Synaptic m⁶A Epitranscriptome Reveals Functional Partitioning of Localized Transcripts for Dynamic Tripartite Synapse Modulation

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41

42 **Abstract**

43 A localized transcriptome at the synapse facilitates synapse-, stimulus-, and
 44 transcript-specific synthesis of the local proteome in response to neuronal
 45 activity. While enzyme-mediated mRNA modifications have been shown to
 46 regulate cellular mRNA turnover and translation, the role of these
 47 modifications in regulating synaptic RNA has not been studied. We
 48 established low-input m⁶A-seq of synaptosomal RNA to determine the
 49 chemically modified local transcriptome in healthy adult mouse forebrain and
 50 identified 4,329 selectively enriched m⁶A RNA peaks in 2,987 genes, which
 51 we refer to as the synaptic m⁶A epitranscriptome (SME). SME is functionally
 52 enriched in synthesis and modulation of tripartite synapses, and in pathways
 53 implicated in neurodevelopmental and neuropsychiatric diseases. Interrupting
 54 m⁶A-mediated regulation via knockdown of reader YTHDF1 in hippocampal
 55 neurons alters expression of SME member *Apc*, and causes synaptic
 56 malfunctions manifesting immature spine morphology and dampened
 57 excitatory synaptic transmission concomitant with decreased PSD-95
 58 clustering and GluA1 surface expression. Our findings indicate that chemical
 59 modifications of synaptic mRNAs critically contribute to synaptic function.

60

61 **Introduction**

62 Synapses connect billions of neurons into functional neuronal and neuroglial
63 circuits that underlie information processing and drive behavior. Remarkably,
64 synaptic connections are highly dynamic, undergoing continuous
65 synaptogenesis and synaptic elimination throughout development and
66 adulthood^{1,2}. Synaptic plasticity, the ability of synapses to change at the level
67 of their molecular components, structure, and transmission efficiency in
68 response to neuronal activity, is a fundamental feature of neural network
69 architecture and brain function³.

70

71 At the center of activity-induced synaptic alteration, local protein synthesis
72 plays a key role in consolidating neuronal activity into persistent structural and
73 functional change^{4–13}. Aberrant translation at synapses has been linked to
74 autism, fragile X syndrome and other intellectual disabilities, identifying
75 dysregulated local translation as a core pathophysiological mechanism in
76 neurodevelopmental and neuropsychiatric disease^{14–17}. In addition to
77 ribosomes, thousands of transcripts are localized to synaptic compartments
78 encoding proteins involved in diverse functions, including synapse- and
79 neuron-specific molecules, as well as components of the protein synthesis
80 and degradation machinery¹⁸.

81

82 Recent advances in understanding specific cell-type contributions to synapse
83 refinement has revealed that glial cells play active roles in signaling and
84 facilitating synapse formation, function, plasticity, and elimination, giving rise
85 to the concept of a “tripartite” synapse that comprises presynaptic and

86 postsynaptic terminals as well as glial cells^{19–21}. The mechanisms of mRNA
 87 trafficking and local translation extend to astrocytes, where selectively
 88 enriched transcripts and translational machinery can be detected in peri-
 89 synaptic processes²². These new findings implicate multicellular contributions
 90 to input-specific, synapse-restricted protein synthesis.

91
 92 Given the significant contribution of local translation to synaptic function both
 93 under physiological and disease conditions, an area of active investigation
 94 concerns the post-transcriptional regulatory mechanisms that support the
 95 rapid, spatio-temporally coordinated alteration of local proteome composition
 96 underlying synaptic plasticity. Current models suggest that signatures carried
 97 by individual RNAs, such as sequence motifs, secondary and tertiary
 98 structures affect specific interactions with RNA binding proteins and
 99 microRNA-mediated regulatory networks^{23–28}. Recent studies of the cellular
 100 transcriptome have focused attention on epitranscriptional regulation via
 101 enzymatic modification as a mechanism that can dynamically regulate the fate
 102 of individual RNA transcripts^{29,30}.

103
 104 Between 0.1 and 0.4% of all adenosines in mammals are modified by the
 105 addition of a methyl group to the N6 position of the adenine to form N⁶-
 106 methyladenosine (m⁶A), the most abundant mRNA internal modification. m⁶A
 107 is reversibly regulated by methyltransferases (“writers”) and demethylases
 108 (“erasers”), and has been shown to affect the splicing, trafficking, stability and
 109 translation of individual RNAs³¹. In contrast to A-to-I editing, which alters the
 110 coding sequence of AMPA or 5HT receptors to regulate signaling, m⁶A alters

111 the local structure and protein binding properties of the modified RNA³². The
 112 brain contains the highest level of m⁶A expression among the major organs³³,
 113 and many neuronal genes, including genes involved in locomotion, circadian
 114 clock regulation, dopaminergic midbrain circuitry, and consolidation of cued
 115 fear memory, contain specific modification sites^{34–38}. Although evidence for
 116 m⁶A as a key post-transcriptional regulator continues to grow, few studies
 117 have been performed in neurons and there has been, to our knowledge, no
 118 investigation of its presence in the synaptic compartment. We therefore
 119 sought to address whether m⁶A could localize to synapses and regulate the
 120 local transcriptome.

121

122 Here we report an m⁶A epitranscriptome, which we term “SME”, in the
 123 synaptic compartment of the adult mouse forebrain. We report that m⁶A is
 124 present in the synaptic transcriptome, selectively modifying diverse transcripts
 125 in all cellular domains of tripartite synapses. Although elevated methylation
 126 has a negative impact on synaptic distribution and abundance of the
 127 methylated mRNA in a general way, select mRNAs are hypermethylated at
 128 synapses. Localized hypermethylated mRNAs showed strong functional
 129 enrichment in synaptic pathways, in sharp contrast to hypomethylated
 130 transcripts. We report nuclear and somatodendritic distribution of m⁶A
 131 readers, writers and erasers in cortical pyramidal neurons *in vivo* as well as in
 132 cultured primary hippocampal neurons. To interrupt m⁶A-mediated regulation
 133 in cultured hippocampal neurons, we knocked down dendritically localized
 134 reader YTHDF1. YTHDF1-deficient neurons had aberrant spine morphology,
 135 with decreased spine volume and dampened spontaneous excitatory synaptic

transmission concomitant with decreased PSD-95 clustering and surface expression of AMPAR subunit GluA1. Translation of at least one dendritically localized, highly methylated SME member mRNA encoding APC, a microtubule plus-end tracking protein, was inhibited by YTHDF1 deficiency, further demonstrating m⁶A-mediated regulation of synaptic gene expression and function.

Results

m⁶A RNA modification in adult mouse forebrain synaptosomes

We initially crossed two m⁶A-databases with 12 lists of mRNAs localized to axons, dendrites, and neuropils. This comparison revealed substantial overlap (14.0%~56.7%, Supplementary Table S1), strongly suggesting synaptic m⁶A localization. Next, we isolated a synaptosome (SYN) fraction from the forebrains of adult ICR mice (Supplementary Fig. S1a). SYN was enriched for synaptic proteins (Synaptophysin and PSD-95) and dendritically targeted mRNA of *Camk2a*, and depleted of soma-restricted markers (Supplementary Fig. S1b and S1c). An integrity check confirmed intact rRNA bands in all samples and enriched mitochondrial rRNA bands in SYN fractions (Supplementary Fig. S1d); m⁶A dot-blot detected strong m⁶A immunoreactivity in poly(A) RNA isolated from homogenized tissue (HOM) and pooled F3 and F4 (SYN), but not in an *in vitro* transcribed unmodified RNA (Fig. 1a, Supplementary Fig. S1e). Densitometry measured that m⁶A abundance in SYN was ~50% that of HOM (Fig. 1b, Supplementary Fig. S1e).

Low-input m⁶A-seq protocol and synaptic enrichment of select RNA

161 We then sought to identify and characterize the full synaptic m⁶A-
 162 epitranscriptome using m⁶A-seq³⁹. The limited yield of RNA from
 163 synaptosomes presented challenges using existing m⁶A-seq protocols. Total
 164 yield from a single forebrain was approximately 2μg, 1/125th that required by
 165 standard protocols³⁹. We established a protocol as described in Online
 166 Methods to use submicrogram input, by enhancing sample quantification and
 167 optimizing IP, and conducting SMARTer amplification prior to cDNA library
 168 construction. Our protocol reliably produced INPUT and IP libraries using
 169 ribo(-) RNA from 8 mouse forebrains per replicate (Fig. 1c).

170

171 Four libraries (INPUT and IP for HOM and SYN) with biological replicates
 172 were sequenced (Supplementary Table S2; reproducibility was evaluated
 173 using Pearson's *r* squared, Supplementary Fig. S2). To focus on cytoplasmic
 174 RNA species, we computationally removed abundant nucleus-restricted and
 175 mitochondrial genome-derived RNA using SortMeRNA (Supplementary Table
 176 S3). The remaining uniquely-mapped sequences were used for downstream
 177 analysis (Supplementary Table S4). Comparing INPUT libraries (HOM:
 178 17,470 RNA; SYN: 15,767 RNA) revealed 3,067 significantly enriched genes
 179 at the synapse ($p < 0.05$ FDR, $\log_2FC > 0.51$) including many known localized
 180 mRNA, supporting successful enrichment of synaptic transcripts (Fig. 1d,
 181 Supplementary Table S5).

182

183 **Identification and characterization of synaptic m⁶A peaks**

184 We called exon-based m⁶A peaks in HOM and SYN, using recurrent peaks in
 185 biological replicates as high-confidence m⁶A sites. We identified 17,009 peaks

186 annotated to 9,600 genes in HOM, and 16,539 peaks annotated to 9,323
187 genes in SYN (Fig. 2a, Supplementary Table S6). HOM peaks showed high
188 and significant concurrence with previously published peaks in mouse brain
189 tissue^{33,40,41} (Fig. 2b, statistical analysis in Supplementary Fig. S3a),
190 indicating robust and reliable peak detection using the low-input protocol.
191 HOM and SYN peaks showed extensive overlap (Fig. 2c), suggesting a
192 common methylation site selection pathway used by both transcriptomes. The
193 established “RRm⁶ACH” consensus motif (R: A or G; H: A or C or U) was
194 detected in both HOM and SYN m⁶A peaks (Fig. 2d); the distance from peak
195 summit to the discovered motifs was typically less than 100 bases,
196 associating the motif with the antibody binding site (Fig. 2e for SYN peaks,
197 Supplementary Fig. S3b for HOM peaks). In agreement with previous studies,
198 the majority of peaks were detected in CDS and 3’UTR (Fig. 2f). The peak
199 distribution along mRNA showed characteristic accumulation at the STOP
200 codon (Fig. 2g, ^{33,42}). Peaks associated with START and STOP codons were
201 identified (Fig. 2h, Supplementary Table S7). Notably, genes with peaks
202 associated with STOP codon, but not START codon, were functionally
203 enriched in human phenotypes of “intellectual disability”, “microcephaly”,
204 “muscular hypotonia”, “hypoplasia of the corpus callosum”, and “seizures,”
205 highlighting their relevance to human brain development and cognitive
206 functions (Fig. 2i and Supplementary Fig. S4; GO analyses using expressed
207 genes in Supplementary Tables S8 and S9).

208

We identified peaks specifically associated with SYN or HOM. Visualization with IGV, shown in Fig. 2j and Supplementary Fig. S5, consistently confirmed differential methylation of RNAs at synapses.

Methylation negatively impacts synaptic location and abundance of transcripts

We next investigated the relationship between methylation and synaptic RNA concentration, observing weak but significant negative correlation between transcript abundance and methylation (Supplementary Fig. S6a). Furthermore, the concentration of transcripts at synapses relative to whole cells was also negatively correlated with methylation (Supplementary Fig. S6b). This result is consistent with the overall decreased m⁶A level in synaptosomal mRNA (Fig. 1a), suggesting there may be a combinatorial result of local degradation, and retention of m⁶A-mRNA in the soma. Interestingly, methylation in the 5'UTR was not significantly correlated with the synaptic location of transcripts (slope -0.02, Pearson's *r* -0.02, *p* value 0.073). This position-specific feature may explain the elevated peak frequency in 5' region of synaptosomal mRNAs (Fig. 2g).

A synaptic m⁶A epitranscriptome (SME)

Defining that synaptic m⁶A RNA should not only be detected in SYN, but also enriched in SYN, we next aimed to identify the full synaptic m⁶A epitranscriptome. We merged all m⁶A peaks in HOM and SYN and scored the coverage in the merged peak regions. After normalization to transcript length, 4,329 significantly enriched peaks in SYN in 2,987 genes were identified

234 (p<0.05 FDR, log2FC>0.14, Fig 3a, Supplementary Table S10). We refer to
235 these methylated transcripts as the synaptic m⁶A epitranscriptome (SME).

236

237 High concordance between relative expression and (de)enrichment of
238 methylation between m⁶A-seq and qPCR measurement was detected
239 ($R^2=0.80795$ for HOM and 0.87021 for SYN, Fig. 3b, Supplementary Fig. S7),
240 validating the results of bioinformatic analysis. Inspection of individual peaks
241 showed more prominent m⁶A peaks in SYN (Fig. 3c). Gene ontology (GO)
242 analysis identified the most significant ontology cluster associated with SME
243 as “central nervous system development” (Fig. 3d), demonstrating the strong
244 functional specificity of the localized m⁶A epitranscriptome. A subset of
245 functional clusters was converted into a network layout to illustrate their
246 functional relationships (Supplementary Fig. S8).

247

248 Known functions of top candidates based on peak abundance in SYN were
249 examined using manual annotation and data mining in Pubmed. Genes with
250 the top differentially methylated peaks were associated to regulatory
251 mechanisms of synapse formation, function, and elimination in response to
252 local environment, mediated by neuron, astrocytes, and microglia. A recurring
253 theme was neurological disorders such as schizophrenia, autism, anxiety, and
254 intellectual disability (Table 1).

255

256 **Functional partitioning of synaptic transcriptome by m⁶A modification**

257 To investigate the functional distinction between hypermethylated and
258 hypomethylated transcripts at synapses, we compared SME to the synaptic

transcriptome (ST, Fig. 1d). 50% of SME genes overlapped with ST (1,505 overlapping genes), while 1,562 non-overlapping ST-specific genes represent a hypomethylated population, and 1,482 SME-specific genes represent a synaptically hypermethylated population of transcripts (Fig. 4a and Supplementary Table S11). GO analysis revealed that the synaptically hypomethylated genes are functionally polarized to cellular metabolic processes such as “formation of a pool of free 40S subunits”, “oxidation-reduction process”, “metabolism of lipid and lipoproteins”, and “lysosome”. Transcripts enriched both by expression and methylation had greater functional applicability to CNS, such as “regulation of cell migration”, “cell morphogenesis”, and “response to growth factor”. The synaptically hypermethylated genes, however, had a striking functional delineation toward synapses, exemplified by the terms “central nervous system development”, “synapse organization”, and “anterograde trans-synaptic signaling”, all within the top 10 functional clusters with significant q-values (Fig. 4b). Furthermore, significance heat-map showed a compelling distinction between hypo- and hyper-methylated genes associated with the terms in cellular metabolism and synaptic function (Fig. 4c). These results suggest that m⁶A methylation predicts functional partitioning of transcripts at the synapse, and could thereby potentially affect the synthesis of critical synapse-specific components that alter synaptic organization and transmission (GO analyses using expressed genes as background in Supplementary Tables 12 and 13).

281

282 **Dendritic localization of m⁶A regulatory proteins**

283 Selective enrichment of methylated transcripts at synapses raises the
 284 possibility of synapse autonomous regulatory mechanisms. We next asked
 285 whether m⁶A regulators could be detected near synapses. Using
 286 immunocytochemistry, we found wide distribution of m⁶A writers, erasers and
 287 readers in adult mouse brain slices in the cortex, thalamus, hippocampus and
 288 other brain regions. In cortical pyramidal neurons, METTL14, FTO, and
 289 YTHDF1, YTHDF2, and YTHDF3 were readily detectable in extra-somatic
 290 regions (Fig. 5a). These *in vivo* localization patterns were replicated in
 291 dissociated hippocampal neuronal cultures (Fig. 5b). Co-staining with
 292 phalloidin detected METTL14, FTO, and all three YTHDF readers in dendritic
 293 shafts adjacent to F-actin-rich spines (Supplementary Fig. S9). Particularly, all
 294 three m⁶A readers YTHDF1, YTHDF2, and YTHDF3 were localized to
 295 dendrites in a non-diffuse pattern that partially overlapped with MAP2-positive
 296 microtubules (Fig. 5c). Co-staining with presynaptic markers (synapsin I or
 297 synaptophysin) demonstrated that all three readers were localized to the
 298 vicinity of synapses. Among the three readers, poly(A) RNA colocalized the
 299 best with YTHDF1, then YTHDF3, and the least with YTHDF2 in dendrites
 300 (Fig. 5c). These results suggest that YTHDF1 may bind to m⁶A-mRNA in
 301 dendrites near synapses, thus may regulate their metabolism locally. In
 302 perfused brain slices, YTHDF1 was detected in the MAP2 positive layers,
 303 albeit the densely packed cortical environment did not resolve clear
 304 colocalization (Supplementary Fig. S10).

305

306 **Reduced expression of m⁶A reader YTHDF1 causes synaptic**
 307 **dysfunction**

308 We first attempted to reduce m⁶A activity by knocking down methyltransferase
 309 METTL3 in cultured neurons and observed massive cell death, which
 310 prohibited further functional analysis (Supplementary Fig. S11). We next
 311 sought to manipulate m⁶A-mediated regulatory pathways by expressing
 312 YTHDF1 shRNAs in dissociated hippocampal neurons (Fig. 6a,
 313 Supplementary Fig. S12). In YTHDF1-KD neurons, altered spine morphology,
 314 characterized by increased spine neck length (1.69 ± 0.17 in shYTHDF1-1,
 315 $1.10 \pm 0.09 \mu\text{m}$ in shYTHDF1-2, $0.73 \pm 0.06 \mu\text{m}$ in shScramble) and decreased
 316 spine head width (0.32 ± 0.01 in shYTHDF1-1, $0.31 \pm 0.02 \mu\text{m}$ in shYTHDF-
 317 21, $0.51 \pm 0.02 \mu\text{m}$ in shScramble) was detected, as well as significantly
 318 reduced PSD-95 cluster intensity (1.9- to 2.2-fold decrease in shYTHDF1-1
 319 and shYTHDF1-2, respectively, Fig. 6b-d). Although spine density was not
 320 significantly affected, the percentage of spines containing visible PSD-95
 321 clusters decreased (36.8% and 58.6% in shYTHDF1-1 and shYTHDF1-2,
 322 95.0% in shScramble). In parallel experiments, expressing CRISPR/Cas9
 323 targeting YTHDF1 in hippocampal neurons recapitulated the abnormal spine
 324 morphology (Supplementary Fig. S13), supporting specific association of the
 325 phenotypes with reduced YTHDF1 expression. These data suggest that m⁶A
 326 recognition/binding protein YTHDF1 is required for normal spine development
 327 and post-synaptic density formation.

328

329 Lack of PSD-95 clustering in spines indicated dysregulation of postsynaptic
 330 signaling complex assembly and synaptic transmission at the excitatory
 331 synapses. We therefore measured surface expression of AMPA-type

glutamate receptors, which mediate fast excitatory transmission. Using an antibody recognizing extracellular N-terminus of GluA1 subunit under membrane non-permeable staining conditions, we detected significantly reduced surface expression of GluA1 (fluorescence intensity 53.4 ± 23.7 in shScram vs 29.8 ± 11.0 in shYTHDF1) but unaltered cluster density (14.5 ± 4.73 in shScram vs 16.7 ± 6.55 clusters per $10 \mu\text{m}$ in shYTHDF1), indicating impaired trafficking of GluA1 to the synaptic membrane and/or anchoring (Fig. 6e and 6f). Such deficits can result in compromised synaptic transmission. We therefore proceeded to record miniature excitatory post-synaptic currents (mEPSCs) and found a significant reduction of amplitude (9.88 ± 0.70 pA in shScram vs 5.92 ± 0.27 pA and 6.86 ± 0.35 pA in shYTHDF1-1 and shYTHDF1-2, respectively) but not of frequency (6.19 ± 0.57 Hz in shScram vs 4.93 ± 0.78 Hz and 6.25 ± 1.34 Hz in shYTHDF1-1 and shYTHDF1-2, respectively, Fig. 6g-i). These results collectively demonstrate that reduced expression level of m⁶A reader YTHDF1 in neurons causes both structural and functional deficits of excitatory synapses.

SME member APC expression is altered in YTHDF1-KD neurons

The functional deficits of synapses may be a product of altered SME expression in YTHDF1-KD neurons. YTHDF1 has been previously shown to facilitate translation of m⁶A mRNAs⁴³. Toward this end, we surveyed potential candidates by crossing SME to a previously published YTHDF1 CLIP/IP dataset⁴³. Among 345 common mRNA, an interesting target was APC

(adenomatous polyposis coli), whose mRNA was stabilized but translational efficiency was decreased in siYTHDF1 cells⁴³. Given the critical role of APC in regulating microtubule dynamics, we suspected that its altered expression in YTHDF1-KD neurons may have contributed to the synapse malfunction.

360

To test this possibility, we performed single-molecule staining of *Apc* mRNA simultaneously with YTHDF1 protein in cultured hippocampal neurons. GFP expression was used for single neuron morphology labeling. *Apc* mRNA was detected in dendrites including distal sites, and partially colocalized with YTHDF1 protein (Fig. 7a). In YTHDF1-KD neurons, a remarkable cell-wide decrease in APC immunoreactivity was detected, including depletion from dendrites near synapses; in contrast, APC2, another top SME candidate and APC homologue, showed unaltered expression (APC: 168.9 ± 31.5 vs 25.4 ± 11.3 A.U., APC2: 129.5 ± 16.4 vs 130.6 ± 14.5 A.U. Fig. 7b and 7c, Supplementary Fig. S14). Importantly, *Apc* mRNA level was comparable between YTHDF1-KD and control cells (10.3 ± 9.90 vs 7.00 ± 7.15 fluorescence puncta per cell, Fig. 7d and 7e). These data suggest that expression of localized SME members is modulated by YTHDF1 expression, exemplified by *Apc* mRNA, whose translation, but not stability or dendritic targeting, is dependent on m⁶A reader YTHDF1.

376

SME highlights cell surface-associated intercellular signaling in neuroglia network

379 Within the SME-enriched functional terms “synapse” and “cell junction,”
 380 surface receptor pathways involved in cell-cell signaling were among the most
 381 abundant methylated transcripts. To further dissect SME function, we crossed
 382 our data set to a recently identified collection of synaptic cleft residents⁴⁴, and
 383 found that 85% of synaptic cleft proteins at both excitatory and inhibitory
 384 synapses were encoded by methylated transcripts. Thus methylation-
 385 mediated regulation may have a wide impact on the biosynthesis of this
 386 functional class of proteins (Fig. 8a). In addition to highly abundant astrocytic
 387 methylated transcripts *Sparcl1* and *EAAT2*, whose proteins promote synapse
 388 formation and remove glutamate from synapses, we identified complement
 389 cascades in microglia surveillance in SME. The mRNAs encoding CX₃CL1,
 390 secreted by neurons potentially as a “find me” signal, and CX₃CR1, the
 391 receptor for CX₃CL1 expressed exclusively by resident microglia in enhancing
 392 synapse refinement, were both highly methylated (Fig. 8b, Supplementary
 393 Fig. S15). By further data mining and assigning the annotated SME genes to
 394 different cell-types, a preliminary yet exciting tripartite SME network emerged
 395 (Fig. 8c). Critical components in all synaptic domains such as pre-synaptic
 396 protein bassoon, post-synaptic CamK2n1, a large portfolio of pre- and post-
 397 synaptic adhesion proteins, secreted proteins and reciprocal receptors
 398 involved in neuron-astrocyte and neuron-microglia communication were
 399 encoded by methylated mRNAs at the synapse, of which many have been
 400 identified as playing critical roles in sculpting synaptic connections.

401

402 Discussion

403 The protein composition of synapses is highly dynamic and regulated in an
 404 activity-dependent manner. mRNA trafficking and local protein synthesis at a
 405 synapse plays an essential role, supplying the initial changes in protein
 406 abundance required for maintaining and modifying connections^{18,45}. A rich
 407 cellular toolbox of post-transcriptional regulatory mechanisms such as
 408 microRNA, nonsense mediated decay, RNA binding proteins, and post-
 409 translational modification of translation elongation factors has been shown to
 410 regulate activity-induced translation at synapses. The SME identified in the
 411 current study reveals another layer of complexity in the regulation of the
 412 synaptic proteome. This enzyme-mediated, reversible modification, and its
 413 impact on the metabolism and translation of individual mRNA, comprises a
 414 highly flexible regulatory mechanism, effective at the single copy level.

415
 416 SME consists of 4,329 m⁶A peaks that are found selectively enriched at the
 417 synapse; half of the 2,987 genes carrying these peaks are not preferentially
 418 localized to synapse. This suggests the existence of mechanisms for
 419 differential methylation or, alternatively, selective mRNA trafficking which have
 420 yet to be identified. Functional enrichment analysis revealed that the 1,482
 421 hypermethylated genes are enriched for synapse-related functions such as
 422 synapse maturation, organization, assembly, and modulation of synaptic
 423 transmission, in sharp contrast to the localized hypomethylated transcripts,
 424 which are primarily involved in cellular metabolic pathways. The functional
 425 impetus for this deliberate partitioning is not clear. An intriguing possibility is
 426 that m⁶A-mediated spatiotemporal control of gene expression is required for
 427 coordinating activity-dependent translation of a cohort of mRNA. In support of

428 this, a rapid and site-specific surge in m⁶A level was shown in response to
 429 UV-triggered DNA damage, indicating that changes in modification status can
 430 occur in a subset of RNAs involved in DNA repair pathways⁴⁶. Moreover, m⁶A
 431 can exert control over translation via direct recruitment of ribosomes^{47,48}
 432 and/or co-translational interaction dynamics between the protein products⁴⁹.
 433 Together these observations support a localized, activity-dependent dynamic
 434 proteomic network regulated by m⁶A at the synapse.

435

436 We found writer METTL14 and eraser FTO distributed to the distal processes
 437 of pyramidal neurons and, although the methylation activity of standalone
 438 METTL14 is under debate³¹, their presence raises the possibility of local
 439 enzyme-mediated (de)methylation. In future studies, this possibility could be
 440 tested using in vitro measurement of RNA methylation after stimulating
 441 isolated synapses. We also detected all three cytoplasmic readers (YTHDFs)
 442 distributed distally, suggesting versatile downstream signaling potential.
 443 Previous studies have demonstrated that binding of YTHDF1-3 to m⁶A
 444 mRNAs enhances translation efficiency and RNA degradation^{43,47,48,50,51}, and
 445 in general, m⁶A is associated with shorter RNA half-life^{51,52}. These apparently
 446 conflicting roles of m⁶A has led to a working model of sequential binding of
 447 YTHDF1 and YTHDF2 to help methylated mRNA achieve steady-state levels
 448 rapidly, with YTHDF3 acting as an enhancer of both degradation and
 449 translation^{53,54}, thereby decreasing response time between stimulus and
 450 phenotypic output⁴³. This model may also help reduce expression “noise” by
 451 degrading excess mRNA and enhancing translation of scarce transcripts.
 452 Both mechanisms can benefit synaptic stability and plasticity in response to

453 activity, and we predict the localized readers act synergistically with the local
454 m⁶A epitranscriptome to achieve robust production of synaptic responses.

455

456 Knockdown of reader YTHDF1 in cultured primary neurons resulted in
457 synaptic transmission deficits and immature morphology of spines. Such
458 phenotypes can be associated with synaptic pathophysiology. We identified a
459 downstream molecular target of YTHDF1, SME member *Apc mRNA*, whose
460 abundance and dendritic localization were not affected, but expression
461 decreased with YTHDF1 knockdown. Previously, the APC cKO mouse model
462 has shown abnormality of central synapses (increased spine density,
463 frequency of miniature excitatory synaptic currents, and long-term
464 potentiation), leading to a multi-syndromic neurodevelopmental disorder⁵⁵,
465 suggesting that altered APC expression and subsequent cytoskeletal
466 regulation might partially contribute to the synaptic dysfunction. Dysregulated
467 m⁶A mRNA translation at synapses may underlie disrupted long-term synaptic
468 plasticity, learning and memory, and neurodevelopmental disorders. In
469 support of this, human genetic research has identified the m⁶A reader
470 YTHDC2 as one of the risk factors for autism spectrum disorder⁵⁶. The
471 functional analysis of synapses in the current study therefore warrants further
472 investigation in m⁶A-mediated local translation and its relation to
473 neuropsychiatric disorders.

474

475 A fascinating aspect of SME is its invoking of the tripartite local translation
476 model, implicating mRNA methylation-mediated local translation in
477 multicellular interactions between neurons, astrocytes, microglia, and

oligodendrocytes. Methylated mRNA encoded proteins that coordinate intercellular cross-talk, such as postsynaptic membrane receptors (Ptpz1), synaptogenic proteins secreted from peri-synaptic astrocyte processes (Sparcl1), neuronal regulators of synapse turnover (Paqr8 and Igfbp5), chemokines secreted by neurons to attract microglia (CX₃CL1), and the reciprocal receptors on the microglial membrane (CX₃CR1). Advanced understanding of molecular communication pathways is required to elaborate how local translation supports combined contributions of synaptic components to the activity and plasticity of tripartite synapses. SME provides an initial candidate list for future mechanistic dissections, highly enriched in specified synaptic functions. Interestingly, of 224 recently identified peri-synaptic astrocyte mRNAs, 180 (80%) overlapped with ST and 152 (68%) overlapped with SME, in contrast to only 23% overlap between the 116 soma-derived astrocyte mRNA and ST²². Thus, RNA metabolism in peri-synaptic astrocyte processes may be directly regulated by m⁶A. Future molecular dissection of m⁶A regulation in tripartite synaptic function requires site-directed manipulation of the methylated adenosine residue and/or adjacent residues to disrupt methylation dynamics on individual transcripts. A recent single nucleotide-resolution m⁶A-seq method may provide valuable information in this regard⁵⁷.

498

Ultimately, m⁶A may be one of many covalent RNA modifications regulating synaptic epitranscriptome^{30,58}. 5mC methylase NSun2 has been shown to colocalize with FMRP in dendrites, where it may exert control over synthesis of a subset of mRNA in an activity-dependent manner⁵⁹. NSun2 mutations

have been associated with intellectual disability as well as other neurodevelopmental deficits^{60,61}. The diverse functional impact of modified transcripts suggests that RNA modification can greatly enhance customization of the local proteome. Such mechanisms are perhaps indispensable for generating context-dependent responses at synapses with spatiotemporal coordination; and aberrant modification events may therefore lead to the pathogenesis of neurodevelopmental and neuropsychiatric disorders.

METHODS

Methods and any associated references are available in the online version of the paper. Accession code PRJNA388019.

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Author Contributions

Wang DO conceived and designed the project. Hong WT purified synaptosomes, performed RNA extractions and m⁶A dot-blot, data mining. Wang DO and Ohara T performed library construction; Merkurjev D, Iida K, Goldie BJ, Yamaguchi H, Pellegrini M performed bioinformatics analysis; Ohara T performed immunostaining; Oomoto I constructed shRNA and performed

KD in cultured hippocampal neurons. Kawaguchi S performed electrophysiology. Wang DO and Goldie BJ wrote the manuscript, Martin K and Hirano T supervised parts of the project. All authors participated in data analysis and interpretation, and made indispensable contributions.

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691

692 **Figure and Table legends**

693 **Figure 1. Low-input m⁶A-seq protocol and synaptic enrichment of**

694 **localized mRNA.** a. m⁶A dot-blot of poly(A) RNA purified from HOM

695 (homogenate lysate) and SYN (synaptosomes) starting from 25ng in a two-

696 fold dilution series. An equal quantity of *in vitro* transcribed RNA using

697 unmodified nucleotides was used as a negative control. b. Representative dot

698 density plots for the first four dots show linear relationship to the quantity of

699 RNA applied. Approximately 50% m⁶A immunoreactivity was observed in SYN

700 compared to HOM. A.U.: Arbitrary Units. c. Workflow of low-input m⁶A-seq

701 protocol. Four sequencing libraries HI, SI, HB, SB representing HOM input,

702 SYN input, HOM IP, and SYN IP were constructed and characterized. d.

703 Scatter plot comparing HOM and SYN gene expression demonstrates SYN-
 704 enriched (orange), SYN-depleted (blue), and genes that are equally
 705 represented between HOM and SYN (black). Known localized mRNA species
 706 (+) are found in the SYN-enriched gene group, supporting successful
 707 enrichment of localized transcripts. TPM: Transcripts per million.

708

709 **Figure 2. Characterization of m⁶A peaks in HOM and SYN.** a. Summary of
 710 peaks identified in this study. Numbers in () are counts after joining peaks
 711 spanning introns. Fold change represents differential coverage density range
 712 between IP and INPUT samples. b. Pairwise comparison of m⁶A peaks
 713 identified in HOM to three published studies using mouse brain RNA. The
 714 number of peaks identified in each study (grey box), number of overlapping
 715 peaks in groups to the left (darker blue), and number of overlapping peaks in
 716 groups on the top (light blue). c. Venn diagram of peaks identified in HOM and
 717 SYN reveals extensive overlap. () indicates co-occurrence in SYN PEAKS. d.
 718 *De novo* motif discovery identified m⁶A consensus motif “RRACU” in both
 719 HOM and SYN peaks with variation in adjacent nucleotides. e. Frequency plot
 720 of motif per 5nt per peak (y axis) against distance from peak summit (x axis)
 721 in SYN. f. Percentage of peaks in HOM and SYN associated with annotated
 722 transcript features. TTS: transcription termination site; UTR: untranslated
 723 region; TSS: transcription start site. g. Metagene profiles depicting relative
 724 frequency of peak coverage along transcripts in HOM and SYN. h. Number of
 725 peaks associated with START and STOP codons in HOM and SYN. i.
 726 Enriched human phenotypes among genes with stop codon-associated SYN
 727 peaks (ToppGene). j. IGV view of peaks in representative synaptically

hypermethylated and hypomethylated genes. Ckap5: cytoskeleton associated protein 5; Lysmd4: LysM domain containing 4.

Figure 3. A comprehensive synaptic m⁶A epitranscriptome. a. Scatter plot of normalized reads in HOM peaks (x axis) and SYN peaks (y axis) delineates SYN-enriched peaks (orange), SYN-depleted peaks (blue), and peaks expressed at equal levels in HOM and SYN (grey). Genes carrying the most abundant and enriched peaks at synapses are labeled. b. Peak quantification was validated by qPCR of cDNA libraries. Scatter plot of log2 fold change (PCR, x axis) and log2 fold change (RPKM, y axis) reveals high concordance. c. IGV views of selected abundant and synaptically enriched peaks. Peaks are represented as subtracted read densities (IP minus Input) in SYN (orange) and HOM (blue). d. Top 20 clusters with their representative enriched terms in SME.

Figure 4. m⁶A functionally partitions synaptic transcripts for dynamic synapse formation and modulation. a. Venn diagram relating synaptic transcriptome (ST) and synaptic m⁶A epitranscriptome (SME). b. Top 10 clusters with their representative enriched terms in synaptically hypomethylated and hypermethylated gene lists. c. Significance heat-map (by p value, FDR) showing enrichment of each term category in synaptically hypo- and hypermethylated gene lists, significance cutoff, 0.05.

Figure 5. m⁶A regulatory proteins are distributed to dendrites and near synapses both in brain slices and in cultures. a. Confocal images of m⁶A

753 writers, erasers, and readers in pyramidal cell layers of perfusion-fixed mouse
754 cortical slices. DAPI: cyan; Immunoreactivity: magenta; Scale bar, 20 μ m. b.
755 Replicate of a in cultured hippocampal neurons. Scale bar, 40 μ m. c. Confocal
756 images of dendritic processes of dissociated hippocampal neuronal cultures
757 immunostained with m⁶A readers YTHDF1-3, co-stained with dendritic marker
758 MAP2, presynaptic marker synapsin I or synaptophysin, and poly(A) RNA.
759 Scale bar, 3 μ m.

760

761 **Figure 6. Reducing YTHDF1 expression in hippocampal neurons causes**
762 **structural and functional synaptic deficits.** a. Confocal images of DIV19
763 dissociated hippocampal neurons expressing shScramble-GFP (left panel) or
764 shYTHDF1-GFP (right panel). DAPI (blue), GFP (green), YTHDF1 (red).
765 Arrowheads point to GFP(+) shScram or shYTHDF1 transfected neurons.
766 Scale bar, 10 μ m. b. Confocal images of dendritic processes of DIV19
767 dissociated hippocampal neurons expressing shScramble-GFP or
768 shYTHDF1-GFP (1 or 2). Left, GFP labels morphology of dendritic shaft and
769 spines; Right, PSD-95 staining in the same samples to label post-synaptic
770 density. Contour of affected dendrites was traced as the white lines through
771 GFP expression. Fluorescence PSD-95 signals outside of the contour were
772 masked to restrict quantification to the affected neurons. Scale bar, 5 μ m. c.
773 Group quantification of spine neck length, spine head diameter, and spine
774 density; d. Group quantification of PSD-95 cluster staining intensity in spines.
775 e. Confocal images of surface GluA1 staining in DIV20 dissociated
776 hippocampal neurons expressing shScramble-GFP or shYTHDF1-GFP.
777 Contour of affected dendrites is indicated by white line. Scale bar, 5 μ m. f.

778 Group quantification of fluorescence intensity of surface GluA1 cluster and
 779 GluA1 cluster density along 10 μ m dendrites. g. Representative mEPSC traces
 780 recorded from neurons transfected with shScramble or shYTHDF1 (sh1 or
 781 sh2). h. Cumulative plots of mEPSC amplitude distribution. i. Group
 782 quantification of amplitudes and frequencies of mEPSCs (n = 12 cells per
 783 condition).

784

785 **Figure 7. Dendritically localized SME member *Apc*, but not *Apc2* mRNA**
 786 **is dependent on YTHDF1 for translation.** a. Confocal images of DIV7
 787 dissociated hippocampal neurons transfected with pCAG-EGFP. Left, merged
 788 images of GFP (white), YTHDF1 immunostaining (magenta) and *Apc* mRNA
 789 (green); Right, dual-color images of YTHDF1 (magenta) and *Apc* mRNA
 790 (green) in insets a1 and a2; Contour of GFP(+) dendrites is indicated by white
 791 lines. Scale bar, 10 μ m and 5 μ m. b. Confocal images of DIV6 neurons with
 792 merged GFP (green) and APC (top, magenta) or APC2 (bottom, magenta).
 793 Scale bar, 10 μ m. c. Group quantification of APC and APC2 immunostaining
 794 intensity in GFP(+) neurons. d. Confocal images of DIV7 dissociated
 795 hippocampal neurons transfected with shScramble-GFP or shYTHDF1-GFP
 796 (sh1) neurons with merged DAPI (blue), GFP (white) and *Apc* mRNA FISH
 797 (green). Scale bar, 10 μ m. e. Quantification of *Apc* mRNA staining clusters per
 798 cell in GFP(+) neurons.

799

800 **Figure 8. SME encapsulates surface-associated intercellular signaling**
 801 **network involving tripartite synapse.** A. Venn diagram of methylated
 802 transcripts in SYN (SYN_m⁶A), transcripts enriched in SYN (ST), and

803 excitatory synaptic cleft proteins (left) and inhibitory synaptic cleft proteins
804 (right) recently identified by Loh et al., 2016. 165 of 194 (85%) excitatory, and
805 35 of 41 (85%) inhibitory, synaptic cleft proteins are encoded by methylated
806 mRNA identified in SYN. B. IGV view of peak coverage of mRNAs encoding
807 chemokine-mediated neuron-microglia communication pathway CX₃CL1-
808 CX₃CR1. C. Curated assignment of proteins encoded by methylated mRNA
809 encoding synaptic cleft proteins and by mRNAs carrying the most abundant
810 methylation sites in SME at excitatory and inhibitory tripartite synapse.

811

812 Supplementary tables 1. Results summary of cross-database comparison of
813 m⁶A mRNA and localized mRNA lists.

814 Supplementary Table S2. Sequencing summary.

815 Supplementary Table S3. Percentage of reads removed after RNA sorting.

816 Supplementary Table S4. QC reads mapped with STAR to mm9 refseq.GTF

817 Supplementary Table S5. Differentially expressed gene lists between HOM
818 and SYN.

819 Supplementary Table S6. HOM peaks and SYN peaks.

820 Supplementary Table S7. Methylated gene lists with peaks associated with
821 5'UTR, START codon, STOP codon, and 3'UTR.

822 Supplementary Table S8. GO analysis of genes associated with STOP codon
823 peaks using all genes expressed either in HOM or SYN as background
824 (supplementary to Fig. 2i).

825 Supplementary Table S9. GO analysis of genes associated with STOP codon
826 peaks using genes expressed in SYN as background (supplementary to Fig
827 2i).

828 Supplementary Table S10. Synaptic m6A epitranscriptome (SME).
 829 Supplementary Table S11. Gene lists associated with synaptic transcriptome
 830 (ST, Fig. 1d) and SME.
 831 Supplementary Table S12. GO analysis of hypermethylated genes using SYN
 832 input genes as background (supplementary to Fig. 4).
 833 Supplementary Table S13. GO analysis of hypomethylated genes using SYN
 834 input genes as background (supplementary to Fig. 4).
 835 Supplementary Table S14. Primers used in q-PCR validation experiments.
 836

837 **Supplementary Figure S1. Preparation and characterization of purified**
 838 **synaptosomes.** a. Synaptosome purification from adult mouse forebrains
 839 using percoll-sucrose gradients. F3 and F4 fractions were pooled as
 840 synaptosomal fraction (SYN). Homogenate lysate without fractionation was
 841 used as control for comparison (HOM); b. Relative (de)enrichment of a panel
 842 of synaptic and somatic markers probed using western blot demonstrate
 843 synapse-enrichment. c. Separation of RNA populations was confirmed by
 844 qRT-PCR examination of nuclear lncRNA Malat1, somatic RNA beta-3 tubulin
 845 and known synaptically localized transcript CaMKII α . d. Bioanalyzer analysis
 846 of RNA integrity in prepared fractions. HOM, homogenate; Cyto, cytoplasm,
 847 F3 and F4 (SYN), synaptosomes. e. A biological replicate of m⁶A dot-blot
 848 presented in Fig. 1b.

849
 850 **Supplementary Figure S2. Correlation between biological replicate**
 851 **sequencing libraries.** Pairwise comparison of biological replicate sequencing
 852 libraries demonstrates strong, linear correlation between replicates.

853

854 **Supplementary Figure S3. Validation of peak calling in low input m⁶A-**

855 **seq.** a. hypergeometric tests on peak overlapping to three previously
856 published databases (supplementary to fig. 2b); b. Frequency plot of motif per
857 5nt per peak (y axis) against distance from peak summit (x axis) in HOM.

858

859 **Supplementary Figure S4. GO analysis of m⁶A-mRNA.** Enriched human
860 phenotypes among genes with START codon-associated SYN peaks
861 (ToppGene). Supplementary to Fig. 2i.

862

863 **Supplementary Figure S5. IGV confirmation of peak calling.** Top panel,
864 synapse-enriched peak in Ckap5 transcript; bottom panel, synapse-depleted
865 peak in Lysmd4. Red, IP tracks; blue, INPUT tracks; black, peak location
866 track. Supplementary to Fig. 2j.

867

868 **Supplementary Figure S6. Negative impact of methylation on synaptic**
869 **mRNA stability and synaptic localization.** a. (left) Box plots depicting
870 synaptic concentrations of genes in four groups with increasing methylation
871 level. For each gene, methylation level (ML) was calculated as IP/INPUT
872 reads ratios in each designated RNA region (full length, 5'UTR, CDS, and
873 3'UTR) and divided into four groups from the least methylated to the most
874 methylated (a; $ML < -2$, b; $-2 \leq ML < 0$, c; $0 \leq ML < 2$, d; $2 \leq ML$). S.TMP:
875 transcripts per million at SYN. (right) Scatter plot and regression model
876 labeled with slope, p-value testing the quality of model fitting, and Pearson's
877 correlation coefficient r ; b. Analogous plots representing transcript enrichment

at synapse compared to whole cell. Y-axis and X-axis represent relative expression values and relative methylation values, respectively. For box plots, genes were classified into one of the four groups as follows: e; rel. ML < -1, f; -1 <= rel. ML < 0, g; 0 <= rel. ML < 1, h; 1 <= rel. ML. Genes with TPM less than 1 were excluded from the analysis.

Supplementary Figure S7. qPCR validation of synaptic (de)enriched methylation sites detected using m⁶A-seq. Left, relative (de)enrichment estimated by qPCR; Right, relative (de)enrichment estimated by m⁶A-seq; log2 fold change (IP/INPUT) shows positive values for methylated sites and negative values for demethylated sites. Log2 fold change (IP/INPUT) values in SYN and HOM are presented together for comparison. Supplementary to Fig. 3a and 3b.

Supplementary Figure S8. A subset of enriched functional clusters in Fig. 3d are depicted in a network layout (Metascape). Each node represents a single term; node size is proportional to number of input genes in that term; nodes colored by p value. Terms with a similarity score > 0.3 are linked by an edge (edge thickness reflects similarity score).

Supplementary Figure S9. m⁶A regulatory proteins in dendritic processes of dissociated hippocampal neuronal cultures. Confocal images of m⁶A regulatory proteins (magenta) and counter-stained with phalloidin (green) to label F actin-rich spines. Scale bar, 5 μm.

903 **Supplementary Figure S10. m⁶A reader YTHDF1 in perfused mouse brain**
 904 **slices.** Top, cortical cortex; Bottom, hippocampus and CA1 and CA3 regions
 905 of hippocampus. Scale bars, 100 μ m and 50 μ m.

906 **Supplementary Figure S11. Knockdown METTL3 in dissociated**
 907 **hippocampal neuronal cultures induces cell death.** a.
 908 Immunofluorescence images of hippocampal neuronal cultures transfected
 909 with shMETTL3 vectors. DAPI (blue), GFP (green), METTL3 immunostaining
 910 (red). b. Decreased METTL3 protein expression was detected within a day
 911 after transfection by immunofluorescence staining.

912
 913 **Supplementary Figure S12. Knock-down efficiency estimation of**
 914 **YTHDF1-sh1 and YTHDF1-sh2.** a. Normalized YTHDF1 mRNA expression in
 915 shScramble, sh1, and sh2 cells (normalized to beta actin mRNA); b. Western-
 916 blots of YTHDF1 proteins in shScramble, sh1, and sh2 cells lysates. β -actin
 917 was blotted on the same membrane as loading control. Supplementary to Fig.
 918 6.

919
 920 **Supplementary Figure S13. Replicated neuron phenotypes of YTHDF1-**
 921 **KD cells using CRISPR/Cas9 technology.** a. GFP fluorescence images of
 922 control cells (expressing pCAG-EGFP and pX330) and YTHDF1-KD cells
 923 (expressing pCAG-EGFP and pX330-guide RNA sequences g1 or g2). Scale
 924 bar, 5 μ m; b. Group quantifications of spine neck length and spine head width
 925 showed similar phenotypes to YTHDF1-KD using shRNA vectors.
 926 Supplementary to Fig. 6.

927

928 **Supplementary Figure S14. APC protein expression was altered in**
 929 **YTHDF1-KD neurons.** a. Confocal immunofluorescence images of APC
 930 using APC N-terminus antibody (top) or APC c-terminus antibody (bottom).
 931 GFP labels neurons expressing the shRNAs (shScramble or shYTHDF1).
 932 Both antibodies consistently detected decreased APC protein expression in
 933 YTHDF1-KD neurons; DAPI (blue), GFP (green), APC protein (magenta).
 934 Scale bar, 10 μ m.

935

936 **Supplementary Figure S15. IGV view of peaks in transcripts involved in**
 937 **neuron-microglia signaling at tripartite synapse.** Red, synapse tracks;
 938 blue, homogenate tracks; black, peak location track. In support of Figure 8b.

Figure 1

Figure 2

Figure 3

Figure 4

a

b Top 10 clusters with their representative enriched terms

Synaptically hypomethylated (n=1562)

GO	Description	%	-Log10(q)
R-MMU-72689	Formation of a pool of free 40S subunits	4.32	53.33
GO:0055114	Oxidation-reduction process	11.41	36.68
GO:0044712	Single-organism catabolic process	7.90	18.07
mmu05012	Parkinson's disease	3.11	17.79
R-MMU-168249	Innate Immune System	9.05	16.91
R-MMU-556833	Metabolism of lipids and lipoproteins	6.55	14.75
GO:1901135	Carbohydrate derivative metabolic process	8.85	13.91
mmu04142	Lysosome	2.30	10.73
GO:0042255	Ribosome assembly	1.55	10.45
ko01200	Carbon metabolism	2.16	9.88

Synaptically hypermethylated (n=1482)

GO	Description	%	-Log10(q)
GO:0044087	Regulation of cellular component biogenesis	7.05	8.24
GO:0007417	Central nervous system development	6.98	8.06
GO:0060341	Regulation of cellular localization	6.77	5.67
GO:0000902	Cell morphogenesis	7.41	5.67
GO:0050808	Synapse organization	3.06	5.30
GO:0006325	Chromatin organization	5.77	5.06
GO:0051640	Organelle localization	4.13	4.92
GO:1905114	Cell surface receptor signaling pathway involved in cell-cell signaling	4.27	4.88
GO:0098916	Anterograde trans-synaptic signaling	4.70	4.39
GO:0050905	Neuromuscular process	1.71	4.16

c

Figure 5

a

b

c

Figure 6

Figure 7

Figure 8

Table 1: Top methylated transcripts in synaptic m⁶A epitranscriptome^{a,b}		
Gene Symbol Protein name	Location	Known functions and references
<i>Ptpnz1</i> Ptpnz1, RPTPβ/ζ	Central nervous system, spines	Multi-ligand protein tyrosine phosphatase receptor localized to postsynaptic membrane of dendritic spines (Hayashi et al., 2005); Associated with schizophrenia (Buxbaum et al., 2008); Transgenic mice overexpressing <i>Ptpnz1</i> show cellular and behavioral changes characteristic of schizophrenia patients (Takahashi et al., 2011)
<i>Sparcl1</i> Sparcl1, hevin	Astrocyte, synaptic cleft	Glycoprotein localized to synaptic cleft of excitatory synapses (Johnston et al., 1990). Reduced expression observed in autism and depression (Purcell et al., 2001; Zhurov et al., 2012). Plays critical role in formation and plasticity of thalamo-cortical synapses by bridging presynaptic NRX1α and postsynaptic NL1 interaction (Risher et al., 2014, Singh et al., 2016).
<i>Slc1a2</i> Slc1a2, EAAT2	Astrocyte, perisynapse	High affinity glutamate transporter responsible for removing most (95%) glutamate from synaptic cleft through uptake in adult forebrain. Crucial in maintaining ambient glutamate low concentration and ensuring high signal-to-noise ratio in synaptic transmission (Rothstein et al., 1996). Protects neurons from glutamate excitotoxicity, and regulates glutamate dynamics during Hebbian synaptic plasticity (Valtcheva and Venance, 2015).
<i>Paqr8</i> mPRβ	Broadly throughout brain	Membrane progesterin receptor. Transduces progesterone signals synergistically with estrogen to promote induction and retraction of spines in estrous cycle in wide brain regions (McEwen and Wooley, 1994, Brinton RD et al., 2008). Neuroprotection and myelin repair functions link progesterone to prevention of nerve diseases (Schumacher, 2008).
<i>Apc</i> Apc	Nervous system	Microtubule plus-end scaffolding protein that binds to hundreds of mRNA and proteins; required for neuron polarization, migration, morphogenesis, and synapse maturation, thus a key regulator of neuron and synapse development (Rosenberg et al., 2008, Preitner et al., 2014). Mutations in APC correlate with autism and mental retardation in human (Raedle et al., 2001; Zhou et al., 2007).
<i>Apc2</i> Apc2	Nervous system	APC-like molecule, whose C-terminal region diversifies significantly from APC. Preferentially expressed in the CNS, key cytoskeletal regulator in axonal projections, migration, growth cone responsiveness to guidance molecules (Yamanaka et al., 2002, Shintani et al., 2009, Shintani et al., 2012). Loss of function of APC2 is associated to etiology of Sotos syndrome, a genetic neurological disorder characterized by intellectual disability and characteristic facial features (Almuriekh et al., 2015).
<i>Bsn</i> Basson	Neuron, presynaptic active zone	Presynaptic cytomatrix scaffold protein involved in organizing cytoskeleton and active zones for neurotransmitter release at both excitatory and inhibitory synapses (Richter et al., 1999, Garner et al., 2000, Goldstein et al., 2008). Recruited at early stages to nascent synapses on Piccolo-Bassoon transport vesicles that may represent

		precursors of the active zonal plasma membrane (Zhai et al., 2001). Photoreceptor ribbons lacking Bassoon are not anchored to the presynaptic active zones and thus causes impaired photoreceptor synaptic transmission (Dick et al., 2003).
S1pr1 S1pr1	Astrocyte foot processes	G protein-coupled receptor that transduces intracellular signals from sphingolipid metabolites. Shown to play important roles in both nervous system development and glutamatergic transmission (Chun et al., 2000; Kimura et al., 2007). Structural analogue of sphingosine has been used to treat multiple sclerosis (Kappos et al., 2010).
Igfbp5 Igfbp5	Cell membrane, cellular matrix	Alters interaction of IGFs with their cell surface receptors. This signaling pathway is altered in spinocerebellar ataxia (Gatchel J et al., 2008). Promotes formation of mature excitatory synapses, thus restores synaptic deficits in iPS-derived neurons from Phelan-McDermid syndrome patients (Shcheglovitov et al., 2013).
Mfap3l Mfap3l, Nyl-sp9	Cell membrane	Single-pass transmembrane protein primarily found in testis but also in human cerebral cortex. GWAS have associated Mfap3l to anxiety disorders (Otowa et al., 2014). The intracellular region of Mfap3l suggests involvement in PI3K/AKT signal transduction (Kang et al., 2008)
Pcdh10 Ol-pc	Neuron	Protocadherin family member linked to autism spectrum disorders (Morrow et al., 2008). Expressed primarily in the brain, essential for elongation of striatal axons and patterning of the putative guidance cues for thalamocortical projections (Hirano et al., 1999; Uemura M et al., 2007); contributes to activity-dependent synapse elimination by linking synaptic structural proteins to degradation (Tsai NP et al., 2012).
Dag1 Dag1, DG	Glia, neuron	Links extracellular matrix and cytoskeleton to stabilize membranes and contribute to extracellular matrix-dependent signaling cascades. In CNS, Dag1 deficient mice show loss of inhibitory synapses between basket neurons and pyramidal neurons and mediates homeostatic synaptic plasticity at GABAergic synapses. (Pribiag et al., 2013, Fruh et al., 2016).
Fat3 Fat3	Neuronal dendrites, axons	Cadherin predominantly expressed in CNS (Mitsui et al., 2002). In retina, Fat3 determines dendrite number, orientation, and migration of amacrine cells (Deans et al., 2011). In developing olfactory bulb, Fat3 is distributed along the dendrites of mitral cells (Nagae et al., 2006).
Camk2n1 Camk2n1	Neuron	Potent inhibitor of Camk2 enzyme activity (Chang et al., 2001). Camk2 is a leading regulator of Ca ²⁺ dynamics in dendritic spines and key molecule in synaptic plasticity and memory (Holtmaat A et al., 2009).
Ppp1r9b Neurabin-2, Spinophilin,	Neuron	Highly enriched in dendritic spines and regulates excitatory synaptic transmission and spine morphology (Feng et al., 2000) Reduced expression in hippocampal formation, and associated dendritic pathology, is found in schizophrenia and mood disorder patients (Law AJ., 2004).
^a Genes were ranked by abundance of peak coverage in SME.		
^b Reference list can be found in Online Methods.		

Figure 1

a

b

d

c

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8