m^6A mRNA Methylation in the Mammalian Brain: Distribution, Function and Implications for Brain Functions

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Contents

Abstract RNA is abundantly modified by a range of covalent modifications, collectively termed the epitranscriptome. Of these modifications, N^6-methyladenosine (m^6A) is the most prevalent internal chemical tag in eukaryotic mRNA. Being cotranscriptionally deposited, it regulates almost all aspects of mRNA's lifetime

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S. Jurga, J. Barciszewski (eds.), *The DNA, RNA, and Histone Methylomes*, RNA Technologies, https://doi.org/10.1007/978-3-030-14792-1_15

including maturation into mRNA, stability, distribution and protein translation. While m^6A is likely present in all developing and adult mammalian tissues, here we highlight its distribution and reported functions in the mammalian brain. Additionally, we describe its potential to act as an encoding mechanism for activity- and experience-dependent adaptation and memory-formation. Such alterations may be positive when adjusting to outer challenges or negative when involved in maladaptive processes of the brain such as in the development of psychopathologies.

Consequently, studying this layer of gene expression control in the brain, alongside posttranslational regulation of proteins and epigenetics may inform us as to the molecular mechanisms underlying normal and pathological behaviors. Unfortunately, measuring m^6A levels, patterns and especially dynamics still poses a major technological challenge especially in such a complicated organ as the brain.

Keywords Epitranscriptome · N^6-methyladenosine · m^6A · Methyltransferase · Demethylase · m^6A reader · Brain functions · Psychiatric disorders

1 Introduction

Over 100 covalent base modifications have been found in all domains of life including prokaryotes and eukaryotes but also archaea and viruses. They appear on almost all types of RNA including mRNA, tRNA, rRNA and snRNA (Boccaletto et al. 2018). Although many of these modifications and their potential to post-transcriptionally regulate gene expression have been known since the 1960s and 1970s, the field—now known as epitranscriptomics—attracted little attention until recent technological developments. In mammals, the most diverse RNA species regarding modified nucleotides are tRNA and rRNA, while only a very limited set of modifications is present on mRNA. The most abundant internal modification in mammalian mRNA is N^6-methyladenosine, abbreviated to m^6A, but many others exist including N^1-methyladenosine, abbreviated to m^1A (Dominissini et al. 2016; Li et al. 2016), pseudouridine Ψ (Carlile et al. 2014; Schwartz et al. 2014a; Li et al. 2015), 5-methylcytosine m^5C (Dubin and Taylor 1975; Squires et al. 2012), and A-to-I editing (Levanon et al. 2004; Li et al. 2009).

2 m^6A mRNA Methylation

The biochemistry and cellular regulation of m^6A has been described before in great detail here (Jia 2016) and elsewhere (e.g., Zhao et al. 2017). Internal mRNA m^6A usually occurs in a fairly defined consensus motif DRm^6ACH (with D = A, U or G; R = G or C; and H = A, U, C) (Wei et al. 1975; Schibler and Perry 1977). m^6A is preferentially localized to the 3′UTR near the stop codon and in the 5′UTR of mRNAs, and to some degree, in the coding sequence (exon), the transcription start

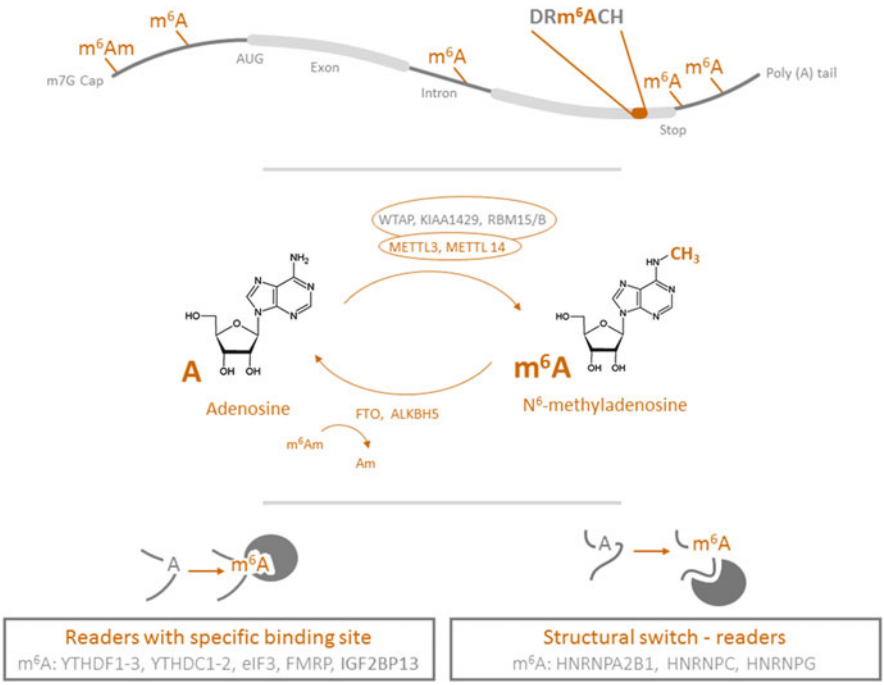

Fig. 1 The m^{6}A regulatory system including writers, erasers and readers; distribution of m^{6}A on mRNA; and m^{6}A reader proteins (adapted from Engel and Chen 2018)

site (TSS) and in long internal exons (Dominissini et al. 2012; Meyer et al. 2012) (Fig. 1).

Adenosine methylation at the N^6-position, in contrast to e.g., N^1-methylation in m^{1}A, does not impair the Watson-Crick pairing with U but works via creating binding motifs increasing the accessibility for RNA-binding proteins (RBP; Liu et al. 2015, 2017), and modulating the mRNA secondary structure (Roost et al. 2015; Liu et al. 2015; Spitale et al. 2015).

The existence of both writer and eraser networks adding and removing m^{6}A (as described in the following) has been widely accepted to indicate that m^{6}A methylation is highly dynamic and a readily reversible system (Fig. 1).

2.1 Writing m^{6}A: The Methyltransferase Complex

A multiprotein methyltransferase complex transfers a methyl group from the donor substrate, S-adenosyl methionine, to the target RNA adenosines creating methylated adenosine (Bokar et al. 1994, 1997). This complex consists of two subunits with catalytic MT-A70 domains occurring in a heterodimer: METTL3 and METTL14 (Liu et al. 2014). Additional complex components include WTAP (Liu et al. 2014;

Ping et al. 2014), KIAA1429 (Schwartz et al. 2014b), RBM15/B (Patil et al. 2016), and others, which enable target tethering and specificity as well as establishing the distinct nuclear localization pattern of the complex. Deposition of m^6A likely occurs co-transcriptionally, i.e., on nascent pre-mRNA that is still tethered to genomic DNA (Slobodin et al. 2017; Ke et al. 2017). These latter studies however argue for rather static levels of m^6A, i.e., conclude that once hnRNA has been released from the chromatin, m^6A can only be removed by demethylation or mRNA decay. Posttranslational regulation of the methyltransferase proteins e.g., by phosphorylation has been described but may not necessarily regulate methylation activity per se (Schöller et al. 2018). Finally, conflicting roles for certain methyltransferase complex components aside from methylating nascent hnRNA or even those occurring in the cytoplasm have also been described but may be restricted to very special circumstances (Chen et al. 2015; Alarcón et al. 2015a; Lin et al. 2016).

2.2 *Erasing m^6A: FTO and ALKBH5*

There are two known m^6A demethylating enzymes enabling a potentially reversible and thus fully dynamic regulation of m^6A: FTO (Jia et al. 2011) and ALKBH5 (Zheng et al. 2013). Interestingly, both were reported to have distinct subcellular and tissue distributions and thus potentially encode target- and tissue-specific regulation of m^6A (Gerken et al. 2007; Vujovic et al. 2013; Zheng et al. 2013; Hess et al. 2013). Many reports even indicate that cellular regulation of e.g., FTO expression and activity is regulated thus enabling active regulation of m^6A levels. However, recent results have dampened the original excitement over dynamic demethylation of m^6A including the observation that FTO may preferentially demethylate a closely related and often co-detected modification, N^6,2′-O-dimethyladenosine m^6Am, in vitro and in vivo (Schwartz et al. 2014b; Linder et al. 2015; Mauer et al. 2017; Engel et al. 2018). These data suggest that reversibility of m^6A may be less extensive than originally thought especially within physiological systems (Mauer et al. 2017; Mauer and Jaffrey 2018). Similar conclusions may be drawn from the fact that all known full mouse knockouts for FTO and ALKBH5 are, in contrast to all knockouts of the methyltransferases, viable after birth (Fischer et al. 2009; Zheng et al. 2013). In favor of active demethylation, localization of the enzymes and stoichiometry of m^6A and m^6Am, may allow FTO to target m^6A in significant amounts in vivo with more recent data showing demethylation of all three mRNA methylated adenosines, m^6A, m^6Am, m^1A, by FTO (Wei et al. 2018).

2.3 *Readers of m^6A*

Given the wide abundance of m^6A and even more diverse cellular functions of m^6A (detailed below), a large part of functional specificity has to be achieved by the diverse range of m^6A-interacting RBPs, the so called m^6A readers. The most

important family of m^{6}A-readers consists of the YTH-domain-containing proteins, which bind directly to m^{6}A. Currently known mammalian members of this family are YTHDF1-3 and YTHDC1-2. They have been assigned very diverse, often contradictory, yet sometimes cooperative cellular functions including promotion and inhibition of translation and decay (recently reviewed e.g., in Roundtree et al. 2017; Patil et al. 2018). The very diverse functions of the different YTH-family members may be regulated by several factors including cellular sub-localization, target-specificity and posttranslational regulation of the readers. Next to YTH-domain readers, m^{6}A has also been reported to directly recruit eIF3 leading to a promotion of cap-independent translation (Meyer et al. 2015). Other proteins lacking a YTH domain, e.g., the hnRNP proteins HNRNPA2B1, HNRNPC, and HNRNPG (Liu et al. 2015, 2017; Alarcón et al. 2015a), may bind m^{6}A instead via m^{6}A-specific structural features. Finally, additional classes of direct binder proteins without a YTH-domain or m^{6}A-specific structural features may exist including IGF2BP1–3 (Huang et al. 2018) and FMRP (Arguello et al. 2017; Edupuganti et al. 2017).

2.4 Cellular Functions of m^{6}A

m^{6}A cellular functions include the regulation of all stages of mRNA's lifetime and thus establish a layer of secondary gene expression regulation (recently reviewed e.g., in Roundtree et al. 2017; Patil et al. 2018). Starting at the very beginning of mRNA's life, m^{6}A has been described to regulate the maturation of pre-mRNA into mature mRNA including 5′ capping, 3′ polyadenylation, splicing, nuclear processing and nuclear export of mRNAs. Thereby, m^{6}A catalyzes differential splicing (Liu et al. 2015; Xiao et al. 2016; Ke et al. 2017) and differential polyA site usage (Ke et al. 2015; Molinie et al. 2016). m^{6}A further promotes and also inhibits mRNA translation depending on the respective mRNA-m^{6}A target and bound m^{6}A-reader (Wang et al. 2015; Zhou et al. 2015; Meyer et al. 2015; Li et al. 2017a; Shi et al. 2017). Indicating the end of mRNA lifetime, methylation generally appears to accelerate mRNA decay (Wang et al. 2014) yet even this relationship is likely more complex than seen on first sight. m^{6}A recognized by other effector proteins beyond the classical YTH-domain readers may have completely different effects on mRNA via third effector proteins. This includes the described interactions with ELAV-like RNA binding protein 1 (ELAV1/HuR) (Wang et al. 2014), intersections with miRNA biogenesis (Chen et al. 2015; Alarcón et al. 2015b), and interactions with the Toll-like receptor (TLR) family protein members TLR3 and TLR7 (Karikó et al. 2005).

3 m^{6}A Distribution and Function in the Brain

The brain is one of the most complexly structured and regulated mammalian organs both during development and in adulthood. The adult brain especially, is a unique organ regarding gene expression regulation as it not only consists largely of

postmitotic cells with very limited regeneration- and repair-capacity, but also because of the huge diversity and specialization of those cells. Thus, a mechanism of secondary gene expression regulation such as RNA methylation may be especially crucial in such a system.

Several studies have begun to uncover the functional significance of m^6A regulation in the central nervous system (CNS) and its role in normal brain physiology during all stages of life from development to adulthood and encoding cellular plasticity in the adult brain. Thereby, m^6A is abundant in the brain during all developmental stages with increasing levels during development (Meyer et al. 2012). In the adult brain, m^6A is likely found in all brain structures of the CNS but also in the peripheral nervous system (PNS) (Weng et al. 2018). Region-specific methylation levels and patterns have been reported (Chang et al. 2017; Engel et al. 2018).

3.1 m^6A in Brain Development

Knockout of the m^6A methyltransferases in embryonic stem cells leads to embryonic lethality in all known cases, usually together with severe nervous system malformation (Fukusumi et al. 2008; Geula et al. 2015). In line, m^6A has been described to be essential for mammalian cortical neurogenesis (Yoon et al. 2017). Loss of m^6A in murine neural progenitor cells by removal of either METTL3 or METTL14 leads to prolonged cell cycle progression and delayed neuronal differentiation via suppression of neuronal lineage markers, thus, extending the cortical neurogenesis widely into postnatal stages (Yoon et al. 2017). Similarly, conditional knockout of *Mettl3* using the prenatally expressing Nestin-Cre causes severe developmental defects both in cortical and cerebellar regions (Wang et al. 2018). Nestin-Cre *Mettl3* conditional knockout mice, i.e., those with a knockout in prenatal brain cells, display cerebellar hypoplasia caused by drastically enhanced apoptosis of newborn cerebellar granule cells in the external granular layer leading to severe motoric deficits and death within the first 3 weeks after birth (Wang et al. 2018). Loss of FTO or FTO function in both mice and human leads to postnatal growth retardation, including microcephaly and increased postnatal lethality (Boissel et al. 2009; Gao et al. 2010). Interestingly, full knockut of the reader genes *Ythdf1* or *Ythdf2* does not lead to any gross brain development abnormalities, lethality or motor deficits (Ivanova et al. 2017; Shi et al. 2018).

m^6A-profiling of human fetal forebrains and human brain organoids has revealed a conserved and unique m^6A landscape similar to that in mouse embryonic forebrains (Yoon et al. 2017). In general, m^6A has been described to be somewhat evolutionary conserved (Ma et al. 2017). Additional to stem cells in the developing brain, m^6A is crucial for at least one of the two populations of neural stem cells (NSCs) remaining in the adult brain, the stem cells in the subgranular zone of the dentate gyrus (Li et al. 2017b). FTO loss in these cells reduces NSC proliferation and neuronal differentiation, reminiscent but not fully equal to the embryonic

neurogenesis defect seen in *METTL3* and *METTL14* knockout mice. Finally, m^{6}A modified RNAs also play a key role in brain cancer (Zhang et al. 2017; Cui et al. 2017).

3.2 *m^{6}A in the Adult Brain*

Several detailed maps of m^{6}A in the adult mammalian brain are available (Meyer et al. 2012; Hess et al. 2013; Chang et al. 2017; Merkurjev et al. 2018; Engel et al. 2018), reporting a total of approximately 10,000–20,000 m^{6}A sites. Additionally, CNS RNA methylation has also been characterized in *Drosophila melanogaster* (Lence et al. 2016). In stark contrast to the deleterious effect of methyltransferases in the developing brain, loss of FTO, METTL3, or METTL14 in postnatal neurons only, e.g., via a conditional knockout using Camk2a-Cre driver lines, does not cause any major brain morphological changes or increase apoptosis of cells (Koranda et al. 2018; Engel et al. 2018; Zhang et al. 2018). The consequence of a loss of these enzymes in cells other than neurons, e.g., astrocytes, has not been investigated yet.

3.3 *Sorting mRNAs in Complex Neurons by m^{6}A?*

m^{6}A is involved in several mechanisms of regulating translocation of mRNA, including nuclear export (Zheng et al. 2013; Fustin et al. 2013) and sorting of mRNA into specific cytoplasmic aggregates like P-bodies and stress granules (Wang et al. 2015; Anders et al. 2018). Neurons are built more complexly than most mammalian cells, including higher polarization, higher fragmentation into specialized components like axons and dendrites, and a higher number of complex cell-to-cell connections. These cell-to-cell connections are highly regulated in the brain including changes of signal transmission efficacy and interactions of neurons with other cell types. Many such cellular changes are often realized via changes of gene expression control including compartmentalized regulation of protein translation e.g., at the synapse (Holt and Schuman 2013). Consequently, it has been speculated that m^{6}A-modification of mRNA may regulate spatial sorting and compartmentalized protein translation control within neurons into axons, pre-synaptic nerve terminals, dendrites, and dendritic spines (Fig. 2). While a final demonstration of such a mechanism is still lacking, enrichment of synaptic and neuronal projection gene ontology terms has been reported repeatedly during the recent efforts to map m^{6}A in the brain (Hess et al. 2013; Widagdo et al. 2016; Yoon et al. 2017; Merkurjev et al. 2018; Engel et al. 2018). Further, m^{6}A has been reported to be localized to axons and to regulate axonal growth via local translation via GAP-43 (Yu et al. 2018) and several components of the m^{6}A-machinery have been observed to be synaptically located including writers and erasers, classically considered to be nuclear proteins (Yu et al. 2018; Merkurjev

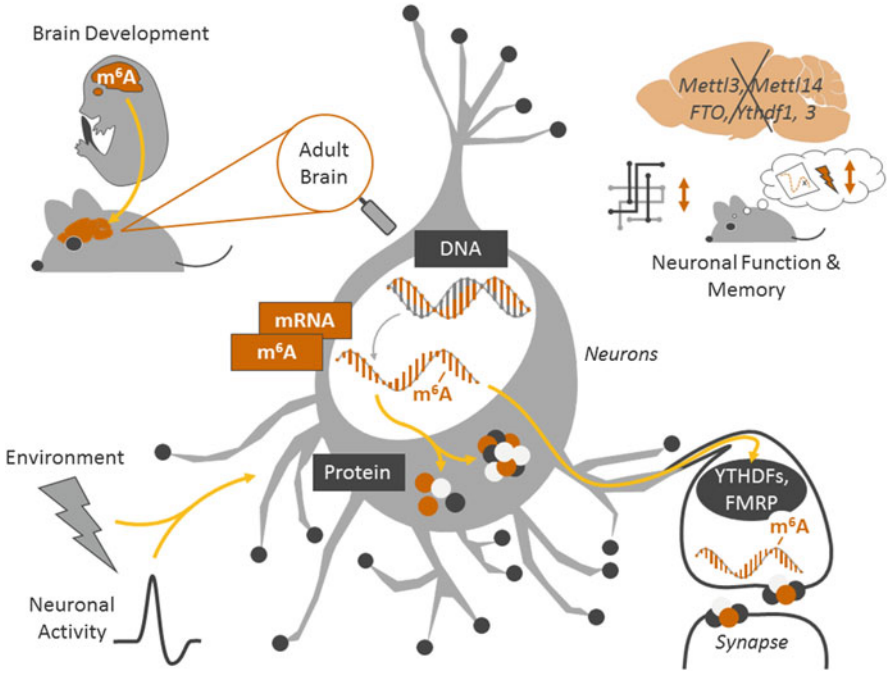

Fig. 2 Functions of m^{6}A in the brain (adapted from Engel and Chen 2018)

et al. 2018). Consequently, synapses and neuronal somas have been reported to harbor their own specific epitranscriptome (Merkurjev et al. 2018).

3.4 FMRP

A potential key player involved in such a mechanism of localized translation regulation in neurons may be the fragile X mental retardation protein (FMRP). FMRP is a neuronal RNA-binding protein known for its role in metabotropic glutamate receptor (mGluR)-dependent signaling and synaptic plasticity (Waung and Huber 2009). It is found in neuronal RNA transport granules and regulates dendritic localization of RNAs. FMRP inhibits RNA local transcript translation including that occuring at the synapse (Holt and Schuman 2013). Overlap of the m^{6}A consensus motif with the one of FMRP and high co-occurrence of FMRP binding sites in m^{6}A modified target sites was observed early on (Anderson et al. 2016; Chang et al. 2017; Engel et al. 2018). Finally, actual binding of FMRP to m^{6}A was shown recently (Arguello et al. 2017; Edupuganti et al. 2017). Competitive binding of m^{6}A between FMRP and YTHDF1 and YTHDF2 was speculated upon as a potential mechanism of m^{6}A-FMRP action (Edupuganti et al. 2017; Zhang et al. 2018).

3.5 Activity Dependent Regulation

A special property of neurons is their constant regulation via electrical and chemical signaling leading to amongst others activity-dependent regulation of gene expression. This enables the critical adaptiveness of the brain to outer and inner stimuli via short- and long-term alterations in gene expression, neuronal morphology, connectivity and ultimately regulation and behavior.

Similarly to m^{6}A being involved in the basic cellular stress response (Dominissini et al. 2012; Zhou et al. 2015; Meyer et al. 2015; Xiang et al. 2017), it has also been described to be involved in cellular processes triggered upon neuronal signaling and activity-dependent regulation (Fig. 2). This includes both regulation in vitro, e.g., in primary neuronal cultures after KCl-induced neuronal depolarization (Widagdo et al. 2016), and in vivo in the adult brain after challenges in several brain regions, e.g., after fear conditioning (Widagdo et al. 2016; Walters et al. 2017; Zhang et al. 2018), stressful challenges (Engel et al. 2018), and also in the PNS after nerve injury (Weng et al. 2018). Levels of activity-dependent regulation include changes within the m^{6}A machinery, altered global and target-mRNA specific m^{6}A levels, and differential translation of downstream-effectors like immediate early genes (IEGs). In line with this, IEG function is widely impaired in *Mettl3* knockout primary cortical neurons after fear conditioning (Zhang et al. 2018).

Furthermore, while activity-dependent gene expression changes for m^{6}A enzymes and readers have been reported, the relation of dynamically regulated mRNA abundancy of e.g., *Mettl3* or *Fto* to the respective active protein levels and cellular consequences of regulated m^{6}A are still mostly unclear. This includes the very limited current knowledge on how the different m^{6}A-enzymes and readers are regulated at the protein level in response to activity, including their subcellular localization, activity, and target specificity. Investigating the posttranslational regulation of the m^{6}A-enzymes and readers, as for example shown or suggested for METTL3 and FTO via SUMOylation and ubiquitination (Tai et al. 2017; Zhu et al. 2018; Du et al. 2018) may provide valuable insight.

3.6 Brain Function, Electrophysiology and Behavior

Consistent with the concept of mRNA methylation being involved in the regulation of gene expression after neuronal activity, alterations of the m^{6}A system via m^{6}A enzyme knockouts were found to change neuronal electrophysiological properties. For example, long term potentiation (LTP) in the hippocampus was decreased after hippocampal knockout of *Mettl3* (Zhang et al. 2018) (but observe conflicting data Engel et al. 2018), *Fto* (Engel et al. 2018), and *Ythdf1* (Shi et al. 2018), while deletion of *Mettl14* in the striatum led to increased neuronal excitability and reduced spike frequency adaptation (Koranda et al. 2018).

While the lack of m^{6}A enzymes during development usually leads to severe developmental defects, enzyme deletion or depletion specifically in the adult brain

causes only limited behavioral phenotypes, usually excluding effects on motor skills, movement properties, or anxiety-like behavior. Adult brain m^{6}A-manipulation, reported to date, leads fairly specifically to memory impairment: Conditional knockout of *Mettl3* in the hippocampus or forebrain excitatory neurons enhances cue-related memory consolidation after fear-conditioning and in the Morris Water Maze (MWM; Engel et al. 2018; Zhang et al. 2018), while full knockout of *Ythdf1* reduces memory consolidation in both of these tests (Shi et al. 2018). Additionally, m^{6}A deficiency via *Mettl14* deletion in striatal neurons impairs learning and performance (Koranda et al. 2018). Conversely, knockout or knockdown of *Fto* in the prefrontal cortex or hippocampus enhances consolidation of cue- and or context-related fear memory (Wang et al. 2015; Widagdo et al. 2016; Walters et al. 2017) while impairing spatial learning and memory of mice in MWM and eight-arm maze test (Li et al. 2017b). Knockout of *Fto* also attenuates the response in cocaine-induced locomotion (Hess et al. 2013).

Together these studies show that although m^{6}A seems to be much less crucial in the adult brain than during development, it is potentially important for specific brain functions. In line with m^{6}A roles as a secondary mechanism of gene expression regulation, it may therefore be especially important for brain functions that require activity-dependent gene expression regulation, like memory formation.

4 m^{6}A: Possible Implications for Psychiatric Disorders

Obesity and type-2 diabetes have been repeatedly associated with genetic polymorphisms in the first intron of the human *FTO* gene (Dina et al. 2007; Scuteri et al. 2007; Frayling et al. 2007), although the variant reported on may actually not affect the *FTO* locus itself but rather neighboring genes (Smemo et al. 2014; Stratigopoulos et al. 2014; Claussnitzer et al. 2015). Consequently, the physiological roles of *FTO* in the context of energy metabolism and expenditure and food intake have been extensively investigated but led to mixed results and as such the mechanisms remain unknown (Hess and Brüning 2014). Beyond such metabolic functions, the *FTO* variant has also been associated with several psychiatric disorders including Major Depressive Disorder (MDD) (Samaan et al. 2013; Milaneschi et al. 2014), Alzheimer's Disease (AD) (Profenno et al. 2010; Keller et al. 2011; Reitz et al. 2012) and Attention Deficit Hyperactivity Disorder (ADHD) (Choudhry et al. 2013). It has further been indicated in memory processing capabilities in humans genome-wide association studies (Ho et al. 2010; Benedict et al. 2011; Keller et al. 2011). Additionally to *FTO*, a polymorphism related to *ALKBH5* has been reported to be associated to MDD in a relatively small candidate gene association study (Du et al. 2015). However, recent more powered genetic association studies and meta-analyses did not report any association of gene variants close to any of the m^{6}A-machinary genes to various psychiatric disorders, including MDD, Post-traumatic stress disorder, ADHD, and AD (Demontis et al. 2017*; Martin et al. 2017*; Purves et al. 2017*; Meier et al. 2018*; Duncan et al. 2018; Wray et al. 2018; Coleman et al. 2018*; Jansen et al. 2019, * not yet peer-reviewed preprints).

Beyond classic genetic association studies, increasing evidence suggests that dysregulation and maladaptation of transcriptional fine-tuning is central to the etiology of psychiatric disorders more so than monogenetic causes (Nestler et al. 2016). In agreement, our recent study comparing m^{6}A of MDD patients and healthy controls in blood and derived cells found hardly any differences between the two groups except for after stimulation of stress-response signaling pathways (Engel et al. 2018). While these measurements were performed only in blood or blood derived cells, peripheral DNA methylation signatures related to neurobiological phenotypes may have some, albeit limited, similarity to central signatures (Davies et al. 2012; Farré et al. 2015; Hannon et al. 2015). Taken together, this indicates that the m^{6}A-system may well be involved in the etiology of psychiatric disorders via changed regulation of gene expression, especially considering the encoding of activity-related gene expression regulation, and thus may lead to long-term changes contributing to psychiatric disorders.

5 Technological Challenges of Measuring m^{6}A in the Brain

Technical approaches to detect m^{6}A and challenges associated to them have been discussed in great detail here (Jia 2016) and elsewhere before (e.g., Schwartz and Motorin 2017; Helm and Motorin 2017; Schaefer et al. 2017). While the field of RNA modifications, especially m^{6}A, has seen an incredible increase in attention over the last 10 years, studying those modifications still poses a major challenge given the limited availability of appropriate molecular tools and methods. This is especially true when aiming to quantify m^{6}A dynamics. In the following section, we will summarize some of the current challenges related to the methods most used to detect m^{6}A in the brain in vivo.

5.1 Global Detection Techniques

Two-dimensional thin layer chromatography (2-D TLC) and high-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (LC-MS/MS) were two of the earliest developed methods to detect and quantify modified nucleotides in RNA. Both require mRNA to be digested into single nucleotides first, which are then separated and detected based on their physico-chemical properties. This enables specific discrimination between different modifications, e.g., m^{1}A and m^{6}A. Stable isotope labelling approaches were developed to optimize these techniques but are mostly not applicable for analyzing intact mice or human organs (Popova and Williamson 2014; Kellner et al. 2014; Paulines and Limbach 2017). Both TLC and LC-MS/MS require comparably large amounts of input material and are usually performed on total RNA or mRNA preparations, and thus report an average global methylation signal derived across all nucleotides with a

loss of sequence and target context. These techniques are thus greatly suited to detect major changes in levels of different modifications e.g., after knockout of enzymes or in some cases of experimental manipulations (Engel et al. 2018), but may fail to detect more subtle changes as often expected after stimulation of the adult brain.

Two additional techniques to measure global m^6A-levels are antibody-based enzyme-linked colorimetric methods like Dot Blots and ELISAs. As with TLC and LC-MS/MS, these methods are commonly performed on total RNA or mRNA preparations but without previous digestion of the RNA. In contrast with the earlier described methods, they allow for low time-consuming and high throughput screening of samples and thus may be an entry point for characterizing m^6A regulation in vivo. However, beyond being only able to report large and global differences in methylation, these methods also potentially suffer from antibody-associated issues (discussed below).

In contrast, a related method not suffering from the problems arising from global averaging of m^6A modification is the SCARLET method (site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography, Liu et al. 2013) which quantifies nucleotides at one specific location with TLC. However, SCARLET also requires very high amounts of input material and radioactive labeling and thus is not readably applicable to study m^6A dynamics in vivo.

5.2 *m^6A-Seq*

The development of mapping m^6A in a transcriptome-wide approach revolutionized the field in 2012 (m^6A-Seq: Dominissini et al. 2012; meRIP-Seq: Meyer et al. 2012). Both, essentially equal, methods are based on affinity purification of fragmented mRNA with m^6A-specific antibodies followed by random primed cDNA library generation, adaptor ligation and high throughput small read sequencing. Antibody-based affinity purification is needed because m^6A, in contrast to other modifications as for example m^1A (Dominissini et al. 2016), does not stop the most common reverse transcription enzymes or lead to base misincorporation. This may be overcome by using specific m^6A-sensitive polymerases (Harcourt et al. 2013) or engineered reverse transcriptase with increased misincorporation in the opposite strand (Aschenbrenner et al. 2018), although both methods are not yet commonly established. This feature of m^6A also limits the current availability of PCR based techniques.

While m^6A-Seq enables a high throughput transcriptome-wide description of m^6A, it comes with many significant problems: First of all, it is dependent on the antibody used with potential differences between the used antibodies and even affinity-differences across different batches of the same antibody. Antibodies may also detect related modifications like m^6Am (Linder et al. 2015). Click chemistry protocols may improve antibody specificity (Hartstock et al. 2018) but are not yet implemented or tested in the majority of protocols. So far, only a limited number of

antibodies have been used, leading to somewhat different signatures (Zeng et al. 2018) but a comprehensive study comparing their detection patterns is not available yet. Furthermore, the binding properties of the antibodies may be influenced by the adjacent nucleotide sequence and RNA secondary structures. Antibodies also may suffer from substantial background signal with sequence-selective capture of certain unmodified fragments (Schwartz et al. 2013). Moreover, all of these biases of the sequence read distributions are mixed and amplified by the structural biases inherent to RNA-Seq itself. The resulting bias-mixture can only be partially remedied by the typical correlation to input RNA-Seq common in m^{6}A-Seq.

Secondly, the original m^{6}A-Seq protocols required substantial quantities of input material (several micrograms of purified mRNA). Recent low input protocols overcome this problem by using library kits optimized for ultra-low input RNA-Seq (Zeng et al. 2018). These low-input protocols may however introduce even more additional biases. Superficial comparisons of data received with such low-input protocols have already showed significant differences from the respective classical m^{6}A-Seq data set but this needs to be further investigated (Zeng et al. 2018).

Thirdly, classic m^{6}A-Seq protocols do not detect the modified nucleotide itself but a pile-up of fragments called m^{6}A-peaks which should, in principle, harbor the m^{6}A site in its center. Such peaks, by design, are around 200 nt long (when using 100 nt RNA fragments) but often experiments result in even bigger peaks likely due to several m^{6}A-sites close by. The m^{6}A-sites appearing in clusters (Ke et al. 2015; Linder et al. 2015) and their potential dynamics will thus be partially lost in m^{6}A-Seq. Further, due to commonly employed method of cDNA synthesis via random hexamer primers, m^{6}A-peaks will not include 5′ ends of the mRNA if methylated, although 5′ mRNA m^{6}A and m^{6}Am methylation may still be inferred from a peak located at the 5′ UTR.

Aiming to improve these protocols, several protocols have been recently developed to enable a nucleotide-specific transcriptome-wide mapping of m^{6}A (m^{6}A-CLIP: Ke et al. 2015; miCLIP: Linder et al. 2015). These methods use UV-crosslinking of an m^{6}A-antibody with mRNA-fragment leading to predictable mutation and truncation patterns in the cDNA strand during reverse-transcription that can be detected later in the sequencing data. Besides being prone to sequence and structural biases again, and the difficulty to map due to short fragments, the resulting data is often more noisy and less consistent than m^{6}A-Seq data with many more replicates needed for consistent mapping and unclear quantitative potential.

In the future, detection of m^{6}A via directly sequencing the RNA in its native form, e.g., while pulling through a nanopore, may overcome these challenges but just begins to be established for a wider audience (Liu et al. 2019).

5.3 Quantification of m^{6}A-Seq

Most bioinformatics pipelines to analyze m^{6}A-Seq data rely on comparisons of the enrichment of m^{6}A-immunoprecipitated fragments over standard RNA-Seq signal, employing cutoffs to defined peak ranges and minimum-occurrence across several

replicates to call the presence or absence of a peak. As a result, analysis results are heavily dependent on the bioinformatics algorithm used and seemingly arbitrary cutoffs chosen by the respective scientist with no consensus yet of best practices. Increasing the number of replicates used may often help to filter out sites that are spuriously detected aiming to increase robustness and reproducibility of the achieved maps. More dedicated analytical approaches are continuously being developed to better identify modified sites, more effectively integrate background levels and better filter out noise.

While binary maps of peaks and non-peaks can then be compared between two conditions, most likely in a physiological regulation of m^6A, for example upon stimulation in the adult brain, quantitative regulation of m^6A may be much more likely. While none of the m^6A-Seq protocols have been originally developed for quantitative comparison of methylation states, methods to compare m^6A-Seq signatures across conditions were proposed soon after the original methods description and are actively developed (Meng et al. 2014; Cui et al. 2015; Liu et al. 2016). For the special case of the mostly postmitotic adult brain, given enough replicates and a mild enough manipulation, underlying changes in mRNA expression may be assumed to be negligible, thus removing the need for extensive normalization to background levels.

Notably, a calibrated pulldown procedure called LAIC-Seq was establish recently as a tool to provide quantitative estimates of methylation stoichiometry (Molinie et al. 2016). However, this protocol uses immunoprecipitation of full-length mRNA rather than fragments with the m^6A-antibody, thus averages m^6A content across the entire transcript and likely suffers from extensive noise due to structural biases. Similar methods have been developed using qPCR instead of RNA-Seq to measure the abundance of m^6A-containing (full-length) mRNA and m^6A- calibrator spike-ins to allow for immunoprecipitation efficiency correction (Engel et al. 2018).

The perhaps biggest challenge to quantifying m^6A in the (adult) brain is the lack of techniques to profile m^6A in different brain cell subpopulations, leaving all currently available m^6A-Seq data to be an average m^6A signal from a widespread cell mixture. Employing knockout animals with conditional removal of m^6A-related genes from distinct cell types may provide some insight about cell-specific m^6A-signatures. Today, not even a comprehensive comparison of, for example, m^6A-signatures in neuron versus astrocytes, the two major brain cell types, is available. The use of neuronal cultures to detect neuron-specific signals was employed before (Widagdo et al. 2016), but may carry limited significance due to neurons normally being highly embedded and structured in vivo.

5.4 Target Manipulation of m^6A-Sites

Almost no reports are available describing the effects of manipulating m^6A at a single target site (except Kane and Beemon 1987; Schwartz et al. 2013). To date, functional significance of RNA methylation has usually been shown by correlation,

or broad manipulation of m^{6}A levels by removal m^{6}A enzymes. As a consequence, significance and cellular function of site-specific m^{6}A is mostly unknown so far, although such manipulation should be easy to achieve using recent CRISPR/Cas9 techniques. One explanation may be that methyltransferases might set compensatory methylation at adjacent sites due to remaining m^{6}A-site context when only removing the target site (Narayan et al. 1994; observe contrary results in Schwartz et al. 2013).

Rather than genetic manipulation of a target site, using CRISPR/Cas9 technology to recruit epitranscriptomic modulators to mRNA, potentially even in a temporally- and cell-type controlled manner, may provide much more insight into the cellular functions of specific m^{6}A-sites (O'Connell et al. 2014).

6 Conclusions

The field of mRNA adenosine methylation has experienced a recent sudden take off, fueled by the transcriptome-wide mapping of m^{6}A and many functional follow-up studies. While the core enzymes and reader protein have been well described, more and more details are added each year highlighting the function of m^{6}A in adapting and fine-tuning gene expression especially after environmental stimulation and, in the brain, neuronal activity. Nevertheless, many questions about the regulatory mechanisms, complex interplay of RNA modifications, and the cellular consequences of RNA methylation remain unanswered. Brute force powered functional studies, based on removing enzymes from whole brain areas, have provided some valuable early insight into potential roles of m^{6}A in the brain, but the actual functions of m^{6}A in the brain and related pathology are still open. Methodological advancements to measure stimulated and activity-related m^{6}A-changes in a time- and cell-specific manner as well as functional assays focusing on specific m^{6}A sites may in the future answer the question of the actual significance of m^{6}A in the brain.

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