Review

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The Role of RNA m5C Modification in Central Nervous System Diseases

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As advances in RNA modification research progress, the significance of 5-methylcytosine (m5C) modification is being increasingly acknowledged. m5C undergoes modification by the methyltransferase NOP2/Sun domain (NSUN) family/DNA methyltransferase (DNMT) family (writer) and is removed by demethylases (eraser), including the ten-eleven translocation (TET) family and Alkb homolog 1 (ALKBH1). Moreover, m5C interacts with RNA-binding proteins (reader), such as Y-box-binding protein 1 (YBX1) and Aly/REF export factor (ALYREF). Expanding on this structural framework, m5C modification possesses the capacity to regulate various physiological and pathological processes. Recent studies indicate that m5C plays a pivotal regulatory role in the central nervous system, and its dysregulation may correlate with the onset and progression of various central nervous system diseases. In this review, we summarize recent research on m5C components and delve into the potential mechanisms of m5C involvement in central nervous system disorders, such as Alzheimer's disease, brain tumors, epilepsy, and stroke.

Keywords: RNA modification; epigenetics; 5-methylcytosine; neurodegenerative diseases; brain tumor; stroke

Introduction

Epigenetics, a discipline that studies the regulation of gene expression without altering the DNA sequence, encompasses fields such as DNA methylation, histone methylation, histone acetylation, and coding and non-coding RNA modifications. With the continuous development of epigenetics, the significance of RNA modifications has increasingly come to the fore [1]. Currently, over 300 RNA modifications have been identified [2], such as N6-methyladenosine (m6A), N1-methyladenosine (m1A), 8-Oxoguanine (o8G), N7-methylguanosine (m7G), pseudouridine (Ψ), and 5-methylcytosine (m5C). RNA modification is a dynamic and reversible post-transcriptional regulatory method that can change the structure of RNA [3,4], thereby regulating RNA stability and translation [5]. m5C, in particular, has emerged as a pivotal RNA modification in recent years, owing to advancements in next-generation sequencing (NGS).

In the field of neuroscience, m6A has been extensively studied due to its abundance at sites and high modification levels in the brain [6]. It has been found to play a crucial regulatory role in brain physiology and is related to various central nervous system diseases [7]. In contrast, m5C has been somewhat overlooked due to its low abundance [8,9]. However, growing evidence shows that m5C also plays an important regulatory role in the central nervous system. For instance, an early report indicated that the deletion of the m5C methyltransferase NOP2/Sun RNA methyltransferase 2 (NSUN2) in flies can cause severe memory deficits and cognitive impairments [10]. Another study pointed out that NSUN2-mediated m5C could regulate neural development through cellular stress, and its dysregulation can lead to syndromes characterized by growth and neural developmental defects in mice and humans [11]. Sequencing results of brain m5C sites have shown that although m5C sites are not highly abundant, they have a unique distribution pattern and are associated with numerous neural functional pathways [12,13]. Nevertheless, there is no comprehensive summary of the relationship between m5C modification and central nervous system diseases. In this review, we summarize recent research on m5C components and discuss the potential mechanisms of m5C in central nervous system diseases such as Alzheimer's disorders, brain tumors, epilepsy, and stroke.

Detection Methods

The bisulfite sequencing method, developed in 1992, was initially employed for DNA methylation detection [14–16]. This method was later adapted for RNA methylation detection, paving the way for the identification and

mapping of m5C sites in RNA at single-nucleotide resolution (Table 1) [17]. RNA-Bis-Seq utilizes sodium bisulfite to convert unmethylated cytosines (C) in single-stranded RNA into uracils (U), which are subsequently converted to thymines (T) during PCR amplification, while the original methylated cytosine (m5C) sites remain unchanged throughout this process [18]. However, the RNA degradation caused by bisulfite sequencing poses a significant challenge for detecting low-abundance RNA methylation sites. Additionally, this method is unable to distinguish between m5C and 5-hydroxymethylcytosine (5hmC) [19].

The m5C-RIP-seq method fragments the total RNA into approximately 100 nt fragments and performing immunoprecipitation using m5C-specific antibodies [19]. This method exhibits high specificity and can use different antibodies to distinguish between m5C and 5hmC. Nevertheless, due to its lower resolution, it is often used in combination with RNA-Bis-Seq. MiCLIP-seq [20] and Aza-IP-seq [21] are also commonly employed techniques that rely on the formation of covalent bonds between methyltransferases and their substrates for immunoprecipitation and sequencing. These methods are primarily used to investigate specific target sites of RNA methyltransferases (RCMTs). However, they differ in the specific mechanisms by which covalent bonds are formed. Notably, Hussain *et al*. [22] found that the overlap of methylation sites detected by Aza-IP, Bis-Seq, and miCLIP was not as high as expected, suggesting that the above methods need further optimization to improve the confidence level of the results.

Furthermore, Liu's team [23] developed a ten-eleven translocation (TET)-assisted pyridine borane sequencing method called TAPS. This method involves a two-step process: in the first step, TET enzymes are used to convert m5C and 5hmC into 5-carboxylcytosine (5caC) as an intermediate, and in the second step, pyridine borane is used to convert 5caC into dihydrouracil (DHU), which is then used for subsequent amplification and detection [23]. Compared to the use of sodium bisulfite, TAPS is milder and preserves single-base resolution. Additionally, they developed two distinct TAPS techniques: TAPS-β and CAPS, which detect 5mC or 5hmC respectively [24]. Wang's team [25] recently developed a direct sequencing method called DM-seq, which utilizes a specialized enzyme-mediated deamination process. This method converts cytosine (C) into 5caC and 5hmC into 5-gluconylhydroxymethylcytosine (5ghmC), thereby preserving m5C for direct sequencing. Although these latest methods offer advantages unparalleled by bisulfite sequencing, they are currently limited to the detection of m5C in DNA.

Distribution

The distribution of m5C modification is widespread across various species, including archaea, bacteria, and eukaryotes [19,26]. Intriguingly, m5C sites demonstrate a species-specific pattern of distribution. In mammals, these sites are predominantly found near the translation initiation site and the 3′ untranslated region (UTR) of mRNA molecules [12,27]. However, in Arabidopsis thaliana, m5C sites in mRNA exhibit enrichment in the coding sequence (CDS) region and the 3′ UTR [28]. A similar specific distribution has also been observed in m6A-modified mRNA [29]. The underlying mechanisms that give rise to this distribution are not fully elucidated, but a recent study has suggested that the exon junction complex (EJC) can inhibit m6A methylation within a certain range within the coding sequence, and the widespread inhibition of EJC is sufficient to suppress the overall m6A modification levels, providing valuable insights into the mechanism of this specific distribution [30].

RNA m5C modification also exhibits differential expression levels across different tissues. Notably, the brain exhibits lower levels of mRNA methylation compared to the heart and muscles. Overall, the majority of sites have low methylation levels, with only a small portion exhibiting moderate or high methylation levels [9]. Analysis of RNA BS-seq results reveals that methylated transcripts in the mouse brain are predominantly associated with ion transport or synaptic function, as opposed to cell cycle, RNA, and chromatin modifications observed in embryonic stem cells [12]. Research conducted by Zachary Johnson *et al*. [13] highlighted an increase in neuronal mRNA 5-methylcytosine levels in mice compared to neural stem cells (NSC). The differentially expressed genes identified in this study were linked to transcriptional regulation and axonal extension functions. Additionally, m5C associated regulators underwent significant changes around the time of birth [13]. In summary, these findings underscore the pivotal regulatory role of RNA 5-methylcytosine modification in the brain.

m5C Composition and Function

Like other RNA modifications, m5C modification consists of three components: writer, eraser, and reader. Writers add methyl groups to RNA targets (Table 2, Ref. [11,31–60]), erasers remove them, and readers recognize and bind to m5C sites on RNA, thereby fine-tuning the transcription of related RNA. Collectively, these components together determine the biological functions of m5C, including nuclear export, cellular stress response, cell development and proliferation, and DNA repair (Figs. 1,2).

Writer

NSUN1/NOP2/p120

Previous investigations have elucidated the localization of NSUN1 in the nucleolus, where it targets the C2870 site in the 25S rRNA of Saccharomyces cerevisiae [61]. The human NOP2/NSUN1 target was further found to cat-

Table 1. Detection methods for RNA m5C modification.

Methods	Principle	Advantages	Disadvantages
Bis-seq	Bisulfite conversion	• Single nucleotide resolution	• Unable to distinguish between m5C and other cytosine modifications
		• Able to accurately predict methylation levels at the m5C site	• RNA damage and exogenous interference make detection of low-abundance RNAs difficult
RIP-seq	RNA immunoprecipitation	• High specificity and sensitivity, and can distinguish between m5C and 5hmC	• Low resolution (about 100 nt) • Unable to detect methylation on low-abundance mRNAs
Aza-IP-seq	Protein immunoprecipitation	• Detect specific target sites of RCMTs • Single nucleotide resolution	• Unable to quantify methylation levels • Cytotoxic and may impact transcriptional profiles
miCLIP-seq	Protein immunoprecipitation	• Detect specific target sites of RCMTs • Single nucleotide resolution • No effect on RNA structure and stability	• Time-consuming and costly • Dependent on RCMTs mutation rate

RCMTs, RNA methyltransferases; m5C, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine.

alyze the deposition of m5C at c4447 of the 28S rRNA and participate in pre-rRNA processing [62]. Interestingly, the m5C-modifying activity does not seem to be indispensable for ribosome processing [63].

Originally identified as p120 [64], a nuclear antigen associated with proliferation, NSUN1 has been observed to be expressed in various types of cancer [65–68]. Juyeong Hong *et al.* [31] found that NSUN1 binds to the T cell factor (TCF) binding element of the cell cycle protein D1 promoter, thereby activating its transcription and sustaining cellular proliferation. Moreover, NSUN1 expression has been detected in astrocytes and mature neurons [32]. Following a stroke, an increase in the co-expression of NSUN1 and nestin, a marker for neural stem cells, was also observed. These findings establish the role of NSUN1 in cell proliferation, but whether these functions are related to m5C modification remains unknown. However, a recent study has shed light on this aspect, revealing that NSUN1-mediated m5C methylation induced *c-Myc* mRNA degradation in an EIF3A-dependent manner, which increased the expression of glycolytic genes and promoted the progression of carcinoma progression [33].

NSUN2

Previous studies have shown that NSUN2 is localized in the nucleus and targets multiple m5C sites in tRNA [69,70], while numerous cancer-related studies suggest its involvement in the methylation of mRNA [39,71,72] and lncRNA [73]. Additionally, Vault ncRNAs were also found to be its substrate [74].

Cellular Stress and Neuroprotection

During stress, angiopoietin cleaves the anticodon loop of mature tRNAs in the cytoplasm, producing tRNA-derived stress-induced RNAs (tiRNAs) [75]. Certain 5' tiRNAs interact with the translational repressor Y-box binding protein 1 (YB-1) to inhibit translation initiation and induce stress granule (SG) assembly [76]. Additionally, these stable DNA analogues of G4-containing tiRNAs spontaneously enter motor neurons and provide cytoprotection against stress. Interestingly, excessively active SG complexes can disturb neuronal function [77], potentially leading to the formation of phosphorylated tau [78]. More importantly, the failure of m5C modification caused by NSUN2 deficiency increases Angiogenin (ANG)-mediated endonucleolytic cleavage within tRNAs, leading to the accumulation of small RNA fragments derived from 5' tRNAs. This accumulation reduces the rate of protein translation and activates stress pathways, ultimately contributing to cell size reduction and increased apoptosis in cortical, hippocampal and striatal neurons [11]. Moreover, Blaze *et al.* [34] found that the diminished levels of tRNA m5C in prefrontal cortex neurons, resulting from the removal of NUSN2, have been linked to impaired translation of tRNA (GLY) and reduced quantities of glycine-rich proteins, disrupting synaptic transmission and affecting complex behaviors.

Cell Proliferation and Differentiation

Initial investigations have identified NSUN2 as a downstream target gene of the proto-oncogene Myc [79], a pivotal transcription factor that orchestrates a myriad of biological processes, including cell cycle regulation, apoptosis, mRNA translation, and stress responses [80]. NSUN2-mediated m5C modifications play a crucial role in regulating the cell cycle. It has been reported that NSUN2 methylates cyclin-dependent kinase inhibitor *p16* mRNA at the 3′ UTR, thereby enhancing the stability of *p16* mRNA and triggering the expression of p16 protein under stress conditions [35]. NSUN2 also fosters cell growth by amplifying the translation of cell cycle protein-dependent kinase 1 (CDK1) [36], and a deficiency in NSUN2 has been shown

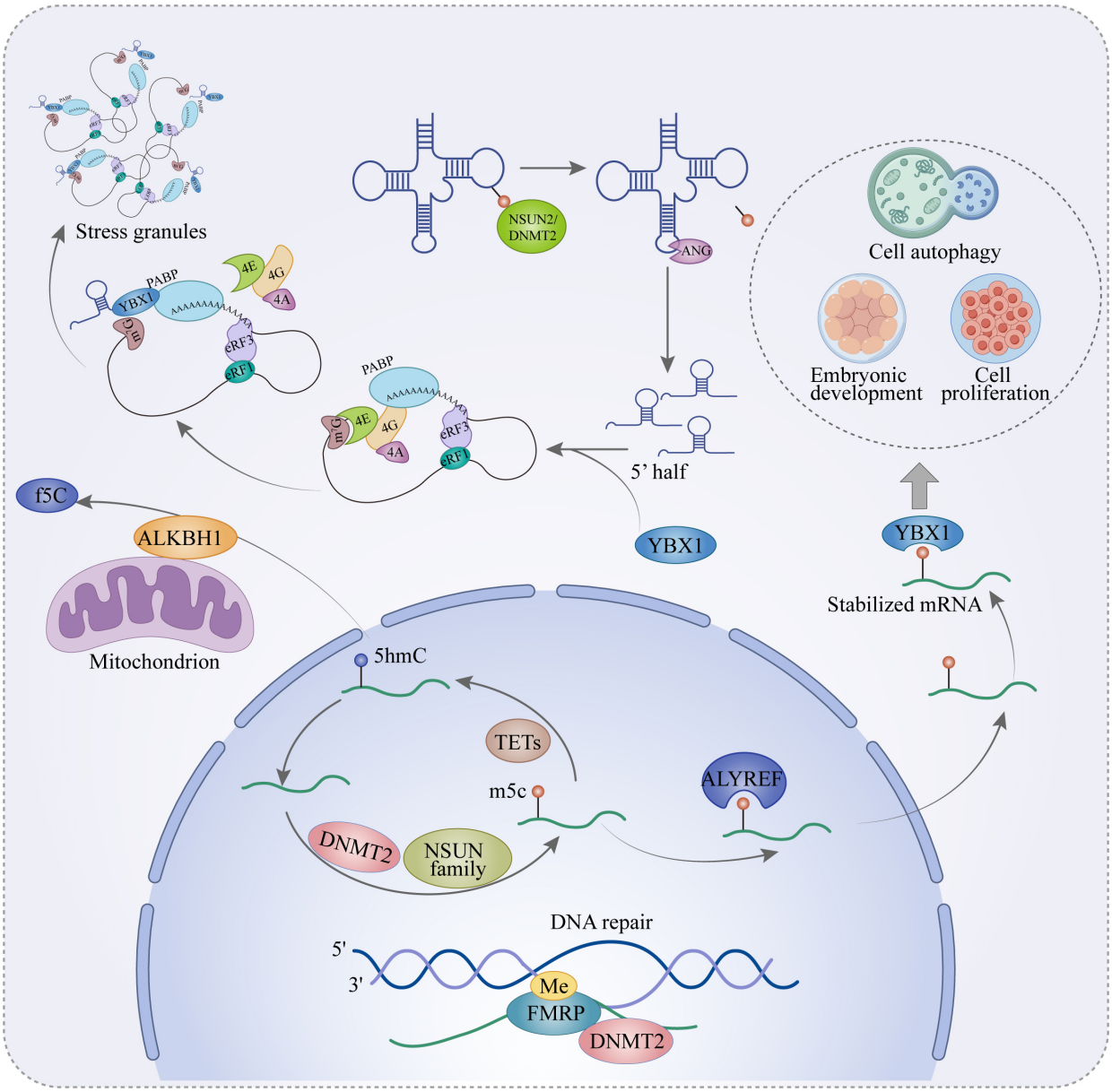

Fig. 1. m5C compositions and their biological functions (this figure was drawn by Adobe Illustrator 2024, 28.2, Adobe Inc., San Jose, CA, USA). YBX1, Y-box-binding protein 1; PABP, poly(A)-binding protein; eRF, eukaryotic translation release factor; NSUN, NOP2/Sun domain; DNMT, DNA methyltransferase; ANG, Angiogenin; f5C, 5-formylcytidine; ALKBH1, Alkb homolog 1; TET, ten-eleven translocation; ALYREF, Aly/REF export factor; FMRP, fragile X mental retardation protein; 5hmC, 5-hydroxymethylcytosine.

to extend the resting phase of epidermal hair follicle stem cells in mice [37]. Additionally, NSUN2 is involved in the methylation of Src homology and Collagen (*SHC*) mRNA, subsequently leading to premature cell death under stress conditions [81]. SHC proteins are integral to a plethora of signaling pathways, including mitosis, Myc activation, cell proliferation, differentiation, and oxidative stress [82,83].

Immunity and Inflammation

Guo *et al.* [41] discovered that the expression of NSUN2-mediated m5C was significantly reduced in $CD4^+$ T cells of patients with systemic lupus erythematosus (SLE). Furthermore, they identified that hypermethylated m5C and upregulated genes in SLE were significantly associated with immune-related and inflammatory pathways [41]. In a recent study, it was demonstrated that the coupling of NSUN2 to RAR-related orphan receptor gamma t (RORγt) enhances the formation of m5C on Th17-specific cytokines, thereby promoting the development of colitis [42]. Th17, a subset of $CD4^+$ helper T cells, plays a crucial role in inflammation and autoimmunity [84]. These findings underscore the importance of NSUN2-mediated m5C modification in immune regulation and its potential implications in autoimmune diseases.

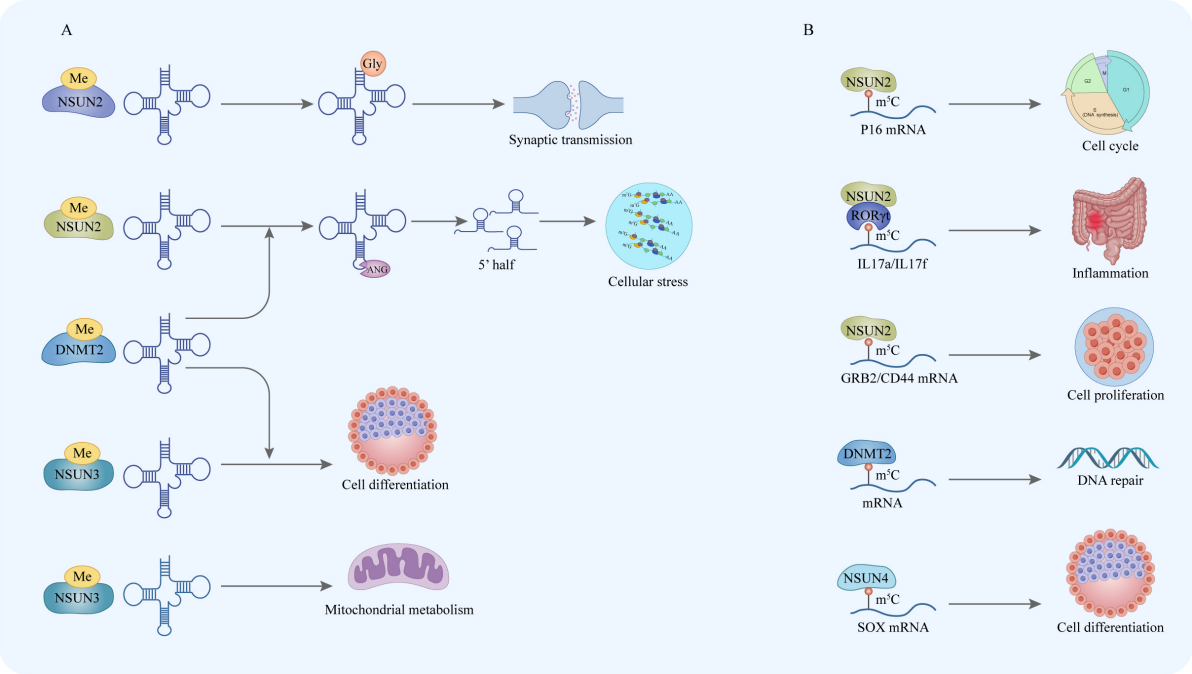

Fig. 2. The m5C modifications of mRNA and tRNA (this figure was drawn by Adobe Illustrator 2024, 28.2, Adobe Inc., San Jose, CA, USA). RORγt, also known as RORγ2, RAR-related orphan receptor gamma t; IL17, Interleukin-17; GRB2, Growth factor receptor-bound protein 2; SOX, Sry-box transcription factor.

DNMT2

DNMT2 was originally classified as a DNA methyltransferase, but with the advent of bisulfite sequencing, it has been redefined as an RNA methyltransferase [85,86]. Interestingly, DNMT2 shares similar function with NSUN2. It targets the C38 position within the anticodon loop of tRNA Asp and has the ability to reduce stress-induced tRNA cleavage [45,87]. DNMT2-mediated m5C modification also regulates cell growth. Knockdown of DNMT2 in zebrafish embryos leads to differentiation defects in organs such as the liver and retina, which can be effectively rescued by human DNMT2 derivatives [43]. In human embryonic kidney cells HEK293, depletion of DNMT2 significantly inhibits its proliferation and migration [44].

A distinctive feature of DNMT2 is its involvement in DNA repair processes. A recent study has demonstrated that DNMT2 is recruited to sites of DNA damage, where it facilitates homologous recombination through m5C modification of mRNA at the damage site [46]. Reduced levels of DNMT2 promoted oxidative stress and DNA damage in cells, which also leads to the upregulation of proliferation-related miRNA [88].

NSUN3

NSUN3 is located within human mitochondria and is responsible for methylation at position C34 of MT-tRNA, which is then oxidized to f5C by Alkb homolog 1 (ALKBH1). Mutations in the NSUN3 protein can lead to a loss of methylation and formylation at position C34 of MT-tRNA, resulting in impaired mitochondrial translation. This impairment can manifest as a range of mitochondrial symptoms, including developmental dysfunctions, microcephaly, developmental delays, muscular weakness, and convergent nystagmus [47,89,90].

The importance of NSUN3 extends to its role in cell growth. Defects in NSUN3 have been associated with increased mortality in mouse embryos [91]. Additionally, NSUN3-mediated methylation is integral to the regulation of embryonic stem cell differentiation by modulating mitochondrial activity. In the absence of NSUN3, embryonic stem cells are more likely to differentiate towards mesoderm and endoderm lineages, while differentiation into neural ectoderm is significantly hindered [48]. In the context of cancer, NSUN3 has been implicated in the metabolic reprogramming of tumor cells. The absence of mitochondrial m5C and f5C modifications has been shown to elevate glycolysis in tumor cells, thereby facilitating tumor cell invasion and metastasis [49].

NSUN4

NSUN4 methylation can autonomously mark the C911 site in the 12S rRNA of the ribosomal small subunit. It can create a protein complex with MTERF4 to target the ribosomal large subunit and coordinate mitochondrial protein assembly [50,92]. NSUN4 knockdown results in embryo mortality in mice, and targeted disruption of NSUN4 in the heart tissue leads to cardiomyopathy

Table 2. Biological functions of different m5C writers.

Writer	Target	Function	Results	References
NSUN1	rRNA	Cell proliferation	Bind to cyclin d1 promoter	[31]
			Co-expression of NSUN1 and Nestin (neural stem cell marker) increases after stroke	[32]
		Glycolysis	NSUN1-mediated m5C regulates c-Myc expression to promote glycolysis	[33]
NSUN2	tRNA	Cellular stress	Accumulation of 5' tiRNA caused by NSUN2 deficiency activates stress pathway	[11]
		Synaptic transmission	NSUN2-mediated m5C deficiency results in the absence of GLY-rich proteins and impacts synaptic signaling	[34]
	mRNA	Cell cycle	Methylates the p16 3′ UTR, enhancing *p16* mRNA stability	[35]
			NSUN2 enhances cell growth by amplifying the translation of cell cycle protein-dependent kinase 1 (CDK1)	[36]
			Deficiency in NSUN2 extends the resting phase of epidermal hair follicle stem cells in mice	[37]
		Cell proliferation	m5C on GRB2 and CD44 may regulate cell proliferation	[38]
		Tumor	m5C on *GRB2* mRNA enhances its stability and promotes esophageal squamous cell carcinoma progression	[39]
			NSUN2-mediated m5C enhances TEAD1 expression and promotes HPSC proliferation and migration	[40]
		Immunity and inflammation	NSUN2-mediated m5C expression significantly reduced in SLE $CD4^+$ T cells	[41]
			NSUN2 binding to RoRγt enhances m5C formation on Th17-specific cytokines to promote colitis	[42]
DNMT2	tRNA	Cell differentiation	Knockdown of DNMT2 in zebrafish embryos leads to differentiation defects in organs such as the liver and retina	[43]
			DNMT2 depletion significantly inhibits proliferation and migration in human embryonic kidney cells (HEK293)	[44]
		Cellular stress	DNMT2-mediated m5C protects tRNAs against ribonuclease cleavage	[45]
	mRNA	DNA repair	DNMT2-mediated m5C promotes homologous recombination (HR)	[46]
NSUN3	MT-tRNA	Mitochondrial translation	Mutations in the NSUN3 protein cause loss of m5C and f5C of MT-tRNA, which impairs mitochondrial translation.	[47]
		Cell differentiation	NSUN3-mediated m5C deficiency results in impaired neural ectodermal differentiation	[48]
		Tumor	Reduced mitochondrial m5C expression increases glycolysis in tumor cells and promotes tumor invasion	[49]
NSUN4	rRNA	Mitochondrial metabolism	Coordinates mitochondrial ribosome assembly	[50]
	mRNA	Cell proliferation	NSUN4-mediated m5C and Mettle3-mediated m6A on *SOX9* mRNA co-promote BMSC chondrogenic differentiation	[51]
		Tumor	Increased in hepatocellular carcinoma	[52]
NSUN5	rRNA	Protein synthesis	NSUN5-mediated m5C deficiency impairs global protein synthesis	[53]
		Cellular stress	Loss of Rcm1 (the yeast homologue of NSUN5) alters the structural conformation of the ribosome and favours recruitment of oxidative stress-responsive mRNAs	[54]
		Tumor	NSUN5 promotes protein synthesis and tumorigenic phenotypes in glioblastoma	[55]
	—	Brain development	NSUN5 deficiency impairs the development of cerebral cortex	[56]
			NSUN5 deficiency leads to hypoplasia of the corpus callosum and defective myelination in mice	[57]
NSUN6	mRNA	Tumor	Decreased in pancreatic cancer, enhancing the growth rate of pancreatic cancer cell	[58]
			NSUN6-mediated m5C deficiency reduces drug resistance in neuroblastoma cells	[59]
NSUN7	eRNA	Metabolic Stress	NSUN7-PGC-1α-mediated m5C increases the stability of enhancer RNAs	[60]

tiRNA, tRNA-derived stress-induced RNAs; UTR, untranslated region; m6A, N6-methyladenosine; GLY, Glycine; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1-alpha.

with mitochondrial dysfunction, demonstrating the involvement of NSUN4 in the control of mitochondrial metabolism [93]. Furthermore, NSUN4 was significantly upregulated in patients with hepatocellular carcinoma, with Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealing its enrichment in signaling pathways such as adhesion junction, RNA degradation, and mammalian target of rapamycin (mTOR) signaling pathway [52]. Furthermore, a study has suggested that NSUN4-mediated m5C modification, in conjunction with methyltransferase like 3 (METTL3)-mediated m6A modification, co-regulates *SOX9* mRNA translation in the 3′ UTR. This synergistic action promotes chondrogenic differentiation in bone marrow mesenchymal stem cells [51], emphasizing the intricate interplay between different RNA modifications in regulating cellular differentiation processes.

NSUN5

By targeting the C3782 site of the ribosome [94], NSUN5 is associated with protein synthesis [53]. NSUN5 is selectively expressed in radial glial cells (RGCs) of the cerebral cortex, which are the progenitors of neurons and glial cells. NSUN5 deletion not only hinders the migration and distribution of cortical neurons, but also effects myelin formation and oligodendroglial development, leading to significant cognitive dysfunction in mice [56,57]. Whether this function is mediated by m5C is not clear, but a recent study has revealed that NSUN5-mediated 5-Methyltransferase controls the maternal-to-zygotic transition by regulating maternal mRNA stability [95].

Moreover, NSUN5 has been implicated in tumor development. Several studies have shown that NSUN5 increases protein synthesis in tumor tissue and promotes tumor proliferation [55,96]. However, contrasting findings were reported by Janin *et al*. [94] who found NSUN5 was transcriptionally silenced in gliomas due to its hypermethylation, and the restoration of NSUN5 expression *in vivo* led to a reduction of tumor weight and volume. Although the epigenetic silencing of NSUN5 led to a reduction in protein synthesis, NSUN5 can activate the stress-responsive translational program and enhance the translation efficiency of specific proteins. Similar conclusions were obtained by Schosserer *et al*. [54] who also found that deletion of NSUN5 homologue Rcm1 altered ribosome conformation and translational fidelity in yeast, favored the recruitment of oxidative stress-responsive mRNAs, and thus improved the longevity and stress resistance of yeast. These results suggest that NSUN5 plays a crucial role in regulating the stress response program [54].

NSUN6

Besides targeting mRNAs [97], NUSN6 can also specifically target tRNAs (Thr) and tRNAs (cys) at the C72 site [98]. The expression of MSUN6 is considerably reduced in pancreatic cancer tissues and its silencing leads to an accelerated growth rate in pancreatic cancer cells. Conversely, overexpression of NSUN6 inhibits cell growth, indicating the potential regulatory role in cell growth and proliferation [58]. Loss of NSUN6-mediated m5C modification reduces the drug resistance of neuroblastoma cells [59]. Interestingly, although NSUN6 exhibits a regulatory role in tumor tissues, its role in normal tissues appears to be limited, as normal mice show no abnormal phenotypes and significant changes in RNA stability following NSUN6 knockout [99]. However, NSUN6 double allele variants can cause neurodevelopmental disorders [100].

NSUN7

NSUN7, together with peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α), can promote the deposition of m5C on specific enhancer RNAs, which increases the stability of these enhancer RNAs [60]. PGC-1α is a major regulator in mitochondrial biosynthesis and is associated with pathways such as oxidative stress, metabolism, and inflammatory responses [101]. For instance, microglia can reduce neuroinflammation after ischemic encephalopathy by increasing PGC-1α [102]. Furthermore, PGC-1α plays a critical role in the formation and maintenance of neuronal dendritic spines [103]. The dysregulation of PGC-1α in neurodegenerative diseases [104] suggests that the NSUN7/PGC-1α pathway may be a potential mechanism in central nervous system (CNS) disorders.

Reader

ALYREF

Before its identification of the mRNA export complex TRanscription-Export (TREX) [105], the integral role of Aly/REF export factor (ALYREF) in facilitating nuclear export had already been unveiled [106]. Acting as a crucial mediator during mRNA export, ALYREF facilitates the clustering of adjacent exon junction complexes (EJCs) to compact the mRNP (messenger ribonucleoprotein) structure, while also engaging with the Transcription/export complex (THO) complex to expedite the export of mRNAs [107]. In the realm of RNA modifications, ALYREF is recognized as a reader of m5C. With a higher affinity of mRNA with m5C modifications, ALYREF and m5C methyltransferase NSUN2 work together to regulate mRNA export [27]. Despite these insights, the intricacies of the cooperative mechanism employed by ALYREF and NSUN2 in the encapsulation and subsequent transfer of mRNAs remain shrouded in mystery. Adding another layer of complexity, recent studies have illuminated the propensity of ALYREF to predominantly bind to the 5’ and 3’ regions of mRNA *in vivo*. Intriguingly, these regions are often found in close proximity to sites rich in m5C modifications, further underscoring the potential interplay between ALYREF and m5C in the regulation of mRNA export [108].

ALYREF is also an important oncogenic factor and has been found to be upregulated in various types of malignancies [109]. In bladder cancer, ALYREF binds to m5C sites in the 3'-untranslated region of pyruvate kinase M2 (*PKM2*) mRNA, ultimately enhancing its stability. This stabilization of PKM2 mRNA subsequently facilitates cell proliferation in tumor tissues through the PKM2-mediated glycolytic pathway [110]. Furthermore, the ALYREF-myelocytomatosis proto-oncogene (MYC) axis has been implicated in the progression of neurologic tumors, with the pathways governed by this axis playing a crucial role in tumor growth [111,112]. These findings underscore the multifaceted role of ALYREF in cellular processes and its potential implications in various pathological conditions, including cancer.

YBX1

As a nucleic acid-binding protein, Y-box-binding protein 1 (YBX1) possesses a highly conserved cold shock domain (CSD) sequence and is capable of interacting with RNA through π-π stacking interactions [113]. The biological role of YBX1 has been extensively studied, especially in tumor development. Elevated YBX1 expression has been linked to various cancers, such as glioblastoma [114], prostate cancer [115], breast cancer [116], lung cancer [117], and liver cancer [118]. As a m5c reader, YBX1's CSD structure selectively binds to mRNA oligonucleotides modified with m5C, thereby enhancing the stability of the bound mRNAs. This interaction plays a pivotal role in regulating a myriad of biological processes, including cell proliferation [119], cellular autophagy [120], and embryonic development [121]. Evans *et al.* [122] found that knockout of YBX1 led to a significant downregulation of genes associated with neuronal differentiation and morphogenesis, indicating the crucial role of YBX1 in brain development. Moreover, YBX1 is integral to the NSUN2-mediated tRNA stress response, with its binding to tiRNAs being a crucial step in the assembly of SGs [123].

Eraser

Currently, two kinds of m5C demethylases have been identified: the TET family and ALKB1. Both of them belong to the 2-oxoglutarate-dependent oxygenases [124] and have the ability to oxidize m5C to 5hmC when binding to RNA [125,126]. The TET family consists of TET1, TET2, and TET3, all sharing common structural features [127]. Functional studies of TET enzymes on RNA are limited, but evidence has suggested that TET-mediated deposition of 5hmC can reduce mRNA stability [128,129]. Furthermore, Yang *et al.* [130] found that fragile X mental retardation protein (FMRP) and TET1 together mediate the demethylation of m5C RNA modifications in DNA: RNA hybrids, thereby promoting transcriptional homologous recombination. TET2 has been found to encourage infection-induced myelopoiesis in an mRNA oxidation-dependent manner [131]. Another report suggests that TET2 is upregulated in low-grade gliomas, indicating its potential role in regulating tumor development [132]. Researches on ALKBH1 demethylation is currently focused primarily on m6A rather than m5C [133,134]. However, studies have found that ALKBH1 can act on both cytoplasmic and mitochondrial tRNA. It first hydroxylates m5C34 to form hm5C34 and then further oxidizes it to f5C34 [126]. The function of this conversion still needs further investigation.

Expression and Role of m5C in Central Nervous System Diseases

As mentioned above, RNA m5C modifications have been shown to play important regulatory roles in a range of diseases. Herein, we describe the m5C alterations that have been elucidated in CNS diseases (Table 3, Ref. [94, 132,135–144]), which may help in the discovery of potential molecular mechanisms and therapeutic approaches for these diseases.

Alzheimer's Disease (AD)

Alzheimer's disease (AD) is the most common chronic neurodegenerative disorder, characterized by progressive dementia that encompasses memory loss, diminished learning abilities, language impairments, decision-making difficulties, and a loss of daily living skills. The hallmark pathological features of the disease are amyloid Aβ deposition and neurofibrillary tangles within the brain [145]. Aβ deposition is believed to be a catalyst for the initial stages of AD [146], while neurofibrillary tangle is composed of abnormally phosphorylated tau protein [147,148]. Tau is a microtubule-associated protein with numerous phosphorylation sites [149]. Abnormal phosphorylation of tau decreases its ability to bind to microtubules [150,151]. In addition, it causes the misplacement of tau proteins, thus interfering with the glutamate receptor transport and disruption of the synaptic function [145]. Recently, Kim *et al.* [135] found that deficiency in NSUN2 led to hyperphosphorylation of tau, while overexpression of NSUN2 reduced the phosphorylation and cytotoxicity of tau. Although the effect is partly attributed to the m6a modification on miRNA-125b, Han *et al.* [152] also confirmed the abnormal elevation of the m6A methyltransferase METTL3 in AD mice. Additionally, the synergistic role of METTL3/m6A with NSUN2/m5C has also been reported [153].

Microglia-mediated innate immune response and neuroinflammation are also reported to be involved in the pathogenesis of AD [154,155]. Through multiple pattern recognition receptors (PRRs) pathways, m5C influenced the Aβ pathological process in AD and induced inflammatory responses [156]. NSUN5 has been shown to bind to the retinoic acid-inducible gene (RIG)-I receptor in the PRR, thereby enhancing the antiviral immune response initiated by RIG-I [157]. Zhang *et al.* [158] found that depletion

Table 3. Altered m5C modification levels in central nervous system diseases.

Disease	Proteins	Change	References
AD	NSUN2	NSUN2 deficiency leads to hyperphosphorylation of tau	[135]
	NSUN6/NSUN7	NSUN6 expression increases in AD brains, while NSUN7 expression decreases	[136]
	ALYREF	ALYREF expression decreases with disease progression	[136]
Brain tumor	NSUN2	Increased in low-grade gliomas, and NSUN2-mediated m5C enhanced *ATX* mRNA translation and export	[132,137]
	DNMT2	Increased in low-grade gliomas	[132]
	DNMT2	Decreased in glioblastomas, which decreased drug sensitivity and the aging of cells caused by drugs	[138]
	NSUN3	Increased in low-grade gliomas	[132]
	NSUN5	Silenced in gliomas, reflecting tumor suppressor properties	[94]
	ALYREF	Increased in low-grade gliomas	[132]
Epilepsy	NSUN3	Mutations in NSUN3 cause Epilepsy Phenotypes	[139]
Stroke	TET2	Increased after IRI	[140]
	TET1	Decreased in delayed cerebral ischemia	[141]
	TET1	Cyclic RNA 0025984 mitigate ischemic stroke injury via miR-143-3p/TET1/ORP150 pathway	[142]
ALS	ALYREF	Increased in human ALS motor neurons	[143]
	ALYREF	Knockdown of Ref1 alleviates the neurotoxicity of TDP-43	[144]

AD, Alzheimer's disease; ATX, Autotaxin; IRI, ischemia/reperfusion injury; ALS, Amyotrophic Lateral Sclerosis; TDP, TAR DNA-binding protein.

of m5C due to NSUN2 deficiency enhances the transcription of polymerase III-transcribed noncoding RNAs. These RNAs are recognized by the RIG-I receptor, which in turn amplifies the type I interferon (IFN) response. Wang *et al*. [159] found that NSUN2 mediated m5c modification of interferon regulatory factor 3 (*IRF3*) mRNA, accelerated its degradation and resulted in lower levels of IRF3 and IFN-β production. Endogenous NSUN2 levels were also decreased during viral infections to enhance antiviral responses [159]. These findings highlight the potential of m5C modification in innate immune responses.

Lipid metabolism disorder is another pathological feature of AD [160]. In the early stages of AD in the brain, levels of various lipid classes are altered, which is associated with the progression of the disease [161,162]. Furthermore, numerous high-risk genes, identified in sporadic AD, such as *APOE CLU*, *ABCA1*, and *TREM2*, are linked to lipid transport or metabolism [163]. NSUN2 and ALYREF have been previously discovered to regulate lipid metabolism by transporting metabolism-related factors. Silencing of NSUN2 considerably increased the mRNA expression of lipid-accumulating and adipogenic transcription factors [164,165]. Interestingly, a recent study found a significant decrease in ALYREF gene expression as the degree of AD progressed [136], suggesting that m5c modification is likely to be a potential driver of lipid accumulation in AD. Moreover, a previous transcriptomics study has revealed reduced NSUN6 gene abundance in the brains of AD patients in STG and white matter regions, while NSUN7 is elevated in hippocampal regions [136]. However, the resulting effects of these proteomic changes remain unclear. Taken together, the findings above give significant clues to understand the pathogenesis and potential targets of AD.

Brain Tumor

RNA-Bis-Seq data has shown that the methylation level of mRNAs in tumor tissues is significantly increased and enriched in many oncogene and oncogenic pathways. Moreover, genes linked with cancer spreading exhibited higher methylation levels [71], indicating a potential association between m5c and the invasion of tumors.

Glioma is a common CNS tumor and accounts for 80% of all malignant brain tumors [166]. High-throughput sequencing data analysis showed that m5C regulators, like NOP2, NSUN2, NSUN3, DNMT2, and ALYREF were upregulated in low-grade glioma [132]. NSUN2-mediated m5C on the 3′-UTR of *ATX* mRNA enhanced its translation and promoted the export of *ATX* mRNA from nucleus to cytoplasm via an ALYREF-dependent manner [137]. Autotaxin (ATX) is a potent tumor motogen that significantly enhances tumorigenicity. A reduced level of DNMT2 was observed in glioblastomas, which decreased drug sensitivity and the aging of cells caused by drugs. Interestingly, methyltransferases appear to interact with each other, as the knockdown of DNMT2 increased the expression of NSUN6 in glioblastoma [138], while a previous report claimed that NSUN6 expression inhibited tumor development [58]. NSUN5 methylation is silenced in gliomas, which reflects tumor suppressor properties [94]. NSUN7 was reported to be highly expressed in low-grade glioma (LGC) and its

high expression was associated with significantly shorter survival [167]. However, the specific mechanisms of these effects need to be further investigated.

Epilepsy

Epilepsy is a common disorder of CNS that causes recurring convulsions [168]. The primary triggers for epileptic seizures are abnormal activities of cortical neurons, although the specific pathophysiological mechanisms remain elusive [169]. Respective biological processes have been identified as potential targets for epilepsy, including oxidative stress, neuroinflammation, ion and water homeostasis, neurotransmitter transmission, energy metabolism, and neuron-glia interactions [170–172]. It is evident that epigenetic modifications, such as DNA methylation, may play a role in epilepsy. For instance, an increase in DNA methylation levels has been observed in the brains of epileptic rats [173,174], and the use of DNMT inhibitor can reduce seizure susceptibility and epilepsy acquisition [175]. Nevertheless, studies related to m5C in epileptic mechanisms are very limited, especially the m5C modification map under epileptic condition.

Remarkably, mitochondrial dysfunction also often manifests as seizures [176]. Mitochondria are responsible for energy production, and energy failure during seizures has been closely linked to mitochondrial dysfunction [177, 178]. In addition, excessive reactive oxygen species (ROS) and Ga^{2+} overload caused by mitochondrial dysfunction may also induce and exacerbate epilepsy [179]. Both NSUN3 and NSUN4 modify m5C and are related to the function of mitochondria. Meanwhile, mutations and deletions of NSUN3 have been shown to lead to epileptic phenotypes [90,139], suggesting that m5C modifications may be a potential target for epilepsy.

Stroke

Ischemic stroke is a CNS disorder characterized by a high incidence and mortality rate. The primary treatments for this disease are intravenous thrombolysis and endovascular thrombectomy, which both aim to rapidly restore the blood flow [180,181]. Nevertheless, cerebral ischemia/reperfusion injury (IRI) often results in permanent brain damage and neuronal injury, which may be triggered by a variety of pathological processes such as oxidative stress, inflammation, and mitochondrial autophagy [182,183]. Epigenetics also plays an important role in post-stroke conditions, particularly regarding DNA methylation and m6a modification [184–186]. However, investigations concerning m5c are still limited.

After analyzing the samples treated with oxygen-glucose deprivation/reoxygenation (OGD/R), Jian *et al.* [187] found that upregulated transcripts with high m5C methylation levels were significantly enriched in processes related to cell apoptosis and neurodegenerative diseases [187], suggesting a potential regulatory function of RNA m5C modification in IRI. Moreover, Yin *et al.* [140] found that the demethylase TET2 was upregulated after IRI injury. Knockdown of TET2 *in vitro* mouse model reduced the methylation level of lncRNA TUG1 and attenuated the OGD/R damage through miR-200a-3p/NLRP3 axis [140]. Furthermore, TET1 expression was found to be decreased in delayed cerebral ischemia [141], and a recent research further demonstrated that cyclic RNA 0025984 may ameliorate ischemic stroke injury and protect astrocytes via the miR-143-3p/TET1/ORP150 pathway [142], indicating that the role of m5C demethylation in stroke is equally significant.

Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the degeneration of upper and lower motor neurons and is associated with numerous physiological processes, such as protein homeostasis, mRNA processing, DNA repair, mitochondrial function, and neuroinflammation [188]. Aggregation and accumulation of ubiquitinated proteins is a major pathological feature of the disease, and the DNA/RNA-binding protein TAR DNA-binding protein (TDP)-43 has been identified as a key component of these disease proteins [189]. In a Drosophila model, mutations of TDP-43 have been shown to increase protein aggregation and neurotoxicity [190]. Importantly, Berson and colleagues [143] found that ALYREF protein levels were elevated in human ALS motor neurons. Knockdown of Ref1 (fly orthologue to human ALYREF) can effectively reduce the expression of TDP-43 and alleviate its neurotoxicity [143]. Like YBX1, TDP-43 is also involved in the assembly of SG particles under stress [144], suggesting that TDP-43 is likely to be a novel m5-creaser playing an important regulatory role in ALS.

Clinical Potential

m5C modifications have been proven important regulators of RNA metabolism and related protein expression. In neoplastic diseases, various m5C factors serve as diagnostic and prognostic biomarkers [191–193]. However, an increasing number of studies are uncovering alterations of m5C-associated factors in non-neoplastic diseases, indicating the great potential of m5C modifications as biomarkers for disease diagnosis and prognosis. Furthermore, the development of m5C-targeted drugs holds promise as a therapeutic strategy for the future. While no m5C-specific inhibitors have been developed yet, small molecule inhibitors targeting the m6A methyltransferase METTL3 and m6A demethylase FTO have shown effectiveness in inhibiting cancer progression [194–196]. Notably, a METTL3 inhibitor named STC-15 is currently undergoing a phase 1 clinical trial for patients with advanced malignancies [197]. It is important to note that research on m5C modifications is still in its early stages. In the coming years, the focus will

likely shift toward investigating the response of m5C modifications in various diseases and understanding the underlying mechanisms, rather than immediate clinical translation.

Conclusions

m5C modification is a crucial epigenetic modification that plays a key role in various physiological processes, including mitochondrial biogenesis, cell growth and development, immune and inflammatory regulation, and cellular stress response. The reader ALYREF has been found to promote the development of cancer by affecting nuclear export and RNA stability. However, despite the numerous functions of m5C that have been revealed, its association with diseases is still not fully understood. For example, previous studies have shown that RNA-binding proteins (RBPs) can play a role in neurodegenerative diseases by regulating the formation of SGs [198]. Although the protein YBXI, as an m5C reader, has been implicated in the formation of SGs, empirical evidence linking it directly to the progression of neurodegenerative diseases remains elusive.

Furthermore, in comparison to research focusing on cancer, investigations into m5C in CNS diseases remain relatively scarce. Particularly noteworthy is the absence of transcriptional maps delineating m5C in specific CNS ailments. Earlier studies have revealed disease-specific alterations in m5C modifications and their associated components, yet the specific mechanisms governing these changes remain largely unexplored. What specific modifications transpire in m5C within CNS diseases? What genes do they target? How might these variances impact disease progression? These inquiries stand as valuable avenues for forthcoming research. Moreover, due to the overall low level of m5C methylation, many low-abundance m5C sites may be overlooked, but these sites may also play important regulatory roles in CNS diseases. Therefore, it becomes imperative to develop detection methods with higher sensitivity and fidelity in the future.

In summary, investigations into m5C modifications within the CNS are nascent. The refinement of RNA site detection methodologies, coupled with the development of transcriptional profiles tailored to specific CNS disorders, harbors potential to demystify the contributions of m5C modifications to CNS pathologies. Such advancements are poised to revolutionize our approach to diagnosis and therapy for these complex diseases in the forthcoming era.

Availability of Data and Materials

Not applicable.

Author Contributions

PYW, JXG, GML, and YCW contributed to the study conception and design. The first draft of the manuscript was written by PYW and JXG. PYW drew the figures and collected the related references. GML and YCW supervised and revised the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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