulates neuron excitability. Regarding the second function, CamkII can also be recruited and bound by diverse proteins that allow its localization, such as NMDA NR2B subunit (Strack and Colbran 1998; Bayer et al. 2001), F-actin (Shen et al. 1998; Khan et al. 2016) and synapsin (Benfenati et al. 1992) and thus have a more structural role. In mouse, AMPAR hippocampal synaptic transmission requires CamkII kinase activity, whereas NMDAR synaptic transmission does not, but relies on CamkII interaction domain (Incontro et al. 2018). Furthermore, in hippocampal neurons, CamkII translocation to dendrites has been reported to promote proteasome redistribution in post-synaptic sites (Bingol et al. 2010). There, CamkII translocation, but not its kinase activity, is essential for activity-dependent degradation of poly-ubiquinated proteins (Bingol et al. 2010).

Functionally, CamkII has long been known for its role in learning and memory establishment (hence the title of “unforgettable kinase” given by Leslie Griffith,) (Griffith 2004), but evidence also starts arising of its critical role in memory storage and maintenance (Rossetti et al. 2017; Vigil and Giese 2018). For instance, affecting CamkII function leads to erasure of pre-established memories after fear conditioning in mouse (Cao et al. 2008).

In *Drosophila*, only one *CamkII* gene is present and this gene is alternatively spliced in 8 isoforms, which all differ in the region separating the regulatory and association domains (Griffith and Greenspan 1993). Nevertheless, all the functional domains are much conserved throughout evolution as well as the key residues crucial for the activation of the enzyme. Furthermore, altering CamkII function leads to altered behavioural and structural plasticity, impacts neuronal excitability and intrinsic properties of neurons and affects neuronal cytoskeletal dynamics (Griffith et al. 1993; Park et al. 2002; Andersen et al. 2005; Malik and Hodge 2014; Nesler et al. 2016; Kuklin et al. 2017).

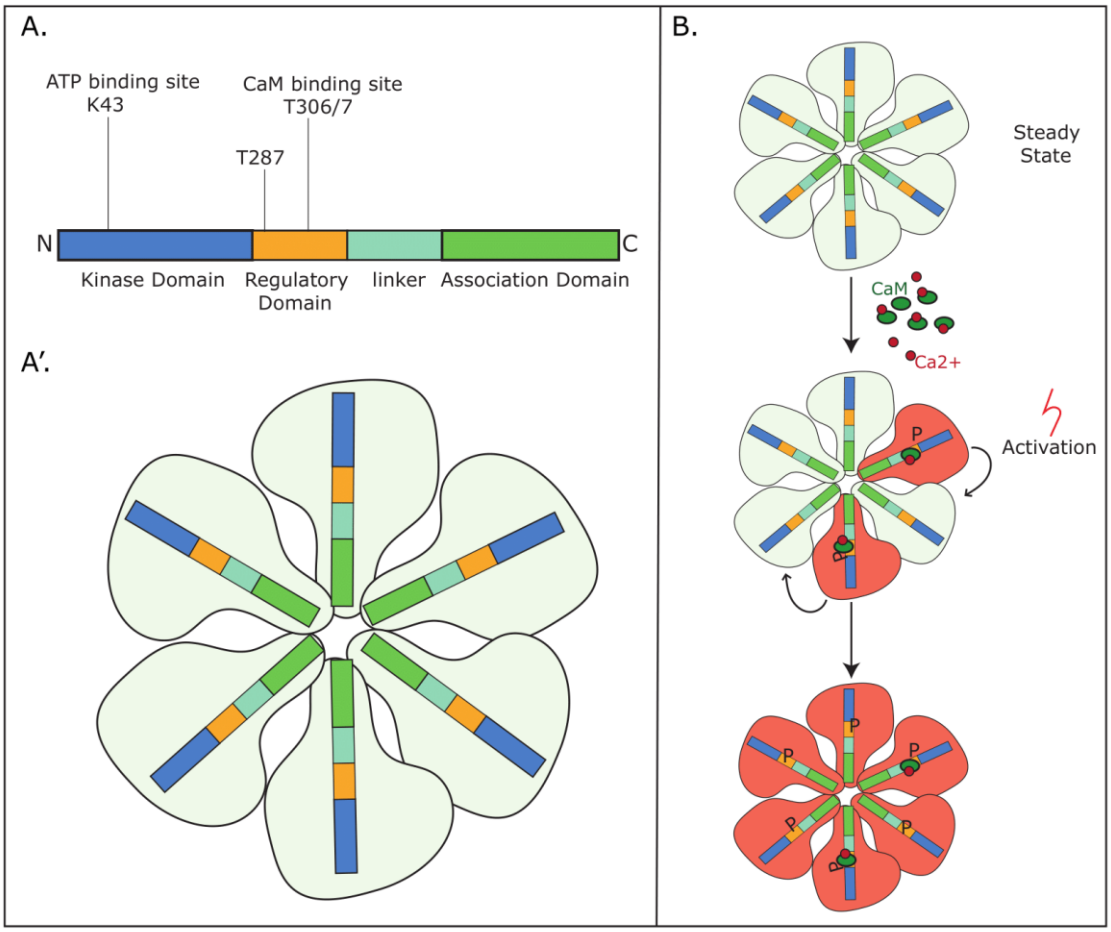

Figure 7. CamkII structure and activation mechanism.
A) CamkII is constituted by an kinase domain (blue), followed by a regulatory domain (orange), a linker (light green) whose length is variable and an association domain (green) which allows the multimerization of the different subunits. Key residues are shown in the different domains (positions numbered according to the Drosophila CamkII sequence, which is shifted of one aminoacid compared to vertebrates). A') CamkII is organized as a holoenzyme composed of two rings of six subunits each, where the kinase domain is exposed to the outside and the association domains interact forming a central hub.
B) CamkII activation mechanism. Upon increase in intracellular Ca2+, Ca2+ ions bind and activate Calmodulin (CaM). The Ca2+:CaM complexes bind CamkII subunits, inducing CamkII autophosphorylation on T287. Once these subunits are active, phosphorylation of the neighbouring subunits is induced too, generating a CamkII form able to function independently of Ca2+.

5. Neuronal RNP Granules

Transcripts to be localized do not travel naked toward their destination. They are coated by various regulatory RBPs and packaged into supramolecular assemblies called RiboNucleoProtein (RNP) granules. These macromolecular complexes exhibit peculiar features such as the lack of a membranous boundary and can contain up to hundreds of proteins. Although their size is highly variable, their diameter ranges in the order of hundreds of nanometres.

5.1 setting the scene

RNP granules can be found in different cellular compartments: the nucleus, the nucleolus and the cytoplasm (Figure 8). In the nucleus, paraspeckles, Cajal Bodies (CBs) and small nuclear RNPs (snRNPs) are found (Figure 8). Paraspeckles are nuclear bodies involved in gene expression regulation by sequestering RNA and proteins in the nucleus (Fox et al. 2018). Their structural backbone is a long non-coding RNA (lncRNA) present in their core, around which a shell containing distinct RBPs forms (Naganuma et al. 2012; Fox et al. 2018). Cajal Bodies, on the other hand, consist of proteins such as coilin, fibrillarin and SMN and contain target snRNAs that are enzymatically modified therein (Lafarga et al. 2017). Another important class of nuclear RNP granules is the one composed by the snRNPs, 5 Uridin-rich RNPs characterized by a common Sm protein set that binds U-rich noncoding RNAs and is the core component of the spliceosome (Papasaikas and Valcarcel 2016; Singh and Singh 2019). In the nucleolus, RNP granules form too (Figure 8). The small nucleolar RNPs (snoRNPs) contain RBPs, enzymes and small nucleolar RNAs (snoRNAs), a class of molecules able to drive chemical modifications of other RNAs such as rRNAs, tRNAs, snRNAs and mRNAs (Puerta 2008; Angrisani et al. 2014).

The cytoplasmic ensemble of RNP granules is probably the most heterogenous one (Figure 8). Among the cytoplasmic RNP granules, processing bodies (P-bodies) and stress granules (SGs) have been largely studied. While P-bodies are constitutive and thought to store mRNAs to prevent their translation, SGs do form in response to stress, for instance, to heat shock, ageing or oxidative stress (Buchan and Parker 2009; Kedersha et al. 2013; Hubstenberger et al. 2017; Guzikowski et al. 2019). Transport RNP granules, specialized in storage and transport, are also part of the RNP cytoplasmic pool. These RNPs can contain or transiently bind motor proteins to move along cytoskeletal tracks and transport specific RNAs towards the synaptic sites, either in dendrites or axons (Knowles et al. 1996; Bassell et al. 1998; Krichevsky and Kosik 2001; Zhang et al. 2001; Zhang et al. 2003; Kanai et al. 2004; Richter 2004; Kiebler and Bassell 2006; Wells 2006; Mitsumori et al. 2017; De Graeve and Besse 2018).

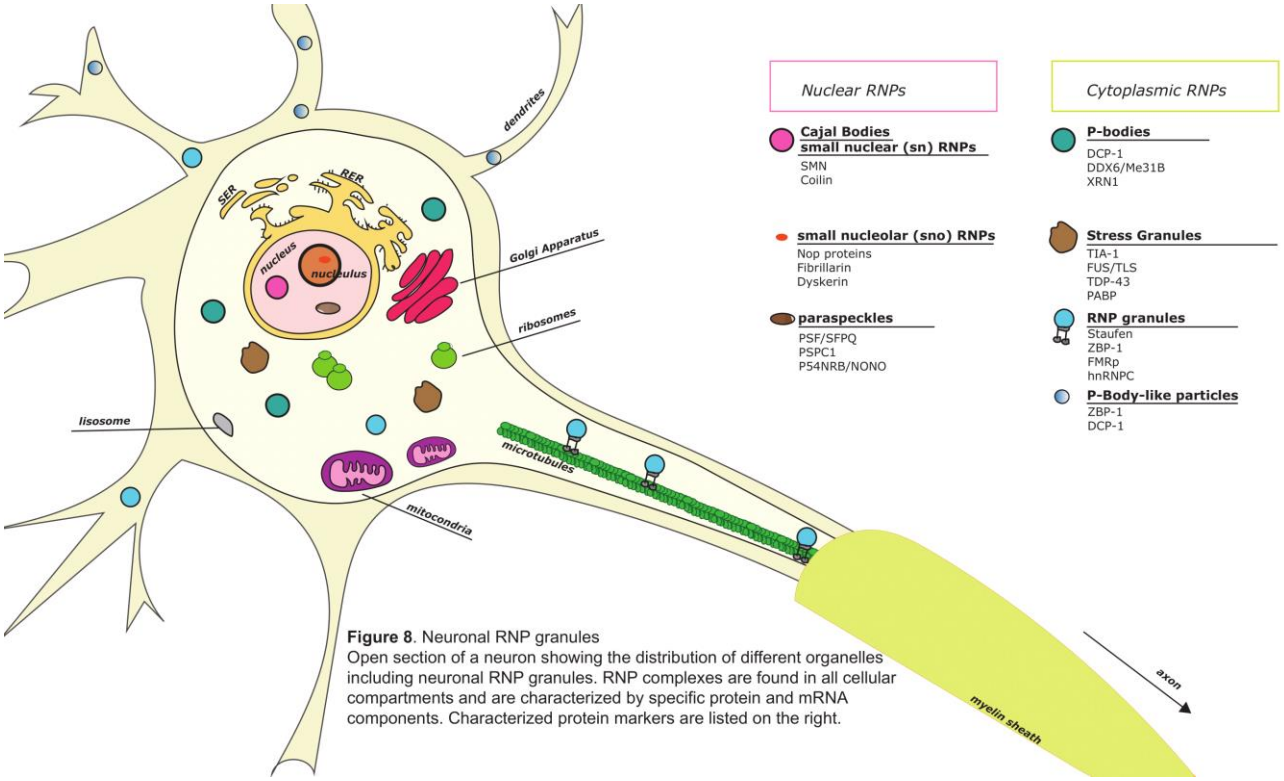

Figure 8. Neuronal RNP granules
Open section of a neuron showing the distribution of different organelles including neuronal RNP granules. RNP complexes are found in all cellular compartments and are characterized by specific protein and mRNA components. Characterized protein markers are listed on the right.

5.1.1. Interaction between RNP granules: a fair trade

Although distinct RNP granules share many protein components, they have also been reported to show differential enrichment in proteins involved in certain processes (Figure 8). For instance, mammalian P-bodies and SGs do share components (*e.g.* AGO2 and UPF1 in human) (Jain et al. 2016; Hubstenberger et al. 2017; Guzikowski et al. 2019), but at the same time contain specific proteins (*i.e.* Tia1, G3BP, eiF3 and ATXN2 for SGs and YTHDF2 and LSM14A for P-bodies) (Kedersha and Anderson 2007; Guzikowski et al. 2019).

In addition, a close relationship seems to interconnect P-bodies with transport RNP granules in neurons. For instance, in rat hippocampal and hypothalamic neurons, dendritic P-body-like complexes (Figure 8) have been observed: these granules are positive for P-body markers such as DCP-1 but also for other proteins such as FMRP, and they are motile in the dendrites, yet excluded from axons (Cougot et al. 2008). Accordingly, markers of P-bodies (Dcp-1, DDX6) and RNP granules (Staufen, FMRp) were also reported to colocalize by Barbee and collaborators in *Drosophila* cultured motor neurons (Barbee et al. 2006). However, when Zeitelhofer and colleagues looked at rat hippocampal neurons, they could not observe the same trend: DDX6/p54 and DCP1 – the P-body markers – did not show significant colocalization with the RNP granule markers used in this work (*e.g.* Staufen1/2 and Barentz) (Zeitelhofer et al. 2008). Nevertheless, the authors showed that P-bodies and RNP granules are frequently found located very close to each other along the dendrites, in an active and dynamic process termed docking (Zeitelhofer et al.

2008). During docking, P-bodies and transport RNP granules are tightly juxtaposed for at least one minute, and experience cycles of dissociation/association (Zeitelhofer et al. 2008). This highly specific event – which is not observed with organelles other than transport RNP granules and Stress Granules – could be a means for specific granules to exchange components and rearrange their content.

Importantly, transport RNP granules also likely exhibit a range of compositions. For instance, the purification of Staufen and Barentsz transport RNP granules from rat brain reported that, while one third of the interactors was shared (Fritzsche et al. 2013), other components probably provide specific regulatory features. In this regards, Barentsz granules were shown to contain components of the Exon Junction Complex (EJC), absent in Staufen complexes (Fritzsche et al. 2013).

5.2 Assembly of RNP granules

The unique property of RNP granules to assemble and disassemble very rapidly in response to cellular crowding and environmental stimuli, has recently raised a lot of interest in the processes underlying RNP granule assembly.Their dynamics can rely on two main connected axes: a biophysical one, based on the liquid-like behaviour of these complexes and their ability to transition in higher-order structures in specific conditions; and a molecular one, which evaluates the protein and RNA and their interaction with each other at the basis of this coalescence (Figure 9).

The two following paragraphs summarize the principles related to RNP granule assembly, relying on research performed in different cell types or *in vitro*. Nevertheless, these discoveries are common for all RNP complexes and can thus be applied to neuronal RNP granules.

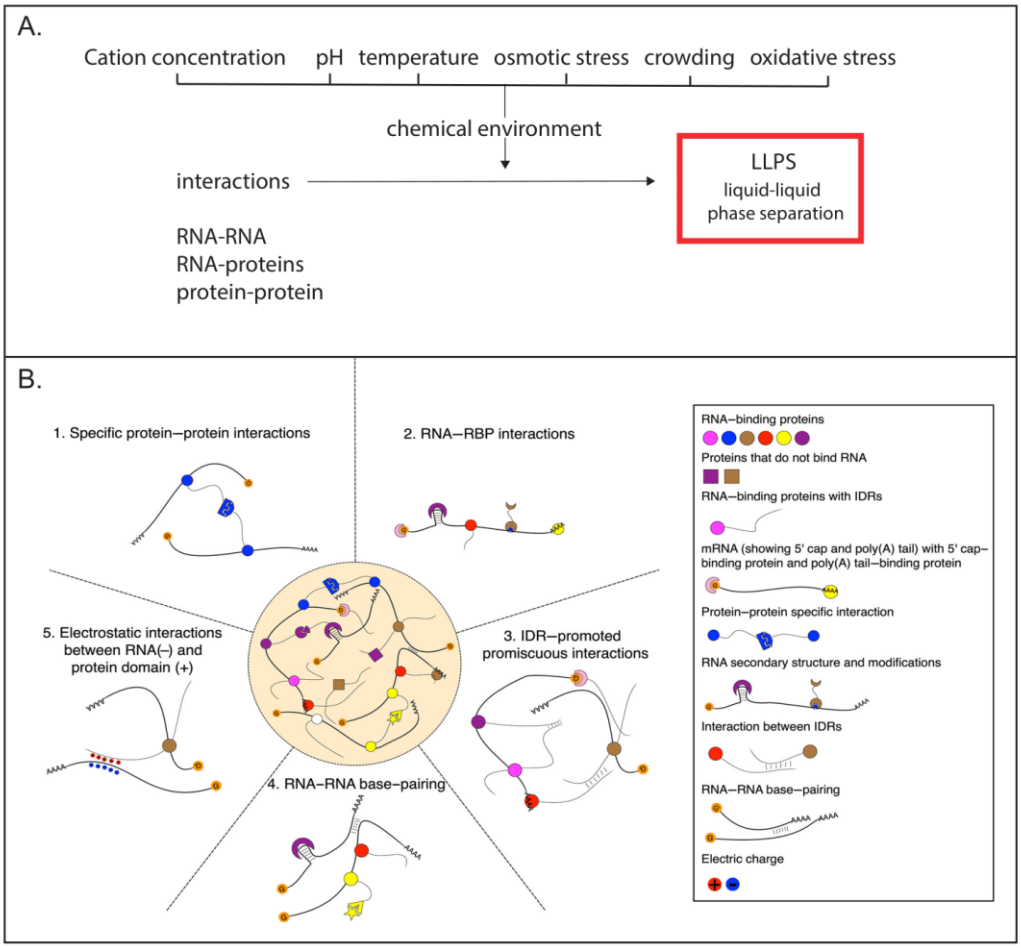

Figure 9. Driving forces guiding RNP granule assembly.
A) RNP granules are formed via a biophysical process called LLPS (Liquid-liquid phase separation) that is nucleated by the establishment of a network of protein and/or RNA interactions. LLPA is modulated by the state of the cell and its chemical environment. B) Diverse RNA-RNA, RNA-protein and protein-protein interactions contribute to RNP phase transition. The nature of these interactions is various as they can be strong or weak, specific or promiscuous.
References:
B) Guzikowski AR, Chen YS, Zid BM. 2019. Wiley Interdiscip Rev RNA 10: e1524.

5.2.1. Phase separation drives RNP granule assembly

RNP granules can assemble within minutes in the cell cytoplasm, meaning that the different granule components, normally diffuse in the highly crowded cytoplasmic environment, can rapidly coalesce. Furthermore, RNP granules can show liquid-like properties including fusion and rapid relaxation to spherical shapes, dynamic assembly/dissolution, capability to deform in response to shear stress and dynamic rearrangement. TDP-43 RNP granules exhibit all these features in different neuronal types (Gopal et al. 2017; Gasset-Rosa et al. 2019) and furthermore re-arrange their content via fusion and fission events (or docking, see § 5.1.2.).

The biophysical process underlying RNP granule assembly is called liquid-liquid phase separation (LLPS). This demixing of cytoplasm components occurs when a critical concentration is reached and has a strong thermodynamic drive. In fact, the energy of the interaction between macromolecules must be higher that the loss in entropy that the demixing causes, leading to an energetically favourable event that does not need cellular energy. This physical phenomenon - extensively studied *in vitro* - is reversible and sensitive to changes in pH, temperature, cation concentration, crowding agents and ionic strength (Hyman et al. 2014; Courchaine et al. 2016; Sfakianos et al. 2016; Alberti 2017; Standart and Weil 2018) (Figure 9A).

Importantly, the dynamic liquid-like state of organelles can vary depending on different conditions and transition to solid states. This latter case has been linked to the formation of solid-like aggregates in neurodegenerative diseases, such as ALS (Amyotrophic Lateral Sclerosis), Alzheimer Disease and Tauopathies (Gabanella et al. 2007; Lenzken et al. 2014; Li et al. 2014; Moschner et al. 2014; Murakami et al. 2015; Donlin-Asp et al. 2017; Price et al. 2018; Ravanidis et al. 2018).

5.2.2. A dense interaction network as the basis for RNP granule assembly

Establishment of a dense network of weak RNA-RNA, protein–protein and RNA-protein interactions represents the key trigger of liquid-liquid phase separation and highly contributes to the dynamics and flexibility of RNP assemblies (Figure 9B). Within an RNP granule, proteins establish these interactions using both folded domains and intrinsically disordered domains, creating a dense yet dynamic network of connections with other proteins or RNA molecules. On one hand, “classically” structured domains are believed to mediate specific interactions. Notably, proteins can establish energetically favourable protein-protein interactions (Banani et al. 2017) by exhibiting Multiple Copies of Interaction Domains (MCIDs) that allow the establishment of protein-protein interactions (Figure 9B). These interactions can range from being relatively weak to being comparable to covalent bindings (Li et al. 2012; Su et al. 2016). On the other hand, intrinsically disordered regions, enriched in RBPs (Hennig et al. 2015; Harrison and Shorter 2017), and

strikingly overrepresented in phase-separating proteins (Mitrea and Kriwacki 2016), are involved in interactions with a broad range of different partners *via* multivalent weak bindings (such as electrostatic, dipole-dipole, π-π) (Pak et al. 2016) (Figure 9B).

Phase separation studies have shown that these regions tend be both necessary and sufficient for phase-transition *in vitro* (Elbaum-Garfinkle and Brangwynne 2015; Patel et al. 2015; Zhang et al. 2015) and can even form hydrogels (Han et al. 2012; Kato et al. 2012). Accordingly, PLDs can be essential for RNP granule formation *in vivo*. Andrusiak and colleagues have very recently shown that the Carboxyl-terminal PLD of Tia1 is necessary and sufficient to form Tia1 granules in *c. elegans* PLM mechanosensory axons (Andrusiak et al. 2019). Strikingly, a subset of disordered regions called Prion-Like Domains (PLDs) displays a biased composition characterized by a high content of uncharged polar aminoacids and glycines. These residues can be organized either in stretches of single aminoacid repeats or with peculiar aminoacidic combinations (Simon and Hancock 2009) (Figure 9B). Biased aminoacid compositions might represent a means to drive assembly of membraneless compartments, as specific aminoacids have been shown to promote phase transition (Wang et al. 2018).

Although the contribution of protein networks to phase-transition is essential, increasing evidence highlights the importance of RNA-RNA interaction in RNP granule formation, shedding new light on a process that has been historically protein-centric (Figure 9B). RNA can both act as a molecular seed, triggering RNP nucleation, and positively or negatively tune phase transition depending on concentration (Schwartz et al. 2013; Langdon et al. 2018; Maharana et al. 2018; Yamazaki et al. 2018) (Figure 9B). Indeed, RNA can self-assemble independently of proteins *in vitro* (Van Treeck and Parker 2018; Van Treeck et al. 2018) (Figure 9B). The exact mechanisms by which RNA can trigger phase separation remain still unclear. Nevertheless, multivalent RNA-RNA interactions could act as seeds to further recruit subsets of RNAs/RBPs in specific conditions, as shown for NORAD lnRNA, which exhibits numerous binding sites for Pumilio proteins and hence acts as a platform to recruit cytoplasmic PUM proteins (Lee et al. 2016; Garcia-Jove Navarro et al. 2019). How would RNAs self-assemble? Secondary structure of RNAs is likely a crucial trigger of RNA-mediated interactions and was shown to (i) determine self-assembly of RNA and (ii) discriminate between RNAs that are contained in a molecular condensate or excluded from it (Langdon et al. 2018; Maharana et al. 2018). Very interestingly, modifications of RNAs – which further modulate RNA interactome - are emerging now as powerful means to modulate phase separation potential of mRNA (Ries et al. 2019).

5.3 RNP granules as sensors of external stimuli

5.3.1. RNP remodelling in response to activity

A remarkable feature of membraneless organelles is their ability to rapidly assemble, and reversibly reorganize or reset in response to external stimuli or environmental fluctuations. This unique property makes them particularly well suited for locally processing transient stimuli in distal neuronal compartments. In dendrites, for example, synaptic activation was shown to induce dramatic changes in the material properties of neuronal RNP granules. As first revealed by ultrastructural analysis, indeed, a transitioning of dense granules into less compact structures is observed upon KCl-induced neuronal activation (Krichevsky and Kosik 2001) (Figure 10A). As further shown by live-imaging experiments, such a reorganization is accompanied by changes in granule dynamics. While a decreased frequency of splitting and merging of *β-actin*-containing granules is observed upon KCl treatment of hippocampal neurons (Park et al. 2014), increased turnover of dendritic granule components was observed in FRAP experiments upon activation of the NMDAR receptor (Cougot et al. 2008). Remarkably, such activity-dependent changes in granule material properties are associated with a remodelling of granules thought to underlie translation activation. In cultured mammalian neurons, for example, chemical induction of long-term potentiation triggers a transient unmasking of the dendritically localized β-actin mRNAs that likely reflects their release from RNP granules (Buxbaum et al. 2014). mRNA unmasking occurs concomitantly to ribosome unmasking and *β-actin* local translation, suggesting that degranulation in response to activity may trigger the translational activation of mRNAs stored at synapses. Transient dissolution of neuronal RNP granules enriched in translational repressors has also been observed upon NMDAR stimulation and linked to the increased translation of associated dendritic mRNAs such as *CamkII-α* mRNA (Zeitelhofer et al. 2008; Baez et al. 2011) (Figure 10B). Interestingly, specific responses are observed in response to distinct stimuli: while Smaug-containing dendritic granules rapidly dissolve upon activation of ionotropic (NMDAR), but not metabotropic (AMPAR) glutamate receptors, FMRP-containing granules only dissolve upon activation of AMPAR (Baez et al. 2011; Luchelli et al. 2015). In these different paradigms, RNP granule dissolution is a reversible phenomenon, as reassembly is observed *ex vivo* in a timescale of dozens of minutes to hours (Zeitelhofer et al. 2008; Baez et al. 2011). Furthermore, re-assembly occurs independently of granule component synthesis (Baez et al. 2011), consistent with a role of phase behaviour in this dynamic de-condensation and re-condensation processes. Although this remains to be demonstrated *in vivo*, neuronal RNP granules may thus be able to undergo repeated cycles of assembly/disassembly, thus providing acute responses to dynamic neuron firing patterns or constantly changing environmental stimuli. Stimuli-induced changes in neuronal RNP granule properties are not only transient, as longer-term re-organization of granules has also been

reported, especially after repeated neuronal stimulations. Repeated KCl stimuli, for example, induce a structural rearrangement of dendritically localized granules manifested by an increased partitioning of distinct RBPs (e.g. FMRP and TDP-43) into common granules (Wang et al. 2008). Furthermore, long-term self-sustaining changes in the conformational state of granule-associated RBPs were shown to be induced by repeated cycles of neuronal stimulation (Si and Kandel 2016; Sudhakaran and Ramaswami 2017).

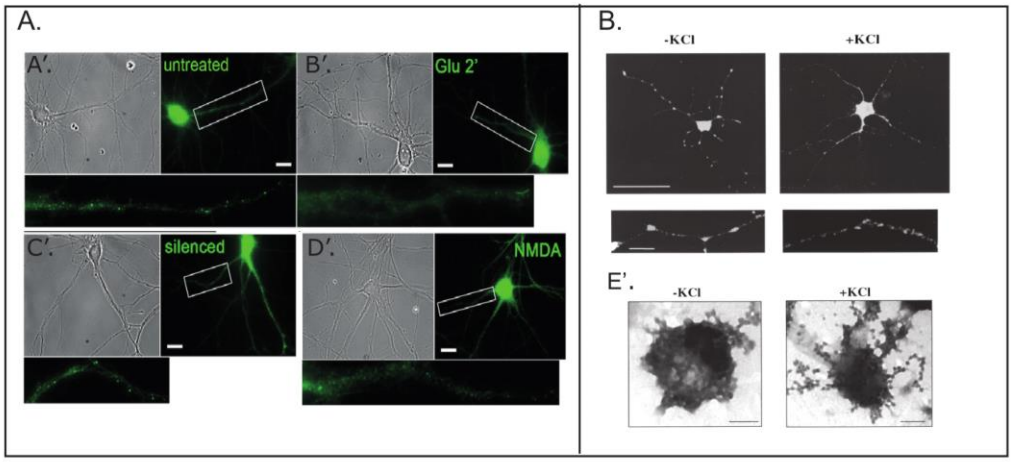

Figure 10. RNP granules disassemble in response to chemical stimulation.
A) DCP-1 dendritic P-bodies in mature hippocampal neurons disassemble in response to Glutamate (B') and NMDA (D') but are not affected by silencing drugs (TTX, CNQX, D-APV or strychnine) (C'). Scale bar: 10 µm.
B) RNP granules (staining for ribosomal protein rpS6) disassemble upon 2h KCl treatment in cultured cortical neurons. RNA granules are visible by electron microscopy in E' and show a less compact structure with ribosomes going out from the granules. Scale bar in B: 50 µm, scale bar in E': 100 nm.
References:
A) Zeitelhofer M, Karra D, Macchi P, Tolino M, Thomas S, Schwarz M, Kiebler M, Dahm R. 2008. Dynamic interaction between P-bodies and transport ribonucleoprotein particles in dendrites of mature hippocampal neurons. J Neurosci 28: 7555-7562.
B) Krichevsky AM, Kosik KS. 2001. Neuronal RNA granules: a link between RNA localization and stimulation-dependent translation. Neuron 32: 683-696.

5.3.2. mechanistic insights into RNP remodelling

Stimuli-driven changes in the properties of neuronal RNP granules are associated with changes in the translational profile of granule-associated mRNAs, namely a switch from translational repression to translational activation. How is such a switch molecularly triggered? Two main models have been proposed to account for local translation activation. In the first one, external stimuli induce the release of translational repressors from their target mRNAs, enabling the local recruitment of released transcripts to active ribosomes. In the second one, external stimuli induce conformational changes that convert translational repressors into translation activators, thus transforming granules into crucibles promoting active translation. Consistent with the first model, phosphorylation of ZBP1, that assembles with its target *β-actin* mRNA also in axons, is induced during axon navigation by the guidance molecule Sonic Hedgehog (Shh) (Lepelletier et al. 2017). Phosphorylation of ZBP1 both decreases its affinity for *β-actin* mRNA (Huttelmaier et al. 2005) and promotes initiation-dependent translation of β-actin synthesis in the axons of cultured neurons (Huttelmaier et al. 2005; Sasaki et al. 2010). Functionally, it is required *ex vivo* for Shh-mediated growth cone turning and *in vivo* for guidance of commissural axons (Sasaki et al. 2010; Lepelletier et al. 2017). Whether ZBP1 phosphorylation triggers the disassembly of β-actin-containing granules localized at the axon tip remains to be demonstrated, but increased splitting:fusion ratio may be induced, as shown for dendritically localized *β-actin* granules in response to external stimuli (Buxbaum et al. 2014). The second mechanism is nicely illustrated by the oligomerization of CPEB/Orb (see § 4.3.1.). This oligomerization is driven by a phosphorylation event that stabilizes the low-abundance Orb2a isoform responsible for the nucleation of amyloid-like oligomers and is controlled by a tight balance between localized kinases and phosphatases (White-Grindley et al. 2014).

Together, these examples have highlighted the importance of PTMs in switching the activity and the conformation of neuronal granules. They, however, likely represent only the top of the iceberg, as *in vitro* studies have indicated that PTMs can modulate the nucleation and the material properties of phase-separated molecular condensates (Hofweber et al. 2018), as well as the translational repression activity of neuronal RBPs (Tsang et al. 2019). Phosphorylation has been proposed to favour aggregate disassembly (Bogaert et al. 2018), while SUMOylation has been implicated in RNP formation (Gao et al. 2008). In addition, ubiquitin has also been shown to modulate phase transition by disrupting multivalent bindings (Dao et al. 2018). The repertoire of PTMs involved in neuronal RNP granule regulation is likely to extend further, as illustrated by the recent discovery that mGlu5R-induced sumoylation of the FMRP protein promotes the dissociation of FMRP from dendritic RNP granules (Khayachi et al. 2018).

Thus, neuronal cells may take advantage of the large variety of available PTMs to trigger reversible, granule-specific, behaviour in response to a combination of external cues. Although physiological evidence is still lacking, PTM-independent switch mechanisms are likely to also be at play in neuronal cells. For example, changes in cation concentration or pH have been reported in distinct neuronal compartments in response to local stimuli (Chesler and Kaila 1992; Parekh 2008; Sutherland et al. 2014), and were shown to dramatically modulate phase behavior *in vitro* (Nott et al. 2015; Franzmann et al. 2018; Rayman et al. 2018; Onuchic et al. 2019). Variations in Ca^{2+} concentration, in particular, induce a switch-like behavior between heterotypic and homotypic RNA droplets, thus dictating component partitioning and droplet composition (Onuchic et al. 2018).

6. The Imp RNA-binding protein family

The IMP RBP family was identified in humans about 20 years ago (Nielsen et al. 1999) as composed by three members able to bind to the *Insulin-like growth factor II (IGF)* mRNA (IGF2BPs – IGF2BP1/2/3). These proteins were found to be ortholog to the chicken ZBP1, the mouse CRD-BP, the *Xenopus* Vg1RBP/Vera and the *Drosophila* Imp (Nielsen et al. 1999; Nielsen et al. 2000) (Table 1) (Figure 11A).

Imp proteins throughout species share about 56% of similarity, notably concerning their RNA binding domains, suggesting a common biochemical function (Bell et al. 2013). Human IMP proteins exhibit two RNA-Recognition Motifs (RRM) and four K-Homology (KH) domains (Nielsen et al. 1999) (Figure 11B). These latter ones are more conserved evolution-wise and within the family (Nielsen et al. 1999). Conservation studies have shown that IMP RNA binding domains cluster together two by two (the two RRMs, the KH1 and 2 and the KH3 and 4, respectively), suggesting that they might function as di-domains (Git and Standart 2002).

IMP proteins are mainly cytoplasmic and assemble into hundreds of nanometre large RNP granules (Nielsen et al. 2002). The repertoire of mRNAs bound by IMP proteins in human has been studied in different cell lines, such as HEK cells, pluripotent stem cells but also glioblastoma cell lines using PAR-CLIP and eCLIP analysis (Hafner et al. 2010; Conway et al. 2016; Degrauwe et al. 2016; Ravanidis et al. 2018). This inventory accounts for up to thousands of protein-coding transcripts (Hafner et al. 2010). These studies have identified CAUH (H = A, U, or C) and CA-rich motifs as IMP target sequences in transcript 3'UTRs, although one of the human IMP protein – IMP3 – rather recognizes sequences in the coding region (Hafner et al. 2010; Conway et al. 2016; Degrauwe et al. 2016; Ravanidis et al. 2018). Strikingly, human IMP proteins were recently shown to recognize RNAs marked by N^6-methyladenosine modifications (E.g. *myc*), thus revealing that

IMP proteins can be defined as m^6a-readers (Huang et al. 2018). Specifically, IMPs were shown to recognize GG(m^6A)C sequences in human tumoral cell lines (Huang et al. 2018).

IMP protein family member	**Species**	**described mRNA target**	**reference**
IGFBP1-3	*Homo sapiens*	*IGF-II*	Nielsen et al, 1999
Vg1RBP/Vera	*Xenopus laevi*	*Vegetal-1*	Yaniv and Yisraeli, 2002
ZBP-1	*Gallus gallus*	*β-actin (via zipcode)*	Ross et al, 1997
CRDBP	*Mus musculus*	*myc*	Doyle et al, 1998
Imp	*Drosophila melanogaster*	*profilin (chickadee)*	Medioni et al, 2014

Table 1. Imp protein family members across species, with their first described mRNA target.

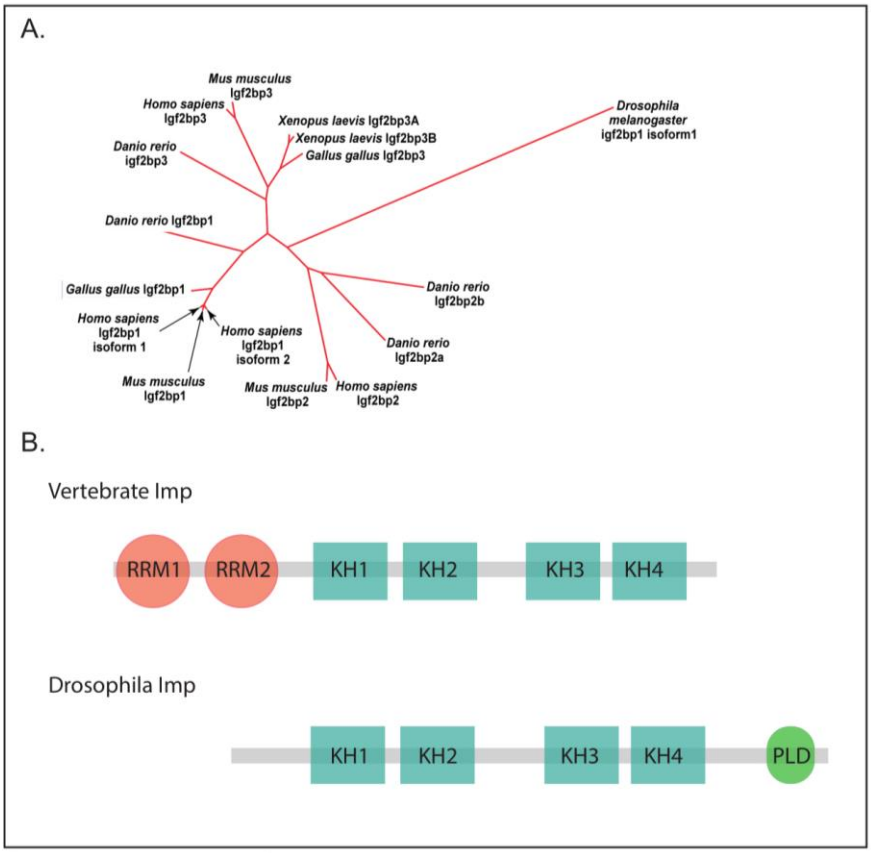

Figure 11. Imp protein family
A) philogenetic tree showing Imp protein family orthologs. B) Imp proteins in vertebrates exhibit two RNA Recognition Motifs (RRMs) and four K-Homology (KH) domains. The main *Drosophila* Imp protein does not possess any RRM, whereas all the four KH domains are conserved. In addition, dImp presents a C-terminal PLD, which is absent in the other species.
References:
A) Gaynes JA, Otsuna H, Campbell DS, Manfredi JP, Levine EM, Chien CB. 2015. PLoS One 10: e0134751.

6.1. IMPs in neurons

IMP proteins were shown to control different aspects of nervous system function, ranging from early neuronal development to neurodegenerative processes.
For example, it was recently shown in humans that plasma levels of IMP2 can be associated with neurodegenerative conditions such as Alzheimer Disease and brain atrophy and that IGFBP-2 is in fact able to interact with biomarkers of these two diseases and correlates with decrease hippocampal volume (Lane et al. 2017).

IMP proteins are also required during early development. In murine models, Imp deletion impacts the brain volume as a result of alterations in neural stem cell (NSC) differentiation (Nishino et al. 2013). Imp exerts an orchestrated control on NSCs also in *Drosophila*, notably by regulating neuronal fates (Liu et al. 2015). Imp proteins can also regulate other processes during early neuronal development, as shown for neural crest migration In *Xenopus* and synaptogenesis in *Drosophila* (Yaniv et al. 2003; Boylan et al. 2008).

A further proof of IMP protein involvement in nervous system development relies on evidence connecting Imp to dendrite and axons morphogenesis, function and guidance (Perycz et al. 2011; Kalous et al. 2014; Gaynes et al. 2015; Preitner et al. 2016; Lepelletier et al. 2017). For instance, *in vivo* expression of a dominant negative truncated form of Vg1RBP (*X. laevis* Imp) reduced axon branching in retinal *Xenopus* neurons while axon outgrowth was found to be regulated by Igf2bp1 in *Danio rerio* (Kalous et al. 2014; Gaynes et al. 2015).

A question arises: how might Imp regulate these different processes during neuronal development? Although few direct targets of Imp have been functionally characterized, it likely controls various processes through its capacity to bind, store in RNP complexes and regulate various mRNAs. For example, commissural axon guidance is a Sonic hedgehog-mediated process which requires ZBP1-dependent local translation of *β-actin* mRNA *in vivo* (Lepelletier et al. 2017) (see § 5.3.3.) and a similar mechanism (Igf2bp1-dependent transport and local regulation on *β-actin*) has been proposed to underly Igf2bp1-dependent RGC axon outgrowth in Zebrafish (Gaynes et al. 2015). Another example is the Imp-dependent post-transcriptional regulation of *chinmo* mRNA, a mechanism that underlies the temporal neural fate specification of *Drosophila* mushroom body (Liu et al. 2015).

6.2. Imp RNP complexes and function in mRNA fate determination

Imp proteins assemble into RNP complexes, detected as granules localizing in dendrites and axon growth cones (Zhang et al. 2001; Tiruchinapalli et al. 2003). These granules undergo active, bi-directional transport along MT (Nalavadi et al. 2012; Medioni et al. 2014; Urbanska et al. 2017).

The Imp RNP granule composition analysis in HEK cells revealed that these granules are unique entities different from SGs, P-Bodies or hStaufen granules (Jonson et al. 2007). The list of components gave many insights about their functional state: they are enriched in RBPs such as several hnRNPs and RNA helicases, they comprise Exon Junction Complex components, PABP and CBP80 but they do not contain translational initiation factors or ribosomal subunits (Jonson et al. 2007; Weidensdorfer et al. 2009). All this suggests that the mRNAs contained within these granules are not translationally active but rather stored as repressed.

The mechanisms through which Imp proteins have been proposed to regulate their target mRNAs are:

1) Translational regulation. ZBP1 was shown in reconstituted systems and in cells to inhibit translation initiation of *β-actin* by blocking the recruitment of the 80S ribosomal subunit (Huttelmaier et al. 2005). In axons, release of ZBP1 through Y396 phosphorylation is required for cue-induced local translation of *β-actin* mRNA (see § 5.3.3.) (Huttelmaier et al. 2005; Welshhans and Bassell 2011; Lepelletier et al. 2017). Translation activation of Imp target mRNAs was also found to be promoted by mTORC1-dependent phosphorylation of IMP1 in mouse fibroblasts, where IMP1 S181 phosphorylation – contrarily to the previous example - is associated to an increased binding and promotion of translational initiation of *IGF2* mRNA (Dai et al. 2013).

2) Stabilisation and protection of mRNAs from degradation. Binding of IMP proteins to target mRNAs can protect these transcripts from decay. The binding of Imp to c-*myc* mRNA, for instance, protects c-*myc* from degradation by polysome-associated endonucleases (Sparanese and Lee 2007) in human cells. Imp proteins can protect mRNAs also from the action of miRNAs, as was shown with *Ccdn2* and *Hgma* mRNAs, targets of let7-miRNA in mice neural stem cells (Nishino et al. 2013).

6.3. *Drosophila* Imp

The *Drosophila* genome codes for only one IMP family member: dImp. In flies, the first described protein isoform lacks the two RRMs found in vertebrates but still carries the four conserved K-homology domains plus a prion-like domain in the carboxy-terminal region (Figure 11B). Similarly to IMP family members in other species, dImp assembles with other proteins and RNA molecules in cytoplasmic RNP granules, whose RNA target repertoire has been characterized in cultured S2R cells by Hansen and collaborators in 2015. This work, based on iCLIP and PAR-CLIP experiments, demonstrated that Imp binding sites were mainly found within the transcript 3'UTRs, notably in UA- and CA-rich regions (Hansen et al. 2015). Interestingly, among the mRNAs found as Imp targets,

several are involved in actin cytoskeleton dynamics (e.g. *Cdc42*, a conserved polarity player, and *chickadee*, the fly homolog of *profilin*) or with neuronal development and differentiation (e.g. *Hrp48*, *pAbp* and *squid*, coding for three RBPs).

However, if on one hand, the RNA targets of *Drosophila* Imp have been studied, the atlas of Imp protein interactome is still missing, as to date, few protein interactors of dImp have been characterised, such as Squid and Hrp48 in the oocyte (Geng and Macdonald 2006).

Flies homozygous for *imp* are lethal and die before hatching in the pharate state, suggesting that Imp plays a pivotal function for the organism survival. In fact, Imp is widely expressed throughout cell types and has been involved in several processes such as oogenesis and spermatogenesis where it acts by controlling RNA stability and localization (Geng and Macdonald 2006; Fabrizio et al. 2008; Toledano et al. 2012). However, Imp is also highly expressed in the *Drosophila* brain, in both the central and the peripheral nervous systems (Adolph et al. 2009). Here, Imp plays a relevant function at different levels: for instance, Imp function is required for correct synaptogenesis in the neuromuscular junction (Boylan et al. 2008) and is also important for the development of the Mushroom Body, the center for learning and memory in flies.

6.3.1. dImp in Mushroom Bodies

In mammals, the structures involved in memory formation and storage are the hippocampus and the cortex (Redondo and Morris 2011). In particular, the hippocampus has been defined as the seat of memory formation whereas the storage of memory would be happening at the level of the temporal cortex.

In the fruit fly, structures called Mushroom Bodies can be considered as analogous to the hippocampus and are critical for learning and memory (Roman and Davis 2001; Waddell and Quinn 2001; Akalal et al. 2006). Complete ablation of the Mushroom Bodies (induced either pharmaceutically or genetically) does not induce lethality but leads to learning impairment (de Belle and Heisenberg 1994). These structures are composed of different neuronal populations, called respectively alpha/beta (α/β), alpha'/beta' (α'/β') and gamma (γ), that are generated at different stages during development (Lee et al. 1999) (Figure 6B). Remarkably, on one hand, MBs receive several inputs, as sensory inputs deriving from hundreds of olfactory projection neurons and modulatory inputs coming from dopaminergic neurons projecting in different regions of the MB (Aso et al. 2014; Keleman et al. 2012; Cervantes-Sandoval et al. 2017; Cognigni et al. 2018; Zhao et al. 2018). On the other hand, MB neurons themselves act as input neurons convaying signals onto the different classes of Mushroom Body Output Neurons (MBONs) and regulating activity therein (Aso et al. 2014; Cognigni et al. 2018; Zhao et al. 2018). Intriguingly, the tripartite neuronal

circuits established seem to be essential to trigger complex fly behaviors and memory formation (Cognini et al. 2018; Zhao et al. 2018).

Imp role in the development of MBs is complex and exerted at different levels by controlling the fate of different mRNAs. In the MB neuroblast, Imp regulates the expression of E93, a steroid-hormone-induced transcription factor (Pahl et al. 2019) that controls neurogenesis termination. The levels of Imp – which show a gradient opposite to that of the RBP Syncrip (Syp) – correlate with a ping-pong post-transcriptional regulation of the transcription factor Chinmo, thereby controlling MB lineage (Liu et al. 2015). Remarkably, Imp is also important in post-mitotic neurons, for the remodelling of MB γ neurons. This specific MB neuronal subpopulation is generated during embryogenesis and then undergo a tightly regulated remodelling during metamorphosis, where the larval γ axonal branches degenerate and then re-grow to form adult-specific projections (Lee et al. 1999) (Figure 12A). Very interestingly, Imp localizes just in In γ neuron axons, whereas it is excluded from α/β axons and not expressed at all in the α'/β' neuronal population, suggesting a specific Imp function in γ neurons. Coherently, in *imp* mutant context, the regrowth and branching of adult γ neuron is specifically affected (Medioni et al. 2014) (Figure 12B-C), a process relying on the Imp target mRNA *chickadee/profilin* (Medioni et al. 2014) (Figure 12D).

This mRNA is bound directly by Imp *via* its 3'UTR and is assembled together with Imp in RNP granules that are formed in MB cell bodies and then transported to γ axons in a developmentally-regulated manner (Medioni et al. 2014; Vijayakumar et al. 2019) (Figure 12D). The transport of Imp RNP granules to axons relies on MTs and is dependent on the presence in Imp sequence of a prion-like domain (PLD) at its C-terminal extremity (Vijayakumar et al. 2019) (Figure 13A,B). Remarkably, Imp PLD interestingly challenges the current acknowledged function for intrinsically disordered regions since it is not required for RNP granules assembly (Vijayakumar et al. 2019) (Figure 13C). Indeed, CRISPR/Cas9-engineered flies endogenously expressing a form of Imp deleted of its PLD are homozygous viable and still able to form RNP granules, without any impact on their composition (Vijayakumar et al. 2019).

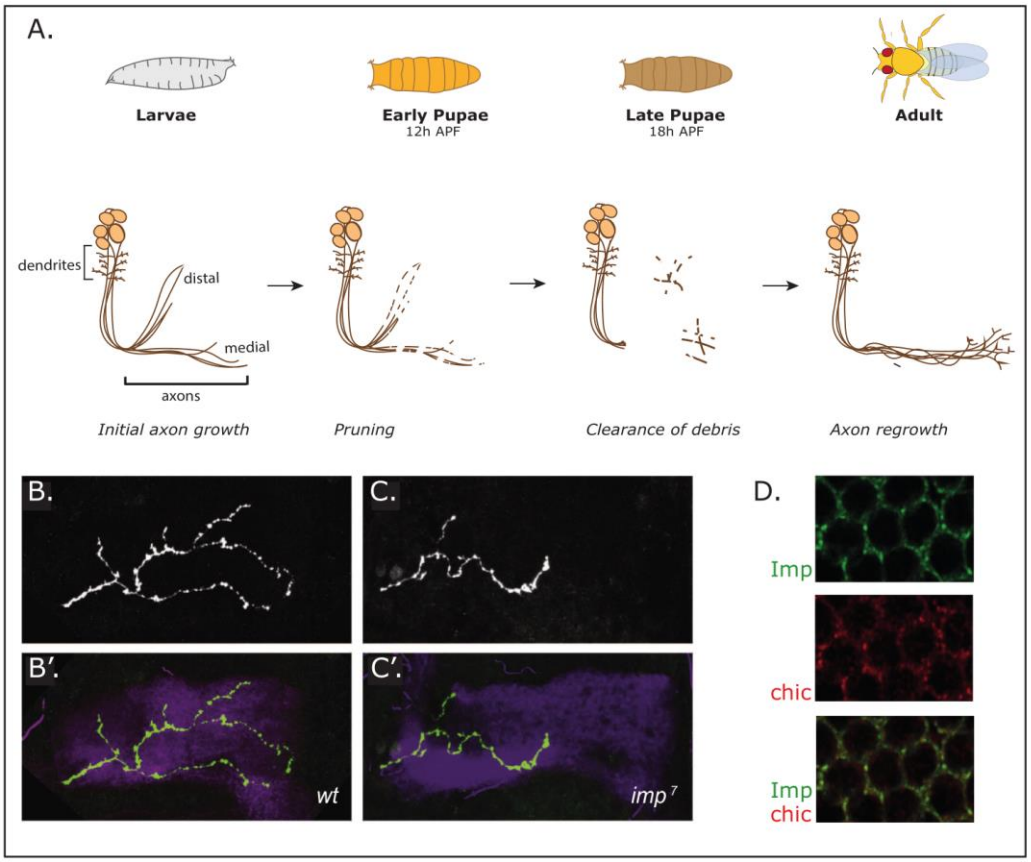

Figure 12. imp is required for MB γ neuron regrowth.
A) stereotypic remodeling of MB γ neurons: in larval stage, axons extend both medially and dorsally; at early metamorphosis (pupal stage), these branches degenerate through pruning and all the debris are cleared; during mid-metamorphosis, axon re-extend medially to form the mature branches. (B-C) imp mutant γ neurons (C) show defective axon regrowth compared to control neurons (B). Images show a single axon with its lateral branches (in green) and a γ lobe marker (magenta) (B'-C'). D) *Drosophila* Imp RNP granule contain *chickadee* (*Drosophila* profilin) mRNA. smFISH experiments with chickadee probes on MB γ cell bodies show colocalization of *chickadee* mRNA (red) within Imp RNP granules (green).
References:
A) inspired by Yaniv and Schuldiner, 2016. Wiley Inderdiscip Rev Dev Biol.doi: 10.1002/wdev241.
B-C) Medioni C, Ramialison M, Ephrussi A, Besse F. 2014. Curr Biol 24: 793-800
D) Vijayakumar J, Perrois C, Heim M, Bousset L, Alberti S, Besse F. 2019. Nat Commun 10: 2593.

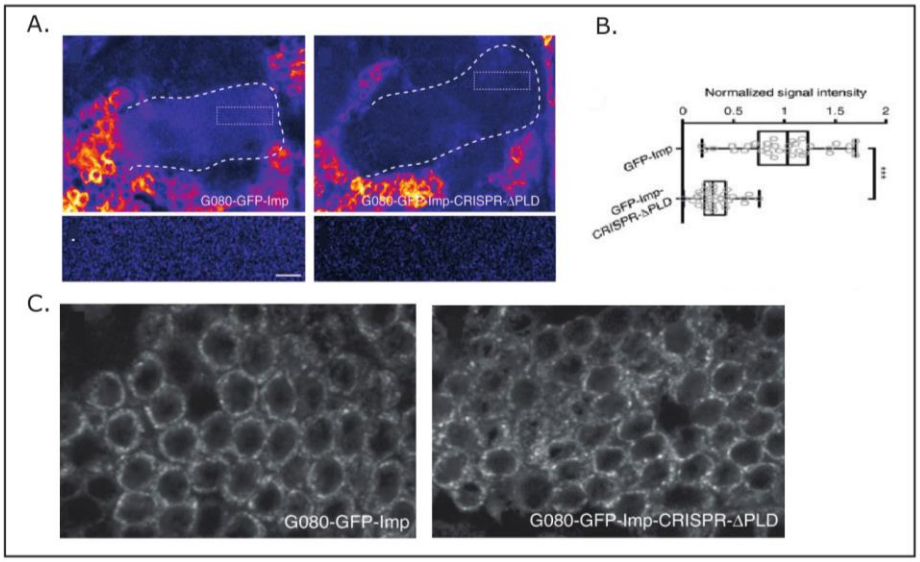

Figure 13. Drosophila Imp prion-like domain (PLD) promotes Imp axonal transport and is not required for Imp RNP granule assembly.
A) Imp axonal transport is perturbed upon endogenous deletion of Imp PLD. Imp signal in MB γ lobes is shown for a control GFP-Imp line (left, within the dotted line) and GFP-Imp-ΔPLD (right, within the dotted line). Magnifications of boxed regions are shown below. The images are presented using the "Fire" Lookup Table of ImageJ. B) Quantification of axonal Imp signal in the two conditions represented in A. C) Imp PLD is dispensable for Imp RNP granule formation. Imp RNP granules is the MB y cell bodies are shown in control conditions (endogenously GFP-tagged wild-type Imp, left panel) and in the ΔPLD condition (endogenously GFP-tagged version of Imp without the PLD). The lack of Imp PLD (right) does not affect Imp RNP granule formation.
References:
Vijayakumar J, Perrois C, Heim M, Bousset L, Alberti S, Besse F. 2019. Nat Commun 10: 2593.

7. Aim of the thesis

Research performed in our group has unravelled the role of *Drosophila* Imp in the morphological remodelling of Mushroom Body (MB) γ neurons, the seat of memory formation in flies, and has further established Imp as a key component of neuronal RNP granules. These assemblies are transported to MB γ axons along MT tracks in a developmentally-regulated fashion and their transport is promoted by the C-terminal PLD of Imp.

Although the axonal localization of Imp RNP granules seems to be highly relevant for axonal remodelling during nervous system maturation, whether it is required in adults was unclear. Furthermore, the mechanisms and the players involved in Imp granule regulation are largely unknown.

Recent models have proposed that RNP granules can function as reservoirs of quiescent mRNAs, which are transported to synapses in order to undergo local translation in response to external cues and activity firing, eventually contributing to nervous system proper function, synaptic plasticity and memory formation.

My PhD project shaped around the hypothesis that Imp neuronal RNP granules could respond to neuronal activation and lead to subsequent changes in translational profile of target mRNAs eventually leading to memory establishment. Hence, the objectives of my PhD were to: 1) investigate the *in vivo* mechanisms underlying activity-dependent remodelling of Imp neuronal RNP granules; 2) test the hypothesis that RNPs could be involved in LTM-underlying mechanisms by regulating gene expression.

To address these points, I first studied the impact of neuronal activation on Imp RNP properties. To this end, I developed a protocol to induce neuronal stimulation by treating *Drosophila* brain explants with a generical depolarizing chemical – KCl - and different neurotransmitters and neuromodulators. I further characterized Imp RNP activity-dependent remodelling by questioning the involvement of Imp PLD, studying how activity impacts granule composition and identifying its regulators.

To investigate the functional relevance of Imp RNP remodelling, I tested whether translation was affected by looking at the translation of Imp mRNA targets. Finally, I investigated in collaboration with the group of Krystyna Keleman the role of Imp in both short- and long-term memory formation.

II – Results

Part 1 - Manuscript

The neuromodulator Tyramine induces the disassembly of neuronal RNP granules in *Drosophila* brains

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Results

Tyramine induces the declustering of Imp molecules

In resting Mushroom Body (MB) γ neurons, Imp molecules are found within hundreds nanometer-sized cytoplasmic granules that contain Imp target mRNAs such as *profilin* (Figure 1A) (Vijayakumar et al. 2019). To monitor with high spatial resolution, and in intact brains, the response of Imp RNP granules to neuronal activation, we first treated brain explants expressing functional GFP-Imp from the endogenous locus with KCl. As shown in Figures 1C-E, neuronal depolarization induced a significant decrease in the number of Imp RNP granules found in cell bodies. Previous work has suggested that RNP granules respond with high specificity to distinct neuronal activation pathways (Baez et al. 2011; Pimentel and Boccaccio 2014; De Graeve and Besse 2018). To then identify the specific trigger inducing granule remodelling, we analyzed the behavior of Imp RNP granules upon treatment of brain explants with different neurotransmitters or modulators. While treatments with dopamine or acetylcholine did not induce any change in granule properties (Figure S1C,D and data not shown), Tyramine induced a near complete declustering of Imp molecules (Figures 1B,D,E). The diffuse cytoplasmic localization of Imp observed in the presence of Tyramine did not result from changes in Imp expression, as similar levels of Imp were observed with and without Tyramine treatment (Figure 1F). Furthermore, subcellular relocalization was not observed for any RNA binding proteins as the distribution of FMRP and PABP was not affected after Tyramine treatment (Figure S2). Together, these results thus suggest that Tyramine-induced depolarization of MB γ neurons triggers a specific release of Imp molecules into the cytoplasm.

Tyramine-induced Imp declustering is blocked by neuronal silencing.

Tyramine treatment induced a strong increase in the activity of MB neurons, characterized by a rise in intra-cellular calcium concentration visualized using the Homer-GCaMp3 reporter (Figure S1E-G). To test whether the Tyramine-induced declustering of Imp molecules requires MB neuron firing, we silenced MB neurons by expressing the inward-rectifying potassium channel Kir 2.1 (Paradis et al. 2001) *via* the GAL4/UAS system. To avoid chronic hyperpolarization and potential subsequent homeostatic regulation, we used the TARGET system, which relies on a temperature-sensitive GAL80 variant repressing GAL4 transcriptional activity exclusively at low temperatures (McGuire et al. 2004). As shown in Figure 2, expression of Kir2.1 significantly (although not completely) suppressed Tyramine-induced degranulation, suggesting that MB neuron electrical activity is required for the cytoplasmic release of Imp.

Tyramine-induced Imp declustering is reversible.

Neuronal RNP granules are dynamic phase-separated condensates that are formed through demixing of granule components from the cytoplasm (Weber and Brangwynne 2012; Mittag and Parker 2018; Van Treeck and Parker 2018; Formicola et al. 2019). To test whether the observed changes in the phase behavior of Imp were reversible, we treated brain explants for 30 minutes with Tyramine, then washed out Tyramine and let the brains recover for 30 more minutes (Figure 3A). Strikingly, while a significant declustering of Imp molecules was visible at the end of the Tyramine treatment (Figures 3C,E,F), bright cytoplasmic Imp granules were observed back after the recovery period (Figures 3D,E,F). This result suggests that Tyramine induces reversible changes in the phase separation process underlying Imp RNP granule assembly.

Imp prion-like domain is not required for Tyramine-induced changes in phase behavior.

Intrinsically disordered prion-like domains (PLDs), because they are prone to interact with RNA, RNA-binding domains or other low-complexity domains enriched in RNP granule components, play a critical role in the phase behavior of RNA binding proteins (Franzmann and Alberti 2018). While most prion-like domains studied so far tend to promote phase separation and granule formation (Kato et al. 2012; Lin et al. 2015; Protter et al. 2018), Imp PLD appears to be dispensable for the formation of cytoplasmic Imp granules (Figure 4A) (Vijayakumar et al. 2019). To test whether Imp PLD may still modulate the solubilization of Imp molecules induced by Tyramine, we treated brain explants expressing exclusively, and from the endogenous promoter, truncated GFP-Imp-ΔPLD proteins lacking Imp C-terminal PLD (Vijayakumar et al. 2019). As shown in Figure 4C, cytoplasmic release of Imp proteins was also observed upon Tyramine treatment in this context, although the response of Imp-ΔPLD granules to Tyramine was less reproducible than that of wild-type Imp granules (Figure 4D, compare with Figure 1E). This result thus suggests that Imp PLD is not critically required for the response of Imp granules to Tyramine.

Tyramine induces RNP granule disassembly and specific relocalization of granule components.

Neuronal RNP granules contain various proteins, many of them involved in the repression of associated mRNA translation (De Graeve and Besse 2018; Formicola et al. 2019). To analyze the behavior of components other than Imp, we stained brains subjected or not to Tyramine with antibodies recognizing the DEAD-box helicase Me31B. Me31B and its orthologs are found in various types of RNP assemblies, and were shown in different contexts to repress the translation of their target mRNAs and/or promote RNA decay (Nakamura et al. 2001; Coller and Parker 2005; Weston and Sommerville 2006; Wang et al. 2017). In the cell bodies of mature adult MB γ neurons, Me31B proteins cluster into distinct granules (Figure 5A'), the vast majority of which also contain Imp (Figures 5A'' and Figure S3A). As shown in Figures 5B' and C, Tyramine induced a dramatic loss of Me31B granular staining in the cell bodies of MB γ neurons. Strikingly, Me31B degranulation was accompanied by a massive relocalization of Me31B proteins to the calyx, a region located below cell bodies and enriched in dendritic processes (Figure 5D-F). Indeed, while defined Me31B-

positive punctae were observed in the calyx in the absence of Tyramine (Figure 5E, boxed region), a strong homogeneous Me31B signal was observed after Tyramine treatment (Figure 5F, boxed region). Such a dramatic change in localization was not observed for Imp, indicative of a specific sorting of RNP granule components between cell bodies and dendrites. Furthermore, it was not associated with a visible increase in Me31B levels, as similar overall levels were observed in the presence and absence of Tyramine (Figure S3B). Together, these results suggest that Tyramine induces the disassembly of neuronal RNP granules containing Imp and Me31B, leading to the differential sorting of granule components into cell bodies and dendrites.

RNP granule disassembly is associated with an increased translation of granule-associated mRNAs

Neuronal RNP granules are thought to maintain associated mRNAs in a translationally silenced state (Krichevsky and Kosik 2001; Fritzsche et al. 2013; El Fatimy et al. 2016; De Graeve and Besse 2018). To investigate whether the observed release of granule components leads to the translational derepression of granule-associated mRNAs, we monitored the translation of reporters in which the 3'UTR of *profilin*, known to be directly bound by Imp (Medioni et al. 2014) and included into Imp granules (Figure S4C) (Vijayakumar et al. 2019), is fused to the coding sequence of EGFP. Constructs generated with the *SV40* 3'UTR were used as a negative control. Remarkably, treating brains for 30 minutes with Tyramine induced a significant increase in GFP signal intensity for the construct containing *profilin* 3'UTR, but not for that containing *SV40* 3'UTR (Figure 6A-E). Increased GFP protein levels were not associated with an increase in the number of *profilin* molecules, as estimated by smFISH experiments (Figure S4A,B,D). Together, this suggests that the Tyramine-induced disassembly of Imp RNP granules leads to an increased translation of target mRNAs.

CamkII is required for Tyramine-induced RNP granule disassembly.

To identify proteins involved in the dynamic regulation of Imp RNP granules, we purified Imp-containing complexes by immuno-precipitating from adult head lysates GFP-tagged Imp proteins expressed specifically in MB γ neurons. Co-precipitated proteins were identified by Mass-Spectrometry, and heads expressing sole GFP were used as a specificity control. In total, 69 protein partners were reproducibly identified, distributing into various functional categories (Figure S5A). As expected, RNA binding proteins were strongly enriched in the bound fraction (26/69 proteins; $p<0.001$). Among the identified Imp partners, we focused our attention on Ca2+/calmodulin-dependent protein kinase II, a conserved kinase activated in response to synaptic stimulation (Coultrap and Bayer 2012). To first validate the physical interaction between Imp and CamkII and test whether it depends on the presence of RNA, we performed co-immunoprecipation experiments in cultured S2R+ cells. As shown in Figure S5B, CamkII co-immunoprecipitated with GFP-Imp, but not with sole GFP, in the presence and absence of RNAse, indicating that the Imp/CamkII interaction is RNA-

independent. To assess whether Imp and CamkII colocalize in MB γ neurons, we analyzed the relative sub-cellular distributions of Imp, CamkII and phospho-CamkII (the active form of CamkII). Both CamkII and phospho-CamkII were found diffusely localized in MB γ neuron cytoplasm (Figure 7A',A''). Although CamkII signal did not accumulate into Imp RNP granules, it overlapped in many instances with Imp signal, a result compatible with the existence of cytoplasmic complexes containing both Imp and CamkII. To then investigate whether the function of CamkII is important for the response of Imp RNP granules to Tyramine, we expressed in MB γ neurons the ala peptide, a known inhibitor of CamkII activity (Griffith et al. 1993). Remarkably, expression of the ala peptide prevented the disassembly of Imp RNP granules observed upon Tyramine treatment (Figure 7B-D), indicating that CamkII is required cell-autonomously downstream of Tyramine to promote Imp RNP granule disassembly.

Materials and methods

Drosophila lines and genetics

Flies were raised on standard media at 25°C. The fly stocks used were the following: w^{1118} ; GFP-Imp G080 (gift from L. Cooley, described in Medioni et al, 2014); GFP-Imp-CRISPR-ΔPLD (Vijayakumar et al, 2019); tubulin-$Gal80^{ts}$,OK107-Gal4; UAS-EGFP:kir2.1 (BSC #6595); UAS-CamkII ala (BSC #29666); MB247-Homer-GCaMp3 (gift of Gaia Tavosanis); $CaMKII^{CPTI000944}$ (DGGR #115127), UASp-EGFP:*profilin* 3'UTR; UASp-EGFP:SV40 3'UTR. The UASp-EGFP:3'UTR constructs were generated by LR recombination using pENTR:D/TOPO donor plasmids containing either *SV40* 3'UTR or *profilin/chickadee* 3'UTR and a UASp-EGFP-W destination vector. The UASp-EGFP-W plasmid was obtained by ligating into a KpnI-PstI blunted doubled-digested pPW plasmid, a KpnII-SacII insert obtained after subcloning into pBluescript (KS) KpnI-EGFP-XhoI and SpeI-Gateway Cassette-SacII fragments. The *SV40*-3'UTR and *chickadee* 3'UTR sequences were PCR amplified using the following primers: SV40_up (5'-CACCTAGAGGATCTTTGTTGAAGG-3') and SV40_low (5'-GATCCAGACATGATAAGATAC-3'), chic_up (5'-CACCGAGAATAGATCAACAC-3') and chic_low (5'-CGTGTGGATTTATGTACG-3').

The EGFP:kir2.1 and EGFP:3'UTR constructs were expressed under the control of tubulin-$Gal80^{ts}$,OK107-Gal4. Flies were kept for 6 days after hatching at 21°C and then switched for 3 days at 29°C to trigger transgene expression.

Ex vivo treatments of Drosophila brains

Brains of 9-12 day-old flies were dissected in cold Haemolymph-Like saline solution 3 (HL3) (Kuklin et al, 2017) (NaCl 70 mM, KCl 5 mM, $MgCl_2$ 4 mM, trehalose 5 mM, sucrose 115 mM, HEPES 5 mM, $NaHCO_3$ 10 mM, pH 7.2-7.3) and transferred into Nunc™ Lab-Tek™ II Chamber Slide™ (thermofisher, #154526) with either 500 µl of HL3 or HL3 + neurotransmitter/KCl for 30' at 25°C. Brains were protected from light during incubations. Neurotransmitters were used at the following final concentrations: Acetylcholine, 35 mM

(sigma, #A6625); Tyramine, 10 mM (sigma, #T2879). KCl was used at a 10 mM final concentration.

After the treatment, brains were collected, fixed with 4% formaldehyde in HL3 for 25 minutes, washed thrice with PBS/Triton-X (PBT) 0.5% and either directly mounted in vectashield (Vector Laboratories) to look at endogenous fluorescence or further immunostained.

smFISH

Drosophila brains were fixed for 1 hour in 4% formaldehyde in PBS and dehydrated overnight in ethanol 70%. Brains were briefly rinsed in Wash Buffer (10% formamide in 2x SSC, 2x SSC) before an overnight incubation at 45°C in Hybridization Buffer (100 mg/ml dextran sulfate, 10% formamide in 2x SSC, 2x SSC) with Quasar® 570-labeled Stellaris® Probes (Cf: 0.06 µM). Brains were then washed twice in pre-warmed wash buffer, stained with DAPI 5 $\mu g.mL^{-1}$, briefly incubated in 2x SSC and mounted in vectashield (Vector Laboratories).

Immunoprecipitation-Mass Spectrometry

201Y-Gal4, UAS-GFP-Imp and 201-Gal4, UAS-GFP flies were amplified at 25°C in bottles. 3-5 day-old flies were collected and immediately frozen. Heads were collected at 4°C using two prechilled sieves of different mesh sizes (630 µm on top and 400 µm at the bottom), and crushed into powder using prechilled mortar and pestle. The head powder (3g for each experiment) was then transferred to a prechilled 15 mL glass Dounce Tissue Grinder and homogenized in 10 mL DXB buffer (25mM Hepes, pH 6.8, 50mM KCl, 1mM MgCl2, 1mM dithoithreitol (DTT), 250mM sucrose, 1/100 Halt™ Protease & Phosphatase Inhibitor Cocktail, Thermo Scientific). The homogenate was cleared by two consecutive centrifugations at 10,000 rpm for 10 min at 4°C.

250 µL of GFP-Trap®_A beads (ChromoTek, Germany) were then added to the cleared lysate in a 15 mL and incubated on a rotator for 1h30 at 4°C. Beads were pelleted by mild centrifugation (2,000 rpm for 2 min at 4°C and washed 2 times with DXB buffer and 1 time with DXB buffer + 0.1% NP 40. Proteins bound to the beads were eluted by addition of 1.6 mL of 0.2M glycine, pH 2.5 and incubation for 10 min on a rotator. The eluate was then collected and neutralized with 400 µL of 1M Tris HCl, pH 8. The 2 mL total eluate was concentrated on an Amicon® Ultra-2 mL centrifugal filter, MWCO 10 kDa (Merck Millipore, USA) to obtain a final volume of 50 µL.

GO analysis of bound fraction was performed using the PANTHER classification system (http://pantherdb.org/).

Subloning of CamkII coding region

CamkII sequence was amplified by PCR from cDNA obtained from flies carrying a UAS-CamkII.R3 construct (BSC #29662), using the following primers: CaMKII_Gtw_for (5'-CACCATGGCCGCACCAGCAGC-3') and CaMKII_Gtw_rev (5'-TATTTTTGGGGTATAAAATCG-3'). The obtained sequence was subcloned into pENTR-D/TOPO vector (Life Technologies), fully sequenced and then LR-recombined into pAFW vectors.

Immuno-precipitation experiments

Drosophila S2 cells were plated in 6-well plates at a density of 5.10^6 cells/ well and incubated for 24 hours at 25°C. Cells were then transfected with 600 ng of plasmids using the Effectene transfection reagent (Qiagen, 301425) and resuspended after 12 hours in Schneider's Insect Medium supplemented with 10% Fetal Bovine Serum and Penicillin/streptomycin (1%). After 3 days of expression at 25°C, cells transfected with either pAGW + pAF-CamkII or pAGImp + pAF-CamkII were harvested and lysated in DXB buffer (HEPES pH 6.8, 2.5 mM; KCl, 50 mM; $MgCl_2$, 1mM; DTT, 1 mM; sucrose, 250 mM; NP-40, 0.05%) supplemented with Halt™ Protease Inhibitor Cocktail 1:100 (Thermofisher, #78429). Cell lysates were treated or not with 10 mg/ µL RNase A/T1 Cocktail (ThermoFisher scientific, #EN0551) and incubated with ChromoTek GFP-Trap® beads (ChromoTek, gt-10, #70112001A) for 2 hours at 4°C.

Immunostainings

After fixation and washes in PBS/Triton-X (PBT) 0.5%, brains were blocked overnight in PBS/Triton-X (PBT) 0.5% supplemented with BSA 1% and then incubated with the following antibodies: α-Imp (rabbit, 1:1000, Medioni et al., 2014), α-ME31B (rabbit, 1:500, gift from C. Lim), α-pCamkII (rabbit, 1:1000, Santa Cruz Biotechnology, sc-12886-R), α-Fmrp (mouse, 1:50, DSHB, 2F5–1 clone), α-PABP (rabbit, 1:1500, gift from C. Lim). After incubation with primary antibodies, brains were washed with PBT 0.5% and incubated with either Alexa Fluor (AF)568-conjugated α-rabbit (1:1000) or AF488-conjugated α-mouse (1:1000) antibodies. DAPI was used at 5 $\mu g.mL^{-1}$ and incubated for 5 minutes. Samples were mounted in vectashield (Vector Laboratories).

Western-blots

15 brains were lysed in 40 µl of RIPA Buffer (0.1% Na deoxycholate, 1% Triton-X, 0.1% SDS, NaCl 150 mM, Tris-HCl pH 7-7.5 50 mM) supplemented with Halt™ Protease Inhibitor Cocktail 1:100 (Thermofisher, #78429) and SDS 5x. Lysates were left in agitation for 30 minutes at 4°C and then incubated for 5 minutes at 95°C for denaturation prior to loading.

Protein extracts were run on 10% polyacrylamide gels, blotted to PVDF membranes and incubated with the following antibodies: rabbit anti-GFP (Torey Pines, 1:2,500), mouse anti-FLAG (sigma, 1:2,500), mouse

anti-tubulin (sigma, 1:5,000).

Imaging, intra-cellular calcium measurements, RNP granule quantification and colocalization analysis

Images were acquired on a Zeiss LSM880 Fast Airy Scan inverted confocal microscope, using either a Plan-Apo 20x NA 0.8 dry (entire Mushroom Bodies), a C-Apo 40x NA 1.4 oil (calyx) or a Plan Apo 63X NA 1.4 oil (cell bodies) objectives. Concerning the 3'UTR reporter experiments, GFP intensity was measured within two cell body regions of 300x300 pixels from maximum intensity projections. To quantify the intra-cellular calcium concentrations, GFP fluorescence intensities were measured in areas of 1312,253 μm^2 in the MB calyx from maximum intensity projections and background intensity subtracted. Values were normalized to control samples. For Imp RNP quantifications, 244x244 pixel ROIs containing 6-7 cells were cropped from single z slices, treated with a Gaussian Blur filter, resized using the Image Pyramid plugin and converted from 32-bit to 16-bit images, all using ImageJ. Granules were detected and quantified using the Small Particle Detection (SPADE) tool described in Cedilnik et al, 2018 (https://hal.inria.fr/hal-01867805/document). The threshold used for RNP granule detection was set as 0.6234 and chosen according to the best measured F1 score (De Graeve et al, 2019). F1 scores were calculated by comparing the spatial coordinates of manually annotated RNP granules in .csv files with the binary masks generated by SPADE with different thresholds. Minimal granule size was set as 4 pixels. Thresholds for Me31B granules and *profilin* mRNA detection were respectively set as 0.4234 and 0.2234. To measure degrees of colocalization, RNP binary masks generated by SPADE for Imp and Me31B granules were analyzed with the JACoP plugin using object-based methods (Bolte and Cordelieres, 2006). All statistical analyses were performed using GraphPad Prism version 6.

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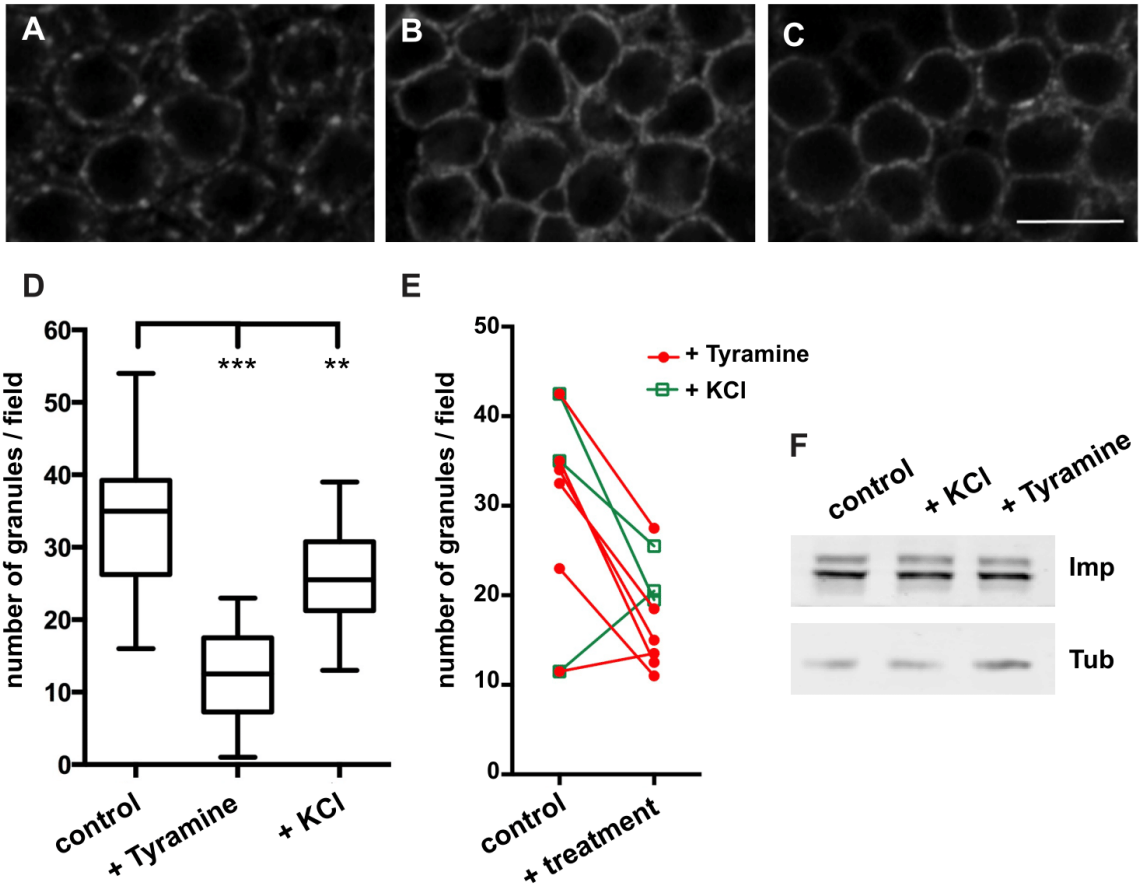

Figure 1. Imp RNP granules disassemble in response to neuronal activation.
(A-C) Cell bodies of adult Mushroom Body g neurons expressing endogenously produced GFP-Imp proteins (G080 protein-trap line). Brain explants were incubated in HL3 buffer (A, control) or HL3 supplemented with 10 mM Tyramine (B) or 10 mM KCl (C) for 30 minutes. GFP-Imp signals are shown in A-C. Images correspond to single confocal sections. Scale bar: 5 mm. (D) Distributions of the numbers GFP-Imp granules per imaged field. Granules were detected with the SPADE algorithm (see Fig. S1). The results of a single replicate are shown. Numbers of imaged fields: 26 (control); 20 (+ Tyramine); 20 (+ KCl). **, $P<0.01$; ***, $P<0.001$ (Mann-Whitney test). (E) Median number of GFP-Imp granules per imaged field for each replicate. (F) Western-Blot performed from adult G080-GFP-Imp protein-trap brains treated, or not, with KCl or Tyramine. a-GFP antibodies were used to detect GFP-Imp fusions and a-Tub antibodies for a loading control.

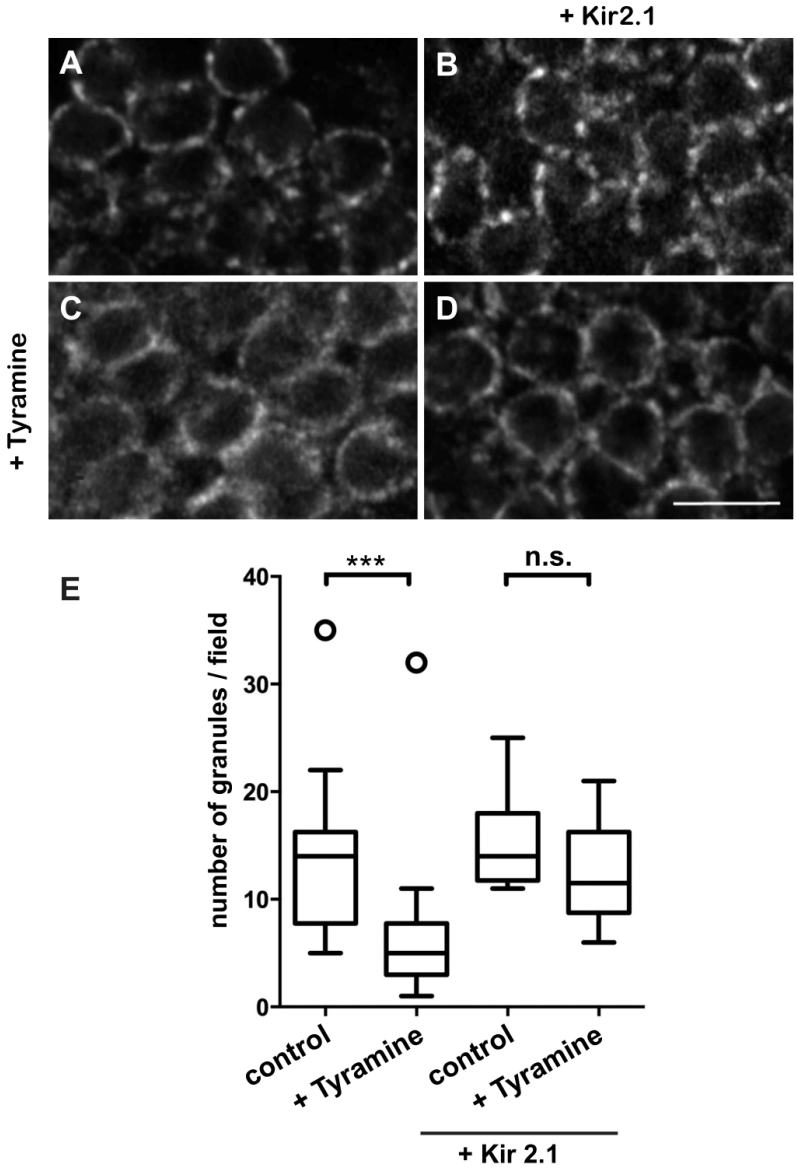

Figure 2. Imp RNP granules disassembly is inhibited by hyperpolarizarion of Mushroom Body neurons.
(A-D) Cell bodies of adult Mushroom Body g neurons stained with a-Imp antibodies. Brain explants were incubated in HL3 buffer (A, B, control) or in HL3 supplemented with 10 mM Tyramine (C, D) for 30 minutes. Imp signals are shown in A-D. Scale bar in A-D: 5 mm. (E) Distributions of the numbers of Imp granules per imaged field. Granules were detected with the SPADE algorithm (see Fig. S1). The results of a single replicate are shown. Numbers of imaged fields: 18 (control); 18 (+ Tyramine); 10 (control + Kir2.1); 18 (+ Tyramine + Kir2.1). ***, $P<0.001$ (Mann-Whitney test). n.s. stands for not significant. The UAS-Kir2.1 construct was expressed for 3 days before the treatment, using the OK107-Gal4 combined with a tub-Gal80ts construct.

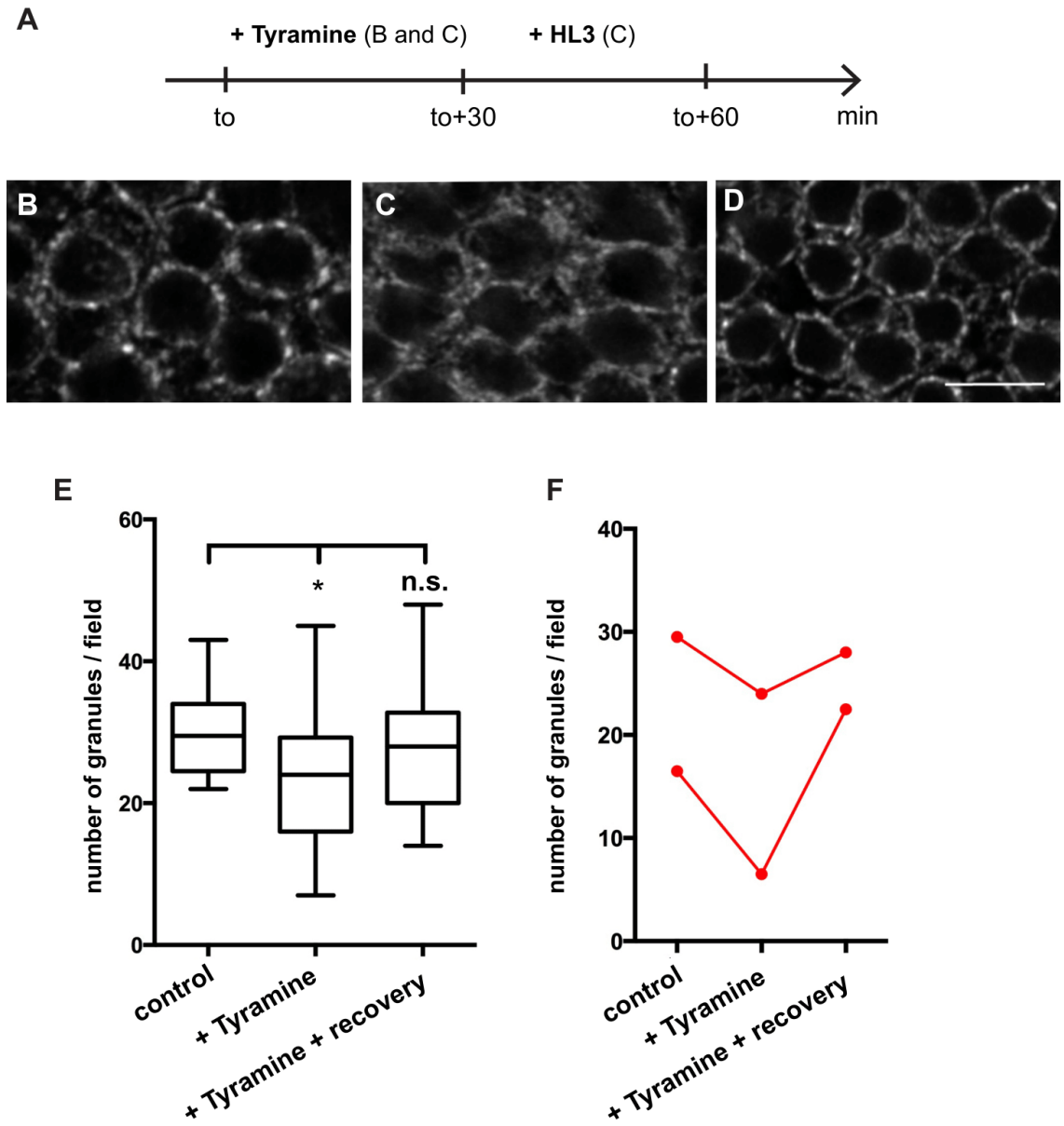

Figure 3. Imp RNP granules disassembly is reversible.
(A) Schematic representation of the experimental procedure used. Brain explants were incubated in HL3 buffer (control) or HL3 supplemented with 10 mM Tyramine for 30 minutes. Tyramine was washed out and replaced by HL3 for 30 minutes (Tyramine + recovery). Note that samples shown in B and C were fixed at to+30 and sample shown in D at to+60. (B-D) Cell bodies of adult Mushroom Body g neurons expressing endogenously produced GFP-Imp proteins (G080 protein-trap line). GFP-Imp signals are shown in B-D. Images correspond to single confocal sections. Scale bar in B-D: 5 µm. (E) Distributions of the numbers of Imp granules per imaged field. Granules were detected with the SPADE algorithm (see Fig. S1). The results of a single replicate are shown. Numbers of imaged fields: 20 (control); 22 (+ Tyramine); 20 (+ Tyramine + recovery). *, $P<0.05$ (Mann-Whitney test). n.s. stands for not significant. (F) E) Median number of GFP-Imp granules per image field for each replicate.

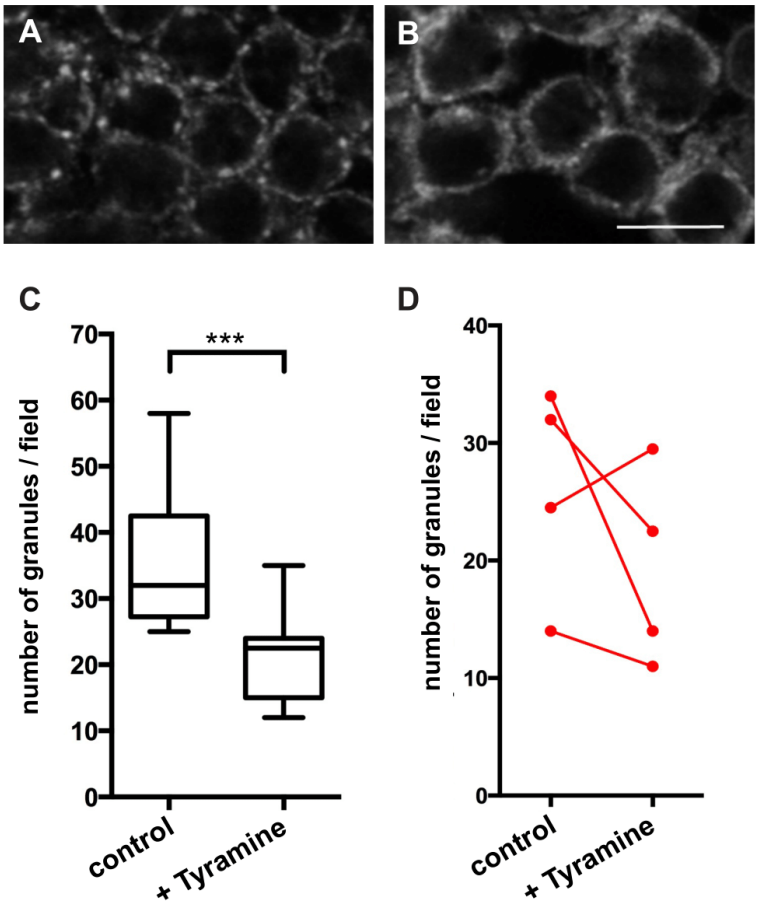

Figure 4. Imp RNP granule remodeling does not involve Imp prion-like domain (PLD).
(A,B) Cell bodies of adult Mushroom Body γ neurons expressing endogenously produced GFP-Imp-ΔPLD proteins (CRISPR-modified protein-trap line). Brain explants were incubated in HL3 buffer (A, control) or HL3 supplemented with 10 mM Tyramine (B) for 30 minutes. GFP-Imp signals are shown in A,B. Images correspond to single confocal sections. Scale bar: 5 µm. (D) Distributions of the numbers GFP-Imp granules per imaged field. Granules were detected with the SPADE algorithm (see Fig. S1). The results of a single replicate are shown. Numbers of imaged fields: 20 (control) and 18 (+ Tyramine). ***, P<0.001 (Mann-Whitney test). (E) Median number of GFP-Imp granules per imaged field for each replicate.

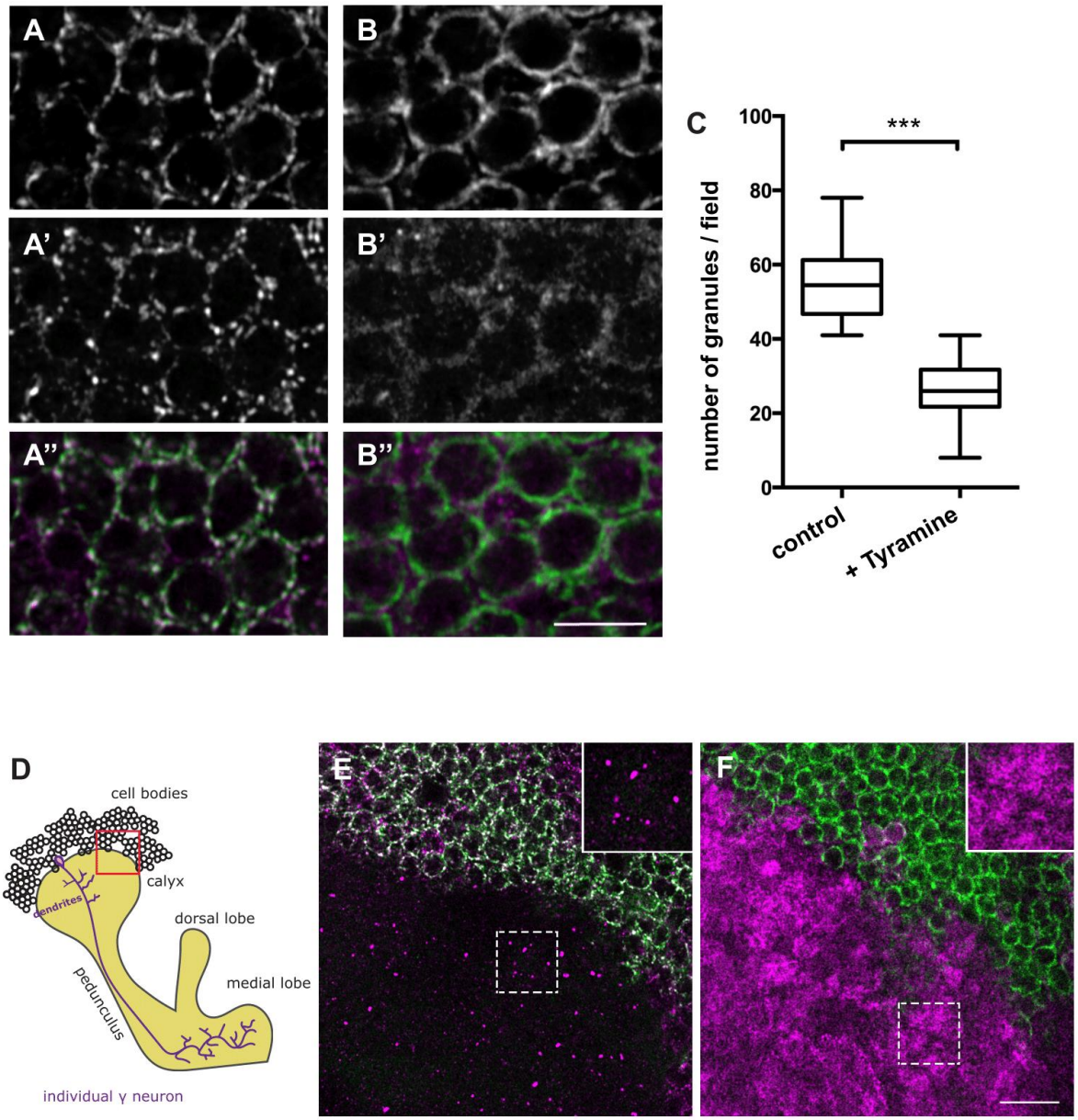

Figure 5. Me31B/DDX6, a component of Imp RNP granules, massively relocalizes to dendrites upon Tyramine treatment. (A,B) Cell bodies of adult Mushroom Body γ neurons expressing endogenously produced GFP-Imp proteins (G080 protein-trap line). Brain explants were incubated in HL3 buffer (A, control) or HL3 supplemented with 10 mM Tyramine (B) for 30 minutes, and then stained with a-Me31B antibodies (white signal in A',B' and magenta in A'',B''). GFP-Imp signals are shown in white in A,B and in green in A'', B''. Images correspond to single confocal sections. Scale bar: 5 µm. (C) Distributions of the numbers Me31B-positive granules per imaged field. Granules were detected with the SPADE algorithm (see Fig. S1). The results of a single replicate are shown. Numbers of imaged fields: 10 (control); 12 (+ Tyramine). ***, P<0.001 (Mann-Whitney test). (D) Schematic representation of an adult Mushroom Body. The red square indicates the region imaged in F,G. Adapted from Vijayakumar et al., 2019. (E,F) Cell bodies and calyx region of adult Mushroom Bodies expressing endogenously produced GFP-Imp proteins (G080 protein-trap line, in green). Brain explants were incubated in HL3 buffer (E, control) or HL3 supplemented with 10 mM Tyramine (F) for 30 minutes, and then stained with a-Me31B antibodies (in magenta). Insets show magnifications of indicated boxed regions. Scale bar: 10 µm.

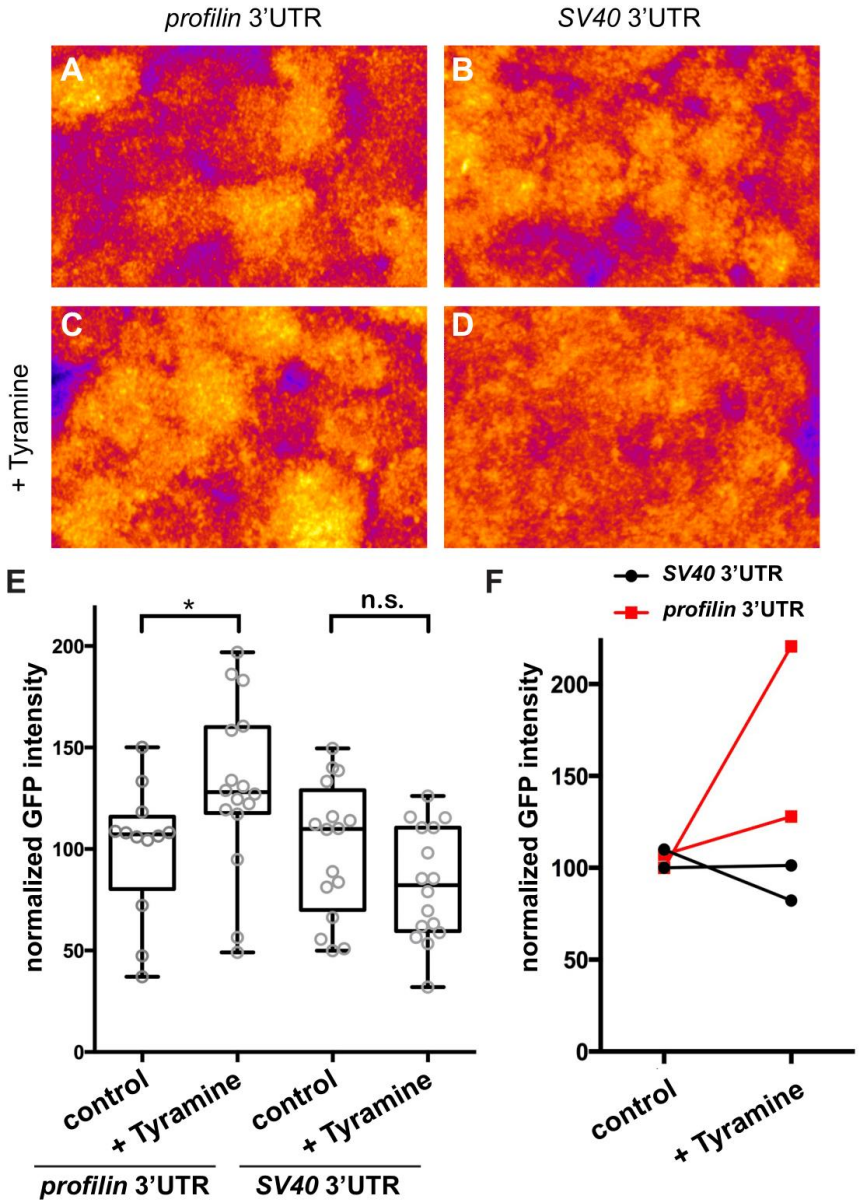

Figure 6. The transation of Imp target mRNAs is increased upon Tyramine treatment.
(A-D) Cell bodies of adult Mushroom Body γ neurons expressing GFP-profilin 3'UTR (A,C) or GFP-SV40 3'UTR (B,D). Brain explants were incubated in HL3 buffer (A, B, control) or HL3 supplemented with 10 mM Tyramine (C,D) for 30 minutes. GFP-Imp signals are displayed using the Fire color code of ImageJ. Images correspond to maximum intensity projections. Scale bar: 5 µm. (E) Distributions of normalized GFP signal intensities. The results of a single replicate are shown. Numbers of imaged fields: 16 (SV40 3'UTR, control); 16 (SV40 3'UTR+ Tyramine); 12 (profilin 3'UTR, control); 16 (profilin 3'UTR+ Tyramine). *, P<0.05 (Mann-Whitney test). (F) Median number of GFP-Imp granules per imaged field for each replicate.

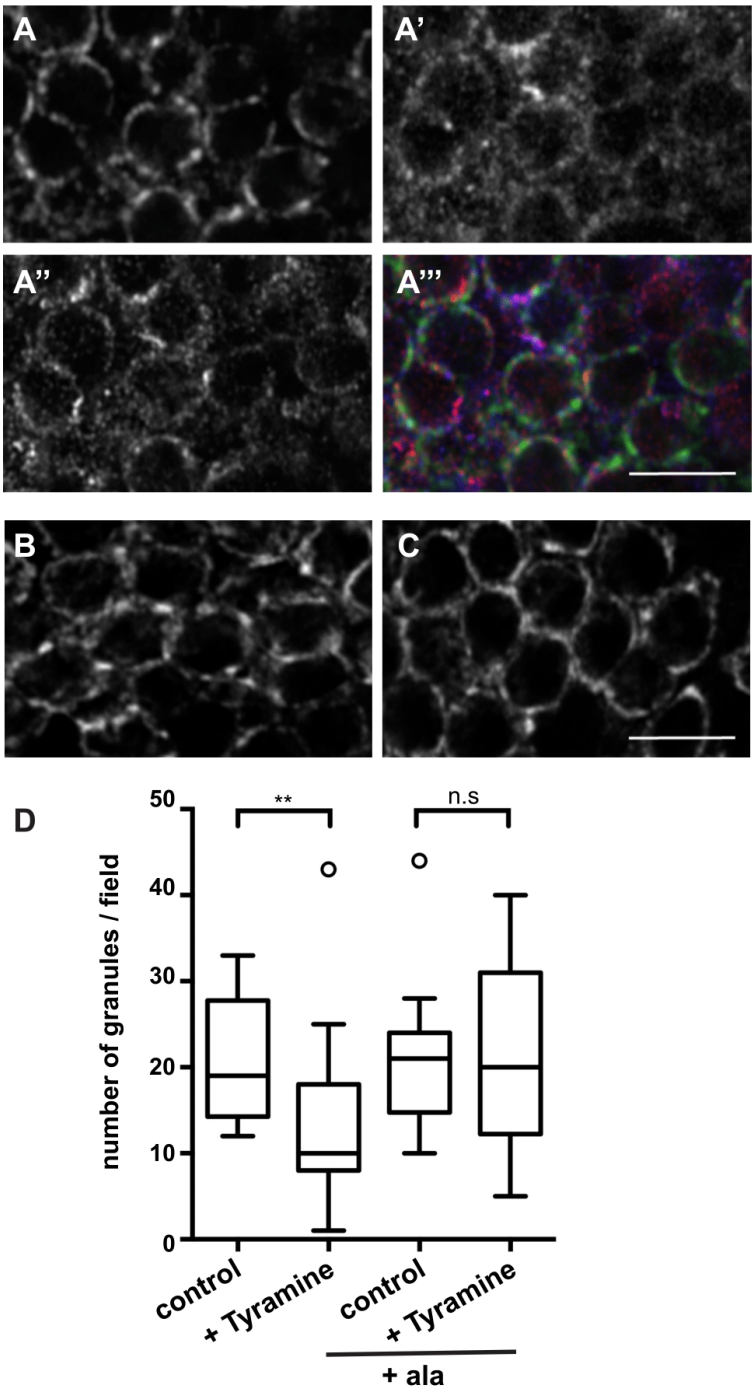

Figure 7. CamkII activity is required for Tyramine-induced RNP granule disassembly. (A) Cell bodies of adult Mushroom Body γ neurons expressing endogenously produced GFP-CamkII proteins (A, red in A''') and stained with a-Imp (A, green in A''') and a-phospho-CamkII (A'', red in A'''). (B,C) Cell bodies of adult Mushroom Body γ neurons expressing endogenously produced GFP-Imp proteins (G080 protein-trap line) together with the CamkII inhibitory peptide ala. Brain explants were incubated in HL3 buffer (B, control) or HL3 supplemented with 10 mM Tyramine (C) for 30 minutes. GFP-Imp signals are shown in B,C. Genotype: G080-gfp-imp/+; tub-Gal80ts/UAS-ala;;OK107-Gal4/+. Images in A-C correspond to single confocal sections. Scale bar in A-C: 5 µm. (C) Distributions of the numbers GFP-Imp granules per imaged field. Granules were detected with the SPADE algorithm (see Fig. S1). The results of a single replicate are shown. Numbers of imaged fields: 24 (control); 19 (+ Tyramine); 16 (+ ala, control) and 14 (+ Tyramine + ala). **, P<0.01 (Mann-Whitney test). n.s. stands for not significant.

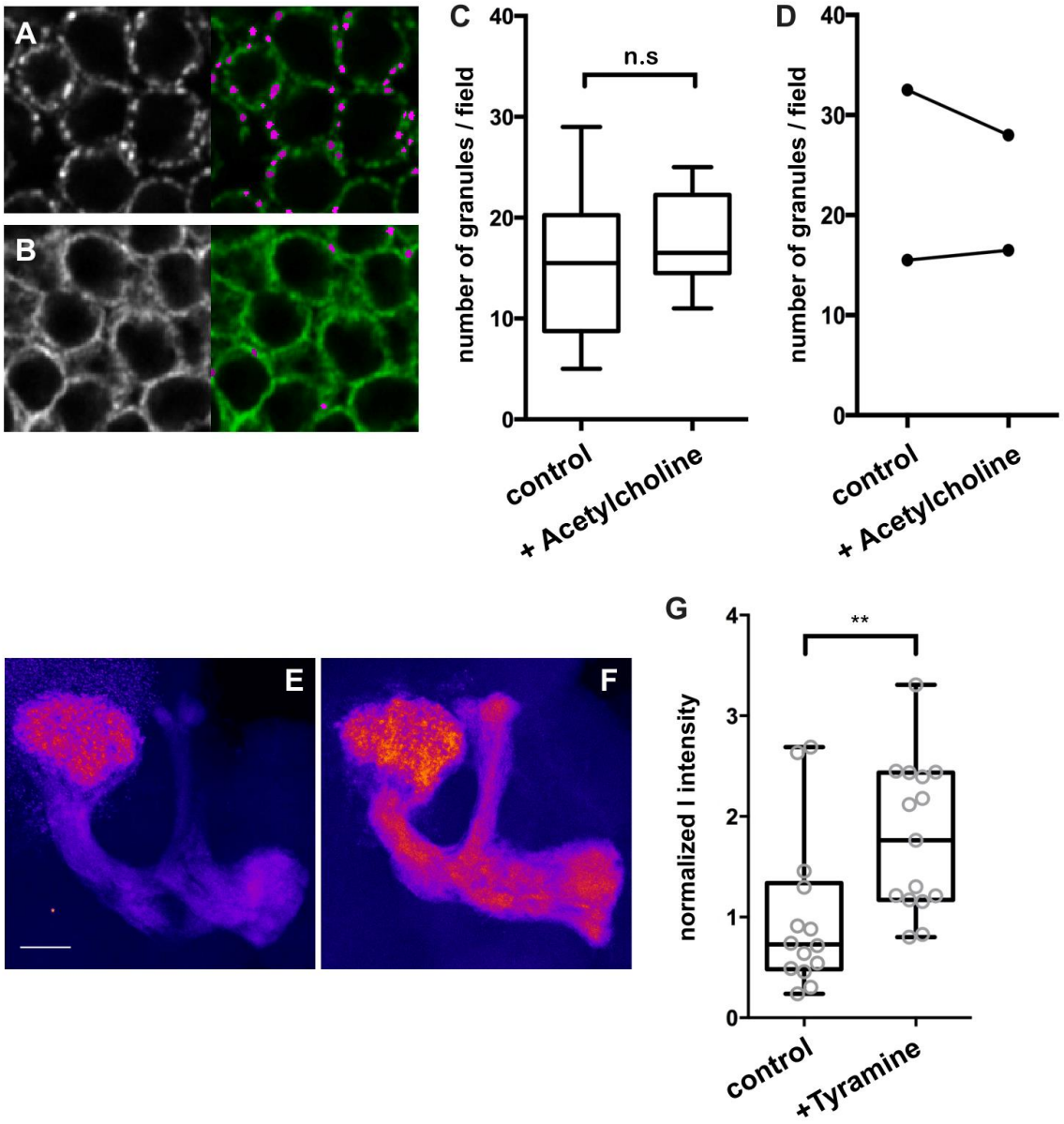

Figure S1. Response to Tyramine treatment - related to Figure 1.
(A,B) Cell bodies of adult Mushroom Body γ neurons expressing a GFP-Imp fusion from the endogenous locus (G080 protein-trap line). Brain explants were treated (B) or not (A) with 10 mM Tyramine. Left: Image field used for detection. Right: objects detected with the SPADE algorithm (in magenta). Single confocal sections were used for analysis. (C) Distributions of the numbers of GFP-Imp RNP granules per imaged field in control brain explants (left) and brain explants treated with 35 mM Acetylcholine (right). A single replicate is shown. Number of fields imaged: 8 (control); 10 (+ Acetylcholine). (D) Median numbers of GFP-Imp RNP granules per field for each replicate. (E,F) Adult Mushroom Bodies expressing a MB247-Homer-GCaMP3 construct and treated (F) or not (E) with 10 mM Tyramine. Maximum intensity projections color-coded using the ImageJ "Fire" lookup table are shown. Scale bar in E,F: 40 µm. (G). GCaMP3 signal intensities (normalized to the control). Data points correspond to individual imaged fields. **, P<0.01 (Mann-Whitney test).

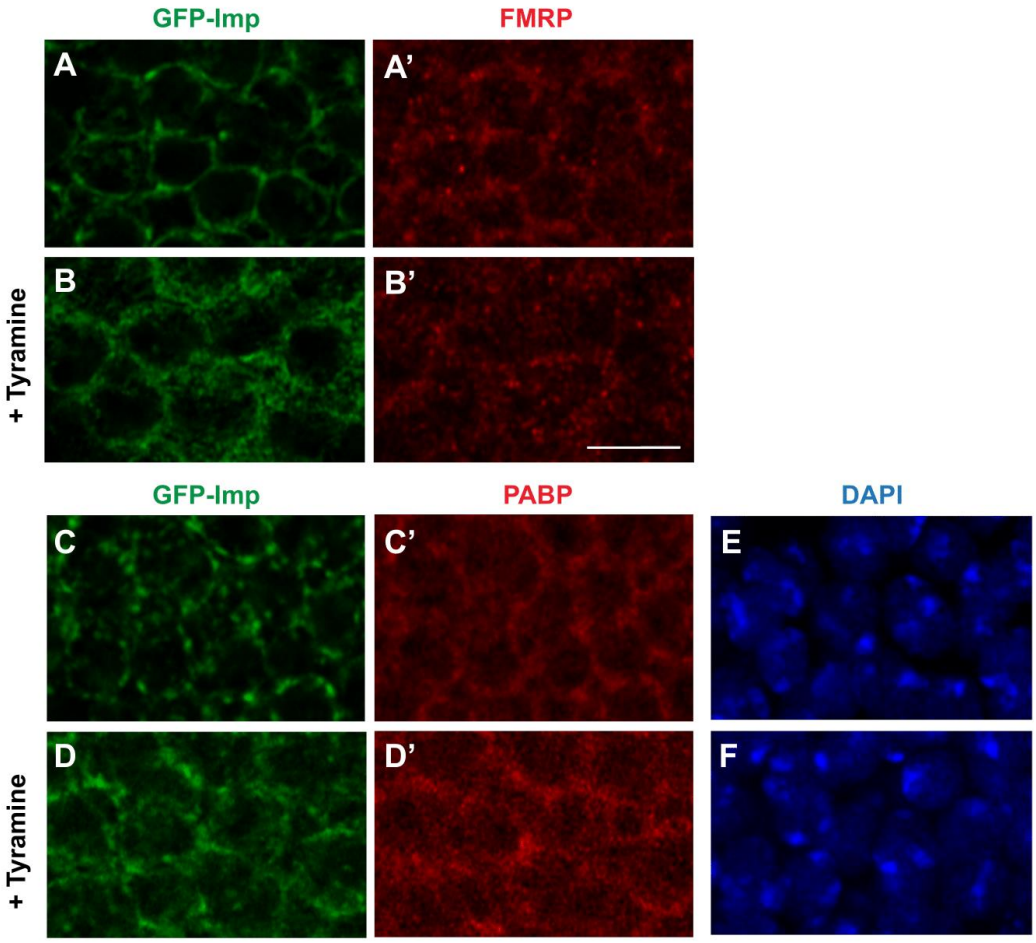

Figure S2. Response of other RNP granule markers to Tyramine treatment - related to Figure 1.
(A-F) Cell bodies of adult Mushroom Body y neurons expressing a GFP-Imp fusion from the endogenous locus (G080 protein-trap line). Brain explants were treated (B,D,F) or not (A,C,E) with 10 mM Tyramine and stained with a-FMRP (red in A',B') or a-PABP (red in C',D') antibodies. GFP-Imp signals are showin in green in A-D. DAPI staining is shown in E,F. Note that cell bodies shown in E and F are different from those shown in C,D. Scale bar in A-F: 5 µm.

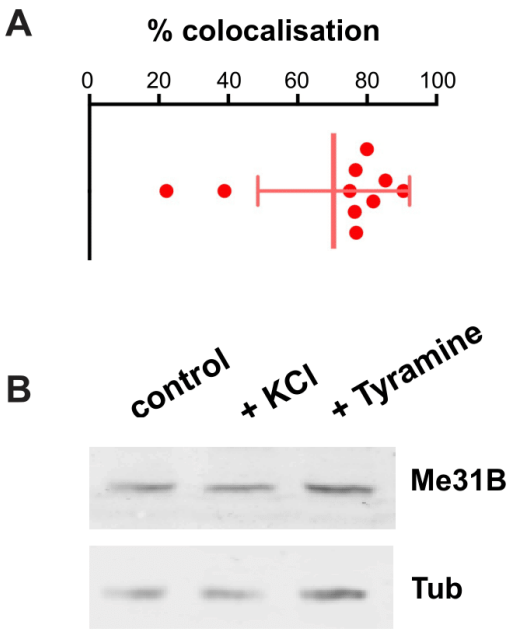

Figure S3. Me31B levels and distribution - related to Figure 5.
(A) Percentage of Imp RNP granules containing Me31B. Individual data points correspond to different imaged fields. Co-localization was quantified using the Fiji JACoP tool (object-based method). (B) Western-Blot performed from adult G080-GFP-Imp protein-trap brains treated, or not, with KCl or Tyramine. Both a-Me31B and a-Tub (loading control) antibodies were used.

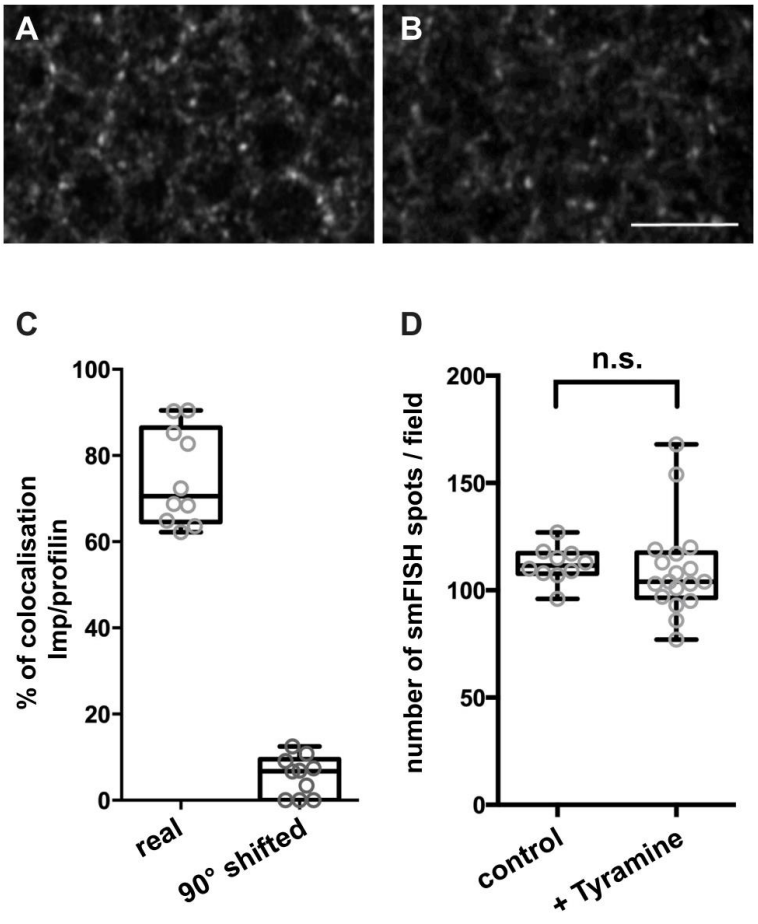

Figure S4. *profilin* **mRNA levels are not changing upon Tyramine treatment - related to Figure 6.**
(A,B) Cell bodies of adult Mushroom Body γ neurons stained with profilin stellaris probes. Images correspond to single confocal sections. Scale bar in A,B: 5 µm. (C) Distributions of the percentage of Imp granules containing *profilin* mRNA. Colocalization between Imp granules and smFISH spots was calculated using the Fiji JACoP tool (object-based method). The results of a single replicate are shown. (D) Distributions of the numbers of profilin smFISH spots per imaged field. Spots were detected with the SPADE algorithm (see Fig. S1). The results of a single replicate are shown. Numbers of imaged fields in C,D: 10 (control) and 18 (+ Tyramine). n.s. stands for not significant.

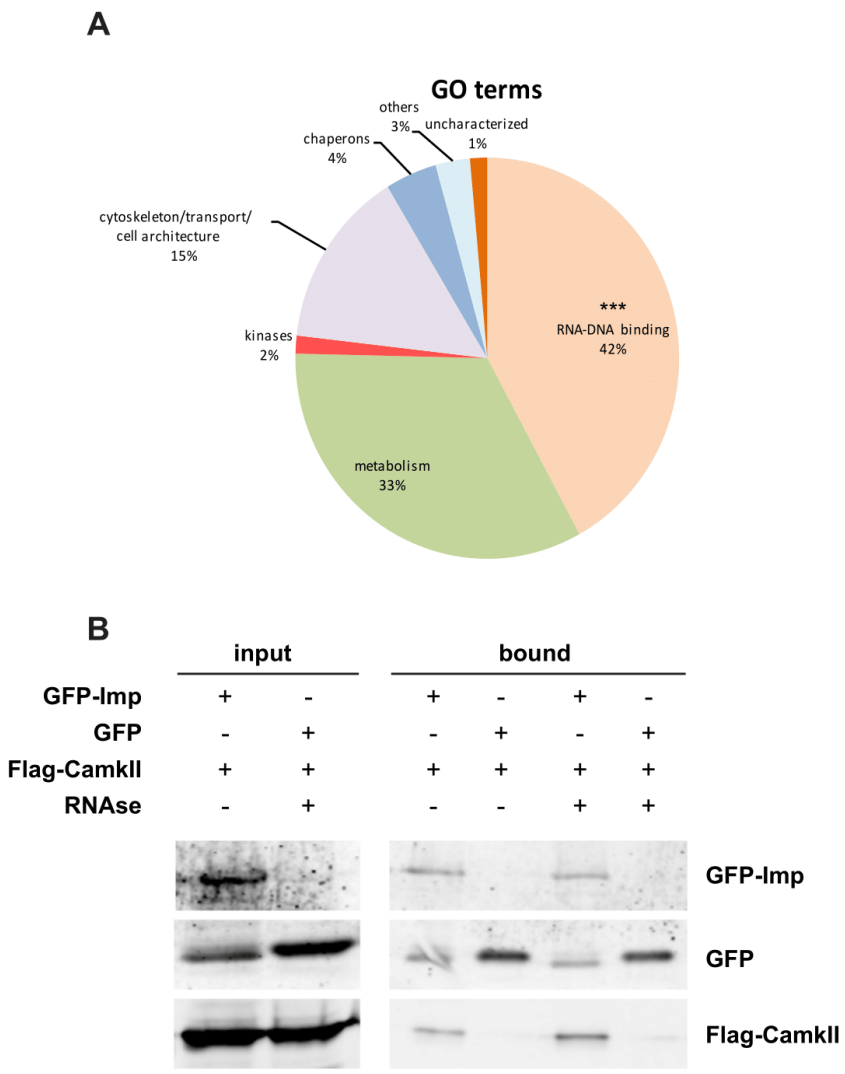

Figure S5. CamkII: a physical partner of Imp.
(A) Pie chart representing the distribution of Imp protein partners in function of their biological GO category. 69 protein partners were reproducibly identified by Mass-Spectrometry analysis of the bound fraction recovered upon immuno-precipitation of GFP-tagged Imp proteins expressed in Mushroom Body neurons (201Y-Gal4, UAS-GFP-Imp). Lysates of adult heads were used for the IP-MS experiments and control IPs (201Y-Gal4, UAS-GFP) were performed in parallel. ***, P<0.001. (B) Imp and CamkII physically interact in S2R+ cells. Flag-Imp constructs were transfected together with GFP-Imp or GFP (negative control), and GFP proteins immunoprecipitated using GFP-trap beads. The Western-Blot was performed from input (left) and bound (right) material. Cell lysates were treated (+) or not (-) with RNAse prior to immunoprecipitation. a-GFP and a-Flag antibodies were used to detect GFP-Imp (or GFP) and Flag-CamkII fusions.

II – Results

Part 2 – Complementary Results

8. Complementary Results

8.1. Summary

The work described in the previous section demonstrates that Imp RNP granules remodel in response to neuronal activation in the context of intact brains. Notably, Imp RNP complexes undergo a reversible disassembly that is functionally associated with an increase in the translation of target mRNAs. Granule disassembly requires CamkII, a partner to which Imp binds in an RNA-independent manner.

In the next section, I further summarize results related to the following three main aspects:

1. ***The Imp/CamkII interaction and the impact of CamkII activity modulation on Imp RNP properties in γ cell bodies.*** In particular, I tested if CamkII activity is required for Imp/CamkII interaction and studied the impact of either CamkII inhibition or constitutive activation on Imp RNP granule features *in vivo*.

2. ***Imp distribution in γ axons and the possible involvement of CamkII in its transport and/or docking***. Here, I first assessed the sub-cellular distribution of CamkII within *γ* axons, where Imp is localized and visible as punctae. I then analyzed if Imp transport to axons was perturbed upon inhibition or constitutive activation of CamkII. Finally, I investigated the possible interaction of Imp and CamkII to the actin cytoskeleton, by testing the capacity of the two proteins to bind F-actin.

3. ***Imp role in memory processes.*** In collaboration with the Keleman laboratory, we investigated the role of Imp in the formation of both short- and long- term memory using the courtship conditioning paradigm.

8.2. Impact of CamkII activity on Imp RNP granules

To dissect the molecular interaction between CamkII and Imp, I tested whether inhibiting CamkII

activity modulated its binding to Imp. To this end, I generated a kinase dead version of CamkII unable to hydrolyse ATP by mutating lysine 43 (K43) to a methionine, using site-directed mutagenesis. Previous work performed in mammals revealed that homozygous K42M mice showed normal *CamkII* mRNA and protein patterns but a 60-70% reduction in kinase activity, whose loss impacts LTP formation, dendritic spine enlargement and learning (Yamagata et al. 2009). Moreover, expression of kinase dead versions of CamkII did not impact the capability of CamkII to bind Calmodulin, to oligomerize into functional dodecamers and to be transported to post-synaptic densities upon activity increase (Hanson et al. 1994; Mukherji and Soderling 1994; Shen and Meyer 1999). As revealed by Co-IP experiments performed in *Drosophila* S2R+ cells, expressing K43M-CamkII nearly abolished interaction between the two proteins (Figure R1A), suggesting that CamkII kinase activity is required for the Imp-CamkII association.

Hence, we hypothesized that this interaction might occur upon activity-dependent CamkII auto-activation and might modulate the properties of Imp RNP granules. To test this, I chronically expressed a constitutively active form of CamkII (Jin et al. 1998; Park et al. 2002) in MB γ neurons and analyzed the Imp RNP complexes found in cell bodies. This UAS-driven form of CamkII is characterized by a point mutation of the threonine 287 to aspartic acid, thus mimicking a constitutive phosphorylated state (Jin et al. 1998). In these conditions, no disassembly was observed, suggesting that CamkII activation is not sufficient to induce Imp RNP dissolution (Figure R1B,C).

In parallel, I tested the impact of basal CamkII activity on Imp RNP granules by expressing the inhibitory peptide ala throughout development (Griffith et al. 1993). Inhibiting CamkII activity did not induce major changes in Imp RNP granule morphology (Figure R1D) compared to control condition (Figure R1B), as no detectable changes in RNP size or number could be observed (data not shown). This result indicates that constitutively decreasing CamkII activity does not have a major impact on Imp RNP granules in MB γ cell bodies.

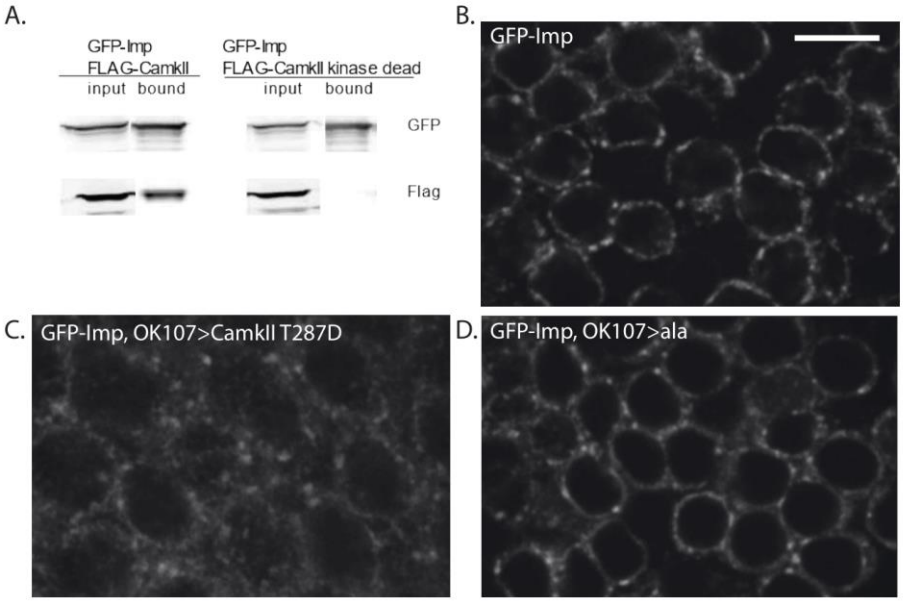

Figure R1. Impact of CamkII activity of Imp/CamkII interaction and Imp RNP granules. A) CamkII kinase dead (K43M) form does not interact with Imp in S2R+ cells. GFP-Imp constructs were co-transfected with either Flag-CamkII wild-type or Flag-CamkII:K43M in S2R+ cells. GFP alone was used as a negative control (data not shown). Immunoprecipitation was performed using GFP-trap beads. Input (left) and bound (right) fractions were used for western blot and detection was performed using a-GFP (for GFP-Imp) and a-Flag (for FLAG-CamkII) antibodies. (B-D) Distribution of GFP-Imp in adult mushroom body neurons upon OK107 Gal4-dependent expression of a constitutively active form of CamkII (C, T287D) or an inhibitory peptide (D, ala). Upon constitutive activation of CamkII, Imp RNP granules are not disassembled (C). CamkII inhibition does not impact Imp RNP morphology (D). Scale bar: 5 µm.

8.3. Imp RNP granules and CamkII in γ axons

Previous research has established that Imp is transported as particles toward MB γ axons from metamorphosis onwards (Medioni et al. 2014). To observe axonal Imp RNP granules with high-resolution, I dissected in Haemolymph-Like buffer 3 (HL3) adult brains carrying endogenously tagged GFP-Imp and then imaged GFP fluorescence in γ axons, using Airyscan microscopy. There, Imp RNP granules were detectable as round 300-400nm-diameter entities (Figure R2B). We reasoned that if Imp RNP complexes are regulated by CamkII in axons, CamkII should be found in axons and activated there. To check if this was indeed the case, I dissected brains from flies carrying a functional GFP insertion in the endogenous *CamkII* locus and stained them with phospho-CamkII antibodies, which recognizes the activated CamkII. Interestingly, pCamkII strongly stained the population of γ -axons (Figure R2C''), whereas pan-CamkII staining was rather low within the γ lobes (Figure R2C').

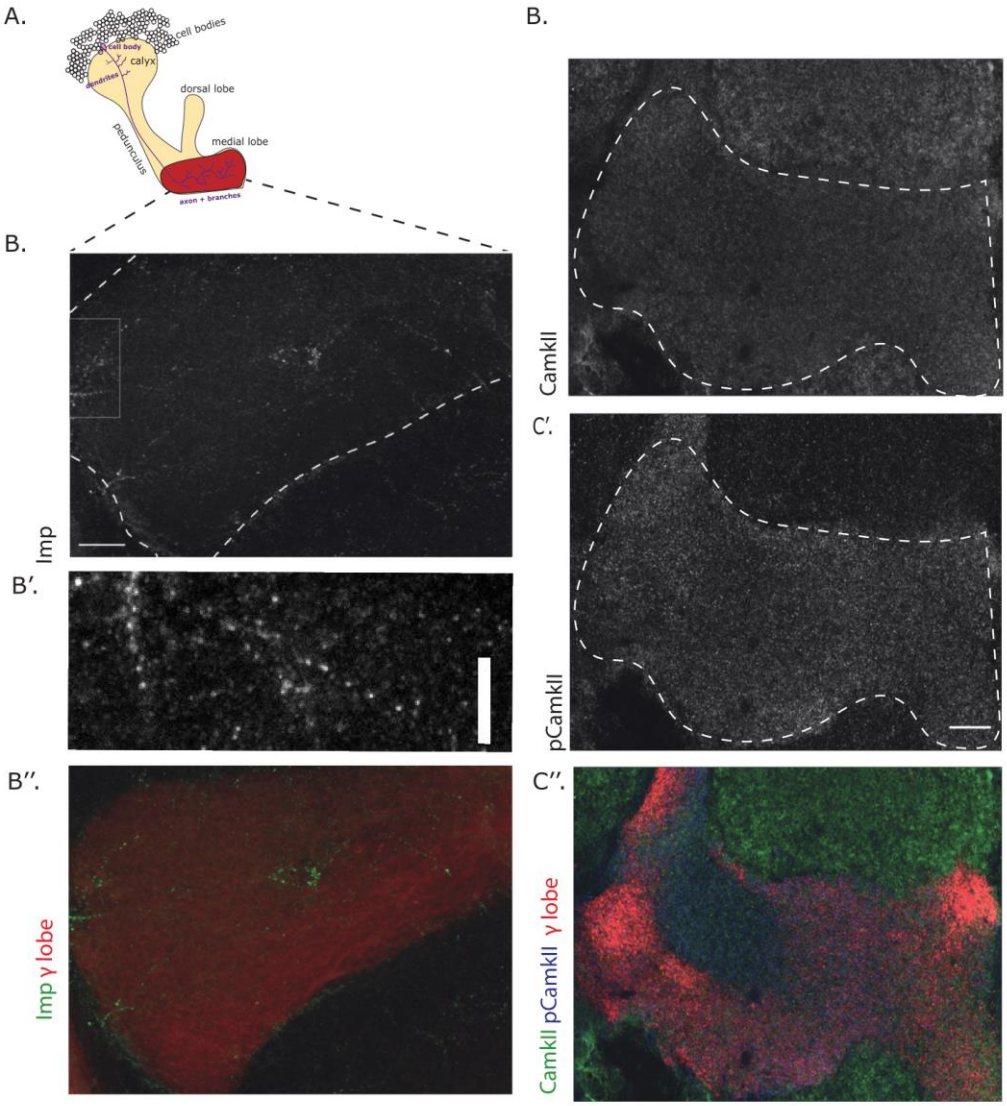

Figure R2. Imp RNP granules and CamkII distribution in γ axons.
A) schematic representation of an adult mushroom body, a single γ neuron is drawn in violet. All γ axons (about 600 in total) project within the γ lobe, here shown in red. B-B'') Imp RNP granules in γ axons, visible as punctae. The endogenous GFP signal from the G080 Imp protein-trap line was imaged and OK107Gal4 driven RFP expression was used as a marker of the γ lobe (B''). B' represents the magnified boxed region in C. Scale bar in B': 5 μm. C-C'') CamkII distribution in the adult γ lobe. Brains from flies carrying a homozygous-viable GFP insertion within the CamkII locus were dissected and stained for GFP (C, CamkII) and pCamkII (C', active CamkII). pCamkII is enriched in the γ axonal region (C'). FasII was used as a marker for the γ lobe (C'', red). Scale bar in all other images: 10 μm.

8.4. CamkII promotes Imp localization to MB γ axons

Although different aspects of Imp translocation to axons have been elucidated (Medioni et al. 2014; Vijayakumar et al. 2019), the players involved in both RNP transport and anchoring are still undescribed. CamkII has been shown to interact with kinesin motors and myosin and to regulate cargo release in different systems (Karcher et al. 2001; Guillaud et al. 2008; Lemieux et al. 2012). Thus, we investigated the role of CamkII in Imp axonal transport. Expression of the ala and T287D constructs was triggered from fly hatching onwards using the TARGET system (McGuire et al. 2003). After 8-10 days of transgene expression at permissive temperature (29°C), I quantitatively analyzed the distribution of endogenous GFP-Imp protein in γ axons in the presence or absence of CamkII transgenes. Interestingly, a 30% reduction in GFP-Imp axonal accumulation was observed upon CamkII inhibition (Figure R3D), whereas the constitutive activation of CamkII had no effect (Figure R3A-C). These results suggest that CamkII may promote the transport or the stabilization of Imp to γ axons.

As previously demonstrated, mammalian CamkII directly binds and regulates F-actin in both dendrites and axons (Okamoto et al. 2007; Khan et al. 2019; Xi et al. 2019). Although Imp RNP granules were shown to travel to axons in a MT-dependent manner during metamorphosis (Medioni et al. 2014), localization of RNAs to axons can rely on both microtubules and actin tracks for long- or short-range movements, respectively (Muslimov et al. 2002; Lopez de Heredia and Jansen 2004). Moreover, the actin cytoskeleton has also been involved in mRNA docking in axons (Yoon et al. 2016). To thus investigate whether Imp and CamkII may associate with F-actin for localization, I performed F-actin-precipitation experiments *in vivo* in *Drosophila* heads. While both Imp and CamkII were found in the F-actin-bound fraction, Tubulin, which was used as a negative control, was not (Figure R3E). Thus, our results show that both Imp and CamkII interact with F-actin *in vivo* (Figure R3E), although we could not assess if CamkII directly bridges Imp to the F-actin cytoskeleton, as CamkII knock-out lines are homozygous lethal. Whether interaction with F-actin is involved in the accumulation of Imp to axons remains to be demonstrated.

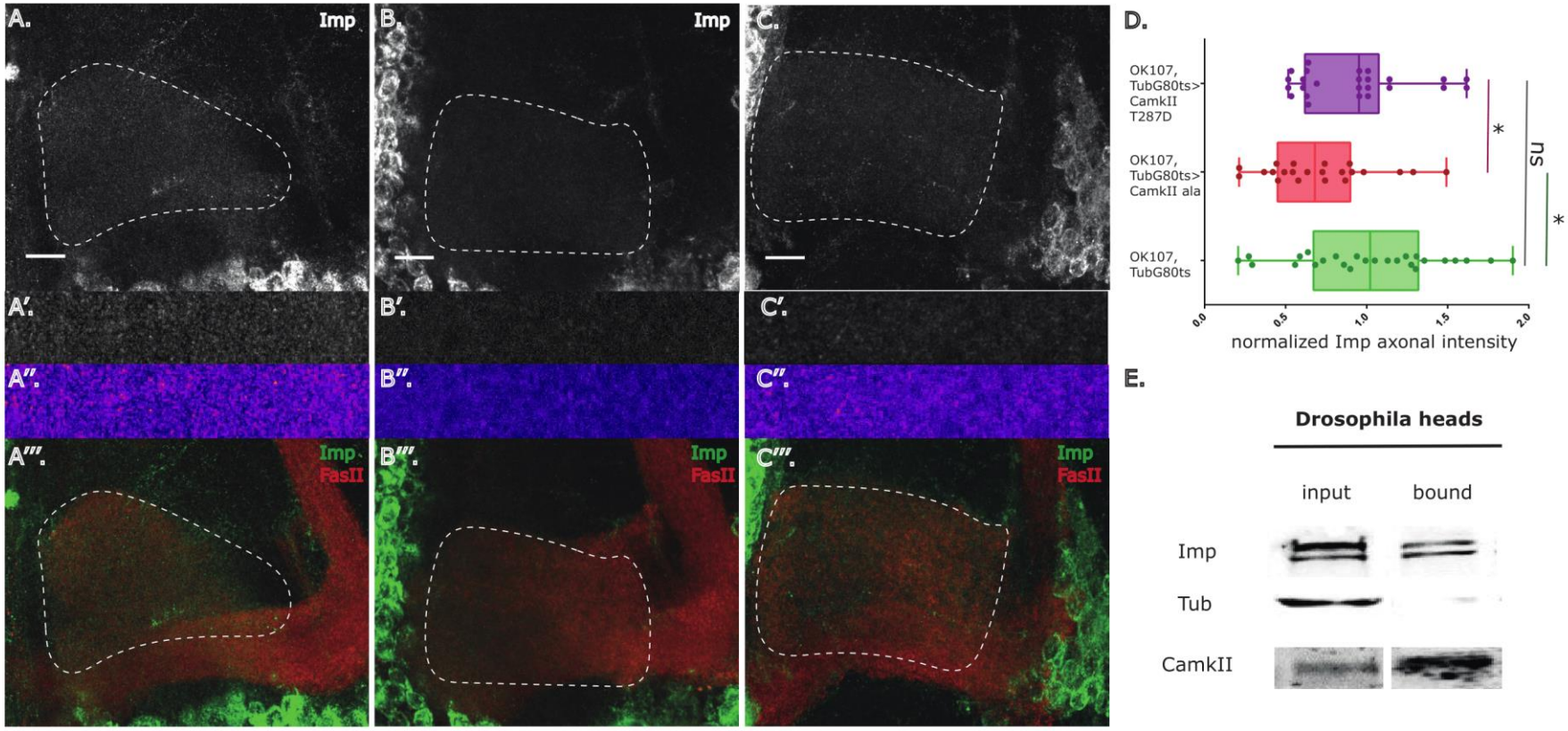

Figure R3. CamkII inactivation leads to decreased localization of Imp in MB γ axons.
A-C) medial lobes of adult brains expressing GFP-Imp and transgenes either inhibiting (B) or constitutively activating (C) CamkII. FasII staining was used as a marker of MB lobes (red in A‴-C‴). Genotypes: A) TubG80ts;OK107-Gal4, B) TubG80ts;OK107-Gal4> CamkII ala and C) TubG80ts;OK107-Gal4> CamkII T287D. Five-fold magnifications of GFP-Imp axonal staining corresponding are shown in grey levels (A'-C') or using the "Fire" look-up table of ImageJ (A''-C''). D) Normalized GFP signal distribution in distal axons (min to max box plot). Number of MB analyzed: GFP-Imp, 26; GFP-Imp + UAS-CamkII ala, 22; GFP-Imp + UAS-CamkII T287D, 25. *, $p<0.05$ (Mann-Whitney test). ns, not significant. Scale bar in B1-D1: 10 µm. E) F-actin precipitation experiments performed in *Drosophila* fly heads. Both Imp and CamkII co-precipitate with F-actin. Input and bound fractions were blotted using α-Imp and α-CamkII antibodies on western blot. Tubulin is used as a negative control.

8.5. Imp is required for LTM *via* its PLD

LTM formation – in contrast to STM – requires *de novo* protein synthesis (Kang and Schuman 1996), involving local translation of dormant mRNAs previously transported and stored at synapses. For this reason, proteins involved in mRNA regulation and transport to neurites such as Imp represent strong candidate regulators of LTM establishment. As we wanted to investigate the role of Imp in memory formation, we teamed up with the group of Krystyna Keleman (Janelia Farm, USA) and performed courtship conditioning assays (Keleman et al. 2007). This robust conditioning procedure was particularly suited for our purpose, as (i) it relies on MB γ neurons, where Imp is expressed and localized (Akalal et al. 2006; Kruttner et al. 2015) and (ii) it can produce both short- and long-term memory. Interestingly, the memory elicited by this test has also been shown to depend on protein synthesis (Kruttner et al. 2015). The test is based on the natural behavior of *Drosophila* adult males, that are naturally inclined to court females to ensure their reproductive success. If males are put in the presence of a pre-mated female, they experience rejection (Siegel and Hall 1979), as the mated female runs away from the male and can even kick him. This rejection acts as a conditioned stimulus on the male and suppresses his courting behavior, leading to a decreased interest in females (Siegel and Hall 1979). *Drosophila* courtship conditioning relies on chemosensory cues, olfaction and pheromones (Mehren et al. 2004; Ejima et al. 2005; Siwicki et al. 2005). In fact, a chemical compound marks mated females but not virgins: the cis-vaccenyl acetate (cVA), an anti-aphrodisiac pheromone that dissuades males from courting (Brieger and Butterworth 1970; Guiraudie-Capraz et al. 2007). A few hours after copulation, females eject the cVA from their reproductive tract and restore their attractiveness to males (Laturney and Billeter 2016). This classic paradigm has been adapted to serve as an efficient assay to test learning and associative memory: Here, a male is trained with a mated female that is going to reject his courtship attempts, then kept in isolation to be further tested with another mated female. If the male remembers the rejection he was subjected to during the training, he will reduce his courtship behavior. In contrast, if he cannot recall the memories related to the previous training, he will court the female tirelessly. The possibility to induce both short- and long-term memory relies on the nature of the training: while a 30 minute-long training induces memories lasting few hours (STM), a 1.5 hour-long training induce memories lasting for up to days (LTM) (Figure R4). This assay is then translated in numbers by calculating i) the percentage of time that the male spends courting and that corresponds to the so-called “Courtship Index” (CI) and ii) the reduction of the CI in flies that have been trained compared to naïve untrained males “Suppression Index”. To knock-down Imp exclusively in adulthood, we induced the expression of deGradFP nanobodies (Caussinus and Affolter 2016), that target endogenously expressed GFP-tagged Imp proteins (G080) using the TARGET system. With this approach, we obtained an adult tissue-specific knock-down of Imp in mushroom bodies upon temperature-switch to 30°C. Conditional *imp* mutant males were tested for

their capacity to form short- or long-term memory (Figure R4 A-A'), revealing that temperature-dependent *imp* knock-down did not impact short-term memory, but dramatically affected long-term memory (Figure R4 B-B').

In these experiments, Imp levels were decreased in both cell bodies and axons, rendering it impossible to conclude about where the function of Imp is required for LTM. Interestingly, recent work showed that a prion-like domain (PLD) located at the C-terminus of Imp is involved in the transport of Imp RNP granules to axons (Vijayakumar et al. 2019). While flies endogenously lacking the Imp PLD are homozygous viable and express Imp proteins at wild-type levels, localization of Imp to axons is impaired in both pupae and adults. Remarkably, courtship conditioning assays performed on these flies recapitulated the phenotype observed upon conditional Imp knock-down (Figure R4C-C'). Altogether, our results thus show that Imp is necessary specifically for LTM and suggest this function may require Imp localization to axons.

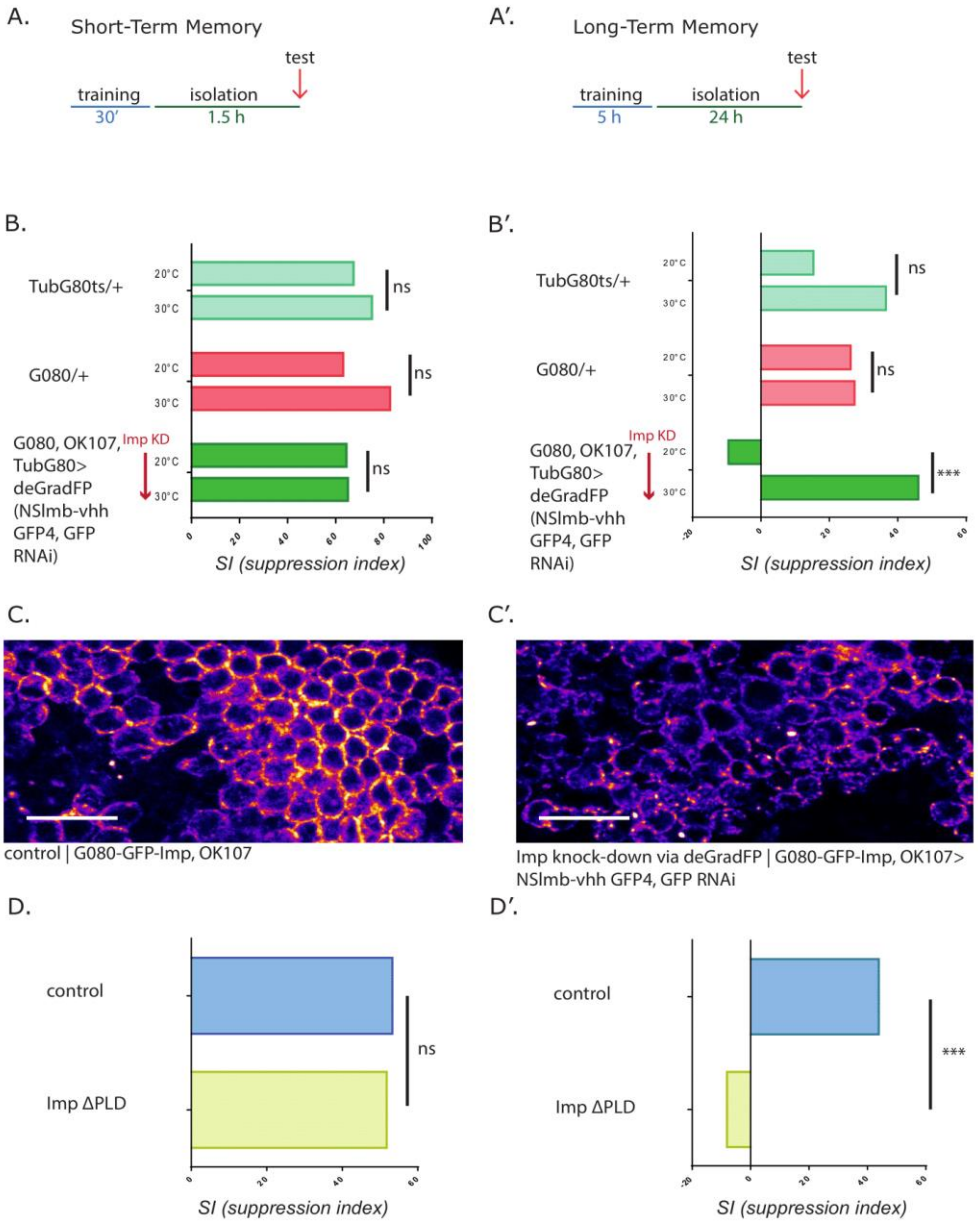

Figure R4. Imp is required for LTM formation in *Drosophila* Mushroom Body neurons.
A-A') schematic description of the different training and isolation protocols used to produce short- and long-term memory in courtship conditioning assays. Freshly hatched males are kept isolated until the training (STM, 30', A/LTM, 5 hours, A'), during which they experience r ejection from a pre-mated female. After the training, males are placed back in isolation (STM, 1.5 hours, A/LTM, 24 hours, A') before being tested in the presence of another pre-mated female. B-B') temperature-induced knock-down of Imp in MB neurons leaves short-term memory unaffected (B) but dramatically impairs long-term memory , compared to controls (B'). Genotypes are indicated. The red arrow indicates the temperature swich necessary to induce Imp KD. C-C') Extent of Imp down-regulation upon degradFP expression triggered by OK107 Gal4 (right panel) compared to control (left panel), visualized in MB γ cell bodies. Images are represented using the "Fire" LUT of ImageJ. Scale bar: 10 µm.
D-D') Imp PLD is required for LTM but not for STM. Behavioural test were performed on control flies carrying a wild-type form of Imp (G080-GFP-Imp) as a control and on flies endogenously lacking the C-terminal Imp PLD (G080-GFP-Imp ΔPLD). Median LI values from pooled independent experiments are plotted and analized by permutation tests, ***:p<0.001. Total number of flies analyzed for each condition is comprised between 30 and 55. Tests were performed by Ugur Dag (Krystyna Keleman group, Janelia Farm - HHMI, Ashburn, USA).

8.6 Materials and Methods

Drosophila lines and genetics

Flies were raised at 25°C on standard media. The following additional stocks were used: UAS-CamkII.T287D (BSC #29664) and GFP-Imp-G080;;UAS-NSlmb-vhh GFP4, UAS-RNAi-GFP, a fly stock carrying the G080 and the deGradFP constructs (Caussinus and Affolter 2016). The CamkII ala and T287D constructs were expressed either chronically under the control of the OK107-Gal4 for assessing their impact on Imp RNPs in cell bodies, or specifically during adulthood with a tubulin-Gal80ts,OK107-Gal4 line to observe Imp axonal distribution. In the latter case, flies were raised at 21°C until hatching and then switched for 8-10 days at 29°C.

Generation of CamkII Kinase Dead construct

To obtain a Kinase Dead version of CamkII, the K43 ATP binding domain of the kinase was mutated to a methionine via site-directed mutagenesis using the pENTR-D/TOPO-CamkII as template. Mutagenesis was performed using the following primers: CamkII_sdm_forward (5'-GTTAATTTTTTTGTATTGATAATCATTGCAGCAAATTCAAAGCCA-3') and CamkII_sdm_reverse (5'-CTGGCTTTGAATTTGCTGCAATGATTATCAATACCAAAAAATTAA-3'). The pENTR-D/TOPO-CamkIIKD obtained was fully sequenced and LR-recombined into a pAFW vector.

F-actin precipitation

20 adult flies were collected per condition, plunged into liquid nitrogen for 2-3 minutes and then decapitated. The obtained heads were lysed in Homogenisation Buffer (HB -100 mM Na_2HPO_4–NaH_2PO_4 pH 7.2, 2 mM ATP, 2 mM $MgCl_2$) containing 1:100 Halt™ Protease Inhibitor Cocktail (ThermoFisher scientific, # 78429). The lysates were incubated for 30 minutes at room temperature with 0.5 units of Biotin-xx Phalloidin (ThermoFisher scientific, # B7474). Streptavidin-coated magnetic beads were blocked with PBS-BSA 5% and then added to the extracts for 30 minutes to allow isolation of biotinylated-phalloidin bound complexes. After washes, all samples were denaturated briefly at 95°C prior to analysis *via* western blot. For membrane blotting 1:1000 α-CamkII antibody (gift from Bih-Hwa Shieh) was used.

Imaging of adult brains, immunostaining and quantifications

9-11 days old adult *Drosophila* brains were dissected in cold PBS 1x or HL3 (for axonal imaging) medium. Brains were fixed for 25 minutes at RT in 4% formaldehyde and washed thrice in PBS/Triton-X (PBT) 0.5%. Samples were either mounted in vectashield (Vector Laboratories) or immunostained. For immunostaining, brains were blocked overnight in PBS/Triton-X (PBT) 0.5%-

BSA 1% and then incubated using α-FasII antibody (mouse, DSHB, 1D4, 1:15). An AF568-conjugated mouse secondary antibody was used.

Images were acquired on a Zeiss LSM880 Fast Airy Scan inverted confocal microscope using either a C-Apo 40x NA 1.4 or a Plan Apo 63X NA 1.4 oil objective. Imp axonal signal was measured from ROIs of 215,41 μm^2 selected using FasII staining as a template and background signal was subtracted. Obtained values were normalized to control samples.

Courtship conditioning assays

Behavioural assays were performed in the Keleman lab, at Janelia Research Campus (Ashburn, Virginia). Freshly hatched males were kept in isolation for 5-6 days and then trained (during day time) with (males defined as trained) or without (males considered as naïve) a single mated female. After training, males were put back in isolation before being tested for memory with fresh mated females. The duration of training and isolation were 1 hour – 30 minutes and 6 hours – 24 hours for STM and LTM tests respectively. Tests were performed in 10 mm diameter chambers and recorded for 10 minutes (Prosilica GT cameras, Allied Vison Technologies). The videotape was used to derive a courtship index (CI) for each male and corresponds to the percentage of time that the male spends courting the female in the 10 minutes recorded. Memory measures refer to courtship suppression index (SI), which accounts for the reduction in the mean courtship indices of trained (CI^+) versus naïve (CI^-) populations ($SI = 100*[1-CI^{train+}/CI^{train-}]$).

III - Discussion

9. Discussion and perspectives

Altogether, my PhD work has uncovered a novel feature of Imp RNP granules: their ability to remodel in response to neuronal activation. This remodelling consists in a rapid yet transient disassembly, associated with an increased translation of Imp target mRNAs. These results are in line with previous models considering RNP granules as sources of dormant mRNAs that would be released and relieved from translational repression in response to specific cues. Although few previously published studies have described activity-induced RNP disassembly events, much of the research up to now has concentrated on *in vitro* neuronal cultures. This study thus further provides proof of stimulation-induced RNP remodelling, and – more importantly – extends this evidence to intact brains, highlighting the specificity of the response and the differential response of granule components.

In this chapter, I discuss the main results obtained during my PhD. The first part will cover Imp RNP granule remodelling, mainly focusing on activity outcomes on cell bodies and dendrites (§ 9.1). Next, I will discuss the potential role of Imp in MB γ axons (§ 9.2). Finally, the last part of the discussion will be dedicated to the function of Imp in memory formation and our speculations about it (§ 9.3).

9.1 Imp RNP remodelling in response to neuronal activation: dynamics, rearrangement and mechanics

Neurons need to respond to localized external cues by regulating their protein content in a compartment-specific manner. One of the strategies adopted by neuronal cells to fulfil this need is to store dormant mRNAs and regulatory proteins in macromolecular RNP complexes, which can act as veritable local reservoirs of translatable transcripts (Kiebler and Bassell 2006; De Graeve and Besse 2018; Formicola et al. 2019). These poised transcripts would then be promptly translated in response to external stimuli, providing the required protein supply. This model implies that, in response to stimulation, RNP complexes would undergo dramatic rearrangements, ultimately leading to transcript de-repression. A key question is: what are these rearrangements? In neuronal cell cultures, previous research has successfully shown that neuronal stimulation can

have different consequences on RNP granules: RNPs can remodel by changing their composition (Mitsumori et al. 2017), dynamics (Antar et al. 2004; Cougot et al. 2008) or by de-compacting into looser forms (Krichevsky and Kosik 2001; Buxbaum et al. 2014), but can also undergo dissolution (Zeitelhofer et al. 2008; Baez et al. 2011; Wong and Tsai 2019). Nevertheless, *in vivo* evidence of analogous activity-dependent RNP rearrangement events is still lacking. In this context, my PhD work has shown that Imp RNP granules undergo activity-induced disassembly *ex vivo,* in entire *Drosophila* brains. This data not only is in line with previous research reporting RNP complexes decompaction in response to neuronal stimulation in cultured neurons, but provides – to our knowledge - unprecedented proof of activity-dependent RNP disassembly in intact tissues. Notably, I showed that Imp RNP granules undergo reversible disassembly in response to a general depolarizing chemical – KCl – but also – and more relevantly - upon treatment with an endogenous neuromodulator, tyramine. Genetic neuronal silencing baffled this process, confirming its activity-dependence. Furthermore, I have demonstrated that granules dissolution is functionally related to increased translation levels of target mRNAs (Figure D1A).

Overall, this work provided insights into four main complementary aspects that will be discussed below: 1) the dynamics and internal activity-induced rearrangements within RNP granules; 2) the mechanisms behind it and 3) the associated functional consequence; and 4) the specificity of the stimuli triggering Imp RNP de-clustering.

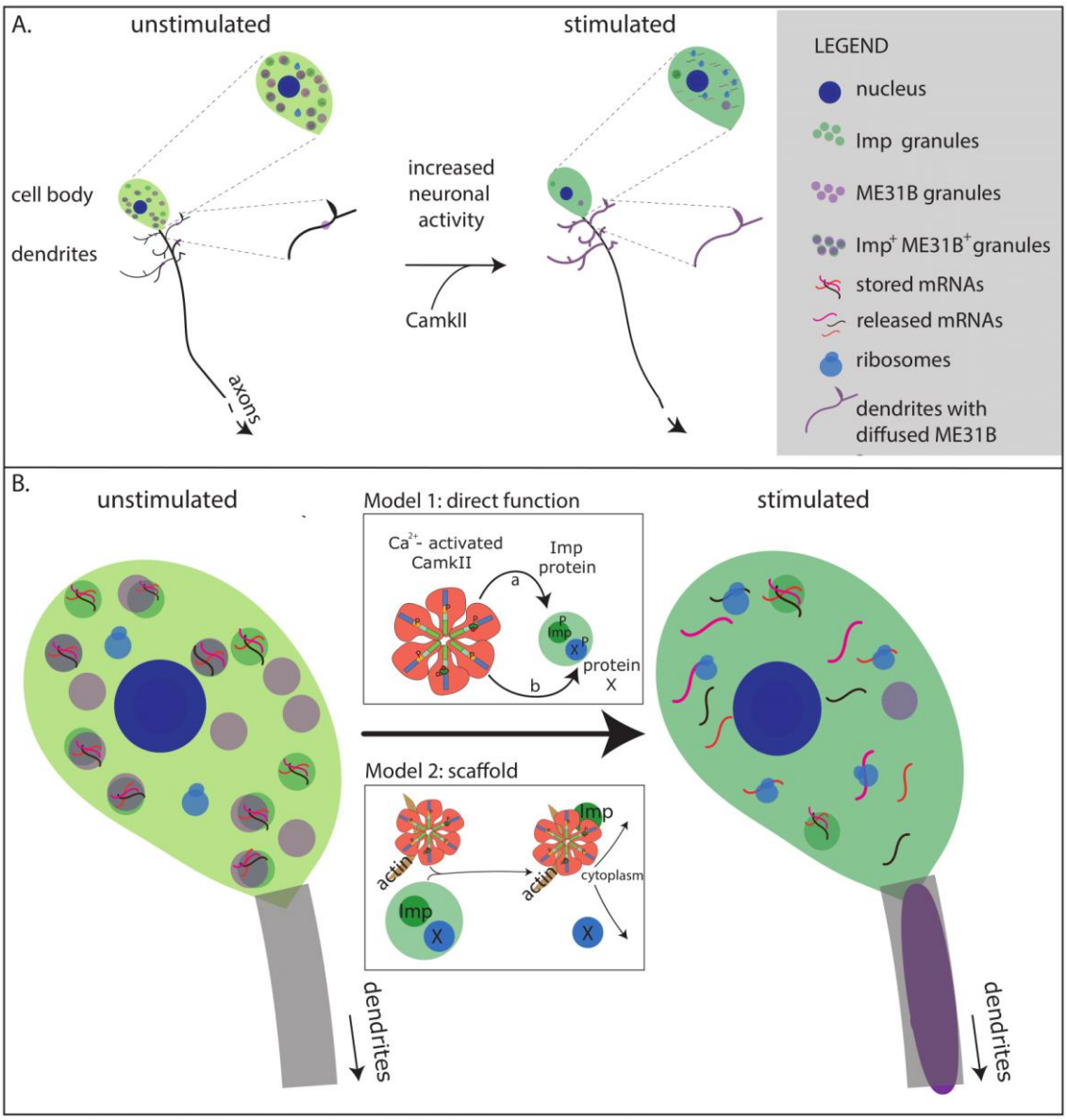

Figure D1. Model for Imp RNP activity-induced remodelling
A) Imp RNP complexes undergo activity-dependent disassembly upon stimulation. Functionally, Imp RNP disassembly is associated with an increased translation of target mRNAs, which are likely released from the complexes and translated. This remodeling requires CamkII. In dendrites, ME31B+ Imp - granules are disassembling and Me31B is found diffusively localizing throughout the calyx. A single neuron is illustrated, arrows point at axonal regions which are not portrayed. B) Model for CamkII function in activity-dependent remodelling. CamkII has been shown to be required for stimulation-induced Imp RNP disassembly. In our model, we propose that CamkII could have an enzymatic or a structural function. In the first case (Model 1), CamkII would act through its kinase activity, by phosphorylating either Imp (a) or another Imp RNP granule component (b). As for the second hypothesis (Model 2), CamkII would serve as a scaffold, regulating Imp interaction with other proteins or with cytoskeleton/cytoskeleton -associated proteins. In the boxed regions, the RNA is omitted.

1) Dynamics and rearrangements within Imp RNP granules

1a. activity-induced RNP disassembly

My results showed that Imp respond to KCl and tyramine treatments by de-clustering and diffusing into the cytoplasm. This phenomenon is observed within 30 minutes after tyramine treatment and can last for up to 90 minutes without inducing cellular stress (data not shown). The disassembly of Imp granules is further supported by the fact that the punctate distribution of Me31B (DDX6/RCK/p54), a *bona fide* component of Imp RNP granules, is also lost upon stimulation (Figure 1D).

Moreover, our data indicates that RNP disassembly is transient and can be reverted in 30 minutes by removing tyramine from the medium. This result suggests that (i) the effect is indeed triggered by tyramine, (ii) granule disassembly is a dynamic process which could be used as a means to respond to environment transient fluctuations. This result is also in line with previous research reporting that granules were restored to basal levels within periods of time above 30 minutes from the initial stimulation pulse (Zeitelhofer et al. 2008; Baez et al. 2011). To better define the dynamics of Imp RNP granules and check whether their activity-induced remodelling might be triggered even more rapidly, it would now be interesting to perform live-imaging experiments (Medioni et al. 2015).

1b. Imp and ME31B in somata

RNP granules are membraneless organelles whose general composition consists of stored mRNAs and regulatory RBPs. However, a vast repertoire of RBPs has been found within different kind of RNP granules, signifying that RNP content is much more heterogeneous than previously thought (Macchi et al. 2003; Barbee et al. 2006; Cougot et al. 2008; Miller et al. 2009; Fritzsche et al. 2013; Fallini et al. 2014; Mitsumori et al. 2017; Guzikowski et al. 2019). Assuming this, an interesting possibility is that RNP content is rather regulated by finely-tuned mechanisms that mould RNP composition and hence RNP properties according to external cues. Although evidence to support this model is still largely scarce, it was shown that dendritic P-bodies exchange components and increase their dynamicity in response to synaptic activation (Cougot et al. 2008). In our system, Me31B granules show a high degree of colocalization with Imp RNP complexes in physiological conditions in the cell bodies of MB γ neurons, whereas they do not contain Imp at all in dendrites. This result is in line with previous work in *Drosophila* projection neurons, where Hillebrand and collaborators reported different compositions in somatic and synaptic ME31B granules (Hillebrand et al. 2010).

Upon stimulation, we observed ME31B de-clustering in both soma (as happens for Imp) and dendrite compartments (Figure 1D). The relationship between ME31B and Imp – and their different

compartment-wise behaviour - led us to wonder what might be the respective roles of these two proteins in RNP granule assembly and maintenance. In fact, RNPs are known to be composed of proteins serving as scaffolds – which are essential for RNP formation – and other proteins, called clients, which are rather transiently recruited to the RNP complexes (Ditlev et al. 2018; Xing et al. 2018). Recent unpublished results obtained in our lab show that interfering with ME31B function leads to Imp RNP granule loss (Kavya Vinayan and Florence Besse, unpublished), suggesting that ME31B acts as a scaffold rather than as a client. Nevertheless, performing the complementary experiment and investigate the effect of Imp knock-down on ME31B granule formation would interestingly show whether Imp itself is a co-scaffold or a client. For their role in RNP regulation, scaffold proteins are also thought to be more stably associated to the RNP (Panas et al. 2016; Ditlev et al. 2018). In line with this assumption, clients would rapidly exchange from granules, easily shuttling in and out, while scaffolds would exhibit slow molecular dynamics (Kedersha et al. 2005; Panas et al. 2016; Xing et al. 2018). The use of the FRAP technique, that was recently further optimized in our lab to be performed on *Drosophila* brain explants (Viyajakumar et al. 2019), would be beneficial to detail the dynamics and exchange rate of ME31B and Imp to further elucidate their respective role in granule homeostasis and regulation.

1c. biophysical insights into RNP disassembly

RNP assemblies are supramolecular condensates with liquid-like behavior that exhibit regulated yet acute responsiveness to cellular environment. By virtue of their fluid features, RNP can rapidly exchange components, resulting in dynamic assembly/disassembly. Regulated RNP disassembly has been proposed to occur in two steps, notably the RNP shell dissipation followed by clearance of the core (Wheeler et al. 2016). To test this hypothesis in our system – once the relationship of scaffold/client is established between Imp and Me31B – it would be interesting to test upon stimulation which one is the first one to disassemble.

Very interestingly, RNP phase separation largely relies on electrostatic interactions, consistently with a crucial role for cation concentrations in LLPS (Banerjee et al. 2017; Mittag and Parker 2018; Rayman et al. 2018; Onuchic et al. 2019). In particular, increased Ca^{2+} concentrations have been associated with RNP condensate disassembly (Banerjee et al. 2017; Onuchic et al. 2019), consistently with my data showing RNP dissolution upon dramatical increase in Calcium levels.

However, the molecular trigger responsible for Imp RNP granule dissolution remains currently obscure. Several findings point at PTMs as suitable candidates for such function. First, PTMs – and notably phosphorylation - have been largely associated with regulation of LLPS and have been shown to impact the relationship between scaffolds and clients (Banani et al. 2016; Banani et al. 2017; Lepelletier et al. 2017; Ditlev et al. 2018; Hofweber and Dormann 2019). Second, evidence has shown nor only that the mammalian Imp protein can be phosphorylated but that this Src-mediated phosphorylation underlies Imp RNP granule disassembly (Huttelmaier et al. 2005;

LePellettier et al. 2017), suggesting that an akin mechanism could occur in *Drosophila*. Third, *Drosophila* Imp interacts with the Ca^{2+}-dependent kinase II (CamkII) and this interaction relies on CamkII activity (this work). The CamkII-Imp interaction and its functional relevance are under investigation (see point 2) with the aim of understanding if Imp RNP disassembly depends on phosphorylation.

As for Me31B, evidence is still lacking regarding its possible regulation *via* PTMs. However, although no kinase nor PTM-mediating enzyme has been shown to date to interact with Me31B, the possibility of this interaction cannot be excluded. Performing a comprehensive Me31B interactome study may help provide insights into the potential biochemical pathways Me31B could be regulated by. Following such a screening, potential candidate regulators could be identified and investigated in order to dissect the molecular mechanisms underlying Me31B granule assembly/disassembly in both cell bodies and dendrites.

1d. Me31B recruitment to dendrites

Importantly, we also unexpectedly observed a massive recruitment of ME31B to dendrites (Figure 1D) upon tyramine-mediated stimulation. This strong relocalization is very unlikely a consequence of *de novo* activity-induced Me31B protein synthesis, as no striking increase in ME31B overall protein levels was observed upon stimulation. Very interestingly, a related phenomenon was reported by Mitsumori and collaborators in hippocampal neurons (Mitsumori et al. 2017). In fact, they observed that Staufen1- and Pur-α granules colocalize in dendritic shafts in unstimulated conditions, whereas stimulation does not affect Staufen1 localization yet induces a translocation specific to Pur-α complexes to dendritic spines (Mitsumori et al. 2017). However, in the case of Pur-α and Staufen1 complexes, Mitsumori and colleagues did not observe RNP granule disassembly. My findings indicating Me31B strong diffused localization to dendrites could interestingly reflect a massive activity-induced de-repression of target mRNAs.
But how would Me31B be transported to dendrites specifically upon activity? An intriguing possibility is that Me31B would normally be stably associated to RNP granules, serving as a scaffold protein. Upon stimulation, client proteins present in the RNP shell would promptly respond to increased neuronal activation and disassemble, leaving core proteins such as Me31B exposed. At this point, previously hidden interaction domains present within Me31B could recruit transport-associated proteins or motors and be targeted to dendrites. Interestingly, in *Drosophila* ovary, Me31B has been shown to interact with cytoskeletal proteins and dynein and kinesin motors (DeHaan et al. 2017). Several questions remain open: once activity is restored to basal levels, is the content of Imp RNP granules restored as well? Do the components temporarily change? Here, observing the behaviour of ME31B protein upon Tyramine treatment and following withdrawal would be very interestingly, as ME31B re-localization to the Imp RNP granules found in cell bodies

would require a backward transport to somata. Furthermore, a methodical examination of several Imp RNP components in recovery experiments would be necessary for a more comprehensive view of the extent of granule remodelling.

2) CamkII as a molecular player of Imp RNP regulated disassembly

To determine the molecular mechanisms and players involved in activity-dependent Imp RNP granule remodelling, we purified Imp complexes by immunoprecipitation from *Drosophila* head lysates and identified Imp protein partners by mass-spectrometry. GO analysis of the bound fraction revealed an expected enrichment of RBPs, coherent with the nature of Imp RNP granules. Remarkably, we found just one kinase among the 69 reproducibly and specifically bound proteins: CamkII. This kinase has been extensively studied for its role in neuronal excitability, synaptic plasticity, learning and memory (Griffith et al. 1993; Park et al. 2002; Griffith 2004; Malik and Hodge 2014; Nesler et al. 2016; Oka et al. 2017; Zalcman et al. 2018; Zalcman et al. 2019). Because of its capacity to get activated by Ca^{2+} spikes induced by activity, CamkII represented a perfect candidate for mediating activity-dependent Imp RNP remodelling. Remarkably, transient, genetically-induced, inactivation of CamkII activity inhibited Imp RNP granule disassembly in response to stimulation, suggesting that CamkII may indeed play a role in such process (Figure D1). On the contrary, chronic constitutive activation of CamkII did not induce Imp RNP disassembly *per se*, suggesting that CamkII stimulation-dependent activation is not sufficient to drive RNP remodelling. Of note, we cannot exclude that chronic activation of CamkII throughout fly development might result in homeostatic regulatory processes or secondary long-term effects that may mask or prevent constant disassembly. To assess this possibility, experiments in which expression of CamkII constitutively active form is triggered in a time-restricted manner would be crucial.

The exact function exerted by CamkII and underlying Imp RNP dissolution remains to be determined. Based on mechanistic studies performed on the mammalian protein, CamkII could act (i) as a kinase or (ii) as a scaffold, the two functions being non-mutually exclusive (Kim et al. 2016) (Figure D1B). In support of the first hypothesis, my co-immunoprecipitation experiments showed that association of CamkII with Imp is reduced when the catalytic activity of CamkII is impaired by replacement of a single amino acid necessary for ATP binding. Furthermore, Imp RNP granule remodelling was impaired upon expression of the ala peptide, that mimics CamkII autoinhibitory sequence and was shown to selectively interfere with CamkII kinase activity and autoactivation (Griffith et al. 1993). Whether CamkII phosphorylates Imp directly, or other Imp RNP components instead/in addition to Imp remains to be demonstrated. Mutagenesis of the 4 CamkII consensus sites (RXXS/T) (Czernik et al. 1987) present in the Imp protein is underway to test for a direct role of CamkII. Interestingly, two proteins found in our IP-mass spec analysis of Imp complexes are known to be CamkII phosphorylation targets. CamkII may also have structural functions in RNP

granule remodelling, in particular through its role in binding and crosslinking F-actin (Okamoto et al. 2007; Kim et al. 2015). CamkII, indeed, might establish interactions with RNP components and the F-actin cytoskeleton, thus indirectly regulating Imp interaction networks and granule property. Consistently, CamkII scaffolding features have been reported to be important to recruit molecules in specific neuronal compartments, such as dendritic spines (Bingol et al. 2010). Furthermore, CamkII association with the F-actin cytoskeleton was shown to depend on stimulation and be regulated by CamkII own activity (Lin and Redmond 2008; Kim et al. 2015).

How to determine if one of the two models is *actually* valid? The generation and *in vivo* analysis of Imp forms carrying point mutations within the different CamkII consensus sites would greatly enhance our knowledge in this regard. A complementary experiment could make use of CamkII kinase dead form in an *in vivo* context, to help discriminating between the kinase-dependent and -independent effects of CamkII on Imp RNP properties and activity-induced remodelling. However, (i) affecting CamkII kinase function might be leading to detrimental and/or indirect effects independent of Imp and (ii) it cannot be excluded that CamkII might play a role as a scaffold with the help of its kinase domain (Bingol et al. 2010; Kim et al. 2015).

3) A cue-specific granule remodelling?

As we sought to investigate Imp RNP granule response to neuronal activation in the mushroom body γ neurons – where Imp is specifically localized -, we decided on one hand to make use of KCl, a known generical depolarizing chemicals, and on the other hand to look for endogenous neuropeptides and neuromodulators able to elicit activity increase in Mushroom Body neurons. MBs are complex structures innervated by numerous and diverse neuronal populations and thus able to respond to different neurotransmitters. Dopaminergic, octopaminergic and tyraminergic neurons (producing respectively dopamine, octopamine and tyramine, three chemical products derived from tyrosine, Figure D2A) have been shown to project towards different areas of the mushroom body γ neurons, suggesting that they might play peculiar individual functions (Figure D2) (Busch et al. 2009; Mao and Davis 2009; Keleman et al. 2012; Aso et al. 2014). In addition to these three populations, acetylcholine (ACh) is also able to evoke activity within the mushroom bodies, which indeed express both the ACh receptor and the ACh-processing proteins (Barnstedt et al. 2016). Hence, to increase neuronal activation in the mushroom body γ neurons, I made use of KCl, dopamine, acetylcholine and tyramine. Remarkably, Imp RNP disassembly was not observed in all conditions, as acetylcholine-treated brains had no effect on Imp RNP complexes. Moreover, although KCl stimulation induced Imp RNP dissolution, the neuromodulator Tyramine had a stronger impact on Imp RNPs, causing nearly complete granule loss. The reasons explaining these differences in Imp RNP response remain to be elucidated. If on one hand we cannot exclude that the results might be impacted by the concentrations used for the different neurotransmitters/neuromodulators or the duration of the treatment (that might lead to different

responses, or even system saturation), on the other hand this result might reflect a genuine tyramine-specific Imp RNP response, recapitulated by the KCl-mediated generical depolarization. To further confirm that Imp RNP granule disassembly depends specifically on tyramine, and not on a consequent increase in octopamine levels, stimulation of *Drosophila* explants should be performed on flies lacking the enzyme converting tyramine to octopamine (Tyramine β-Hydroxylase, Figure D2A) or using octopamine rather than Tyramine treatments. An additional proof for a tyramine-specific response would be a loss of Imp RNP remodelling phenotype in flies carrying a null mutation for TyrR, a receptor specific for tyramine (Cazzamali et al. 2005; Huang et al. 2016). Such finding would serve as another example of specific function exerted by Tyramine independently of octopamine, after the evidence of octopamine-independent tyramine control of male courtship drive (Huang et al. 2016). Describing the Tyramine-driven pathways leading to RNP disassembly would represent a very exciting achievement that would shed light on the enigmatic function of Tyramine as a neuromodulator. However, as tyramine has been reported to act rather as a negative neuromodulator in different contexts (Nagaya et al. 2002; Pirri et al. 2009; Ormerod et al. 2013; Huang et al. 2016; Schützler et al. 2019), the activation of MB neurons – and hence the Imp RNP disassembly - is likely happening in an indirect fashion. To elucidate tyramine neuromodulatory function, investigating both tyramine-activated receptors' functioning and related downstream pathways is indeed crucial. More broadly, further insights into Tyramine functions would be of interest, notably considering that tyramine concentration has been reported as altered in several neurological disorders (Borowsky et al. 2001).

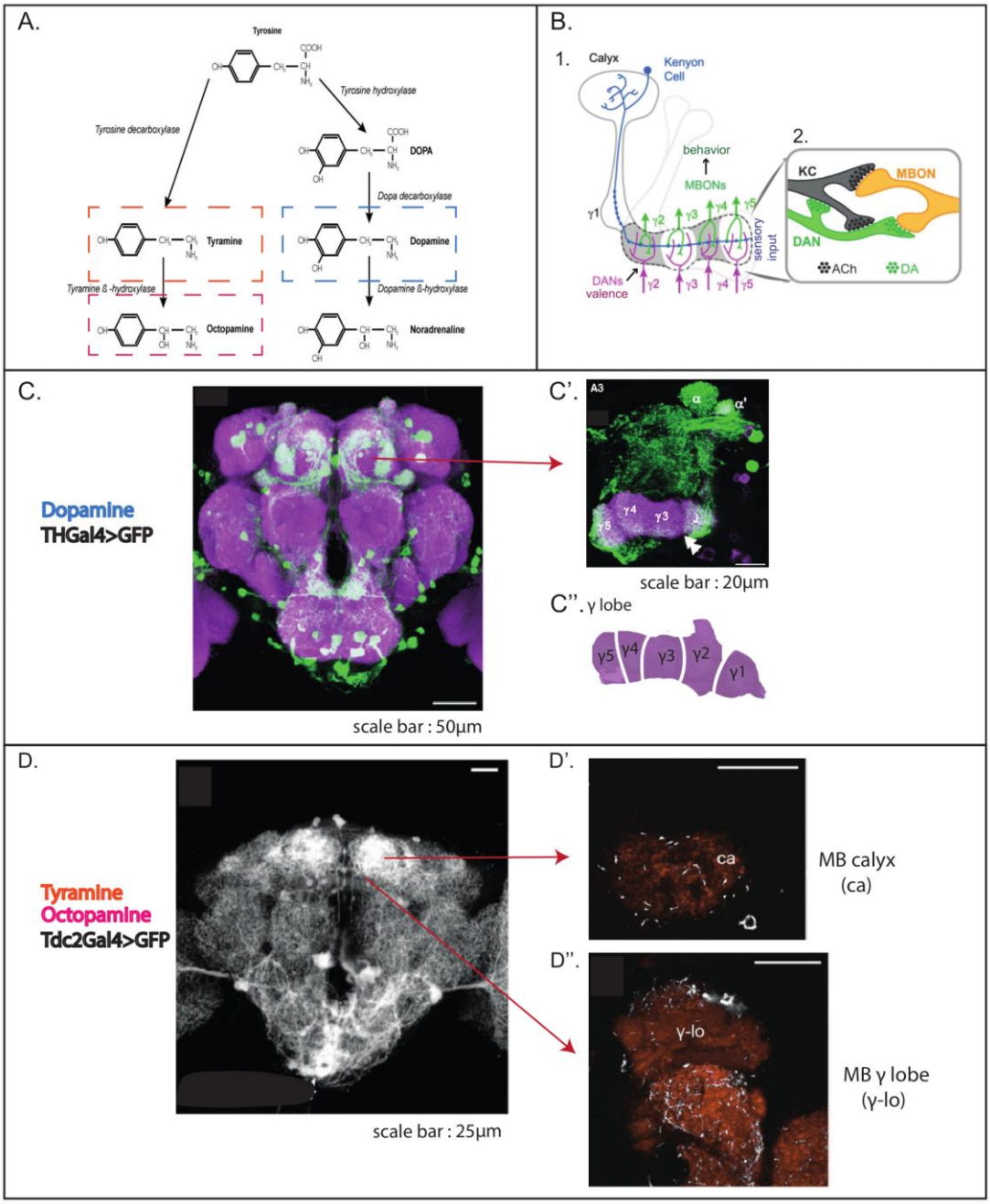

Figure D2. dopaminergic, tyraminergic and octopaminergic circuitries in *Drosophila* brains.
A) Biosynthetic pathway leading to tyramine, octopamine and dopamine formation. B) scheme of a *Drosophila* MB, where (1) the γ lobe receives information from dopaminergic neurons (DANs) to further convay them into mushoom body output neurons (MBONs), thus acting as a tripartite unit (2) eventually leading to behavior responses. C) Dopaminergic neurons labeled with a membrane-tagged GFP protein driven by the TH-Gal4 (in green). Synaptic structures are labeled in magenta. (C') PAM dopaminergic neurons (green, stained with anti-Tyrosine Hydroxylase (TH) antibodies) innervate the γ lobe (magenta, observed using NP1131Gal4-driven GFP) (C'). Note that the γ lobe can be divided into 5 different subregions (PAM γ1, γ2, γ3, γ4 and γ5, in C''), where specific subclasses of dopaminergic neurons project. D) Tyraminergic/octopaminergic neurons revealed by expression of a membrane-GFP triggered using the Tdc2 Gal4 (in white). These neurons (grey) innervate the MB calyx (D') and the γ lobe (D''), as indicated by the left magnified panels where synapsin stains the neuropil (in orange).
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D) Busch, S., M. Selcho, K. Ito and H. Tanimoto (2009). J Comp Neurol 513: 643-667

4) Functional relevance of Imp RNP granule remodelling

Neurons respond to stimulation by locally regulating their protein content (Biever et al. 2019). Consistently, translation has been shown to occur upon stimulation in various systems, in both soma and neurites (Ashraf et al. 2006; Kruttner et al. 2015; Suhl et al. 2015; Baj et al. 2016; Na et al. 2016; Yoon et al. 2016; Jones et al. 2018; Feuge et al. 2019; Hafner et al. 2019; Wong and Tsai 2019). Transcripts stored in RNP complexes represent a likely source of mRNAs for activity-dependent translation. Hence, we wondered whether Imp RNP remodelling would functionally be associated with an increased translation of mRNAs stored within. To tackle this question, I monitored *ex vivo* in MB γ neurons the changes in translational levels of a direct Imp target (*chickadee/profilin)* (Medioni et al. 2014; Vijayakumar et al. 2019) upon Tyramine stimulation. Strikingly, I could observe using translational reporters an up to 2-fold increase in *chickadee* translation levels upon tyramine addition. This result, together with the evidence that the total number of *chickadee* mRNA spots revealed by smFISH does not increase in response to the treatment, led us to conclude that Imp RNP remodelling is accompanied by an increased translation of target transcripts. Further experiments, including QRT-PCR, are required to carefully quantify the actual impact of the stimulation procedure on transcript levels. Indeed, recent *in vivo* work on *Arc* transcription dynamics upon neuronal activation revealed that 30 minutes were necessary to observe newly transcribed and mature *Arc* molecules predominantly in the cytoplasm, which represents the entire duration of our treatment (Das et al. 2018). Furthermore, we cannot exclude that, in addition to the mRNAs released from the RNPs, also the mRNAs present within the cytoplasmic pool could contribute to the translational increase. Using the SunTag technique would allow the estimation of the fraction of repressed *chickadee* mRNAs in the cytoplasm and within the granules in unstimulated and stimulated condition. By enabling spatial analysis of translation patterns, this technique would also allow for a better understanding of where (cell bodies, dendrites, axons) translation is happening.

9.2. Imp RNP granules in MB γ axons: local implications?

During *Drosophila* development, Imp RNP complexes are transported to MB γ axons in a developmentally-regulated fashion using MT tracks as travel rails (Medioni et al. 2014). These “axons” do exhibit peculiar features, as they receive inputs from modulatory neurons innervating the MB lobe (such as dopaminergic and octopaminergic neurons) but are also pre-synaptic to other neurons called MBONs (Mushroom Body Output Neurons), thus acting as pre- and post-synaptic compartments at the same time (Aso et al. 2014; Cognigni et al. 2018; Zhao et al. 2018) (Figure D2B). Tripartite circuits involving (i) modulatory neurons, (ii) mushroom body γ neurons and (iii) MBONs (Figure D2B) have been shown to underlie memory formation (Zhao et al. 2018), suggesting a key role for MB γ neurons in signal integration. In the adult γ axons, Imp RNP

particles can still be observed as punctae of a diameter of 300-400 nm. Understanding what happens to these axonally-localized granules in response to neuronal activity is of strong interest as (i) mRNA localization to neurites can be enhanced upon stimulation (Rook et al. 2000; Tiruchinapalli et al. 2003; de Solis et al. 2017; Bauer et al. 2019) and (ii) both pre- and post-synapses have been shown to dynamically translate mRNAs (Hafner et al. 2019; Biever et al. 2019; Sahoo et al. 2018). To determine whether activity may affect Imp RNP properties in axons, I looked at Imp granule distribution and morphology therein. No striking alteration in Imp axonal localization could be observed upon stimulation with KCl, Tyramine or Dopamine (data not shown), suggesting that transport largely remains unaffected. While no dramatic effect on the properties of axonal Imp RNP granules could be observed, testing the effect of activity on axonal RNP morphology has revealed to be a very challenging task. In fact, although Imp RNP granules can be observed as distinct punctae throughout the lobe, their high density throughout the γ lobe, combined with the difficulty of identifying background spots renders the development of a method to quantify their parameters very complex. Development of optogenetic-based protocols to activate spatially restricted sub-compartments along the γ lobe may represent a suitable strategy to tackle this question and overcome technical challenges. Activating upstream dopaminergic neurons that specifically innervate the distal portion of MB γ lobe (Aso et al. 2014; Keleman et al. 2012; Kruttner et al. 2015), for example, should provide internal controls within the lobe, enabling a more reliable analysis of activity-dependent remodelling of Imp complexes within a precise sub-compartment. Furthermore, the combined utilization of optogenetics together with methods enabling the visualization of *on-site* translation, such as global puromycylation, or transcript-specific SunTag tagging, should also provide important insights regarding the possible activity-dependent local translation of mRNAs in γ axons. Of note, the greater signal:noise ratio provided by the SunTag technology may help overcome the classical difficulties in unambiguously detecting mRNA in γ axons linked to (i) the inaccessibility of the lobe, (ii) the background noise typical of whole mount preparations, (iii) the probable low amount of axonal RNA molecules.

9.3. Dissecting the role of Imp in LTM processes

An adult *Drosophila* brain contains 100.000 neurons, a million times less than humans. Nevertheless, fruit flies exhibit an incredibly wide range of sophisticated behaviors and are capable of both short- and long-term behavioral plasticity. Flies have been shown to retain numerous and various information: they can memorize visual and spatial cues, they can learn to avoid odors that they have been trained to associate to electric shocks and they can adjust their courtship behavior depending on their mating possibilities (Quinn et al. 1974; Quinn et al. 1979; Siegel and Hall 1979; Tempel et al. 1983; Waddell and Quinn 2001; Bellen et al. 2010; Ofstad et al. 2011; Chouhan et al.

2015; Ostrowski et al. 2015; Hige 2018). Remarkably, flies can form short-term memories, that can be further consolidated in long-lasting memories. These two forms of memory molecularly rely respectively on modification of already circulating proteins – and hence impact on signalling cascades – and *de novo* protein synthesis.

A robust type of associative memory translating into strong behavior changes is elicited during male courtship, through the rejections experienced by males confronted to pre-mated females (see § 8.5). As demonstrated by conditional silencing and phenotypic rescue experiments, long-term courtship conditioning requires the activity and function of MB γ neurons (Keleman et al. 2007; Kruttner et al. 2015). Further investigations have revealed that neurons projecting to the MB do also have an essential role in courtship-related memory formation. Tyraminergic, octopaminergic and dopaminergic neurons do project on γ neurons and have been involved in courtship behavior (Figure 2D) (Keleman et al. 2012; Zhou et al. 2012; Kruttner et al. 2015). While the precise role of the octopamine circuitry has not been investigated in detail, how dopaminergic neurons impact on the establishment of LTM has been dissected. In particular, a sub-class of dopaminergic neurons projecting onto the most distal part of the MB γ lobe, the PAM γ5/aSP13 neurons (Figure D2C), has been shown to be crucial both for establishment of STM and for LTM (Keleman et al. 2012; Kruttner et al. 2015). Furthermore, flies in which the function of the dopamine receptor R1 was knocked down showed strong LTM deficits, that could be rescued by expressing the DopR1 back in the MB γ population (Kruttner et al. 2015). Intriguingly, stimulation of PAM γ5/ aSP13 neurons 8-10 hours after a short training session - normally inducing only STM - was demonstrated to be sufficient to mediate the consolidation of STM into LTM (Kruttner et al. 2015). This consolidation relies on protein synthesis, as concomitantly feeding flies with the translation inhibitor cycloheximide abolished LTM consolidation, a result consistent with previous work on rodents demonstrating that STM elicited by training could be consolidated into LTM by a novel experience triggering protein synthesis (Moncada and Viola 2007).

Altogether, these findings have highlighted the importance of protein synthesis-based mechanisms in LTM formation and have in addition demonstrated that precise activation of MB γ neurons is a major driver for these mechanisms. Given that the dopaminergic neurons involved project to the γ lobe, where γ axons are located, and based on previously published work, it can be speculated that newly synthesized proteins would be the product of (i) localized and (ii) activity-induced axonal translation. In this model (Figure D3), tightly regulated mechanisms controlling mRNA storage in neurites and activity-dependent translation would be finely orchestrated and converge in the control of experience-induced translation and long-term memory establishment. Furthermore, proteins involved in RNA regulation would represent key players in LTM-underlying processes.

Consistent with this view, Orb2, the fly homolog of the CPEB RBPs, has been shown to be involved in LTM (both formation and consolidation), but not in STM, and to regulate translation by

switching from a monomeric state (associated to translational repression) to an oligomeric self-sustaining prion form (associated to translational activation) (Keleman et al. 2007; Majumdar et al. 2012; Khan et al. 2015; Kruttner et al. 2015; Stepien et al. 2016). This transition is induced in synaptic fractions by neuromodulators such as dopamine and tyramine and relies on the presence of a PLD in Orb2 protein (Majumdar et al. 2012; Kruttner et al. 2015). To date, however, local changes in the properties of Orb2 complexes and in the translation of Orb2 targets have not been documented.

Here, we report that the conserved RBP Imp is also involved specifically in LTM, without affecting STM. However, we believe that the mechanism by which Imp acts on LTM establishment might be dissimilar to the one proposed for Orb2. In particular, although both Imp and Orb2 likely act as translational repressors at steady state, we speculate that release of Imp-dependent translational repression upon stimulation may occur *via* activity-regulated disassembly, akin to what was observed in cell bodies, rather than *via* PLD-dependent oligomerization, as shown for Orb2. Although Imp contains a PLD, indeed, this domain is not sufficient to trigger aggregation or recruitment to Imp granules (Vijayakumar et al., 2019). Furthermore, my results suggest that activity-dependent remodelling of Imp granules can occur in the absence of Imp PLD. Still, as revealed by experiments performed on flies lacking the Imp PLD, Imp function in LTM depends on the presence of this domain. Interestingly, given that Imp PLD was recently reported to promote localization of Imp RNP granules to MB γ axons without affecting overall protein levels (Vijayakumar et al. 2019), these results may suggest that LTM defects may result from a limited amount of axonally-localized Imp RNP granules and thus represent the first *in vivo* evidence for a local function of an RBP in LTM formation. Whether Imp function is required similarly to Orb2 downstream of PAM γ5 neurons, for consolidation of STM into LTM, remains to be demonstrated. First, Imp requirement for memory consolidation should be assessed. To this end, interfering with Imp function specifically in the time window where memory consolidation occurs and quantify LTM formation would be beneficial. Thereafter, to test if Imp functions downstream of dopaminergic neurons, LTM could be assessed in Imp ΔPLD flies fed or not with dopamine, as dopamine feeding for 24 hours has been shown to correlate with increased LTM formation (Kruttner et al. 2015). Finally, in a scenario where local Imp RNP remodelling underlies LTM memory formation, one should check if axonal Imp RNP disassembly is induced by PAM γ5 activation and blocked by inactivation of Dopamine receptor (DopR1) function in MB neurons. Given the specific remodelling of Imp granules observed in brain explants in response to tyramine, it would also be interesting to investigate the role in LTM of this neuromodulator already shown to control courtship behavioral drive (Huang et al. 2016).

In perspective, the functional relevance of Orb2- and Imp-mediated translational de-repression upon stimulation might represent a crucial step in memory formation process as it could lead to

translation of transcripts relevant for memory establishment or neuronal proper functioning. Orb2 target mRNAs for instance have been reported to be enriched in memory-related genes, such as *tequila*, which is involved in both short- and long-term memory (Chen et al. 2012; Khan et al. 2015; Stepien et al. 2016). Imp binds up to hundreds of mRNAs involved in neuronal function in MB γ neurons (Florence Besse, unpublished data). Among these, *chickadee/profilin,* codes for an essential protein mediating F-actin polymerization and shown in different species to control brain development and synaptic plasticity (Reeve et al. 2005; Ojelade et al. 2013; Medioni et al. 2014; Michaelsen-Preusse et al. 2016).

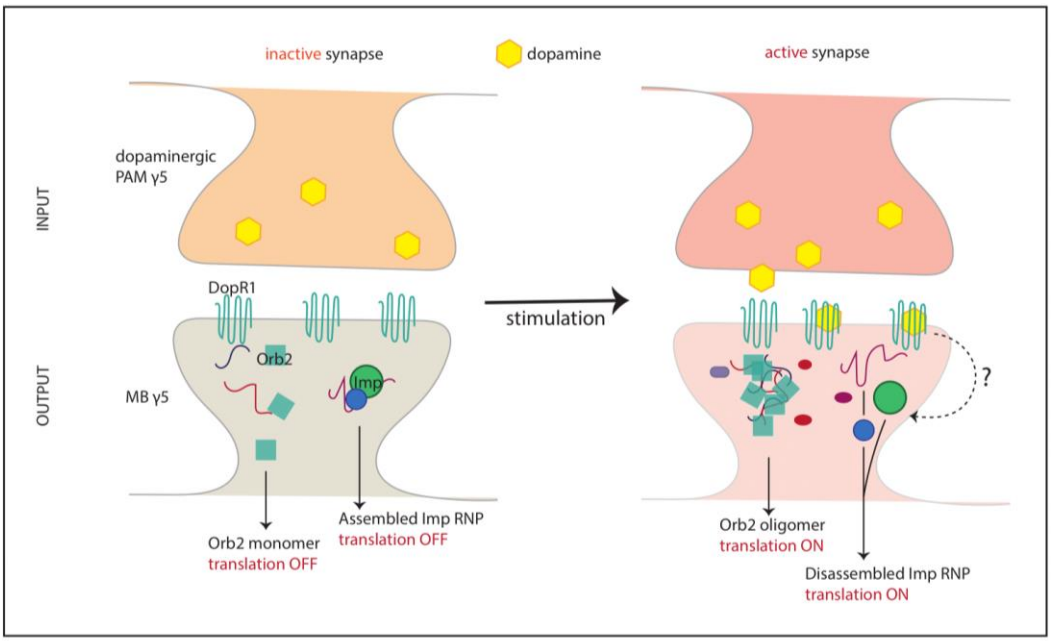

Figure D3. Putative model of the mechanisms underlying Imp and Orb2 RNP granule remodeling in activity-induced translation and LTM formation in Drosophila mushroom body γ neurons. A subset of dopaminergic neurons called PAM γ5 project to the most distal part of mushroom body γ lobe (MB γ5). In unstimulated condition (drawn on the left), Orb2 is present as a monomer (associated to translation repression), while Imp is assembled with target mRNAs and other protein in RNP complexes, thus impeding translation of associated transcripts. Upon stimulation, PAM γ5 neurons trigger dopamine release, thus locally activating MB γ5 synapses via Dopamine Receptor 1 (DopR1). This results in the induction of the Orb2 oligomer formation, which promotes translation. Simultaneous disassembly of Imp RNP granules, leading to translation of previously stored mRNAs, is represented although its dependency to Dopamine signaling remains to be investigated.

10. Conclusions

Long-term memory is what defines who we are and is established *via* long-lasting changes at synapses relying on *de novo* protein synthesis. Transcripts stored as dormant in RNPs represent a suitable reservoir of translatable mRNAs, which can be released in the cytoplasm in response to external cues such as neuronal activation, eventually allowing translation events underlying LTM. Despite the attractiveness of this model, demonstrating it is difficult and evidence is still extremely scarce regarding its validity. Here, we report the first proofs of RNP granule disassembly induced by neuronal stimulation in intact *Drosophila* brains and we further show that (i) this disassembly correlates with an increase in associated mRNAs translation and that (ii) one of the main components of these RNPs plays a pivotal role in LTM formation without affecting STM.

However, several questions remain still unanswered. Do all neuronal RNP granules behave similarly and respond to stimulation by releasing mRNAs? If not, what might contribute to give to each RNP granule this specificity? Is it due to RNP composition/biophysical structure? How is actually this structure defined? What does RNP granule content heterogeneity *actually* mean? And in a context of memory formation, how specific combinations of neural stimuli are able to trigger just as specific molecular responses eventually impacting on RNP homeostasis? Recent findings are gradually and partially answering to these questions, presenting a fascinating scenario where RNA-based mechanisms represent sophisticated means to establish memory formation. Furthermore, recent efforts to overcome technical limits and develop efficient tools enabling quantitative analysis of local translation will greatly help to deepen our knowledge about these processes in *in vivo* systems and in real-time. The role of RNA in learning and memory processes, indeed, remains – as Kenneth Kosik stated – the “kernel of what many neurobiologists are interested in today”.

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REVIEW

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Neuronal ribonucleoprotein granules: Dynamic sensors of localized signals

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Abstract

Membrane-less organelles, because of their capacity to dynamically, selectively and reversibly concentrate molecules, are very well adapted for local information processing and rapid response to environmental fluctuations. These features are particularly important in the context of neuronal cells, where synapse-specific activation, or localized extracellular cues, induce signaling events restricted to specialized axonal or dendritic subcompartments. Neuronal ribonucleoprotein (RNP) particles, or granules, are nonmembrane bound macromolecular condensates that concentrate specific sets of mRNAs and regulatory proteins, promoting their long-distance transport to axons or dendrites. Neuronal RNP granules also have a dual function in regulating the translation of associated mRNAs: while preventing mRNA translation at rest, they fuel local protein synthesis upon activation. As revealed by recent work, rapid and reversible switches between these two functional modes are triggered by modifications of the networks of interactions underlying RNP granule assembly. Such flexible properties also come with a cost, as neuronal RNP granules are prone to transition into pathological aggregates in response to mutations, aging, or cellular stresses, further emphasizing the need to better understand the mechanistic principles governing their dynamic assembly and regulation in living systems.

KEYWORDS
biological condensates, local translation, neuronal compartments, neuronal RNA granule, RNP complex

1 | INTRODUCTION

Neurons are highly polarized cells specialized in complex information processing, transfer and local storage. They are compartmentalized into macroscopic functional domains—the dendrites, soma and axons—themselves further divided into operational subcompartments locally integrating external signals.[1] These subcompartments are not defined by structural boundaries, but rather by their enrichment in specialized supramolecular complexes. Axon growth cones, for example, are enriched in signaling molecules, components of the protein synthesis and degradation machineries, and cytoskeletal regulators, that together promote physical turning or collapse in response to chemical or mechanical guidance cues.[2,3] Dendritic spines are enriched in postsynaptic proteins such as transmembrane receptors, channels and downstream signal transduction components modulating synaptic strength in response to neuronal activation or inhibition.[4-7] Remarkably, neuronal subcompartments are highly plastic and undergo extensive remodeling of their proteomes in response to external cues.[8,9] Such a remodeling involves local protein synthesis of new proteins, a process achieved via the translation activation of localized mRNAs transported from the soma in a translationally silent

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state.[10-12] Whereas the precise mechanisms underlying the selective trafficking of mRNAs to dendrites or axons, and their translational regulation still remain to be identified, it was shown in different instances that the recognition of specific RNA signatures by neuronal RNA-binding proteins (RBPs) and the subsequent formation of regulatory ribonucleoprotein (RNP) complexes is key.[13-15] Localized mRNAs and associated RBPs assemble into high-order macromolecular complexes forming hundreds nanometer-scale structures visible by light microscopy and termed neuronal RNP granules (Figure 1).[16,17] As shown by seminal studies,[18-23] these granules are membrane-less organelles that are dynamically transported along neuronal processes and contain at rest translationally-repressed mRNAs. As revealed by more recent biophysics studies,[24-29] RNP assemblies form through liquid-liquid phase separation, that is, by demixing of components from the cytoplasm, to form condensates with liquid-like properties. Demixing of RNP granule components is triggered by the establishment of a dense network of RNA-RNA, RNA-protein and protein-protein interactions, which involves structure-based RNA-RNA association, multivalent RNA-RNA-binding domain interactions, as well as intrinsically disordered low-complexity domains enriched in RNP granule-associated proteins.[28,30-36] In contrast to membrane-bound organelles, neuronal RNP granules thus lack a physical barrier separating their components from the cytoplasm. Rather, they are delimited by a phase boundary enabling both the selective concentration of RNA and proteins and the continuous exchange of molecules with the surrounding.[37] This phase boundary is highly sensitive to local concentrations in ions or cellular hydrotropes, and to posttranslational modifications (PTM) of granule components,[37-39] thus providing RNP granules with the capacity to remodel, dissolve and/or re-assemble in a time scale of minutes, a process essential for the dynamic and local regulation of mRNA translation in response to external cues.

In this review, we first describe our current understanding of the biophysical properties of neuronal RNP granules. We then highlight the diversity of neuronal RNP granules and discuss how these condensates are assembled and dynamically remodeled in response to external stimuli. We finally provide a general overview of the connections established over the past years between defective RNP granule assembly and neurodegenerative diseases. For a more detailed description of the mechanisms underlying the transport of neuronal RNP granules and the mechanisms underlying their translational repression, we refer the reader to our previous recent review.[16]

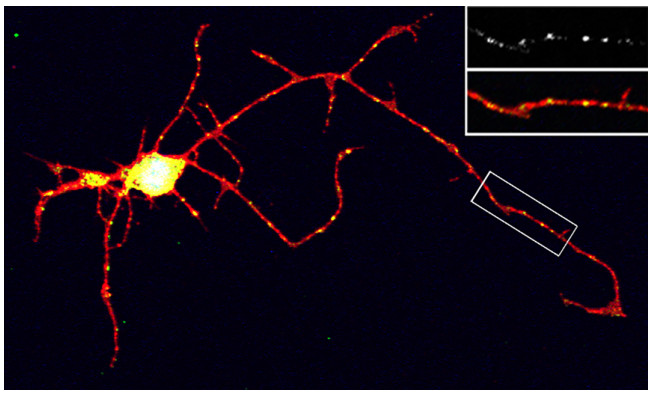

FIGURE 1 Neuronal RNP granules localize to neuronal extensions. Cultured *Drosophila* mushroom body neuron stained with HRP (red, to visualize membranes) and anti-ZBP1/Imp antibodies (green). ZBP1/Imp accumulates into granules that localize as punctae along neuronal processes. The inset corresponds to a magnification of the boxed region. Reprinted from Reference 1 with permission from De Gruyter Publishers, © 2018

2 | NEURONAL RNP GRANULES ARE DYNAMIC MEMBRANE-LESS CONDENSATES

In contrast to the various vesicular cargoes or membrane-delimited organelles actively transported to the axon or dendrites of neuronal cells, neuronal RNP granules are not bounded by a membrane, an observation established by a variety of approaches. First, ultrastructural analyses performed on SYTO14-labeled granules from cultured neurons,[20] or on granules purified from entire brains by density gradient centrifugation,[22,40,41] have identified neuronal RNP granules as electron-dense round-shaped structures not surrounded by a membrane. Second, characterization of neuronal granule proteomes by mass spectrometry revealed a strong enrichment in conserved RBPs, cytoskeleton-associated proteins and ribosomal proteins, but the near absence of transmembrane proteins.[40,42] Third, measurement of the biophysical properties of neuronal RNP granules using high-resolution imaging demonstrated that these assemblies behave as phase-separated liquid-like compartments in cultured neurons.[25,43] Consistent with surface tension dictating their shape, indeed, granules are spherical at rest, exhibiting an aspect ratio close to one. Under the shear stress induced by fast axonal transport, however, they undergo a dramatic deformation not observed for membrane-bound organelles.[25] Further characteristic of a liquid state, neuronal granules are subject to rapid internal rearrangements, as visualized in fluorescence recovery after photobleaching (FRAP) experiments after half-bleaching of granules. Intra-granule diffusion of molecules is also associated with exchange of molecules with the surrounding cytoplasmic environment, typically on the time-scales of seconds to minutes,[25,44-47] although turnover rates appear to depend on granule type and/or component.[48]

Thus, neuronal RNP granules can be considered as metastable condensates that behave like dense liquid-like droplets coexisting with the cytoplasm, a model further confirmed by the frequent occurrence of both fusion and splitting events observed upon real-time imaging of cultured neurons.[21,25,40,45,49] Interestingly, fusion events can be associated with a transfer of properties. As shown for Fragile X Mental Retardation Protein (FMRP) containing granules, for example, fast-moving granules can impose their kinetics upon coalescence with slowly moving granules.[40] Fast exchange of components has also been observed in the context of more transient interactions or docking of distinct RNP granules,[25,50] enabling dynamic changes in granule composition. Although splitting of granules may also represent a means to segregate components into distinct entities, differential sorting of molecules has so far not been observed upon fission. Splitting may,

however, represent a means to adapt to dendrite spatial constraints, as budding of small FMRP-positive granules from large ones was proposed to enable the translocation of granule components through the narrow spine neck, into the synapse-enriched spine head.[40]

Remarkably, the material properties of neuronal granules are not homogenous within a given neuronal cell, but exhibit some degree of compartmentalization. TDP43-containing granules found in the proximal axon compartment, for example, were shown to be less dynamic than distal ones after photobleaching and more resistant to disruption of weak hydrophobic interactions.[25] Such regionalization might reflect differences in granule maturation states,[51] changes in local environments, and/or variations in granule composition.[28,49,52]

3 | A HETEROGENOUS COLLECTION OF GRANULES

Neuronal RNP granules are not uniform in composition and properties, but rather belong to a heterogeneous and dynamic collection of entities that coexist to regulate with a high spatiotemporal resolution the fate of associated target mRNAs.[16,17,53] Pioneer characterization of the protein content of neuronal RNP granules isolated by differential centrifugation enabled the identification of granule markers,[40,41,54] but did not characterize the diversity of individual granule contents. As revealed by various further colocalization experiments, granule-associated RBPs do not all colocalize within the same structures.[41,48,52,55-59] Although multiplexing analyses would be required to obtain an extensive cartography of the exact repertoire of granules present at a given time point in neuronal compartments, these experiments have rather revealed that multiple types of granules, containing varying overlapping subsets of proteins, are present along neuronal extensions. Further consistent with this idea, only a partial overlap was observed in the protein content of RNP complexes exogenously assembled on distinct axonally-localized RNAs,[60] or in the protein content of two distinct types of dendritic RNP granules purified biochemically based on the selective presence of the Staufen 2 and Barentsz RBPs.[42] Together, these results suggested that a restricted number of proteins (eg, Pur-α, or DDX-6 and Yb1 in dendrites) may be present in various types of granules, potentially representing a core set of constitutive factors required for granule assembly/maintenance, while a larger number of RBPs (eg, Staufen 2, Barentsz, FMRP, ZBP1, HuR or cytoplasmic polyadenylation element binding (CPEB)) may be recruited to specific granule species for defined regulatory functions.[17,42] Notably, heterogeneity in granule protein content is also associated with heterogeneity in RNA content. As first shown by fluorescent in situ hybridization experiments in cultured neurons, indeed, distinct dendritically localized mRNA species are sorted into distinct neuronal granules.[61-63] Furthermore, characterization of the RNA content of Staufen 2 and Barentsz neuronal RNP granules revealed no significant overlap between the transcriptomes of these two entities,[42,64] suggesting that individual neuronal RNP granules may be considered as distinct regulatory units.

Remarkably, differences in translational status are observed in addition to differences in protein and RNA contents.[16] At rest, translationally active RNP granules can be observed along neurites, at a higher frequency proximally than distally.[65] The vast majority of RNP granules, however, appears to be translationally inactive.[22,65-67] Depending on RNP granule type, translation can be blocked either before the recruitment of the translation initiation machinery, as suggested by the presence of the nuclearly loaded cap-binding complex,[42,68] or at the elongation step, as characterized by the presence of stalled ribosomes.[16,40,69,70] While the origin of such a variability in the mode of translational repression is unclear, this may provide the neurons with a flexible means to respond selectively to various extracellular cues.[16]

4 | HOW AND WHERE ARE NEURONAL RNP GRANULE ASSEMBLED?

Addressing such a question requires understanding the molecular principles underlying RNP granule nucleation, a process that has been so far best studied in in vitro reconstituted systems. Strikingly, pioneer work has focused on the role of RBPs and their multivalent-binding domains in the assembly of supramolecular assemblies, revealing the importance of establishing homo- and hetero-protein-protein interactions in this process.[30,71,72] This early work particularly highlighted the role of low complexity domains, among which prion-like domains, that are found at high frequency in RNP granule-associated proteins and are characterized by their lack of defined structure and biased amino acid composition.[72-75] These domains, because they are prone to interact with RNA, RNA-binding domains, or other low-complexity domains enriched in RNP granule components, play a critical role in defining and modulating the network of interactions driving the phase separation of granule components into droplets in vitro.[32] As most of the prion-like domains studied so far trigger phase separation in vitro, the prevalent view is that they may function as autonomous modules for RNP granule assembly.[36,76,77] More recent work has, however, suggested that they may rather have chaperone-like functions, behaving as modifiers of phase separation and granule material properties.[32,47,78] Although such a protein-centric way of considering RNP assembly fits with the fact that neuronal RNP granules have historically been defined based on their characteristic protein markers (eg, FMRP, ZBP1, Staufen 2, Barentsz, CPEB, TDP-43),[16,17] it does not take into account the contribution of a major player in the system: RNA. Intermolecular RNA-RNA interactions were indeed recently shown to be a key driver of RNP granule self-assembly, leading to a model in which a weighted combination of RNA-RNA and RNA-proteins contribute to granule assembly and dynamics.[31] While this model explains how the dense network of interactions underlying granule component coalescence may be established, it does not explain where specificity and component sorting originate from. Particularly interesting in this context is the recent discovery made in the filamentous fungus *Ashbya gossypii* that RNAs found in distinct granules in vivo partition into distinct and immiscible droplets in vitro, while RNAs co-enriched in similar granules in vivo self-assemble in vitro.[34] This result thus suggests that RNA-RNA interactions may

underlie the sorting of specific mRNAs into diverse granules, and thus that granule identity may be largely encoded by mRNAs. Whether this model applies to neuronal RNP granules remains to be demonstrated, as mRNAs were suggested to largely travel individually, as single copies, in both dendrites[61,79] and axons.[80] A way to reconcile these different results may be to consider that neuronal RNP granules are initially assembled in neuronal cell bodies, where concentrations of RNA and other RNP granule components are high. There, RNA-based differential sorting may dictate the differential targeting of RNP granules to dendrites or axons, while further maturation along neuronal processes may refine their content and regulatory properties (Figure 2). Consistent with this hypothesis, a progressive decrease in the number of RNA copies per granule was observed along dendritic proximo-distal axis.[49] Furthermore, differences in granule protein content were observed along dendritic processes,[52] and when comparing granules found in cell bodies and at synapses.[81] How would such a maturation process be spatially regulated? Based on in vitro and in silico work, one could speculate that spatial gradients of neuronal RNP granule maturation may reflect gradients of molecules regulating phase behavior, such as posttranslational modifiers,[38,82] divalent cations[83,84] or ATP.[85,86] As described further in the next section, local and specific disassembly or remodeling of RNP granules in response to environmental signals (eg, synaptic activity or guidance molecules) may also lead to local changes in soluble protein/RNA concentrations, and RNP granule composition. By analogy to the "scaffold and client" model proposed for in vitro condensates,[87,88] such remodeling may promote the recruitment or exchange of nonconstitutive, dynamically exchanging molecules (or clients), thus enabling local and rapid changes in granule composition.

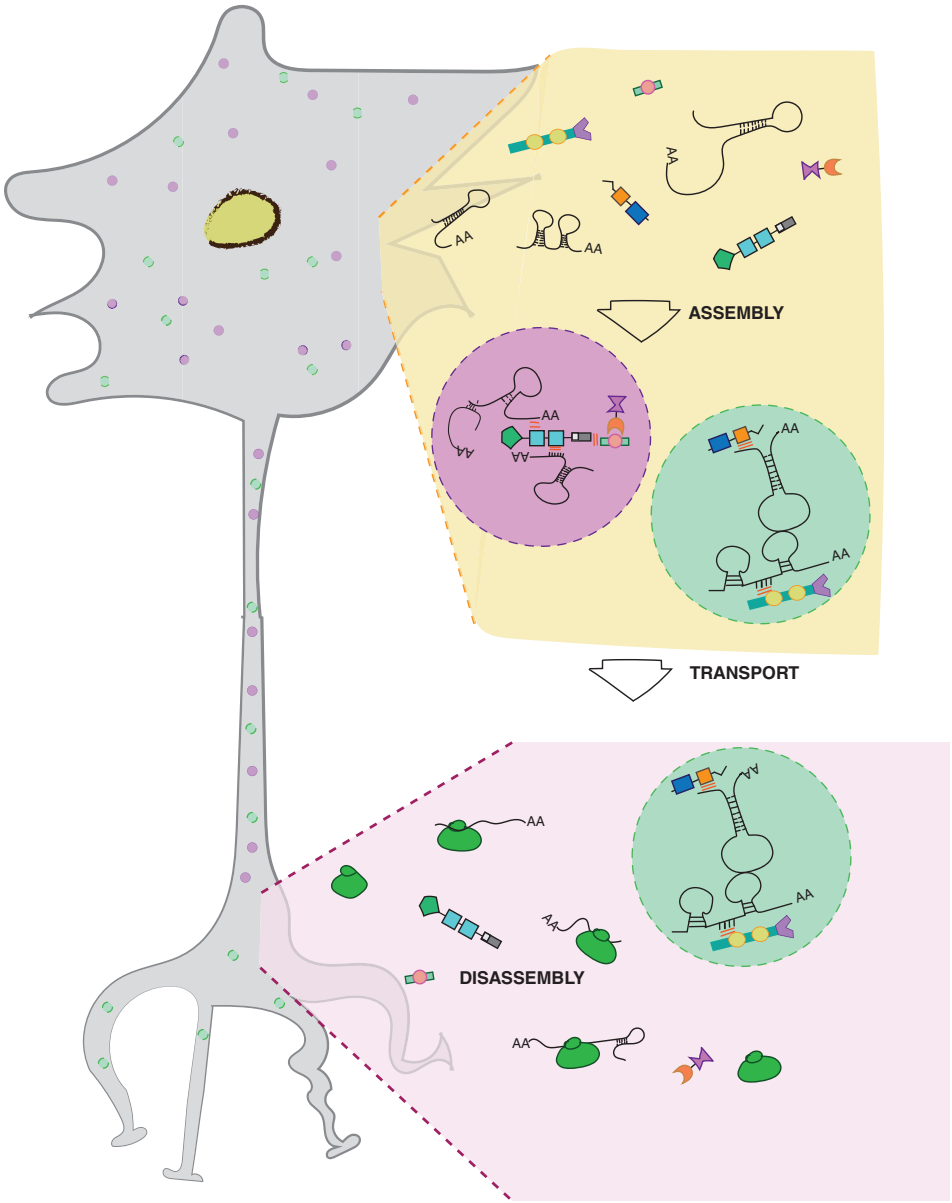

FIGURE 2 Assembly and maturation of neuronal RNP granules. RNA-RNA, RNA-protein and protein-protein interactions drive the proximal assembly of RNP granules. RNP granules then undergo maturation and are transported distally to dendrites. There, they are remodeled in response to specific stimuli, releasing their mRNAs for local translation

5 | NEURONAL RNP GRANULES ARE REMODELED IN RESPONSE TO EXTERNAL SIGNALS

A remarkable feature of membrane-less organelles is their ability to rapidly assemble, and reversibly reorganize or reset in response to external stimuli or environmental fluctuations. This unique property makes them particularly well suited for locally processing transient stimuli in distal neuronal compartments. In dendrites, for example, synaptic activation was shown to induce dramatic changes in the material properties of neuronal RNP granules. As first revealed by ultrastructural analysis, indeed, a transitioning of dense granules into less compact structures is observed upon KCl-induced neuronal activation.[22] As further shown by live-imaging experiments, such a reorganization is accompanied by changes in granule dynamics. While a decreased frequency of splitting and merging of *β-actin*-containing granules is observed upon KCl treatment of hippocampal neurons,[49] increased turnover of dendritic granule components was observed in FRAP experiments upon activation of the N-methyl-D-aspartate receptor (NMDAR) receptor.[48] Remarkably, such activity-dependent changes in granule material properties are associated with a remodeling of granules thought to underlie translation activation. In cultured mammalian neurons, for example, chemical induction of long-term potentiation triggers a transient unmasking of the dendritically localized *β-actin* mRNAs that likely reflects their release from RNP granules.[79] mRNA unmasking occurs concomitantly to ribosome unmasking and β-actin local translation, suggesting that degranulation in response to activity may trigger the translational activation of mRNAs stored at synapses. Transient dissolution of neuronal RNP granules enriched in translational repressors has also been observed upon NMDAR stimulation and linked to the increased translation of associated dendritic mRNAs such as *CamkII-α* mRNA.[50,89] Interestingly, specific responses are observed in response to distinct stimuli: while Smaug-containing dendritic granules rapidly dissolve upon activation of ionotropic (NMDAR), but not metabotropic (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid receptor (AMPAR)) glutamate receptors, FMRP-containing granules only dissolve upon activation of AMPAR.[58,89] In these different paradigms, RNP granule dissolution is a reversible phenomenon, as re-assembly is observed ex vivo in a timescale of dozens of minutes to hours.[50,89] Furthermore, re-assembly occurs independently of granule component synthesis,[89] consistent with a role of phase behavior in this dynamic de-condensation and re-condensation processes. Although this remains to be demonstrated in vivo, neuronal RNP granules may thus be able to undergo repeated cycles of assembly/disassembly, thus providing acute responses to dynamic neuron firing patterns or constantly changing environmental stimuli.

Stimuli-induced changes in neuronal RNP granule properties are not only transient, as longer-term re-organization of granules has also been reported, especially after repeated neuronal stimulations. Repeated KCl stimuli, for example, induce a structural rearrangement of dendritically localized granules manifested by an increased partitioning of distinct RBPs (eg, FMRP and TDP-43) into common granules.[90] Furthermore, long-term self-sustaining changes in the conformational state of granule-associated RBPs were shown to be induced by repeated cycles of neuronal stimulation.[91,92] Interestingly in this case, persistent conformational changes were proposed to underlie synaptic tagging, a process that selectively marks activated synapses and restricts the competence to undergo long-lasting changes to these synapses.[91-94]

6 | MOLECULAR MECHANISMS UNDERLYING NEURONAL RNP GRANULE REMODELING

Stimuli-driven changes in the properties of neuronal RNP granules are associated with changes in the translational profile of granule-associated mRNAs, namely a switch from translational repression to translational activation. How is such a switch molecularly triggered? Two main models have been proposed to account for local translation activation. In the first one, external stimuli induce the release of translational repressors from their target mRNAs, enabling the local recruitment of released transcripts to active ribosomes. In the second one, external stimuli induce conformational changes that convert translational repressors into translation activators, thus transforming granules into crucibles promoting active translation. In both models, PTM of granule-associated components were proposed to molecularly trigger translational activation. Consistent with the first model, phosphorylation of ZBP1, a translational repressor that assembles with its target *β-actin* mRNA into axonal granules, is induced during axon navigation by the guidance molecule Sonic Hedgehog (Shh).[95] Phosphorylation of ZBP1 both decreases its affinity for *β-actin* mRNA[96] and promotes initiation-dependent translation of β-actin synthesis in the axons of cultured neurons.[96,97] Functionally, it is required ex vivo for Shh-mediated growth cone turning and in vivo for guidance of commissural axons.[95,97] Whether ZBP1 phosphorylation triggers the disassembly of *β-actin*-containing granules localized at the axon tip remains to be demonstrated, but increased splitting:fusion ratio may be induced, as shown for dendritically localized *β-actin* granules in response to external stimuli.[79] Consistent with the second model, the neuronal granule component CPEB was shown to switch from soluble monomer to an oligomeric prion-like form in response to neuronal activation in *Aplysia*, mouse and *Drosophila*.[91,92,98-101] Whether CPEB oligomerization reflects the formation of functional self-sustaining amyloïds or rather prion-like domain-dependent changes in phase behavior is still under debate. However, the monomeric to oligomeric transformation is clearly required for persistence of long-term plasticity and long-term memory[98,99] and induces a dramatic change in CPEB activity. As shown in *Drosophila*, indeed, the monomeric form of CPEB/Orb2 represses translation and enhances deadenylation of bound mRNAs, while the oligomeric form enhances translation and elongates poly(A) tails.[102] Phosphorylation triggers oligomerization by stabilizing the low-abundance Orb2a isoform responsible for the

nucleation of amyloid-like oligomers, and is controlled by a tight balance between localized kinases and phosphatases.[103]

Together, these examples have highlighted the importance of PTMs in switching the activity and the conformation of neuronal granules. They, however, likely represent only the tip of the iceberg, as in vitro studies have indicated that various PTMs can modulate the nucleation and the material properties of phase-separated molecular condensates,[38] as well as the translational repression activity of neuronal RBPs.[104] For example, phosphorylation of the Serine and Tyrosine residues enriched in the prion-like domains of granule-associated RBPs modulates phase behavior, either positively or negatively, while methylation of the Arginine residues found in the RGG/RG repeats of RBPs inhibits liquid-liquid demixing and enhances droplet dynamics in reconstituted systems.[38,104-106] The physiological importance of Arginine methylation was further demonstrated in *Xenopus* retinal neurons, where hypomethylated fusedin sarcoma (FUS) forms a higher number of more solid axonal granules compared to methylated FUS, a change correlating with impaired axonal translation.[106] The repertoire of PTMs involved in neuronal RNP granule regulation is likely to extend further, as illustrated by the recent discovery that mGlu5R-induced sumoylation of the FMRP protein promotes the dissociation of FMRP from dendritic RNP granules.[46] Thus, neuronal cells may take advantage of the large variety of available PTMs to trigger reversible, granule-specific, behavior in response to a combination of external cues.

Although physiological evidence is still lacking, PTM-independent switch mechanisms are likely to also be at play in neuronal cells. For example, changes in cation concentration or pH have been reported in distinct neuronal compartments in response to local stimuli,[107-109] and were shown to dramatically modulate phase behavior in vitro.[78,83,84,110] Variations in Ca^{2+} concentration, in particular, induce a switch-like behavior between heterotypic and homotypic RNA droplets in vitro, thus dictating component partitioning and droplet composition.[83]

7 | FROM PHYSIOLOGICAL TO PATHOLOGICAL ASSEMBLIES

Clinical and fundamental studies have converged over the past years to establish strong connections between alterations in the assembly and material properties of neuronal RNP granules and progression of age-related neurodegenerative diseases, leading to the concept of hypo- and hyper-assembly diseases of RNA-protein complexes.[51,111-115] Hypo-assembly diseases are part of a diffuse spectrum of disorders associated either with RNA degradation or loss of molecular chaperones.[111] To date, the best described example is spinal muscular atrophy (SMA), a motor neuron degenerative disease caused by the inactivation of the *SMN1* gene, which encodes an RNA-associated protein involved in the assembly of spliceosomal small ribonucleoprotein complexes (snRNPs), and in the efficient recruitment of RBPs into axonally transported neuronal RNP granules.[116,117] Although the pathological mechanisms underlying specific motor neuron degeneration are still under debate, they may include defective splicing of critical neuronal genes and defective axonal transport of neuronal RNP granules.[118]

The concept of hyper-assembly diseases initially came from the discovery that a hallmark feature of clinically related neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) or multisystem proteinopathy (MSP), is the accumulation of pathological inclusions enriched in RNP granule-associated RBPs.[119-123] A further direct molecular link between pathological progression and altered RNP homeostasis was clearly established for ALS and FTD, with the identification of disease-causing mutation in the granule-associated RBPs TDP-43, FUS and hnRNPA1.[29,113,120,124] Strikingly, ALS/FTD mutations were shown to cluster in the prion-like domains of these proteins[72-75] and to alter the material properties of condensates formed in vitro, promoting the transition of liquid-like condensates into solid aggregates reminiscent of the pathological inclusions seen in ALS patients.[26,27,29,125] Disease-causing variants also induce the formation of ectopic aggregates when expressed in cells,[29,126,127] which led to the proposal that mutations in prion-like domains promote the switch of reversible assemblies into irreversible pathological inclusions. In the absence of efficient clearing mechanisms, these inclusions may lead to cellular toxicity by sequestering RBPs and preventing the long-range transport or translation activation of associated mRNAs.[112,128] Because TDP-43, FUS and hnRNPA1 are found in stress granules, and because patient inclusions contain stress granule markers, it is commonly assumed that ALS/FTD toxic inclusions evolve from stress granules.[114] Whether the increased aggregation of mutant FUS or TDP-43 into stress-granules is causative in ALS/FTD patients, and not purely a consequence of the cellular stress responses induced in degenerating neurons, however, remains to be demonstrated. Recent work relying on optogenetic control of stress granule coalescence took a step in that direction, revealing that chronic intermittent stress granule assembly triggered by repeated cycles of illumination induces the formation of abnormal inclusions characteristic of ALS/FTD pathology in neuronal cells.[129] These results are reminiscent of previous in vitro work demonstrating that wild-type FUS proteins can assemble into fibrillar stable aggregates in response to repeated cycles of assembly and disassembly as well as upon molecular aging, although at a lower frequency.[26,27,51,76] Together, these studies have suggested a mechanistic framework in which neuronal RNP granules, because they concentrate molecules prone to aggregation, may constantly be on the verge of transitioning into solid inclusions. Transition into pathological inclusions is promoted by mutations generating mislocalized or hyperaggregative variants, but may also occur stochastically, with risk factors including aging and repeated exposures to stress[130,131] (Figure 3). Interestingly, such a framework provides mechanistic insights into the etiology of both familial and sporadic forms of ALS/FTD, and can also account for the pathological signature of repeat expansions RNAs.[132] Indeed, ALS/FTD-associated nucleotide expansions of the *C9orf72* mRNA were shown in vitro and in cells to trigger aggregation of RNP components into solid-like condensates.[133] This process may be mediated by RNA molecules themselves, via recruitment of cellular mRNAs and RBPs and nucleation of neomorphic assemblies,[134] and/or through the production of arginine-rich dipeptide repeats that bind to the prion-like domains of endogenous RBPs, promoting phase behavior

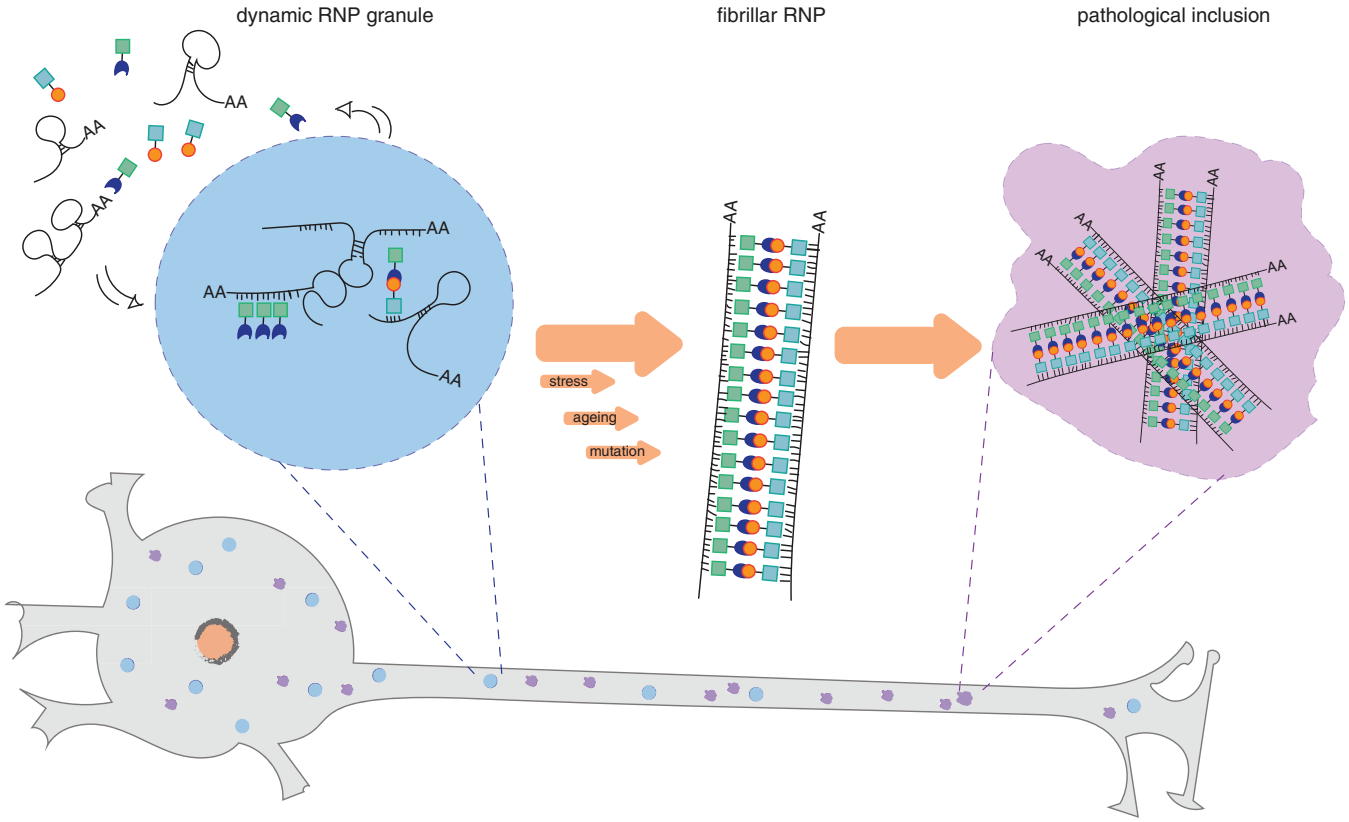

FIGURE 3 Transition of dynamic RNP granules into solid fibrillar aggregates in hyper-assembly diseases. Upon disease-causing mutation, cellular stress or aging, dynamic RNP granules transition into more solid assemblies that eventually further transition into toxic pathological inclusions trapping RBPs and associated RNAs into static aggregates

and decreasing granule dynamics.[135,136] Finding treatments for hyper-RNP assembly diseases is now a main challenging task, and may involve different strategies, including boosting clearing mechanisms, and identifying small molecules specifically modulating RBP conformation and/or RNA structures.[111]

8 | CONCLUSIONS

Although the membrane-less nature of neuronal RNP granules has been described for 20 years,[20,22] the physical and molecular principles underlying their dynamic assembly and regulation have only recently started to emerge. Theoretical and experimental biophysics work based on reconstituted systems, on one hand, has generated a framework to understand how the demixing of granule components is achieved and regulated.[37,39] Biochemical studies combined with point mutagenesis, on the other hand, have started decrypting the molecular code underlying selective molecular interactions between RNP granule components.[34,137,138] To date, however, a number of challenges still remain to be addressed when considering the complex population of RNP granules found in the dendrites and/or axons of living neurons, and their interaction with locally fluctuating subcellular environments. A first important challenge will be to describe with improved spatiotemporal resolution the regulated or pathological changes in the material properties or phase behavior of physiological RNP granules, and most importantly to mechanistically couple them with changes in neuronal functions. This should be tested very locally and at a multi-scale level, by analyzing both short-term responses such as localized activation of granule-associated mRNA translation and longer-term responses such as modification of synaptic strength or consolidation of memories. In this context, it will be particularly interesting to unravel how local and self-sustained changes in granule properties are, to determine the half-lives of neuronal RNP granules and the number of assembly/disassembly cycles they can they undergo. An additional future challenge will be to understand to which extent, and at which scale, the assembly and remodeling of distinct RNP granules are coupled. Addressing such a question will require understanding how the selective sorting of RNAs and RBPs is mediated, if distinct neuronal RNP granules freely exchange components, or if there are immiscibility barriers preventing mixing. Furthermore, interactions may not be limited to neuronal RNP granules, but may also involve other membrane-less condensates that were recently shown to define functionally important pre- and postsynaptic microcompartments.[139,140] Docking of dendritic RNP granules at postsynaptic densities, for example, has been observed[90,141,142] and may promote the switch from translational silencing to translational activation and the reversible tagging of active synapses.[93] Interaction of RNP granules with cytoskeletal assemblies or condensates induced by activated transmembrane receptors may also be important, as docking of

RNP granules along the axon shaft was, for example, shown to precede translation activation and branch emergence.[143-145] Addressing these questions will be possible with the advent of new high resolution live-imaging methodologies, and will benefit from quantitative analysis and modeling of interacting systems.

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