Development of a Viral Toolbox to Study the Role of m6A RNA Methylation in Memory

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Physiology
University of Toronto

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Abstract

Memory relies critically on local synthesis of proteins at synapses. How cells are able to precisely and independently coordinate memory-related translation at each of their synapses is not fully understood. Just as transcription is modulated by epigenetic modifications of DNA during memory formation, local translation may be regulated by mRNA modifications, like N6 methylation of the adenosine base (m6A). In this project, we investigate the m6A pathway and find that the demethylase Fto is highly expressed in the mouse brain, and regulated in the CA1 of the hippocampus following a contextual memory task, along with several other pathway components and overall levels of m6A in mRNA. To probe the role of m6A in memory formation, we then develop herpes simplex virus (HSV) vectors to rapidly and selectively manipulate Fto levels in cells, including the first HSV-mediated CRISPR/Cas9 knockdown vector, which will increase our ability to flexibly target multiple genes in vivo.
Acknowledgments

This thesis project represents over a year of important growth in my scientific research skills which would not have been possible without the humour, advice and patience of many people.

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Contributions of Collaborators

Several members of the Josselyn laboratory were involved in producing the work presented in this thesis. Their contributions are listed here.

Figure 3.1
Brandon Walters: Designed qPCR primers for Alkbh5 and Fto.

Figure 3.2
Brandon Walters/Matthew Yip: Ran experiments for Alkbh5 and Fto.
Brandon Walters: Helped analyse the data.

Figure 3.3
Brandon Walters/Matthew Yip: Ran experiments for m6A in total and mRNA enriched samples.

Figure 3.4
Brandon Walters: Designed the sgRNA targeting Fto in the Cas9 knockdown virus. Prepared and analysed some of the samples to validate the shRNA and Cas9 knockdown viruses in cell culture.
Rachael Neve lab (MIT): Cloned the Cas9 virus plasmids, and packaged them into virus particles.
Mika Yamamoto: Packaged the Fto overexpression and shRNA knockdown virus plasmids into virus particles.

Figure S1
Brandon Walters/Matthew Yip: Ran experiments for Alkbh5 and Fto.
Brandon Walters: Helped analyse the data.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ACTB</td>
<td>beta-actin</td>
</tr>
<tr>
<td>ALK</td>
<td>alpha-ketoglutarate-dependent dioxygenase</td>
</tr>
<tr>
<td>ALKBH5</td>
<td>alkB homolog 5</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARC</td>
<td>activity-regulated cytoskeleton-associated protein</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CA1</td>
<td>cornu Ammonis area 1</td>
</tr>
<tr>
<td>CA3</td>
<td>cornu Ammonis area 3</td>
</tr>
<tr>
<td>CamKII</td>
<td>calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CamKIIA</td>
<td>alpha subunit of CamKII</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CaN</td>
<td>calcineurin</td>
</tr>
<tr>
<td>CCAC</td>
<td>Canadian Council on Animal Care</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CFC</td>
<td>contextual fear conditioning</td>
</tr>
<tr>
<td>CPEB1</td>
<td>cytoplasmic polyadenylation element binding protein 1</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>CT</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
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<td>EC</td>
<td>entorhinal cortex</td>
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<tr>
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<td>ELAV-like RNA binding protein 1</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>E-LTP</td>
<td>early phase LTP</td>
</tr>
<tr>
<td>f6A</td>
<td>N6-formyladenosine</td>
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<tr>
<td>FMRP</td>
<td>fragile X mental retardation protein</td>
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<tr>
<td>FTO</td>
<td>fat mass and obesity-associated protein</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>hm6A</td>
<td>N6-hydroxymethyladenosine</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early gene</td>
</tr>
<tr>
<td>L-LTP</td>
<td>late phase LTP</td>
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<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
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<td>5-methylcytosine</td>
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<td>m6A</td>
<td>N6-methyladenosine</td>
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<tr>
<td>m7G</td>
<td>N7-methylguanosine</td>
</tr>
<tr>
<td>MAP2</td>
<td>microtubule-associated protein 2</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>mESC</td>
<td>murine embryonic stem cell</td>
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<tr>
<td>METTL14</td>
<td>methyltransferase-like 14</td>
</tr>
<tr>
<td>METTL3</td>
<td>methyltransferase-like 3 (or MT-A70)</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro RNA</td>
</tr>
<tr>
<td>MIT</td>
<td>Massachusetts Institute for Technology</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>N2a</td>
<td>neuro2a</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PpI</td>
<td>protein phosphatase I</td>
</tr>
<tr>
<td>PRP</td>
<td>plasticity-related protein</td>
</tr>
<tr>
<td>PSD</td>
<td>postsynaptic density</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real time PCR</td>
</tr>
<tr>
<td>RELN</td>
<td>reelin</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNP</td>
<td>ribonucleoprotein particles</td>
</tr>
<tr>
<td>shRNA</td>
<td>small hairpin RNA</td>
</tr>
<tr>
<td>sgRNA</td>
<td>single guide RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Sub</td>
<td>subiculum</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>WTAP</td>
<td>Wilms' tumour 1-associating protein</td>
</tr>
<tr>
<td>YTH</td>
<td>YT521-B homology</td>
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<td>ZBP1</td>
<td>zip-code-binding protein 1</td>
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<tr>
<td>ZIF268</td>
<td>zinc finger protein 225 (or Egr-1)</td>
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Chapter 1
Introduction

1.1 Summary of the Research Question

Memory formation requires strengthening and weakening connections between different neurons in the brain, a process called synaptic plasticity known to depend on both gene transcription and translation (Bourtchuladze et al., 1998; Castellucci et al., 1970, 1986; Frey et al., 1988, 1996; Mayford et al., 2012). It was long thought that gene translation occurs mostly in the cell soma, and that during memory formation, plasticity-related proteins (PRP) are trafficked to their synaptic targets to enable plasticity (Frey & Morris, 1997; Redondo & Morris, 2011). How neurons, with their highly complex dendritic arbours, are able to specifically target proteins only to those synapses undergoing plasticity remains unclear. The discovery that protein synthesis also occurs in dendrites and spines, and that this local translation is necessary for memory formation suggested an additional mechanism for regulating local plasticity, namely regulating local translation (Miller et al., 2002; Ouyang et al., 1999). Indeed, trafficking transcripts to synapses instead of proteins may be advantageous for neurons, as it allows them to produce proteins only when and where they are needed in the cell. However, a similar question emerges, namely how are neurons able to independently and precisely regulate local translation of messenger ribonucleic acids (mRNA) at each of their synapses? The mechanisms regulating local translation would have to include a stimulus-induced, dynamic and flexible pathway. In particular, such a pathway would have to allow for a large number of differentiable signals, to enable fine-tuned control over local translation at the individual mRNA transcript level. Lastly, to ensure that each transcript may be processed independently of others, such a signal would likely have to be present in the actual transcripts themselves. The mechanisms identified to date account well for the broad regulation of local translation, but do not provide transcript-level precision. In light of this, the N6-methyl adenosine (m6A) modification that occurs in RNA emerges as a promising candidate, due to its wide prevalence, flexibility and dynamic nature, for the fine regulation of local protein synthesis in memory (Fu et al., 2014a).
1.2 The Hippocampus

As a first step in investigating the potential role of m6A in memory, I first review the key brain regions involved in learning and memory. Implicit memory, which includes the abilities to subconsciously remember procedures, perceptions and motor tasks, is principally supported by the striatum, neocortex and cerebellum respectively (Squire, 2004). Declarative memory, the ability to remember facts and events, relies primarily on the hippocampus, and neighbouring regions, including the amygdala and fornix, as well as the entorhinal (EC), perirhinal and parahippocampal cortices (Simons & Spiers, 2003). As declarative memories age however, they become increasingly independent of the hippocampus, depending mainly on neocortical and prefrontal regions (for review, see Frankland & Bontempi, 2005; Simons & Spiers, 2003).

Among these key memory regions, the hippocampus has been most widely studied, not only because its dysfunction results in profound memory impairments (Morris et al., 1982; Rudy et al., 2002; Scoville & Milner, 1957), but also because it constitutes a central hub for information flow in the brain during the acquisition of declarative memories (Battaglia et al., 2011; Wheeler et al., 2013). The hippocampus thus emerges as a promising candidate region in which to begin our investigation of the role of m6A in memory.

1.2.1 Memory in the hippocampus

The first strong clinical evidence for the role of the hippocampus and neighbouring regions in memory came from patients suffering from epilepsy or schizophrenia who, in severe cases, had been treated with surgical brain tissue resections of these commonly epileptogenic regions. The most famous patient to undergo bilateral resection from these regions was H. M. (Milner 1968; Scoville & Milner 1957). Following surgery, he suffered severe anterograde, and graded retrograde amnesia, losing the ability to form new memories, or recall memories from several years preceding his surgery. His case was particularly severe, as the region resected included not only large portions of the hippocampus, but also the subiculum (Sub), amygdala and EC (Squire, 1992; Squire & Zola-Morgan 1991). By comparing the regions resected in patients suffering from permanent post-surgical amnesia, Scoville & Milner (1957) identified the hippocampus as the common factor. Squire et al. (1990) arrived at the same conclusion using magnetic resonance imaging (MRI) in patients having developed amnesia following a stroke or other brain injury.
In light of the early clinical findings pointing strongly to a critical role for the hippocampus in declarative learning, Hirsh (1974) reviewed hippocampus memory lesion studies in animals and was the first to propose a specific role for the hippocampus in contextual memory. Since then, the hippocampus has been shown to be necessary not only for the acquisition of contextual memory (Fanselow, 2000; Martin & Clark, 2007; Rudy et al., 2002), but also for spatial memory tasks (Girardeau et al., 2009; Morris et al., 1982) and trace conditioning (McEchron & Disterhoft, 1999; Misane et al., 2005; Solomon et al., 1986). As these studies show, inhibiting or disrupting activity in the hippocampus during or shortly after learning significantly impairs memory recall performance. Together, these findings strongly support the choice of the hippocampus as a starting point for investigating potential mechanisms underlying memory formation, like m6A RNA methylation.

1.2.2 Hippocampal anatomy

The ability of the hippocampus to coordinate the complex task of storing and retrieving memories may in part be due to its highly organized structure which is well conserved from rodents to humans (Simons & Spiers, 2003). This feature has enabled researchers to distinguish the different roles of hippocampal subregions in memory. In investigating a potential role for m6A in memory, it is helpful to focus on specific subregions, as the regulation of m6A may differ depending on the role a subregion plays in memory, and thus the function for which m6A may be harnessed (van Gemert et al., 2009).

Located in the medial temporal lobe, the hippocampus is most commonly subdivided into cornu Ammonis fields (CA1 and CA3) and the dentate gyrus (DG) (Fig. 1.1). The primary connections between these regions are comprised in a tri-synaptic circuit, first described by the Spanish neuroscientist Santiago Ramón y Cajal in 1893, and later refined by Swanson et al. (1978). The principal input of the circuit begins with the neighbouring EC, which receives input from cortical sensory association areas, and projects onto the DG via the perforant pathway. DG granule cells then synapse onto CA3 pyramidal cells via the mossy fiber pathway. Lastly, CA1 receives inputs from CA3 cells via the Schaffer collaterals (Fig. 1.1). CA1, acting as the main output of the hippocampus, then projects back onto the EC and the Sub. In addition to these commonly described connections, many additional inputs and outputs exist, contributing to the complexity of the hippocampus (for review, see Amaral & Witter, 1989).
Through their different structures and composition, CA1, CA3 and the DG each contribute differently to memory formation. Whereas the DG is thought to be particularly important for ensuring that similar memories are stored distinctly, a process called pattern separation (Deng et al., 2013; Leutgeb et al., 2007), CA3 is thought to function as an autoassociative network, binding together different components of a memory. This property of CA3 is thought to underlie pattern completion, a process that allows an incomplete memory cue to trigger recall of an entire memory (Lisman, 1999; Kesner, 2007). Last in the circuit, CA1 encodes cognitive maps of new environments, and plasticity in this region is particularly critical for spatial memory formation (Moser et al., 2008; O’Keefe & Dostrovsky, 1971; Tsien et al., 1996; Yiu et al., 2011). For its critical role in spatial memory and position as the main output of this circuit, we have chosen to focus on CA1 in investigating the role of m6A in memory in the hippocampus.

![Figure 1.1. The tri-synaptic circuit.](image)

**Figure 1.1. The tri-synaptic circuit.** Sagittal section of the rodent hippocampus drawn by Santiago Ramón y Cajal. Arrows show information flow through the tri-synaptic circuit: EC → DG → CA3 → CA1 → Sub/EC (Adapted from Ramón y Cajal, 1954).

### 1.2.3 CA1 and spatial memory

Evidence from both human and rodent studies has shown the CA1 subregion of the hippocampus, and particularly the dorsal region (Moser et al., 1993), to be particularly important in memory. Among patients who have suffered hippocampal damage, one patient (R. B.)
sustained a bilateral ischemic injury largely restricted to CA1. Even with only CA1 affected, R. B. displayed a moderately severe anterograde declarative memory impairment, wherein he retained most memories from before his injury, but showed severe, although not complete, amnesia for events following his injury (Zola-Morgan et al., 1986). CA1 neurons have since been shown to be particularly affected in Alzheimer’s disease (van Hoesen et al., 1999), providing further evidence for a critical role for CA1 in memory in humans.

In rodents, the cognitive maps encoded by place cells in dorsal CA1 are necessary for spatial memory, and inhibiting their reactivation after learning impairs long-term memory (Carr et al., 2011; Jadhav et al., 2012; Wilson & McNaughton, 1994). Deng et al. (2013) showed that during spatial memory recall, many of the cells active during learning are reactivated in dorsal CA1 in particular, indicating that these neurons are critical components of the memory. Ablation of only CA1 cells or disruption of their firing is sufficient to impair performance in spatial memory tasks (Auer et al., 1989; Lenck-Santini et al., 2001; Volpe et al., 1992). Based on these findings, it is clear that CA1, and dorsal CA1 in particular, plays a key role in spatial memory.

A commonly used spatial task is contextual fear conditioning (CFC) in which rodents learn to associate an aversive stimulus, typically a foot shock, with a specific context. Similarly to other spatial tasks, CFC induces synaptic plasticity in CA1 (Hall et al., 2000; Levenson et al., 2002) and is impaired by hippocampal lesions including CA1 (Chen et al., 1996; Maren et al., 1997). Since CFC can be learned after a single session, it is particularly conducive to studying mechanisms underlying memory formation, as these can be temporally restricted to the period immediately following the single learning session (Sekeres et al., 2012; Wheeler et al., 2013). For these reasons, we selected CFC as a learning paradigm with which to study the potential role of m6A in memory formation in dorsal CA1.

1.3 Local Translation Regulates Memory Formation

During formation of memories such as context fear memories, neurons in CA1 and other regions of the hippocampus undergo synaptic plasticity. Through this process which relies on both gene transcription and protein translation, synaptic connections between neurons are strengthened or weakened to encode the new memory (Bourtchuladze et al., 1998; Castellucci et al., 1970, 1986;
Frey et al., 1988, 1996; Mayford et al., 2012). Considering that each pyramidal neuron in CA1 is estimated to have over 30,000 synapses (Megías et al., 2001), the task of ensuring that only those synapses involved in a new memory are strengthened or weakened is not trivial. The ability of neurons to do this is partially accounted for by their ability to engage in local protein synthesis, whereby genes are translated locally in dendrites and spines. However, how cells are able to traffic individual transcripts to specific dendritic segments or locally label them for translation or degradation remains an important gap in our understanding of the mechanisms underlying synaptic plasticity, and memory formation.

1.3.1 Synaptic plasticity in memory
In addition to his early description of the tri-synaptic circuit, Ramón y Cajal (1894) was among the first to have the insight that the strengthening of connections between neurons might underlie memory formation in the brain. Almost half a century later, Donald Hebb (1949) formulated his now famous theory of learning in which he proposed that when a neuron A repeatedly contributes to the firing of a neuron B, the connection between these neurons is strengthened. Research in invertebrate systems investigating the relatively simple synaptic connections underlying reflexes like the gill-withdrawal reflex in Aplysia confirmed these insights, showing that learning relies on synaptic plasticity, the strengthening and weakening of synaptic connections between neurons (Castellucci et al., 1970; Kandel & Tauc 1965; Mayford et al., 2012). Shortly thereafter, the most widely accepted model for activity-dependent strengthening of synapses, long-term potentiation (LTP), emerged. Indeed, in a critical experiment, Bliss & Lømo (1973) were able to induce long-lasting potentiation of DG synapses in anaesthetized rabbits through high-frequency stimulation of the perforant pathway. In other words, after this potentiation stimulation, inputs from presynaptic neurons excited postsynaptic neurons more strongly, and this increase was maintained for a prolonged period of time. Almost twenty years later, Dudek & Bear (1992) showed that the counterpart to LTP, long-term depression (LTD) in which synaptic connections are weakened, could be produced using low-frequency stimulation. The discovery of LTP and LTD has provided a useful model for investigating the mechanisms underlying memory formation at the cellular level. For simplicity, I will focus here on LTP, which has been most studied for its relationship to memory formation.
As pointed out by Lynch (2004), the mechanisms at play in LTP closely parallel those observed in memory. First, LTP is most readily induced in the hippocampus, a region critically involved in memory. Second, inhibiting LTP in the hippocampus impairs learning of hippocampal-dependent tasks. Third, the high-frequency stimulation used to induce LTP resembles the theta rhythms that occur in the brain naturally during learning. Fourth, similar biochemical changes occur in cells after LTP as during learning, including gene transcription and translation. Lastly, just as memories can be stored temporarily or long-term, LTP has an early phase (E-LTP) lasting two to three hours, and a late phase (L-LTP) lasting hours to weeks (Lynch, 2004). While there are differences between L-LTP and the mechanisms through which synaptic plasticity occurs naturally in the brain during learning (for discussion of this topic, see Martin et al., 2000), these strong parallels make L-LTP an excellent candidate for studying memory at the cellular level.

Typically, as in CA1 where it has been most extensively studied, L-LTP requires activation of N-methyl-D-aspartate (NMDA) receptors at the postsynaptic terminal of stimulated neurons. Calcium flowing into the terminal through NMDA channels initiates the signaling cascades which lead to potentiation or depression of a synapse, through translation and activation of a large number of PRPs and molecules (Bliss & Collingridge, 1993; for review, see Malenka & Bear, 2004; for review in CA1, see Luscher & Malenka, 2012). Gene transcription and translation are key components of these cascades and required for both L-LTP and memory formation (Bourtsouladze et al., 1998; Castellucci et al., 1986; Frey et al., 1988, 1996). Although it is clear that these processes are tightly regulated, how neurons are able to coordinate local translation in particular to ensure that only the appropriate synapses are strengthened or weakened during memory formation is unknown.

1.3.2 Gene transcription in memory

Similar to gene translation, gene transcription is critical for L-LTP and long-term memory (lasting at least 24 hours). Inhibiting RNA synthesis, or gene transcription, has been shown to block L-LTP in Aplysia and rat hippocampal slices (Castellucci et al., 1986; Frey et al., 1996) and impair memory for spatial and contextual tasks when the hippocampus is targeted (Da Silva et al., 2008; Igaz et al., 2002; Thut & Lindell, 1974). Two time windows have been identified during which transcription is required in CA1 for memory formation: the first at the time of training, and the second three to six hours after training (Igaz et al., 2002). This biphasic pattern
is a key feature of memory formation, as it is also seen in immediate early gene (IEG) activity, gene translation and LTP (Ramírez-Amaya et al., 2005).

Two general mechanisms for regulating gene transcription during memory have been identified. The first is the translation or activation of IEGs, many of which are transcription factors, which bind to promoter sequences of PRP genes like brain-derived neurotrophic factor (*Bdnf*)\(^1\) and nerve growth factor (*Ngf*) (Barco et al., 2005; Castrén et al., 1993), and stimulate their transcription. These IEGs notably include cyclic adenosine monophosphate (cAMP) responsive element binding protein (*Creb*), zinc finger protein 225 (*Zif268*, also known as *Egr-1*) and activity-regulated cytoskeleton-associated protein (*Arc*) (Lynch, 2004). Knocking down or blocking activation of IEGs in the hippocampus impairs both L-LTP and memory formation (Bourtchuladze et al., 1994; Bozon et al., 2003; Guzowski et al., 2000; Pittenger et al., 2002). Although efficient, this mechanism for regulating gene transcription during memory lacks flexibility and gene-specificity as it relies on hard-coded promoter sequences that are common to a large number of genes.

More recently, a second more flexible and gene-specific mechanism for regulating gene transcription during memory was uncovered, namely epigenetic modifications. Indeed, following learning, dynamic changes are seen in the deoxyribonucleic acid (DNA) methylation patterns around PRP genes. Through these changes in methylation patterns, neurons are able to flexibly and quickly regulate the expression of individual PRPs, like *Bdnf*, reelin (*Reln*), protein phosphatase I (*Ppi*), *Arc* and calcineurin (*CaN*) (Lubin et al., 2008; Miller et al., 2010; Miller & Sweatt, 2007; Zovkic et al., 2013). Blocking DNA methylation by inhibiting methyltransferase activity in the hippocampus impairs synaptic plasticity and learning (Levenson et al., 2006; Miller & Sweatt, 2007), suggesting that epigenetic modifications are critical for regulating gene transcription in memory. Importantly, the existence of two parallel mechanisms regulating gene transcription, and in particular the flexibility and reversibility of epigenetic markers, may provide a hint as to how gene translation is regulated during memory.

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\(^1\) Following standard mouse nomenclature, in this document, italics (e.g., *Bdnf*) are used for genes or mRNA transcripts, and uppercase letters (e.g., BDNF) are used for proteins.
1.3.3 Gene translation in memory

Gene translation is critical to memory formation and inhibiting protein synthesis in the hippocampus impairs L-LTP (Frey et al., 1988; Kelleher et al., 2004; Stanton & Sarvey 1984), L-LTD (Manahan-Vaughan et al., 2000; Sajikumar & Frey, 2003) and memory. This is particularly the case during two critical time windows which it shares with IEG activation, gene transcription and LTP, namely one occurring at the time of training, and the other three to six hours after (Bourtchuladze et al., 1998; Quevedo et al., 1999).

Protein synthesis was long thought to occur only in the soma of neurons. This raises an important question for memory formation: with their thousands of synapses, how are neurons able to traffic newly synthesized PRPs only to the specific synapses to be potentiated or depressed? One candidate mechanism proposed by Frey & Morris (1997) is synaptic tagging, whereby recently activated synapses may be able to preferentially sequester PRPs travelling non-specifically in the neuron (for discussion of this topic, see Redondo & Morris, 2011). More recently, however, the discovery that several PRPs are synthesized locally in dendrites and synapses suggests that a more important question may be how neurons coordinate local translation of proteins during memory. Indeed, trafficking mRNA to synapses instead of proteins provides several advantages. First, it saves neurons energy by ensuring that proteins are only translated when needed. Second, it reduces the risk of proteins producing off-target effects during transport to their dendritic targets. Last, if a localization or translation signal is present in mRNA, it can occur without being translated, and thus two mRNAs with different signals can produce the same functional protein. Nonetheless, the same question remains: how are mRNA transcripts targeted to or translated at only those synapses involved in a memory?

1.3.4 Local gene translation in memory

The first evidence suggesting local gene translation occurs in neurons was the discovery of translational machinery, particularly polyribosomes, in dendrites and beneath synaptic junctions in the hippocampus (Steward & Levy, 1982; Steward & Reeves, 1988). Tiedge & Brosius (1996) later confirmed that dendrites were competent for protein synthesis, as they contain critical translation elements, like transfer RNAs (tRNAs), initiation and elongation factors, and cotranslational signal recognition components. More recently, it was shown that following high-frequency stimulation, there is an influx of polyribosomes from dendritic shafts into spines in the
hippocampus, including CA1 (Bourne et al., 2007; Ostroff et al., 2002). Furthermore, spines that have received an influx of polyribosomes have enlarged postsynaptic densities (PSDs) as compared to those that have not, indicating synaptic potentiation at these spines (Ostroff et al., 2002). Moreover, many mRNA transcripts, including several PRPs, have been found in high levels in dendrites, spines or synaptosomes, including the alpha subunit of calcium/calmodulin-dependent protein kinase II (CamKIIA) (Mayford et al., 1996; Paradies & Steward, 1997), Arc (Link et al., 1995), beta-actin (Actb) (Tiruchinapalli et al., 2003), microtubule-associated protein 2 (Map2) (Garner et al., 1988), and NMDA receptor subunits (Grooms et al., 2006; Miyashiro et al., 1994).

Although these findings strongly indicate that dendrites and spines are competent for translation, they do not demonstrate that local gene translation does in fact occur during learning. In 1992, Torre & Steward were able to show that local protein synthesis does occur in live cultured dendrites isolated from their cell bodies. Furthermore, in vivo in the hippocampus, synaptic activity or high-frequency stimulation enriches levels of transcripts such as Arc and CamKIIA in dendrites, as well as local protein synthesis (Havik et al., 2003; Link et al., 1995). The increase in CAMKII protein levels that follows high-frequency stimulation depends on local protein synthesis as it is blocked even when synthesis is only inhibited at the dendrites (Ouyang et al., 1999). Additionally, selective localization of Arc to activated dendritic segments in the DG, depends on NMDA receptor activity providing a further link to memory and L-LTP (Steward et al. 1998; Steward & Worley, 2001). Finally, in investigating the functional role of local protein synthesis, Bradshaw et al. (2003) specifically inhibited protein synthesis at apical or basal dendrites, and showed that L-LTP was only impaired at the targeted locations. Together, these findings strongly suggest that L-LTP requires local gene translation.

Confirming that local protein synthesis is required in vivo for memory formation has proved harder to tackle technically. Nonetheless, Ainsley et al. (2014) recently showed that CFC leads to rapid association of dendritic mRNA with local ribosomes. Most notably however, Miller et al. (2002) disrupted dendritic localization of CAMKIIA during learning by mutating the localization signal present in its 3' untranslated region (UTR). Although the protein’s function was unimpaired, mice with this mutation showed an impairment in L-LTP, as well as in spatial, associative fear conditioning and object recognition memory. These findings further corroborate the critical role of local gene translation in memory formation.
Given the evidence that local translation occurs during learning and is required for memory formation, one can identify two general approaches neurons may use to coordinate protein synthesis at individual synapses (Fig. 1.2). The first is that neurons regulate local translation by specifically targeting each mRNA transcript to the correct spine, via unique labels (Fig. 1.2A). The second is that each mRNA present in a spine is locally and dynamically labelled for processing, e.g., translation, silencing or degradation, as needed (Fig. 1.2B). It is also possible that mechanisms of both types are engaged in coordinating local translation together. Importantly however, among the mechanisms known to regulate both mRNA transport and local translation,
none appear to have the flexibility and dynamic nature required to regulate plasticity at the level of the individual spine and transcript.

1.3.4.1 Regulation of local mRNA transport in memory

Local transport of mRNA occurs primarily via RNA-containing granules like ribonucleoprotein particles (RNPs) which are transported along microtubules to dendrites (Dynes & Steward, 2007; Knowles et al., 1996; Vessey et al., 2006). When transported in RNPs, mRNA is typically maintained in a translationally repressed state to be released following arrival at the destination (Bramham & Wells, 2007).

How specific mRNAs are labelled for transport is not well understood. One of the best studied examples is the case of Actb, which possesses a 54-nucleotide element termed "RNA zipcode" in its 3' UTR (Kislauskis et al., 1994). This zipcode is recognized by zip-code-binding protein 1 (ZBP1), which is thought to coordinate RNP transport of Actb mRNA to dendritic segments. Indeed, ZBP1 has been shown to colocalize with Actb in dendritic RNPs (Tiruchinapalli et al., 2003) and silencing ZBP1 prevents granule formation (Farina et al., 2003), impairing Actb transport to dendrites (Hüttelmaier et al., 2005; Oleynikov & Singer 2003). When bound to Actb, ZBP1 inhibits translation. However, upon phosphorylation, ZBP1’s binding affinity for RNA is reduced, relieving translation repression (Hüttelmaier et al., 2005). Thus, ZBP1 appears to function both as a transport and translation regulator for Actb. Importantly, neuronal depolarization has been shown to stimulate movement of ZBP1-containing granules to dendrites in an NMDA receptor mediated way, suggesting that the Actb zipcode may indeed regulate dendritic localization during LTP and memory formation (Tiruchinapalli et al., 2003).

Other mRNAs have been found to possess similar zipcodes in their 3' UTRs, like CamKIIA (Miller et al., 2002), which binds cytoplasmic polyadenylation element binding protein 1 (CPEB1) (Huang et al., 2003). Similar to ZBP-1, CPEB1 forms RNPs in cultured hippocampal neurons (Huang et al., 2003) and following neuronal stimulation, the number of CamKIIA mRNA-containing granules localized to dendrites increases (Rook et al., 2000). Several other zipcode-containing mRNAs, like Arc and Bdnf, are also known to be transported to dendrites, but the binding proteins involved are unknown (for review, see Andreassi & Riccio, 2009). In the case of Bdnf, transcripts possessing a longer version of the 3' UTR are more likely to localize to dendrites of hippocampus neurons (An et al., 2008). Furthermore, Flavell et al. (2008) saw some
evidence that, following neuronal stimulation, there is a relative increase in shorter versions of mRNA transcripts with truncated 3' UTRs, however whether this is the case for PRPs in particular is unknown. If PRPs are among these genes, this could partially account for activity-related regulation of transcript localization. Thus, altogether, these findings support a role for 3’UTR zipcodes in determining whether certain transcripts are kept in the soma or nucleus, or transported to dendrites.

However, several lines of evidence show that zipcodes cannot fully account for the complexity of dendritic localization. First, evidence from Ainsley et al. (2014) suggests that the number of gene transcripts present in dendrites may be several orders of magnitude greater than previously thought. Indeed, following CFC, they found over 2,000 mRNAs bound to ribosomes in dendrites of CA1 pyramidal neurons. With this many dendritically localized transcripts, it is unlikely that each possesses a different hardcoded signal recognized by a different localization protein. Nonetheless, no widespread shared dendritic localization signal has been identified to date that could account for the dendritic localization of so many mRNAs. Second, different mRNAs are dendritically expressed in different cells, and at different developmental stages. In fact, even within individual cells, certain mRNAs, like Map2 and the InsP3 receptor are differentially expressed in proximal and distal dendrites (Steward & Schuman, 2001). Each zipcode thus would have to exist in multiple versions to allow for this level of precision. Thirdly and most importantly for memory formation, although zipcodes may signal for dendritic localization with some precision, they simply cannot generate enough differentiable signals to specify the exact dendritic segment or synapse to which a transcript should be transported. Overall, it is clear that mRNA targeting in neurons is too complex to be entirely regulated by hardcoded signals like zipcodes.

An important parallel can be drawn here with gene transcription where transcription factors allow broad changes in gene expression during memory, and epigenetic markers enable fine-tuned regulation at the level of the individual gene. A similar dynamic and flexible system must be regulating local mRNA transport during LTP and memory formation (Fig. 1.2A).

1.3.4.2 Regulation of local mRNA translation in memory

Although neurons may primarily regulate local gene translation by controlling which transcripts are targeted to which synapse, it is also possible that each transported transcript bears a signal
indicating whether it should be translated, silenced or degraded (Fig. 1.2B). Generally, when mRNA transcripts are trafficked to spines for local translation, they remain dormant until a signaling cascade is initiated prompting their translation, as occurs during L-LTP (Bramham & Wells, 2007). How different genes are translated at different rates and how some are kept silent, while others are translated is unknown. A mechanism must exist to precisely and dynamically label different transcripts for translation or degradation during learning.

Promising candidates for transcript-specific regulation of local protein synthesis include CPEB1, fragile X mental retardation protein (FMRP) and miR-134, a brain-specific micro RNA (miRNA). All three molecules localize to synapses (Schratt et al., 2006; Wu et al., 1998; Zalfa et al., 2003) and have been shown to be involved in translation repression (Kim & Richter, 2006; Schratt et al., 2006; Stebbins-Boaz et al., 1999; Zalfa et al., 2003; Zhang et al., 2001) and memory processes. Specifically, knocking out CPEB1 and FMRP respectively impairs spatial memory in mice (Berger-Sweeney et al., 2006) and alters synaptic morphology and plasticity of cultured hippocampal neurons (Braun & Segal, 2000). miR-134 negatively regulates dendritic spine size (Schratt et al., 2006) and the miRNA signaling pathway is involved in long-term memory acquisition in Drosophila (Ashraf et al., 2006). Furthermore, CPEB1, FMRP and miRNAs are regulated by memory-related events like release of BDNF and glutamate receptor activation (Ashraf et al., 2006; Huang et al., 2002; Shin et al., 2004; Wells et al., 2001; Weiler et al., 1997). These findings point strongly to a role for these molecules in regulating local mRNA translation during memory formation.

It is likely that many other translation regulators exist in addition to CPEB1, FMRP and miRNAs. Several of these may regulate local translation like transcription factors, by binding to hard-coded signals in mRNA, thus broadly silencing or activating translation at synapses during memory. Additional mechanisms, like the phosphorylation of certain elongation factors, also enable cells to regulate overall translation levels (Costa-Mattioli et al., 2009). However, as mentioned previously, it is also likely that certain translation regulators enable individual mRNAs to be distinctly regulated, by recognizing more flexible and dynamic signals in mRNA. This would allow neurons to target certain transcripts at a synapse for translation and others for silencing or degradation in response to external stimuli. As was suggested for local mRNA transport, epigenetic modifications of DNA may provide a hint as to the nature of these dynamic signals.
To conclude, memory formation in tasks such as CFC relies on synaptic plasticity in many regions of the brain, including the dorsal CA1 region of the hippocampus. Long-term synaptic plasticity in turn depends on both gene transcription and translation, including local translation in spines and dendrites. In order to specifically strengthen the synaptic connections that underlie a memory, neurons must be able to carefully regulate local gene translation through dynamic and transcript-specific regulation of transport and/or translation. How transport and translation are specifically regulated at the individual transcript- and spine-level remains unknown. We propose that a flexible and rapidly modifiable regulatory system must exist that is able to distinctly label transcripts to be targeted to different dendritic segments and/or to be translated at a specific synapse during memory formation. Just as epigenetic DNA modifications flexibly and quickly regulate gene transcription during learning, modifications of mRNA may fulfill the same role in translation regulation in memory.

1.4 m6A mRNA Methylation as a Potential Regulator of Local Gene Translation

1.4.1 Common mRNA modifications

Similar to genes which are surrounded by promoters and operators that regulate transcription, mRNAs contain non coding regulatory components in their 3' and 5' UTRs, as well as in their introns (for review, see Mignone et al., 2002; Wilkie et al., 2003). These hardcoded regions regulate mRNA splicing, translation, degradation and even broad subcellular localization, as in the previously described case of CamKIIA. Although these elements can differ between transcripts, precursor mRNA undergoes only a few specific sequence modifications, namely capping, polyadenylation and splicing (for review, see Moore & Proudfoot, 2009), and mature mRNA is subject to few sequence changes prior to degradation (Coller & Parker, 2004; Dunn & Cowling, 2014). As a result, it is clear that hardcoded signals like zipcodes in mature mRNA cannot be readily modified during memory formation to finely regulate local translation at the individual synapse.

However, similarly to DNA whose nucleotides can be epigenetically modified, RNA nucleotides can undergo chemical modifications. In fact, more than one hundred such modifications exist,
most of which involve methylation, namely the substitution of an atom or group by a methyl group \((\text{CH}_3)\) on a nucleotide (Czerwoniec et al., 2009). The most well studied modification is the cap which is critical for the stability of most mRNAs, but also involved in regulating translation initiation, export, polyadenylation and splicing of mRNA (for review, see Dunn & Cowling, 2014). Although capping is reversible, decapping is generally followed by degradation, as it destabilizes transcripts (Dunn & Cowling, 2014). Another common modification is 5-methylcytosine (\(\text{m5C}\)), which plays a key role in epigenetically regulating gene transcription during memory, but is much less prevalent and not well studied in mRNA (Squires et al., 2012).

One of the main reasons these modifications have remained poorly understood is the lack of appropriate tools to detect and locate these sites in transcripts. Recent advances in high throughput sequencing has allowed in-depth characterization of many of these modifications. It is through this work that the most prevalent modification of mRNA, \(\text{m6A}\), was discovered in the 1970’s (Desrosiers et al., 1974). However, the more recent discovery that \(\text{m6A}\) is dynamically regulated is what has brought it to the forefront of research into mRNA modifications (Dominissini et al., 2012).

### 1.4.2 \(\text{m6A}\) modification of mRNA

The \(\text{m6A}\) modification of mRNA is characterized by the replacement of a hydrogen by a methyl group on the nitrogen in the N6 position in adenosine (Fig. 1.3) (Kierzek & Kierzek, 2003). Accounting for around 50% of mRNA modifications (Wei et al., 1975), 0.1-0.4% of RNA adenosines are estimated to be methylated, with one m6A site occurring per 2,000 ribonucleotides (Fu et al., 2014a; Wei et al., 1975). In human cells, m6A is present in over 7,000 mRNAs, and enriched around stop codons, in 3’ UTRs and within long internal exons (Dominissini et al., 2012; Meyer et al., 2012), and binds to a well-conserved consensus sequence: RRACH (where \(R\) is a purine, \(A\) is the methylated adenosine, and \(H\) is adenosine, cytosine or uracil). Although the consensus sequence is strong, most instances of it in mRNA are not methylated (Dominissini et al., 2012). Taken together, these findings suggest that m6A patterns in different copies of the same gene could vary enough to signal for a variety of different fates, as would be needed to finely regulate local transport or translation of mRNA.

In studying the role of m6A in mRNA, it is important to note that m6A is also found in long non coding RNAs, as well as in ribosomal, small nuclear and transfer RNAs (Fu et al., 2014a). In
contrast, in mammalian genomic DNA, where methylation typically occurs on cytosine, m6A is barely detectable (Fu et al., 2014a; Jia et al., 2011). Together, these instances of m6A account for less than 6% of m6A sites, demonstrating that m6A is largely restricted to mRNA (Meyer et al., 2012).

Figure 1.3. The m6A pathway. The m6A methyltransferase complex (including METTL3, METTL14 and WTAP) catalyzes methylation of the adenosine (A) base. m6A binding proteins like ELAVL1 and YTHDF1, 2 and 3 bind to or near m6A, influencing mRNA processing (e.g. translation, stability, splicing and transport). m6A demethylases FTO and ALKBH5 catalyze demethylation of the adenosine base. Whereas demethylation by ALKBH5 is direct, demethylation by FTO generates two sequential intermediates, hm6A and f6A, each of which can directly hydrolyze to adenosine (Fu et al., 2014a).

One of the early indicators that m6A does not occur statically in mRNA was the discovery that although many m6A peaks are well-conserved between humans and mice, certain sites are methylated only in a subset of transcripts, whereas other transcripts completely lack m6A even at
consensus sites (Cory et al., 1976; Dominissini et al., 2012; Horowitz et al., 1984). However, it was the discovery in 2011 by Jia et al. that m6A can be demethylated, and is thus a reversible modification that brought the study of m6A to the forefront. Dominissini et al. (2012) then showed that external stimuli like ultra-violet light, heat shock, cell specific growth factors or interferon-gamma can induce a dynamic shift in the m6A patterns of mRNA in cells, affecting 5-30% of m6A peaks. Importantly, this regulation of m6A is accompanied by changes in gene expression and splicing, suggesting an important functional role for m6A in regulating cellular responses to external stimuli.

These studies provided the first evidence for m6A as a rapid and reversible regulator of mRNA processing, and led to a rapid growth of interest in m6A. Additional research has since suggested that m6A is involved in regulating not only mRNA transport, but also splicing and translation (Dominissini et al., 2012; Fustin et al., 2013). Specifically, Zhou et al. (2015) showed that following heat shock, increased methylation in the 5' UTR of certain transcripts enables accelerated translation. Together, these findings point to a dynamic and flexible role for m6A in regulating a variety of mRNA processing steps, making it a prime candidate for finely regulating local protein synthesis in neurons during memory formation.

1.4.3 m6A pathway

In the next few sections, an overview is provided of the research conducted to date looking at the various components known to be involved in the m6A pathway. These components can be subdivided into three groups: methyltransferases, demethylases and methylation binding proteins (Fig. 1.3). It is important to note that research in this field has only just begun to gather momentum. As a result, it is very likely that new components and functions will be discovered in the next few years, filling in many of the gaps in our current understanding of the m6A pathway. Nonetheless, significant progress has been made in sketching a preliminary account of the functioning of the m6A pathway.

1.4.3.1 m6A methyltransferases

m6A RNA methylation is catalyzed by a methyltransferase complex, comprising three well conserved components: two methyltransferases (METTL3 or MT-A70, and METTL14) and one interacting protein (Wilms’ tumour 1-associating protein (WTAP)) (Fu et al., 2014a) (Fig. 1.3).
METTL3 and 14 may appear redundant in the complex, as they are highly homologous and each possesses a binding site for S-adenosylmethionine, a key substrate for catalyzing methylation (Bokar et al., 1994; Chiang et al., 1996; Ping et al., 2014; Wang, Y. et al., 2014). However, the fact that METTL14 shows ten times higher methyltransferase activity suggests that METTL3 may also undertake different functions in the complex (Liu et al., 2014). WTAP, for its part, is a known regulator of mammalian pre-mRNA splicing (Horiuchi et al., 2013; Ping et al., 2014), and shows no methyltransferase activity (Liu et al., 2014). Instead, it interacts with many proteins involved in RNA splicing, stability, polyadenylation, and export (Horiuchi et al., 2013), and thus, may be responsible for recruiting enzymes to the complex to modulate methyltransferase activity (Fu et al., 2014a).

Evidence for the role of this complex in methylating mRNA comes first from the fact that all three components of the complex bind mRNA at sequences consistent with the m6A consensus sequence, mostly in coding sequences or 3' UTRs, where m6A peaks occur in mammalian mRNA (Dominissini et al., 2012; Liu et al., 2014; Meyer et al., 2012). Furthermore, knocking down the levels of each methyltransferase component in vitro has repeatedly been shown to deplete m6A in a variety of cell types, with the biggest effects occurring when WTAP or METTL14 is knocked down (Bokar et al., 1997; Dominissini et al., 2012; Geula et al., 2015; Liu et al., 2014; Wang, Y. et al., 2014) (For a detailed list, see Table 1.1). Conversely, overexpressing METTL3 or METTL14, but not WTAP, also increases m6A levels, with a synergistic effect occurring when both are overexpressed together (Liu et al., 2014). Interestingly, one study in porcine adipocytes found no effect of knocking down METTL3 on m6A levels, but did see an increase in m6A following overexpression (Wang et al., 2015b). This lends further support to the hypothesis that METTL3 may have functions additional to transcript methylation. It is clear nonetheless from these studies that the methyltransferase complex critically regulates m6A levels in mRNA. This regulation is in turn important for cellular function, and in particular, coordinating several stages of mRNA processing, including translation, splicing and transport.

The importance of m6A for normal cell function is shown by the effects of dysregulating the levels of methyltransferase complex components. For instance, knocking down METTL3 in vitro leads to apoptosis in certain cells (Dominissini et al., 2012), whereas overexpression reduces adipogenesis in porcine adipocytes (Wang et al., 2015b). Similarly, knocking down METTL3 or
METTL14 in murine embryonic stem cells (mESC) leads to loss of self-renewal capability and aberrant lineage priming resulting in premature cell death (Geula et al., 2015; Wang, Y. et al., 2014). A comparable effect is seen following knockdown of METTL3 or WTAP in zebrafish embryos (Ping et al., 2014) (see Table 1.1). Thus, it is clear that interfering with m6A patterns in mRNA has severe consequences on cellular function. This dysregulation could be due to effects on gene translation, splicing and/or transport, all of which have been shown to be affected when m6A methyltransferase complex components are targeted.

When it comes to gene translation and stability, knocking down METTL3 upregulates some genes while downregulating others, suggesting that m6A can have bidirectional effects on its targets (Dominissini et al. 2012). Wang, Y. et al., 2014 found that developmental regulator genes targeted by the methyltransferase complex show an inverse correlation between their m6A methylation levels and transcript stability, as seen by enriched mRNA levels when m6A is depleted (Wang, Y. et al., 2014). Expression of developmental regulators in mESCs may therefore be regulated by m6A to maintain a pluripotent state (Wang, Y. et al., 2014). Recently, however, Lin et al. (2016) found that a catalytically inactive METTL3 mutant lacking methyltransferase activity was still able to promote mRNA translation in human cancer cells by associating directly with ribosomes (see Table 1.1). Thus, although METTL3 is certainly involved in RNA methylation, it is possible that some of the effects on translation of manipulating METTL3 are independent of m6A.

In addition to regulating translation, m6A patterns in mRNA have also been linked to splicing. Indeed, modulating mRNA m6A levels by knocking out METTL3 leads to differential expression of splice variants (Dominissini et al., 2012). Furthermore, all three components of the methyltransferase complex are enriched in nuclear speckles (Bokar et al., 1997; Ping et al., 2014), structures specifically involved in splicing regulation (Lamond et al., 2003). WTAP depletion reduces this enrichment for all three enzymes, indicating that its presence is required for the methyltransferase complex to localize to nuclear speckles (Ping et al., 2014) (see Table 1.1). Together, these findings also support a role for m6A in regulating splicing.

Lastly, in investigating the role of m6A in regulating the cellular circadian clock, Fustin et al. (2013) showed that overexpressing METTL3 in human cells and mouse embryonic fibroblasts (MEF) shortens the circadian period, whereas knocking down METTL3 elongates it (see
Further investigation suggested that the period elongation was linked to delayed export of mature mRNAs involved in regulating the cellular circadian clock. These findings provide preliminary evidence for a role for m6A in dynamically regulating mRNA transport.

Overall, the m6A methyltransferase complex is clearly involved in regulating m6A levels in RNA and may play a role in regulating translation and stability, splicing and transport of mRNA (see Table 1.1). Specifically, it seems that METTL3 and METTL14 directly regulate m6A levels, whereas WTAP, and perhaps METTL3 as well, through their interactions with other enzymes, provides an interface for cellular signals involved in mRNA processing to respond to m6A levels. Although these findings support a potential role for m6A and the methyltransferase complex in regulating processes like local protein synthesis in memory formation, no research has been conducted investigating the role of these enzymes in vivo, and particularly in memory. For this research project, we investigate how the methyltransferase complex is regulated during memory, with a focus on METTL14, as it is the main active component of the complex, and METTL3, as it has been shown to regulate translation.

1.4.3.2 m6A demethylases

In 2011, Jia et al. identified the fat mass and obesity-associated protein (FTO) as the first m6A demethylase. Two years later, Zheng et al. (2013) discovered that AlkB Homolog 5 (ALKBH5) also demethylates m6A in RNA (Zheng et al., 2013). These discoveries were of great importance, as they demonstrated that m6A is reversible, and therefore could dynamically regulate mRNA processing. Since then, several studies have shown that FTO and ALKBH5 expression in vitro inversely correlates with m6A, further demonstrating their roles as m6A demethylases (Jia et al., 2011; Zheng et al., 2013) (see Table 1.2 for more details).

Previous to this discovery, FTO had been primarily studied for its possible link to obesity (Church et al., 2010; Dina et al., 2007; Frayling et al., 2007; Fredriksson et al., 2008; Gerken et al., 2007; McTaggart et al., 2011; Scott et al., 2007; Stratigopoulos et al., 2008; Tung et al., 2010), but its precise function remained unknown. It had also been linked to memory, first as it is well expressed in the brain (Church et al., 2010), and second, because several of the mutation sites in FTO correlate with an increased risk for Alzheimer’s disease, cognitive decline and reduced brain volume in humans, independently of obesity-related measures (Bressler et al., 2013; Keller et al., 2011). If m6A is indeed involved in regulating
plasticity in the brain, this could account for FTO’s link to cognitive function, and perhaps even obesity. ALKBH5, for its part, is enriched in testes and has been primarily linked to fertility (Zheng et al., 2013), suggesting it may not play a prominent role in brain-related functions (see Table 1.2).

In addition to being expressed in different tissues, FTO and ALKBH5 catalyze oxidative demethylation of m6A in different ways, as FTO produces oxidation intermediates between m6A and adenosine, whereas ALKBH5 does not (Chen et al., 2014) (Fig. 1.3). These two intermediates, N6-hydroxymethyladenosine (hm6A) and N6-formyladenosine (f6A) are stable in vitro under physiological conditions with half-lives of approximately three hours each and show reduced binding affinities to m6A binding proteins (Fu et al., 2013). Given that mammalian mRNAs have median half-lives of around five hours, it is possible that FTO-generated m6A intermediates are recognized by a different set of binding proteins and play a functional role in regulating mRNA processing (Fu et al., 2014a) (Fig. 1.3). Together, these findings strongly suggest that ALKBH5 and FTO, although both regulators of m6A, have different functions and can thus produce distinct downstream effects on mRNA processing.

As expected based on the findings from manipulating methyltransferase levels in vitro, altering demethylase levels also impairs cellular function. Indeed, just as knocking down METTL3 and METTL14 in mESC leads to aberrant premature differentiation, knocking down FTO in mouse preadipocytes produces the inverse effect, inhibiting differentiation (Zhang et al., 2015). In male mice, knocking down ALKBH5 impairs fertility due to maturation arrest and apoptosis in spermatocytes (Zheng et al., 2013) (see Table 1.2). Again, this is likely due to effects of m6A on mRNA translation and stability, splicing and transport.

First, as seen when methyltransferase components are knocked down, overexpressing FTO in mice differentially affects different groups of transcripts. This supports a nuanced role for m6A in regulating mRNA translation, implying that transcripts can be targeted distinctly for processing based on the needs of a cell (Merkestein et al., 2014). Second, both FTO and ALKBH5 localize to nuclear speckles, and depleting FTO leads to modifications in splicing patterns of methylated transcripts (Jia et al., 2011; Zhao et al., 2014; Zheng et al., 2013). Third, whereas knocking down METTL3 levels delays nuclear export of certain transcripts, knocking down ALKBH5 accelerates nuclear export, while increasing the rate of RNA synthesis (see
Table 1.2). Together these findings demonstrate the flexibility of the m6A pathway and further corroborate the hypothesis that m6A is used by cells to regulate mRNA processing and cellular functions dynamically in response to external stimuli. Based on the link between FTO and memory, one of these functions could be synaptic plasticity.

To date, only one study has investigated the m6A pathway in the brain, focusing on FTO which is highly expressed in the hypothalamus, cerebellum and hippocampus (Church et al., 2010), and localizes to neurons (McTaggart et al., 2011). They found that knocking out FTO leads to a decrease in IEG levels, implying a change in cellular activity (Hess et al., 2013). Importantly, they also saw a specific increase in methylation of certain PRP transcripts, including glutamate and dopamine receptor subunits. While no research has been done to further investigate the contribution of FTO and m6A to synaptic plasticity, it is clear from these studies that both may play a key role in memory formation. Investigating how FTO and ALKBH5 are regulated following memory formation will provide insight into this link, and may indeed uncover a difference between the roles of these two m6A demethylases in memory.

1.4.3.3 m6A binding proteins

The third group in the m6A pathway comprises the proteins that recognize m6A sites in RNA (Fig. 1.3). Given that the presence of m6A only minimally affects the secondary structure of mRNA, it is likely that its functions are primarily carried out through recognition by these binding proteins, and not direct chemical effects on mRNA structure (Kierzek & Kierzek, 2003). Furthermore, several of these proteins have been linked to mRNA stability, translation, splicing, and localization, further supporting a role for m6A in mRNA processing.

Among the proteins found to bind m6A sites in mammalian cells, several are members of the YT521-B homology (YTH) domain family (YTHDF1, 2 and 3) (Dominissini et al., 2012; Wang et al., 2015a; Wang, X. et al., 2014; Zhang et al., 2010). The most well-studied, YTHDF2, has been linked to bidirectional changes in target mRNA translation, depending on its binding site. On the one hand, Wang, X. et al. (2014) showed that YTHDF2 binding in 3’UTRs and coding sequences of transcripts results in the translocation of mRNA from the translatable pool to decay sites, like processing bodies. Knocking down YTHDF2 prevents this translocation, leading to an accumulation of non-translating m6A-containing mRNAs that would typically be degraded (Wang, X. et al., 2014) (see Table 1.3). On the other hand, Zhou et al. (2015) found that heat
shock upregulates YTHDF2 levels in the nucleus, as well as the translation of stress-induced transcripts via an increase in m6A levels in their 5’UTRs. These increases are abolished when YTHDF2 is silenced, but potentiated when FTO is knocked down, suggesting that YTHDF2 binding to m6A in stress-related transcripts blocks their demethylation by FTO, and enables their translation (Zhou et al., 2015) (see Table 1.3). Given that heat shock typically leads to a broad suppression of translation, m6A thus enables cells under these conditions to specifically upregulate translation of stress-related proteins only. Together, these findings show that by binding m6A at different sites, YTHDF2 dynamically regulates mRNA translation.

In addition to YTHDF2, YTHDF1 has also been shown to promote protein synthesis by translocating its target mRNA to ribosomes and interacting with translation initiation factors (see Table 1.3). Although both binding proteins share many target transcripts, YTHDF2 binds RNA at a later stage in processing than YTHDF1, suggesting complementary rather than competitive roles (Wang et al., 2015a). Further research is needed to elucidate the different roles of YTHDF1, 2 and 3 in regulating translation and stability, and perhaps also splicing and transport of mRNA. Nonetheless, the research to date demonstrating the ability of cells to exercise fine-tuned control over cellular responses to external stimuli by regulating the expression of m6A pathway components like FTO and YTHDF2 is already very promising, and certainly supports the potential for this pathway to finely regulate processes like synaptic plasticity in neurons.

In addition to YTHDF1-3, Dominissini et al. (2012) also identified ELAV-like RNA binding protein 1 (ELAVL1 or HuR) as significantly associated with methylated mRNA. Likely the best studied proteins in the m6A pathway, ELAVL1 is involved in many aspects of mRNA processing that have also been linked to m6A, including enhancing stability (for review, see Brennan et al., 2001), modulating translation levels (Durie et al., 2011), and possibly regulating splicing (Lebedeva et al., 2011; Mukherjee et al., 2011). Unlike YTHDF1-3 however which bind the m6A consensus site, ELAVL1 typically binds uracil-rich regions in 3’ UTRs, increasing the stability of transcripts. In fact, although ELAVL1 is associated with methylated mRNA, its ability to bind mRNA is sensitive to the proximity of m6A, showing different binding affinities at different intervals from m6A (Wang, Y. et al., 2014). Like YTHDF2, ELAVL1 also binds to 5’UTR regions where it promotes transcript translation (Durie et al., 2010). This appears to be partially due to its ability to block binding of miRNAs (for review, see Srikantan et al., 2012), which have been shown to regulate local gene translation during memory formation (Kosik,
25

2006; Schratt et al., 2006), reducing the stability and suppressing the translation of certain mRNAs (for review, see Fabien et al., 2010), while promoting the translation of others (Vasudevan et al., 2010) (See Table 1.3). These findings not only suggest a more complex interaction between ELAVL1 and m6A than with YTHDF1, 2 and 3, but also support the ability of m6A to flexibly induce a large number of distinct downstream effects on mRNA depending on its location in a transcript, a property necessary for regulating complex mechanisms like local gene translation in memory.

More research is needed to identify new m6A binding proteins and elucidate their effects on mRNA translation, stability, splicing and transport, in particular in vivo. For instance, heterogeneous nuclear RNPs which form mRNA granules are also associated with methylated mRNA, but their potential role in regulating transcript localization and transport based on m6A patterns has yet to be investigated (Fu et al., 2014a). Nonetheless, the large variety of proteins and molecules linked to m6A demonstrates a great potential for m6A binding proteins to finely regulate mRNA processing, and confirms the flexible and dynamic nature of this pathway, as well as its potential as a regulator of local translation during memory formation. In particular, YTHDF2, which shows a rapid response to external stimuli like heat shock, and ELAVL1, which is already known to regulate many aspects of mRNA processing also affected by m6A, are promising candidates for a role in memory formation in the brain. This project will thus focus on YTHDF2 and ELAVL1 in investigating the effects of memory formation on m6A binding proteins.
**Table 1.1. Role of m6A methyltransferase components.**

<table>
<thead>
<tr>
<th>Component</th>
<th>m6A levels</th>
<th>Cellular function</th>
<th>mRNA expression, stability and translation</th>
<th>mRNA splicing</th>
<th>mRNA transport</th>
<th>Memory</th>
</tr>
</thead>
<tbody>
<tr>
<td>METTL3</td>
<td>- Reduced after knockdown in HepG2 cells, HeLa cells, 293FT cells, mESCs and zebrafish embryos (Bokar et al., 1997; Dominissini et al., 2012; Geula et al., 2015; Liu et al., 2014; Ping et al., 2013; Wang, Y. et al., 2014) - No effect after knockdown in porcine adipocytes (Wang et al., 2015b) - Increased after overexpression in porcine adipocytes (Wang et al., 2015b) - Increased after overexpression in HeLa and 293FT cells (Liu et al., 2014)</td>
<td>- Knockdown impairs self-renewal capability and lineage priming, leading to cell death in mESCs (Geula et al., 2015; Wang, Y. et al., 2014) - Knockdown elongates circadian period in U2OS cells and MEFs (Fustin et al., 2013) - Overexpression shortens circadian period in U2OS cells and MEFs (Fustin et al., 2013)</td>
<td>- Knockdown has bidirectional effects on transcript levels in HepG2 cells (Dominissini et al., 2012) - Knockdown increases developmental transcript levels in mESCs (Wang, Y. et al., 2014) - Promotes translation in a methyltransfer-independent way (Lin et al., 2016)</td>
<td>- Enriched in nuclear speckles (Bokar et al., 1997) - Knockout leads to differential expression of splice variants in HepG2 cells (Dominissini et al., 2012)</td>
<td>- Knockdown delays nuclear export in U2OS cells and MEFs (Fustin et al., 2013)</td>
<td>No findings to date</td>
</tr>
<tr>
<td>METTL14</td>
<td>- Reduced after knockdown in HeLa cells, 293FT cells and mESCs (Liu et al., 2014; Wang, Y. et al., 2014) - Increased after overexpression in HeLa and 293FT cells (Liu et al., 2014)</td>
<td>- Knockdown impairs self-renewal capability and lineage priming, leading to cell death in mESCs (Wang, Y. et al., 2014)</td>
<td>- Knockdown increases developmental transcript levels in mESCs (Wang, Y. et al., 2014)</td>
<td>- Enriched in nuclear speckles (Ping et al., 2014)</td>
<td>No findings to date</td>
<td>No findings to date</td>
</tr>
<tr>
<td>WTAP</td>
<td>- Reduced after knockdown in HeLa cells, 293FT cells and zebrafish embryos (Liu et al., 2014; Ping et al., 2013) - No effect after overexpression in HeLa and 293FT cells (Liu et al., 2014)</td>
<td>No findings to date</td>
<td>No findings to date</td>
<td>No findings to date</td>
<td>Interacts with stabilizing proteins (Horiuchi et al., 2013)</td>
<td>No findings to date</td>
</tr>
</tbody>
</table>
### Table 1.2. Role of m6A demethylases.

<table>
<thead>
<tr>
<th>ALKBH5</th>
<th>FTO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>m6A levels</strong></td>
<td><strong>m6A levels</strong></td>
</tr>
<tr>
<td>- Increased after knockdown in HeLa cells (Zheng et al., 2013)</td>
<td>- Increased after knockdown in HeLa cells (Jia et al., 2011)</td>
</tr>
<tr>
<td>- Reduced after overexpression in HeLa cells (Zheng et al., 2013)</td>
<td>- Reduced after overexpression in HeLa cells (Jia et al., 2011)</td>
</tr>
<tr>
<td><strong>Cellular function</strong></td>
<td><strong>Cellular function</strong></td>
</tr>
<tr>
<td>- Knockdown leads to maturation arrest and apoptosis in spermatocytes, and impairs fertility (Zheng et al., 2013)</td>
<td>- Knockdown impairs differentiation in mouse 3T3-L1 preadipocytes (Zhang et al., 2015)</td>
</tr>
<tr>
<td><strong>mRNA expression, stability and translation</strong></td>
<td><strong>mRNA expression, stability and translation</strong></td>
</tr>
<tr>
<td>- Knockdown increases RNA synthesis rate in HeLa cells (Zheng et al., 2013)</td>
<td>- Overexpression differentially affects levels of different transcript groups in HepG2 cells (Merkestein et al., 2014)</td>
</tr>
<tr>
<td>- Demethylation of transcripts in MEFs during heat shock induces degradation (Zhou et al., 2015)</td>
<td>- Demethylation of transcripts in MEFs during heat shock induces degradation (Zhou et al., 2015)</td>
</tr>
<tr>
<td><strong>mRNA splicing</strong></td>
<td><strong>mRNA splicing</strong></td>
</tr>
<tr>
<td>- Enriched in nuclear speckles (Zheng et al., 2013)</td>
<td>- Enriched in nuclear speckles (Jia et al., 2011)</td>
</tr>
<tr>
<td>- Knockdown leads to differential expression of splice variants in mouse 3T3-L1 preadipocytes (Zhao et al., 2014)</td>
<td>- Knockdown leads to differential expression of splice variants in mouse 3T3-L1 preadipocytes (Zhao et al., 2014)</td>
</tr>
<tr>
<td><strong>mRNA transport</strong></td>
<td><strong>mRNA transport</strong></td>
</tr>
<tr>
<td>- Knockdown accelerates nuclear export in HeLa cells (Zheng et al., 2013)</td>
<td>No findings to date</td>
</tr>
<tr>
<td><strong>Memory</strong></td>
<td><strong>Memory</strong></td>
</tr>
<tr>
<td>No findings to date</td>
<td>- Point mutations linked to reduced brain volume and increased risk for Alzheimer’s disease and cognitive decline in humans (Bressler et al., 2013; Keller et al., 2011)</td>
</tr>
<tr>
<td>- Enriched in mouse hypothalamus, cerebellum and hippocampus (Church et al., 2010)</td>
<td>- Enriched in mouse hypothalamus, cerebellum and hippocampus (Church et al., 2010)</td>
</tr>
<tr>
<td>- Expressed in neurons in the mouse hypothalamus, cerebellum and hippocampus (McTaggart et al., 2011)</td>
<td>- Expressed in neurons in the mouse hypothalamus, cerebellum and hippocampus (McTaggart et al., 2011)</td>
</tr>
<tr>
<td>- Knockout in mice decreases cellular activity in the midbrain (Hess et al., 2013)</td>
<td>- Knockout in mice decreases cellular activity in the midbrain (Hess et al., 2013)</td>
</tr>
<tr>
<td>- Knockout in mice increases m6A in PRP transcripts (Hess et al., 2013)</td>
<td>- Knockout in mice increases m6A in PRP transcripts (Hess et al., 2013)</td>
</tr>
<tr>
<td>- Knockout in mice increases PRP mRNA levels (Hess et al., 2013)</td>
<td>- Knockout in mice increases PRP mRNA levels (Hess et al., 2013)</td>
</tr>
<tr>
<td>- Knockout in mice decreases PRP levels (Hess et al., 2013)</td>
<td>- Knockout in mice decreases PRP levels (Hess et al., 2013)</td>
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</tbody>
</table>
Table 1.3. Role of m6A binding proteins.

<table>
<thead>
<tr>
<th>mRNA expression, stability and translation</th>
<th>ELAVL1</th>
<th>YTHDF1</th>
<th>YTHDF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Binding promotes translation and blocks miRNA binding (Dürre et al., 2010)</td>
<td>- Binding promotes translation and blocks miRNA binding (Dürre et al., 2010)</td>
<td>- Binding induces degradation in HeLa cells (Wang, X., et al., 2014)</td>
<td>- Binding reduces translation efficiency in HeLa cells (Wang, X., et al., 2014)</td>
</tr>
<tr>
<td>- Binding enhances RNA stability (Brennan et al., 2001).</td>
<td>- Binding enhances RNA stability (Brennan et al., 2001).</td>
<td>- Binding during heat shock prevents degradation and promotes translation in MEFs (Zhou et al., 2015)</td>
<td>- Binding during heat shock prevents degradation and promotes translation in MEFs (Zhou et al., 2015)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>mRNA splicing</th>
<th>ELAVL1</th>
<th>YTHDF1</th>
<th>YTHDF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Possible role in regulating splicing (Lebedeva et al., 2011; Mukherjee et al., 2011)</td>
<td>No findings to date</td>
<td>No findings to date</td>
<td>No findings to date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Memory</th>
<th>ELAVL1</th>
<th>YTHDF1</th>
<th>YTHDF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No findings to date</td>
<td>No findings to date</td>
<td>No findings to date</td>
<td>No findings to date</td>
</tr>
</tbody>
</table>
1.4.4 m6A in the brain

As this overview showed, research to date investigating the m6A pathway has been conducted almost exclusively *in vitro*, with only one study looking at the effects of modulating m6A in the brain on PRP transcripts (Hess *et al.*, 2013). It is known however that m6A is particularly enriched in the brain, and present in the kidney, liver and lung. Furthermore, the over 4,500 transcripts in the brain that show enrichment in m6A include many PRPs, like *Bdnf*, and these transcripts are preferentially targeted by miRNAs expressed at high levels in the brain (Meyer *et al.*, 2012). Together, this evidence points toward a potential role for the m6A pathway in providing fine-tuned regulation of mRNA processing in response to external stimuli in the brain, and possibly in memory formation.

1.5 Study Rationale

In summary, although research on memory has demonstrated that local gene translation is required in dendrites and spines to enable synaptic plasticity, how neurons are able to independently regulate translation of different transcripts at different spines when encoding new memories is not fully understood. The regulatory system that underlies this ability must be dynamic, allow for a large number of different signals and respond quickly to external stimuli. The m6A modification of RNA possesses all of these characteristics, and has been shown to play a key role in dynamically and flexibly regulating gene translation, stability, splicing and transport. Together, these findings suggest m6A as an excellent candidate for regulating fine-tuned memory-related processes, like local gene translation.

Given that the m6A pathway has not been studied extensively in the brain or in memory, this project aims to lay the groundwork for future research into the possible role of m6A in regulating local protein synthesis during memory formation. This is accomplished first by establishing whether certain m6A pathway components are enriched in the brain as compared to other organs, and second by investigating how these expression levels are affected in the dorsal CA1 of the hippocampus following a memory task. Lastly, in order to enable future investigation of the role of m6A in memory, a viral toolbox is designed and validated to probe the effects of dysregulating the m6A pathway during memory formation.
1.6 Hypothesis

The overarching hypothesis for this project is that the m6A pathway is involved in memory formation. Specifically, we hypothesize that among the m6A pathway components investigated, specifically Mettl3, Mettl14, Alkbh5, Fto, Elavl1 and Ythdf2, some show enrichment in the brain as compared to other organs. We further hypothesize that the levels of these components, and of m6A are regulated during memory formation. We then design viral tools to manipulate the levels of Fto and validate their effects in vitro on Fto mRNA and m6A levels.

1.7 Review of Chosen Gene Manipulation Methods

1.7.1 Herpes Simplex Virus (HSV)

For the tool development component of this project, the herpes simplex virus (HSV) was selected to deliver transgenes to neurons and behaviourally investigate their effects on memory formation. This choice is based on the many advantages HSV offers as compared to other commonly used transduction tools, including adeno-associated virus (AAV). The first consideration is cell-type specificity, as we are primarily interested in generating tools to study the role of m6A in neuronal plasticity. Although AAVs can be targeted specifically to neurons through the use of different serotypes or promoters (Shetsova et al., 2005; van den Pol et al., 2009), HSVs are naturally neurotropic and consistently infect only non-dividing mature neurons, making them a superior choice for neuron-specific work (Fink et al., 1996; Neve et al., 2005).

The second consideration is time course of transgene expression. As the m6A pathway is clearly involved in a broad range of cellular mechanisms, it is preferable to employ a system that allows rapid expression, reducing the risk that neurons will engage compensatory mechanisms to counteract the effects of the transgene, thus confounding any results. HSVs are thus ideal, as maximal transgene expression is reached within 24 to 72 hours, with expression dissipating completely by seven days post-infection (Carlezon et al., 1998). This transient expression also allows behaviour to be specifically correlated to the level of transgene expression, which is particularly useful in establishing whether the effects of the transient manipulation are permanent or temporary. In contrast, AAVs are better suited to long-term studies, as maximal transgene
expression is typically reached several weeks after infection, and persists for many months, if not permanently (Lo et al., 2004; van den Pol et al., 2009).

Lastly, the consideration of packaging size is of particular importance for this project. Indeed, as will be discussed in the next section, one of the viral tools we have developed uses the RNA-guided endonuclease Cas9 to knockout FTO. Given that Cas9 mRNA spans 4.1 kilobases (kb) (Senís et al., 2014), and the maximum packaging capacity of most viruses is 4.0-8.0 kb, with AAVs at the low end (Neve et al., 2005), viral delivery of Cas9 can be particularly difficult, as additional components like a reporter gene, promoter regions and virus packaging elements must also be included in viral vectors. Certain groups have attempted to circumvent this problem using AAVs by co-transducing cells with two viruses (Swiech et al., 2015) or using smaller orthologues of Cas9 (Ran et al., 2015). HSV avoids this problem entirely, as its packaging capacity can reach up to 40 kb.

It is important to note that due to the use of helper virus in packaging HSV vectors, HSVs have a higher toxicity level than AAVs. However, improvements in packaging methods have enabled minimal uses of helper virus, largely reducing cytotoxic effects (Neve et al., 2005). HSVs have indeed been successfully used in multiple studies to investigate the role of different genes in memory in different brain regions, including the hippocampus (Cole et al., 2012; Josselyn et al., 2001; Han et al., 2009; Sekeres et al., 2010, 2012).

Thus, for its ability to specifically target neurons, as well as its rapid and transient expression, and significantly larger packaging size, we have chosen to design HSV vectors to deliver transgenes targeting the m6A pathway to neurons in the hippocampus.

1.7.2 Cas9-based Gene Manipulation

Understanding the role of different genes in regulating brain function is a key goal of neuroscience research, and typically requires the use of gene editing tools. A common approach to manipulating gene expression in vivo is viral-mediated delivery of genetic material to overexpress or knockdown a gene of interest. Traditionally, overexpression has been achieved by delivering copies of the gene’s coding sequence expressed under a constitutive promoter, whereas knockdown is typically mediated by RNA interference molecules, like small hairpin RNAs (shRNAs) that target a gene’s transcripts for degradation (Wilson & Doudna, 2013).
Although effective, these tools have certain important limitations. First, since a vector overexpressing a gene must contain its entire coding sequence, which typically spans several kbs, targeting several genes at once rapidly increases viral vector size and is thus limited by maximum virus packaging size, even in HSV (Neve et al., 2005). Second, a gene expressed from a viral vector does not undergo the same mRNA processing as its endogenous counterpart, due in part to the absence of the intronic and intergenic regulatory regions that typically surround a gene. This limits the insight overexpression can provide into the endogenous function of a gene. In the case of shRNA knockdown, designing a shRNA that effectively targets the transcript of interest without inducing off-target effects is complex, as the efficiency and specificity of a shRNA depend on its secondary structure, as well as the structure of the target mRNA region (Moore et al., 2010). Thus, although virally-mediated overexpression and knockdown are useful for probing gene function, it will be helpful to develop new methods that circumvent these disadvantages. For these reasons, although the toolbox developed for targeting the m6A pathway in this project includes traditional overexpression and shRNA viruses, we have also developed a Cas9 knockdown virus in order to open the door to developing new, more effective and flexible gene editing tools.

Identified in the mid-2000s as a natural adaptive immune system in bacteria, the clustered RNA guided regularly interspaced short palindromic repeats (CRISPR) Cas9 system has quickly become one of the most popular tools for gene editing (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). This is largely due to the elegance of this system where an endonuclease, Cas9, is guided by a single guide RNA (sgRNA) spanning approximately 40 base pairs to cleave a complementary region of DNA (Jinek et al., 2012). Given that cellular repair of double-stranded breaks in DNA is prone to error, Cas9 cleavage is able to rapidly and efficiently introduce frameshift mutations leading to loss of function in target genes (Heidenreich et al., 2003) (Fig. 1.4A).

Compared to shRNA, sgRNA design is very simple and off-target effects can more easily be avoided by ensuring that the designed sgRNA shows minimal homology with non-target DNA sites (Fu et al., 2014b). However, since Cas9 acts by mutating DNA sequences, its effects on a cell are permanent, even when delivered by a virus that allows transient gene expression like HSV. To increase the flexibility of the Cas9 system, Gilbert et al. (2014) designed a mutant Cas9 lacking nuclease activity (dCas9). Instead of cutting the target DNA region, dCas9 can act as a
scaffold, targeting repressors or promoters to a gene, and temporarily modulating its endogenous expression. As described by Walters et al. (2015), this can be achieved by including in the sgRNA a stem loop sequence recognized by certain RNA-binding proteins which in turn recruit effectors to the dCas9 complex (Fig. 1.4B). Like Cas9, dCas9 can easily be targeted to several genes by including several different sgRNAs in the viral vector, at relatively little cost in terms of vector size. If different stem loops are included in different sgRNAs, the target genes can even be modulated in different directions within the same cell, which may be particularly helpful in investigating complex pathways like the m6A pathway.

Thus, the advantages associated with using Cas9 and the rapid expansion of its use in gene editing make it an excellent choice for developing new viral tools to investigate the role of the m6A pathway memory. To date, however, Cas9 has only been used once in the brain of adult mice and it was delivered using two AAVs, targeting three genes (Swiech et al., 2015). Knockdown rates ranged from 17-60%, and were sufficient to produce a behavioural effect. Given the advantages of HSV over AAV for our purposes detailed in the previous section, we have elected to express Cas9 and its associated sgRNA using an HSV vector. As a result, this project constitutes one of the first uses of HSV to deliver Cas9 to cells. If successful, it will pave the way for the rapid development of a variety of HSVs using dCas9 to target different m6A pathway components in different directions, allowing for extensive interrogation of the role of this pathway in memory.
**Figure 1.4. Gene editing with Cas9 and dCas9.** A. Cas9 binds to the target region in gene A, complementary to the targeting portion of the sgRNA, and causes a double stranded break, which is likely to lead to the introduction of a loss-of-function mutation during repair. B. dCas9 binds to the target region in gene B, complementary to the targeting portion of the sgRNA. Gene activators or repressors bound to stem loops in the sgRNA are thus recruited to gene B leading respectively to an increase or decrease in gene expression.
Chapter 2
Materials and Methods

2.1 Mice

Adult (10-14 weeks old) male F1 hybrid (C57 BL/6NTac × 129S6/SvEvTac) mice were used for all experiments. Mice were group housed (2-5 mice per cage) on a 12h light/dark cycle and provided with food and water ad libitum. All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC), National Institutes of Health (NIH), and approved by the Animal Care Committee at the Hospital for Sick Children.

2.2 Behavioural Training

Mice were handled daily for three days prior to CFC. During CFC, mice were placed in a unique fear conditioning chamber for 5 min. The first 2 min of habituation were followed by three 0.5 mA foot shocks delivered 1 min apart, and an additional minute after the final shock. Mice were then returned to their home cages in a holding chamber for 30 min, 1h, 2h, or 4h, after which they were anaesthetized with isofluorane and their brains were flash frozen in isopentane and stored at -80°C. Hippocampi were later dissected from the whole brain on ice, and dorsal CA1 was isolated and stored at -80°C (Fig. 3.2, S1).

For gene enrichment studies, three control groups were included to establish whether any effects found were due to contextual memory formation specifically, or simply exposure to a novel environment or stress alone: (1) Naïve mice, (2) Shock-only mice, (3) Context-only mice. Naïve mice were kept in their home cages in the animal colony. Both the shock-only and context-only control mice underwent similar procedures to the CFC mice. However, instead of CFC, the shock-only mice received an immediate 0.5 mA foot shock upon entering the fear conditioning chamber, and were immediately returned to their home cages. Under these conditions, mice are not able to form a contextual memory, due to a lack of exposure to the context in which the
shock is received (Wiltgen et al., 2006). The context-only control mice were exposed to the fear conditioning chamber for 5 min without foot shocks. All three control groups were age-matched to the CFC group.

2.3 Cell Culture

Neuro2a (N2a) mouse neuroblastoma cells (ATCC, CCL-131) were grown in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, non-essential amino acids and penicillin/streptomycin, and maintained in an atmosphere of 5% CO₂ and 95% air at 37°C. N2a cells were seeded at different concentrations in 6-well plates, such that wells would reach approximately 70% confluency when harvested. One day after seeding, cells were infected with 1-2 µL of virus (p1005-FTO, p1005(+), see details below) or 2-4 µL (Cas9-FTo, Cas9-ctrl, shRNA-FTo or shRNA-scr, see details below) in 6-well and 12-well plates respectively. Cells were harvested in the appropriate lysis buffer for quantitative real time polymerase chain reaction (qPCR) and m6A enzyme-linked immunosorbent assay (ELISA) (Fig. 3.4) 12h or 2-4 days after transduction.

2.4 Sample Analysis

2.4.1 RNA isolation and complementary DNA (cDNA) synthesis

RNA from whole organs (Fig. 3.1), dorsal CA1 (Fig. 3.2, 3.3, and S1) or N2a cells (Fig. 3.4) was isolated using the EZ-10 RNA isolation kit (Bio-Basic, BS82322). For mRNA enrichment (Fig. 3.3A), 26 of the 30 µl of isolated RNA were further purified using the MagJet mRNA enrichment kit (Thermo Scientific, K2811) and concentrated to 14 µl using the RNEasy MinElute cleanup kit (Qiagen, 74204). Total RNA was quantified using a Nanodrop 1000 (Thermo Scientific). To ensure that the Fto mRNA sequence present in the p1005-FTO viral plasmid would not affect gene enrichment results, RNA from cells or tissue infected with p1005-FTO or p1005(+) (Fig. 3.4A-C) underwent DNase treatment using the DNA-free DNA removal kit (Ambion, AM1906). For gene enrichment studies or mRNA normalization, RNA was
converted into cDNA (400 ng for Fig. 3.1, 7, 9, S1; 2 µL mRNA for Fig. 3.3B) using the High Capacity cDNA synthesis kit (Thermo Scientific, 4368814).

### 2.4.2 qPCR

qPCR was performed on 6 ng of cDNA, using the EvaGreen Mastermix (Diamed, ABM Mastermix-S) in a 10 µL reaction and run on a CFX96 real-time qPCR detection system (BioRad) using 500 nM primer dilutions (see below for sequences). To normalize m6A-containing mRNA, 2 µL of cDNA were loaded in a standard 10 µL qPCR reaction (Fig. 3.3B).

Relative gene enrichment was calculated from threshold cycles ($C_T$) using the $2^{-\Delta\Delta C_T}$ method (see below for equation) (Schmittgen & Livak, 2008), and two internal controls for normalization: glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and hypoxanthine-guanine phosphoribosyltransferase (Hprt). Control samples used for normalization were brain for organ analysis (Fig. 3.1), naïve mice for CFC analysis (Fig. 3.2, 3.3, and S1) and cells treated with control viruses for virus validation (Fig. 3.4).

$2^{-\Delta\Delta C_T}$ measure of relative enrichment, where $\Delta\Delta C_T = \left[ \left( C_T \text{~gene of interest} - \text{~geometric mean of } C_T \text{~internal controls} \right) \text{~for sample A} \right] - \left[ \left( C_T \text{~gene of interest} - \text{~geometric mean of } C_T \text{~internal controls} \right) \text{~for sample B} \right]$, with sample A being the treated sample, and sample B being the control sample.

Primers (5’-3’, forward-reverse):

- **Alkbh5**: (GGCGTTCTTAATGTCTCTGA, AGTTCCAGTTCAAGCCCATC)
- **Elavl1**: (GACACCAFAAAATCCCACTCAT, GCCAATCCCAACCAGAACA)
- **Fto**: (CTCAGCCACTCAAACCTCCAC, TCTTAGAACGCTGTCAGTTGG)
- **Gapdh**: (GTGGAGTCATACTGGAACATGTA, AATGGTGAAAGGTCGGTGTG)
- **Hprt**: (GGAGTCTTGTTGATGTGTCAGTA, GGGACGCAACTGACATTTTCTA)
- **Mettl3**: (GTTCCCTTGCTTGTTGTGGTAT, CTGCTCCGATGTTGATCTG)
- **Mettl14**: (ATTCTCCTGGAGCCCTCCTCT, ACCCCACTTTCGCAAGCATC)
- **Ythdf2**: (CAGTCTATGCAGAACCCTT, ACACTATGAGAACGCGAAGAG)
2.4.3 m6A ELISA

m6A-containing RNA was quantified using the EpiQuik RNA methylation quantification kit (Epigentek, P-9005). Either 200 ng of total RNA (Fig. 3.3A, Fig. 3.4C, F, I) or 8 µl of concentrated mRNA (Fig. 3.3B) were run and fit to a standard curve. Total RNA samples were then normalized against the total amount (ng) of RNA (Fig. 3.3A, Fig. 3.4C, F, I), whereas mRNA samples were normalized to the geometric mean of two internal controls (Hprt, Gapdh) determined using qPCR (Fig. 3.3B), as the concentration of mRNA was below the detection threshold of the Nanodrop 1000.

2.5 HSV Viral Vector Design

In order to establish in future experiments whether regulation of the m6A pathway is critical to memory formation, viruses were designed to manipulate the levels of a central pathway component in neurons: the demethylase FTO. An FTO overexpression virus and two Fto knockdown viruses (shRNA- and Cas9-based) were designed to enable us to investigate the effects of counteracting or enhancing the natural regulation of Fto following CFC on memory.

2.5.1 Design of the FTO overexpression HSV vector (p1005-FTO)

Primers were designed to amplify the mouse Fto mRNA coding sequence (NCBI Reference Sequence: NM_011936.2) with KpnI (5’) and XhoI (3’) restriction sites (5’-3’, forward-reverse: ATAGGTACCCTTTAGTAGCAGCATGAAGCGC, CCTCTCGAGTGCTTCCCTAGATGGATCTTGCTT). Fto was amplified from whole brain cDNA using the Phusion flash high-fidelity PCR master mix (Thermo Scientific, F548S), and cloned into the pENTR1A gateway entry vector (Invitrogen, A10462). Positive clones, as shown by digestion and sequencing analyses, were then cloned into the HSV p1005(+) vector (Clark et al., 2002, kindly provided by Dr. Rachael Neve, Massachusetts Institute of Technology (MIT), MA) using the Gateway LR Clonase II enzyme mix (Invitrogen, 11791100), placing Fto under an IE4/5 promoter. Notably, p1005(+) co-expresses green fluorescent protein (GFP) under a CMV promoter. The resulting plasmid (Fig. 2.1A) was sequenced and midi-prepped from a positive clone and packaged into virus particles using a helper virus. The virus was then purified on a sucrose gradient, pelleted and resuspended in 10% sucrose. The average titer obtained for the
recombinant virus stocks was typically 1.8 x 10^9 infectious units/ml. p1005(+) was used as a control for all experiments using p1005-FTO (Fig. 3.4A-C).

2.5.2 Design of Fto sgRNAs

Three sgRNAs targeting the different exons of Fto were designed using Desktop Genetics Ltd. to ensure maximal on-target and minimal off-target activity. These guides were synthesized (Integrated DNA Technologies) with flanking sites corresponding to BbsI restriction sites (see sequences below), phosphorylated (NEB, M0201), annealed and cloned into the pSpCas9(BB)-2A-GFP (PX458) validation plasmid from Dr. Feng Zhang, MIT, MA (Addgene, 48138) (Ran et al., 2013) using the BbsI restriction enzyme. Plasmids were then transfected into NIH 3T3 embryonic murine fibroblasts (ATCC, CRL-1658) using Lipofectamine 3000 (Invitrogen, L3000001). Cells were harvested and processed for qPCR as described above. The sgRNA that generated the highest level of Fto knockdown, namely the one targeting exon 3, was selected for the viral plasmid design (data not shown).

sgRNA (5’-3’, 3’-5’):

- targeting exon 1: CACCG TTCCCGCTCTCGTTCTTCCG,
  AAAC CGGAGGAACGAGAGCGGGAA C
- targeting exon 2: CACCG CCAAGAAGACTGAGGCTCCTTG,
  AAAC CAAGGAGCCTCAGTTTCTGG C
- targeting exon 3: CACCG GCAGTGTGAGAAAGGCCTC,
  AAAC GAGGCCTTTCTCACACTGC C

2.5.3 Design of the Fto and control sgRNA Cas9 vectors (Cas9-Fto and Cas9-ctrl)

The sgRNA targeting exon 3 of Fto was cloned by collaborators (Dr. Rachael Neve, MIT, MA) into an HSV vector expressing Cas9-2A-GFP under a Synapsin promoter and the sgRNA under a U6 promoter (Fig. 2.1B). A control vector containing no sgRNA was also designed. The average titer obtained for these recombinant virus stocks was 6.0 x 10^8 infectious units/ml.
2.5.4 Design of the Fto and scrambled shRNA vectors (shRNA-Fto and shRNA-scr)

A shRNA targeting Fto (Sigma, SHCLNG-NM_011936) and a scrambled shRNA (Addgene, 1864) were obtained. Primers were designed to amplify the U6 promoter and shRNA regions with BamHI (5’) and NotI (3’) restriction sites (5’-3’, forward-reverse: GTCCCGGTCTTCACGCAGGGGCTAT, TATGCGGCCGCTGATTCGGTCAACGAGG). Regions were amplified from the pLKO.1 vector using the Phusion flash high-fidelity PCR master mix (Thermo Scientific, F548S) and 0.25 M Betaine solution (Sigma, B0300) to reduce hairpin formation. The sequences were then cloned into p1005(+) and the resulting vector (Fig. 2.1C) was packaged as described for p1005-FTO. The average titer obtained for these recombinant virus stocks was 2.2 x 10⁹ infectious units/ml.

shRNA sequences (5’-3’):

Fto shRNA: CCGGGTTCTCGTTGAAATCCTTTGATCTCGAGATCAAAGGATTTCA ACGAGACTTTTGT

Scrambled shRNA: CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTA ACCTTAGG

Figure 2.1. Virus designs. Orange wedges show where sequences were cloned into the p1005(+) vector for p1005-FTO and shRNA-FTO. AmpR = ampicillin resistance gene; oriS = HSV origin of replication. A. p1005-FTO overexpression vector (9.5 kb). Fto coding sequence (CDS) inserted under an IE4/5 promoter. eGFP expressed under a CMV promoter. B. Cas9-Fto knockdown vector (9 kb). Fto sgRNA expressed under a U6 promoter. Cas9-2A-GFP expressed under a Synapsin promoter. C. shRNA-Fto knockdown vector (9 kb). Fto shRNA inserted under U6 promoter after the IE4/5 promoter. eGFP expressed under a CMV promoter.
2.6 Statistical Analysis

For organ and CFC enrichment experiments, one-way analyses of variance (ANOVA) were used to test for population differences (Fig. S1), followed either by Tukey honest significant differences (Fig. 3.1) or Dunnett (Fig. 3.2 and 3.3) post-hoc analyses. Specifically, the Dunnett post-hoc test was used for tests where only comparisons between the four or six different conditions and the naïve control group were considered (Dunnett, 1955). No significant differences were observed in shock-only or context-only groups between different time points for any of the genes analyzed (Fig. S1), and thus these groups were collapsed into a single context-only or a single shock-only group (Fig. 3.2). For virus validation experiments, unpaired t-tests were conducted to compare the cells infected with viruses manipulating Fto levels to control cells, for each time point (Fig. 3.4), with Welch’s corrections when required for unequal variances (Fig. 3.4A-B). Adjusted p-values below 0.05 are considered significant. Statistical analyses were performed using GraphPad Prism 7.00.
Chapter 3
Results

3.1 m6A Pathway Components Show Distinct Tissue Expression Patterns

In order to establish whether any m6A pathway components were enriched in the brain compared to other regions, we measured the mRNA levels of the different components in the brain, liver, kidney and lung. Levels of the m6A methyltransferase Mettl3 were similar across all different tissues (Fig. 3.1A, $F_{3,10} = 1.12$, $p > 0.05$), whereas Mettl4 (Fig. 3.1B, $F_{3,10} = 7.82$, $p < 0.05$) and both m6A binding proteins, Elavl1 (Fig. 3.1E, $F_{3,10} = 3.97$, $p < 0.05$) and Ythdf2 (Fig. 3.1F, $F_{3,10} = 3.94$, $p < 0.05$), showed differences as revealed by one-way ANOVAs. Tukey post-hoc analyses revealed that Mettl4 expression in the brain was higher than in the lung, whereas Elavl1 and Ythdf2 expression was higher in the liver than in the lung. The same analyses revealed that both m6A demethylases displayed opposite patterns of expression from one another. Whereas Alkbh5 showed higher expression in the liver and kidney than in the brain and lung (Fig. 3.1C, $F_{3,10} = 25.24$, $p < 0.05$), Fto expression in the brain was enriched in the brain as compared to all three other organs, but also higher in the liver than in the lung (Fig. 3.1D, $F_{3,10} = 25.11$, $p < 0.05$).

3.2 m6A Pathway Component Levels are Regulated During Learning

To determine whether m6A pathway components are regulated during learning, mice underwent CFC and the mRNA levels of the different components in dorsal CA1 were measured at different time points following CFC. For the control groups that underwent context-only or shock-only treatments, none of the m6A pathway components investigated showed differences in expression levels at the different time points as revealed by one-way ANOVA (Fig. S1). Thus, all time points for each treatment were merged into one group for comparison to the CFC time points.
Figure 3.1. m6A pathway components show distinct tissue expression patterns. A. No tissue enrichment found for Mettl3 (F_{3,10} = 1.12, p > 0.05). B. Mettl14 is enriched in the brain compared to the lung (F_{3,10} = 7.82, p < 0.05). C. Alkbh5 is enriched in the liver and kidney compared to the brain and lung (F_{3,10} = 25.24, p < 0.05). D. Fto is enriched in the brain compared to the liver, kidney and lung, and enriched in the liver compared to the lung (F_{3,10} = 25.11, p < 0.05). E. Elavl1 is enriched in the liver compared to the lung (F_{3,10} = 3.97, p < 0.05). F. Ythdf2 is enriched in the liver compared to the lung (F_{3,10} = 3.94, p < 0.05). n = 4 (brain, kidney), 3 (liver, lung). *p < 0.05.
Figure 3.2. m6A pathway components are regulated during learning. A. *Mettl3* is upregulated 30 min after CFC compared to naïve controls ($F_{6,114} = 7.33$, $p < 0.05$). B. *Mettl14* is downregulated 30 min after CFC compared to naïve controls ($F_{6,102} = 7.51$, $p < 0.05$). C. No changes found for *Alkbh5* ($F_{6,82} = 1.94$, $p > 0.05$). D. *Fto* is downregulated 30 min and 2h after CFC compared to naïve controls ($F_{6,117} = 6.61$, $p < 0.05$). E. *Elavl1* is downregulated 30 min and 2h after CFC compared to naïve controls ($F_{6,114} = 3.41$, $p < 0.05$). F. *Ythdf2* shows changes in regulation, but pairwise comparisons did not reveal differences ($F_{3,10} = 2.75$, $p < 0.05$). Dashed black line represents naïve control levels. $n = 19-23$ (naïve), 18-37 (context-only), 18-33 (shock-only), 8-10 (30 min after CFC), 7-8 (1h after CFC), 8-11 (2h after CFC), 7-8 (4h after CFC). *$p < 0.05$.}
Following CFC, both methyltransferases showed changes in expression levels as revealed by one-way ANOVA (Mettl3: Fig. 3.2A, F_{6,114} = 7.33, p < 0.05; Mettl14: Fig. 3.2B, F_{6,102} = 7.51, p < 0.05). Dunnett post-hoc analyses revealed that both Mettl3 and Mettl14 levels were different 30 min after CFC as compared to naïve mice, with Mettl3 levels increasing by nearly 70%, and Mettl14 levels decreasing by just over 30%. Fto levels also showed changes in expression (Fig. 3.2D, F_{6,117} = 6.61, p < 0.05) with a decrease at both 30 min and 2h after CFC of just over 35% and 50% respectively as compared to naïve mice. In contrast, the other demethylase, Alkbh5, did not show changes in expression following CFC (Fig. 3.2C, F_{6,82} = 1.94, p > 0.05). Lastly, both m6A binding proteins showed changes in expression (Elavl1: Fig. 3.2E, F_{6,114} = 3.41, p < 0.05; Ythdf2: Fig. 3.2F, F_{3,10} = 2.75, p < 0.05). Lastly, Elavl1 showed a 40% decrease at 30 min following CFC. However, pairwise comparisons of Ythdf2 levels in the different conditions with naïve mice did not reveal any differences.

3.3 m6A Levels in mRNA are Regulated During Learning

As the levels of the m6A pathway components were regulated following CFC, we then investigated whether m6A levels in total RNA and mRNA were regulated at different time points following CFC. Whereas m6A levels in total RNA did not show changes following CFC as revealed by one-way ANOVA (Fig. 3.3A, F_{4,22} = 0.02, p > 0.05), m6A levels in mRNA enriched samples did (Fig. 3.3B, F_{4,22} = 5.48, p < 0.05). Specifically, Dunnett post-hoc analyses revealed a change in m6A levels in mRNA both 30 min and 4h following CFC, corresponding to an approximately 140% increase compared to naïve mice.

3.4 In Vitro Validation of HSVs

Given that Fto showed enrichment in the brain, and was regulated during learning, we selected the demethylase FTO as the target for the HSVs we designed to manipulate the m6A pathway. These viruses were then validated in vitro in N2a cells, a line of immortalized neural precursor cells, to verify their effects on Fto and m6A.
3.4.1 p1005-FTO overexpression virus

As compared to cells infected with the control virus, p1005(+), N2a cells infected with the p1005-FTO overexpression virus had higher levels of Fto both 12h (Fig. 3.4A, t_{5} = 3.67, p < 0.05) and 2 days (Fig. 3.4B, t_{7} = 6.05, p < 0.05) after infection. Specifically, Fto levels in p1005-FTO infected cells were approximately 1200% and 600% higher at 12h and 2 days respectively, as compared to control cells. m6A levels in total RNA were 40% lower after 2 days in p1005-FTO cells as compared to control cells (Fig. 3.4C, t_{4} = 16.0, p < 0.05).

3.4.2 Cas9-Fto knockdown virus

As compared to cells infected with the control virus, Cas9-ctrl, N2a cells infected with the Cas9-Fto knockdown virus had lower levels of Fto both 2 (Fig. 3.4D, t_{10} = 4.0, p < 0.05) and 4 days (Fig. 3.4E, t_{10} = 3.31, p < 0.05) after infection. Specifically, Fto levels in Cas9-Fto infected cells were approximately 25% and 55% lower at 2 and 4 days respectively, as compared to control cells. m6A levels in total RNA were 50% higher after 4 days in Cas9-Fto cells as compared to control cells (Fig. 3.4F, t_{4} = 2.84 , p < 0.05).
Figure 3.4. *In vitro* validation of HSVs. A. *Fto* levels were upregulated compared to controls 12h after infection with the p1005-FTO overexpression virus ($t_{5} = 3.67$, $p < 0.05$). B. *Fto* levels were upregulated compared to controls 2 days after infection with the p1005-FTO overexpression virus ($t_{7} = 6.05$, $p < 0.05$). C. m6A levels were downregulated compared to controls 2 days after infection with the p1005-FTO overexpression virus ($t_{7} = 16.0$, $p < 0.05$). D. *Fto* levels were downregulated compared to controls 2 days after infection with the Cas9-*Fto* knockdown virus ($t_{10} = 4.0$, $p < 0.05$). E. *Fto* levels were downregulated compared to controls 4 days after infection with the Cas9-*Fto* knockdown virus ($t_{10} = 3.31$, $p < 0.05$). F. m6A levels were upregulated compared to controls 4 days after infection with the Cas9-*Fto* virus ($t_{4} = 2.84$, $p < 0.05$). G. *Fto* levels were downregulated compared to controls 2 days after infection with the shRNA-*Fto* knockdown virus ($t_{8} = 3.59$, $p < 0.05$). H. *Fto* levels were downregulated compared to controls 3 days after infection with the shRNA-*Fto* knockdown virus ($t_{10} = 2.99$, $p < 0.05$). I. m6A levels were upregulated compared to controls 3 days after infection with the shRNA-*Fto* virus ($t_{4} = 5.97$, $p < 0.05$). n = 7-9 (p1005-FTO, mRNA), 4-6 (mRNA Cas9- and shRNA-*Fto*), 3 (m6A). *$p < 0.05$. 
3.4.3 shRNA-\textit{Fto} knockdown virus

As compared to cells infected with the control virus, shRNA-scr, N2a cells infected with the shRNA-\textit{Fto} knockdown virus had lower levels of \textit{Fto} both 2 (Fig. 3.4G, $t_8 = 3.59$, $p < 0.05$) and 3 days (Fig. 3.4H, $t_{10} = 2.99$, $p < 0.05$) after infection. Specifically, \textit{Fto} levels in Cas9-\textit{Fto} infected cells were approximately 70\% and 30\% lower at 2 and 3 days respectively, as compared to control cells. m6A levels in total RNA were 35\% higher after 3 days in Cas9-\textit{Fto} cells as compared to control cells (Fig. 3.4I, $t_4 = 5.97$, $p < 0.05$).
Chapter 4
Discussion

Memory formation critically relies on local mRNA translation (Miller et al., 2002; Ouyang et al., 1999). However, the mechanism regulating the trafficking and translation of individual transcripts to and at specific spines in neurons during memory formation is poorly understood. Such a mechanism must be stimulus-induced, dynamic and flexible, like a modifiable signal present in mRNA transcripts that enables differential processing of individual transcripts. The m6A modification of RNA emerges as a promising candidate as it appears to possess all these characteristics (Fu et al., 2014a), but has not yet been studied in memory. This study aimed to begin this investigation by establishing whether certain components of the m6A pathway are enriched in the brain and regulated during memory formation, and developing tools for future probing of the role of m6A in memory. Our results show that the m6A demethylase Fto is particularly enriched in the brain, and that several m6A pathway components are regulated following CFC. Overexpression and knockdown viruses targeting Fto were designed and validated in vitro for future probing of the role of m6A in memory.

4.1 Fto is Enriched in the Brain

We hypothesized that some of the components of the m6A pathway would be enriched in the brain, as compared to other organs, and this is indeed the case for the demethylase Fto which shows over 50% higher expression in the brain than in the lung, kidney, or lung (Fig. 3.1D). Although the other components are not enriched in the brain, they do show different relative gene expression patterns from one another across the different organs. Specifically, the other demethylase, Alkbh5, displays an almost opposite pattern to Fto, as it is expressed 200-300% more in the liver and kidney respectively than in the brain (Fig. 3.1C). In addition, many components show much lower relative enrichment in the lung (Fig. 3.1). As m6A has not been studied extensively in the lung, it is unclear whether this finding reflects a less dynamic engagement of the m6A pathway in this organ or whether further investigation of the pathway...
will reveal additional components enriched in the lung. Altogether, therefore, these findings suggest that the m6A pathway is engaged differently by different organs, possibly enabling them to harness m6A for distinct purposes. In addition, the enrichment patterns of Fto suggest it may be particularly important for brain-related m6A functions.

In supporting distinct roles for FTO and ALKBH5 in the brain, these results complement previous findings that showed that FTO may play a role in brain-related functions (Bressler et al., 2013; Church et al., 2010; Keller et al., 2011), whereas Alkbh5 is particularly expressed in testes and involved in spermatogenesis (Zheng et al., 2013). Indeed, as with Alkbh5, expression patterns across organs and cells often hint at the function of specific genes (Cahoy et al., 2008; Zetterström et al., 1996; Zheng et al. 2013). Given that m6A is involved in many distinct cellular functions, like differentiation (Geula et al., 2015), adipocytosis (Zhang et al., 2015) and circadian rhythm regulation (Fustin et al., 2013), it is possible that the expression levels of its pathway components are critical to allowing it to have different functions in different tissues. This shifting of the balance between the demethylases toward Fto, for instance, might enable the m6A pathway in the brain to exploit its particularities, like the particular transcripts it targets or the intermediates, hm6A and f6A, that are produced during demethylation by FTO, but not ALKBH5 (Chen et al., 2014; Fu et al., 2013). For instance, if, as suggested by the evidence from Hess et al. (2013), FTO does specifically target PRP transcripts in the brain, its enriched levels in the brain might be key to allowing the m6A pathway to regulate memory-related processes like synaptic plasticity. Thus, altogether, our findings further corroborate a potential role for FTO in the brain and in memory.

Although the methylation and binding components of the m6A pathway that were investigated did not show any specificity in the brain, they are well expressed in this organ and may nonetheless be involved in shaping brain-related m6A function, not through their basal expression, but through their dynamic regulation during memory formation. Thus, in addition to supporting a particular role for FTO in regulating m6A-related functions in the brain, our results also support a possible role for the other pathway components, as these are also well expressed in the brain. If these components are indeed involved in memory, it is likely that, similar to many PRPs and IEGs (Barco et al., 2005; Miller & Sweatt, 2007; Ramírez-Amaya et al., 2005), their levels are regulated in memory-related brain regions like dorsal CA1 during memory formation.
4.2 The m6A Pathway is Extensively Regulated During Memory Formation

Our second hypothesis was that m6A pathway components are regulated during memory formation. In agreement with the hypothesis, several components show changes in gene expression in the dorsal CA1 region of the hippocampus at different time points following CFC. This is not the case for shock-only and context-only controls (Fig. S1), suggesting that the regulation of the m6A pathway seen after CFC is not simply due to stress or exposure to a novel environment, but rather to the formation of a contextual memory.

As Fig. 3.2 shows, the m6A pathway is most widely regulated 30 min after CFC. Indeed, the methyltransferase Mettl14, the demethylase Fto and the binding protein Elavl1, are all downregulated, whereas Metl3 is upregulated. Furthermore, as seen in Fig. 3.3, these changes are accompanied by an increase in overall m6A levels in mRNA, but not total RNA. Then, 2h after CFC, both Fto and Elavl1 are downregulated again, whereas m6A levels remain at baseline levels until they rise again 4h after CFC. By relating these changes to what is known from the literature about the different components, one can start to piece together the dynamics of the m6A pathway.

4.2.1 Regulation of m6A by methyltransferase and demethylase levels

The overall increase in mRNA m6A at 30 min (Fig. 3.3B) may be accounted for by both the upregulation of Metl3 and the downregulation of Fto. Indeed, although METTL3 and METTL14 are thought to methylate m6A as part of a complex (Fu et al., 2014a), overexpressing either component alone leads to an increase in m6A levels (Liu et al., 2014; Wang et al., 2015b), and knocking down either reduces m6A levels (Bokar et al., 1997; Dominissini et al., 2012; Geula et al., 2015; Liu et al., 2014; Ping et al., 2013; Wang, Y. et al., 2014). Thus, both methyltransferases appear to be able to function independently from one another. This would allow them to have distinct functions in memory, as suggested by the fact that they are regulated in opposite directions 30 min after CFC (Fig. 3.2A-B).

For instance, if METTL3 and METTL14 target different groups of transcripts in the brain, such as PRPs and proteins involved in baseline cell metabolism respectively, their differential regulation at 30 minutes could enable a specific increase in synaptic targeting and translation of
PRPs during memory formation. Indeed, although one study found that METTL3 is able to act independently of its ability to methylate adenosine by directly promoting mRNA translation (Lin et al., 2016), it has also clearly been implicated in methylating RNA (Bokar et al., 1997; Dominissini et al., 2012; Geula et al., 2015; Liu et al., 2014; Ping et al., 2013; Wang et al., 2015b; Wang, Y. et al., 2014). Thus METTL3 could be primarily driving the enrichment in mRNA m6A levels seen at 30 minutes, targeting transcripts involved in memory formation specifically, and compensating for the decrease in METTL14.

The other factor that could explain the upregulation of m6A at 30 min is the downregulation of the demethylase Fto 30 min after CFC (Fig. 3.2D). Interestingly, Alkbh5 does not show any regulation (Fig. 3.2C), fitting with the idea that its role in the m6A pathway is less prominent in the brain than in other tissues. It is also possible that ALKBH5 is critical to maintaining baseline transcript processing in the cell, whereas FTO is involved in memory-related processing. Importantly however, Fto is downregulated again at 2h (Fig. 3.2D), when no change is seen in m6A levels, and returns to baseline at 4h when m6A is enriched again (Fig. 3.3B). This could be accounted for if major changes in m6A occurring in the nucleus drown out local changes in m6A at the synapses involved in a memory. For instance, if the methyltransferase complex functions primarily in the nucleus, methylating transcripts for transport to the synapses, and Fto acts primarily at synapses, demethylating arriving transcripts, an increase in mRNA m6A levels at the synapse at 2h caused by Fto downregulation could be undetected if m6A levels are held constant in the nucleus. Then at 4h, the observed increase in m6A levels could be driven by an increase in methylation in the nucleus. Since neither Mettl3 nor Mettl14 is upregulated at 4h, it is likely that an additional methyltransferase component is upregulated or activated at this time, and possibly enabling specific increases in m6A in PRP transcripts required for memory formation. Such components could include WTAP, although it does not catalyze methylation and its overexpression has not been shown to increase m6A levels in vitro (Liu et al. 2014), or additional as yet unidentified methyltransferase components.

Overall, these findings show that at least three, and possibly more regulators of m6A levels, each of which may target different sets of transcripts, are all regulated during memory formation. This demonstrates the remarkable flexibility of the pathway, further supporting a potential role in regulating complex cellular functions. How these changes in m6A levels in different transcripts
affect memory formation depends, however, on how these changes are recognized by m6A binding proteins and enable changes in transcript processing.

4.2.2 Downstream effects of m6A regulation on transcript processing

In changing the levels of m6A in transcripts during memory formation, cells are likely enabling changes in transcript processing by m6A binding proteins like ELAVL1 and YTHDF2. The regulation of these proteins following CFC provides indications of how this may be occurring. During the first wave of m6A enrichment, at 30 min, neither of the binding proteins is upregulated. Elavl1, which has independently been linked to promoting mRNA translation and stability (Brennan et al., 2001; Durie et al., 2010), and regulating splicing (Lebedeva et al., 2011; Mukherjee et al., 2011), is in fact downregulated at this time point (Fig. 3.2E). This indicates that ELAVL1’s functions in the m6A pathway are being suppressed at this stage in memory formation, in favour perhaps of YTHDF2, or other binding proteins like YTHDF1 or 3. Indeed, YTHDF2 has been shown to compete with FTO and promote translation during heat shock (Zhou et al., 2015). Thus, it is possible that the decrease in Fto releases the brake on YTHDF2, allowing it to bind m6A-containing transcripts and increase their translation. However, depending on where m6A occurs in transcripts, YTHDF2 binding may also promote degradation (Wang, X. et al., 2014). Thus, the location of m6A in transcripts could enable translation of PRPs at 30 min and degradation of transcripts suppressing synaptic plasticity via YTHDF2.

Interestingly, when m6A levels rise again at 4h, neither Elavl1 nor Ythdf2 is upregulated (Fig. 3.2E-F). This may be due to the functions of both components being equally engaged at this stage in memory formation. An alternative explanation, however, is that just as changes in m6A may occur primarily in the nucleus, ELAVL1 and YTHDF2 may operate primarily at synapses during memory formation. Depending on the length of the dendritic arbour, it can take RNPs containing mRNA an hour or two to travel to spines (Dynes & Steward, 2007; Knowles et al., 1996; Steward et al., 1998), and thus Fto and Elavl1 may be modulated at 2h in response to the arrival of mRNAs affected by the increase in m6A levels in the nucleus at 30 min. It would then follow that the increase in m6A levels at 4h would be linked to changes in Fto and Elavl1 levels at 5 or 6h. This possibility is further discussed in the model presented in Fig. 4.1.

Lastly, it is possible that it is the location of new m6A sites in mRNA, and not the levels of different binding proteins, that allows some proteins to be engaged rather than others. Both
YTHDF2 and ELAVL1 are sensitive to the location of m6A in a transcript (Wang, X. et al., 2014; Wang, Y. et al., 2014; Zhou et al., 2015), and thus may not need to be regulated themselves in order to drive an increase in translation of PRPs and decrease in translation of plasticity-suppressing proteins. This feature of m6A adds an important layer of complexity to the m6A pathway, and further supports its flexibility and ability to finely regulate complex cellular functions in memory.

4.2.3 Biphasic regulation of m6A and other memory-related processes

Evidently the m6A pathway is highly flexible, and dynamically regulated during memory formation. Although causal evidence is needed to confirm the role of m6A in memory, its biphasic regulation during memory formation provides a strong parallel with many key memory-related processes. Indeed, IEG expression, gene transcription, protein synthesis and LTP all show biphasic regulation during memory formation, with a first wave typically occurring in the first three hours following a memory task, and the second wave, three to six hours after (Lynch, 2004; Igaz et al., 2002; Quevedo et al., 1999; Ramírez-Amaya et al., 2005). The biphasic pattern observed for m6A fits well within these windows, occurring at 30 min and 4h. Although, the waves of Fto and Elavl1 downregulation waves are closer together, occurring at 30 min and 2h, their lack of synchrony with m6A could be accounted for if, as proposed, they are regulated at spines, and m6A is upregulated in the nucleus. Thus, neurons could first upregulate m6A at the nucleus at 30 min to promote transport of PRP transcripts involved in the first wave of memory-related translation, while enabling local translation of transcripts already present in spines by decreasing Fto and Elavl1 levels. The downregulation of Fto and Elavl1 at 2h might then enable translation of newly arrived PRP transcripts (Fig. 4.1). This process might then be repeated at 4h for PRPs involved in the second wave of memory-related translation (Lynch, 2004). Thus, in demonstrating the temporal similarities between the regulation of the m6A pathway, and that of transcription and translation during memory formation, our results further corroborate the potential of m6A as a regulator of fine-tuned local mRNA translation in memory. Importantly, this hypothesis is also supported by the fact that the changes in m6A levels following CFC occur specifically in mRNA.
4.3 m6A is Specifically Regulated in mRNA During Memory Formation

As shown in Fig. 3.3, both increases in m6A levels appear to be specific to mRNA. This finding points strongly to a role for m6A in regulating transcript processing during memory formation, and possibly local translation. Importantly, the fact that these increases are not reflected in total RNA is not surprising given that 94% of m6A sites occur in mRNA (Meyer et al., 2012) and, by weight, mRNA constitutes only about 4% of the total RNA isolated from a mouse brain (Alberts et al., 2015). Thus, any changes in m6A levels specific to mRNA would be drowned out in the total RNA sample.

To date, none of the methyltransferases or demethylases have been shown to specifically target mRNA over other RNAs such as tRNA and rRNA. These findings demonstrate however that such targeting is indeed occurring. The ability of FTO to target non coding RNA in addition to mRNA, at least in vitro, is clearly demonstrated by our findings in Fig. 3.4 where manipulating Fto levels affected overall m6A levels in total RNA. Whether this is also the case in the brain, or whether it is other components, like METTL3, that specifically enable an increase in mRNA m6A levels remains to be established. Nonetheless, it is clear that m6A pathway components are able to target mRNA specifically, and it is also possible that they are able to target specific transcripts, like PRPs, as well.

All in all, the finding that both increases in m6A in CA1 following CFC are specific to mRNA certainly implicates m6A in regulating memory-related mRNA processing, and possibly in coordinating critical aspects of local mRNA translation, like transport and local translation of PRPs. This is further supported by Hess et al. (2013)’s finding that FTO may primarily target PRP transcripts in the brain, and several studies that have linked m6A to transcript transport and translation in vitro (Dominissini et al., 2012; Durie et al., 2010; Fustin et al., 2013; Merkestein et al., 2014; Wang, X. et al., 2014; Wang, Y. et al., 2014; Wang et al., 2015b; Zheng et al., 2013; Zhou et al., 2015). Thus, the enrichment of Fto in the brain, and dynamic regulation of m6A pathway components, and of mRNA m6A levels in dorsal CA1 during memory formation all corroborate a potential role for m6A in regulating plasticity, and possibly the local translation of PRPs. To bring together our findings and the ideas proposed here into a
cohesive picture, I propose a speculative model of how m6A may be regulating local mRNA translation during memory formation.

### 4.4 Speculative Model of the Role of m6A in Regulating Local mRNA Translation During Memory Formation

As simplifications of and speculations about reality, most models are false, and some more false than others. Nonetheless, it can be useful to build a speculative model when observing novel phenomena. Indeed, models provide a framework in which disparate pieces of a puzzle are brought together to produce specific hypotheses. These hypotheses can then be tested to establish more realistic models (Wimsatt, 2007). Thus, in order to explore the possible role the m6A pathway could play in regulating local mRNA translation, I have devised a speculative model of their interactions in Fig. 4.1. Specifically, this model shows how the changes found in Fig. 3.2 and 3.3 in m6A pathway component levels during memory formation could contribute to regulating mRNA transport to spines and local translation of mRNA at synapses during memory formation or L-LTP. The example given is of a pyramidal neuron and one of its dendritic spines located in the CA1 region of a mouse learning a memory task. As previously suggested, this model proposes that in regulating memory, the methyltransferase complex may function primarily in the nucleus, adding m6A signals to individual transcripts as they are produced, whereas the demethylase FTO and binding proteins may operate primarily at the synapse in spines, responding to the m6A signals when transcripts arrive.

In this model, upon transcription, each mRNA is labelled with two m6A signals by the methyltransferase complex: one synapse specific transport signal, and one translation-related signal. The colours used to distinguish the signals from one another in Fig. 4.1 correspond to different patterns of m6A sites in transcripts. In the naïve state (Fig. 4.1A), when all pathway components are at basal states, transcripts receive an m6A transport signal specifying their synaptic destination and an m6A translation suppressing signal. They are then packaged into RNPs (Bramham & Wells, 2007), likely through recognition by m6A binding proteins (not shown in Fig. 4.1), and shipped to their synaptic destination. At the spine, FTO suppresses translation of transcripts by removing m6A translation promoting signals from transcripts, and preventing YTHDF2 or other m6A binding proteins from recruiting the transcripts to the
translation machinery (Merkestein et al., 2014; Zhao et al., 2014; Zhou et al., 2015). ELAVL1 binds to the m6A translation suppressing signal on certain transcripts, stabilizing and silencing them (Brennan et al., 2001).

At the 30 min time point (Fig. 4.1B), after the memory task, the upregulation of METTL3 and downregulation of METTL14 change the composition of the methyltransferase complex in the nucleus, leading to an increase in the number of transcripts bearing an m6A signal and being sent to synapses. Furthermore, these transcripts, involved in the first wave of plasticity-related translation, now bear an m6A translation promoting signal, such that they may be translated immediately upon arrival. At the synapse, the downregulation of FTO and ELAVL1 releases translation suppression, allowing PRP transcripts that are already present to be recruited to the translation machinery, through binding to YTHDF2 or independently (Zhou et al., 2015).

At the 1h time point (Fig. 4.1C), m6A pathway components return to baseline levels, and the rate of transcript methylation in the nucleus slows down to a normal rate in the presence of basal METTL3 and METTL14 levels. Newly generated transcripts receive an m6A translation suppressing signal, as in the naïve state. At the spine, translation is silenced by the return of FTO and ELAVL1. Recently translated PRPs strengthen the synapse (Bliss & Collingridge, 1993; Grooms et al., 2006; Lynch, 2004; Mayford et al., 1996; Miyashiro et al., 1994; Paradies & Steward, 1997), shown in Fig. 4.1 as an increase in receptor numbers at the synapse. Meanwhile, RNPs travel through the dendritic arbour toward their synaptic destination (Dynes & Steward, 2007; Knowles et al., 1996; Steward et al., 1998).

At the 2h time point (Fig. 4.1D), the state in the nucleus remains the same. At the spine, FTO and ELAVL1 are downregulated again, as RNPs reach their destination and deliver a new batch of mRNA. Transcripts are recruited to the translation machinery, through binding to YTHDF2 or independently, and PRPs are locally synthesized (Ramírez-Amaya et al., 2005).
Figure 4.1. Speculative model of the role of m6A in regulating local mRNA translation during memory formation. A pyramidal neuron and one of its dendritic spines (the target of orange m6A synapse specific targeting signals) located in the dorsal CA1 region of the hippocampus of a mouse learning a memory task are shown. In this model, methyltransferase complexes operate primarily in the nucleus, adding m6A signals to individual transcripts as they are produced, whereas the demethylase FTO and binding proteins operate primarily at spines, responding to the m6A signals when transcripts arrive. Upon transcription, each mRNA is labelled with two m6A signals by the methyltransferase complex: one synapse specific transport signal, and one translation-related signal. A. Naïve state: pathway components are at basal states, transcripts with m6A translation suppressing signals are packaged and shipped in RNPs to synaptic destinations. At the spine, translation is suppressed due to transcript demethylation by FTO, preventing YTHDF2 from inducing translation, and ELAVL1 binding to m6A translation suppression signals. B. 30 min after memory task: changes in METTL3 and METTL14 levels change the composition of the methyltransferase complex, leading to an increase in transcript methylation and trafficking, and adding of m6A translation promoting signals to transcripts. At the spine, FTO and ELAVL1 are downregulated, releasing translation suppression. PRPs transcripts are then recruited to the translation machinery, through binding to YTHDF2 or independently. C. 1h after memory task: m6A pathway components return to baseline levels, transcript methylation in the nucleus slows down, and switches back to adding m6A translation suppressing signals to transcripts. At the spine, FTO and ELAVL1 inhibit translation. Recently translated PRPs strengthen the synapse. This is shown as an increase in receptors inserted at the synapse. Meanwhile, RNPs travel through the dendritic arbour toward their synaptic destination. D. 2h after memory task: At the spine, FTO and ELAVL1 are downregulated again. RNPs reach their destination and deliver a new batch of mRNAs, which are recruited to the translation machinery, through binding to YTHDF2 or independently. PRPs are locally synthesized. E. 4h after memory task: FTO and ELAVL1 return to baseline in the spine, inhibiting protein translation. The second batch of PRPs contribute to strengthening the synapse. This is again shown as an increase in receptors inserted at the synapse. In the nucleus, the second phase of transcription is initiated and additional PRP transcripts are methylated and shipped to their synaptic destination. F. Longer-lasting synaptic potentiation critical to memory formation is solidified through arrival and translation of the second batch of transcripts. Through m6A pathway regulation, the spine targeted by orange m6A synapse specific targeting signals has thus specifically been potentiated to help encode the memory task.

At the 4h time point (Fig. 4.1E), FTO and ELAVL1 return to baseline at the spine, halting protein translation, and the second batch of PRPs contributes to strengthening the synapse. In the nucleus, the second phase of transcription is initiated and additional transcripts involved in the second wave of plasticity-related translation are methylated and shipped to their synaptic destinations (Igaz et al., 2002). As time points beyond 4h were not explored in this study, the specifics of the model end here. However, it is assumed that upon arrival at the spine, these transcripts are likely translated and contribute to the longer-lasting potentiation observed in synapses critical to a new memory (Bliss & Collingridge, 1993; Bradshaw et al., 2003; Lynch,
Thus, by regulating m6A component levels in PRP transcripts, the neuron was able to specifically target this synapse to be potentiated and encode the new memory (Fig. 4.1F).

4.5 Overview of the Potential Role of m6A in Memory

As mentioned previously, it is likely that key components of the m6A pathway are missing from the model, and that the details proposed in Fig. 4.1 are different from the actual mechanisms at play in real neurons. Nonetheless, the model provides a general idea of how m6A might fine-tune regulation of local mRNA translation, and more importantly demonstrates how powerful and flexible the dynamics of such a richly regulated pathway are.

If m6A pathway components are indeed involved in regulating local mRNA translation, it is likely through interactions with known broad regulators of dendritic mRNA transport and local protein synthesis. By recognizing m6A patterns in mRNA, m6A binding proteins, for example, could be directing these regulators to specific transcripts. Specifically, if ELAVL1 and YTHDF2 are indeed involved in regulating translation in spines, it is likely that they interact with previously identified local protein synthesis regulators like CPEB, FMRP and miR-134 (Kim & Richter, 2006; Schratt et al., 2006; Stebbins-Boaz et al., 1999; Zalfa et al., 2003; Zhang et al., 2001). If these or other m6A binding proteins are acting in the nucleus, they may also direct dendritic transport proteins, like RNPs (Dynes & Steward, 2007; Knowles et al., 1996; Vessey et al., 2006), to transcripts specifically labelled by m6A. Thus, m6A may provide the mechanism by which broad mRNA processing mechanisms can be targeted specifically to those transcripts required for memory formation.

Furthermore, although the speculative model presented in Fig. 4.1 suggests that some m6A pathway components are active and regulated in spines, it is possible that the pathway is entirely regulated in the cell body. Indeed, since the findings presented in Fig. 3.2 and 3.3 reflect overall changes in m6A pathway component levels in dorsal CA1, and not changes within different neuronal compartments, one cannot conclude that the m6A pathway is acting in spines. In fact, expression patterns of m6A pathway components have not yet been well studied within neurons, and thus it is not yet known which components are expressed in dendrites. If it is in fact the case that m6A components are indeed primarily expressed and regulated in the cell body, this would
be compatible with the role proposed in Fig. 4.1 for m6A of regulating mRNA transport from the nucleus, but not the proposed role of locally regulating translation in spines. However, as protein synthesis is also required in the soma during memory formation (Huber et al., 2000; Lasek et al., 1970), m6A pathway components could still be involved in fine-tuned regulation of somatic translation. A similar model to that presented in Fig. 4.1 could therefore be considered in which FTO, ELAVL1 and YTHDF2 promote degradation or translation of transcripts in the soma. In this model, m6A would enable fine-tuned regulation of the levels of specific PRPs before they are trafficked to their cellular destination, for example through synaptic tagging, and enable neuronal plasticity and memory formation (Frey & Morris, 1997; Redondo & Morris, 2011). Further research looking at where m6A pathway component levels are regulated, and what stage of mRNA processing they affect in memory will help establish which of these two models best describes the role of m6A.

However, before these models and their proposals can be tested, a causal link must first be established showing that m6A regulation is indeed critical for memory formation. Second, it will be important to identify which transcripts show changes in m6A levels during memory formation and how these changes affect their processing. Third, establishing where in the cell different m6A pathway components are regulated during memory formation will provide important insight into whether m6A is indeed involved in local regulation of mRNA processing in memory, or whether it is acting primarily in the cell body.

To begin tackling the first question, it is critical to develop tools to target the pathway directly and observe the effects on memory. Given its enriched levels in the brain, links to cognitive function in humans (Bressler et al., 2013; Keller et al., 2011), and regulation during memory formation, Fto is an excellent target for which to begin developing these tools.

### 4.6 Development of HSV Gene Manipulation Tools to Probe the Role of m6A in Memory

#### 4.6.1 Overexpression and knockdown vectors

Our third aim was to develop HSVs to manipulate Fto, and m6A levels in cells. Three viruses were developed, namely one overexpression virus and two knockdown viruses, and their effects
on Fto levels were measured, as well as their functional effects on manipulating m6A levels. All viruses express well in N2a cells, as seen by the proportion of cells expressing GFP which was approximately 80-90% for cells infected with p1005(+), p1005-FTO, shRNA-scr and shRNA-Fto, and 60-70% for cells infected with Cas9-Fto and Cas9-ctrl, likely due to the two-fold lower titer of these viruses. The p1005-FTO overexpression virus robustly increases Fto levels, and decreases overall m6A levels, demonstrating its functional effect (Fig. 3.4A-C). The Cas9-Fto and shRNA-Fto viruses show the opposite effects, both leading to a decrease in Fto levels, and a corresponding increase in m6A levels in total RNA (Fig. 3.4D-I). Thus, all viruses worked as expected in vitro.

It is important to note first that, as the mechanisms of action of the different viruses are different, distinct time points were selected for each virus. The shortest time points (12h and 2 days) were used for p1005-FTO which increases FTO directly by increasing Fto levels. Longer time points (2 and 3 days, or 2 and 4 days respectively) were used for the shRNA-Fto and Cas9-Fto viruses to allow basal FTO levels to decrease naturally, and reveal the effects of knocking down Fto mRNA on m6A. Lastly, since Cas9 must first produce a mutation in the Fto gene for a knockdown to occur, an extra day was added to the second time point for the Cas9-Fto virus.

At 2 days, the knockdown of 25% generated by Cas9-Fto seems lower than the 75% knockdown produced by shRNA-Fto (Fig. 3.4D, G). This may be due to the lower titer of the Cas9-Fto virus, as well as the additional step in its knockdown mechanism. As expected, at 4 days the Cas9-Fto knockdown increases, reaching approximately 50% (Fig. 3.4E). Contrary to expectations, however, there is a substantial drop of approximately 50% in the level of Fto knockdown from 2 to 3 days for shRNA-Fto (Fig. 3.4G-H). A similar drop is seen in the efficiency of the Fto overexpression from 12 hours to 2 days for p1005-FTO (Fig. 3.4A-B). This drop in efficiency may be due to the cytotoxicity caused by the HSV helper virus which is involved in packaging the viral vectors and is present, albeit at low concentrations, in the viral stocks (Fink et al., 1996; Neve et al., 2005). Indeed, an increase in cell death was seen in transduced cells, expressing GFP, particularly for p1005(+), p1005-FTO, shRNA-scr and shRNA-Fto. Cells infected with Cas9-Fto and Cas9-ctrl may have been spared due to the lower titers of these viruses.

Furthermore, as cytotoxicity affects normal metabolic processes in cells like gene expression, it is likely that viral gene expression is also affected (Lim, 2013). If this is the case, cells infected with Cas9-Fto should be less sensitive, as persistent viral gene expression is not required for the
knockdown to persist. In fact, once Cas9 has effectively generated a loss of function mutation in the \textit{Fto} gene, the \textit{Fto} knockdown is permanent in that cell and its daughter cells.

Nonetheless, given that the viruses were still effective in manipulating \textit{Fto} levels at the later time points, these time points were selected for the m6A analyses in order to allow sufficient time for the changes in \textit{Fto} mRNA levels to induce changes in FTO protein levels, and consequently in m6A levels. In all three cases, the expected changes in m6A were observed, with a possibly stronger effect seen for Cas9-\textit{Fto} compared to shRNA-\textit{Fto} (Fig. 3.4F, I). This may be due to the difference in days from infection, and the better persistence of the Cas9 knockdown.

Overall, all viruses functioned generally as expected, and their rapid and robust effects support the future use of these viruses \textit{in vivo} to target the m6A pathway during learning and investigate the effects on memory. Given that HSV infects neurons differently from cells \textit{in vitro} (Fink \textit{et al}., 1996; Lim, 2013), it is likely that the overexpression and knockdown levels observed \textit{in vivo} will be different from those observed here. Thus, further validation of these viruses in cultured neurons and \textit{in vivo} will be critical to verifying and measuring their impact on \textit{Fto} and m6A levels in the brain.

4.6.2 Cas9-based HSV

The Cas9-based HSV in particular not only constitutes the first use of Cas9 in HSV, but will also enable one of the first uses of Cas9 in the brain (Swiech \textit{et al}., 2015). One of the principal advantages of using Cas9 to investigate the m6A pathway, is that once the effects of knocking down \textit{Fto} on memory have been investigated, it will be relatively simple to expand our investigation of the pathway by designing new sgRNAs targeting different m6A pathway components, and adding them to the viral vector in order to knockdown several components at once (Walters \textit{et al}., 2015). Eventually, the vector may be redesigned to express dCas9, which has no endonuclease activity, but can be used to recruit activators and repressors to different genes (Gilbert \textit{et al}., 2014). This will enable an even greater flexibility in targeting and modulating gene expression levels. Overall, the successful validation of this first Cas9-based HSV is very promising and will likely enable faster progress not only in understanding the role of the m6A pathway in the brain and in memory, but also in addressing other gene-based research questions.
4.7 Limitations

4.7.1 Missing pieces in the m6A pathway puzzle

As mentioned previously, it is likely that key components of the m6A pathway in the brain are missing. First, as research into m6A has only recently begun to gather momentum, many components of the m6A pathway have probably not yet been identified. Second, the dynamics observed in the levels of methyltransferases and demethylases do not fully account for the increases in m6A seen at 30 min and 4h (Fig. 3.3B), or the lack of change at 2h when the demethylase Fto is downregulated (Fig. 3.2D). Third, none of the components identified in the m6A pathway to date have been shown to specifically target mRNA. Thus, although some, like METTL3, may indeed show specificity in the brain, it is also possible that additional components confer this specificity during memory formation. Fourth, although only two m6A binding proteins were investigated, namely ELAVL1 and YTHDF2, if m6A is indeed involved in regulating complex mechanisms like local mRNA translation, it is very likely that many additional binding proteins are involved in the pathway, and dynamically regulated during memory formation. Thus, although this study provides a first overview of how the m6A pathway is regulated during memory formation, many pieces of the puzzle may still be missing, some of which will likely provide key insight into the role of the m6A pathway in memory. Further research identifying new m6A pathway components and investigating how they are regulated during memory is required to help fill in the pieces of the puzzle.

4.7.2 m6A pathway regulation may not be occurring primarily in principal neurons

Due to our dissection technique, in which the entire dorsal CA1 is dissected and processed, the pool of RNA in the samples used for Fig. 3.2, 3.3 and S1 comes not only from principal neurons, but also from glial cells and interneurons. As a result, the changes observed in Fig. 3.2 and 3.3 may not reflect regulation of the m6A pathway in neurons, as is assumed in the proposed model linking m6A to local mRNA translation in neurons (Fig. 4.1). To specifically verify in which cells the m6A pathway is being regulated, future experiments could be run on RNA isolated from manually sorted fluorescently labeled pyramidal or glial cells, and could compare regulation of the m6A pathway in the different cell types (Hempel et al., 2007).
Regardless, the HSVs designed in this project to target *Fto* expression levels will likely shed some light on this question. Indeed, as HSVs are neurotropic, infecting only non-dividing, mature neurons (Fink *et al.*, 1996; Neve *et al.*, 2005), any effects seen on memory following manipulation of *Fto* using these viruses will reflect changes in FTO and m6A occurring in neurons, and thus reflect the role of the m6A pathway in neurons in regulating memory.

### 4.7.3 m6A regulation in other brain regions during learning

The dorsal CA1 region of the hippocampus was selected as a starting point for investigating the possible role of m6A in memory in the brain, due to its critical role in spatial memory and position as the main output of the hippocampus (Amaral & Witter, 1989; Auer *et al.*, 1989; Lenck-Santini *et al.*, 2001; Moser *et al.*, 1993; Volpe *et al.*, 1992). If m6A is indeed involved in memory, it is likely that the m6A pathway is regulated in different memory tasks, as well as in several other regions involved in memory, like the DG and prefrontal cortex, both of which play critical roles in memory and undergo synaptic plasticity during learning (Laroche *et al.*, 2000; O’Malley *et al.*, 2000; Saxe *et al.*, 2006; Steward & Worley, 2001). In order to develop a stronger understanding of the role of m6A in memory and establish whether it is involved in basic memory-related functions, like regulating local mRNA translation, which are critical to all cells undergoing plasticity, it will be helpful to explore its role in different tasks and regions involved in learning and memory.

### 4.7.4 Measuring mRNA levels versus protein levels

In order to evaluate the variations in the levels of m6A pathway components, we chose to measure mRNA levels instead of protein levels. Indeed, compared to other techniques like Western blotting, qPCR enables higher throughput, and more precise and sensitive measurements of enrichment levels (Aebersold *et al.*, 2013; Svec *et al.*, 2015). However, given that the m6A pathway components investigated in this study are of interest here for their functions in protein form, it is important to ensure that the changes in mRNA levels observed are in fact reflected at the protein level. To do this, one could use Western blot analyses or ribosome profiling, and compare the findings to the qPCR results (Cho *et al.*, 2015). However, a study looking at over 1000 genes by Cho *et al.* (2015) showed that during memory formation, changes in protein levels in the brain are primarily driven by increases in protein synthesis in the first 5 to 10 minutes, then by changes in gene transcription from 30 min to 4h. In other words, after 30
min, changes in protein levels are well reflected by changes in mRNA transcript levels. As all of our time points occurred within this second window, the use of qPCR to measure gene levels was deemed appropriate to measure changes in m6A pathway components following CFC, and likely accurately reflects protein levels. Future studies could nonetheless confirm these findings by measuring protein levels as well.

As for the virus validation component of this project, in order to ensure that the changes induced in Fto transcript levels were having a functional effect in the cells, we measured the effects on m6A levels. As m6A levels should only be affected if the FTO protein is being properly overexpressed or knocked down, and all three viruses showed the expected changes in m6A levels (Jia et al., 2011), we concluded that the viruses were manipulating FTO levels as intended.

4.7.5 Limitations related to the use of HSVs

4.7.5.1 Toxicity of HSVs in cell culture

As mentioned above, there was a substantial drop in the level of Fto overexpression from 12h to 2 days for p1005-FTO (Fig. 3.4A-B), as well as in the efficiency of the Fto knockdown from 2 to 3 days for shRNA-Fto (Fig. 3.4G-H). Given that increased levels of cell death were also seen at these time points, for both control and Fto manipulating viruses, it is likely that this is due to the cytotoxicity of the HSV helper virus used. Helper virus is used to package transgene vectors into viral particles, as it contains the necessary HSV genes, some of which induce cell lysis. It is then diluted to a minimal level, but not completely eliminated from the final viral stock, and as such can be toxic and affect the health and metabolism of cells in vitro (Fink et al., 1996; Lim, 2013; Neve et al., 2005). Importantly, when infecting non-dividing cells such as neurons, many HSV-1s, including the type used in this project, enter a latent state in which lytic genes are not expressed (Fink et al., 1996; Lim, 2013). As a result, cell death is much less of a concern in vivo, and viruses of this type are widely and successfully used for transgene expression in a variety of brain regions, including the hippocampus (Cole et al., 2012; Han et al., 2009; Josselyn et al., 2001; Sekeres et al., 2010, 2012). Future validation of the viruses in vivo will thus be important to confirm the effects seen on Fto and m6A in vitro, establish the size of these effects in the brain, and verify that the increased cell death and drop in efficiency are indeed due to helper virus, as neither effect should be seen in neurons.
4.7.5.2 Limited infection rate using HSVs

HSVs offer several advantages for our investigation of the role of the m6A pathway in memory over other viruses used for gene delivery, like AAVs. As mentioned above, they are neurotropic, enable rapid and short-term expression of transgenes (Carlezon et al., 1998), and allow sufficiently large packaging sizes for large proteins like Cas9 (Neve et al., 2005; Senís et al., 2014). However, although this varies according to the region targeted and vector used, HSVs typically infect only 6-20% of cells in a brain region (Sekeres et al., 2012; Yiu et al., 2014). Depending on the memory-related mechanism targeted, manipulating gene expression in such a small proportion of cells may be sufficient to produce an effect on memory (Han et al., 2009; Hsiang et al., 2014). Indeed, if manipulating Fto levels in approximately 20% of CA1 principal neurons impacts memory formation, this will strongly support a critical role for FTO and m6A in memory. However, if no effect is seen, it may be necessary to verify that this is due to FTO and not the size of the infection by confirming the results using viruses with higher infection rates, such as AAVs (Swiech et al., 2015) which can be engineered to specifically target neurons through different promoters or serotypes (Shetsova et al., 2005; van den Pol et al., 2009).

4.7.6 Effects on m6A levels of targeting Fto are not restricted to mRNA

Lastly, as mentioned previously, none of the demethylases or components of the methyltransferase complex have been shown to specifically target m6A in mRNA. Furthermore, as Fig. 3.4 shows, overexpressing or knocking down Fto in vitro leads to observable changes in m6A levels in total RNA, confirming that FTO also demethylates non-coding RNA (Fu et al., 2014a). Thus, although these HSVs can be used to establish a causal link between FTO and memory, the findings will not provide an answer as to whether regulation of m6A in mRNA specifically is critical for learning and memory, as suggested in the proposed model linking m6A to local mRNA translation (Fig. 4.1).

In order to address this question more specifically, and establish whether m6A is indeed involved in regulating mRNA processing in memory, and possibly local translation, it will be necessary to identify which components of the m6A pathway enable specific targeting of mRNA. Indeed, it is clear from Fig. 3.4 that it is possible to specifically increase m6A levels in mRNA. Once the components involved in regulating this effect during memory formation are identified, new HSVs can be designed to target them with relatively little difficulty, by adding or replacing
sgRNAs in the Cas9-\textit{Fto} vector (Walters et al., 2015). These viruses can then be used to manipulate m6A levels specifically in mRNA during memory formation, and begin to answer questions specifically about the potential role of m6A in regulating mRNA processing in memory.

4.8 Future Aims

Overall, the results presented here show that the m6A pathway is extensively regulated during memory formation. Furthermore, they show that both the m6A demethylase \textit{Fto} and m6A levels in mRNA display a biphasic regulation similar to many other critical memory-related processes like IEG activation, gene transcription, protein synthesis and LTP. Our results also indicate that mRNA is particularly targeted by the m6A pathway during memory formation, supporting a role for m6A in regulating components of memory-related mRNA processing, like transport and translation. Lastly, they demonstrate the flexibility of the m6A pathway, supporting its ability to regulate complex processes like local mRNA translation. Altogether, these findings strongly support a role for m6A in memory, and further suggest a possible role in regulating local mRNA translation. Nonetheless, as this study is the first to explore the m6A pathway in the brain during memory, more experiments must be conducted before the link between m6A and memory can be established.

The first of these proposed experiments uses the HSVs designed in this project to test whether manipulating \textit{Fto} levels in CA1 during learning affects memory. If this is the case, it would support a critical role for m6A in memory formation. The second experiment aims to further explore the result showing an increase in m6A in mRNA during memory formation, in order to identify the mRNA transcripts in which m6A levels are increased. If these transcripts are primarily PRPs, it would strongly support a role for m6A in regulating memory-related mRNA processing. The last experiment aims to establish whether the m6A pathway is in fact engaged at the synapse during memory formation. If this is the case, it would corroborate the potential role of m6A in regulating local mRNA translation during memory formation. Thus, each experiment will help fill in a piece of the puzzle linking m6A to memory.
4.8.1 Test whether manipulating FTO and m6A levels impacts memory

One of the principal aims of this project was to develop HSVs to target the m6A pathway during learning. Using these tools, we will be able to establish whether, as we hypothesize, m6A regulation is critical in memory formation. Indeed, although our results show that the m6A pathway is regulated during learning, it is possible that these changes are not critical to memory, or reflect basic cellular mechanisms independent of memory.

The first step in testing our hypothesis will be, as mentioned previously, to validate the HSVs designed in this project and establish the size of their effects in vivo. To do this, we will infuse them into dorsal CA1, collect the tissue three to five days later (Sekeres et al., 2010) and measure Fto and m6A levels in vivo. Results should be qualitatively similar to those obtained in culture (Fig. 3.4). Once the HSVs are fully validated, the next step will be to verify how manipulating FTO levels affects memory formation. Since our results showed that Fto levels are downregulated following CFC (Fig. 3.2), and m6A levels are upregulated (Fig. 3.3), we hypothesize that (1) knocking down Fto levels will enhance the naturally occurring regulation, producing a stronger memory, whereas (2) overexpressing Fto will act counter to natural regulation of m6A, and thus impair memory.

To test these hypotheses, we will first perform stereotaxic infusions of the HSVs into area CA1 of the hippocampus of adult mice (Sekeres et al., 2010). Following recovery, mice infused with a knockdown virus (1) will be trained with a weak CFC protocol, receiving for example one 0.5 mA shock over the course of a 5-min exposure to a new context. Mice will then be returned to the context 24h later and their levels of freezing will be recorded as measures of the strength of their memory (Chen et al., 1996; Maren et al., 1997). Based on our hypothesis, mice infused with knockdown viruses should freeze more than mice infused with the control virus, showing an enhancement of a weak memory. Mice infused with the overexpression virus (2) will be trained in a strong CFC protocol, receiving for example three 0.5 mA shocks over the course of a 5-min exposure to a new context. Based on our hypothesis, upon testing 24h later, mice infused with the overexpression virus should freeze less than mice infused with the control virus, showing an impairment of a strong memory.

If the results of these experiments do not show an effect of manipulating Fto on memory, it will suggest that although m6A is regulated during memory formation, it is not critical. If however
the results of these experiments are as hypothesized, they would provide the first causal evidence demonstrating a role for m6A regulation in memory. Given the flexibility of the Cas9 system, the viruses targeting *Fto* could quickly be redesigned to target other pathway components and start to piece together their distinct roles in regulating m6A in memory.

4.8.2 Identify mRNA transcripts showing enriched m6A levels during memory formation

As shown in Fig. 3.3, there is an upregulation of m6A in mRNA specifically following CFC. During heat shock, both Dominissini *et al.* (2012) and Zhou *et al.* (2015) observed an increase in m6A sites and translation of transcripts encoding stress-related proteins. Similarly, we hypothesize that if the upregulation of m6A is indeed related to memory, it will primarily affect memory-related transcripts.

To test this hypothesis, we will collect RNA from similar cohorts to those in Fig. 3.2, namely naïve mice, and mice sacrificed 30 min to 4h after CFC. We will then run a methylated RNA immunoprecipitation assay on the samples, allowing only transcripts containing m6A to be retained. These samples will then be sequenced to identify which transcripts show an increase in m6A 30 min and 4h following CFC (Dominissini *et al.*, 2012). Assuming that the transcripts showing higher m6A levels are also preferentially translated, we would expect to see many locally translated PRP transcripts on the list, like *CamKIIA* (Mayford *et al.*, 1996; Paradies & Steward, 1997), *Arc* (Link *et al.*, 1995), *Actb* (Tiruchinapalli *et al.*, 2003), *Map2* (Garner *et al.*, 1988), and NMDA receptor subunits (Grooms *et al.*, 2006; Miyashiro *et al.*, 1994).

However, the m6A system is very flexible, which is why we proposed that it could be involved in the fine-tuned processing of mRNA. As a result, the location of m6A in a transcript affects which binding proteins recognize it, and thus whether it is translated or degraded, for example, (Durie *et al.*, 2010; Wang, X. *et al.*, 2014; Wang, Y. *et al.*, 2014; Zhou *et al.*, 2015). Thus, it is likely that not all transcripts showing increased m6A levels will show an increase in translation. Ribosome profiling could thus be used to identify those transcripts recruited to the translation machinery following methylation (Cho *et al.*, 2015) and establish whether they are primarily PRPs.
Altogether, the results of this experiment will provide key insight as to what transcripts are being targeted by the m6A pathway during memory formation for processing. If the transcripts are not PRPs, it will suggest that m6A may be regulating more basic cellular functions than memory. However, if they are primarily PRPs, it would strengthen the hypothesis that m6A regulates mRNA processing of PRP transcripts during memory formation.

4.8.3 Establish whether m6A is playing a role in memory at the synapse

Local mRNA translation is known to be critical to memory formation (Miller et al., 2002; Ouyang et al., 1999), and yet the underlying mechanisms governing it remain unknown. The flexibility and dynamic nature of the m6A pathway makes it an excellent candidate for regulating complex processes like mRNA translation, as proposed in Fig. 4.1. It is for this reason that its potential role in memory is of particular interest, and thus this final experiment proposes to establish whether m6A is indeed playing a role in memory at the synapse.

In order to do this, we will look at where m6A pathway components are expressed and regulated in neurons. If the pathway is important for local mRNA translation, some of its components must be active at the synapse where local translation occurs. In our model (Fig. 4.1), we propose that m6A binding proteins, like Elavl1, and the demethylase Fto are active and regulated at the synapse during memory formation. In order to verify whether this is the case, we will first use antibodies against ELAVL1, FTO and m6A to establish the subcellular distribution of these pathway components in hippocampal cultured neurons.

If there is basal expression of these components in dendrites and spines, we will collect the hippocampi of mice having undergone CFC protocol as in Fig. 3.2. Samples will be separated into nuclear, cytoplasmic and synaptosomal fractions (Boyl et al., 2007), and processed for a Western blot analysis of protein abundance. Using appropriate internal controls for each fraction, we will first compare the level of components, like ELAVL1, FTO and m6A, in the nucleus, cytoplasm and synaptosomes of naïve mice, then investigate whether any changes in distribution occur 30 min after CFC. If ELAVL1, FTO or m6A is regulated at the synapse specifically during memory formation, there should be a relative change in the abundance of the components in the synaptosomal fraction compared to the cytoplasmic or nuclear fraction 30 min after CFC compared to naïve controls.
If none of the components of the m6A pathway are regulated at the synapse, it will suggest that m6A is primarily involved in regulating processes like mRNA transport and possibly somatic translation during memory formation (Dominissini et al., 2012; Fustin et al., 2013; Zhao et al., 2014). If however some of the components are regulated locally, this would strongly support a role for m6A in regulating local mRNA translation.
Memory relies critically on the synthesis of plasticity-related proteins, not only in the soma, but also locally at synapses. How cells are able to precisely and independently coordinate memory-related translation at each of their synapses remains unknown. Recently, m6A, the most abundant modification of RNA, was shown to be dynamically regulated and involved in translational control. Similar to epigenetic modifications of DNA which enable precise control over transcription in the nucleus during memory formation, mRNA modifications may provide fine-tuned control over translation throughout the neuron, including locally at individual synapses.

In this project we investigated the role of the m6A RNA methylation pathway in memory. Firstly, we found that components of the m6A pathway are enriched in different tissues, with the demethylase Fto being highly expressed in the mouse brain. We then showed that during formation of a contextual memory, levels of several m6A pathway components, including Fto, are transiently modulated in the dorsal CA1 of the hippocampus. Correspondingly, there is a biphasic increase in overall levels of m6A in mRNA. To probe the role of m6A in memory formation, we then developed and validated HSV vectors to rapidly and selectively overexpress or knockdown FTO in cells. Among these is the first HSV-mediated CRISPR/Cas9 knockout. Harnessing this tool will allow increased flexibility and rapid integration of new gene targets to more thoroughly investigate the role of the m6A pathway in memory.

As this project is one of the first to look at the possible role of m6A in memory, the results presented here remain exploratory, but promising. Indeed, we show not only that the m6A pathway is remarkably flexible, but also that it is dynamically regulated during memory formation, in particular in mRNA. Together, these findings provide the first compelling evidence showing that m6A may be critically involved in regulating highly complex processes like local mRNA translation in memory. FTO has already been linked to dementia and cognitive impairments. Gaining deeper insight into the role of m6A in memory may not only improve our
ability to treat diseases involving memory dysfunction, but also strengthen our grasp of the intricate biological processes that shape who we are and what we do.
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Appendix

m6A methyltransferases

**A. Mettl3**

**B. Mettl14**

m6A demethylases

**E. Alkbh5**

**F. Fto**

m6A binding proteins

**I. Elavl1**

**J. Ythdf2**

**Fig. S1. m6A pathway components levels in context-only or shock-only controls.** None of the pathway components showed changes at different time points following context-only exposure or shock-only exposure. A. Mettl3 context ($F_{3,34} = 0.87$, $p < 0.05$), shock ($F_{3,33} = 0.82$, $p < 0.05$). B. Mettl14 context ($F_{3,27} = 1.51$, $p < 0.05$), shock ($F_{3,26} = 1.11$, $p < 0.05$). C. Alkbh5 context ($F_{3,14} = 0.10$, $p < 0.05$), shock ($F_{3,14} = 3.156$, $p < 0.05$). D. Fto context ($F_{3,28} = 1.86$, $p < 0.05$), shock ($F_{3,28} = 2.09$, $p < 0.05$). E. Elavl1 context ($F_{3,30} = 0.11$, $p < 0.05$), shock ($F_{3,27} = 0.70$, $p < 0.05$). F. Ythdf2 context ($F_{3,24} = 1.59$, $p < 0.05$), shock ($F_{3,23} = 1.33$, $p < 0.05$). All time points normalized to naïve levels. Time points for context-only or shock-only conditions were collapsed into single groups for each gene for analysis in Fig. 3.2. n = 5-11 (30 min, context-only), 5-8 (1h, context-only), 4-10 (2h context-only), 4-8 (4h context-only), 5-13 (30 min, shock-only), 5-8 (1h, shock-only), 4-8 (2h shock-only), 4-8 (4h shock-only). *$p < 0.05$. 

n = 5-11 (30 min, context-only), 5-8 (1h, context-only), 4-10 (2h context-only), 4-8 (4h context-only), 5-13 (30 min, shock-only), 5-8 (1h, shock-only), 4-8 (2h shock-only), 4-8 (4h shock-only). *$p < 0.05$. 

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