

Imaging Molecular Processes of Neural Plasticity

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Abstract

Memories are believed to be encoded by changes in the efficacy of synaptic connections between neurons in the brain. Neurons are highly specialised cells which receive input and send outputs through morphologically and functionally distinct cellular compartments known as axons and dendrites. This polarised morphology requires that neurons asymmetrically distribute cellular components between compartments. To achieve this, neurons regulate the asymmetric distribution of specific mRNA transcripts as a means of spatially and temporally regulating gene expression within functional compartments. In recent years, the development of single-molecule mRNA imaging techniques has revolutionised the study of mRNA biology. Such techniques include single molecule fluorescent *in situ* hybridisation (smFISH) and MS2 labelling. smFISH involves labelling an mRNA of interest with a set of fluorescently-labelled oligonucleotide probes. While the MS2 system involves the binding of fluorescent coat proteins to RNA hairpin structures engineered into an mRNA of interest, enabling mRNA to be visualised live. In this thesis, both techniques were applied to visualise mRNA in the brain of the fruit fly *Drosophila melanogaster*. mRNA localised within axonal and dendritic compartments of memory-relevant neurons in the whole *Drosophila* brain were visualised at single-neuron resolution. Specific mRNAs were found to localise within neuronal compartments in a manner that recapitulated the localisation of the encoded protein product. Moreover, dynamic behaviours were visualised in real-time. These developments enable the molecular correlates of plasticity to be visualised within the well described memory circuitry of the *Drosophila* mushroom body.

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Chapter 1

Introduction

1.1 Background

Learning induces lasting changes in an animal's nervous system. These changes allow animals to store information in the form of memories which are used to guide behaviour adaptively. At the cellular level, the physiological manifestation of memory coding is believed to be implemented through changes in the number and/or efficacy of synaptic contacts between neurons. The ability of neurons to modify patterns of functional interconnectivity in response to experience is known as plasticity and underlies the physiological mechanism of information storage in the nervous system.

In addition to their highly specialised function, neurons are also morphologically elaborate structures with specialised functional compartments for receiving, integrating and transmitting information. This unique combination of features requires that neurons asymmetrically distribute cellular components to the appropriate subcellular compartments. Moreover they possess the ability to individually tune synaptic weights in a synapse-specific manner. An understanding of the molecular mechanisms that underlie this specialised biology and enable neurons to translate experience into physiological change is of outstanding scientific interest.

1.1.1 Properties of neurons

Structure and function

Neurons are a morphologically and functionally diverse cell type (Cajal, 1894). Their structures are organised into distinct cellular compartments which receive and transmit information. These are known as dendrites and axons, respectively. This specialised morphology thus reflects functional compartmentalisation within this cell type. The presynaptic contacts of one neuron with the dendrites of another form the functional unit of communication between neurons, the synapse. The number of synaptic contacts a neuron makes can be vast, often hundreds to thousands. Furthermore, neurons are also some of the largest known cells, extending meter-long processes in the case of large mammals (Smith, 2009).

The specialised morphological and functional complexity of neurons presents a plethora of cell biological challenges. One such challenge is the requirement of efficient mechanisms of intra- and inter-cellular communication. The fast transmission of information throughout the animal nervous system is achieved by the propagation of a relative change of electrical potential across the cell's plasma membrane. This electrical communication is relayed between neurons mainly via the transmission of chemical neurotransmitters across synapses. This form of electrochemical relay serves fast information propagation and has been preserved across the animal kingdom. The spatio-temporal patterns of neuronal activity form biological representations of the outside world, which enable animals to meaningfully interact with each other and their environment.

Heterogeneity

A further challenge arises from the polarised structure of neurons. Individual functional compartments possess a unique molecular composition which reflects the requirements of their specific function. For example, dendrites require the relevant neurotransmitter receptors to detect input from their synaptic partners, while presynaptic outputs require the machinery for appropriate neurotransmitter synthesis and release. The molecular

composition of neuronal compartments is therefore heterogeneous and reflects functional compartmentalisation. The targeting of specific molecules to their appropriate sub-cellular destination is critical in establishing functional heterogeneity. However the mechanisms detailing how neurons achieve this are not completely understood.

Autonomy

A further element of complexity arises from the relative autonomy of distal neuronal compartments. Isolated synaptic compartments, for example, are capable of stimulus-specific physiological change independent of the cell body (Corera et al., 2009; Kang and Schuman, 1996). The functional independence of isolated neuronal compartments highlights the degree of autonomy with which they can function. This is important when considering the synaptic specificity of memory formation. The implementation of plasticity-related change occurs in a synapse-specific manner. This could be achieved by one of three mechanisms. The structural and functional changes required to alter synaptic weights may be broadly distributed and selectively captured by activated synapses, or specifically targeted to activated synapses that have been marked as targets. Alternatively, activated synapses may be responsible for independently synthesising the required molecular components for plasticity themselves (Mayford et al., 2012; Schuman, 1997). It seems perfectly plausible that a combination of these mechanisms contributes to the establishment of synapse-specific plasticity. However, the semi-autonomous functioning of isolated functional neuronal compartments suggests that the capacity to undertake the molecular modifications required, at least, for short-term physiological change reside within individual compartments.

The unique morphological character of neurons, their functional and molecular heterogeneity, along with the ability of independent compartments to function semi-autonomously hints at the cell biological complexities facing neurons. One mechanism that permits compartment-specific molecular targeting as well as enabling local responses to extrinsic stimuli is the localisation of mRNA transcripts coupled with decentralised post-transcriptional regulation of gene expression. This will be the topic of the proceeding discussion.

1.2 Cellular and molecular correlates of change

1.2.1 Protein synthesis and plasticity

The functional modification of interconnections between neurons requires that they adapt biochemically, physiologically and structurally in response to both external and internal cues. Such physiological changes often require changes in patterns of gene expression. It therefore follows that dynamic regulation of protein synthesis is an important mechanism by which neurons regulate their physiology. An important first step in progressing towards an understanding of the biological mechanisms underpinning memory coding in the nervous system was the knowledge of a specific temporally-defined requirement for protein synthesis.

The widely accepted view of memory formation is that it occurs in at least two temporally defined and physiologically distinct phases (McGaugh, 1966). Newly learned information gives rise to an initial labile memory, known as short-term memory, which is characteristically susceptible to disruption and persists from minutes to hours. While a second phase of memory, known as long-term memory, is produced following a time-dependent period of stabilisation known as consolidation (Dudai et al., 2015). During this period, the initial labile form of the memory is converted into a stable memory trace which is both enduring (in some cases persisting for days or longer) and resistant to disruption (Nader and Hardt, 2009). The physiological correlates of these distinct memory states are incompletely understood. However it is thought that short-term changes in synaptic strength result from post-translational modifications of existing proteins, while long-term changes require structural and functional synaptic modifications that involve *de novo* protein synthesis (Hernandez and Abel, 2008).

The requirement of protein synthesis for the implementation of long-term memory was discovered over 50 years ago following the discovery of reversible pharmacological inhibitors of protein synthesis (Flexner et al., 1963, 1965). The majority of these compounds function as inhibitors of translation with varying molecular modes of action (Alberini, 2008;

Davis and Squire, 1984). Administration of pharmacological protein synthesis inhibitors during a defined temporal window shortly after learning yields amnesiac phenotypes. Crucially, learning or short-term memory is not affected. Genetic manipulations that interfere with protein synthesis similarly perturb the formation specifically of long-term memory (Alberini, 2008; Hernandez and Abel, 2008). Thus, the requirement of protein synthesis is temporally defined and specifically limited to the physiological state of long-term memory.

In addition to the behavioural expression of memory, long-term memory formation can also be assessed at the level of the physiological neuronal unit of change, that is, the synapse. Activity-dependent changes in the efficacy of synaptic communications are believed to be the physical neuronal changes that underlie memory coding (Malenka and Nicoll, 1999) and the two principle mechanisms thought to implement such changes in synaptic efficacy are long-term potentiation (LTP) and long-term depression (LTD). In LTP induction protocols, repetitive presentations of glutamate and postsynaptic spikes at single dendrites of glutamatergic synapses produces both an immediate and a gradual phase of spine enlargement, the second of which is dependent on protein synthesis (Tanaka et al., 2008). *Ex-vivo* preparations in which LTP or LTD is induced in isolated synaptic compartments while genetically disrupting regulators of translation initiation impedes plasticity (Banko et al., 2006; Costa-Mattioli et al., 2005). Therefore a temporally defined requirement for *de novo* protein synthesis in behavioural plasticity is mirrored at the level of synaptic plasticity.

That various interventions to disrupt protein synthesis converge on the same behavioural output, the specific impediment of long-term memory, lends support to the idea that protein synthesis is an indispensable prerequisite for synaptic plasticity and the formation of long-term memory. This understanding has provided the foundation for studying the mechanistic cellular and molecular basis of plasticity and memory coding in the nervous system.

1.2.2 Transcriptional regulation of gene expression in neurons

The requirement of protein synthesis for synaptic plasticity and long-term memory is pervasive throughout the animal kingdom and many different learning paradigms (Davis and Squire, 1984). This suggests it is a fundamental evolutionarily conserved mechanism by which nervous systems bring about the molecular changes required for information storage. The requirement of protein synthesis for long-term memory indicates that remodelling of the proteome underlies behavioural and physiological plasticity. In order to bring about such changes, it follows that the cell must also remodel its pattern of gene expression. Indeed, the structural and functional changes that occur in response to learning are known to require complex changes in gene expression (Alberini and Kandel, 2015). Such changes in gene expression in response to learning have been most extensively studied at the level of transcription and its regulation in response to learning (Alberini, 2009).

It is thought that the transcriptional responses to learning that underlie the formation of long-term memory occur in two temporally-distinct waves (Alberini, 2009; Lefer et al., 2012; Li et al., 2018). The first wave occurs around the time of learning and primarily involves the transcription of immediate-early genes, which mostly encode transcription factors and DNA binding proteins (Saha and Dudek, 2013). The protein products of immediate early genes then regulate the second wave of transcription several hours later. The targets of this second wave of transcription involve an array of genes responsible for the molecular reorganisations that contribute to plasticity.

A particularly well-studied example of transcriptional responses to neuronal activity is the learning-induced regulation of the transcriptional activator cAMP Response Element Binding Protein (CREB). Synaptic signalling via the postsynaptic detection of neurotransmitter release from presynaptic terminals is relayed intracellularly by elevating intracellular Ca^{2+} (Brini et al., 2014). Elevated intracellular Ca^{2+} contributes to the synthesis of cyclic AMP (cAMP) by Ca^{2+} /calmodulin-sensitive adenylate cyclases (Eliot et al., 1989). In addition to activity-dependant (i.e. Ca^{2+} -mediated) activation of adenylate cyclases, receptor-dependant activation also occurs via G-protein coupled receptor mediated regula-

tion of the G protein $GS\alpha$ (Wolfgang et al., 2004). In response to increases in intracellular cAMP triggered by neuronal activity, specific protein kinases (including PKA, MAPK and CaMK) phosphorylate and thereby activate CREB, which in turn regulates the transcription of genes containing cAMP response elements (CREs) within their regulatory DNA enhancer sequences (Kandel, 2012; Montminy, 1997). The requirement of this transcriptional regulator for long-term memory has been well-studied in synaptic preparations of *Aplysia californica* neurons (Kandel, 2012), but has also been demonstrated in other invertebrate (Bartsch et al., 1995; Yin et al., 1994) and mammalian systems (Bourtchuladze et al., 1994), suggesting that this learning-induced gene regulatory mechanism has been conserved throughout evolution.

Transcriptional responses to learning in *Drosophila*

The fruit fly *Drosophila melanogaster* has been an invaluable tool for delineating the contributions of specific genes to complex behaviours, including learning and memory (Tully et al., 1994). This is due to the neural systems that specify complex *Drosophila* behaviours being readily amenable to genetic analysis and manipulation. The use of forward genetics approaches in which random mutagenesis is used to generate disruptive lesions in single genetic loci, which are subsequently screened for behavioural abnormalities (Benzer, 1973), has identified many genes required for learning and memory. These include *dunce*, which encodes a cAMP phosphodiesterase (Dudai et al., 1976), and *rutabaga*, which encodes an adenylate cyclase (Livingstone et al., 1984). The identification of mutations in components of the cAMP signalling pathway leading to perturbations in *Drosophila* memory substantiates the importance of this signalling cascade in the mechanistic underpinnings of memory formation. Moreover, it highlights the striking conservation of memory-relevant molecular cascades across the animal kingdom.

1.2.3 Translational regulation of gene expression

The regulation of modifications to cellular proteomes through changes in patterns of gene expression as a means of bringing about physiological change have been traditionally considered at the level of transcription. Until the end of the twentieth century the prevailing

view of gene expression regulation in neurons was that of the central dogma of DNA transcribed into RNA then translated into protein occurring centrally in the soma of the cell (Holt and Schuman, 2013). Indeed, following the discovery of the signal peptide (Blobel and Dobberstein, 1975) it was considered that the primary biological mechanism for targeting of proteins to their required subcellular destination occurred at the level of the protein via localisation information encoded in the polypeptide sequence. However, protein localisation can also be manifested via localisation of the mRNA transcript encoding it. In this way, the spatial regulation of gene expression is determined by the translation of localised mRNA transcripts. Recently, the prevalence and biological importance of subcellular mRNA localisation has begun to overturn the central dogma of gene expression regulation being confined to the nucleus.

Examples of translational regulation of gene expression

The localisation of mRNA transcripts as a means of spatially restricting translation and hence the expression of encoded protein products has been described in a number of biological contexts serving various biological functions. Perhaps the best-studied function of mRNA localisation is in the establishment of morphogen gradients during embryonic development (Driever and Nusslein-Volhard, 1988; Ephrussi et al., 1991). This example is spectacularly exemplified by the patterning of mRNA transcripts that decorate the developing *Drosophila* embryo (Lécuyer et al., 2007). Other biological functions of mRNA localisation as a means of spatially regulating gene expression include the targeting of proteins to specific organelles (Weis et al., 2013) and subcellular compartments (Mili et al., 2008). In addition, the localisation of specific mRNA transcripts is used as a means of partitioning cell fate determinants between daughter cells of the budding yeast *Saccharomyces cerevisiae* (Takizawa et al., 1997). The focus of the proceeding discussion will focus on translational regulation of gene expression in the context of neurons.

1.3 Translational regulation of gene expression in neurons

The morphological and functional complexity of neurons coupled with the capacity of individual distal synaptic compartments for localised information processing provides an intuitive logic for the decentralised regulation of gene expression. The localisation and translation of mRNA transcripts at or near functionally independent neuronal sub-compartments permits the spatial and temporal regulation of *de novo* protein synthesis autonomously.

Benefits of localised translation

Localised mRNA translation confers several advantages for a biological system. Firstly, it enables post-transcriptional spatial regulation of gene expression. Importantly, in the context of neurons, this provides individual synaptic compartments with the ability to autonomously modify the local proteome independent of the cell body (Martin and Zukin, 2006). Secondly, activity-regulated translation of localised mRNA transcripts allows greater temporal precision of gene expression when compared to relying on regulation of gene expression at the transcriptional level which requires signal transduction from activated synapse to nucleus, transcription, mRNA export to the cytoplasm, translation, and targeting of the protein to the appropriate synapse (Martin and Ephrussi 2009). Thirdly, off-target effects of erroneous protein function in inappropriate cellular destinations are avoided by localising the synthesis and therefore function of a protein to its appropriate subcellular destination. Finally, localised mRNA transcripts can be translated multiple times to generate multiple proteins (Wu et al., 2016; Yan et al., 2016), without requiring the targeted transport of many copies of a particular protein.

1.3.1 Protein synthesis machinery in neural processes

The implementation of localised translation in neural processes requires the localisation and function of a number of cell biological systems. These include systems to target, localise and functionally regulate mRNA transcripts, in addition to outposts of the appropriate cell biological machinery required to translate and produce functional mature proteins. The ability of isolated synaptic compartments to perform protein synthesis de-

pendent forms of plasticity (Corera et al., 2009), along with their ability to incorporate radiolabelled amino acids into protein (Rao and Steward, 1991; Torre and Steward, 1992) suggests that these components do in fact reside within neuronal compartments.

Ribosomes

The presence of polyribosome structures located at or near synaptic boutons has been established with electron micrograph studies (Bodian, 1964; Steward and Levy, 1982) and later confirmed unequivocally with electron microscopy coupled with immunogold labelling of ribosomal proteins (Gardiol et al., 1999; Pierce et al., 2001). Since the identification of ribosomes proximal to synaptic densities, it has subsequently been shown that these ribosomal populations are highly dynamic, increasing at sites of synaptic activity following stimulation (Ostroff et al., 2002, 2010). In addition to the presence of the major translational machine, the ribosome, regulatory proteins of translation have also been identified. Elongation initiation regulators including eIF-4E and eIF-4E binding proteins are localised in hippocampal dendrites (Tang et al., 2002) and interference with their functioning impairs the formation of long-term memory *in vivo* (Hoeffler et al., 2011) as well as the formation of LTP and LTD *ex vivo* (Banko et al., 2006; Costa-Mattioli et al., 2005).

Endoplasmic reticulum and Golgi

The presence of ribosomes along with regulatory proteins suggests that synaptic compartments contain the necessary components required to translate mRNA into polypeptide chains and thus synthesise soluble proteins. However secreted and integral membrane proteins require the organelles of the endomembrane system for their synthesis. Specifically, these proteins require synthesis in the endoplasmic reticulum, followed by modification within the Golgi network before they can be inserted into the plasma membrane. Immunogold labelling of luminal endoplasmic reticulum and Golgi-associated proteins proximal to synaptic boutons (Gardiol et al., 1999; Pierce et al., 2001) along with the ability of isolated dendrites to incorporate radiolabelled monosaccharides into proteins (Torre and Steward, 1996) suggests the presence of a functional endomembrane system in neural compartments.

Although the classical Golgi apparatus is located in the cell body, it is thought that neural processes have evolved a specialised, more simplified, Golgi system. Neurons employ a simplified microsecretory Golgi satellite system for secretory and membrane protein processing which apparently lacks many of the protein markers commonly associated with the classical Golgi apparatus (Mikhaylova et al., 2016; Hanus et al., 2014). In addition, protein cargo are known to traffic from the ER into a system of tubulovesicular membrane structures known as ER-Golgi intermediate compartments (ERGICs) that extend throughout dendrites (Hanus et al., 2014). The ERGIC network normally shuttles protein cargo onto the Golgi apparatus, however given the lack of canonical Golgi apparatus in dendrites, it is unclear whether this occurs here. Instead, it has been shown that protein can traffic through ERGIC onto recycling endosomes (REs) bypassing transport through classical Golgi apparatus (Bowen et al., 2017). The secretory and membrane protein processing system in dendrites therefore seems to be a more simplified, stripped-back, version of the classical systems found within the cell body. In line with this, many neuronal surface membrane proteins display immature mannose-rich core N-glycosylation patterns, which are hallmarks of an unconventional protein maturation pathway, e.g. Golgi by-pass or reduced Golgi function (Hanus et al., 2016). Nonetheless, neuronal compartments seem to be equipped with the machinery required for the synthesis of secreted and integral membrane proteins.

The presence of synaptically proximal organelles including ribosomes, endoplasmic reticulum and Golgi structures suggests that dendritic compartments are equipped with the necessary cell biological machinery required to locally synthesise proteins. Moreover, the modified Golgi system suggests the capacity to synthesise membrane-associated and secretory proteins that require the endomembrane system for translation, glycosylation and membrane insertion. This is an important point when considering the local synthesis of ion channels and neurotransmitter receptors in neurons.

1.3.2 Localised mRNA transcripts

Following the discovery of polyribosomes located proximally to dendritic spines (Bodian, 1964; Gardiol et al., 1999; Pierce et al., 2001; Steward and Levy, 1982), *in situ* hybridisation techniques revealed the presence of various extrasomatically localised mRNA transcripts, including *CaMKII* (Burgin et al., 1990) *MAP2* (Garner et al., 1988) and *Arc* (Lyford et al., 1995). More recently, deep sequencing approaches have been used to document the nature of the axonal and dendritic transcriptome. A total of 2,250 mRNA species were identified in the synaptic neuropil of the rat hippocampus, encoding a functionally diverse range of proteins from ionotropic and metabotropic neurotransmitter receptors and voltage gated ion channels, to signalling molecules and components of the protein synthesis and degradation machinery (Cajigas et al., 2012). These localised mRNA transcripts have been shown to encode around half of the localised proteome. Moreover, localisation of mRNA transcripts is correlated with protein localisation in neurites, suggesting that the localisation of the mRNA transcript regulates the targeting and localisation of the encoded protein product (Zappulo et al., 2017).

Prevalence throughout the animal kingdom

Localisation of mRNA transcripts has been reported in the peripheral neurites within the nervous systems of very distantly related animal species. These include larvae of the marine annelid worm *Platynereis dumerilii* (Randel et al., 2013), the sea slug *Aplysia californica* (Lyles et al., 2006), the fruit fly *Drosophila melanogaster* (Yang et al., 2017), mouse (Lionnet et al., 2011), rat (Cajigas et al., 2012) and cultured human neurons (Bigler et al., 2017). The presence of mRNA transcripts localised within the axons and dendrites of neurons from evolutionary diverse animal species suggests that this biological phenomenon is an ancient and conserved feature.

Specialised characteristics

Interestingly, extrasomatically localised mRNA in the nervous system seems to employ characteristically unique systems of regulation. In the *Drosophila* and mammalian nervous system, mRNA transcripts contain exceptionally long 3'UTRs, in some cases >18

kb (Hilgers et al., 2011; Miura et al., 2013; Smibert et al., 2012). Moreover, mRNA transcripts in the brain also exhibit intricate mechanisms of activity-dependent post-transcriptional regulation. Cleavage and 3' polyadenylation is a ubiquitous pre-mRNA modification step during eukaryotic gene expression. mRNA transcripts typically contain multiple polyadenylation sites usually found within the 3'UTR but sometimes also within introns. The use of alternative polyadenylation sites thus has the capacity to alter both the coding sequence of the transcript and the length of the 3'UTR. It has recently been shown that LTP induces a general trend towards shortening of 3'UTRs through the use of alternative polyadenylation sites (Fontes et al., 2017). Such regulation of mRNA sequence has the consequence of affecting the coding sequence and/or gene expression by altering the localisation, stability or translation of the transcript. This nervous system specific 3'UTR lengthening and activity-dependent regulation of 3'UTR length seems to reflect an extensive capacity for post-transcriptional regulation of mRNA transcripts in the brain.

1.3.3 Localisation elements and mechanisms

The subcellular localisation of mRNA transcripts requires their delivery from the nucleus to their appropriate subcellular destination. A number of mechanisms that bring about the subcellular localisation of mRNA in various biological contexts have been described (Holt and Bullock, 2009). Firstly, localisation can occur by a diffusion and entrapment mechanism in which mRNA transcripts diffuse passively throughout the cell cytoplasm and are specifically localised in certain subcellular compartments through spatially restricted mechanisms that sequester mRNA. The localisation of *nanos* mRNA to the posterior pole of the *Drosophila* oocyte during embryogenesis occurs via such a mechanism (Forrest and Gavis, 2003). Secondly, localisation can occur by the selective degradation and stabilisation of mRNA whereby mRNA is degraded in most parts of the cell but is selectively protected from degradation in some localised compartment. This mechanism is exemplified by the localisation of *Hsp83* mRNA in the posterior pole of the *Drosophila* embryo. *Hsp83* mRNA is distributed throughout the embryo where it is continually degraded except at the posterior pole where it accumulates (Semotok et al., 2005). Thirdly, in contrast to the aforementioned passive diffusion of mRNA transcripts, in many cases mRNA lo-

calisation involves targeted active transport of transcripts via motor proteins along the microtubules of the cellular cytoskeleton. Many specific subcellular localisation patterns of mRNA transcripts are dependent upon both motor proteins and the proper functioning of the cytoskeleton (Long et al., 1997). In *Aplysia*, kinesin interacts with thousands of mRNAs comprising approximately 2-5% of the synaptic transcriptome (Puthanveetil et al., 2013). Active transport is thus an important and ubiquitous mechanism of subcellular mRNA localisation.

mRNPs in active transport

Active localisation of mRNA along the cytoskeleton requires the association of mRNA with RNA binding proteins (RBPs). Together, mRNA, RBPs as well as regulatory RNAs such as micro RNAs (miRNAs) and long non-coding RNAs (ncRNAs) (Fabian et al., 2010; Yoon et al., 2013), form large complexes known as messenger ribonucleoprotein particles (mRNPs) (Müller-McNicoll and Neugebauer, 2013), which are known to interact with the cytoskeletal active transport machinery (Kanai et al., 2004). It is thought that mRNAs encompassed in mRNPs for localisation purposes are maintained in a translationally repressed state during transit (Krichevsky and Kosik, 2001). Indeed, many RBPs that associate with mRNA and mediate their subcellular localisation function as repressors of translation, including the Fragile X associated RNA binding protein FMRP (Darnell et al., 2011).

The mechanistic details of actively transported mRNA transcripts bundled into mRNPs in neurons are not fully understood. Indeed, the exact molecular composition of mRNPs is as of yet not fully described. The molecular constituents of *in vivo* mRNPs has been assessed using variations of techniques involving the crosslinking of RNA with RBPs followed by RBP immunoprecipitation and identification of captured RNAs. Such approaches have revealed that each mRNA can be bound by multiple RBPs. Moreover, RBPs may have multiple RNA binding domains and consequently may bind thousands of mRNA targets (Ascano et al., 2012; Mitchell and Parker, 2014; Müller-McNicoll and Neugebauer, 2013). *In vivo*, mRNA transcripts are found both as single molecules (Buxbaum et al., 2014;

Femino et al., 1998) and in complex with other mRNA transcripts (Buxbaum et al., 2014; Park et al., 2014; Skinner et al., 2013). However, concomitant visualisation of multiple mRNA species suggested that they tend not to associate within the same mRNP complexes (Batish et al., 2012; Tübing et al., 2010). mRNA transport specificity is little understood. However these observations do suggest a level of selectivity in targeted transport of mRNA. It seems that assemblages of mRNA species are not lumped together in a generalised transport mechanism, but instead are independently packaged and transported in mRNPs.

Recent technological advances, particularly the development of the MS2 system (described in detail below), that enable live imaging of mRNAs *in vivo* has begun to reveal important kinetic features of active mRNA transport along the cytoskeleton. In the case of β -actin, only a small subset of mRNAs are actively transported at any one time, approximately 10% (Park et al., 2014). Of this motile mRNA population, mRNA molecules tend to exhibit processive movements in both anterograde and retrograde directions within neurites (Dynes and Steward, 2007; Park et al., 2014; Tübing et al., 2010).

Localisation elements

The association of mRNA with the specific RBPs that orchestrate localisation is achieved by the interaction of conserved RNA binding domains in the protein either with specific cis-acting localisation elements within the nucleotide sequence, or with the secondary structure it specifies. Most commonly, localisation elements are found in the 3'UTR of the mRNA transcript, however they can also be present within the 5'UTR or coding sequences (Shahbadian and Chartrand, 2012). Moreover, localisation elements are highly variable in length, ranging from a few nucleotides up to 1 kb, and they are often repeated several times across the mRNA transcript (Eliscovich et al., 2013; Doyle and Kiebler, 2011).

The cis-acting localisation elements that direct subcellular mRNA distribution are thus complex and incompletely understood. Owing to this complexity and variability of localisation elements, bioinformatic approaches have not yet been successfully employed

to unambiguously identify or predict localisation sequences or common structural motifs in mRNA sequences (Doyle and Kiebler, 2011). Efforts to locate and identify localisation elements have so far involved fusing putative localisation elements to reporter genes to identify cis-acting sequences that are responsible for directing localisation. Such an approach was used to identify the 3'UTR signal responsible for the dendritic localisation of *CaMKII α* mRNA, given the ability of this sequence to localise an exogenous lacZ transcript (Mayford et al., 1996).

A well-studied example of the mechanisms of a subcellularly localised mRNA is *β -actin*. The *β -actin* mRNA transcript contains a bipartite 54-nucleotide localisation element within the 3'UTR which is recognised and bound by two C-terminal KH domains of the RNA binding protein ZBP1 (Chao et al., 2010). The interaction of ZBP1 with the *β -actin* localisation element is necessary and sufficient for transcript localisation (Kislauskis et al., 1994).

1.4 Visualising mRNA transcripts

In order to study mRNA localisation, it is necessary to be able to visualise transcripts. The first studies to assess mRNA localisation in the brain used *in situ* hybridisation techniques, in which short nucleic acid probes bind DNA or RNA in cells or tissues. In old fashioned versions of the technique, biotinylated or radioactive probes would then be used to detect patterns of mRNA localisation. Using such methods, mRNA transcripts of *MAP2* (Garner et al., 1988) and *CaMKII α* (Burgin et al., 1990) were found to localise in distinct layers in the mammalian brain including regions corresponding to dendritic arborisations of hippocampal neurons. Similar patterns of extrasomatic localisation of mRNA transcripts were also observed using *in situ* hybridisation in cultured hippocampal neurons (Kleiman et al., 1990). Technological advances have recently led to the development of high-resolution single molecule mRNA visualisation techniques (described below) and have revolutionised the study of mRNA biology.

1.4.1 Single molecule *in situ* hybridisation

In recent years, technological advancements have led to the development of fluorescently labelled *in situ* hybridisation probes (FISH), and more recently the use of probe sets composed of multiple short (approximately 20 nucleotides) individually fluorescently pre-labelled oligonucleotide probes which tile a region of the mRNA of interest. Coupled with improvements in the sensitivity of image detection, the use of sets of fluorescently labelled probes has led to the development of single molecule fluorescence *in situ* hybridisation (smFISH). Hybridisation of fluorescent probes to the target mRNA allows detection of single mRNA transcripts and quantification of mRNA levels at single cell resolution (Femino et al., 1998). Multiple copies of mRNA are also visualised, including sites of active transcription in the nucleus. This has allowed the development of computational tools such as BayFish to study specific kinetic parameters of gene expression, including transcription rate and patterns of promoter activation and inactivation (Gómez-Schiavon et al., 2017). Moreover, smFISH has been further developed to enable multiplexed high-throughput imaging based transcriptomics in single cells (Moffitt et al., 2016).

In the context of mRNA localisation in neurons, smFISH has recently been applied to the whole *Drosophila* brain (Long et al., 2017; Yang et al., 2017). In this way, the distribution and subcellular localisation of endogenous mRNA transcripts can be visualised within the spatial context of a whole brain. In addition, smFISH has been used to monitor temporal changes in mRNA transcript abundance, both to visualise cyclical fluctuations in circadian transcripts such as *Drosophila timeless* mRNA (Long et al., 2017), as well as responses of localised β -actin mRNA populations to induced neuronal stimulation (Buxbaum et al., 2014). smFISH is therefore a valuable tool with which endogenous mRNA localisation patterns can be documented and monitored in response to neuronal activity.

1.4.2 MS2/PP7 labelling of mRNA

smFISH has facilitated important insights into many aspects of mRNA biology, including qualitative information regarding the abundance and localisation of individual transcripts,

as well as quantitative information including the dynamics of gene expression and even single cell transcriptomics. However, the smFISH protocol requires fixed tissue and hence temporal information can only be accessed by analysing fixed samples at successive time points, restricting the temporal resolution to relatively broad timescales. Details regarding fine temporal dynamics such as mRNA trafficking are therefore unattainable.

To address this temporal limitation, techniques were developed to fluorescently label mRNA transcripts directly. The basis of such approaches rely on the binding of certain RNA binding proteins (RBPs) to nucleotide sequences within mRNA transcripts encoding specific secondary structures, namely stem loop or hairpin structures. The bacteriophage derived MS2 system has so far been the most extensively used method of directly labelling mRNA molecules. Related systems, including the PP7 system, function very similarly. It involves introducing an exogenous array of RNA hairpin structures derived from the MS2 bacteriophage into the nucleotide sequence encoding an mRNA of interest. The array of RNA hairpin structures is typically composed of between 6 and 24 tandem repetitions of stem loops, sometimes referred to as MS2 binding sites, which are recognised and bound by the MS2 coat protein (MCP). Chimeric versions of the MCP that allow the protein to be visualised consequently enables visualisation of the mRNA transcript once the MCPs bind the MS2 binding sites. MCPs can be fused to naturally fluorescent proteins such as GFP or RFP (Bertrand et al., 1998; Brechbiel and Gavis, 2008), or protein tags such as SNAP or HALO (Carrocci and Hoskins, 2014) that are visualised by covalent binding of a synthetic ligand tagged with a chemical fluorescent dye (Kohl et al., 2014). Chemical fluorescent dyes offer several advantages, particularly from the perspective of single molecule imaging, in that they tend to be much brighter and more photostable compared to fluorescent proteins. Homologous systems of coat proteins binding to specific RNA hairpins have also been adopted for mRNA visualisation, including the bacteriophage PP7 coat protein and its cognate RNA hairpin structure (Chao et al., 2008).

The major advantage of labelling mRNA with hairpin arrays and visualising them via binding of cognate coat proteins is that it permits live monitoring of mRNA, and hence

fine temporal resolution of dynamic behaviour. The ability to monitor individual mRNA molecules in specific cells in real time has revolutionised the study of mRNA biology and provided important insights into the contribution of spatial and temporal regulation of gene expression via mRNA localisation with respect to the physiological functioning of neurons.

1.5 Using the MS2 system to study mRNA biology

The first use of the MS2 system was in the visualisation of *Ash1* mRNA in live budding yeast cells (Bertrand et al., 1998). *Ash1* mRNA is preferentially distributed to the daughter nucleus relative to the mother nucleus as a regulatory mechanism of mating type switching. Visualising *Ash1* mRNA via the MS2 system allowed the authors to follow this localisation in real time and calculate kinetic parameters of migrating transcripts.

mRNA localisation in *Drosophila* development

The power of *Drosophila* genetics allows the relatively straightforward co-expression of both MS2-tagged mRNA and fluorescent MCP with single cell specificity using binary expression systems (e.g. Gal4/UAS, LexA/LexAop). Brechbiel and Gavis (2008) exploited the advantages of this system to label *nanos* mRNA and visualise its expression in larval class IV dendritic arborisation neurons, showing the translational control of dendritically localised *nanos* mRNA transcripts to be functionally important for neural morphogenesis. Other efforts to use the MS2 system for labelling mRNA in combination with the genetic tools available in *Drosophila* have been limited to the oocyte, visualising mRNAs such as *oskar* (Zimyanin et al., 2008), *gurken* (Jaramillo et al., 2007), *bicoid* (Weil et al., 2008) and *nanos* (Forrest and Gavis, 2003) which are well described mRNA transcripts that characteristically decorate the *Drosophila* oocyte and embryo with their specific localisation patterns, contributing to the establishment of morphogen gradients during embryogenesis.

mRNA localisation in neurons

In regards to using the MS2 system to study the physiological significance of mRNA localisation in neurons, the development of two mouse models has been particularly insightful. The first contains an array of MS2 hairpin structures knocked into the 3'UTR of the genomic *β -actin* locus (Lionnet et al., 2011) and the second contains an array of PP7 hairpin structures knocked into the 3'UTR of the genomic *Arc* locus (Das et al., 2018). Such technical developments have enabled endogenous neuronal mRNA to be visualised in real time. *β -actin* mRNA was first visualised by transfecting cultured fibroblasts and hippocampal neurons with a YFP tagged MS2 coat protein (Lionnet et al., 2011). *β -actin* mRNA molecules were imaged live as they moved along the processes of a cultured hippocampal neurons, revealing some populations of mRNA molecules to be stationary and some that exhibited various motile behaviours including both unidirectional and bidirectional transport at speeds of around $0.55 \mu\text{m s}^{-1}$.

The initial strategy for combining MS2 tagged *β -actin* with the part of the system that enables tagged mRNA to be visualised, the fluorescently labeled MCP, required the transfection of the MCP::FP into cultured cells derived from the *β -actin* MS2 knock-in mouse. Park et al. (2014) subsequently crossed the *β -actin* MBS mouse to a line expressing a GFP tagged MCP expressed under the control of a high-expression ubiquitous promoter. The co-expression of both components of the MS2 system ubiquitously in a whole animal in principle enables *β -actin* mRNA to be visualised in any cell type at any point in time. Park et al. (2014) visualised *β -actin* mRNA live in cultured fibroblasts as well as in cultured hippocampal neurons and *ex-vivo* brain slices. In cultured hippocampal neurons, *β -actin* mRNA molecules were present both singly and in multiple copies as part of mRNA transport granules (mRNPs), with $\sim 25\%$ of mRNPs in proximal dendrites containing more than one *β -actin* mRNA molecule. Around 10% of *β -actin* mRNA containing mRNPs were actively transported in both anterograde and retrograde directions with a mean speed of $1.3 \mu\text{m s}^{-1}$. Das et al. (2018) similarly used a genetically-encoded MCP::GFP to label endogenous PP7-labelled *Arc* mRNA in cultured hippocampal neurons. They observed localisation of *Arc* mRNA to dendritic spines and similar motility

dynamics to those reported for β -actin.

The responses of both β -actin and *Arc* mRNA to induced neuronal stimulation were also assessed (Das et al., 2018; Park et al., 2014). In the case of *Arc* mRNA, physiological stimulation of cultured hippocampal neurons did not alter transport velocity (Das et al., 2018). In the case of β -actin, potassium chloride (KCL) depolarisation increased the number of mRNP foci containing a single β -actin mRNA transcript, while the number of foci containing multiple mRNA transcripts decreased. Moreover, the diffusion coefficients of particles exhibiting directed movement tended to decrease, suggesting that the increase in single β -actin mRNA foci was not due to transport of new transcripts from the nucleus. Instead, the authors propose that mRNA molecules undergo assembly and disassembly from mRNP complexes within neural processes in an activity dependent manner. The function of this dynamic association and disassociation of mRNAs to mRNPs may be related to translational accessibility of the transcript, since ZBP1 (a proposed translational repressor) and ribosome binding to β -actin are anti-correlated (Wu et al., 2015).

To gain a more detailed insight into the dynamics of β -actin mRNA in response to localised synaptic activity, a photolytic uncaging method was developed to locally deliver glutamate to individual dendritic spines of cultured hippocampal neurons (Yoon et al., 2016). β -actin mRNA was specifically recruited to activated dendritic spines within 15 mins of postsynaptic stimulation. Neighbouring spines showed a net decrease in β -actin mRNA density while the total average mRNA density per dendrite did not change, suggesting that the distribution of the total neuronal mRNA pool is remodeled upon specific postsynaptic stimulation. Specifically, mRNA is recruited to activated dendritic spines from neighbouring regions. The observed patterns of mRNA relocation to the sites of postsynaptic activation were dependent on NMDA receptor activity, the actin cytoskeleton, and the RNA-binding protein ZBP1 which interacts with the localisation element in the β -actin 3'UTR.

The authors also addressed the question of whether localised mRNA transcripts are

translated at stimulated spines. Using a reporter construct in which the β -*actin* mRNA is labelled using the MS2 system and the protein is labelled with a HALO tag, the authors were also able to show that, in addition to mRNA, newly synthesised β -*actin* protein accumulates at stimulated spines. The accumulation of β -*actin* mRNA and newly synthesised protein at the sites of postsynaptic stimulation presumably contributes to the actin cytoskeletal rearrangements that underlie structural changes in synapses related to plasticity.

The study of mouse β -*actin* and *Arc* mRNA has revealed that distally localised mRNA molecules are not simply static translational outposts. They exhibit bidirectional motile behaviours and the capacity to dynamically reposition towards activated synapses in response to stimulation. Moreover, they appear to undergo cycles of association and dissociation with mRNPs in response to neural stimulation in a manner that suggests mRNA transcripts may be released from protein granule associations to allow activity-dependant local translation to occur. These findings support a model in which dendritically localised mRNAs and their associated regulatory proteins that form mRNPs are mobile in the vicinity of several synapses, upon activation of which results in the recruitment and accumulation of scanning mRNA transcripts for local translation (Doyle and Kiebler, 2011).

1.5.1 Visualising transcription

One area of mRNA biology that has particularly benefited from the advent of single-gene, single-molecule mRNA visualisation, is the study of transcriptional dynamics. In both prokaryotes and eukaryotes, the synthesis of RNA from DNA occurs discontinuously in bursts of transcription (Larson, 2011). A given gene can occupy an active or inactive transcriptional state, and oscillates between the two with variability in burst frequency, duration and RNA synthesis between genes, cells and organisms. The MS2 system has been fruitfully applied to study the spatio-temporal transcription dynamics of the mRNA transcripts which characteristically pattern the *Drosophila* embryo with their spatially localised expression patterns. In particular, *even-skipped* (*eve*) and *hunchback* (*hb*) transcription has been visualised live during *Drosophila* embryogenesis. Both genes are specifically transcribed within the spatial confines of their particular expression pattern and

exhibit transcriptional bursting activity within a strict temporally-defined period during embryogenesis (Berrocal et al., 2018; Bothma et al., 2014; Garcia et al., 2013; Lucas et al., 2013). In the case of *eve*, analysis of nascent transcription foci revealed that high frequency bursts of transcription occur within the pattern of stripes across the *Drosophila* embryo corresponding to *eve* expression (Berrocal et al., 2018). Moreover, burst durations range from 4-10 minutes (Bothma et al., 2014).

The development of the PP7-labelled *Arc* mRNA mouse model also enabled transcriptional responses in neurons to be visualised (Das et al., 2018). *Arc* is a well described mammalian immediate early gene whose expression is characteristically induced by synaptic activity. In cultured hippocampal neurons, Das et al. (2018) visualised the transcriptional responses of *Arc* to induced neuronal stimulation. They show that the increased transcriptional output of the *Arc* locus is achieved by prolonging the transcriptional on state in response to neuronal activity. This work provides an important visual insight into the molecular responses that couple neuronal activity and gene expression.

1.5.2 Visualising translation of localised mRNA transcripts

The ability to visualise molecules of mRNA live in neurons has revolutionised our understanding of the basic biology of decentralised gene expression. It has allowed us to document localisation kinetics of mRNA and probe the responses of these molecules to induced neural activity. However many outstanding questions remain unanswered. The full protein and RNA content of mRNPs is unknown, and the bulk of work has thus far been directed towards very few select mRNA species, namely *β -actin*. Perhaps most importantly, however, is the functional significance of localised mRNA transcripts. Are extrasomatically localised mRNA molecules translated? What are the temporal events that regulate this phenomenon? And do the resulting proteins functionally contribute to the molecular remodelling at synapses that underlies plasticity? Novel imaging technologies that visualise translation events are beginning to be developed to enable us to begin to probe these biological questions.

One approach taken was to use fluorescence fluctuation spectroscopy to quantitate the molecular interactions between single molecule mRNA with RBPs and ribosomes via brightness analysis (Wu et al., 2015). Using this approach, Wu et al. (2012) were able to show anticorrelation of ZBP1 and ribosome binding of β -actin mRNA in cellular regions proximal to the cell body in mouse embryonic fibroblasts and hippocampal neurons. The authors conclude from their findings that the association of the RBP ZBP1 to β -actin mRNA mediates translational repression and mRNAs are subsequently released from RBP complexes to permit translation. For reasons that are not clear, the authors apparently did not extend the approach to dendritically localised β -actin mRNA in hippocampal neurons. Although informative to some extent, the study used ribosome association as a proxy for translational activity. However this is not necessarily always the case since mRNA-associated ribosomes may be stalled (Wilson et al., 2016). More recent efforts to address the functional significance of extrasomatically localised mRNA transcripts have focused on developing reporter systems that visualise translation events in real time.

Translation reporters using MS2/PP7 labelling

Halstead et al. (2015) were the first to report the development of a reporter construct that enables an individual mRNA as well as the first round of its translation to be imaged. The system is based on concomitant labelling of a reporter mRNA species (fluoregen activating protein dNP799) with MS2 sites within the 3'UTR and PP7 sites at the beginning of and in frame with the open reading frame. The 3'UTR MS2 sites permanently label the mRNA transcript with an RFP tagged MCP. The PP7 sites, when visualised by a GFP tagged PCP, specifically label the mRNA transcript before translation occurs. Upon translation however, traversing ribosomes knock-off associated PP7 coat proteins within the open reading frame. Thus, co-localisation of both MS2 and PP7 coat protein labels indicates an untranslated mRNA transcript. Because the 3'UTR MS2 sites label the mRNA permanently, initiation of the first translation event results in the loss of co-localisation and therefore allows the first round of translation to be visualised. The authors hence named the system translating RNA imaging by coat protein knock-off (TRICK).

A drawback of the TRICK reporter is that it is limited to enabling only the visualisation of the first translation event and as the authors describe, most transcripts are already translated at the point of imaging (Halstead et al., 2015). More recent efforts have combined concomitant mRNA labeling with techniques to visualise nascent polypeptides as they are being translated, thus allowing multiple rounds of translation to be visualised. Such approaches use MS2 or PP7 labelling of the mRNA transcript coupled with systems that label nascent proteins as they are synthesised (Morisaki et al., 2016; Wang et al., 2016; Wu et al., 2016; Yan et al., 2016). Such nascent protein labeling systems, referred to as SunTag or V4, involve the expression of fluorescently labeled antibody fragments that bind multiple short epitopes in the polypeptide sequence in tandem to boost the signal from peptide binding above unbound background noise (Tanenbaum et al., 2014).

This system has been applied to cultured hippocampal neurons to assess whether dendritically localised mRNA transcripts are translated. One approach fused the SunTag or V4 epitope array with a PP7 tagged *Arc* 3'UTR known to instruct dendritic localisation (Wang et al., 2016), while another approach fused the SunTag or V4 epitope array with an MS2 tagged *β -actin* 3'UTR which is also known to instruct dendritic localisation (Wu et al., 2016). Both variations of this translation reporter system visually demonstrated translation of nascent polypeptides in the dendrites of cultured hippocampal neurons in real time. These reporters also enabled quantitative kinetic parameters of translation in neurons to be measured. For example, the elongation rate of translation in hippocampal neurons was measured at between 4-5 amino acids per second and, similarly to transcription, the dynamics of translation show bursting behaviours (Wang et al., 2016; Wu et al., 2016).

Another interesting result to arise from these experiments is that mRNAs can engage in active transport while being translated. Thus translation and transport are not mutually exclusive events. This result challenges the long-held belief that mRNA molecules are transported in mRNPs in translationally repressed states (Krichevsky and Kosik, 2001).

Intuitively, given that the purpose of transporting mRNA molecules for localisation at distal sites is to allow the temporally regulated translation of that particular transcript within the spatial confines of a specific compartment, the translation of mRNA molecules during transit seems incoherent. Whether such reporter constructs, which lack the regulatory elements of an endogenous gene's 5'UTR and open reading frame, correctly assemble into mRNPs with all the relevant regulatory proteins and RNAs seems unlikely and hence perhaps this finding is an artefact of the reporter system.

These recent technological advances have provided the proof of principle data to demonstrate that the translation of dendritically localised single molecule mRNA can be visualised in real time. It will be important to extend such systems to biologically meaningful mRNAs with all the relevant regulatory components within the 5'UTR and coding sequence. Hopefully such approaches will enable us to probe the biological significance of these localised molecules, particularly in relation to synaptic plasticity.

1.5.3 Potential caveats of the MS2 system

A discussion on the use of fluorescent proteins that bind stem loop structures introduced into mRNA molecules to visualise them would of course not be complete without acknowledging the potential caveats of the system. As is often the case in biological research, the visualisation of cells and molecules requires their manipulation. This frequently involves the targeting of fluorescent tags. However, in the effort to visualise and hence study these molecules, the effect of any manipulation on the natural behaviour of the molecules cannot be overlooked.

The use of the MS2 system, and relatives thereof, have been employed for the past 15 years and have provided numerous insights into single-molecule mRNA behaviour that were previously unknown. However the technique is not without criticism. Garcia and Parker (2015, 2016) along with Haimovich et al. (2016) have shown that in the yeast *Saccharomyces cerevisiae*, the introduction of an MS2 array into some mRNA transcripts leads to an accumulation of 3'UTR MS2-tagged fragments. The cause of the accumulated frag-

ments, whether transcription artefact or degradation impairment, was unclear (Haimovich et al., 2016). Naturally this would lead to aberrant interpretations of mRNA localisation behaviours. However, such an artefact has only been observed in yeast for mRNA molecules with particularly short half lives. Nonetheless, many studies have shown using complementary techniques such as smFISH that mRNA molecules tagged with arrays of MS2 stem loops recapitulate both the endogenous cellular and subcellular spatial patterns of expression and localisation (Berrocal et al., 2018; Bertrand et al., 1998; Bothma et al., 2014; Garcia et al., 2013; Long et al., 1997; Lucas et al., 2013; Katz et al., 2012), as well as the temporal dynamics of developmental expression (Berrocal et al., 2018; Bothma et al., 2014; Campbell et al., 2015; Garcia et al., 2013; Lucas et al., 2013). Despite the observation of accumulated 3'UTR MS2-tagged fragments in yeast, the overwhelming majority of studies in various model systems and various mRNA transcripts of interest do not report aberrant effects of the introduction of stem loop arrays. According to studies published to date, the merits of single-molecule mRNA visualisation using stem loop arrays and fluorescent cognate binding proteins seems to outweigh the caveats.

1.6 Conclusion and outlook

The localisation of mRNA transcripts to specific subcellular neuronal compartments as a means of both spatially targeting gene expression and equipping distal synaptic compartments with the capacity for local translation is revolutionising our understanding molecular neuroscience. Recent technical advances are permitting the study of such phenomena in increasingly finer detail. The aim of this thesis was to develop and apply such techniques, including smFISH and MS2 labelling of mRNA, to study mRNA localisation in the context of the well-described memory circuitry of the *Drosophila* mushroom body. Genetic accessibility to single identified memory-relevant neurons enabled the study of mRNA localisation and behaviour at single cell resolution. A schematic anatomical overview of the mushroom body and subsets of output neurons and dopaminergic neurons relevant to this thesis can be found in Figure 1.1.

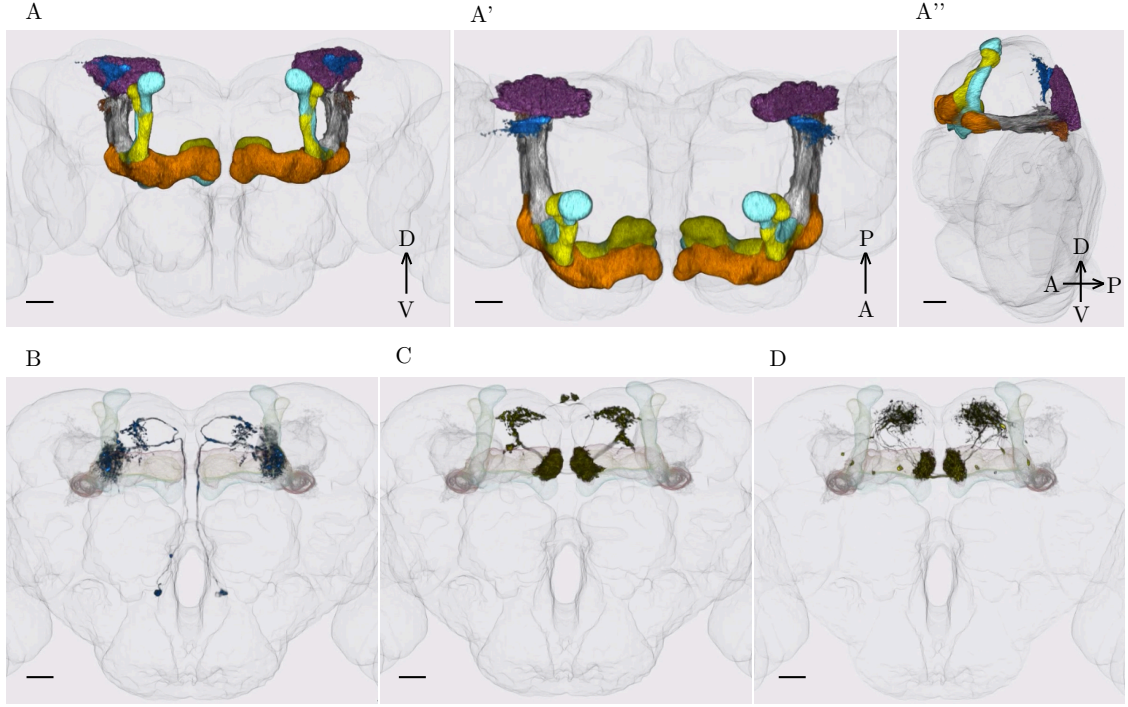


Figure 1.1: Anatomic overview of the *Drosophila* mushroom body. **A-A''**: Intrinsic mushroom body neurons, or Kenyon cells, visualised to indicate the anteroposterior and dorsoventral positioning in the adult brain. The calyces, Kenyon cell dendritic regions, are represented in purple, and the peduncle in grey. Kenyon cell axonal tracts form the lobes of the mushroom body and are divided into three subsets: α/β neurons are represented in light blue, α'/β' in yellow and γ in orange. Genetic tractability enables targeted manipulation of single neurons, including $\gamma2\alpha'1$ (**B**) and M6/ $\gamma5\beta'2a$ (**C**) mushroom body output neurons, and subsets of reinforcing dopaminergic neurons including those innervating the $\gamma5$ region (**D**). Figures adapted from Aso et al. (2014). Scale bar= $25\mu\text{m}$.

Chapter 2

Materials and Methods

2.1 Flies

2.1.1 Fly husbandry

All flies described herein are of the species *Drosophila melanogaster*. The wild type strain used was Canton-S (CS). Flies were raised at 25°C at 40-50% relative humidity. Flies for behavioural experiments were raised on standard cornmeal agar food supplemented with dried yeast in plastic bottles. Flies for imaging experiments were raised on standard cornmeal agar food supplemented with molasses and dried yeast in plastic vials.

2.1.2 Gal4 driver lines and UAS effector lines

All Gal4 driver and UAS effector lines used are described in Table 2.1.

Table 2.1: *Drosophila* strains used.

Gal4 driver lines			
Line	Stock reference	Insertion site (chromosome)	Described by
MB077c-Gal4		AD: VK00027 (III), DBD: attP2 (III)	Aso et al. (2014)

R66C08-Gal4	49412 (BDSC)	attP40 (III)	Pfeiffer et al. (2008)
R48B04-Gal4		attP2 (III)	Jenett et al. (2012)
MB247-GeneSwitch Gal4	50742 (BDSC)	(III)	
UAS effector lines			
UAS-2xeYFP	6660 (BDSC)	(III)	
10xUAS-IVS- mCD8::GFP	32186 (BDSC)	attP40 (II)	Rubin G, Pfeiffer B., Janelia Farm, HHMI
UAS-myr::SNAPf	58376 (BDSC)	attP40 (II)	Rubin G, Pfeiffer B., Janelia Farm, HHMI
UAS-MCP::RFP	27418 (BDSC)	(II)	Gavis L., Princeton
UAS- <i>CaMKII</i> - 24xMS2		attP2 (III)	Mitchell J.
UAS- <i>NMDAR1</i> - 24xMS2		attP2 (III)	Mitchell J.
UAS- <i>DLG1</i> -24xMS2		attP2 (III)	Mitchell J.
UAS- <i>Act5C</i> -24xMS2		attP2 (III)	Mitchell J.
UAS- <i>DOPR1</i> - 12xPP7		attP2 (III)	Mitchell J.
UAS-MCP::SNAPf		VK00018 (II)	Mitchell J.
UAS-MCP::SNAPf		VK00022 (II)	Mitchell J.
UAS-PCP::HALO		VK00018 (II)	Mitchell J.
UAS-PCP::HALO		VK00022 (II)	Mitchell J.
CRISPR/Cas9 generated mutant and tagged lines			
Nep1KO (2)			Mitchell J.
Nep1KO (4)			Mitchell J.
Nep1::GFP			Mitchell J.
Nep3KO (8)			Mitchell J.
Nep3KO (19)			Mitchell J.

Nep3KO (23)			Mitchell J.
YFP insertion lines			
w1118; PBac(602.P.SVS- 1)CaMKII ^{CPTI000944}	115127 (DGRC)		Lowe et al. (2014)
w1118; PBac(681.P.FSVS- 1)Pka-R2 ^{CPTI001580}	115174 (DGRC)		Lowe et al. (2014)
w1118; PBac(544.SVS- 1)Rab11 ^{CPTI00051}	115089 (DGRC)		Lowe et al. (2014)
w1118; PBac(681.P.FSVS- 1)Ten-m ^{CPTI001175}	115131 (DGRC)		Lowe et al. (2014)

2.2 Single Molecule Fluorescence *in situ* Hybridisation (sm-FISH)

2.2.1 Gal4 driver lines, UAS effector lines and YFP insertion lines

For the labelling and visualisation of endogenous mRNA transcripts in memory-relevant neurons in the whole *Drosophila* brain, smFISH was performed as described by Yang et al. (2017). Genes containing YFP or GFP insertions were visualised with YFP and GFP smFISH probe sets, respectively. YFP insertion lines used were CaMKII::YFP, PKA-R2::YFP, Rab11::YFP and Ten-m::YFP. The GFP insertion line used was Nep1::GFP. For single neuron resolution smFISH, YFP tagged genes were co-visualised within the dendrites of $\gamma 2\alpha'1$ MBONs labelled with a UAS-myr::SNAPf transgene driven by the MB077c-Gal4 driver. YFP and GFP insertion lines along with Gal4 driver and UAS effector lines used are documented in Table 2.1.

2.2.2 Probe design

Stellaris smFISH probe sets were purchased from LGC BioSearch Technologies (California, USA). Each set of oligonucleotide probes was designed using the web-based probe design software <https://www.biosearchtech.com/stellaris-designer> (oligonucleotide DNA sequences for each probe set used are described in Appendix Tables 5.2, 5.3 and 5.4). Three smFISH probe sets labelling YFP, GFP and the 24xMS2 cassette were used. Each complement of probes tiled the target RNA sequence of interest: YFP, GFP or the 24xMS2 nucleotide sequence. For all three smFISH probe sets, individual oligonucleotide probes were labelled with a Quasar-670 dye. Individual oligonucleotide probe sequences from YFP and GFP smFISH probe sets were non-redundant. There was a high degree of redundancy in the 24xMS2 smFISH probe set, given the repetitive nature of the 24xMS2 sequence, and hence there were 47 probe sequences in total, but only 5 sequences were unique.

2.2.3 smFISH protocol for whole adult *Drosophila* brain

The whole adult *Drosophila* brain smFISH protocol was carried out essentially as described by Yang et al. (2017). 2-4 day old adult *Drosophila* brains were dissected in RNase-free PBS and fixed in 4% v/v paraformaldehyde in PBS for 20 minutes at room temperature. Brains were washed twice with PBS, followed by 3 x 10 minute washes in 0.3% v/v Triton X-100 in PBS to permeabilise the tissue. Samples were incubated in wash buffer (2x RNase-free SSC + 10% v/v deionised formamide) for 10 minutes at 37°C. The wash buffer was then replaced with hybridisation buffer (2x RNase-free SSC, 10% v/v deionised formamide, 5% w/v dextran sulphate, 250 nM smFISH probes) and incubated overnight at 37°C. Hybridisation buffer was then removed before the samples were washed twice in freshly prepared wash buffer and then incubated for 10 minutes in wash buffer containing Vybrant DyeCycle Violet Stain (1:1000) to label cell bodies. For SNAP labelling of neurons, the SNAP ligand JF549-SNAPTag (500 nM) was also included. Samples were then washed a further three times in wash buffer. Samples were mounted onto a glass slide, covered with Vectashield anti-fade mounting medium, covered with a glass coverslip and sealed with nail varnish. Care was taken to maintain the slide in the dark so as to preserve

the fluorescent signal.

2.2.4 GeneSwitch-Gal4 induction

The GeneSwitch Gal4 system was used to temporally control the expression of a UAS-YFP transgene in order to validate the ability of smFISH to detect changes in mRNA expression. In this system the expression of UAS effector genes is controlled by a chimeric Gal4 protein, which is activatable by the steroid hormone RU486 (mifepristone) (Osterwalder et al., 2001). The MB247-GeneSwitch Gal4 line (Didelot et al., 2006) was used to temporally and spatially control the expression of a UAS-YFP transgene specifically in mushroom body Kenyon cells. Dietary ingestion of RU486 (Sigma-Aldrich, catalogue no. M8046) was used to activate the Gal4. Standard cornmeal-agar fly food was supplemented with 200 μ M RU486 (RU486+). Control food was not (RU486-). Newly emerged flies were transferred into vials containing RU486+ food on days 1, 2, 3, and 4 of the experiment to measure YFP mRNA and protein expression levels after 4, 3, 2 and 1 days of 200 μ M RU486 feeding, respectively. Control flies were fed RU486- food for 4 days.

2.2.5 smFISH following aversive olfactory conditioning

CaMKII mRNA abundance in the mushroom body calyx and the dendrites of $\gamma 2\alpha'1$ MBONs was assessed immediately, 2h and 24h following aversive olfactory conditioning. A non-trained group was used as a negative control. Details of the aversive olfactory conditioning protocol can be found in section 2.6.3. Following conditioning, the 2h and 24h group were returned to 25°C, while the immediate group were immobilised on ice for approximately 10 seconds before heads were removed and fixed in 4% v/v paraformaldehyde in PBS for 20 minutes at room temperature. The 2h and 24h group were similarly processed 2h and 24h after conditioning, respectively. The smFISH protocol was then performed as described above.

2.2.6 Image acquisition and analysis

Imaging

Samples were imaged using a Perkin Elmer UltraView Spinning Disk with 60x 1.35 N.A. Oil UPlanSApo objective.

mRNA and protein quantification

All quantitative smFISH image analyses were carried out using the image analysis software, FIJI. Maximum intensity projections of 10 x 0.2 μm individual z slices, representing 2 μm sections, of mushroom body calyx or $\gamma 2\alpha'1$ dendrites were analysed. Image brightness and contrast were manually adjusted such that the data were visible. Care was taken to adjust all images within each data set equally. For mRNA abundance quantification, single smFISH puncta within the region of interest (ROI) were counted using the FIJI TrackMate plugin. Spots were detected using a Differences of Gaussian (DoG) detector with an estimated particle diameter of 1 μm and manually adjusted threshold. For protein expression quantification, average pixel intensity values were calculated within a defined ROI. Each calyx and $\gamma 2\alpha'1$ dendritic region were sampled only once and each treated as a separate n.

Statistical analyses

All data were analysed using Matlab (version R2015a). An Anderson-Darling test was used to assess the distribution of the data. Normally distributed data were analysed using a one-way ANOVA. Non-normally distributed data were analysed using a Kruskal Wallis test. A post-hoc multiple comparisons test was used to assess differences between specific groups. Statistical significance is defined as $p < 0.05$. For each data set, the p-value, test statistic, and sample size are reported.

2.3 Application of the MS2 system to the *Drosophila* brain

2.3.1 Transgene synthesis

Molecular biology tools and reagents

Plasmids containing 24 tandem copies of the MS2 hairpin sequence (pCR 24xMS2) and 12 tandem copies of the PP7 hairpin sequence pCR (12xPP7) were obtained through personal correspondence from Jeffrey Chao (FMI, Basel). The 24x MS2 cassette and the 12x PP7 cassette were used in subsequent cloning stages to incorporate MS2 or PP7 loops into the cDNA of various genes in order to tag the mRNA transcript. Given the repetitive nature of the 24x MS2 cassette and the 12x PP7 cassette, all cloning steps requiring bacterial transformation were carried out using high efficiency NEB Stable Competent *E. coli* (NEB, catalogue no. C3040H) to prevent recombination of repetitive elements.

PCR amplification was carried out using an Agilent Technologies SureCycler 8800 thermocycler with Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB, catalogue no. M0531S) and manufacturer recommended thermocycling conditions. Gel extraction was carried out using the QIAquick Gel Extraction Kit (Qiagen, catalogue no.28704). All digestion reactions were carried out using restriction enzymes purchased from NEB. Ligation reactions were carried out using T4 DNA ligase (Roche, catalogue no. 10481220110). All oligonucleotide primers were custom synthesised by Sigma-Aldrich. All primer sequences described are listed in Table 5.1 of the Appendix.

pUAST attB *Act5C*-24xMS2

cDNA encoding *Drosophila melanogaster* Act5C isoform E was custom synthesised by Invitrogen GeneArt Gene Synthesis (ThermoFisher Scientific) and cloned into the pMA vector backbone. The cDNA sequence was modified to introduce KpnI and PspOMI restriction sites immediately after the 5'UTR and preceding the start codon, and SacI and NotI restriction sites immediately after the stop codon and preceding the 3'UTR. The introduction of such restriction sites were chosen to enable MS2 and/or PP7 cassettes, as well as SNAP and HALO tags, to be cloned into these sites to tag *Act5C* mRNA and/or

protein.

The synthesised Act5C containing plasmid (pMA Act5C) was linearised between the end of the stop codon and the beginning of the 3'UTR by SacI and NotI restriction. The 24xMS2 cassette was excised from pCR 24xMS2 by digestion with SacI and NotI and cloned into pMA Act5C, resulting in 24xMS2 3'UTR tagging of Act5C. The 3'UTR 24xMS2 tagged Act5C construct was excised from pMA Act5C via AvrII digestion. pUAST attB was linearised by XbaI digestion and treated with alkaline phosphatase (calf intestinal, CIP) to prevent re-circularisation of the plasmid. The 3'UTR 24xMS2 tagged Act5C construct was cloned into the pUAST attB plasmid downstream of the 5x upstream activation sequence (UAS promoter sequence) via compatible restriction overhangs. Correct insertion orientation and construct assembly were verified by sequencing using F pUAST attB and R pUAST attB primers.

pUAST attB *NMDAR1*-24xMS2

cDNA encoding *Drosophila melanogaster* NMDAR1 was obtained as a clone from DGRC (catalogue no. RE12105). The 3'UTR was amplified by PCR introducing a 5' BglII restriction site and a 3' NotI restriction site using the forward primer F NMDAR1 3'UTR and reverse primer R NMDAR1 3'UTR. The amplified 3'UTR was extracted from the PCR product by gel electrophoresis followed by gel purification. The 3'UTR fragment was digested with BglII and NotI. The 24xMS2 containing pCR 24xMS2 plasmid was linearised with the same restriction enzymes. The BglII/NotI digested 3'UTR was cloned into pCR 24xMS2, downstream of the 24xMS2 cassette. The 5'UTR and ORF were then amplified by PCR introducing 5' KpnI and PspOMI restriction sites and 3' HA tag and BamHI restriction site using the forward primer F NMDAR1 and the reverse primer R NMDAR1. The amplified 5'UTR and ORF fragment was extracted from the PCR product by gel electrophoresis followed by gel purification. The 5'UTR and ORF fragment was digested with KpnI and BamHI. The 24xMS2 containing pCR 24xMS2 plasmid was linearised with the same restriction enzymes. The KpnI/BamHI digested 5'UTR and ORF fragment was cloned into pCR 24xMS2, upstream of the 24xMS2 cassette.

The entire 3'UTR 24xMS2 tagged NMDARI construct was excised from the pCR 24xMS2 plasmid via digestion with PspOMI and XbaI restriction enzymes. The construct was then cloned into the multiple cloning site of the pUAST attB plasmid downstream of the 5x upstream activation sequence (UAS promoter sequence) via compatible restriction sites NotI and XbaI. Correct construct assembly was verified by sequencing using F pUAST attB and R pUAST attB primers.

pUAST attB *CaMKII*-24xMS2

cDNA encoding *Drosophila melanogaster* CAMKII isoform H was obtained as a clone from DGRC (catalogue no. FI03620). The 3'UTR was amplified by PCR introducing a 5' BglII restriction site and 3' XhoI and PstI restriction sites using the forward primer F CAMKII 3'UTR and reverse primer R CAMKII 3'UTR. The amplified 3'UTR was extracted from the PCR product by gel electrophoresis followed by gel purification. The 3'UTR fragment was digested with BglII and PstI. The 24xMS2 containing pCR 24xMS2 plasmid was linearised with the same restriction enzymes. The BglII/PstI digested 3'UTR was cloned into pCR 24xMS2, downstream of the 24xMS2 cassette. The 5'UTR and ORF were then amplified by PCR introducing 5' HindIII and PspOMI restriction sites and 3' HA tag and a SacI restriction site using the forward primer F CAMKII and reverse primer R CAMKII. The amplified 5'UTR and ORF fragment was extracted from the PCR product by gel electrophoresis followed by gel purification. The 5'UTR and ORF fragment was digested with HindIII and SacI. The 24xMS2 containing pCR 24xMS2 plasmid was linearised with the same restriction enzymes. The HindIII/SacI digested 5'UTR and ORF fragment was cloned into pCR 24xMS2, upstream of the 24xMS2 cassette. The entire 3'UTR 24xMS2 tagged CAMKII construct was excised from the pCR 24xMS2 plasmid via digestion with PspOMI and XhoI restriction enzymes. The construct was then cloned into the multiple cloning site of the pUAST attB plasmid downstream of the 5x upstream activation sequence (UAS promoter sequence) via compatible restriction sites NotI and XbaI. Correct construct assembly was verified by sequencing using F pUAST attB and R pUAST attB primers.

pUAST attB *DLG1*-24xMS2

cDNA encoding *Drosophila melanogaster* DLG1 isoform F cDNA was obtained as a clone from DGRC (catalogue no. GH01107). The 3'UTR was amplified by PCR introducing a 5' BglII restriction site and a 3' NotI restriction site using the forward primer F DLG1 3'UTR and the reverse primer R DLG1 3'UTR. The amplified 3'UTR was extracted from the PCR product by gel electrophoresis followed by gel purification. The 3'UTR fragment was digested with BglII and NotI. The 24xMS2 containing pCR 24xMS2 plasmid was linearised with the same restriction enzymes. The BglII/NotI digested 3'UTR was cloned into pCR 24xMS2, downstream of the 24xMS2 cassette. The 5'UTR and ORF were then amplified by PCR introducing 5' HindIII and PspOMI restriction sites and 3' HA tag and SacI restriction site using the forward primer F DLG1 and the reverse primer R DLG1. The amplified 5'UTR and ORF fragment was extracted from the PCR product by gel electrophoresis followed by gel purification. The 5'UTR and ORF fragment was digested with HindIII and SacI. The 24xMS2 containing pCR 24xMS2 plasmid was linearised with the same restriction enzymes. The HindIII/SacI digested 5'UTR and ORF fragment was cloned into pCR 24xMS2, upstream of the 24xMS2 cassette. The entire 3'UTR 24xMS2 tagged DLG1 construct was excised from the pCR 24xMS2 plasmid via digestion with PspOMI and XbaI restriction enzymes. The construct was then cloned into the multiple cloning site of the pUAST attB plasmid downstream of the 5x upstream activation sequence (UAS promoter sequence) via compatible restriction sites NotI and XbaI. Correct construct assembly was verified by sequencing using F pUAST attB and R pUAST attB primers.

pUAST attB *DopR1*-12xPP7

cDNA encoding *Drosophila melanogaster* DopR1 isoform D/E/F (identical isoform sequence identity according to NCBI) was custom synthesised by Invitrogen GeneArt Gene Synthesis (ThermoFisher Scientific) and cloned into the pMK-RQ vector backbone. The cDNA sequence was modified to introduce HindIII, KpnI and PspOMI restriction sites immediately after the 5'UTR and preceding the start codon, and SacI, NheI and NotI restriction sites immediately after the stop codon and preceding the 3'UTR. The intro-

duction of such restriction sites were chosen to enable MS2 and/or PP7 cassettes, as well as SNAP and HALO tags, to be cloned into these sites to tag mRNA and/or protein.

The synthesised DopR1 containing plasmid (pMK-RQ DopR1) was linearised between the end of the stop codon and the beginning of the 3'UTR by digestion with SacI and NotI restriction enzymes. The 12xPP7 cassette was excised from pCR 12xPP7 by digestion with SacI and NotI and cloned into pMK-RQ DopR1, resulting in 12xPP7 3'UTR tagging of DopR1. The 3'UTR 12xPP7 tagged DopR1 construct was excised from pMK-RQ DopR1 via AvrII digestion. pUAST attB was linearised by XbaI digestion and treated with alkaline phosphatase (calf intestinal, CIP) to prevent re-circularisation of the plasmid. The 3'UTR 24xMS2 tagged DopR1 construct was cloned into the pUAST attB plasmid downstream of the 5x upstream activation sequence (UAS promoter sequence) via compatible restriction overhangs. Correct insertion orientation and construct assembly were verified by sequencing using F pUAST attB and R pUAST attB primers.

pUAST attB 24xMS2-*DopR1*-12xPP7

pMK-RQ DopR1-12xPP7 was linearised between the end of the 5'UTR and the beginning of the start codon by digestion with restriction enzymes HindIII and PspOMI. The 24xMS2 cassette was excised from pCR 24xMS2 by digestion with the same restriction enzymes and cloned into pMK-RQ DopR1-12xPP7 to generate pMK-RQ 24xMS2-DopR1-12xPP7. The 5'UTR 24xMS2 and 3'UTR 12xPP7 tagged DopR1 construct was excised from pMK-RQ 24xMS2-DopR1-12xPP7 via digestion with the restriction enzyme AvrII. pUAST attB was linearised by XbaI digestion and treated with alkaline phosphatase (calf intestinal, CIP) to prevent re-circularisation of the plasmid. The construct was cloned into the pUAST attB plasmid downstream of the 5x upstream activation sequence (UAS promoter sequence) via compatible restriction overhangs. Correct insertion orientation and construct assembly were verified by sequencing using F pUAST attB and R pUAST attB primers.

pUAST attB SNAPf::*DopR1*-12xPP7

The SNAPf DNA sequence was amplified by PCR from the pENTR4 Snapf (w878-1) plasmid (Addgene catalogue no. 29652) using the forward primer F KpnI-SNAP to introduce a 5' KpnI restriction site and the reverse primer R PspOMI-SNAP to introduce a 3' PspOMI restriction site. The amplified SNAPf sequence was extracted from the PCR product by gel electrophoresis followed by gel purification. The sequence was digested with restriction enzymes KpnI and PspOMI. pMK-RQ DopR1-12xPP7 was linearised between the end of the 5'UTR and beginning of the start codon via digestion with restriction enzymes KpnI and PspOMI. The SNAPf sequence was then cloned into pMK-RQ DopR1-12xPP7 to generate the DopR1 SNAPf-tagged construct pMK-RQ SNAP::DopR1-12xPP7. The SNAP and 3'UTR 12xPP7 tagged DopR1 construct was excised from pMK-RQ SNAP::DopR1-12xPP7 via digestion with the restriction enzyme AvrII. pUAST attB was linearised by XbaI digestion and treated with alkaline phosphatase (calf intestinal, CIP) to prevent recircularisation of the plasmid. The construct was cloned into the pUAST attB plasmid downstream of the 5x upstream activation sequence (UAS promoter sequence) via compatible restriction overhangs. Correct insertion orientation and construct assembly were verified by sequencing using F pUAST attB and R pUAST attB primers.

pUAST attB MCP::SNAP

The MCP::SNAPf fusion sequence was derived from the plasmid MS2-SNAPf (Carrocci and Hoskins 2014, Addgene catalogue no. 65860). The MCP::SNAPf sequence was excised by digestion with restriction enzymes BamHI and KpnI. pUAST attB was linearised by digestion with BglII and KpnI restriction enzymes. The construct was cloned into the pUAST attB plasmid downstream of the 5x upstream activation sequence (UAS promoter sequence) via compatible restriction overhangs. Correct construct assembly were verified by sequencing using F pUAST attB and R pUAST attB primers.

pUAST attB 2xPCP::HALO

The PP7 coat protein sequence was derived from the plasmid pHsp83-NLS-HA-2xPCP-2xGFP (Halstead et al. 2015, Addgene catalogue no. 71243). The NLS-HA-2xPCP sequence was amplified by PCR using the pHsp83-NLS-HA-2xPCP-2xGFP plasmid as a template, forward primer F EcoRI-PCP to introduce a 5' EcoRI restriction site and reverse primer R PCP-NdeI-NotI to introduce 3' NdeI and NotI restriction sites. The amplified fragment, along with the pUAST attB vector, was digested with restriction enzymes EcoRI and NotI and the NLS-HA-2xPCP sequence was cloned into pUAST attB.

The HALO DNA sequence was amplified by PCR using the pENTR4-HaloTag (w876-1) plasmid (Addgene catalogue no. 29644) as a template, forward primer F NdeI-HALO to introduce a 5' NdeI restriction site and reverse primer R HALO-PspXI to introduce a 3' PspXI restriction site. The amplified HALO sequence, along with the pUAST attB 2xPCP vector, was digested with restriction enzymes NdeI and PspXI. The HALO sequence was cloned downstream and in frame of the NLS-HA-2xPCP sequence to generate pUAST attB 2xPCP::Halo. Correct construct assembly was verified by sequencing using F pUAST attB and R pUAST attB primers.

Transgenic flies

Transgenic flies harbouring the constructs described above were generated by BestGene Inc. using ϕ C31-directed targeted integration. All MS2/PP7 tagged constructs were integrated into the same third chromosome landing site (attP2, cytological location 25C6) to ensure comparable mRNA expression levels between lines. Coat protein (UAS-MCP::SNAP and UAS-PCP::HALO) constructs were targeted to two different second chromosome landing sites (VK00018, cytological location 53B2; VK00022, cytological location 57F5).

2.4 Testing MS2 constructs in *Drosophila* neuronal cell culture

2.4.1 Reagents and conditions

SNAP-tag and HALO-tag ligands

JF549-SNAPTag, JF646-SNAPTag, JF549-HaloTag, and JF646-HaloTag were obtained through personal communication with Luke Lavis (HHMI Janelia Farm) and described previously (Grimm et al., 2015). The primary advantage of the Janelia Fluor SNAP and HALO ligands is the improved quantum efficiency and photon yield of the synthetic chemical dye that facilitates higher resolution *in vivo* single molecule imaging. 1 mM stock concentrations were prepared in DMSO and stored at -20°C in dark conditions. Stock solutions were diluted as required.

Cell culture reagents and conditions

ML-DmBG3-c2 cells (DGRC, stock number 68) derived from the central nervous system of *Drosophila melanogaster* 3rd instar larvae (Ui et al. 1994) were used for all cell culture experiments owing to their neuronal-like morphology. Cells were maintained at 25°C in Schneiders medium supplemented with 10% fetal calf serum, 5 μ l/ml penicillin-streptomycin, and 20 μ g/ml insulin.

2.4.2 Transfection protocol

24h prior to transfection, cells were seeded either on 4-well Eppendorf Cell Imaging Slides (catalogue no. 0030742010) for fixed cell imaging or 35 mm Eppendorf glass bottomed Cell Imaging Dishes (catalogue no. 0030740009) for live cell imaging. Cells were transfected with 0.5 μ g each of the appropriate UAS-containing plasmids and 0.5 μ g of a CuSO₄-inducible Gal4-containing plasmid, pRmHa-3-Gal4 (Klueg et al. 2002) using Effectine (Qiagen) according to the manufacturers instructions. 24h post-transfection, Gal4 expression was induced with 1 mM CuSO₄. 24h post-induction, cultured cells were either fixed and stained for fixed cell imaging, or stained for live cell imaging (see below).

2.4.3 HALO-Tag and SNAP-Tag labelling for fixed cell imaging

24h following CuSO₄ induced Gal4 expression the growth medium was removed. Cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature. Fixed cells were then permeabilised with 3 x 10 minute washes in 0.1% Triton X-100 in PBS. SNAP and HALO protein tags were labelled using the appropriate JF549-SNAPTag, JF646-SNAPTag, JF549-HaloTag, or JF646-HaloTag ligand (10 nM) diluted in 0.1% Triton X-100 in PBS incubated for 10 minutes at room temperature. Cells were then washed with 3 x 10 minute washes in 0.1% Triton X-100 in PBS, covered with Vectashield anti-fade mounting medium, covered with a glass coverslip and sealed with nail varnish.

2.4.4 HALO-Tag and SNAP-Tag labelling for live cell imaging

24h following CuSO₄ induced Gal4 expression the growth medium was removed and replaced with fresh medium supplemented with the appropriate JF549-SNAPTag, JF646-SNAPTag, JF549-HaloTag, or JF646-HaloTag ligand (10 nM), incubated for 15 minutes at room temperature. Cells were then washed with fresh media three times and incubated for 30 minutes to allow excess ligand to exit the cells. The medium was then replaced twice more prior to imaging.

2.4.5 Image acquisition and data processing

Imaging

Fixed cells were imaged using a Leica SP5 confocal microscope with 63x 1.40 N.A. Oil HCPLApo objective. Live cultured cells were imaged using a Perkin Elmer UltraView Spinning Disk with 60x 1.35 N.A. Oil UPlanSApo objective.

Data processing

Acquired images were processed using the image analysis software, FIJI. Maximum intensity projections of z-stacks were generated for image visualisation.

2.5 Visualising MS2-tagged mRNA constructs live in the whole adult *Drosophila* brain

2.5.1 Gal4 driver lines and UAS effector lines

All lines used are described in Table 2.1. Gal4 lines used to drive expression of UAS effector transgenes in specific mushroom body output neurons (MBONs) were MB077c-Gal4 and R66C08-GAL4. MB077c-Gal4 labels the $\gamma 2\alpha'1$ MBONs (Aso et al., 2014). R66C08-Gal4 labels the M6/ $\gamma 5\beta'2a$ MBONs (Owald et al., 2015). The Gal4 line used to drive expression of UAS effector transgenes in PAM- $\gamma 5$ neurons was R48B04-Gal4 (Huetteroth et al., 2015).

2.5.2 *Ex plant* brain preparation

Brains of 2-4 day old adult *Drosophila* were dissected in carbogenated external saline (1.5 mM CaCl_2 , 10 mM glucose, 3 mM KCL, 4 mM MgCl_2 , 5 mM N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid, 103 mM NaCl, 1 mM NaH_2PO_4 , 26 mM NaHCO_3 , 7 mM sucrose, 10 mM trehalose) (Wang et al., 2004). pH was adjusted to 7.3 and osmolarity was adjusted to 275 mOsm. Aliquots were stored at -20°C . Prior to imaging, external saline aliquots were thawed and the solution was perfused with carbogen (95% O_2 , 5% CO_2) for 20 minutes. Samples were mounted bathed in carbogenated external saline on a glass slide and sealed with a glass coverslip. Two pieces of double-sided sticky tape flanking the samples were used to raise the coverslip enough to bridge the samples. Care was taken to limit light exposure of the samples in order to preserve fluorescence. Samples were imaged immediately.

2.5.3 Image acquisition and data analysis

Imaging

Live *ex plant* brains were imaged using a Perkin Elmer UltraView Spinning Disk with 60x 1.35 N.A. Oil UPlanSApo objective. GFP signal was imaged with a 488 nm laser at approximately 10% maximum laser power with 400 ms exposure. RFP signal was imaged with a 561 nm laser at approximately 10% maximum laser power with 1000 ms

exposure. Z-sections were collected with 0.2 μm spacing. Live images of single z-sections were collected at a rate of 1 frame s^{-1} . Video data can be viewed via the following URL: <https://www.dropbox.com/sh/psvl7vs1ur2835i/AABpAeyZlghey91aP9XgbD0Na?dl=0>. All videos are projected at 12 frames s^{-1} , scale bar= $2\mu\text{m}$.

Data processing

Z-stack and live images were processed using the image analysis software, FIJI. Z-stack images presented were constructed from a maximum projection of 10 z-sections representing 2 μm sections. Image brightness and contrast were manually adjusted such that the data were visible. Care was taken to adjust all images within each data set equally. For mRNA abundance quantification, spots within the ROI were counted using the FIJI TrackMate plugin. Spots were detected using a Differences of Gaussian (DoG) detector with an estimated particle diameter of 1 μm and manually adjusted threshold. Particle diameter and maximum brightness values were calculated using FIJI. A straight line was drawn through the particle of interest and a pixel intensity histogram along the line was plotted to determine the maximum pixel intensity value as well as the diameter of the spot.

Statistical analyses

All data were analysed using Matlab (version R2015a). An Anderson-Darling test was used to assess the distribution of the data. Normally distributed data were analysed using a one-way ANOVA. Non-normally distributed data were analysed using a Kruskal Wallis test. A post-hoc multiple comparisons test was used to assess differences between specific groups. Statistical significance is defined as $p < 0.05$. For each data set, the p-value, test statistic, and sample size are reported.

Single particle tracking

A custom written Matlab script was developed in collaboration with Carlas Smith (Waddell lab) in order to track the movements of mRNA particles recorded live in the *Drosophila* brain. The analysis presented was undertaken on video microscopy data of *Act5C-24xMS2*

mRNA in $\gamma 2\alpha'1$ dendrites recorded at 1 frame s^{-1} . A total of 11 independent replicate data sets were analysed. Single particles were tracked throughout the recording as described by Smith et al. (2015). Particles were detected using a generalised likelihood ratio test (GLTR) with a false discovery rate of 5%. Spots within 3 pixels (equivalent to the width of a single point spread function) between frames were joined to create a trajectory. Temporal jumps larger than 3 frames were not permitted, based on the mRNA mobility in the range of 1 $\mu m^2 s^{-1}$ for the diffusive fraction (Politz et al., 2006). From the reconstructed single particle tracks, several mobility parameters were calculated. The distance a given particle moved from its starting position (distance from start, DFS) was calculated as:

$$DFS = \frac{\Delta pixels}{frames}$$

Particle velocity was calculated as:

$$Velocity = \frac{pixels}{frames}$$

Mean squared displacement (MSD) was calculated as:

$$MSD(\tau) = \langle \Delta(\tau)^2 \rangle = \langle [r(t + \tau) - r(t)]^2 \rangle$$

where $r(t)$ is the position of the particle at time t , and τ is the lag time between the two positions taken by the particle used to calculate the displacement $\Delta r(\tau) [r(t + \tau) - r(t)]$. The average $\langle \dots \rangle$ indicates an ensemble average over several trajectories.

2.5.4 FRAP imaging conditions

FRAP experiments were performed using a Perkin Elmer UltraView Spinning Disk with 60x 1.35 N.A. Oil UPlanSApo objective. Single z-slices through $\gamma 2\alpha'1$ dendrites were imaged. GFP signal was imaged with a 488 nm laser at approximately 10% maximum laser power with 400 ms exposure. RFP signal was imaged with a 561 nm laser at approximately 10% maximum laser power with 1000 ms exposure. For FRAP experiments, ROI calibration was first performed on a fluorescent slide, and then in the $\gamma 2\alpha'1$ dendrites *in vivo*. A

12 x 7 μm ROI was drawn within the $\gamma 2\alpha'1$ dendrites containing MS2 mRNA signal. The sample was first imaged prior to photobleaching. The ROI was then photobleached using a 561 nm laser at 100% laser power with 100 ms exposure. Successive images were then captured immediately after photobleaching and then every minute for 10 subsequent time points.

2.6 Neprilysin 1 and Neprilysin 3 in *Drosophila* memory

2.6.1 CRISPR/Cas9 generation of mutant alleles

Nep1 and Nep3 mutant alleles were generated commercially via CRISPR/Cas9 targeted knock-out by GenetiVision Corporation (2016-). Guide RNAs (gRNAs) were designed to target the first coding exon in order to knock out the common start codon of all isoforms and presumably nearby regulatory sequences. gRNA sequences targeting the first coding exon of Nep1 and Nep3 can be found in Appendix Table 5.5. A schematic of the Nep1 and Nep3 loci can be found in Figure 2.1. A donor construct containing the selection marker 3xP3-eGFP and flanked by 1 kb homology arms was co-injected with the gRNA constructs into a nos-Cas9 line. F1 progeny were screened for eGFP expression in the eyes and eGFP positive individuals were crossed to an FM7a balancer line. Genomic PCR confirmed the correct insertion of the donor construct. As both Nep1 and Nep3 homozygous mutant females and hemizygous mutant males were viable and fertile, the FM7a balancer chromosome was gradually lost.

2.6.2 RT-qPCR

Total RNA was extracted from 4-6 day old whole adult flies by phenol-chloroform extraction using TriZOL reagent (ThermoFisher Scientific, catalogue no. 15596018). RNA was extracted from 4 biological replicates per sample (10 male and 10 female flies per replicate). RNA was reverse transcribed to produce complementary DNA (cDNA) using Superscript III First-Strand (Thermo Fisher Scientific, catalogue no. 18080400) using oligo(dT)20 primers. 5 μg total RNA per sample was used for cDNA synthesis. RT-qPCR was performed using a LightCycle 480 (Roche), using LightCycler 480 SYBR Green I Master and

gene specific primers (RT-qPCR primer sequences can be found in Appendix Table 5.6). Cycling conditions followed the manufacturers instructions. Technical replicates for each reaction were performed in triplicate. Efficiency of primer sets were assessed by 10 fold dilution series. Primer set specificity was assessed by melt curve analysis and the size of the amplification product was assessed by gel electrophoresis. Nep3 expression was quantified relative to three stably expressed reference genes: 1433 ϵ , Rpl32 and α -Tub48B. Nep1 expression was quantified relative to two stably expressed reference genes: Rpl32 and α -Tub48B, since 1433 ϵ expression was not stable between samples. RT-qPCR data were analysed using the qbase+ software, version 3.1 (Biogazelle, Zwijnaarde, Belgium - www.qbaseplus.com). Values of relative gene expression are calculated using an algorithm based on the $2^{-\Delta\Delta C_T}$ method (Hellemans et al., 2007). Relative expression of Nep1 and Nep3 were normalised to the WT (CS) group.

2.6.3 Aversive olfactory conditioning

Aversive olfactory conditioning was used to assess leaning and memory. The unconditioned stimulus, or aversive reinforcement, was 90 V electric shock. The conditioned stimuli used were the odours 3-octanol (OCT) and 4-methylcyclohexanol (MCH). Odours used for conditioning and for odour acuity tests were prepared at concentrations of 6-8 μ l OCT in 8 ml mineral oil, and 9-11 μ l MCH in 8 ml mineral oil. All aversive olfactory conditioning experiments were performed at 23°C and 70% relative humidity.

Aversive memory was assayed essentially as described by Tully and Quinn (1985). Mixed sex groups of ~ 100 flies were transferred into vials containing standard cornmeal-agar food and a 20 x 60 mm piece of filter paper 24h prior to the experiment. Animals were trained in a T-maze training arm in a copper wire-lined training tube. The following sequence of conditioned and unconditioned stimuli were presented: 1 min odour A (OCT or MCH) plus 12 x 90 V electric shocks with 5 s interstimulus intervals, 45 s odour-free air flow, 1 min odour B (OCT or MCH, the reciprocal of the first presented odour) without reinforcement.

To assay learning, flies were immediately transferred to the testing arms of the T-maze and given 2 min to choose between the two odours (OCT and MCH) encountered previously during training. To assay aversive medium-term memory (MTM), flies were transferred back into their cornmeal-agar food-containing vial for 2 hours at 23°C undisturbed. Flies were then transferred to the testing arms of the T-maze and given 2 min to choose between the two odours, OCT and MCH. Performance index (PI) was calculated as:

$$PI = \frac{(n(CS+) - n(CS-))}{(n(CS+) + n(CS-))}$$

where $n(CS+)$ is the number of flies in the arm of the T-maze containing the conditioned odour and $n(CS-)$ is the number of flies in the arm of the T-maze containing the unconditioned odour. A single performance index value is calculated from the average score from flies tested with the reciprocal reinforced/nonreinforced odour combination.

2.6.4 Shock acuity

Mixed sex groups of ~ 100 flies were transferred into vials containing standard cornmeal-agar food and a 20 x 60 mm piece of filter paper 24h prior to the experiment. Flies were transferred to a T-maze, one arm of which provided 24 90 V electric shocks with 5 s interstimulus intervals, while the other was free of electric shock. Flies were given 2 minutes in the dark to distribute themselves between the arms of the T-maze. Performance index was calculated as:

$$PI = \frac{(n(Shock+) - n(Shock-))}{(n(Shock+) + n(Shock-))}$$

where $n(Shock+)$ is the number of flies in the arm of the T-maze providing electric shock and $n(Shock-)$ is the number of flies in the arm of the T-maze without electric shock. A single performance index value is the average score from flies tested with the reciprocal shocked/non-shocked arm of the T-maze.

2.6.5 Odour acuity

Mixed sex groups of ~ 100 flies were transferred into vials containing standard cornmeal-agar food and a 20 x 60 mm piece of filter paper 24 h prior to the experiment. Flies were transferred to a T-maze, one arm of which contained the odour OCT or MCH while the other was free of odour. Flies were given 2 minutes in the dark to distribute themselves between the arms of the T-maze. Performance index was calculated as:

$$PI = \frac{(n(Odour+) - n(Odour-))}{(n(Odour+) + n(Odour-))}$$

where $n(Odour+)$ is the number of flies in the arm of the T-maze containing OCT/MCH and $n(Odour-)$ is the number of flies in the odour-free arm of the T-maze. A single performance index value is the average score from flies tested in reciprocal arms of the T-maze.

2.6.6 Statistical analyses

All data were analysed using Matlab (version R2015a). An Anderson-Darling test was used to assess the distribution of the data. Normally distributed data were analysed using a two-sample T-test. Non-normally distributed data were analysed using a Wilcoxon rank sum test. Statistical significance is defined as $p < 0.05$. For each data set, the p-value, test statistic, and sample size are reported.

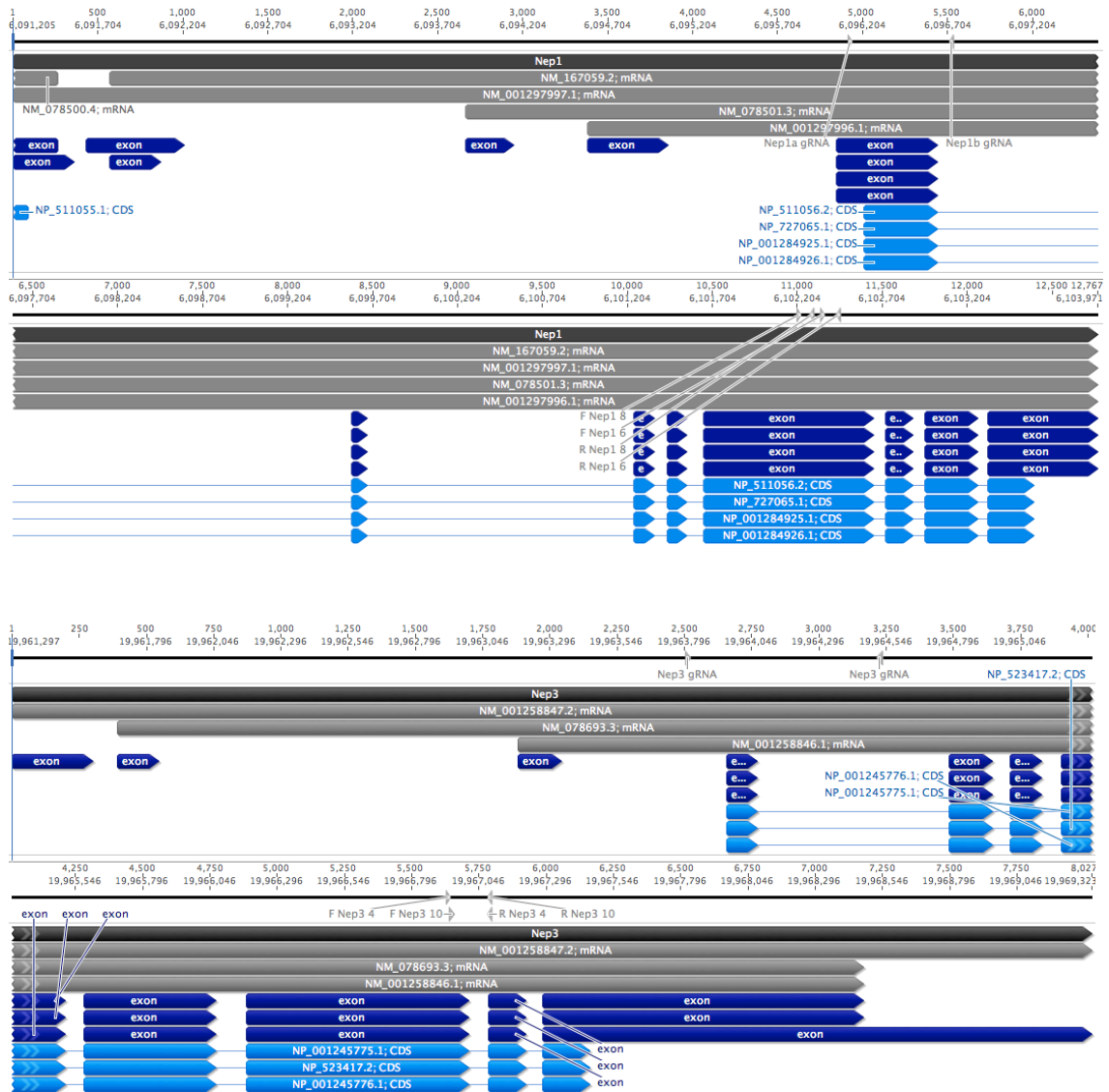


Figure 2.1: *Nep1* and *Nep3* loci targeted for CRISPR/Cas9 mutagenesis. Positions of nucleotide sequences complementary to gRNAs used for Cas9 targeting and primer sequences used subsequently in RT-qPCR experiments are indicated.

Chapter 3

mRNA visualisation with smFISH in the whole *Drosophila* brain

3.1 Background

The post-transcriptional regulation of gene expression in neurons via the subcellular localisation of mRNA transcripts serves both the targeting of proteins as well as the ability of synaptic compartments to independently remodel their proteome in an activity-dependent manner. The presence of localised mRNA transcripts (Burgin et al., 1990; Garner et al., 1988; Kleiman et al., 1990) and translational machinery (Bodian, 1964; Steward and Levy, 1982) suggested the prospect of decentralised regulation of gene expression in neurons over 30 years ago. Until recently however this area of neuroscience has remained relatively understudied. With the development of more sensitive mRNA detection techniques, such as single molecule fluorescence *in situ* hybridisation (smFISH), the localisation of mRNA transcripts can be quantitatively visualised at single-molecule resolution within the spatial context of a whole brain (Femino et al., 1998; Long et al., 2017; Yang et al., 2017). Such technical developments have facilitated a much more detailed insight into neuronal mRNA biology and are revolutionising how we think about molecular neuroscience (Holt and Schuman, 2013).

3.1.1 smFISH overview

smFISH involves the hybridisation of a set of up to 48 individually fluorescently pre-labelled oligonucleotide probes (approximately 20 nucleotides long) that tile a region of an mRNA transcript of interest (Raj et al., 2008) (see Figure 3.1 B for schematic illustration). The use of short oligonucleotide probe sequences improves probe penetration, while the use of a relatively large number of individually fluorescently pre-labelled probes amplifies signal such that individual mRNA transcripts are visualised as bright fluorescent puncta easily discernible among the background signal of any unbound probes (Femino et al., 1998; Feng et al., 2016; Raj et al., 2008). The benefits brought about by the development of smFISH make it almost perfectly suited to probing the spatial distribution and localisation of mRNA transcripts in a relatively small whole brain. To this end, smFISH has recently been applied to visualise mRNA in the whole adult and larval *Drosophila* brain (Long et al., 2017; Yang et al., 2017). One technical hurdle of smFISH however is the variability in efficiency between probe sets. Taking advantage of the *Drosophila* genetic toolkit, both Long et al. (2017) and Yang et al. (2017) used a single YFP smFISH probe set to label the mRNA of genes containing endogenous YFP insertions. In this way, one probe set can be used to visualise any YFP tagged gene and directly compare levels of expression and localisation patterns between genes.

3.1.2 Aims

The aim of this work was to apply smFISH to the whole adult *Drosophila* brain to visualise and quantify subcellularly localised mRNA of a subset of genes in identified, memory-relevant neurons. Using a YFP smFISH probe set, the endogenous expression and localisation of genes containing YFP insertions could be compared. Furthermore, the aim was to probe whether subcellularly localised mRNA within memory-relevant neurons responds dynamically to a learning assay. To this end, four endogenously tagged YFP genes were selected: calcium/calmodulin-dependent protein kinase II (*CaMKII*), the regulatory subunit of cAMP-dependent protein kinase 2 (*PKA-R2*), *Rab11* and *Tenascin major* (*Ten-m*). The functions of the selected genes along with known mRNA localisation patterns in the *Drosophila* brain are summarised in Table 3.1. Genes were selected based on

Table 3.1: Genes chosen for mRNA visualisation with smFISH.

Gene	Function	mRNA localisation
<i>CaMKII</i>	Neuronal activity regulated kinase (Malik and Hodge, 2014)	Pre- and postsynaptic (Ashraf et al., 2006)
<i>PKA-R2</i>	Regulatory subunit of the activity-regulated kinase PKA	Pre- and Postsynaptic (Crittenden et al., 1998)
<i>Rab11</i>	Monomeric small GTPase (Zerial and McBride, 2001)	Unknown
<i>Ten-m</i>	Synaptic-partner-matching molecule (Hong et al., 2012)	Unknown

the availability of YFP insertion lines and their relevance to neuronal physiology. *CaMKII* and *PKA-R2* were selected as important mediators of neuronal activity-regulated cAMP signalling, and their requirement for learning and memory (Giese and Mizuno, 2013; Malik and Hodge, 2014). *Rab11* and *Ten-m* were selected because they were identified in a screen for long-term memory mutants, indicating an important role in some aspect of memory formation (unpublished data: Yanying Wu, Waddell lab).

3.2 Results and discussion

3.2.1 Validating the detection of mRNA by smFISH

Using a YFP smFISH probe set, the detection of mRNA as fluorescent puncta in the mushroom body calyx (Figure 3.1 A and B) was compared between YFP-negative wild-type (CS) flies and flies containing a YFP insertion in the *Rab11* locus. Fluorescent background signal was seen in CS flies, representing unbound probes, but fluorescent puncta were almost never observed (Figure 3.1 C and C'). In contrast, distinct fluorescent puncta were visualised in *Rab11::YFP* flies (Figure 3.1 D and D'). These puncta are visualised as distinct peaks when the intensity values along a traversing line are plotted (Figure 3.1). smFISH can therefore be reliably used to detect mRNA in memory-relevant neuropils in the whole *Drosophila* brain.

Detecting single molecule mRNA

Single molecule mRNA transcripts can be detected using smFISH (Femino et al., 1998), however it is unclear whether the fluorescent smFISH spots detected here represent single molecule mRNA or multiple molecules of mRNA. In cultured cells, the number of photons emitted per probe can be calculated by measuring the light emitted by a known number of probes in a defined imaging volume (Femino et al., 1998). In this way, it is possible to calculate the number of fluorescent probes contributing to the fluorescence emitted from a single smFISH spot, and hence determine the number of mRNA molecules present. In the context of a whole brain however, the depth and complexity of the tissue reduces the number of photons from each probe reaching the objective and hence such a calibration would be unsuitable.

In theory, there are two ways in which mRNA transcript copy number could be calculated here: by size or brightness of the fluorescent spots. The diffraction limit of the dye's emission wavelength (Quasar-670) is 239.29 nm (calculated as $\lambda/2\text{N.A.}$, where λ =wavelength (670 nm), N.A.=numerical aperture (1.4) (Abbe, 1873)). This means that two fluorescent point sources separated by a lateral distance of < 239.29 nm cannot be resolved. Based on the full width half maximum calculation from pixel intensity histograms, the diameter of smFISH puncta were around 400 nm, i.e. they were not diffraction limited. Therefore individual spots may contain multiple mRNA transcripts. However the theoretical diffraction limit does not account for any aberrations introduced from imaging at depth within complex biological tissues, such as the *Drosophila* brain, and hence the real point spread function in this biological context may be greater. Another approach to determine mRNA copy number at fluorescent smFISH spots is based on fluorescence intensity. By plotting fluorescence intensities of a sample of particles, the presence of multiple distinct distributions could be used to infer multiple populations containing varying mRNA copy numbers. However it seems likely that variability in probe set binding efficiency for each mRNA transcript would mask multiple distributions of fluorescent intensity profiles. In

short, determining mRNA copy number from smFISH data collected within the whole *Drosophila* brain is not trivial.

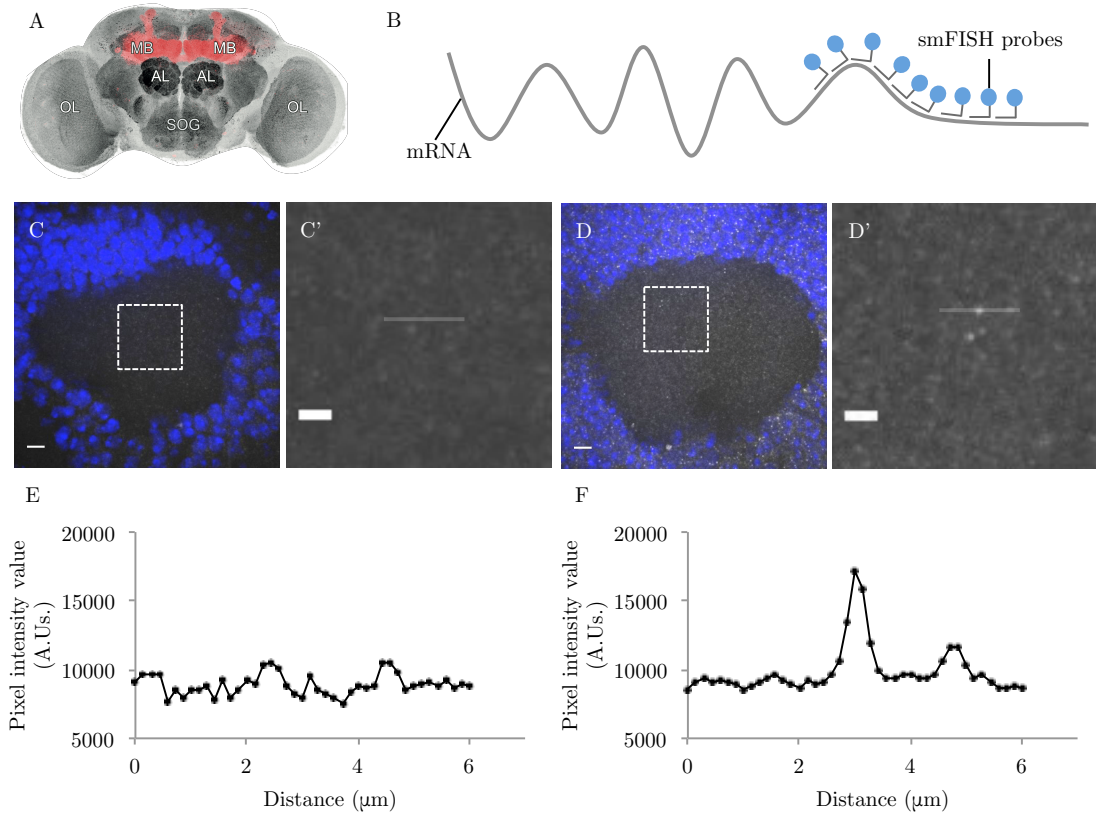


Figure 3.1: smFISH using YFP probes to visualise mRNA of endogenous YFP-tagged genes in the mushroom body calyx. **A**: Schematic illustration of the *Drosophila* brain with mushroom bodies (MB) highlighted in red. AL: antennal lobes. SOG: suboesophageal ganglion. OL: optic lobes. **B**: Schematic illustration of smFISH showing an mRNA transcript and fluorescently-labelled smFISH probes tiling a complementary sequence in the target mRNA transcript. **C-C'**: YFP smFISH signal in a wild-type (CS), YFP negative genotype. **D-D'**: YFP smFISH signal labelling *Rab11::YFP* mRNA. **C, D**: YFP smFISH signal in the mushroom body calyx is visualised in white. Cell bodies are stained with Vybrant DyeCycle Violet Stain and visualised in blue. Scale bar=5μm. **C', D'**: Magnification of white hatched box region in C and D, respectively. Scale bar=2μm. **E**: Pixel intensity values (arbitrary units) plotted along the transparent line in C'. **F**: Pixel intensity values (arbitrary units) plotted along the transparent line in D'.

3.2.2 Visualising endogenous mRNA localisation in the mushroom body calyx

After validating the sensitivity of smFISH to detect endogenously expressed mRNA in the mushroom body calyx, the localisation patterns of four YFP tagged genes (*CaMKII*, *PKA-R2*, *Rab11* and *Ten-m*) were compared (Figure 3.2). Quantitative analysis of mRNA abundance within the mushroom body calyx revealed significant differences in the localisation patterns of the four genes (Figure 3.2 B). Specifically, *CaMKII* and *PKA-R2* were the most abundant mRNA transcripts, while *Rab11* and *Ten-m* were much scarcer. The data suggest that the extent to which the mRNA of various genes are localised within the mushroom body calyx is highly variable, and likely reflects either the requirement of the encoded protein product in mushroom body dendritic compartments and/or the degree of post-transcriptional regulation of gene expression via localised translation.

CaMKII and PKA-R2 are known to be expressed in the mushroom body and are required as neuronal activity regulated kinases for the proper formation of memory (Crittenden et al., 1998; Gervasi et al., 2010; Malik and Hodge, 2014). The abundant localisation of *CaMKII* and *PKA-R2* mRNA transcripts in the mushroom body calyx suggests that a proportion of the regulation of *CaMKII* and *PKA-R2* gene expression occurs post-transcriptionally via the localisation of mRNA transcripts coupled with decentralised translation. The function of such post-transcriptional regulation of *CaMKII* and *PKA-R2* expression may either be related to the targeting of the encoded protein products to specific subcellular regions and/or to provide localised neuronal compartments with the capacity for neuronal activity regulated translation and hence localised proteome remodelling. In regards to the latter function, *CamKII* mRNA abundance has previously been shown to respond dynamically to neuronal activity (Ashraf et al., 2006).

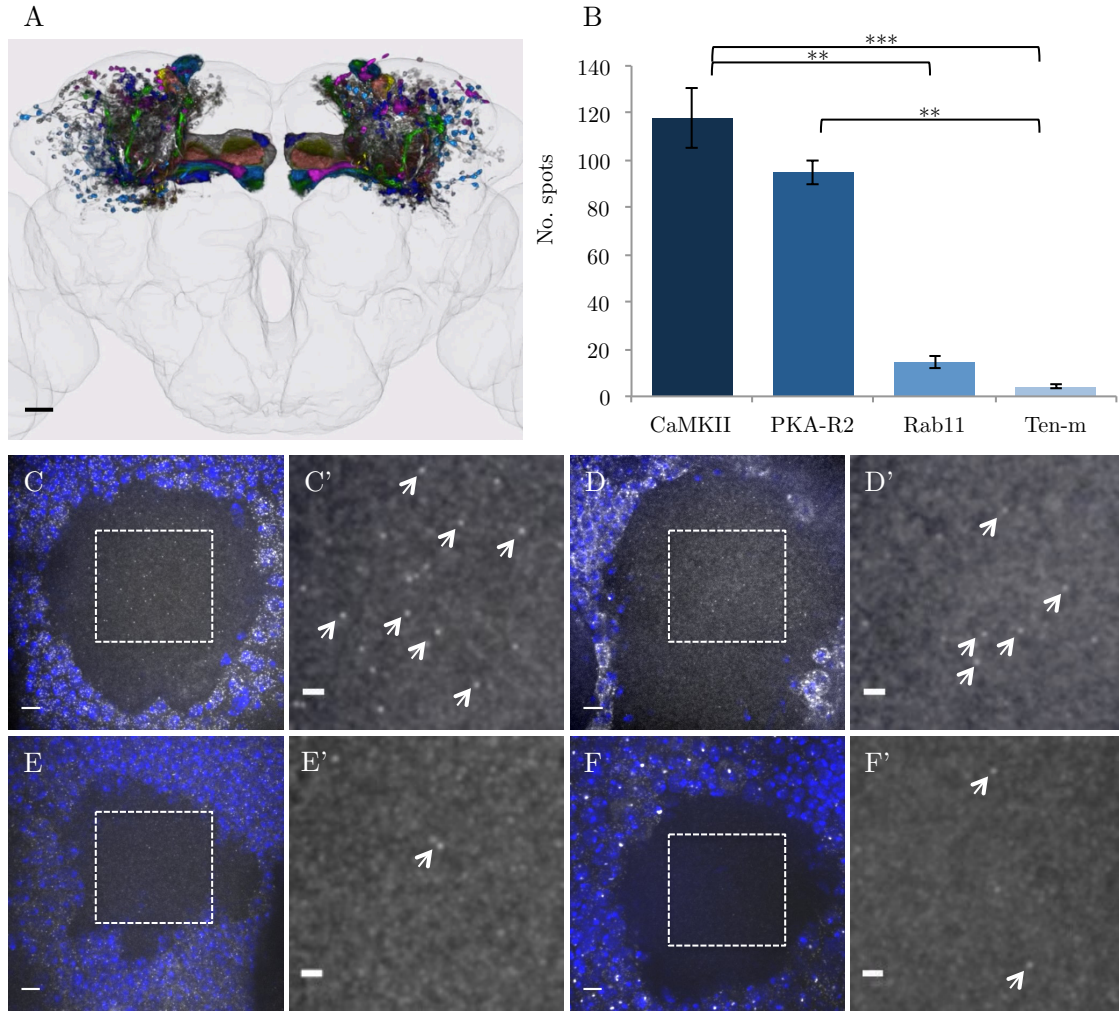


Figure 3.2: smFISH using YFP probes to visualise mRNA of endogenously expressed YFP-tagged genes in the mushroom body calyx. **A**: Schematic illustration of all mushroom body Kenyon cells visualised posteriorly indicating the positioning of the calyces. Figure adapted from Aso et al. (2014). Scale bar= $20\mu\text{m}$. **B**: Mean ($\pm s.e.m$) abundance of *CaMKII*, *PKA-R2*, *Rab11* and *Ten-m* mRNA in the mushroom body calyx. *CaMKII* mRNA abundance was significantly greater than *Rab11* (Kruskal Wallis test: $\chi^2=28.5219$, $p=0.0036$, $n=15$ (*CaMKII*), $n=10$ (*Rab11*)), and *Ten-m* (Kruskal Wallis test: $\chi^2=37.5219$, $p<0.0001$, $n=15$ (*CaMKII*), $n=10$ (*Ten-m*)). *PKA-R2* mRNA abundance was also significantly greater than *Ten-m* (Kruskal Wallis test: $\chi^2=38.6421$, $p=0.0029$, $n=5$ (*PKA-R2*), $n=10$ (*Ten-m*)). All other pairwise comparisons were not statistically different. **C-F'**: Example images of *CaMKII* (**C**), *PKA-R2* (**D**), *Rab11* (**E**), and *Ten-m* (**F**) smFISH signal in the mushroom body calyx. Cell bodies are labelled by Vybrant DyeCycle Violet Stain and visualised in blue. smFISH signal is visualised in white. **C**: *CaMKII::YFP*. **C'**: Magnification of **C**, hatched box region. **D**: *PKA-R2::YFP*. **D'**: Magnification of **D**, hatched box region. **E**: *Rab11::YFP*. **E'**: Magnification of **E**, hatched box region. **F**: *Ten-m::YFP*. **F'**: Magnification of **F**, hatched box region. **C-E**: Scale bar= $5\mu\text{m}$. **C'-E'**: Scale bar= $2\mu\text{m}$.

3.2.3 Visualising endogenous mRNA localisation at single neuron resolution

Imaging the abundance of mRNA transcripts in the mushroom body calyx enabled a quantitative comparison of extrasomatic mRNA localisation between the four genes. However the exact localisation of these transcripts, whether Kenyon cell postsynapses or presynaptic inputs to the calyx (e.g. projection neurons from the antennal lobe) is not known. To pursue single neuron mRNA localisation with smFISH, a membrane marker, myr::SNAPf, was used to label the mushroom body output neuron, $\gamma 2\alpha'1$ (Figure 3.3). This particular neuron was chosen because of its relevance in *Drosophila* memory processes (Felsenberg et al., 2017), as well as the positioning of its compact dendritic tuft fairly superficially in the brain which provided favourable molecular imaging conditions. smFISH signal of *CaMKII*, *PKA-R2*, *Rab11* and *Ten-m* were thus imaged in $\gamma 2\alpha'1$ dendrites. Co-localisation of dendrite signal and smFISH puncta was taken as evidence of localisation of the particular mRNA in $\gamma 2\alpha'1$ dendrites.

Accurate measurements of three-dimensional spatial relationships between molecules using multi-colour fluorescence imaging requires correction for chromatic aberration between channels (Matsuda et al., 2018). In order to address this, samples were co-stained with a dsDNA-binding dye (Vybrant DyeCycle Violet Stain) which has fluorescent excitation/emission maxima of 369/437 nm, respectively, and importantly a long tail of emission up to 600 nm (Smith et al., 2015). The dye was excited with 405 nm and emission was recorded using the appropriate filters for each imaging channel. In this way, each channel could be registered and possible chromatic aberrations corrected for. Based on a preliminary assessment of chromatic aberration, the error in alignment of channels using the Vybrant DyeCycle Violet Stain in x and y is approximately 200 nm $\pm$ 30 nm, while the error in z is approximately 300 nm $\pm$ 70 nm. Although the error in alignment between channels is small, a misalignment of several hundred nanometres could be the difference between the binary assignment of an mRNA molecule within or outside of an intricate neuronal process such as a dendrite. In the analysis presented here, image registration had not yet been performed, and therefore single neuron resolution assignment of mRNA

localisation cannot yet be concluded definitively. It will be important to correct for this error in channel alignment in the future progression of the work and this caveat must be borne in mind during the interpretation of the data.

smFISH spots co-localised within $\gamma 2\alpha'1$ dendrites were counted and compared between the four genes (Figure 3.3 B, C-F'). Statistically significant differences between the total number of mRNA transcripts of some genes were observed. *CamKII* mRNA transcripts were most abundant, more so than *Rab11* and *Ten-m*. While *Ten-m* mRNA transcripts were least abundant. In fact, *Ten-m* transcripts within $\gamma 2\alpha'1$ dendrites were never observed. There was no statistically significant difference between the number of mRNA transcripts of *CaMKII* and *PKA-R2*, or *Rab11* and *PKA-R2* or *Ten-m*. The relatively high abundance of *CaMKII* and *PKA-R2* in $\gamma 2\alpha'1$ dendrites would appear to be consistent with the involvement of their protein products in the transduction of signalling events at active synapses of memory-relevant neurons (Giese and Mizuno, 2013; Malik and Hodge, 2014). That the mRNA transcripts of *CaMKII* and *PKA-R2* are found within the dendritic compartment of the $\gamma 2\alpha'1$ MBONs located approximately 100 μm from the soma suggests that these molecules are transported there specifically for the purpose of localised translation. Conversely, the relatively low abundance of *Rab11* and absence of *Ten-m* would suggest that either the protein products of these genes are not present in $\gamma 2\alpha'1$ dendrites, or that they are present but that the regulation of gene expression does not occur via mRNA localisation and localised translation.

Long et al. (2017) similarly applied smFISH to visualise mRNA transcripts at single neuron resolution in the whole adult *Drosophila* brain. The authors quantified *timeless* mRNA abundance in the circadian pacemaker neurons, known as PDF neurons. Using smFISH they were able to visualise the cyclical daily turnover of *timeless* mRNA abundance in PDF neurons. The application of smFISH to detect mRNA at single neuron resolution in the spatial context of an entire *Drosophila* brain underscores the sensitivity and applicability of the technique to visualise molecular neurobiological processes.

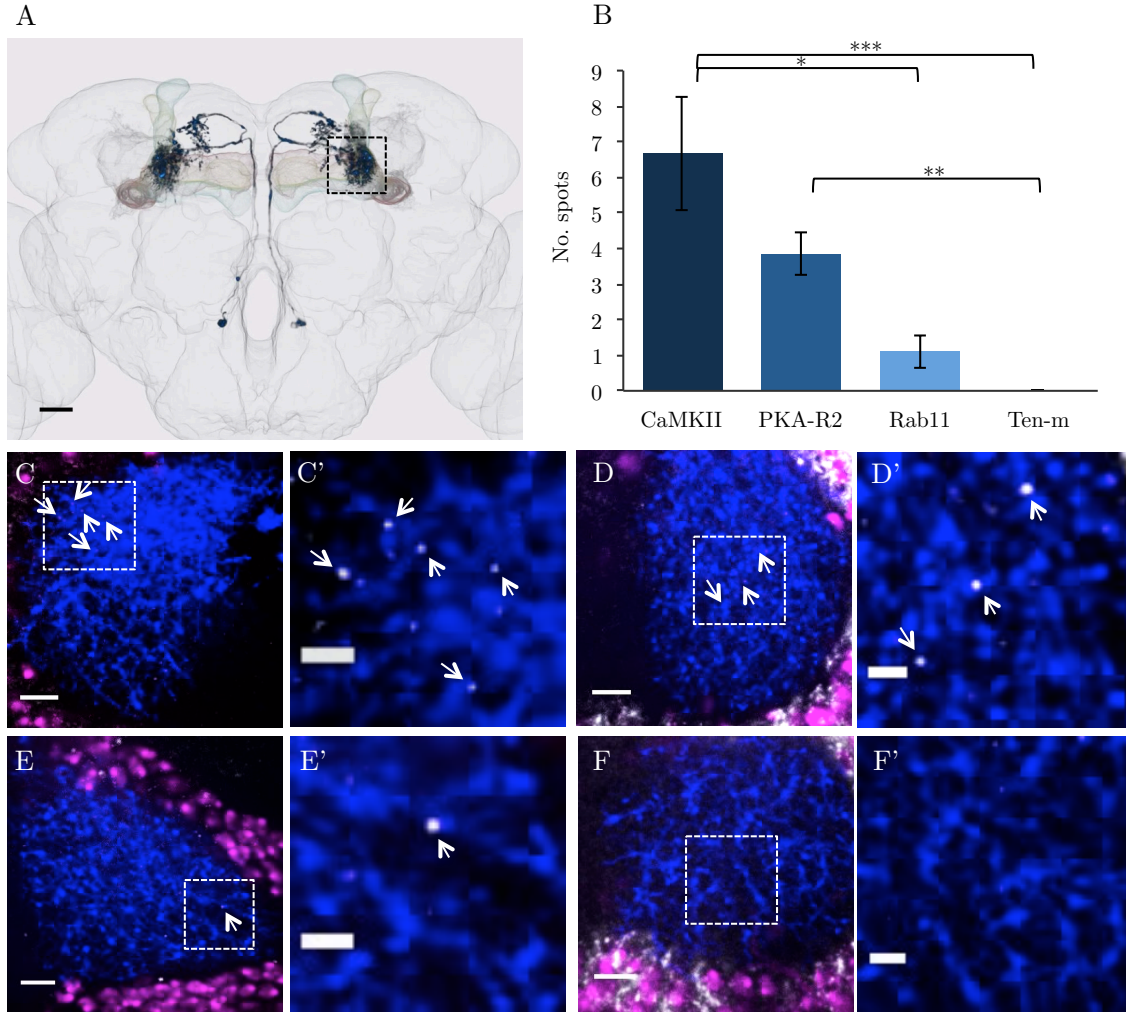


Figure 3.3: smFISH using YFP probes to visualise mRNA of endogenously expressed genes in the dendrites of $\gamma 2\alpha'1$ MBONs. **A**: Schematic illustration of $\gamma 2\alpha'1$ MBONs adapted from Aso et al. (2014). Hatched box indicates dendrites. Scale bar= $20\mu\text{m}$. **B**: Mean mRNA transcript abundance $\pm$ s.e.m. *CaMKII* mRNA abundance was significantly greater than both *Rab11* (Kruskal Wallis test: $\chi^2=24.8055$, $p=0.0231$, $n=10$ (*Rab11*), $n=6$ (*CaMKII*)) and *Ten-m* (Kruskal Wallis test: $\chi^2=30.8555$, $p=0.0002$, $n=10$ (*Ten-m*), $n=6$ (*CaMKII*)). *PKA-R2* mRNA abundance was significantly greater than *Ten-m* (Kruskal Wallis test: $\chi^2=27.9388$, $p=0.0024$, $n=10$ (*Ten-m*), $n=6$ (*PKA-R2*)). All other pairwise comparisons were not statistically different. **C-F'**: Example images of *CaMKII* (C), *PKA-R2* (D), *Rab11* (E), and *Ten-m* (F) smFISH signal in $\gamma 2\alpha'1$ dendrites. Cell bodies are labelled by Vybrant DyeCycle Violet Stain and visualised in magenta. smFISH signal is visualised in white. $\gamma 2\alpha'1$ dendrites are labelled with a JF549-SNAPTag stained myr::Snapf transgene and visualised in blue. **C**: *CaMKII::YFP*. **C'**: Magnification of C, hatched box region. **D**: *PKA-R2::YFP*. **D'**: Magnification of D, hatched box region. **E**: *Rab11::YFP*. **E'**: Magnification of E, hatched box region. **F**: *Ten-m::YFP*. **F'**: Magnification of F, hatched box region. **C-E**: Scale bar= $5\mu\text{m}$. **C'-E'**: Scale bar= $2\mu\text{m}$.

3.2.4 Using smFISH to quantify mRNA transcript abundance

In addition to characterising the presence of mRNA transcripts localised within distal neuronal compartments, a major aim of imaging molecules within neurons is to correlate the dynamics of their behaviour to neural functions, such as plasticity. In order to test the sensitivity of smFISH to detect changes in mRNA abundance in the whole *Drosophila* brain, the mRNA and protein expression of an induced UAS-YFP transgene was quantified over 4 successive days of induced expression. UAS-YFP expression was targeted to the mushroom body using the MB247-GeneSwitch Gal4 driver (Nicholson et al., 2008; Osterwalder et al., 2001). Expression was induced with dietary supplementation of the GeneSwitch Gal4 hormonal activator RU486, and expression of mRNA and protein in the mushroom body calyx was measured over 4 days of RU486 feeding (Figure 3.4). Flies feeding on RU486- food for the duration of the experiment expressed a basal level of YFP protein and very few *YFP* mRNA transcripts were detected in the calyx region (Figure 3.4 A-A"). With progressive number of days feeding on RU486+ food, the number of *YFP* mRNA spots (Figure 3.4 A'-E') and the mean YFP fluorescence (Figure 3.4 A"-E") in the mushroom body calyx increased. Reassuringly, the rate of mRNA increase preceded the rate of protein increase, with mRNA levels plateauing at day 3 and protein levels continuing to increase at day 4 (Figure 3.4 F and G). The sensitivity of smFISH to detect induced changes in gene expression within memory-relevant neurons in the whole *Drosophila* brain provided important validation for using smFISH to probe changes in mRNA abundance in response to induced neuronal activity.

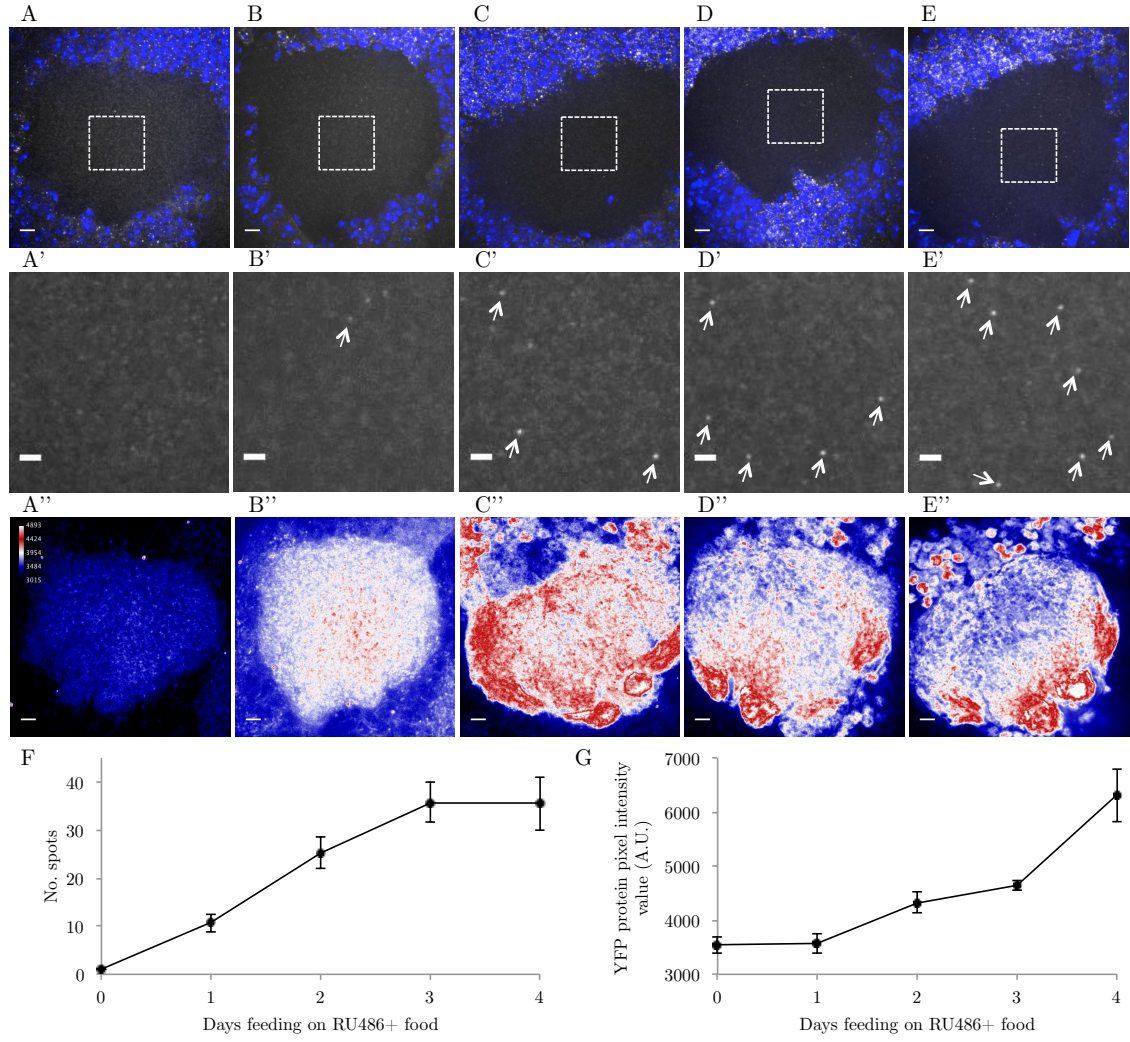


Figure 3.4: Quantification of mRNA and protein expression following induction of a YFP transgene in the mushroom body. Flies were fed RU486- control food (A) or RU486+ food for 1 day (B), 2 days (C), 3 days (D), or 4 days (E). **A-E**: Composite of YFP smFISH signal visualised in white and DAPI stained cell bodies visualised in blue in the mushroom body calyx. Scale bar=5 μ m. **A'-E'**: Magnification of hatched box region in A-E. Scale bar=2 μ m. **A''-E''**: YFP protein expression in the mushroom body calyx. Calibration scale bar indicates pixel intensity values (arbitrary units) and corresponding colours. Scale bar=5 μ m. **F**: YFP mRNA expression quantified as mean number of smFISH spots ($\pm$ s.e.m.) within the calyx region with days feeding on RU486+ food. **G**: YFP protein expression quantified by mean pixel intensity value (arbitrary units, $\pm$ s.e.m.) in the calyx region with days feeding on RU486+ food.

3.2.5 Quantifying *CaMKII* mRNA abundance in $\gamma 2\alpha'1$ dendrites and mushroom body calyx following aversive training

The previous data demonstrate the applicability of smFISH to visualising and quantifying mRNA transcript abundance at single neuron resolution as well as the sensitivity of the technique to detect induced changes in transcript abundance. With this, smFISH was used to probe whether changes in mRNA transcript abundance could be detected at single neuron resolution in response to physiological neuronal activity. Groups of flies were subject to an aversive olfactory conditioning paradigm and *CaMKII* mRNA was visualised in the dendrites of $\gamma 2\alpha'1$ MBONs as well as the mushroom body calyx at various subsequent time points. $\gamma 2\alpha'1$ dendrites overlap with the processes of a subset of aversively reinforcing dopaminergic neurons known as PPL1- $\gamma 2\alpha'1$ (Aso et al., 2014), whose artificial activation reinforces aversive olfactory memory (König et al., 2018; Aso and Rubin, 2016). The $\gamma 2\alpha'1$ dendrites were therefore chosen for imaging as a possible site of integration between Kenyon cell encoded olfactory information and aversively reinforcing dopamine input. *CaMKII* was chosen as the mRNA of interest to visualise for two main reasons. Firstly, of the four genes assessed, *CaMKII* was the most abundant mRNA transcript both in the mushroom body calyx (Figure 3.2) and in $\gamma 2\alpha'1$ dendrites (Figure 3.3). Secondly, *Drosophila CaMKII* mRNA abundance has previously been shown to respond dynamically to induced neuronal activity (Ashraf et al., 2006).

Using a YFP smFISH probe set, *CamKII::YFP* mRNA abundance was quantified in 4 groups of flies: a non-trained (NT) control group, and immediately, 2h or 24h after aversive olfactory conditioning. mRNA abundance was imaged and quantified in $\gamma 2\alpha'1$ dendrites (Figure 3.5 A) as well as the mushroom body calyx (Figure 3.5 B). *CaMKII* mRNA transcript abundance increased significantly in $\gamma 2\alpha'1$ dendrites at all time points following aversive olfactory conditioning, relative to the non-trained control group (Figure 3.5 C). There was also a trend towards time dependent reduction of *CaMKII* mRNA from the immediate timepoint onwards (Figure 3.5 C). In contrast, *CaMKII* mRNA transcript abundance did not change in the mushroom body calyx (Figure 3.5 D). Example images for all experimental conditions can be found in Appendix Figures 5.5 and 5.6. Whether

increased mRNA abundance in $\gamma 2\alpha'1$ dendrites is a result of increased trafficking of new molecules, or the release of transcripts previously contained within mRNPs remains unclear. In the case of β -*actin* mRNA, Buxbaum et al. (2014) and Park et al. (2014) suggest that dynamic responses to induced neuronal stimulation result from the transient dissociation of mRNA molecules from translationally-repressed mRNPs. Therefore there is evidence to suggest that dynamic mRNA responses to neuronal stimulation result from dynamic regulation of mRNP association, presumably for the purposes of regulating localised translation.

Ca^{2+} -mediated autophosphorylation and prolonged activation of CaMKII in response to synaptic activity is essential for synaptic plasticity and learning (Hell, 2014). Moreover, CaMKII protein concentration in dendritic spines is known to increase following LTP induction in mammalian neurons (Otmakhov et al., 2004; Shen and Meyer, 1999; Zhang et al., 2008). According to the data presented here, an increase in *CaMKII* abundance may be mediated at the molecular level by increased accumulation, and hence translation of, localised *CaMKII* mRNA transcripts. Why *CaMKII* mRNA abundance did not return to baseline levels and instead remained elevated in $\gamma 2\alpha'1$ dendrites after 24h is unclear. It is plausible that recurrent network activity persisting beyond training maintained activity in $\gamma 2\alpha'1$ dendrites (Cognigni et al., 2018). Alternatively, persistent increased *CaMKII* mRNA may prime the dendrites for future activity. The time point at which baseline levels would be resumed is also unknown. The specificity of the response to $\gamma 2\alpha'1$ dendrites and not to the mushroom body calyx suggests that the phenomenon is not a ubiquitous neuronal response. Even though the Kenyon cell dendrites would have been stimulated by olfactory input via projection neurons of the antennal lobe during the aversive olfactory conditioning protocol (Ito et al., 2008; Perez-Orive et al., 2002).

The data indicate that the abundance of *CaMKII* mRNA transcripts responds dynamically to activity in the dendrites of defined memory-relevant neurons. Dynamic changes in mRNA abundance have previously been reported in artificial stimulation experiments, for example involving bath neurotransmitter or KCL application (Ashraf et al., 2006; Park

et al., 2014), and chemical LTP induction protocols (Buxbaum et al., 2014). Therefore, dynamic responses to neuronal activity seems to be a common feature of localised mRNA in distal neuronal compartments. However a dynamic mRNA response to a behavioural manipulation to the whole animal has not yet been reported. Whether the response is unique to *CaMKII* mRNA and whether the increase is due to mRNP dissociation or transport of new molecules from elsewhere in the cell remains to be seen.

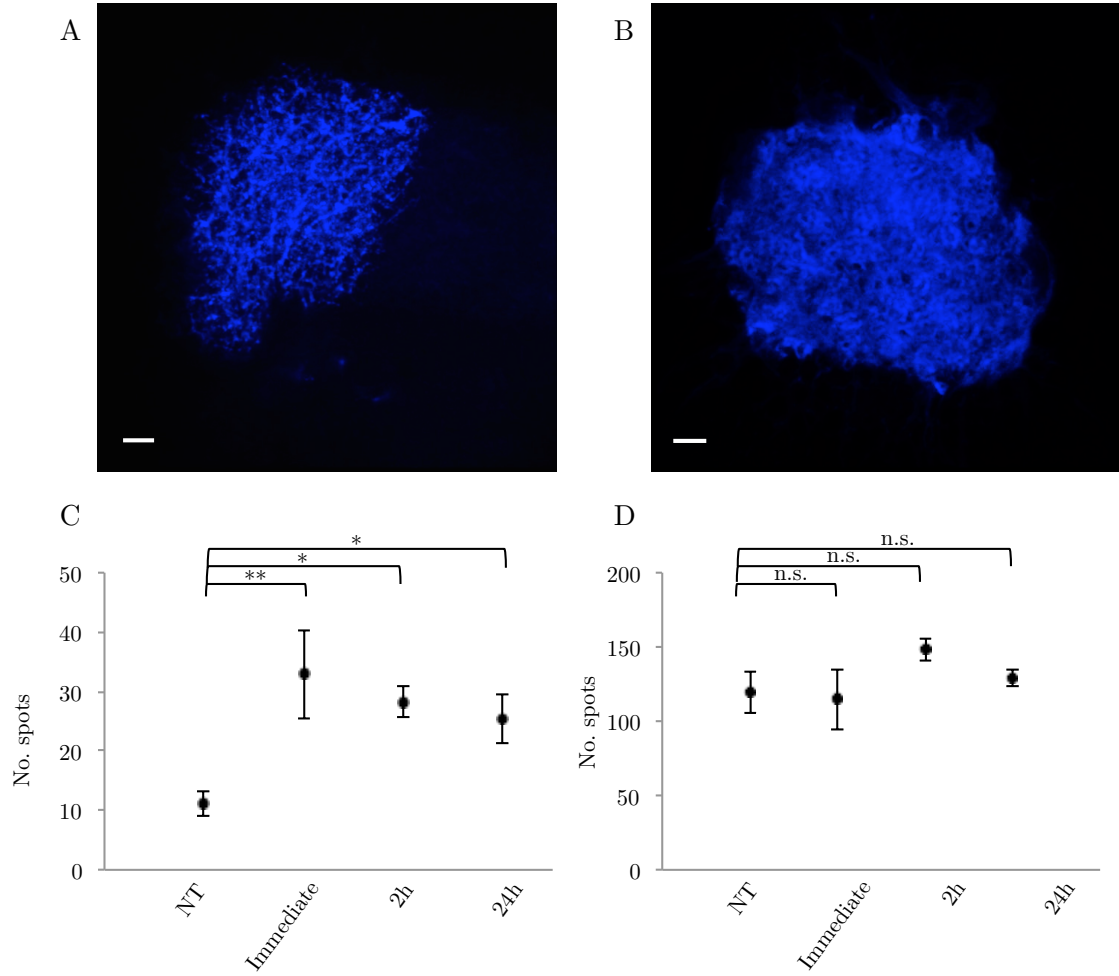


Figure 3.5: Quantification of *CaMKII* mRNA abundance in $\gamma 2\alpha'1$ dendrites and the mushroom body calyx following aversive learning. **A:** $\gamma 2\alpha'1$ dendrites labelled with a UAS-mCD8::SNAP transgene and visualised with JF549-SNAPTag. **B:** Mushroom body calyx labelled with a UAS-mCD8::GFP transgene and visualised with JF549-SNAPTag. Scale bar= $5\mu\text{m}$. **C:** Quantification of *CaMKII* mRNA smFISH spots in $\gamma 2\alpha'1$ dendrites in non-trained (NT) animals and immediately, 2h and 24h after aversive training. *CaMKII* mRNA increased immediately (ANOVA: $f=4.3759$, $p=0.0098$, $n=11$), 2h (ANOVA: $f=0.8791$, $p=0.0361$, $n=10$), and 24h (ANOVA: $f=1.1066$, $p=0.0756$, $n=17$) after aversive olfactory conditioning relative to the NT group ($n=11$). **D:** Quantification of *CaMKII* mRNA smFISH spots in the mushroom body calyx in non-trained (NT) animals and immediately, 2h and 24h after aversive training. *CaMKII* mRNA abundance was not statistically different immediately (ANOVA: $f=52.2229$, $p=0.9938$, $n=10$), 2h (ANOVA: $f=10.8315$, $p=0.2282$, $n=23$), or 24h (ANOVA: $f=32.4096$, $p=0.9356$, $n=18$) after aversive olfactory conditioning relative to the NT group ($n=11$).

3.3 Conclusion

The development and application of smFISH to the whole adult *Drosophila* brain enabled a quantitative assessment of mRNA localisation within identified memory-relevant neurons of interest. The use of a single YFP smFISH probe set enabled a quantitative comparison of the localisation patterns of specific genes of interest containing YFP insertions, in this case *CaMKII*, *PKA-R2*, *Rab11* and *Ten-m*. The extent of dendritic localisation of the four mRNA transcripts to the mushroom body calyx differed between transcripts, and was particularly high for *CaMKII* and *PKA-R2*. Within the whole brain, the use of co-labelled neural subsets meant that mRNA could be detected at single neuron resolution, in the dendrites of $\gamma 2\alpha'1$ MBONs. In the $\gamma 2\alpha'1$ dendrites, *CaMKII* mRNA abundance was highest and moreover responded dynamically to an aversive olfactory conditioning paradigm. In comparison, no such response was evident in the mushroom body calyx. It will be important for channel alignment errors introduced through chromatic aberration to be corrected for in the continuation of this work, for the definitive assignment of mRNA molecules to specific neuronal compartments. Nevertheless, these data substantiate the sensitivity of smFISH to quantitatively detect differences in mRNA localisation patterns of different genes in the whole *Drosophila* brain. Furthermore, the dynamic response of *CaMKII* mRNA to aversive olfactory conditioning in $\gamma 2\alpha'1$ dendrites provides the first evidence that changes in mRNA dynamics can be visualised following a behavioural manipulation of the whole animal.

3.4 Future directions

The localisation patterns of mRNA transcripts in the context of the whole *Drosophila* brain were assessed for just four genes. It will be interesting to know more about such patterns for other memory-relevant genes, especially given the availability of single cell transcriptomics data for the whole *Drosophila* brain (Croset et al., 2018) to inform candidate mRNA and neuron selection. An interesting candidate would be *Arc*, an mRNA that apparently mediates its own intercellular transfer (Ashley et al., 2018; Pastuzyn et al., 2018). At the level of single neuron resolution, the co-application of smFISH with advanced imaging

techniques such as expansion microscopy (Chen et al., 2016) would provide unparalleled spatial resolution within intricate neuronal compartments. For example, the distance of mRNA transcripts from synaptic sites or translational or regulatory machinery could be ascertained. In regards to the dynamic response of *CaMKII* to aversive olfactory conditioning, it will be interesting to know whether the response also occurs for other mRNA types and in other memory-relevant circuitries. In addition it will be interesting to see whether the response is abolished in the absence of *CaMKII* mRNA binding proteins, such as Orb2 (Stepien et al., 2016), and to assess the behavioural consequences of disrupting the accumulation of *CaMKII* mRNA in $\gamma 2\alpha'1$ dendrites.

Chapter 4

Visualising mRNA using the MS2 system

4.1 Background

High resolution single molecule mRNA imaging techniques such as smFISH have contributed enormously to the study of neuronal mRNA biology and the regulation of gene expression. The technique has revealed many aspects of the dynamic nature of mRNA biology, including responses of localised molecules to neuronal activity (Park et al., 2014; Long et al., 2017). However a major limitation of smFISH is that it requires fixed tissues. The visualisation of dynamic behaviours is therefore temporally limited to snapshots in time. Dynamic mRNA behaviour provides a rationale for seeking finer temporal resolution through live molecular imaging. The development and application of the MS2 system has served exactly this purpose.

4.1.1 The MS2 system

The development of techniques to label and hence visualise mRNA molecules live *in vivo* has opened the door to studying many aspects of mRNA biology in real time. The most significant of such techniques has been the MS2 system, and derivatives thereof. Such techniques have enlightened many aspects of mRNA biology, including: dynamics of transcriptional bursting (Berrocal et al., 2018; Bothma et al., 2014; Garcia et al., 2013; Lucas

et al., 2013), spatial patterning of mRNA localisation during development (Bertrand et al., 1998; Forrest and Gavis, 2003; Jaramillo et al., 2007; Weil et al., 2008; Zimyanin et al., 2008), mRNA localisation and motility in neuronal processes (Lionnet et al., 2011; Park et al., 2014; Yoon et al., 2016), and the visualisation of translation events (Halstead et al., 2015; Morisaki et al., 2016; Wang et al., 2016; Wu et al., 2016; Yan et al., 2016).

The technique involves the direct fluorescent tagging of mRNA molecules using the MS2 bacteriophage system, in which the MS2 coat protein binds a unique RNA hairpin structure. Expression of a fluorescently labelled MS2 coat protein along with a multimerised MS2 stem-loop array cloned into an mRNA of interest enables live mRNA imaging (Buxbaum et al., 2014). To increase the signal-to-background ratio of mRNA molecules within neurites, free fluorescently-labelled MS2 coat proteins are sequestered in the nucleus by the presence of a nuclear localisation signal. See Figure 4.1 A for a schematic overview. The application of the MS2 system to study neuronal mRNA localisation has mostly been limited to live imaging of cultured neurons (Das et al., 2018; Lionnet et al., 2011; Park et al., 2014) or cultured *ex vivo* brain slices (Park et al., 2014). To date, however, nobody has visualised mRNA in a whole live brain.

4.1.2 Aims

The aim of this project was to exploit the genetic tractability and relatively small brain of *Drosophila* to image a subset of relevant neuronal mRNAs live *in vivo*. To this end, a number of MS2-tagged transgenes were created. Transgenes placed under the regulation of the UAS promoter sequence could be expressed in a spatially restricted manner using Gal4 drivers to specifically express the components of the MS2 system in defined memory-relevant neural subsets in the *Drosophila* brain. The first aim was to characterise the expression of MS2-tagged transgenes in a number of defined memory-relevant neurons in order to compare neuron and mRNA specific localisation patterns. The second aim was to characterise dynamic mRNA behaviours in the whole live brain *in vivo*.

Four mRNAs were chosen to study: Actin 5C (*Act5C*), NMDA receptor 1 (*NMDAR1*),

calcium/calmodulin-dependent protein kinase II (*CaMKII*) and discs large 1 (*DLG1*). The selection of these candidate mRNA species was based on a number of criteria ascertained through literature research. These criteria included: 1) synaptically localised protein products, relevant for neuronal physiology and plasticity 2) extrasomatic mRNA localisation patterns in neurons, and 3) associations with RBPs with known function in neuronal physiology and plasticity. A summary of these criteria can be found in Table 4.1. All four mRNAs were identified as targets of the *Drosophila* RNA binding protein CPEB (cytoplasmic polyadenylation element binding) known as Orb2 (Stepien et al., 2016), which is required for long-term memory (Keleman et al., 2007). *DLG1* mRNA is regulated by the RNA binding protein Pumilio (Chen et al., 2008), which is also required for *Drosophila* long-term memory (Dubnau et al., 2003). *Act5C*, the *Drosophila* homolog of mouse β -*actin*, was primarily selected for experimental continuity with the extensive existing literature visualising β -*actin* mRNA using the MS2 system in mice (Lionnet et al., 2011; Park et al., 2014). NMDAR1, a *Drosophila* glutamate receptor, CaMKII, a neuronal activity regulated kinase (Malik and Hodge, 2014), and DLG1, a synaptic scaffolding protein (Mendoza-Topaz et al., 2008), all encode physiologically-relevant, synaptically-localised protein products.

4.2 Results and discussion

4.2.1 Applying the MS2 system to visualise mRNA in the *Drosophila* brain

An overview of the MS2 system is displayed schematically in Figure 4.1 A. *Act5C*, *CaMKII*, *NMDAR1* and *DLG1* were tagged with an array of 24xMS2 stem loops positioned between the the end of the open reading frame and the beginning of the 3'UTR. Positioning of the stem loop array between the stop codon and the beginning of the 3'UTR was chosen to minimise interference with both the coding sequence and any localisation sequences within the 3'UTR. MS2 tagged constructs were then cloned downstream of a UAS promoter sequence. The MS2 coat protein was fused to a SNAP tag and also placed downstream of

Table 4.1: mRNA species chosen for visualisation using the MS2 system

Gene	Protein function	Extra-somatic localisation of mRNA transcripts	Synaptic localisation of protein product	RBP association
<i>Act5C</i>	Structural constituent of the cytoskeleton	Mouse homolog of <i>Act5C</i> , β -actin (Buxbaum et al., 2014; Lionnet et al., 2011; Park et al., 2014)	Yes	Orb2 (Stepien et al., 2016)
<i>NMDAR1</i>	Glutamate receptor	Unknown	Yes	Orb2 (Stepien et al., 2016)
<i>CaMKII</i>	Neuronal activity regulated kinase (Malik and Hodge, 2014)	Yes (Burgin et al., 1990; Yang et al., 2017)	Yes	Orb2 (Stepien et al., 2016), FMRP (mouse homologs) (Darnell et al., 2011)
<i>DLG1</i>	Synaptic scaffolding protein (Mendoza-Topaz et al., 2008)	Unknown	Yes	Orb2 (Stepien et al., 2016), Pumilio (Chen et al., 2008), FMRP (mouse homologs) (Darnell et al., 2011)

a UAS promoter sequence. Details of cloning procedures can be found in Materials and Methods section 2.1.

To verify the functioning of the components of the MS2 system that were generated, a pilot experiment in which both components were expressed in cell culture was performed. For this, *CaMKII*-24xMS2 was selected as the test construct. UAS-*CaMKII*-24xMS2 and UAS-MCP::SNAP were co-transfected into cultured *Drosophila* neurons and visualised via labelling of the SNAP protein with JF646-SNAPTag. Fluorescent spots were observed within neural processes (Figure 4.1 C, C') when UAS-*CaMKII*-24xMS2 and UAS-MCP::SNAP were co-expressed, but not when UAS-MCP::SNAP was expressed alone (Figure 4.1 B). Cultured cells transfected with UAS-*CaMKII*-24xMS2 and UAS-MCP::SNAP were also visualised live, revealing these fluorescent puncta to be mobile (See Video 1 using the URL: <https://www.dropbox.com/sh/psv17vs1ur2835i/AABpAeyZlghey91aP9XgbD0Na?dl=0>).

The selective presence of mobile SNAPTag-labelled puncta within neurites when both UAS-*CaMKII*-24xMS2 and UAS-MCP::SNAP were co-expressed was taken as evidence that these puncta were molecules of *CaMKII* mRNA. The results of this pilot experiment were used as verification that the transgenic constructs generated were functional, and could be used for embryonic injection to generate transgenic flies. The visualisation of MS2-tagged mRNA in *Drosophila* cell culture was not pursued further since the use of the MS2 system in cell culture has already been explored (Das et al., 2018; Lionnet et al., 2011) and the aim of the project was to visualise mRNA live in the *Drosophila* brain.

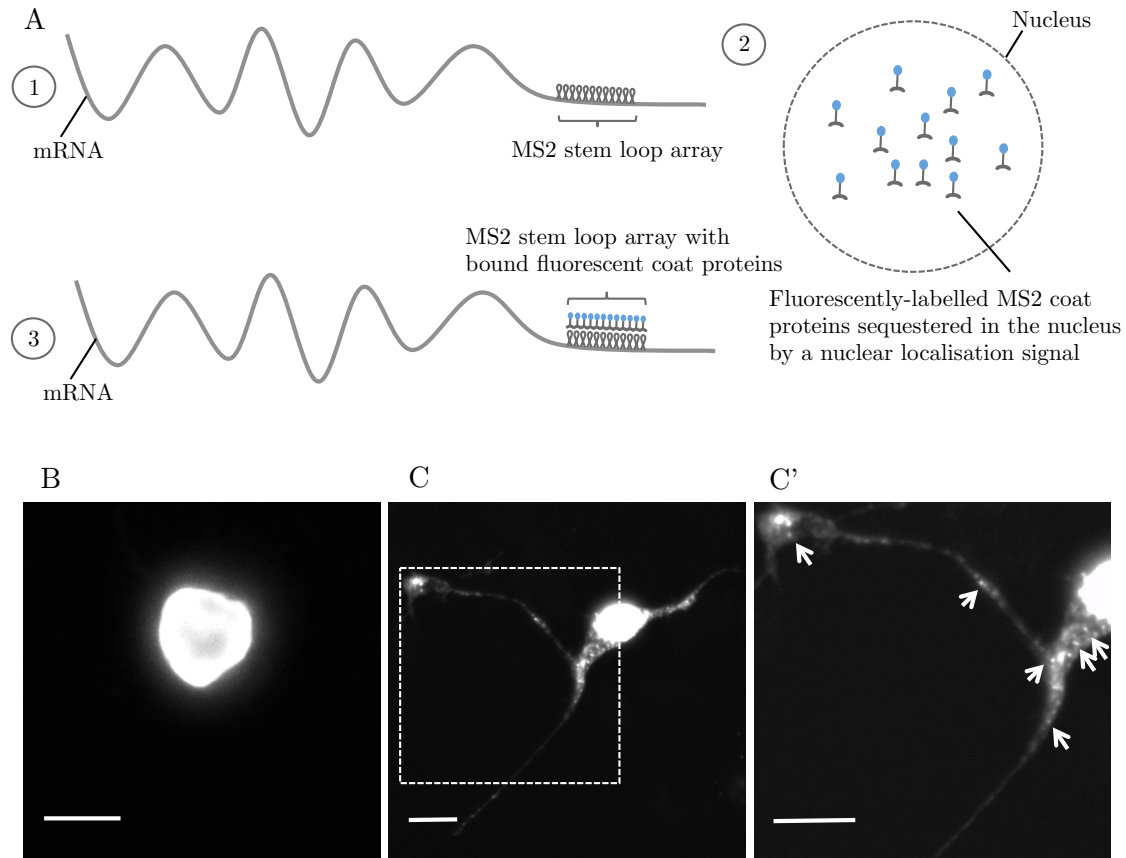


Figure 4.1: The MS2 system. **A**: Schematic representation of components of the MS2 system. 1) mRNA transcript labelled with an MS2 stem loop array. 2) Fluorescently-labelled MS2 coat proteins sequestered in the nucleus by the presence of a nuclear localisation signal. 3) mRNA transcripts exiting the nucleus are labelled via binding of fluorescently-labelled MS2 coat proteins to the MS2 stem loop array and hence visualised. **B-C'**: Expression of UAS-MCP::SNAP and UAS-*CaMKII-24xMS2* in *Drosophila* neuronal cell culture. **B**: Expression of UAS-MCP::SNAP alone. **C**, **C'**: Co-expression of UAS-MCP::SNAP plus UAS-*CaMKII-24xMS2*. **C'**: Magnification of C, hatched box region. White arrows indicate fluorescent *CaMKII-24xMS2* mRNA. Scale bar=5 μ m.

4.2.2 MS2 tagged mRNA transcripts visualised in $\gamma 2\alpha'1$ MBONs

UAS-*Act5C*-24xMS2, UAS-*NMDAR1*-24xMS2, UAS-*CaMKII*-24xMS2, UAS-*DLG1*-24xMS2 and UAS-MCP::SNAP constructs were site-specifically integrated into the *Drosophila* genome to generate transgenic fly lines harbouring the respective transgenes. In order to test whether labelled mRNA puncta could be observed by expressing both components of the MS2 system in the *Drosophila* brain, a Gal4 driver line (MB077c-Gal4) labelling the $\gamma 2\alpha'1$ mushroom body output neurons (MBONs) (Aso et al., 2014) was selected. There were several reasons to select this line. Firstly, the $\gamma 2\alpha'1$ dendrites are physically located distally from the cell bodies (approximately 100 μm , see Figure 4.2 A) meaning that fluorescently-labelled coat protein harboured in the nucleus would not interfere with imaging of discrete puncta in distal portions of the neuron, for example the dendrites. Secondly, the $\gamma 2\alpha'1$ dendrites form a compact tuft located fairly superficially on the anterior of the *Drosophila* brain, providing favourable molecular imaging conditions (see Figure 4.2 B). Thirdly, $\gamma 2\alpha'1$ MBONs are involved in the re-consolidation of learned information in *Drosophila* (Felsenberg et al., 2017) and therefore are physiologically relevant in terms of memory coding and plasticity.

Expression of the UAS-MCP::SNAP construct occurred in the absence of any Gal4 driver (data not shown), i.e. the expression of the transgene was leaky. For this reason, the UAS-MCP::SNAP coat protein was not used and instead a UAS-MCP::RFP coat protein (Brechtel and Gavis, 2008) was used to visualise MS2-tagged mRNA in the adult *Drosophila* brain. The MB077c-Gal4 driver was combined with the neuronal marker UAS-10xIVS mCD8::GFP , and crossed with either the UAS-MCP::RFP coat protein alone or the UAS-MCP::RFP coat protein combined with an MS2-tagged transgene. In each case, $\gamma 2\alpha'1$ dendrites were imaged. Expression of the UAS-MCP::RFP coat protein alone in $\gamma 2\alpha'1$ MBONs revealed only a few fluorescent spots within the dendritic field (Figures 4.1 C, 4.3 A-A"). Similar observations have been previously reported when the UAS-MCP::RFP coat protein was expressed in larval dendritic arborization neurons (Brechtel and Gavis, 2008). Larger fluorescent aggregates around the dendritic field were also observed, however their exact source is not known. The MB077c-Gal4 driver line does not

exclusively label $\gamma 2\alpha'1$ MBONs but also a number of other neurons, including α/β Kenyon cells (personal observations), suggesting that the extraneous RFP fluorescent signal may arise from non- $\gamma 2\alpha'1$ neuronal expression.

Expression of the UAS-MCP::RFP coat protein in $\gamma 2\alpha'1$ MBONs alone provided a negative control against which the introduction of MS2-tagged mRNA could be compared. Discrete fluorescent puncta were observed in $\gamma 2\alpha'1$ dendrites when *Act5C*-24xMS2 (Figure 4.3 B-B"), *NMDAR1*-24xMS2 (Figure 4.3 C-C"), *CaMKII*-24xMS2 (Figure 4.4 A-A") and *DLG1*-24xMS2 (Figure 4.4 B-B") were co-expressed with the MCP::RFP coat protein. Quantification of fluorescent puncta within $\gamma 2\alpha'1$ dendrites showed minimal counts in the absence of MS2-containing mRNA (Figure 4.2 C). Significantly greater numbers of fluorescent puncta were observed with the co-expression of *Act5C*-24xMS2, *NMDAR1*-24xMS2 or *CaMKII*-24xMS2 (Figure 4.2 C). Between the MS2-tagged mRNA types, *NMDAR1*-24xMS2, *CaMKII*-MS2 and *DLG1*-24xMS2 expressed equivalent numbers of fluorescent puncta within $\gamma 2\alpha'1$ dendrites, whereas *Act5C*-24xMS2 expression was highest (Figure 4.2 C).

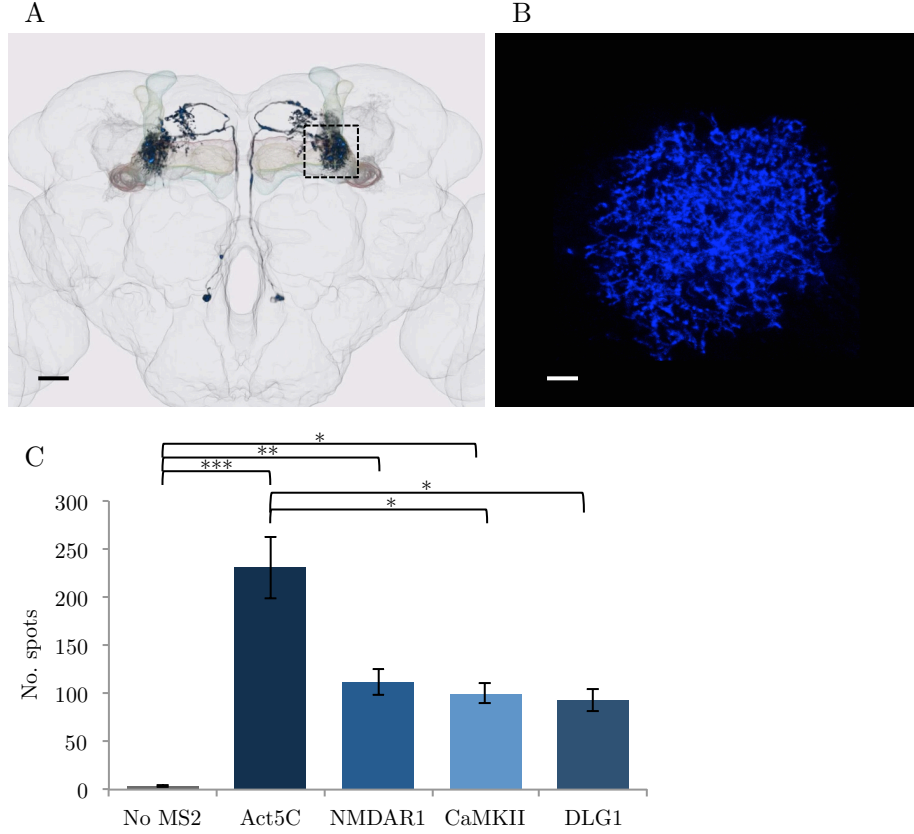


Figure 4.2: Visualising MS2 labelled mRNA in $\gamma 2\alpha'1$ MBONs. **A**: Schematic representation of $\gamma 2\alpha'1$ MBONs. Box indicates dendritic region. Figure adapted from Aso et al. (2014). Scale bar= $20\mu\text{m}$. **B**: Dendrites of $\gamma 2\alpha'1$ MBON visualised by a UAS-mCD8::GFP transgene driven by the MB077c-Gal4 driver. Scale bar= $5\mu\text{m}$. **C**: Mean ($\pm s.e.m$) number of *Act5C*-, *NMDAR1*-, *CaMKII*-, and *DLG1*-24xMS2 tagged mRNA puncta in $2\mu\text{m}$ sections of $\gamma 2\alpha'1$ dendrites. The number of *Act5C*- (Kruskal Wallis test: $\chi^2=63.6949$, $p<0.0001$, $n=10$ (*Act5C*-24xMS2), $n=7$ (No MS2)), *NMDAR1*- (Kruskal Wallis test: $\chi^2=44.6736$, $p=0.0046$, $n=15$ (*NMDAR1*-24xMS2), $n=7$ (No MS2)), and *CaMKII*-24xMS2 (Kruskal Wallis test: $\chi^2=42.3460$, $p=0.0217$, $n=13$ (*CaMKII*-24xMS2), $n=7$ (No MS2)) mRNA puncta was significantly greater than when the MS2 coat protein was expressed alone. The number of *Act5C*-24xMS2 mRNA puncta was significantly greater than *CaMKII*- (Kruskal Wallis test: $\chi^2=2.2715$, $p=0.0182$, $n=13$ (*CaMKII*-24xMS2), $n=10$ (*Act5C*-24xMS2)) and *DLG1*-24xMS2 (Kruskal Wallis test: $\chi^2=2.9466$, $p=0.0148$, $n=9$ (*DLG1*-24xMS2), $n=10$ (*Act5C*-24xMS2)).

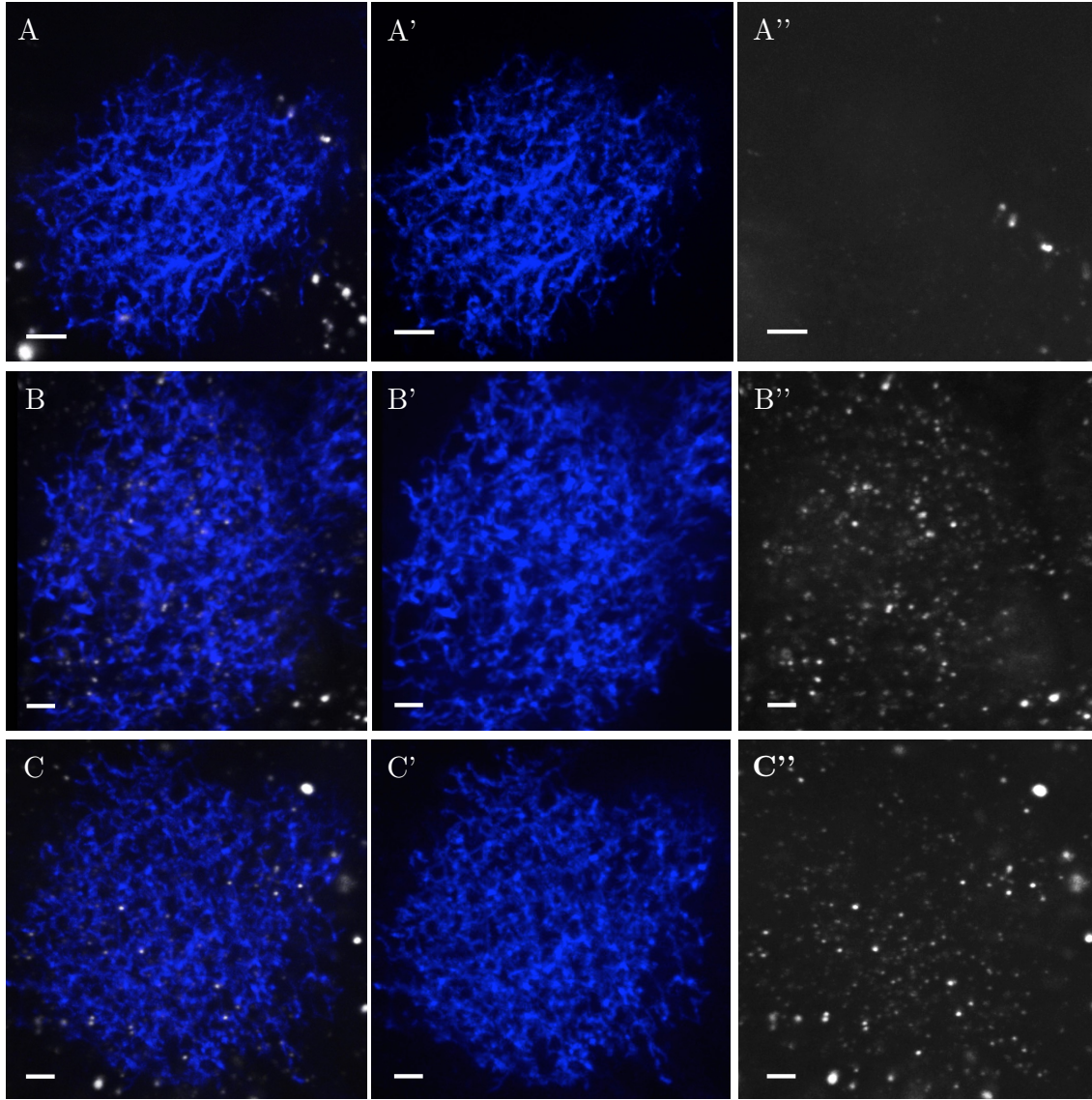


Figure 4.3: MS2 labelled mRNA expressed in $\gamma 2\alpha'1$ MBONs. **A**: Dendrites of $\gamma 2\alpha'1$ MBON expressing the MS2 coat protein MCP::RFP alone, i.e. in the absence of any MS2 containing mRNA. Composite of A' and A''. **A'**: $\gamma 2\alpha'1$ dendrites visualised by 10x-IVS-myr::GFP. **A''**: MCP::RFP. **B**: Dendrites of $\gamma 2\alpha'1$ MBON expressing the MS2 coat protein MCP::RFP along with *Act5C*-24xMS2. Composite of B' and B''. **B'**: $\gamma 2\alpha'1$ dendrites visualised by 10x-IVS-myr::GFP. **B''**: MCP::RFP labelled *Act5C*-24xMS2. **C**: Dendrites of $\gamma 2\alpha'1$ MBON expressing the MS2 coat protein MCP::RFP along with *NMDAR1*-24xMS2. Composite of C' and C''. **C'**: $\gamma 2\alpha'1$ dendrites visualised by 10x-IVS-myr::GFP. **C''**: MCP::RFP labelled *NMDAR1*-24xMS2. Scale bar=5 μ m.

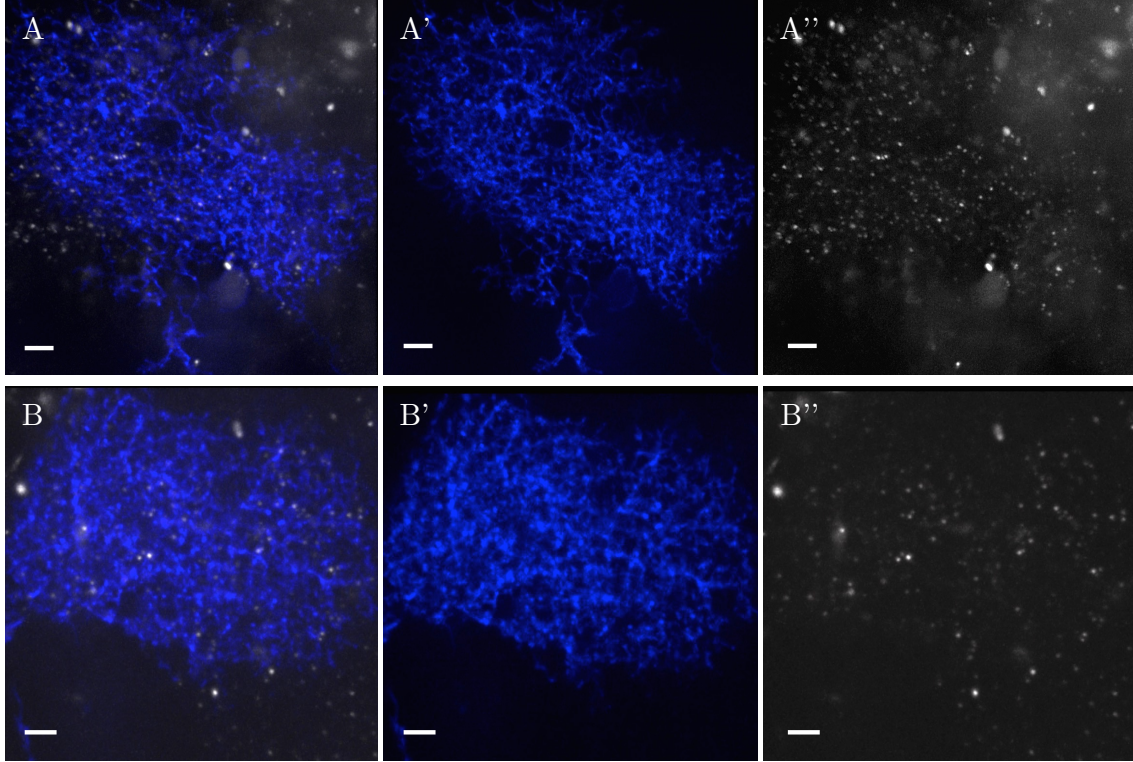


Figure 4.4: MS2 labelled mRNA expressed in $\gamma 2\alpha'1$ MBONs. **A**: Dendrites of $\gamma 2\alpha'1$ MBON expressing the MS2 coat protein MCP::RFP along with *CaMKII*-24xMS2. Composite of A' and A''. **A'**: $\gamma 2\alpha'1$ dendrites visualised by 10x-IVS-myr::GFP. **A''**: MCP::RFP labelled *CaMKII*-24xMS2. **B**: Dendrites of $\gamma 2\alpha'1$ MBON expressing the MS2 coat protein MCP::RFP along with *DLG1*-24xMS2. Composite of B' and B''. **B'**: $\gamma 2\alpha'1$ dendrites visualised by 10x-IVS-myr::GFP. **B''**: MCP::RFP labelled *DLG1*-24xMS2. Scale bar= $5\mu\text{m}$.

4.2.3 Validation of fluorescent MCP::RFP puncta as mRNA

The presence of discrete fluorescent puncta in the dendrites of $\gamma 2\alpha'1$ when MS2-labelled transgenes are co-expressed with an RFP labelled MS2 coat protein could theoretically arise from one of two phenomena. Firstly, from aggregates of the fluorescently-labelled coat protein alone. Secondly, from binding of the fluorescently-labelled coat protein to the MS2 hairpin structures in the MS2-labelled mRNA. The almost complete absence of fluorescent puncta when the RFP tagged coat protein was expressed in the absence of any MS2-containing mRNA supports the second argument (Figure 4.2 C, Figure 4.3 A). However, in order to further validate the presence of fluorescent puncta as molecules of mRNA, smFISH using a probe set targeting the MS2 stem loop array was performed. When *Act5C*-24xMS2 was expressed in $\gamma 2\alpha'1$ dendrites, MS2 smFISH puncta were de-

tectable (Figure 4.5). This result provides further evidence that the fluorescent puncta observed with expression of MS2-containing mRNA are indeed molecules of mRNA. Efforts to combine smFISH labelling of the MS2 stem loop array with concomitant smFISH labelling of another region of the mRNA transcript were hampered by probe set efficiency and fluorophore incompatibility. However, both sets of control experiments performed support the theory that observed fluorescent puncta when both components of the MS2 system are co-expressed represent mRNA.

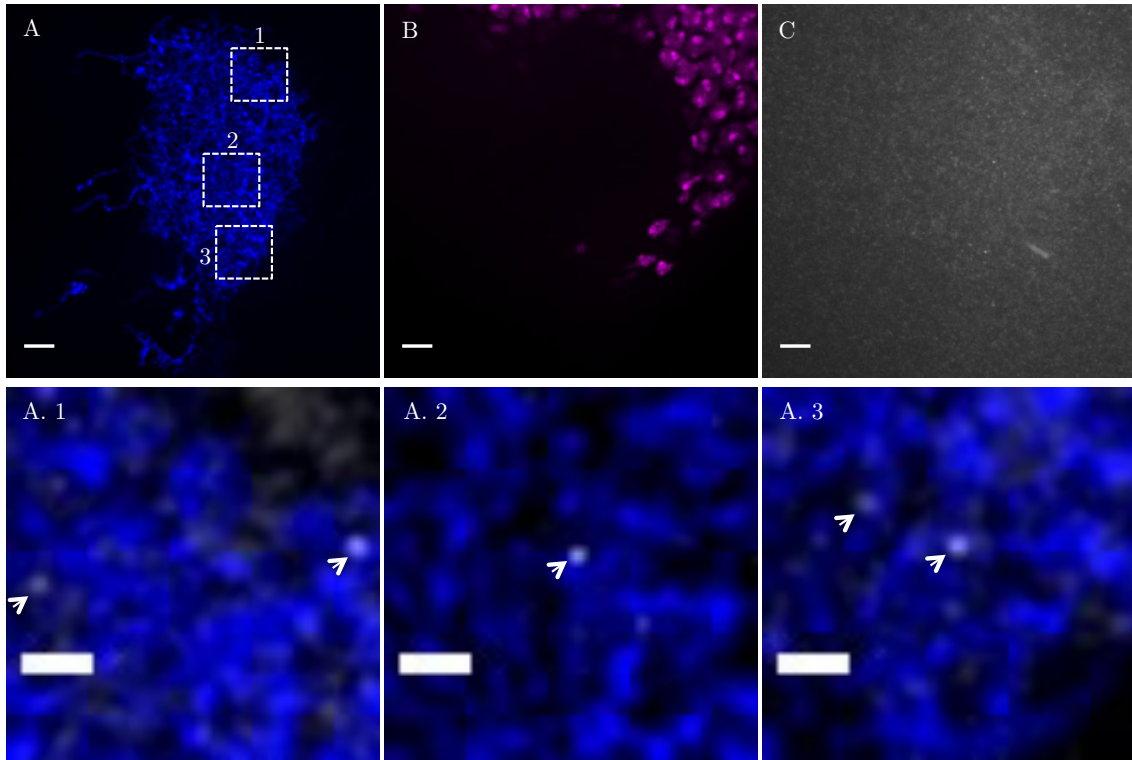


Figure 4.5: *Act5C*-24xMS2 expressed in $\gamma 2\alpha'1$ MBONs and visualised with an smFISH probe set targeting the MS2 array. **A:** $\gamma 2\alpha'1$ dendrites labelled with myr::SNAPf and visualised with JF549-SNAPTag. **B:** Cell bodies labelled with the nuclear dye Vybrant DyeCycle Violet Stain and visualised in magenta. **C:** *Act5C*-24xMS2 mRNA visualised with smFISH probes targeting the 24x-MS2 array. Scale bar A-C=5 μ m. **A.1:** Magnification of white dashed box 1 in A. **A.2:** Magnification of white dashed box 2 in A. **A.3:** Magnification of white dashed box 3 in A. White arrows indicate *Act5C*-24xMS2 smFISH spots. Scale bar A.1-A.3=2 μ m.

4.2.4 Implications of $\gamma 2\alpha'1$ dendritically localised mRNA

The dendritic localisation of *Act5C*, *CaMKII*, and *DLG1* mRNA in $\gamma 2\alpha'1$ MBONs is coherent with previous descriptions of dendritic localisation of these mRNA types and the function of their encoded protein products (Ashraf et al., 2006; Burgin et al., 1990; Buxbaum et al., 2014; Lionnet et al., 2011; Park et al., 2014; Yang et al., 2017). However, the dendritic localisation of *NMDAR1* mRNA in $\gamma 2\alpha'1$ MBONs was surprising. This subset of cholinergic MBONs would be expected to receive excitatory input from cholinergic mushroom body Kenyon cells as well as modulatory input from dopaminergic neurons (Aso et al., 2014). Glutamatergic input and therefore a requirement for NMDA receptors seemed spurious. A possible source of glutamatergic input to $\gamma 2\alpha'1$ dendrites from MBON $\gamma 4 > \gamma 1\gamma 2$ was suggested from overlap of the respective neuron terminals (Aso et al., 2014). Furthermore, the recent electron microscopy volume of a whole fly brain, which has enabled synaptic resolution tracing of memory-relevant neuropils (Zheng et al., 2018), revealed synapses from $\gamma 4 > \gamma 1\gamma 2$ onto $\gamma 2\alpha'1$ dendrites (Nils Otto, Waddell lab, personal communication). The presence of *NMDAR1* mRNA would therefore be consistent with $\gamma 2\alpha'1$ dendrites receiving glutamatergic input from $\gamma 4 > \gamma 1\gamma 2$ presynapses. Functional synaptic connectivity would have to be verified with live 2-photon Ca^{2+} imaging.

4.2.5 MS2 tagged mRNA transcripts visualised in M6/ $\gamma 5\beta'2a$ MBONs

Act5C-24xMS2, *NMDAR1*-24xMS2, *CaMKII*-24xMS2 and *DLG1*-24xMS2 transgenes along with the MCP::RFP coat protein were expressed in another subset of MBONs, the M6/ $\gamma 5\beta'2a$ MBONs, using the R66C08-Gal4 driver (Owald et al., 2015). M6/ $\gamma 5\beta'2a$ MBON dendrites mostly innervate the $\gamma 5$ and $\beta'2a$ zones of the mushroom body (Figure 4.6 A, B) where they most likely receive cholinergic and dopaminergic input (Owald et al., 2015). Their presynaptic output terminals form large anatomically-distinct spherical boutons (Figure 4.6 C) which are glutamatergic (Owald et al., 2015). As well as comparing the expression of MS2-labelled mRNA in another set of memory-relevant MBONs, the M6/ $\gamma 5\beta'2a$ neurons were selected because their anatomically-distinct dendritic and presynaptic compartments are located proximally and superficially in the anterior of the *Drosophila* brain, thereby providing favourable molecular imaging conditions of both com-

partments. In this way, a comparison between the presence of fluorescent mRNA puncta in the physiologically-distinct polar compartments of the same neuron could be made.

Expression of *Act5C*-24xMS2 (Figure 4.8), *NMDAR1*-24xMS2 (Figure 4.9), *CaMKII*-24xMS2 (Figure 4.10) and *DLG1*-24xMS2 (Figure 4.11) along with MCP::RFP in M6/ $\gamma 5\beta'2a$ MBON dendrites and presynaptic boutons revealed sparse, specific labelling within the respective compartments of these neurons. The lack of extraneous RFP fluorescence suggests that observed using the MB077c-Gal4 driver to express the constructs in $\gamma 2a'1$ MBONs is the result of the driver and not the constructs. Expressing the MCP::RFP coat protein in M6/ $\gamma 5\beta'2a$ MBONs alone resulted in minimal fluorescent puncta in both the dendrites and presynaptic boutons (Figures 4.6 D, E, 4.7). Quantitative analysis of MS2-containing mRNA expression within M6/ $\gamma 5\beta'2a$ MBON dendrites revealed expression only of *CaMKII*-24xMS2 (Figure 4.10) and *DLG1*-24xMS2 (Figure 4.11) produced significantly more fluorescent puncta with respect to the expression of the MS2 coat protein alone (Figure 4.6 D, E). In the presynaptic region however, only the expression of *NMDAR1*-24xMS2 produced significantly greater numbers of fluorescent mRNA puncta relative to the expression of MS2 coat protein alone (Figure 4.6 E). In some cases, the expression patterns of MS2 labelled mRNA was very specific. For example, a line of *DLG1*-24xMS2 mRNA was observed in M6/ $\gamma 5\beta'2a$ MBON dendrites (Figure 4.12), which possibly represents mRNA localised within the branching arbour that gives rise to the main dendritic terminal.

4.2.6 Implications of M6/ $\gamma 5\beta'2a$ localised mRNA

The lack of localised *NMDAR1* mRNA in M6/ $\gamma 5\beta'2a$ dendrites is consistent with the notion that they receive cholinergic and dopaminergic input (Owald et al., 2015). However the localisation of *NMDAR1* mRNA in M6/ $\gamma 5\beta'2a$ presynaptic regions was surprising given that they are glutamatergic presynaptic output sites. In order to explain this result, the electron micrograph reconstruction of the whole *Drosophila* brain was consulted to assess whether any synaptic input sites were present onto the M6/ $\gamma 5\beta'2a$ presynaptic region. Interestingly the synapse-resolution connectome has revealed that the postsynaptic

input sites and presynaptic release sites of *Drosophila* neurons are not completely polarised. Often presynapses are present within dendritic regions and conversely postsynapses are present on axonal regions. In the case of the M6/ $\gamma 5\beta'2a$ presynaptic region, there were many input sites, including from the glutamatergic M4/MBON- $\beta 2\beta'2a$ (M4 β) and MBON- $\beta'2mp$ (M4 β') (Nils Otto, Waddell lab, personal communication). The nature of the other inputs had not yet been identified. However, the presence of *NMDAR1* mRNA in these regions appears to indicate these presynaptic terminals also receive glutamatergic input.

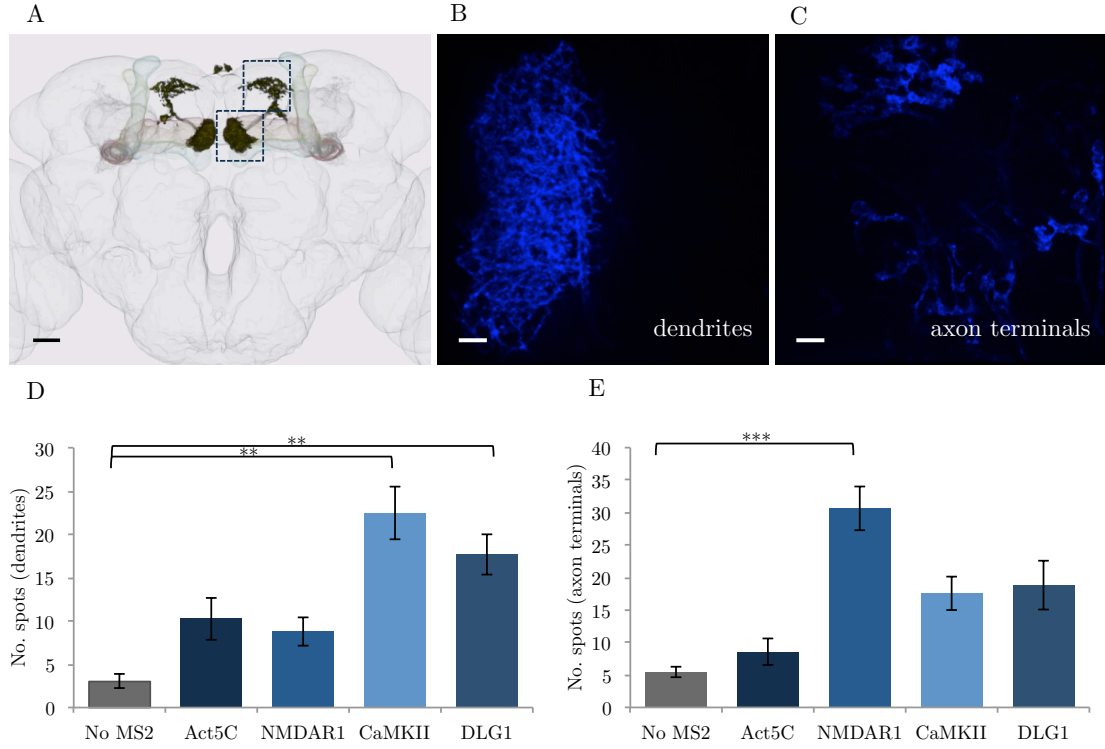


Figure 4.6: **A**: Schematic representation of M6/ $\gamma 5\beta'2a$ MBONs. Lower box indicates dendritic region. Upper box indicates axonal region. Figure adapted from Aso et al. (2014). Scale bar=20 μ m. **B**: Dendrites of M6/ $\gamma 5\beta'2a$ MBON. **C**: Axonal region of M6/ $\gamma 5\beta'2a$ MBON. M6/ $\gamma 5\beta'2a$ MBONs visualised by a UAS-mCD8::GFP transgene driven by the R66C08-Gal4 driver. Scale bar=5 μ m. **D**: Mean ($\pm$ s.e.m) number of *Act5C*-, *NMDAR1*-, *CaMKII*-, and *DLG1*-24xMS2 tagged mRNA puncta in 2 μ m sections of M6/ $\gamma 5\alpha'2a$ dendrites. Significantly greater numbers of *CaMKII*- (Kruskal Wallis test: $\chi^2=39.4356$, $p=0.006$, $n=7$ (*CaMKII*-24xMS2), $n=6$ (No MS2)) and *DLG1*-24xMS2 (Kruskal Wallis test: $\chi^2=35.9356$, $p=0.0059$, $n=7$ (*DLG1*-24xMS2), $n=6$ (No MS2)) puncta were observed in $\gamma 5\alpha'2a$ dendrites relative to the expression of the MS2 coat protein alone. **E**: Mean ($\pm$ s.e.m) number of *Act5C*-, *NMDAR1*-, *CaMKII*-, and *DLG1*-24xMS2 tagged mRNA puncta in 2 μ m sections of M6/ $\gamma 5\beta'2a$ presynaptic regions. Significantly greater numbers of *NMDAR1*-24xMS2 puncta were observed in $\gamma 5\alpha'2a$ presynaptic regions (Kruskal Wallis test: $\chi^2=36.8789$, $p=0.0006$, $n=9$ (*NMDAR1*-24xMS2), $n=5$ (No MS2)) relative to the expression of the MS2 coat protein alone.

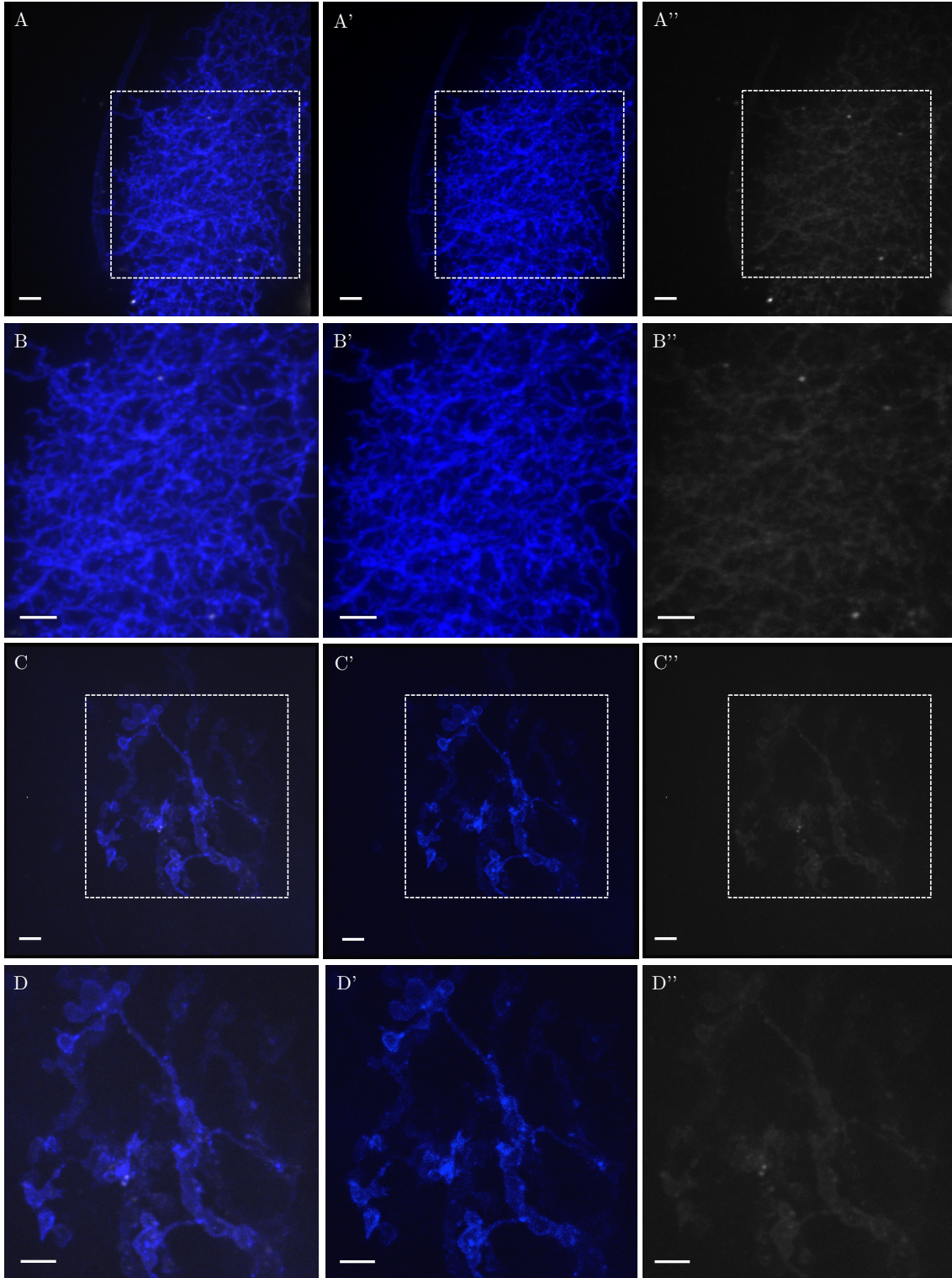


Figure 4.7: Dendrites and presynaptic outputs of M6/ $\gamma 5\beta'2a$ MBONs expressing the MS2 coat protein MCP::RFP alone, i.e. in the absence of any MS2 containing mRNA. **A**: Composite of **A'** and **A''**. **A'**: M6/ $\gamma 5\beta'2a$ dendrites visualised by 10x-IVS-myr::GFP. **A''**: MCP::RFP. **B-B''**: Magnification of hatched box in **A-A''**. **C**: Composite of **C'** and **C''**. **C'**: Presynaptic outputs of M6/ $\gamma 5\beta'2a$ MBON visualised by 10x-IVS-myr::GFP. **C''**: MCP::RFP. **D-D''**: Magnification of hatched box in **C-C''**. Scale bar= $5\mu\text{m}$.

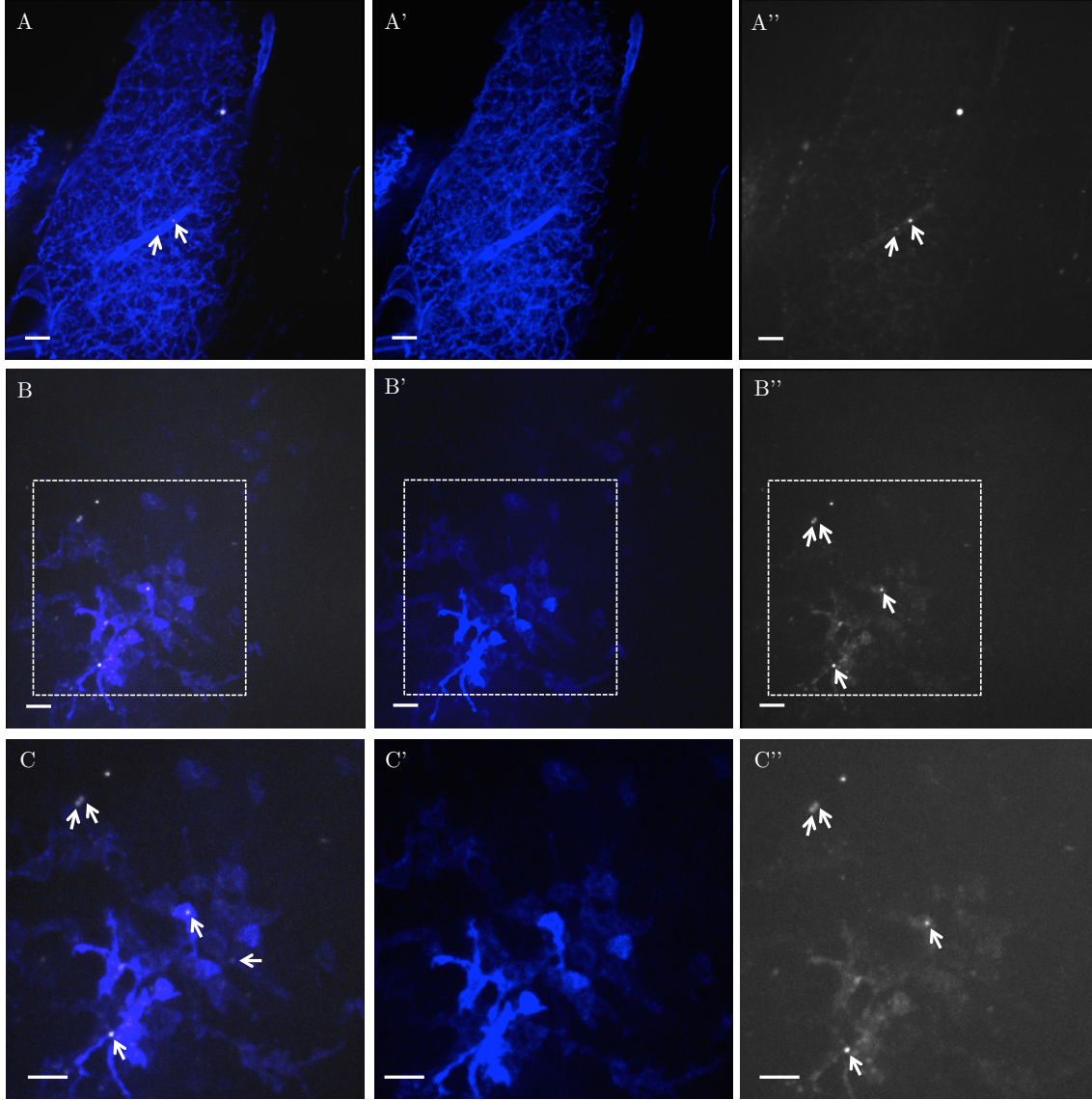


Figure 4.8: Dendritic and presynaptic outputs of M6/ $\gamma 5\beta'2a$ MBONs expressing *Act5C*-24xMS2 along with the MS2 coat protein MCP::RFP. **A**: Composite of A' and A''. **A'**: M6/ $\gamma 5\beta'2a$ dendrites visualised by 10x-IVS-myr::GFP. **A''**: *Act5C*-24xMS2 visualised with MCP::RFP. **B**: Composite of B' and B''. **B'**: M6/ $\gamma 5\beta'2a$ presynaptic outputs visualised by 10x-IVS-myr::GFP. **B''**: *Act5C*-24xMS2 visualised with MCP::RFP. **C-C''**: Magnification of hatched box in B-B''. White arrows indicate MS2-labelled *Act5C* mRNA puncta. Scale bar= $5\mu\text{m}$.

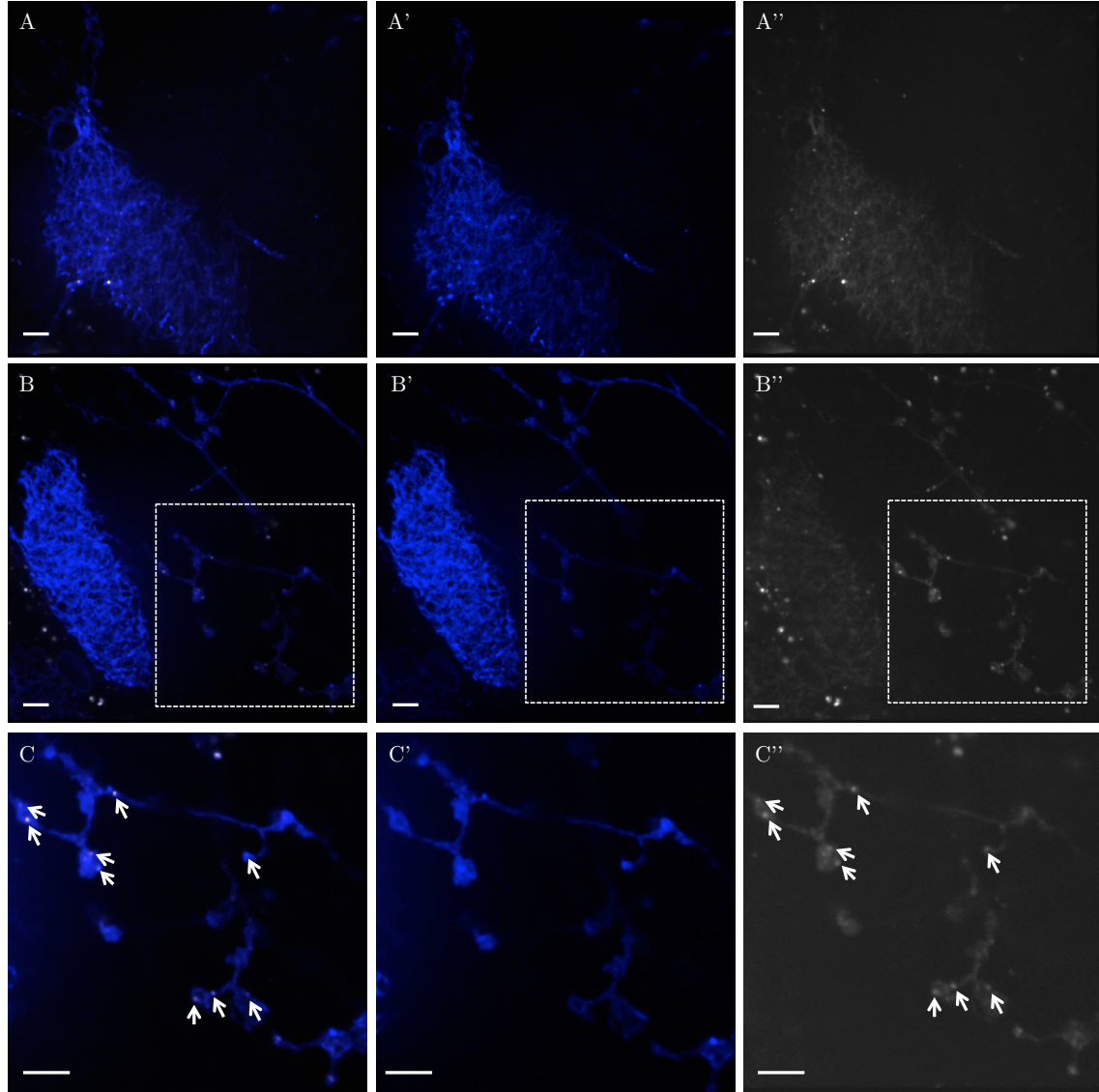


Figure 4.9: Dendritic and presynaptic output regions of M6/ $\gamma 5\beta'2a$ MBON expressing *NMDAR1*-24xMS2 along with the MS2 coat protein MCP::RFP. **A**: Composite of A' and A''. **A'**: M6/ $\gamma 5\beta'2a$ dendrites visualised by 10x-IVS-myr::GFP. **A''**: *NMDAR1*-24xMS2 visualised with MCP::RFP. **B**: Composite of B' and B''. **B'**: Presynaptic outputs and dendrites of M6/ $\gamma 5\beta'2a$ MBON visualised by 10x-IVS-myr::GFP. **B''**: *NMDAR1*-24xMS2 visualised with MCP::RFP. **C**: Magnification of B, hatched box. Composite of C' and C''. **C'**: Magnification of B', hatched box. **C''**: Magnification of B'', hatched box. White arrows indicate MS2-labelled *NMDAR1* mRNA puncta. Scale bar=5 μ m.

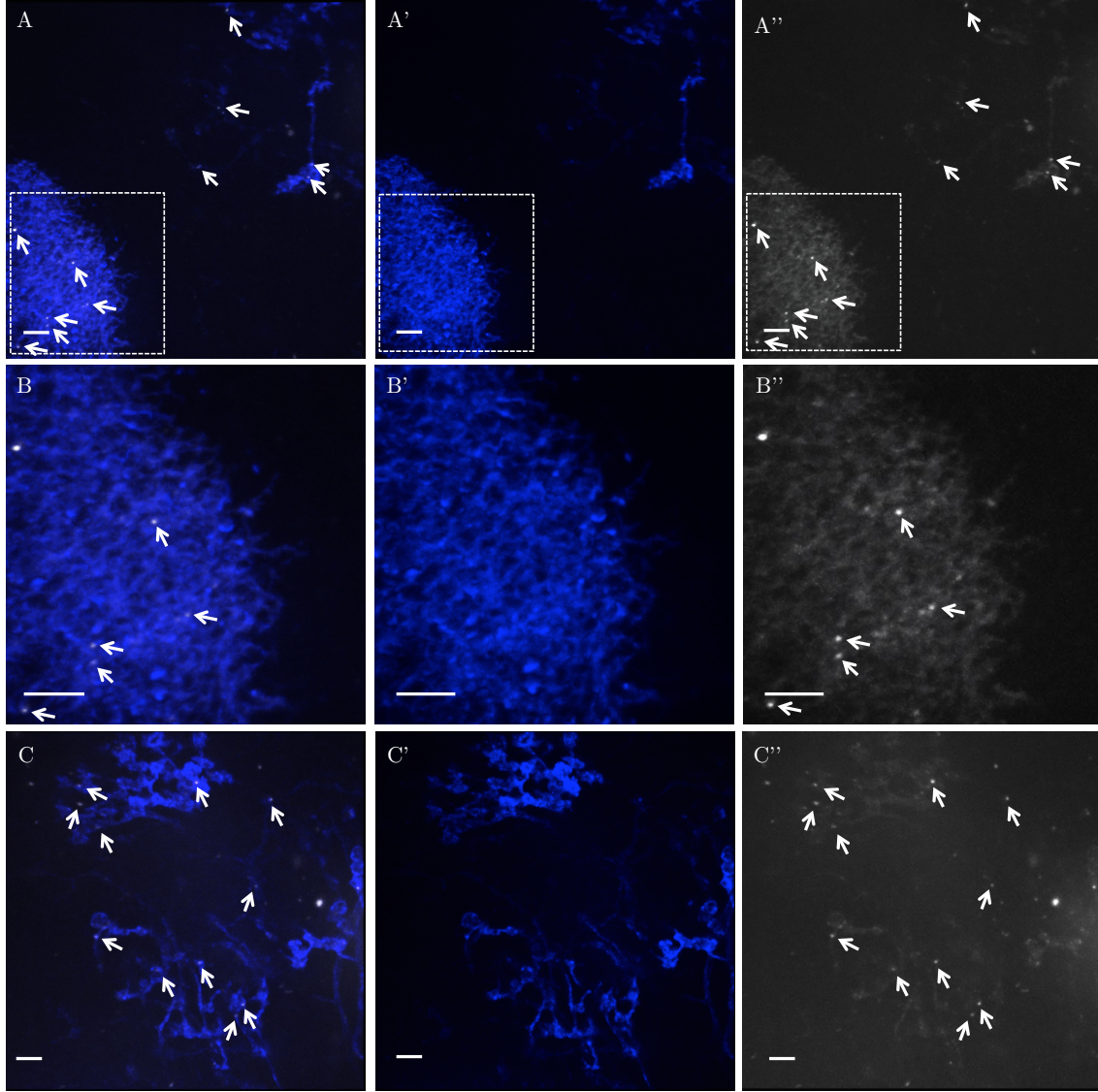


Figure 4.10: Dendritic and presynaptic outputs of M6/ $\gamma 5\beta'2a$ MBON expressing *CaMKII*-24xMS2 along with the MS2 coat protein MCP::RFP. **A**: Composite of **A'** and **A''**. **A'**: M6/ $\gamma 5\beta'2a$ dendrites visualised by 10x-IVS-myr::GFP. **A''**: *CaMKII*-24xMS2 visualised with MCP::RFP. **B-B''**: Magnification of hatched box region in **A-A''**. **C**: Composite of **C'** and **C''**. **C'**: M6/ $\gamma 5\beta'2a$ presynaptic outputs visualised by 10x-IVS-myr::GFP. **C''**: *CaMKII*-24xMS2 visualised with MCP::RFP. White arrows indicate MS2-labelled *CaMKII* mRNA puncta. Scale bar=5 μ m.

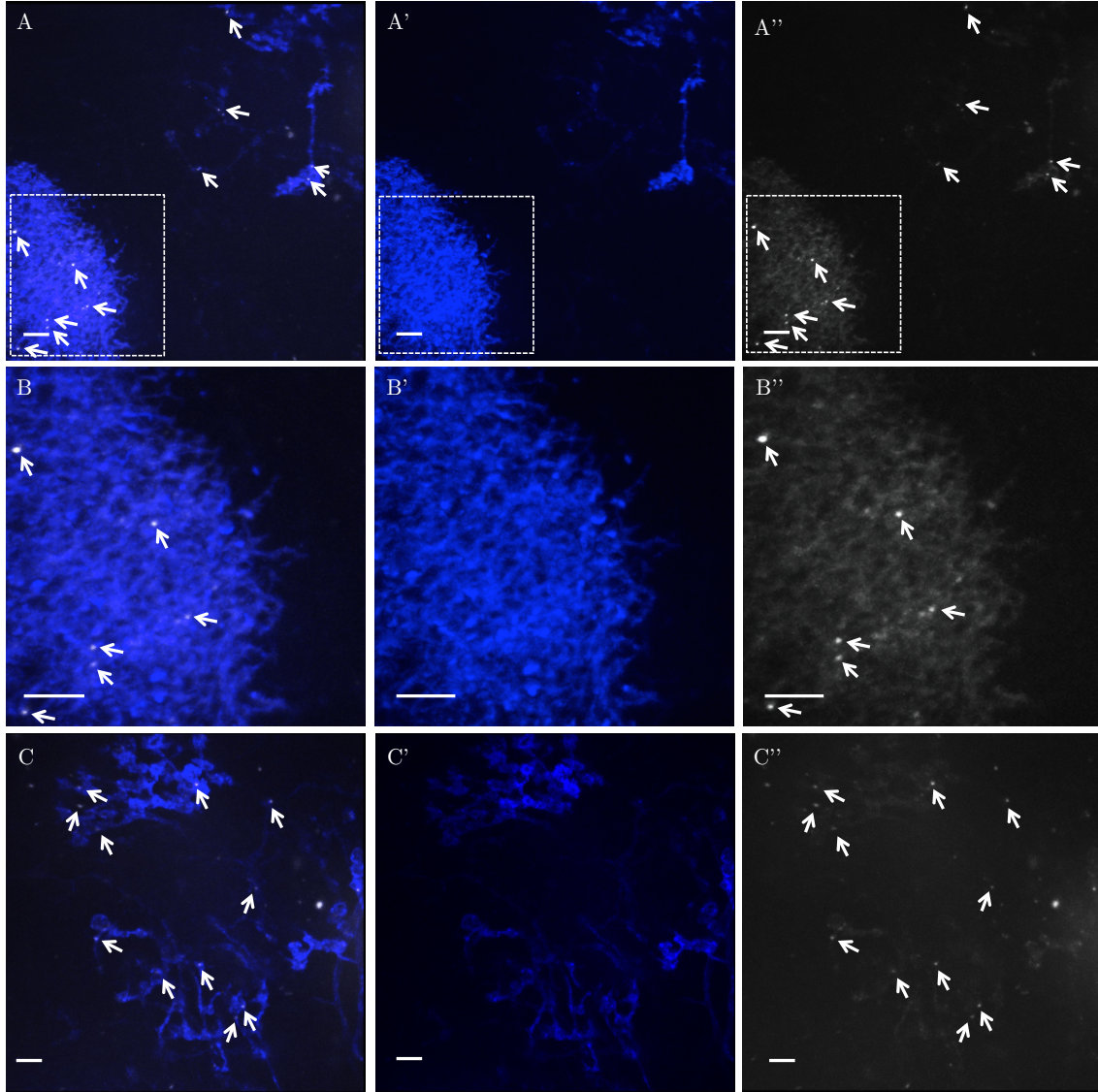


Figure 4.11: Dendritic and presynaptic outputs of M6/ $\gamma 5\beta'2a$ MBON expressing *DLG1*-24xMS2 along with the MS2 coat protein MCP::RFP. **A**: Composite of **A'** and **A''**. **A'**: M6/ $\gamma 5\beta'2a$ dendrites and neighbouring axon terminals visualised by 10x-IVS-myr::GFP. **A''**: *DLG1*-24xMS2 visualised with MCP::RFP. **B-B''**: Magnification of hatched box region in **A-A''**. **C**: Composite of **C'** and **C''**. **C'**: Presynaptic outputs of M6/ $\gamma 5\beta'2a$ MBON visualised by 10x-IVS-myr::GFP. **C''**: *DLG1*-24xMS2 visualised with MCP::RFP. White arrows indicate MS2-labelled *DLG1* mRNA puncta. Scale bar=5 μ m.

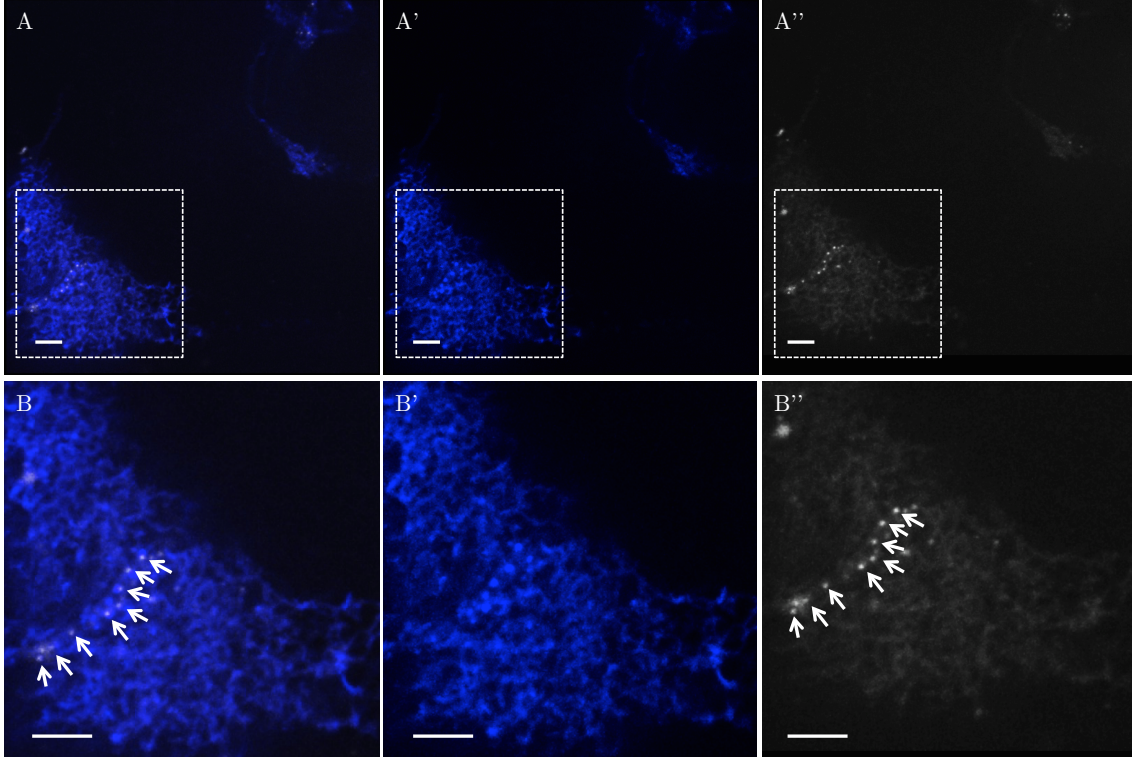


Figure 4.12: Dendrites of M6/ $\gamma 5\beta'2a$ MBON expressing *DLG1*-24xMS2 along with the MS2 coat protein MCP::RFP. **A**: Composite of A' and A''. **A'**: M6/ $\gamma 5\beta'2a$ dendrites visualised by 10x-IVS-myr::GFP. **A''**: *DLG1*-24xMS2 visualised with MCP::RFP. **B-B''**: Magnification of A-A'', hatched box region. Scale bar=5 μ m.

4.2.7 MS2 tagged mRNA transcripts visualised in $\gamma 5$ dopaminergic neurons

The specific localisation of *NMDAR1* mRNA transcripts to M6/ $\gamma 5\beta'2a$ presynaptic regions relative to the dendrites suggests a high level of specificity in the targeting of these molecules. In addition, the presence of *NMDAR1* mRNA in $\gamma 2\alpha'1$ dendrites with predicted glutamatergic inputs is coherent. Such specific targeting of mRNA to certain portions of neurons argues against mRNA localisation patterns arising merely as overexpression artefacts. In order to explicitly test patterns of specific localisation, *NMDAR1*-24xMS2 was expressed in a subset of dopaminergic neurons known as PAM- $\gamma 5$ neurons (Aso et al., 2014) (Figure 4.13). PAM- $\gamma 5$ dopaminergic neurons have presynaptic dopamine-releasing terminals onto the $\gamma 5$ region of the mushroom body and dendritic inputs which lie in close proximity to M6/ $\gamma 5\beta'2a$ axon terminals. M6/ $\gamma 5\beta'2a$ dendrites receive dopaminergic

input from PAM- $\gamma 5$ neurons which in turn receive excitatory glutamatergic input from M6/ $\gamma 5\beta'2a$ in a potentiating recurrent circuit thought to lay the foundation for short-term memory formation (Felsenberg et al., 2018; Zhao et al., 2018). In accordance with this circuit structure, *NMDAR1* mRNA was predicted to be localised specifically in the PAM- $\gamma 5$ dendrites relative to the axon terminals. Compared to MS2 coat protein expression alone, *NMDAR1*-24xMS2 mRNA puncta were specifically localised within the dendritic region of the PAM- $\gamma 5$ neuron relative to the axon terminals (Figure 4.14). The localisation patterns of *NMDAR1*-24xMS2 mRNA therefore seem to recapitulate the spatial localisation patterns of the encoded glutamate receptor.

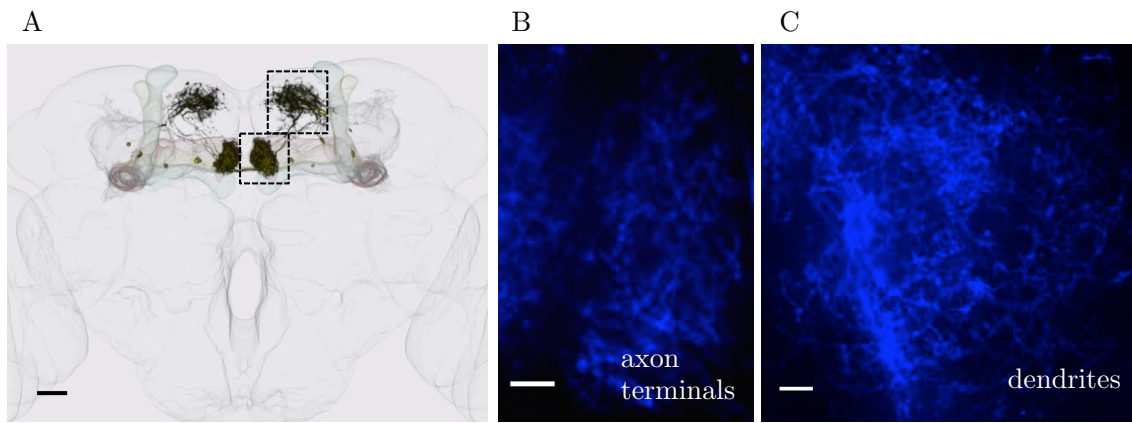


Figure 4.13: **A:** Schematic representation of PAM $\gamma 5$ dopaminergic neurons. Upper dashed box indicates dendritic input region, lower dashed box indicates presynaptic output region onto the mushroom body $\gamma 5$ region. Figure adapted from Aso et al. (2014). Scale bar= $20\mu\text{m}$. **B:** PAM $\gamma 5$ presynaptic outputs labelled with a UAS-10x-IVS-mCD8::GFP transgene driven by the R48B04-Gal4 driver. **C:** PAM $\gamma 5$ dendrites labelled with a UAS-10x-IVS-mCD8::GFP transgene driven by the R48B04-Gal4 driver. Scale bar= $5\mu\text{m}$.

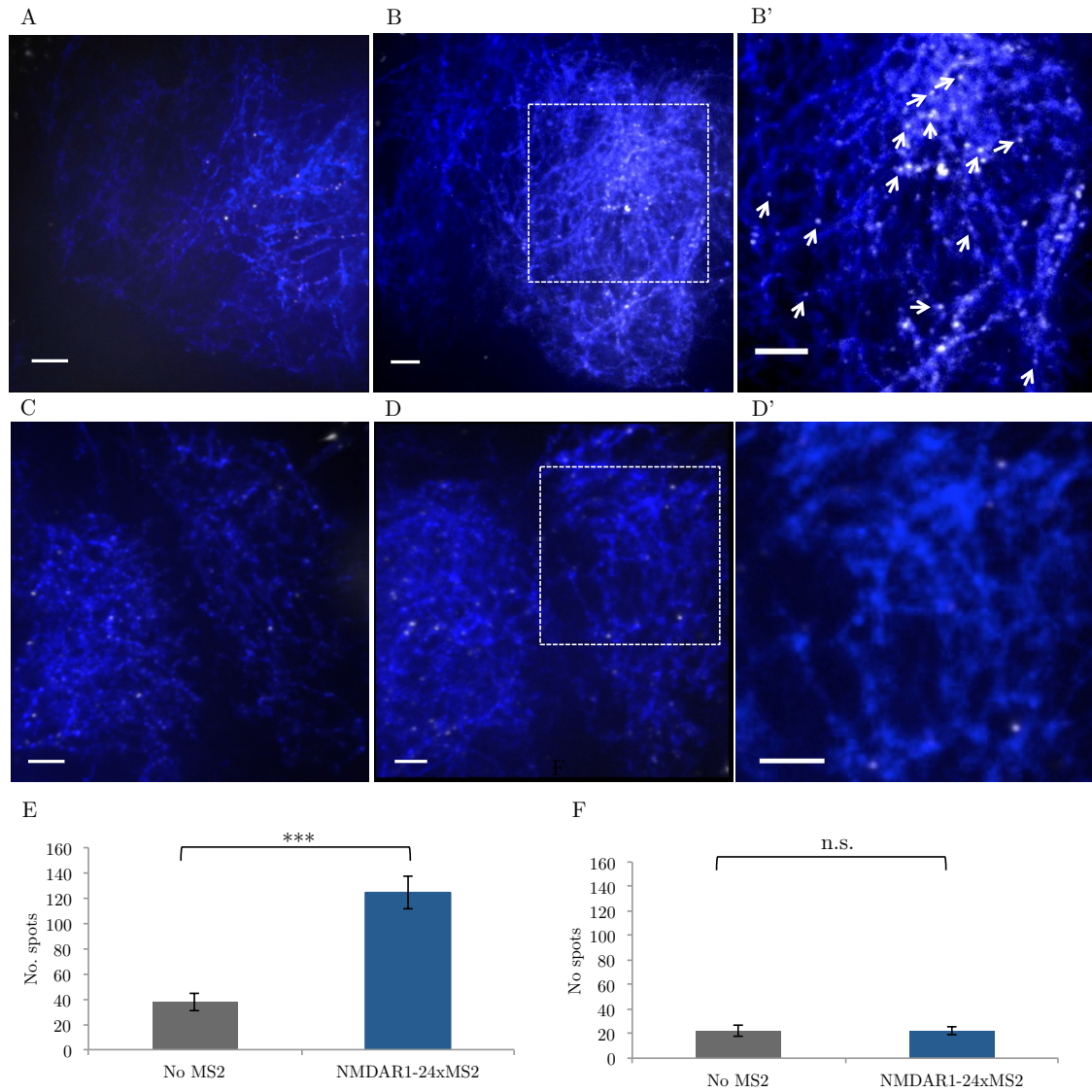


Figure 4.14: *NMDAR1-24xMS2* expression in PAM $\gamma 5$ dendrites and $\gamma 5$ presynaptic outputs. **A**: Expression of MCP::RFP (No MS2) in PAM $\gamma 5$ dendrites. **B**: Co-expression of MCP::RFP along with *NMDAR1-24xMS2* in in PAM $\gamma 5$ dendrites. **B'**: Magnification of B, hatched box region. **C**: Expression of MCP::RFP (No MS2) in PAM $\gamma 5$ presynaptic outputs. **D**: Co-expression of MCP::RFP along with *NMDAR1-24xMS2* in in PAM $\gamma 5$ presynaptic outputs. **D'**: Magnification of D, hatched box region. Scale bar=5 μm . **E**: Mean number of fluorescent puncta ($\pm s.e.m.$) with expression of MCP::RFP (No MS2) and co-expression of MCP::RFP along with *NMDAR1-24xMS2* in PAM $\gamma 5$ dendrites. Co-expression of MCP::RFP along with *NMDAR1-24xMS2* resulted in significantly more fluorescent puncta compared with the No MS2 control (Wilcoxon rank sum test: $p < 0.0001$, $z = -3.6525$, $n = 8$ (No MS2), $n = 14$ (*NMDAR1-24xMS2*)). **F**: Mean number of fluorescent puncta ($\pm s.e.m.$) with expression of MCP::RFP (No MS2) and co-expression of MCP::RFP along with *NMDAR1-24xMS2* in PAM $\gamma 5$ presynaptic outputs. Co-expression of MCP::RFP along with *NMDAR1-24xMS2* did not result in significantly different numbers of fluorescent puncta compared with the No MS2 control (T-test: $p = 0.959$, $t = 0.0525$, $n = 8$ (No MS2), $n = 5$ (*NMDAR1-24xMS2*)).

4.2.8 Variation amongst MS2-tagged mRNA particles

From the experiments described above in which MS2-tagged transgenes were visualised in the whole adult *Drosophila* brain, it was observed that not all fluorescent mRNA puncta were uniform in size and brightness. In order to quantify this variability, the size and brightness of individual MS2-labelled mRNA foci visualised in the dendrites of $\gamma 2\alpha'1$ MBONs were measured. Observed mRNA particle sizes ranged between 0.2-0.5 μm (200-500 nm). This range was fairly consistent between mRNA type (*Act5C*-, *NMDAR1*-, *CaMKII*- and *DLG1*-24xMS2) (Figure 4.15 B).

Since the visualisation of mRNA transcripts using the MS2 system relies on the detection of multiply bound fluorescent proteins, one source of variation in particle size is likely the result of positively correlated variations in fluorescent protein loading of the MS2 stem loop array. Indeed, larger particles tended to be brighter (Figure 4.15 C-F). Beyond variation in fluorescent protein occupancy of the MS2 stem loop array, a further source of variation could arise from mRNA copy number at individual foci. Since standard fluorescence microscopy does not permit two mRNA molecules that are in close proximity or bound together to be resolved, it is likely that larger particles in fact represent multiple mRNA copies either in close physical proximity or bound together within a common mRNP.

The quantification of particle brightness has previously been used to calculate precise mRNA copy numbers (Skinner et al., 2013). Copy number quantification here could be ascertained by quantifying the absolute number of fluorophores present per spot by stepwise photobleaching experiments (Verdaasdonk et al., 2014). In this way, fluorescent MS2 spots containing 24 fluorophores or less could be considered single-molecules. And conversely, spots containing more than 24 fluorophores could be considered aggregates of 2 or more mRNA molecules. From the data collected, several observations (namely mRNA merging events) suggest that larger, brighter, particles may indeed be the result of multiple mRNA transcripts either in close proximity or bound together (see below).

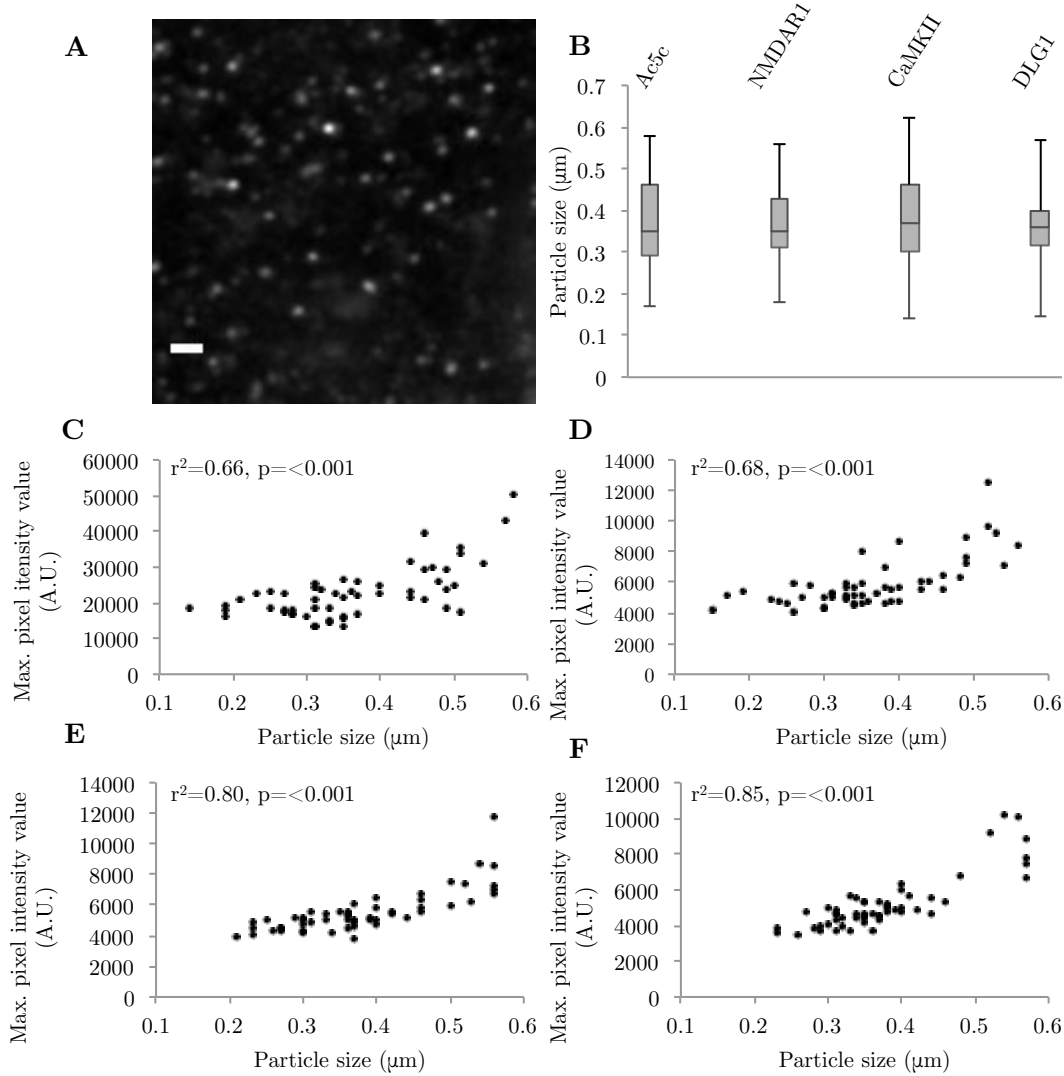


Figure 4.15: Quantification of mRNA particle sizes of *Act5C*-24xMS2, *NMDAR1*-24xMS2, *CaMKII*-24xMS2 and *DLG1*-24xMS2 in $\gamma 2\alpha/1$ dendrites. **A**: Example range of mRNA molecules used for quantification. Scale bar= $2\mu\text{m}$. **B**: Ranges of mRNA particle sizes (μm). Box plots represent the 1st quartile range, the median value and the 3rd quartile range. Whiskers represent the maximum and minimum values. **C**: Correlation of *Act5C*-24xMS2 mRNA size (μm) with maximum pixel intensity value (A.U., arbitrary units.). **D**: *NMDAR1*-24xMS2 mRNA size vs. brightness correlation. **E**: *CaMKII*-24xMS2 mRNA size vs. brightness correlation. **F**: *DLG1*-24xMS2 mRNA size vs. brightness correlation. r^2 and p values represent the correlation coefficient and level of statistical significance, respectively, from Pearson correlation tests.

4.2.9 mRNA copy number may account for variation in particle brightness

The major advantage of the MS2 system is that it allows the visualisation of mRNA molecules live. *Act5C*-24xMS2, *NMDAR1*-24xMS2, *CaMKII*-24xMS2 and *DLG1*-24xMS2 were all visualised live in whole *ex plant Drosophila* brains to assess motility dynamics. An interesting observation made during live mRNA recording was the apparent merging of two particles. Three independent observations of such merging behaviour were recorded. One example is shown in Figure 4.16. To establish quantitative evidence for these merging events, a pixel intensity histogram was generated for each particle (i.e. both individuals and the merged particle). It was reasoned that the sum maximum pixel intensity values of the two individual particles should closely approximate that of the merged particle. Indeed, the sum of the maximum pixel intensity value minus the minimum pixel value (to correct for changes in background intensity), named the Δ pixel intensity, of the two individual particles closely approximated the Δ pixel intensity of the merged particles (Table 4.2). In all three observations, the summed Δ pixel intensity values of the individual particles approximated that of the merged particle with an average error of $\sim 6\%$.

That the summed Δ pixel intensity values of the two individual particles closely approximates that of the merged particle is evidence that these individual molecules come into very close physical proximity. Whether or not these events represent molecular binding events, for example to a common mRNP, is unclear. However, it is clear that one source of variation in particle brightness observed (Figure 4.15) arises from multiple closely associated molecules.

4.2.10 Implications of mRNA merging events

mRNA merging events were not observed frequently. However mRNA splitting, or dissociation, events were never observed. Nonetheless, the biological implications of visualising mRNA molecular association and/or disassociation events in a live brain could be quite significant. Park et al. (2014) report both merging and splitting events of MS2-tagged *β -actin* mRNA in cultured mouse hippocampal neurons. Following depolarisation with

KCL treatment, the number of mRNA puncta were found to increase while the number of molecules of mRNA per puncta (i.e. the number of mRNA molecules presumably contained within an mRNP) decreased. Moreover, the ratio of split to merge events increased. The authors postulate that mRNA molecules undergo continuous assembly and disassembly into mRNP complexes, with neuronal activity favouring disassembly perhaps for accessibility to translational machinery. Buxbaum et al. (2014) also suggest a similar hypothesis. Using smFISH probe binding efficiency as a readout of mRNA transcript accessibility to translation factors, Buxbaum et al. (2014) showed that following chemical LTP induction in cultured hippocampal neurons, *β -actin* mRNA becomes transiently more accessible to smFISH probe binding. The authors propose that the default state for localised mRNA is one in which transcripts are masked by encompassment in RNP granules in a translationally repressed state. Upon synaptic stimulation, localised mRNA transcripts are released for approximately 15 minutes before being repackaged into RNP granules and translationally re-repressed. In this way, localised mRNA may undergo cycles of activity-regulated translation via association and dissociation with RNP granules. It is interesting to postulate whether the observed merging of M2S-tagged mRNA particles represents such an RNP association event and whether this is related to the translatability of the mRNA molecule. It would be particularly interesting to measure whether the frequency of such events, or indeed the occurrence of dissociation events, could be observed following chemical or physiological stimulation of the *Drosophila* brain.

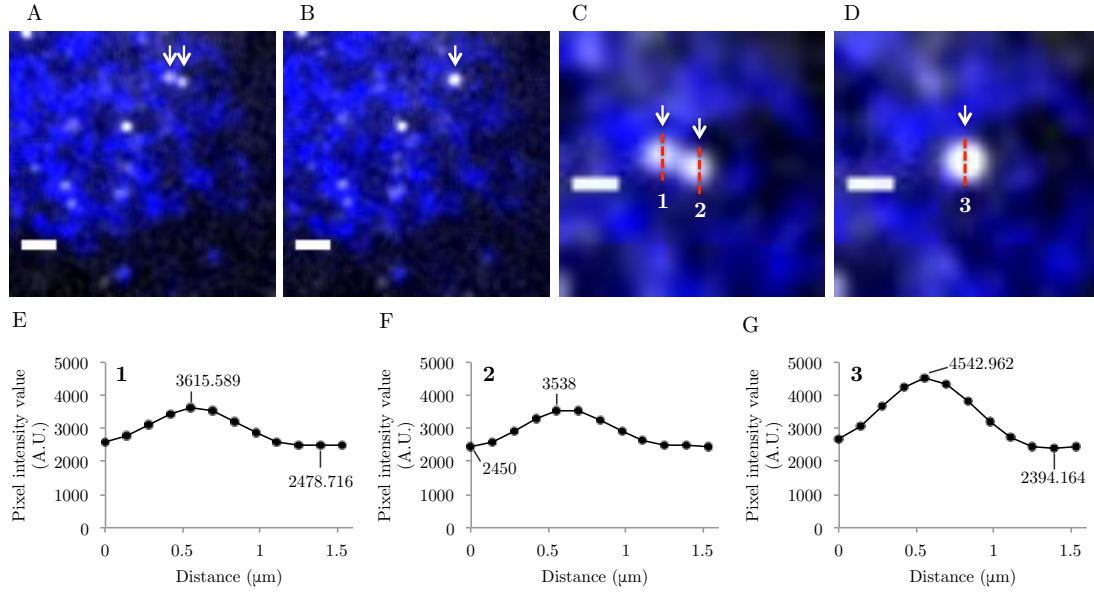


Figure 4.16: *NMDAR1*-24xMS2 mRNA molecules observed merging in $\gamma 2\alpha'1$ dendritic region. **A**: Single frame of a single z-slice captured at 1 frame s⁻¹. **B**: Single frame of a single z-slice captured at 1 frame s⁻¹, 10 frames (10 s) after A. Scale bar A and B=2 μm. **C**: Magnification of A. **D**: Magnification of B. Scale bar C and D=1 μm. **E**: Pixel intensity values (arbitrary units) plotted along the red line drawn through particle 1 in C. **F**: Pixel intensity values (arbitrary units) plotted along the red line drawn through particle 2 in C. **G**: Pixel intensity values (arbitrary units) plotted along the red line drawn through particle 3 in D. Minimum and maximum pixel intensity values are annotated in E-G.

Table 4.2: Minimum, maximum and Δ pixel intensity values from Figure 4.16 E-G.

Particle	Min. pixel intensity	Max. pixel intensity	Δ pixel intensity
1	2478.716	3615.589	1136.873
2	2450	3538	1088
3	2394.164	4542.962	2148.798
1 + 2			2224.873

4.2.11 Dynamic behaviours

A major advantage of the MS2 system is that it permits the visualisation of mRNA *in vivo* in real time. *Act5c*-, *NMDAR1*-, *CaMKII*- and *DLG1*-24xMS2 constructs were thus visualised live in the *Drosophila* brain. Sample videos can be viewed via the following URL: <https://www.dropbox.com/sh/psvl7vs1ur2835i/AABpAeyZlghey91aP9XgbD0Na?dl=0>. Mobile mRNA particles were observed in the dendrites of $\gamma 2\alpha'1$ MBONs (see Videos 2-

5) and in the presynaptic output terminals of M6/ $\gamma 5\beta'2a$ MBONs (See Video 6). From qualitative observations, some particles appeared stationary while others were mobile. Of the mobile fraction, distinct types of motile behaviours were observed: most particles appeared to move in an oscillatory manner (Figure 4.17 and Video 7), while only very few were observed to move in a processive, directed manner (Figure 4.18 and Video 8).

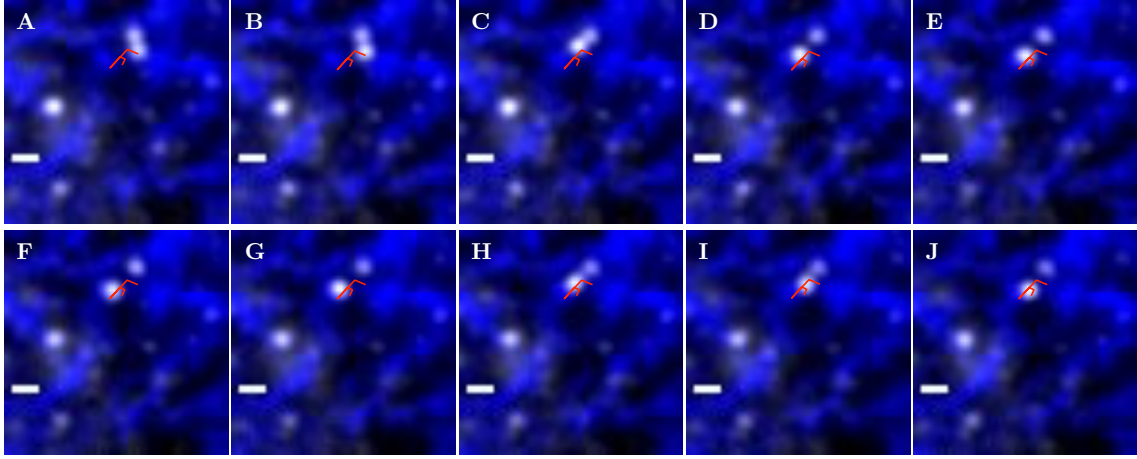


Figure 4.17: *CaMKII*-24xMS2 mRNA molecule observed moving in an oscillatory manner in the dendritic region of the $\gamma 2\alpha'1$ MBON. **A-J**: Single sequential frames of a single z-slice captured at 1 frame s^{-1} . Trajectory of the *CaMKII*-24xMS2 particle is illustrated in red. Scale bar= $2\mu m$.

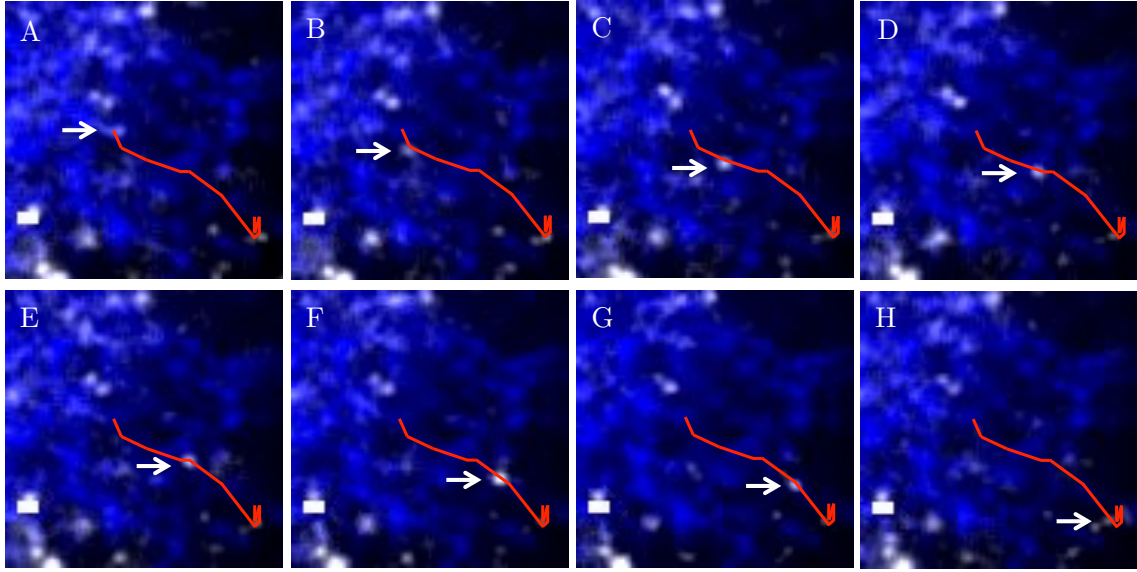


Figure 4.18: *Act5C*-24xMS2 mRNA molecule observed moving directionally in $\gamma 2\alpha'1$ neurite. A-H: Single sequential frames of a single z-slice captured at 1 frame s^{-1} . Trajectory of the *Act5C*-24xMS2 particle is illustrated in red. White arrows indicate the *Act5C*-24xMS2 particle of interest. Scale bar= $1\mu\text{m}$.

4.2.12 Single particle tracking

In order to extract quantitative information from video microscopy data, single particle tracking was used to reconstruct the tracks of individual particles over time. A number of parameters relating to mobile mRNA behaviour could then be extracted. In this case, the particle's deviation from its start position, the velocity of mobile particles and the mean squared displacement were calculated for *Act5C*-24xMS2 mRNA visualised in $\gamma 2\alpha'1$ dendrites (Figure 4.19).

Spatial displacement of particles

In order to assess the distribution of distances travelled by individual particles, the displacement of a particle from its original start position was calculated. The majority of particles were either stationary, or moved less than 20 pixels from their starting position (Figure 4.19 A). With 1 pixel being equal to $139 \times 139 \text{ nm}$, most particles thus moved very small distances, i.e. less than $2 \mu\text{m}$, suggesting that most particles do not engage in long-distance transport. This is in agreement with both qualitative observations which suggest that most particles are either stationary or move in an oscillatory manner (see

video data), as well as previous reports of mobile mRNA populations (Fusco et al., 2003).

Velocity

The velocity of single particle movements was also calculated. The majority of particles travelled at 2 pixels/frame or less (approximately $0.3 \mu\text{m s}^{-1}$) (Figure 4.19 B). mRNA particle velocity is not a particularly well described parameter, and little is known about the velocities of specific types of motion and in specific cell types. The velocities of directed particle movements have been reported to range between $1\text{--}1.5 \mu\text{m s}^{-1}$ (Fusco et al., 2003; Park et al., 2014). In contrast, the velocities calculated here are considerably slower. It is likely this reflects different types of particle motility, since the majority of single particles that were tracked here were not moving in a directed manner.

Mean squared displacement

A commonly calculated parameter from single particle tracking analyses is the mean squared displacement (MSD) as a function of time. The MSD provides a position time series of individual particle trajectories, which can be used to infer information regarding the mechanisms that drive or constrain particle movements. mRNA mobility studies have categorised dynamic behaviours as stationary, passively diffusing, corralled and directed transport (Park et al., 2010). Most commonly, mRNA particles are diffusive and can dynamically transition between various types of motion (Fusco et al., 2003). The slope of MSD curves obtained for *Act5C*-24xMS2 mRNA in $\gamma 2\alpha'1$ dendrites suggests that the ensemble average of particles move by corralled diffusion (Figure 4.19 C) (Park et al., 2010). This is in accordance with track length and velocity measurements (Figure 4.19 A and B), which suggest that although many *Act5C*-24xMS2 mRNA particles in $\gamma 2\alpha'1$ dendrites are mobile, they mostly do not travel long distances. In contrast to passive diffusion, corralled diffusion kinetics imply that single particles are mobile but otherwise tethered to a fixed location, presumably in association with the cell cytoskeleton and/or mRNP complexes involved in maintaining specific patterns of localisation and regulating translation (Müller-McNicoll and Neugebauer, 2013). Corralled diffusion of *Act5C*-24xMS2 mRNA in $\gamma 2\alpha'1$ dendrites may explain the discrepancy between calculated particle velocities reported here

and in previous publications of directed particle movements (Fusco et al., 2003; Park et al., 2014). mRNA actively transported along the cell's cytoskeleton, perhaps intuitively, seem to travel at greater velocities compared to mRNA moving by corralled diffusion.

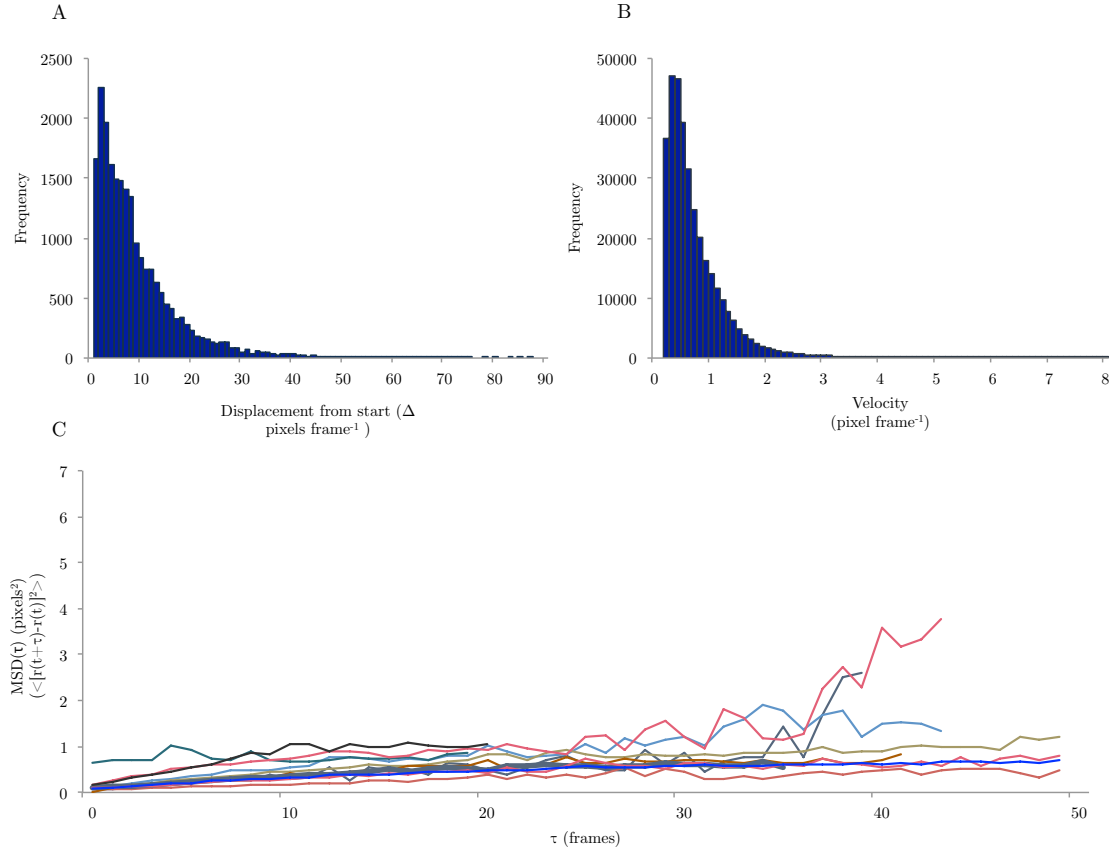


Figure 4.19: Single particle tracking analysis of *Act5C*-24xMS2 mRNA in $\gamma 2\alpha'1$ dendrites. Analyses were performed on a total of 11 independent replicate data sets. **A**: Frequency histogram of *Act5C*-24xMS2 mRNA particle displacement over time (pixels). **B**: Frequency histogram of *Act5C*-24xMS2 mRNA particle velocity (pixels per frame). **C**: Mean squared displacement (MSD) of *Act5C*-24xMS2 mRNA particles over time (τ). Each coloured line represents a separate data set.

4.2.13 Assessing motility of MS2-tagged mRNA in $\gamma 2\alpha'1$ dendrites with FRAP

Single particle tracking analysis revealed most mobile *Act5C*-24xMS2 mRNA movements were via corralled diffusion. Despite this, processive, directed mRNA movements were also observed (Figure 4.18 and Video 8), albeit infrequently. Imaging long-range directed mRNA movements live *in vivo* is experimentally challenging for two main reasons. Firstly,

the intricate branching processes of *in vivo* neurons weave throughout the sample in x, y, and z planes, meaning imaging of a continuous stretch of neurite is rarely a given. Secondly, the RFP-tagged coat protein used here is easily photobleached. The second point can be overcome by switching to using a SNAP- or HALO-tagged coat protein, which can be visualised by binding of the appropriate SNAP or HALO ligand tagged with a chemical dye. However, the fluorescently-labelled SNAP- or HALO-Tag must be externally introduced to the live fly brain to visualise the SNAP or HALO protein. Experimental efforts to supplement the fly's food with SNAP-TagJF525 in order to visualise the SNAP-tagged coat protein in the live *Drosophila* brain are currently a work in progress.

In order to experimentally quantify the long-range motility of *Act5C*-24xMS2, *NMDAR1*-24xMS2, *CaMKII*-24xMS2 and *DLG1*-24xMS2 mRNA in the complex intertwining processes of the $\gamma 2\alpha'1$ dendrites, Fluorescence Recovery After Photobleaching (FRAP) was used to assess the reappearance of mRNA into a photobleached region of interest (Figure 4.20 A, B). MS2-tagged mRNA expressed in $\gamma 2\alpha'1$ dendrites was imaged before photobleaching, immediately after photobleaching, and subsequently one frame min^{-1} was captured for 10 subsequent time points (Figure 4.20 C). An example data set is shown in Figure 4.20 D-F. Here, a region of interest is drawn around several *NMDAR1*-24xMS2 molecules present in $\gamma 2\alpha'1$ dendrites (Figure 4.20 D-D''), which disappear following photobleaching within the ROI (Figure 4.20 E-E''). In this example, a newly appeared *NMDAR1*-24xMS2 molecule is observed 10 minutes later (Figure 4.20 F-F''). The experiment was performed for all MS2-tagged mRNA species. No reappearance events were observed with *CaMKII*-24xMS2, and the overall reappearance frequency for the other mRNA species was low (*Act5C*-24xMS2: 0.2, *NMDAR1*-24xMS2: 0.27, *DLG1*-24xMS2: 0.29, Table 5.4, Figure 4.21). Raw data can be found in Appendix Table 5.4. Although the FRAP laser successfully limits the region of photobleaching in x and y, it does not limit photobleaching in the z plane and consequently the ROI is photobleached throughout the z-plane of the entire $\gamma 2\alpha'1$ dendritic field (Figure 4.20 A, B). Visible mRNA molecules re-entering the ROI following photobleaching must therefore originate from the x and y positions around the ROI. Given the extent of photobleaching in the z plane, and the single particle tracking

data that suggests most motile behaviours are corralled diffusion, the low reappearance frequency may not be particularly surprising.

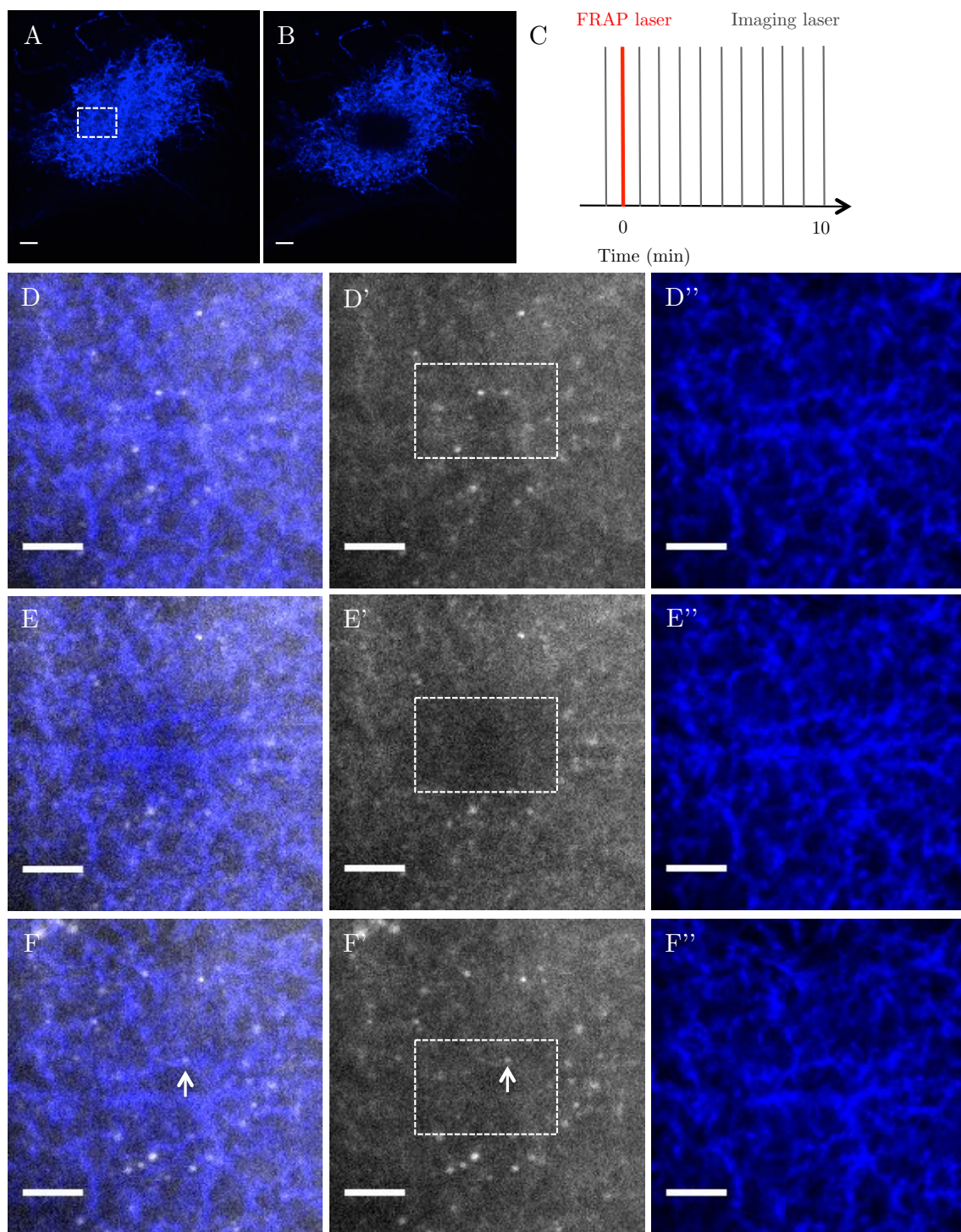


Figure 4.20: FRAP analysis of *NMDAR1*-MS2 mRNA motility in $\gamma 2\alpha'1$ dendrites. **A**: $\gamma 2\alpha'1$ dendrites with FRAP ROI selected. **B**: $\gamma 2\alpha'1$ dendrites photobleached in ROI from A. **C**: Schematic overview of experiment time line. **D**: Pre-photobleaching. Composite image of D' and D''. **E**: immediately post-photobleaching. Composite of E' and E''. **F**: 10 min post-photobleaching. Composite of F' and F''. **D'-F'**: *NMDAR1*-MS2 mRNA. **D''-F''**: $\gamma 2\alpha'1$ dendrites. ROI for photobleaching is indicated by the white dashed box. White arrow indicates newly appeared *NMDAR1*-MS2 mRNA molecule. Scale bar= $5\mu\text{m}$.

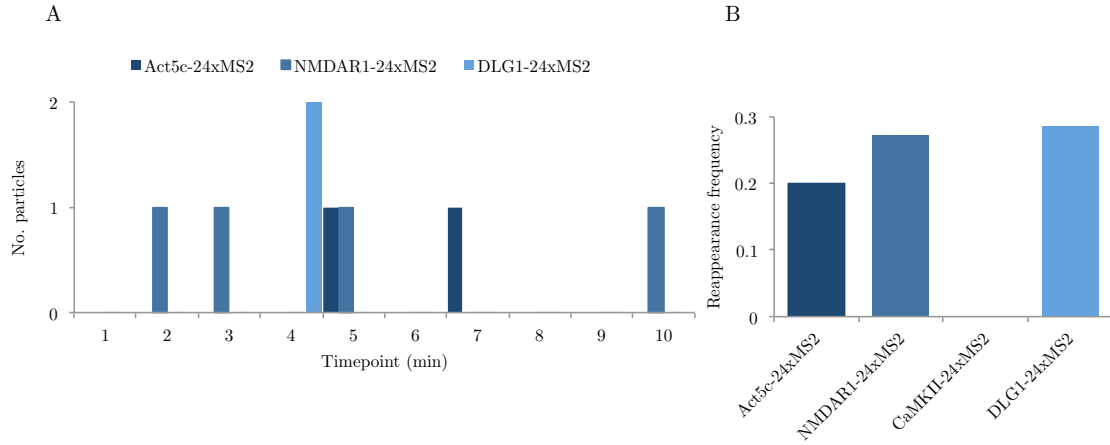


Figure 4.21: Analysis of mRNA motility using FRAP. **A:** mRNA particle reappearance events at 1 minute timepoints for *Act5c*-24xMS2, *NMDAR1*-24xMS2 and *DLG1*-24xMS2. **B:** Reappearance frequency of mRNA particles *Act5c*-24xMS2, *NMDAR1*-24xMS2 and *DLG1*-24xMS2. No reappearance events for *CaMKII*-24xMS2 were recorded. Experiment repeats (n): *Act5C*-24xMS2=10, *NMDAR1*-24xMS2=11, *CamKII*-24xMS2=8 and *DLG1*-24xMS2=8.

4.3 Conclusion

The development and application of the MS2 system described enabled mRNA to be visualised at single neuron resolution live in the *Drosophila* brain. Using an extensive available genetic toolkit, the expression of MS2 system components was targeted to defined memory-relevant neurons in the *Drosophila* brain. These include the $\gamma 2\alpha'1$ and M6/ $\gamma 5\beta'2a$ MBONs, along with the PAM- $\gamma 5$ subset of mushroom body innervating dopaminergic neurons. In each case, fluorescent spots representing mRNA were observable in presynaptic and postsynaptic compartments. smFISH using an oligonucleotide probe set targeting the 24xMS2 array verified these spots as molecules of mRNA. The concomitant use of two smFISH probe sets, one targeting the 24xMS2 array and another targeting a different region of the transcript would be necessary for unequivocal verification of the specific MS2 tagged mRNA.

Specific localisation patterns were also observed. *NMDAR1*-24xMS2 was preferentially localised to M6/ $\gamma 5\beta'2a$ presynaptic output terminals relative to dendrites. This specific

localisation pattern prompted the search for relevant glutamatergic inputs using the newly available whole *Drosophila* brain connectome. Glutamatergic inputs were indeed identified onto M6/ $\gamma 5\beta'2a$ axon terminals, indicating a requirement for glutamatergic receptors here. Moreover, specific localisation patterns were coherent with expected patterns of distribution of the encoded glutamatergic receptor. For example, localisation of *NMDAR1-24xMS2* in PAM- $\gamma 5$ dendrites, where they receive glutamatergic inputs from M6/ $\gamma 5\beta'2a$ presynapses, was observed. *NMDAR1*-MS2 mRNA localisation patterns therefore recapitulated glutamatergic receptor localisation patterns. These observations suggest that mRNA localisation is likely an important neural mechanism for targeting proteins to their appropriate subcellular compartment in memory-relevant neurons in the *Drosophila* brain. It will be important to verify the localisation of endogenous mRNA transcripts to these defined neural compartments with smFISH. Reassuringly however, smFISH was used to demonstrate that YFP tagged *CaMKII* mRNA localises within $\gamma 2\alpha'1$ dendrites (Chapter 3) in a similar manner to the MS2-tagged transgene. Whether these localised transcripts then serve to modify the local synaptic proteome through activity-dependent translation remains an exciting prospect.

Whether observed fluorescent spots represent single molecule mRNA or several transcripts in aggregate is not known, although it could be ascertained through stepwise photobleaching experiments. Variation in MS2 particle size and brightness was observed, however, and suggests in addition to variations in fluorescent protein loading onto the MS2 stem loop array, MS2-tagged mRNA copy number is a further contributing source of variation. mRNA merging events were also observed, suggesting mRNA copy number at individual fluorescent foci does contribute to particle size and brightness variability. The biological significance of mRNA merging events within *Drosophila* neurons remains to be seen. However it is interesting to speculate about such events in relation to both neuronal activity and the translatability of the transcript.

Live MS2-tagged mRNA was visualised in the whole *Drosophila* brain. Single particle tracking analysis revealed many *Act5C-24xMS2* to move short distances ($<2\ \mu\text{m}$) at

relatively slow velocities ($<0.3 \mu\text{m s}^{-1}$). In accordance with such kinetics, MSD analysis revealed most particle mobility to be corralled diffusion, and suggests most *Act5C*-24xMS2 mRNA particles are tethered. Long-range motility dynamics assessed with FRAP indicated such behaviours to be a feature of *Act5C*-24xMS2, *NMDAR1*-24xMS2 and *DLG1*-24xMS2 mRNA transcripts, albeit at low frequency. Thus although MS2 tagged mRNA can engage in long-range types of transport, the majority of particles appear to be tethered and relatively fixed in certain locations.

4.4 Future directions

The sum of the data presented indicate that the MS2 system can be successfully applied to visualise live mRNA at single neuron resolution within the whole *Drosophila* brain. Expression pattern profiling within defined memory relevant neurons and live molecular imaging open the door to many experimental possibilities. Of outstanding interest would be the responses of live mRNA to chemically or physiologically induced neuronal activity. In addition, the development of several untested reporter constructs that would enable the visualisation of mRNA and protein product offers the exciting prospect of probing local translation responses to neuronal activity *in vivo*. The well-described memory circuits of the *Drosophila* mushroom body coupled with MS2-labelled mRNA offer an exciting opportunity to visualise molecular responses to memory processes *in vivo* in real time.

Chapter 5

Neprilysins in *Drosophila* memory

5.1 Background

Neprilysins are a family of M13 zinc-metalloendopeptidases that function as catalysts of peptide hydrolysis. The majority of neprilysins are type II integral membrane proteins composed of an N-terminal cytoplasmic domain, a hydrophobic transmembrane domain and a C-terminal extracellular domain containing the active site (Turner et al., 2001). It is thought that they function primarily in the breakdown of small extracellular peptides, effectively terminating peptide signalling events at the cell surface (Turner et al., 2001). Mammalian neprilysins have been extensively studied for their medical relevance. In particular, they are responsible for the degradation of the amyloid- β peptide (Iwata et al., 2000, 2001), hypothesised to be a major contributor to Alzheimer's disease pathogenesis (Musiek and Holtzman, 2015), and as such have been a target of medical intervention. Indeed, delivery of soluble neprilysin was shown to reduce amyloid- β accumulation and ameliorate pathological symptoms in a mouse model of Alzheimer's disease (Hemming et al., 2007; Park et al., 2013). Outside of the nervous system, neprilysins degrade a diverse array of signalling peptides to regulate cardiovascular (McMurray, 2015; Volpe et al., 2015), renal (McMurray, 2015) and mitotic functions (Rosanò et al., 2013; Stephen et al., 2016).

In accordance with the diverse physiological roles of mammalian M13 endopeptidases,

Drosophila neprilysins are similarly implicated in diverse biological functions. Phylogenetic analyses have identified five *Drosophila* neprilysins (Neps 1-5) which represent orthologs of the single human neprilysin (Bland et al., 2008; Sitnik et al., 2014). The *Drosophila* family of neprilysins has been implicated in development (Hallier et al., 2016; Panz et al., 2012), reproduction (Sitnik et al., 2014), renal function (Thomas et al., 2005), locomotion (Bland et al., 2009), and memory (Turrel et al., 2016, 2017). Thus neprilysins in both mammals and in fruit flies serve as regulators of peptidergic signalling in a broad range of biological systems. This is reflected in their similarly broad range of substrate specificity. Known *Drosophila* neprilysin substrates include sNPF, allatostatin, tachykinins (Hallier et al., 2016), and the *Drosophila* amyloid- β peptide (Turrel et al., 2017).

Within the nervous system, neprilysin functions include the termination of neuropeptidergic synaptic signalling. Within the well-described circuitry of the *Drosophila* mushroom body, neuropeptidergic signalling is known to play several important functions. dNPF input to MB-MP1 neurons is known to signal hunger and promote appetitive memory expression in hungry flies (Krashes et al., 2009), while amnesiac expression in DPM neurons is thought to be required for memory consolidation (Keene et al., 2006; Waddell et al., 2000). Thus there is clear impetus for the requirement of systems to regulate memory-relevant peptidergic signalling in the mushroom body.

For almost 40 years *Drosophila* has been an invaluable model organism with which the contributions of various genes to behavioural phenotypes, including learning and memory, has been delineated (Benzer, 1973; Keene and Waddell, 2007; Quinn et al., 1974; Tully et al., 1994). This is primarily due to the amenability of the fruit fly to genetic manipulation, its relatively small brain (containing approximately 100,000 neurons) and sophisticated behavioural repertoire. *Drosophila* memory can be assessed using an associative olfactory conditioning paradigm in which an odour is paired either with a negatively (e.g. electric shock) or positively (e.g. sugar) reinforcing stimulus, and hence acquires by association the valence of that stimulus (Quinn et al., 1974; Tempel et al., 1983).

A memory of this association is assessed behaviourally by approach or avoidance of the conditioned odour. The neuropil responsible for this association is the mushroom body (Heisenberg et al., 1985), a bilateral structure composed of around 4000 intrinsic neurons called Kenyon cells. The parallel axons of three Kenyon cell subsets form the α/β , α'/β' , and γ lobes of the mushroom body where they synapse with a relatively small number of outgoing mushroom body output neurons (MBONs) (Aso et al., 2014). Mushroom body-innervating dopaminergic neurons are responsible for assigning valence to Kenyon cell odour representations (Waddell et al., 2000). They modulate Kenyon cell-MBON synapses to mediate behavioural output (Owald and Waddell, 2015). The neural architecture of the *Drosophila* mushroom body along with the contributions of defined subsets of intrinsic neurons, reinforcing dopaminergic neurons and output neurons is providing unparalleled insights into the physiological mechanisms of memory coding.

5.1.1 Aims

The aim of this work was to study the involvement of neprilysins in *Drosophila* memory, specifically neprilysin 1 (Nep1) and neprilysin 3 (Nep3). Mushroom body expression of Nep1 had been demonstrated previously (Sitnik et al., 2014) and it was presumed that neprilysins would function as homeostatic regulators of peptidergic signalling. CRISPR/Cas9 was used to site-specifically modify the respective genetic loci to create knock-out alleles and GFP fusion proteins. In this way, the contributions of Nep1 and Nep3 to *Drosophila* memory and the expression patterns of the respective genes could be assessed.

5.2 Results and discussion

5.2.1 Characterisation of mutant alleles

In order to understand the contribution of Nep1 and Nep3 to memory formation in *Drosophila*, CRISPR/Cas9 was used to site-specifically disrupt the respective genetic loci. In both cases, the first coding exon was targeted for disruption since it contains the start

codon, and presumably relevant regulatory sequences, shared by all four isoforms of Nep1 and all three isoforms of Nep3. Details of the CRISPR/Cas9 mutagenesis approach can be found in Materials and Methods section 2.6, subsection 2.6.1 CRISPR/Cas9 generation of mutant alleles. A schematic of the Nep1 and Nep3 loci can be found in Figure 2.6.2 in Materials and Methods.

Two independent knock-out lines for Nep1 were obtained: Nep1KO (2) and Nep1KO (4), and three independent knock-out lines for Nep3 were obtained: Nep3KO (8), Nep3KO (19) and Nep3KO (23). RT-qPCR was used to characterise the effect of targeted deletion of the first coding exon on the expression of Nep1 and Nep3 mRNA using primers targeting downstream exons. In both Nep1 knock-out lines, the expression of Nep1 (Figure 5.1 A and B) was reduced by over 99% compared to wild-type (CS) flies. In all three Nep3 knock-out lines, the expression of Nep3 (Figure 5.1 C and D), was similarly reduced by over 97% compared to wild-type (CS) flies. The removal of the first coding exon of Nep1 and Nep3 effectively knocked-out the expression of the respective mRNA and hence protein. The Nep1 and Nep3 knock-out alleles can therefore be considered null, or amorphic.

5.2.2 Aversive learning and memory

Homozygous Nep1 and Nep3 knock-out lines were tested for learning and memory using an aversive associative olfactory conditioning paradigm. Both lines were tested along with wild-type (CS) flies as the control group. It should be noted that knock-out lines had not yet been back crossed to CS. The genetic backgrounds of the mutant and wild-type strains are therefore not homogeneous and it will be important in the continuation of this work to address this. Nevertheless, as an initial behavioural screen of learning and memory performance these lines were compared. Both Nep1KO lines (2 and 4) were screened while only the Nep3KO (23) was used since Nep1KO behavioural testing began before RT-qPCR experiments confirmed both lines were indeed genetic nulls.

The acquisition phase of memory, or learning, was assessed by testing the relative avoidance of an electric shock-paired odour immediately after training. Both Nep1 and

Nep3 knock-out lines displayed significantly lower performance indices compared to CS flies (Figure 5.2 A and C). Compared to the wild-type CS control therefore, Nep1 and Nep3 knock-outs have a reduced learning phenotype. The formation of medium-term memory (MTM) was then assessed by testing the relative avoidance of an electric shock-paired odour 2h after training. Both Nep1 and Nep3 knock-out lines displayed significantly lower performance indices compared to CS flies (Figure 5.2 B and D). In addition to the learning deficiency phenotype therefore, Nep1 and Nep3 knock-outs also have a 2h memory deficit.

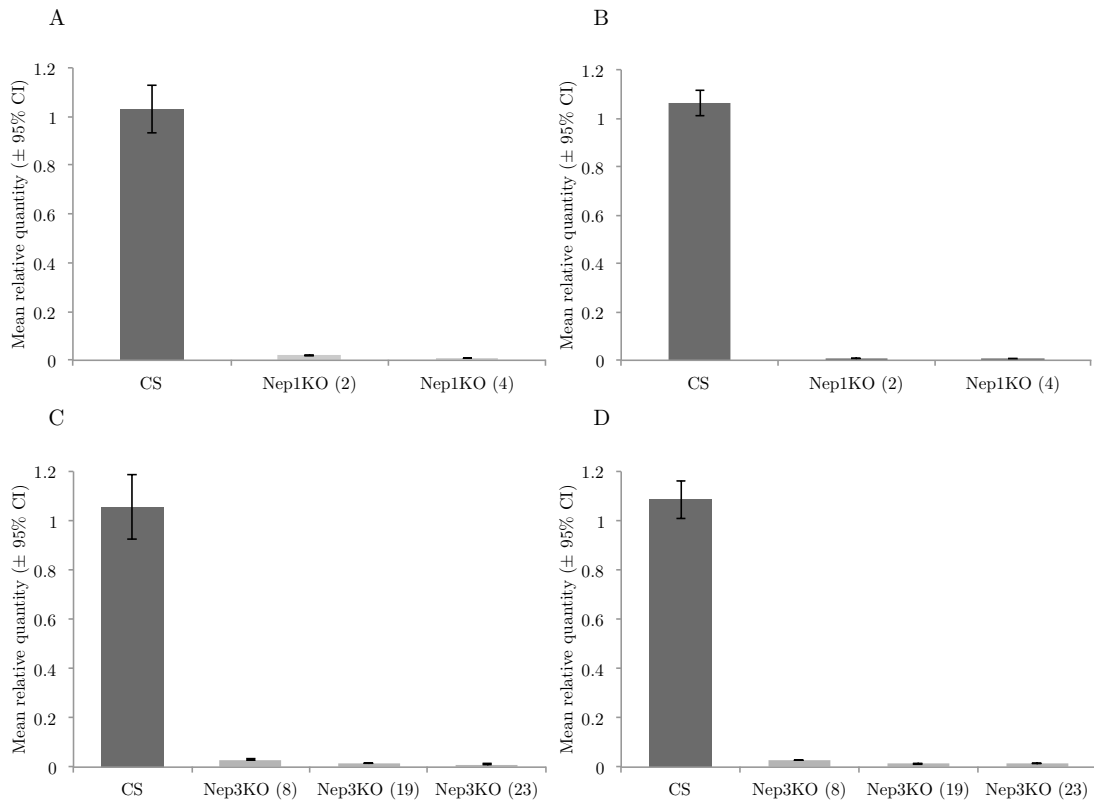


Figure 5.1: Mean relative expression (±95% confidence intervals) of Nep1 in CS, Nep1KO (2) and Nep1KO (4) mutants using primer set Nep1 6 (**A**) and primer set Nep1 8 (**B**). Mean relative expression (±95% confidence intervals) of Nep3 in CS, Nep3KO (8), Nep3KO (19) and Nep3KO (23) mutants using primer set Nep3 4 (**C**) and primer set Nep3 10 (**D**). Primer nucleotide sequences can be found in Appendix Table 5.4.

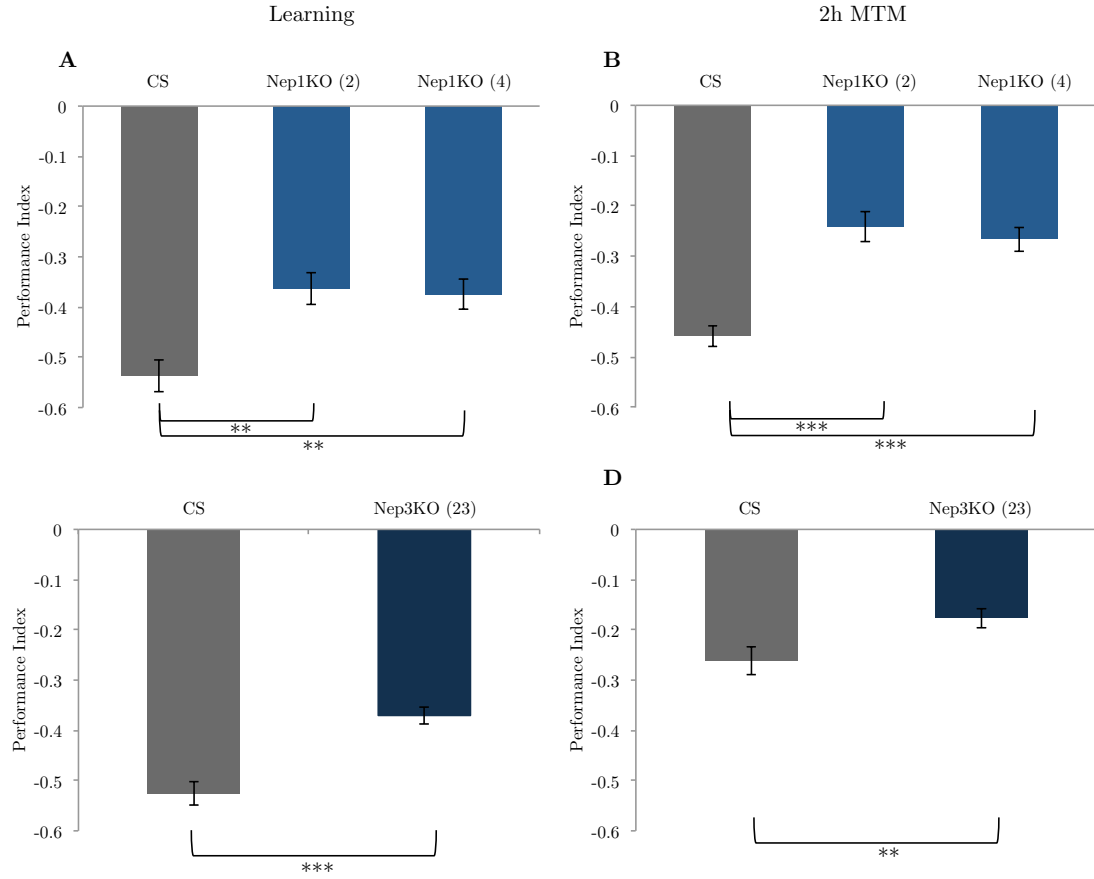


Figure 5.2: Aversive learning (immediate memory) and 2h MTM (mean performance index $\pm$ s.e.m.) in CS (WT), 2 Nep1 amorphic lines, and one Nep3 amorphic line. **A:** Nep1 aversive learning. Performance index of the CS group is significantly different to both Nep1KO (2) (T-test: $p=0.001$, $t=-3.7819$, $n=12$) and Nep1KO (4) (Wilcoxon rank sum test: $p=0.0006$, $z=-3.4352$, $n=12$). **B:** Nep1 aversive 2h MTM. Performance index of the CS group is significantly different to both Nep1KO (2) (T-test: $p<0.0001$, $t=-6.1398$, $n=12$) and Nep1KO (4) (T-test: $p<0.0001$, $t=-5.7710$, $n=12$). **C:** Nep3 aversive learning. Performance index of the CS group is significantly different to Nep3KO (23) (T-test: $p<0.0001$, $t=-5.5194$, $n=14$). **D:** Nep3 aversive 2h MTM. Performance index of the CS group is significantly different to Nep3KO (23) (Wilcoxon rank sum test: $p=0.0032$, $z=-2.9473$, $n=32$).

5.2.3 Acuity to conditioned stimuli

In order to test the acuity of Nep1KO and Nep3KO flies to the conditioned stimuli, the naive avoidance behaviour of electric shock and odours relative to the absence of such stimuli was assessed in a T-maze. For Nep1KO (2), Nep1KO (4) and Nep3KO (23), electric shock avoidance was equivalent to wild-type (CS) performance (Figure 5.3 A and C). However, naive odour avoidance in all three mutant lines was significantly reduced relative to wild-type (CS) (Figure 5.3 B and D).

Equivalent behavioural responses of mutant flies to conditioning stimuli have long been considered evidence that the animal's sensory systems retain the ability to perceive the relevant stimuli (Tully et al., 1994). It follows therefore that an inability to learn must result from a defect in the physiological association of the stimuli as opposed to the sensory perception of the stimuli. However the T-maze avoidance paradigm is merely a readout of the animal's behavioural response to a stimulus, there is no physiological indication of the animal's perception of it. It therefore does not distinguish between the possibility that the animal does not perceive the stimulus or that the animal does not behave equivalently towards it. The reduced odour avoidance behaviour of Nep1 and Nep3 mutants therefore may either be a result of a physiological perturbation of odour perception, or in the behavioural response to the odours which are perceived normally. In order to satisfactorily delineate these two possibilities, it would be necessary to record the odour responses of relevant neurons with two-photon calcium imaging.

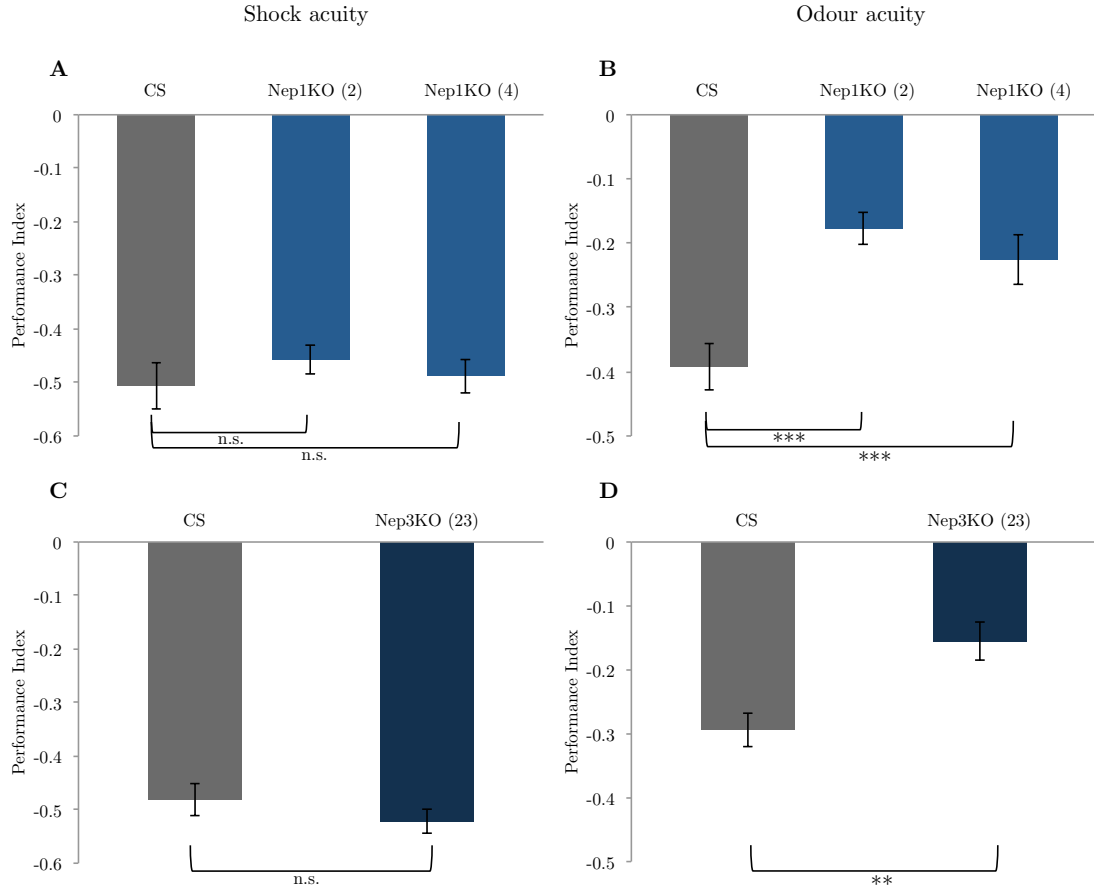


Figure 5.3: Electric shock and odour acuity (mean performance index $\pm s.e.m.$) in CS (WT), 2 Nep1 amorphous lines, and one Nep3 amorphous line. **A**: Nep1 shock acuity. Performance index of the CS group is not significantly different to both Nep1KO (2) (T-test: $p=0.3416$, $t=-0.9769$, $n=12$) and Nep1KO (4) (T-test: $p=0.7255$, $t=-0.3567$, $n=12$). **B**: Nep1 Odour acuity. Performance index of the CS group is significantly different to both Nep1KO (2) (T-test: $p<0.0001$, $t=-4.9076$, $n=12$) and Nep1KO (4) (T-test: $p=0.0056$, $t=-3.1461$, $n=12$). **C**: Nep3 shock acuity. Performance index of the CS group is not significantly different to Nep3KO (23) (Wilcoxon rank sum test: $p=0.2366$, $z=1.18367$, $n=12$). **D**: Nep3 odour acuity. Performance index of the CS group is significantly different to Nep3KO (23) (T-test: $p=0.0021$, $t=-3.4777$, $n=12$).

5.2.4 Nep1::GFP protein and mRNA expression in the *Drosophila* brain

The expression of Nep1 in the adult *Drosophila* brain was visualised by an in-frame CRISPR/Cas9 mediated knock-in of a GFP coding sequence to the N-terminal of the *Nep1* genetic locus. The same approach was taken for Nep3. However, at the time of writing, Nep3::GFP positive flies had not yet been obtained. Relatively high Nep1::GFP expression was seen in the antennal lobes, the ellipsoid body and the mushroom body (Figure 5.4 A). Within the antennal lobes, particularly high expression of Nep1 was observed within the olfactory receptor neurons targeting what appear to be the DL3 or DA1 glomeruli (Laissue and Vosshall, 2008). Mushroom body α/β and γ lobes were specifically labelled (Figure 5.4 A), and Nep1::GFP intensity appeared not to be homogeneous. Particularly high expression was observed in the β lobes and at the tips of the α lobes, while γ lobe expression was weaker. In addition to the lobes, Nep1::GFP was also expressed in the mushroom body calyx (Figure 5.4 B). Using a GFP smFISH probe set, mRNA localisation in the mushroom body calyx was visualised. Fluorescent smFISH puncta were observed both in the cell body and dendritic regions of the calyx (Figure 5.4 B' and B''). The presence of *Nep1* mRNA transcripts localised extrasomatically suggests that decentralised translation of localised mRNA transcripts may be an important mechanism of regulating Nep1 gene expression in the mushroom body.

Neuropeptidergic signalling is widespread in the *Drosophila* central complex, including the ellipsoid body and neurons innervating it (Kahsai and Winther, 2011). The high expression of Nep1 invokes an important function of this peptidase in the regulation of neuropeptidergic signalling in the ellipsoid body. The specificity of Nep1 expression in the DL3 or DA1 glomeruli of the antennal lobe is particularly interesting. sNPF is also highly expressed in these glomeruli relative to other glomeruli (Carlsson et al., 2010), raising the possibility that Nep1 is a regulator of sNPF signalling here.

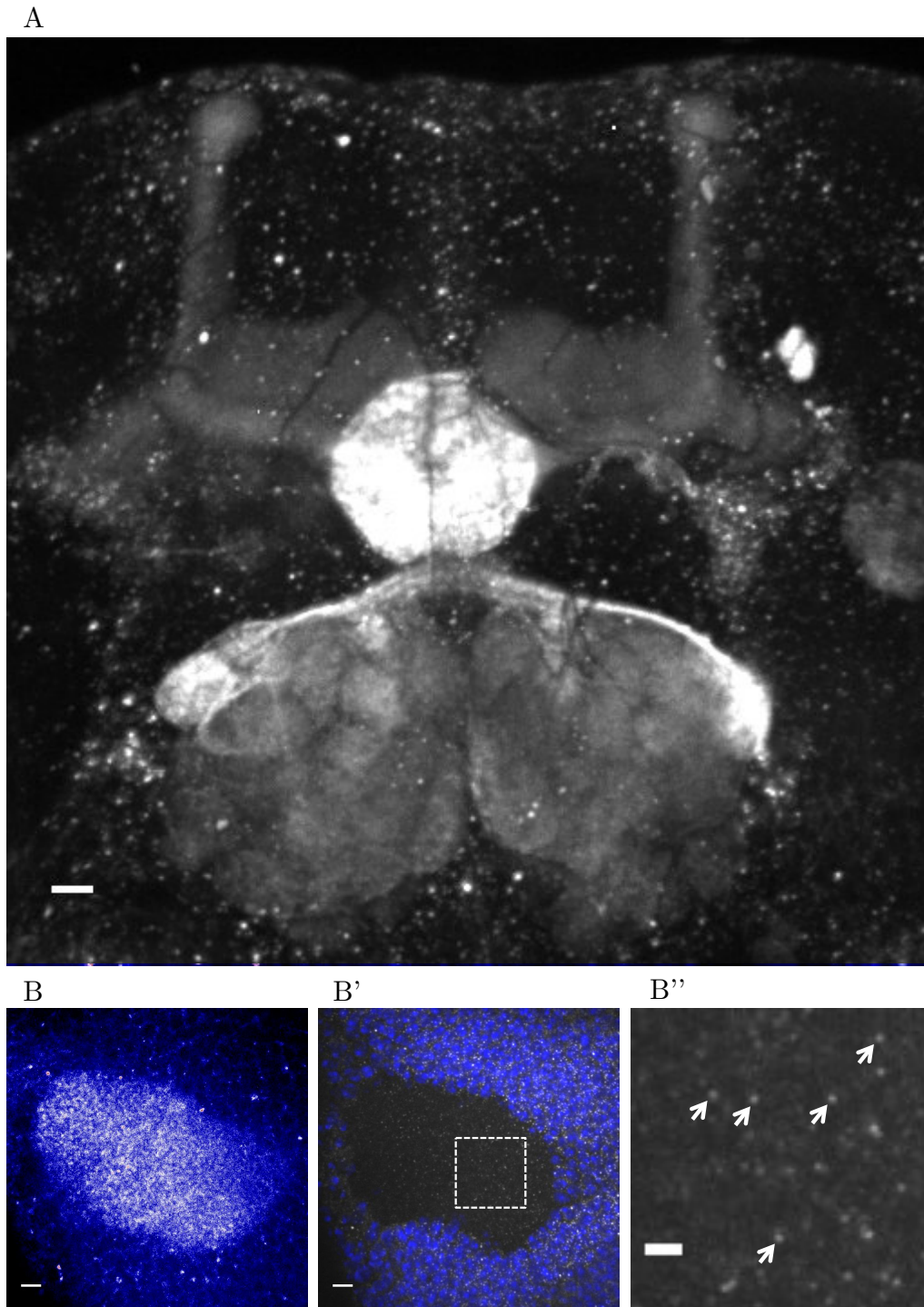


Figure 5.4: **A**: Nep1::GFP expression in the adult *Drosophila* brain. Scale bar=20 μ m. **B-B''**: Nep1::GFP protein and mRNA expression in the mushroom body calyx. **B**: Nep1::GFP protein. Scale bar=5 μ m. **B'**: *Nep1::GFP* mRNA with DAPI stained cell bodies visualised in blue. Scale bar=5 μ m. **B''**: Magnification of white hatched box in B'. White arrows indicate Nep1 mRNA. Scale bar=2 μ m.

5.2.5 The involvement of neprilysins in *Drosophila* memory

During the preparation of this work, Turrel et al. (2016) published their findings on the effect of conditional RNAi-mediated knock-down of *Drosophila* Nep1, Nep2, Nep3 and Nep4 on learning and memory performance. The authors report that Nep1 is the only neprilysin required in α/β Kenyon cells for 2h MTM and that no neprilysin is required in the mushroom body for learning. In addition, they find a role for both Nep1 and Nep3 in MTM and LTM in the DPM neurons, two large mushroom body innervating neurons required for memory consolidation (Keene et al., 2006; Waddell et al., 2000). Interestingly, the expression of Nep1 in α/β Kenyon cells (Figure 5.4) would seem to corroborate its involvement in 2h MTM (Turrel et al., 2016). No deficit in odour acuity is reported for mushroom-body or DPM specific RNAi-mediated knock-down of neprilysin expression (Turrel et al., 2016).

The data presented here are in accordance with the involvement of Nep1 in 2h MTM. However, involvement of both Nep1 and Nep3 in learning, in addition to the involvement of Nep3 in 2h MTM are not consistent. It is important therefore to highlight the differences in experimental approaches and their implications. Spatially and temporally restricting the expression of RNAi constructs as a means of reducing the expression level of a target gene of interest has variable and unpredictable effects. In this case, the strongest RNAi knock-down effect observed was for Nep1 (75%), while Nep3 expression is knocked down by only 30% (Turrel et al., 2016). This complicates any comparison between experiments, given that the nature of the manipulation is not equal. A negative result therefore does not necessarily imply a lack of involvement since small reductions in mRNA levels may be not be sufficient to cause physiological change. Moreover, the authors report some functional redundancy between the neprilysins (Turrel et al., 2016), suggesting that small knock-downs in the expression of one gene may be compensated for by another. Interestingly, Nep1 was concluded as the most important neprilysin for memory formation, and incidentally was the gene whose level of expression was most perturbed. In contrast, targeted manipulation of the Nep1 and Nep3 genetic loci by CRISPR/Cas9 resulted in global knock-out of gene expression. However in addition to the certainty of genetic manipu-

lation there is a concomitant lack of temporal or spatial specificity. This could however be addressed by the reintroduction of rescue constructs in defined cell subsets of interest. Despite differences in experimental approaches and their associated pitfalls, both data sets indicate that neprilysins play an important role in memory processes in *Drosophila*. Moreover expression of Nep1 in the mushroom body substantiates this conclusion.

Olfactory processing

The deficiency in odour acuity of both Nep1 and Nep3 mutants might reasonably be interpreted as confounding any conclusions regarding olfactory learning and memory processes. Indeed this will be an important point for future resolution. The relatively high expression of Nep1 in the antennal lobe does indicate an involvement of this neprilysin in olfactory processing. It is known that the projection neurons which convey odour information from the antennal lobe to higher order brain structures including the mushroom body express neuropeptides that serve neuromodulatory functions (Ignell et al., 2009; Wang et al., 2013; Wilson, 2013). Neprilysins may therefore be required within this circuitry to regulate neuropeptidergic synaptic signalling. Thus any disruption to neprilysin function and hence neuropeptide signalling homeostasis might plausibly affect olfactory processing in the *Drosophila* brain.

5.2.6 Implications for peptidergic signalling systems in the *Drosophila* mushroom body

The expression of Nep1 in the *Drosophila* α/β and γ lobes of the mushroom body, along with the observed memory impairment of Nep1 and Nep3 mutants, suggests an important use of neuropeptides as neurotransmitters and/or neuromodulators in the mushroom body. Neprilysins may also play important roles in the processing of neuromodulatory peptides, in addition to terminating their signalling. The first indication of the involvement of neuropeptides in *Drosophila* memory came from a behavioural screen for memory mutants in which amnesiac was identified (Quinn et al., 1979). Amnesiac mutant flies learn equivalently to wild-type flies, but their memory rapidly decays within 30 minutes (Quinn et al., 1979). The amnesiac mutant was subsequently predicted to encode a neuropeptide

precursor product (Feany and Quinn, 1995), and found to be expressed in the *Drosophila* DPM neurons (Waddell et al., 2000), which are required for memory consolidation (Keene et al., 2006). Curiously the peptide product of the amnesiac gene has never been identified. Despite this, advanced single cell transcriptomics of the *Drosophila* brain does detect amnesiac mRNA transcripts (Croset et al., 2018). The requirement of Nep1 and Nep3 in DPM neurons for MTM and LTM prompted the suggestion of an interaction between amnesiac and Neprilysins in DPM neurons (Turrel et al., 2016). Such a link awaits further investigation.

The mushroom body is also known to express a number of other neuropeptides. In fact, the expression of sNPF in α/β and γ Kenyon cells (Johard et al., 2008; Knapek et al., 2013) was known before the major Kenyon cell neurotransmitter, acetylcholine, was deciphered (Barnstedt et al., 2016). Coincidentally, Nep1 expression in the mushroom body was also localised to the α/β and γ lobes (Figure 5.4). It is tempting to speculate a role for Nep1 in regulating sNPF signalling in α/β and γ Kenyon cells. In addition, monoaminergic neurons have recently been shown to commonly co-express neuropeptides (Croset et al., 2018). A possible role for Nep1 expression in mushroom body α/β and γ lobes could therefore be the termination of peptidergic signalling from dopaminergic neurons that are known to assign valence signals to conditioned stimuli.

5.3 Conclusion

The generation of Nep1 and Nep3 amorphic alleles enabled an assessment of the role of these peptidases in olfactory learning and memory. Defects in olfactory learning, 2h MTM and naive odour avoidance responses were observed in both mutants. Furthermore, the expression of Nep1 was visualised in antennal lobes as well as the α/β and γ lobes of the mushroom body. It is likely that the global knock-out of Nep1, including from the antennal lobes and the mushroom body, disrupted peptidergic signalling concomitantly in both olfactory processing and associative brain structures. Together with the work of

(Turrel et al., 2016) however, the work presented here suggests that *Drosophila* neprilysins 1 and 3 play an important role in the process of memory formation.

5.4 Future directions

It will be interesting to compare Nep1 and Nep3 expression patterns with the generation of an N-terminal GFP tagged Nep3 line. It will be important to delineate antennal lobe and mushroom body neprilysin functions to understand memory functions in the context of normal olfactory processing. This could be achieved by restoring expression levels to the olfactory lobes using Gal4 driven wild-type neprilysin rescue constructs in the relevant Nep1/3 mutant background. Similarly, restoring neprilysin expression levels through targeted expression of rescue constructs in Kenyon cell subsets would enable the mushroom body specific contribution of neprilysins to be assessed. In the long-term, it would also be interesting to characterise the peptide substrates of Nep1 and Nep3, and relate such information to the neuropeptides known to be expressed by the monoaminergic neurons (Croset et al., 2018) which provide modulatory input to the mushroom body, where at least Nep1 is expressed.

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Appendix

Table 5.1: Primer sequences used for cloning.

Primer name	5' - 3' nucleotide sequence
F CAMKII 3'UTR	GCGGCCAGATCTTGGGCATTAATCAATGG
R CAMKII 3'UTR	GCGGCCGCGCGCCTCGAGAAATTAATAAAATTTG
F CAMKII	GCGGCCAAGCTTGGGCCCCGATAATACATTTTCAGAC
R CAMKII	GCGGCCGAGCTCCTAAGCGTAATCTGGAACATCGTATGGGTA TTTTTGGGGTATAAAATCG
F NMDAR1 3'UTR	GCGGCCAGATCTGTTAACATTTTGCCAAAGC
R NMDAR1 3'UTR	GCGCCGCGGCCGCGCAGCTTTAAATATTTATTTTAG
F NMDAR1	CAATTAGGTACCGGGCCCAGTAGACGAGCAAC
R NMDAR1	GTCGCATCTGGTCGTCTACCCATACGATGTTCCAGATTACGC TTAAGGATCCGGCCGC
F DLG1 3'UTR	GCGGCCAGATCTACAACCAACAATCAC
R DLG1 3'UTR	GCTATGCGGCCGCGAGTTTGCTGTTGATTCTTG
F DLG1	GAATCCAAGCTTGGGCCCCGGCACTTGGGCGCGAAG
R DLG1	GCGGCCGAGCTCTCAAGCGTAATCTGGAACATCGTATGGGTA GATGCTCGACTTACG
F pUAST attB	TCAACTGCAACTACTGA
R pUAST attB	TCATCAGTTCCATAGGTTGG
F KpnI-SNAP	TGACCGGGTACCATGGACAAAGACTGCG
R PspOMI-SNAP	TTAATTGGGCCCCACCCAGCCCAGGC

F EcoRI-PCP	GGCGCCGAATTCATGGGCCCAAAAAAGAAAAG
R PCP-NdeI-NotI	TATTAAGCGCCCGCCATATGTCTAGAATCACGGCCCAGCG
F NdeI-Halo	GCGGCCCATATGGCAGAAATCGGTACTGGCTTTCC
R Halo-PspXI	GCGGCCCTCGAGCCTAGATATCTCGAGTGCGGCC

Table 5.2: Nucleotide sequences of YFP smFISH probe set

Probe number	Nucleotide sequence (5'-3')
1	CGGTGAACAGCTCCTCGC
2	GACCAGGATGGGCACCAC
3	GTTTACGTGCGCGTCCAG
4	CCGGACACGCTGAACTTG
5	TTGCCGTAGGTGGCATCG
6	CCGGTGGTGCAGATGAAC
7	AGGGTGGTCACGAGGGTG
8	AAGCACTGCACGCCGTAG
9	ATGTGGTCGGGGTAGCGG
10	TGAAGAAGTCGTGCTGCT
11	ACGTAGCCTTCGGGCATG
12	AGAAGATGGTGCGCTCCT
13	TCTTGTAGTTGCCGTCGT
14	TCGAACTTCACCTCGGCG
15	TCGATGCGGTTACACAGG
16	TGAAGTCGATGCCCTTCA
17	CAGGATGTTGCCGTCCTC
18	TTGTACTCCAGCTTGTGC
19	AGACGTTGTGGCTGTTGT
20	CTGCTTGTCGGCCATGAT
21	TTCACCTTGATGCCGTTC
22	CGATGTTGTGGCGGATCT

23	TAGTGGTCGGCGAGCTGC
24	CGATGGGGGTGTTCTGCT
25	TTGTCGGGCAGCAGCACG
26	GACTGGGTGCTCAGGTAG
27	GTTGGGGTCTTTGCTCAG
28	ACCATGTGATCGCGCTTC
29	CGGTCACGAACTCCAGCA
30	TCCATGCCGAGAGTGATC

Table 5.3: Nucleotide sequences of GFP smFISH probe set

Probe number	Nucleotide sequence (5'-3')
1	AAGTTCTTCTCCTTTACTCA
2	GAATTGGGACAACCTCCAGTG
3	CCCATTAACATCACCATCTA
4	CCTCTCCACTGACAGAAAAT
5	GTAAGTTTTCCGTATGTTGC
6	GTAGTTTTCCAGTAGTGCAA
7	ACAAGTGTTGGCCATGGAAC
8	GCATTGAACACCATAAGAGA
9	TCATGCCGTTTCATATGATC
10	GGGCATGGCACTCTTGAAAA
11	TTCTTTCCTGTACATAACCT
12	GTTCCCGTCATCTTTGAAAA
13	TGACTTCAGCACGTGTCTTG
14	TAACAAGGGTATCACCTTCA
15	ATACCTTTTAACTCGATTCT
16	GTGTCCAAGAATGTTTCCAT
17	GTGAGTTATAGTTGTATTCC
18	GTCTGCCATGATGTATACAT

19	CTTTGATTCCATTCTTTTGT
20	CCATCTTCAATGTTGTGTCT
21	ATGGTCTGCTAGTTGAACGC
22	CGCCAATTGGAGTATTTTGT
23	GTCTGGTAAAAGGACAGGGC
24	AAGGGCAGATTGTGTGGACA
25	TCTTTTCGTTGGGATCTTTC
26	TCAAGAAGGACCATGTGGTC
27	AATCCCAGCAGCTGTTACAA
28	TATAGTTCATCCATGCCATG

Table 5.4: Nucleotide sequences of 24xMS2 smFISH probe set

Probe number	Nucleotide sequence (5'-3')
1	TCGTGCTTTCTTGGCAATAA
2	CGTTTGAAGATTCGACCTGG
3	AATACTGGAGCGACGCGTGA
4	ACCGTAGGATCTGATGAACC
5	TCGTGCTTTCTTGGCAATAA
6	CGTTTGAAGATTCGACCTGG
7	AATACTGGAGCGACGCGTGA
8	ACCGTAGGATCTGATGAACC
9	TCGTGCTTTCTTGGCAATAA
10	CGTTTGAAGATTCGACCTGG
11	AATACTGGAGCGACGCGTGA
12	ACCGTAGGATCTGATGAACC
13	TCGTGCTTTCTTGGCAATAA
14	CGTTTGAAGATTCGACCTGG
15	AATACTGGAGCGACGCGTGA

16	ACCGTAGGATCTGATGAACC
17	TCGTGCTTTCTTGGCAATAA
18	CGTTTGAAGATTCGACCTGG
19	AATACTGGAGCGACGCGTGA
20	ACCGTAGGATCTGATGAACC
21	TCGTGCTTTCTTGGCAATAA
22	CGTTTGAAGATTCGACCTGG
23	AATACTGGAGCGACGCGTGA
24	ACCGTAGGATCTGATGAACC
25	TCGTGCTTTCTTGGCAATAA
26	CGTTTGAAGATTCGACCTGG
27	AATACTGGAGCGACGCGTGA
28	ACCGTAGGATCTGATGAACC
29	TCGTGCTTTCTTGGCAATAA
30	CGTTTGAAGATTCGACCTGG
31	AATACTGGAGCGACGCGTGA
32	ACCGTAGGATCTGATGAACC
33	TCGTGCTTTCTTGGCAATAA
34	CGTTTGAAGATTCGACCTGG
35	AATACTGGAGCGACGCGTGA
36	ACCGTAGGATCTGATGAACC
37	TCGTGCTTTCTTGGCAATAA
38	CGTTTGAAGATTCGACCTGG
39	AATACTGGAGCGACGCGTGA
40	ACCGTAGGATCTGATGAACC
41	TCGTGCTTTCTTGGCAATAA
42	CGTTTGAAGATTCGACCTGG
43	AATACTGGAGCGACGCGTGA
44	ACCGTAGGATCTGATGAACC
45	TCGTGCTTTCTTGGCAATAA

46	GTGATCGTCGTCGTTTGAAG
47	TGAACCCTGGAATACTGGAG

Table 5.5: gRNA sequences used for CRISPR/Cas9 targeted mutagenesis

gRNA name	5' - 3' nucleotide sequence
Nep1a	GCAGCGGTGCGGCGTATCAGCGG
Nep1b	GATACCAGATACTATGTAGTTGG
Nep3a	CGGTATCTATAACAATCCGAGCGG
Nep3b	CTGCATTTTCATGAGTCGTTGCGG

Table 5.6: Primer sequences used for RT-qPCR

Primer name	5' - 3' nucleotide sequence
F Nep1 6	TCCGCAAGATCCTAATGGGC
R Nep1 6	GTTGTCCCGGATGAAGAGGG
F Nep1 8	GGCTGCACTATCTCACCGAA
R Nep1 8	ATCAAGGACATGACCAGGCG
F Nep3 4	AGCGGTTTAAACGAAAAGTCCG
R Nep3 4	CGGCTATATTTTCGCCCAACG
F Nep3 10	AAGTCCGAATGCATTGCCAG
R Nep3 10	GGCTATATTTTCGCCCAACGTC
F 1433 ϵ	GAGCGCGAGAACAAATGTGTA
R 1433 ϵ	TGGAGGCGACCTTCTTCAT
F α -Tub48B	TTGTCGCGTGTGAAACACTT
R α -Tub48B	CCGTGCTCCAAGCAGTAGA
F Rpl32	CGGATCGATATGCTAAGCTGT
R Rpl32	CGACGCACTCTGTTGTCTG

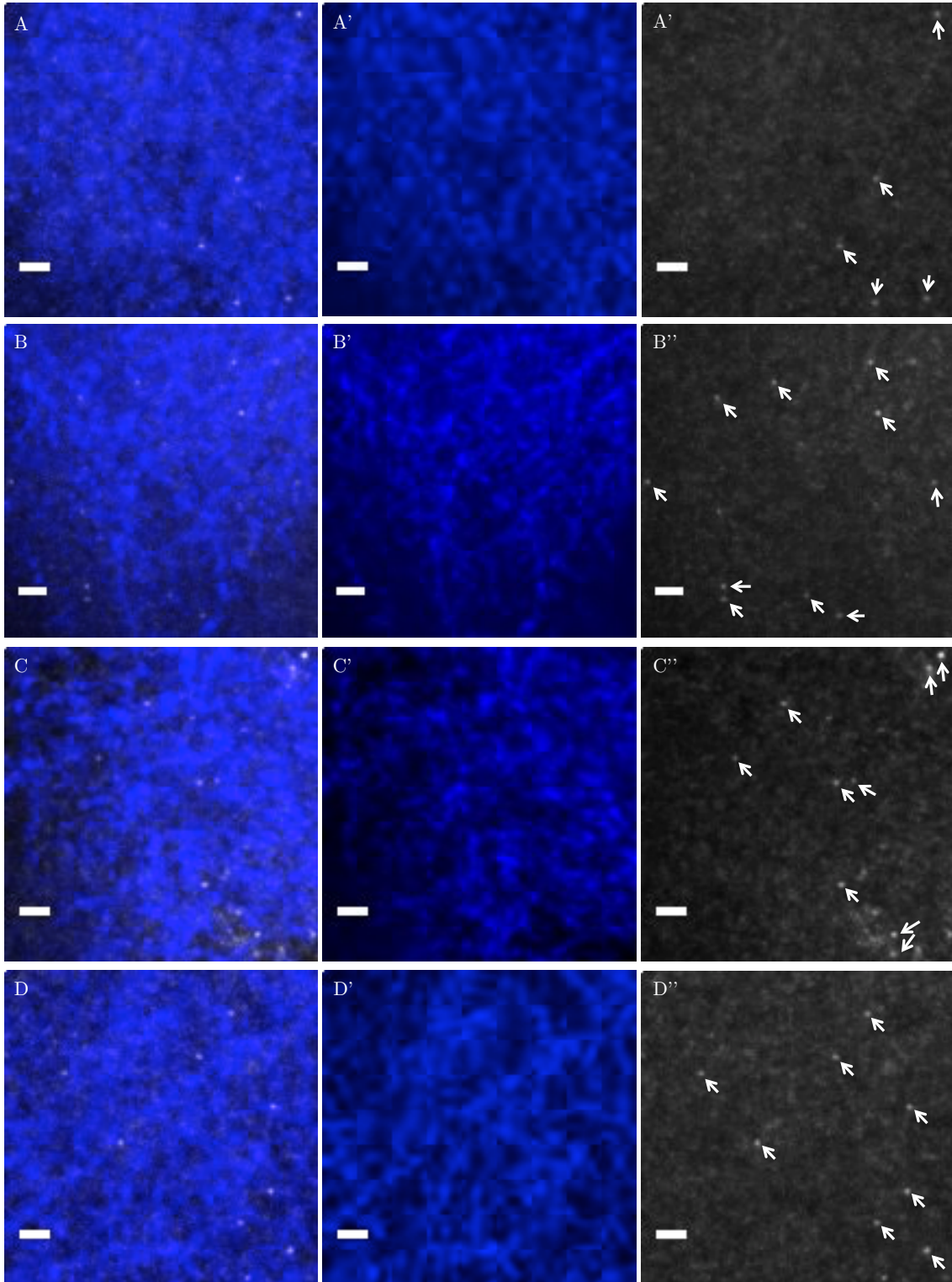


Figure 5.5: *CaMKII* smFISH in $\gamma 2\alpha'1$ dendrites in **A**: Non-trained group, and **B**: Immediately, **C**: 2h, and **D**: 24h following aversive olfactory conditioning. **A-D**: Composite of A'-D' and A''-D''. **A**: $\gamma 2\alpha'1$ dendrites. **A''-D''**: *CaMKII* smFISH. White arrows indicate *CaMKII* smFISH fluorescent puncta. Scale bar=2 μ m.

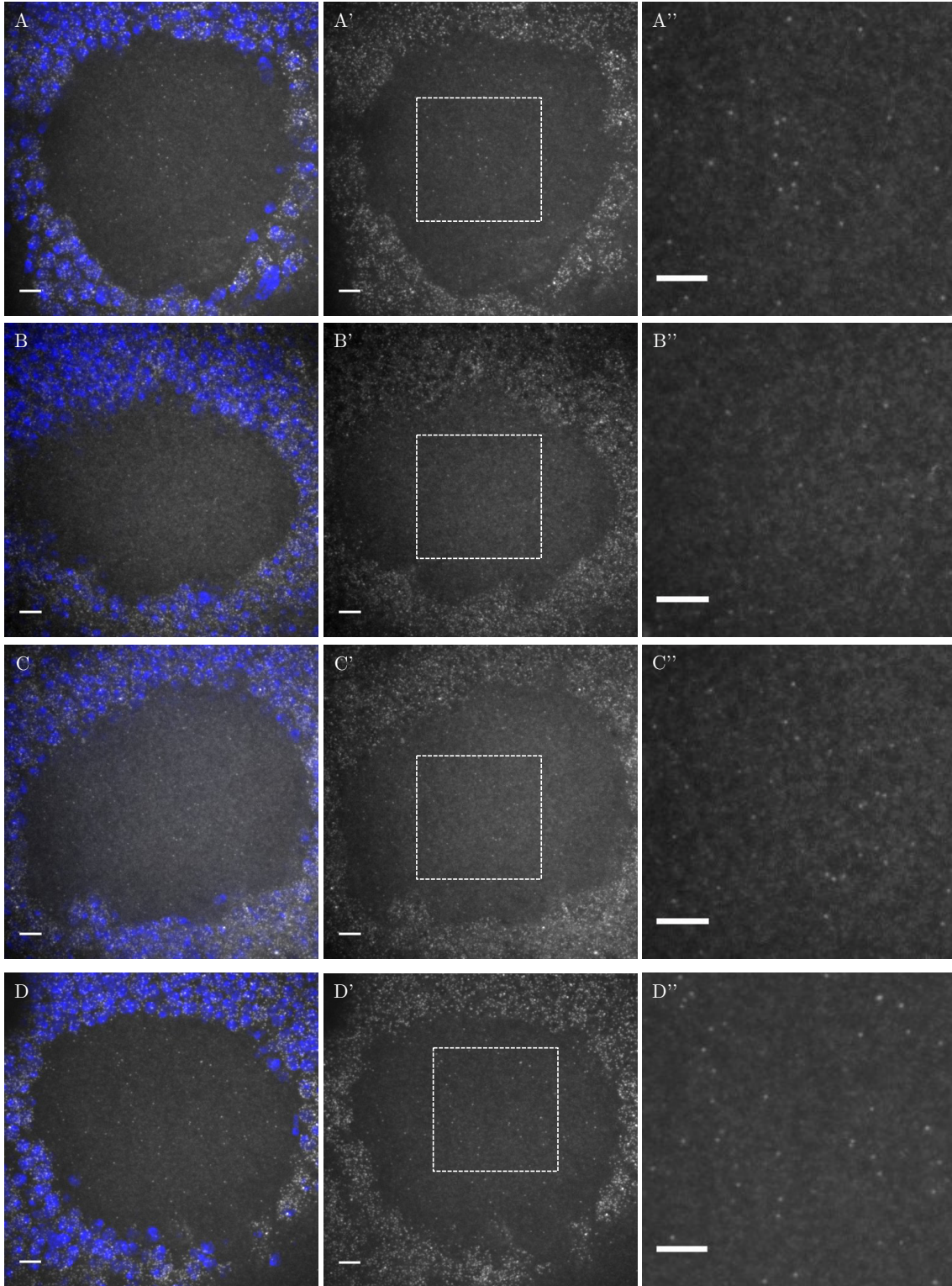


Figure 5.6: *CaMKII* smFISH in mushroom body calyx in **A**: Non-trained group, and **B**: Immediately, **C**: 2h, and **D**: 24h following aversive olfactory conditioning. **A-D**: Mushroom body calyx with cell bodies in blue stained with Vybrant DyeCycle Violet Stain and *CaMKII* smFISH signal in grey. **A'-D'**: *CaMKII* smFISH in mushroom body calyx. **A''-D''**: Magnification of white hatched box in A'-D'. Scale bar= $5\mu\text{m}$.

Table 5.7: Observations of MS2-tagged mRNA reappearance
in $\gamma 2\alpha'1$ dendrites following FRAP

N	mRNA type	Reappearance event (no. fluorescent spots)	Reappearance time point (min)	Total reappearance frequency
1	<i>Act5C</i> -24xMS2	1	5	0.2
2	<i>Act5C</i> -24xMS2	1	7	
3	<i>Act5C</i> -24xMS2	/	/	
4	<i>Act5C</i> -24xMS2	/	/	
5	<i>Act5C</i> -24xMS2	/	/	
6	<i>Act5C</i> -24xMS2	/	/	
7	<i>Act5C</i> -24xMS2	/	/	
8	<i>Act5C</i> -24xMS2	/	/	
9	<i>Act5C</i> -24xMS2	/	/	
10	<i>Act5C</i> -24xMS2	/	/	
1	<i>NMDAR1</i> -24xMS2	/	/	0.27
2	<i>NMDAR1</i> -24xMS2	/	/	
3	<i>NMDAR1</i> -24xMS2	1	2	
4	<i>NMDAR1</i> -24xMS2	/	/	
5	<i>NMDAR1</i> -24xMS2	/	/	
6	<i>NMDAR1</i> -24xMS2	/	/	
7	<i>NMDAR1</i> -24xMS2	1	10	
8	<i>NMDAR1</i> -24xMS2	/	/	
9	<i>NMDAR1</i> -24xMS2	2	1, 5	
10	<i>NMDAR1</i> -24xMS2	/	/	
11	<i>NMDAR1</i> -24xMS2	/	/	
1	<i>CAMKII</i> -24xMS2	/	/	0
2	<i>CAMKII</i> -24xMS2	/	/	
3	<i>CAMKII</i> -24xMS2	/	/	

4	<i>CAMKII</i> -24xMS2	/	/	
5	<i>CAMKII</i> -24xMS2	/	/	
6	<i>CAMKII</i> -24xMS2	/	/	
7	<i>CAMKII</i> -24xMS2	/	/	
8	<i>CAMKII</i> -24xMS2	/	/	
1	<i>DLG1</i> -24xMS2	/	/	0.29
2	<i>DLG1</i> -24xMS2	1	4	
3	<i>DLG1</i> -24xMS2	1	4	
4	<i>DLG1</i> -24xMS2	/	/	
5	<i>DLG1</i> -24xMS2	/	/	
6	<i>DLG1</i> -24xMS2	/	/	
7	<i>DLG1</i> -24xMS2	/	/	
8	<i>DLG1</i> -24xMS2	/	/	

