CMTR1-Catalyzed 2’-O-Ribose Methylation Controls Neuronal Development by Regulating Camk2α Expression Independent of RIG-I Signaling

Highlights

- Every mRNA molecule in neurons is N1 2’-O methylated by CMTR1
- CMTR1 is essential for neuromorphogenesis and brain development
- CMTR1 deficiency does not activate RIG-I and interferon signaling
- CMTR1 promotes Camk2α expression to support dendrite development

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In Brief

Lee et al. demonstrate that CMTR1-catalyzed 2’-O-ribose methylation in mRNAs is important for dendritic morphogenesis and brain development. CMTR1 is dispensable for silencing RIG-I-activated innate immunity in neurons. Transcriptomic profiling and rescue experiments show Camk2α as the most downregulated gene in the absence of CMTR1, suggesting a role in dendrite development.
CMTR1-Catalyzed 2'-O-Ribose Methylation Controls Neuronal Development by Regulating Camk2α Expression Independent of RIG-I Signaling

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SUMMARY

Eukaryotic mRNAs are 5’ end capped with a 7-methylguanosine, which is important for processing and translation of mRNAs. Cap methyltransferase 1 (CMTR1) catalyzes 2'-O-ribose methylation of the first transcribed nucleotide (N1 2’-O-Me) to mask mRNAs from innate immune surveillance by retinoic-acid-inducible gene-I (RIG-I). Nevertheless, whether this modification regulates gene expression for neuronal functions remains unexplored. Here, we find that knockdown of CMTR1 impairs dendrite development independent of secretory cytokines and RIG-I signaling. Using transcriptomic analyses, we identify altered gene expression related to dendrite morphogenesis instead of RIG-I-activated interferon signaling, such as decreased calcium/calmodulin-dependent protein kinase 2α (Camk2α). In line with these molecular changes, dendritic complexity in CMTR1-insufficient neurons is rescued by ectopic expression of CaMK2α but not by inactivation of RIG-I signaling. We further generate brain-specific CMTR1-knockout mice to validate these findings in vivo. Our study reveals the indispensable role of CMTR1-catalyzed N1 2’-O-Me in gene regulation for brain development.

INTRODUCTION

In all eukaryotes and many viruses, the 5’ end of transcripts is modified with a 7-methylguanosine (m7G), rendering the terminal dinucleotide resistant to ribonuclease digestion (Shuman, 2002). This m7G structure, called cap0 (m7GpppNN, N: any nucleotide), is important for RNA stability, splicing, nucleocyttoplasmic transport, and translation initiation (Cheng et al., 2006; Hernández et al., 2010; Ramanathan et al., 2016). Except for yeast mRNAs with the primitive m7G cap, the cap structure in higher eukaryotes has additional methylation at the 2'-O-ribose position of the first and second nucleotides by cap methyltransferase 1 (CMTR1) and CMTR2, respectively, to produce cap1 (m7GpppNmN) and cap2 (m7GpppNmNm) structures (Bélanger et al., 2010; Werner et al., 2011).

Yeast mRNAs are not cap1 modified, so the cap0 structure, which recruits the assembly of the eukaryotic initiation factor (eIF) 4F complex (i.e., eIF4E, 4G, and 4A) by direct binding of eIF4E to the m7G cap, is sufficient for “cap-dependent” translation. An early study indicated that cap1 exists in every mRNA molecule, whereas cap2 is present in ~50% of mRNA molecules in HeLa cells (Furuchi et al., 1975). Although the cap-methyltransferring enzyme activity was first detected in HeLa extracts (Langberg and Moss, 1981), CMTR1, the enzyme catalyzing 2'-O-ribose methylation of the first nucleotide in mRNAs (hereafter called N1 2’-O-Me), was not identified until 2010 (Bélanger et al., 2010). Dom3Z/DXO, a mammalian homolog of yeast Rai1 and Dxo1, possesses decapping, pyrophosphohydrolase, and 5’-3’ exoribonuclease activities to degrade GpppG-RNA and m7GpppG (cap0)-RNA from the 5’ end (Jiao et al., 2013), but it could not degrade cap1-oligoribonucleotide in vitro (Picard-Jean et al., 2018). Moreover, a previous study showed that cap1 modification accompanied with cytoplasmic polyadenylation in c-Mos mRNA promotes translation during oocyte matura-

The 5’ cap composition is also a determinant of self- (host) versus non-self RNA during viral infection (Leung and Amarasinghe, 2016). Viruses with inactive 2'-O methyltransferase (MTase), such as West Nile virus, Poxvirus, and Coronavirus mutants, are attenuated in vivo because interferon (IFN)-induced proteins with tetratricopeptide repeat 1 (IFIT1) bind to cap0 viral RNAs better than cap1 viral RNAs and consequently prevent the recruitment of eIF4E for viral protein synthesis (Daffis et al., 2010; Habjan et al., 2013). CMTR1 is also known as IFN-stimulated gene 95 kDa protein (ISG95), and its expression is upregulated by viral infection in various cells (Geiss et al., 2003; Guerra et al., 2003; Su et al., 2002). Moreover, the cap1 structure is suggested to prevent cellular mRNAs from being recognized...
Figure 1. Nuclear CMTR1 Is Required for Dendritic Development

(A) The chemical structure of capped RNAs. RNMT (mRNA cap guanine-N7 methyltransferase), CMTR1, and CMTR2, which transfer the methyl group from S-adenosylmethionine (SAM) to the corresponding positions, are indicated. SAH, S-adenosylhomocysteine.

(B) Developmental expression of CMTR1 in neurons at the denoted days in vitro (DIV). The level of CMTR1 was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as a relative ratio to DIV2. Data are mean ± SEM from three cultures.

(C–E) CMTR1 domain structure (C). The K239A and ΔNLS mutations render catalytic dead and cytoplasm-localized CMTR1, respectively. DIV2 rat neurons were infected with lentivirus expressing siCTL or siCM1 ± RFP or RFP-tagged human CMTR1 WT or mutants. The infected neurons at DIV7 were used for immunoblotting or immunostaining of RFP and MAP2 (D and E). Scales, 50 μm.

(F) Quantification of dendritic development. siCM1 + RFP-K239A significantly reduced the number of intersections compared to RFP, siCM1 ± RFP, or siCM1 + WT (P < 0.001).

(G) Quantification of total dendritic length. siCM1 + ΔNLS significantly increased the total dendritic length compared to RFP, siCM1 ± RFP, or siCM1 + WT (P < 0.001).

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as non-self molecules by the RNA sensor retinoic-acid-inducible gene-I (RIG-I) (Schuberth-Wagner et al., 2015).

The previous findings suggest that the cap1 moiety may regulate mRNA stability or translation while serving as a molecular signature for self-transcript identification. Nevertheless, none of these findings have been validated in vivo. Here, we investigated CMTR1 function in neurons, in which RNA modifications control molecular diversity to support their complex morphologies and functions (Huang and Lu, 2018; Noack and Calegari, 2018). To avoid any interference from innate immunity, we also studied CMTR1 in RIG-I-knockout (KO) and mitochondrial antiviral signaling protein (MAVS)-KO neurons. We found that impaired dendrite development in CMTR1-knockdown (KD) neurons resulted from downregulated transcription of calcium/calmodulin-dependent protein kinase 2α (Camk2α). Unexpectedly, CMTR1-KD did not trigger innate immune responses in neurons. Moreover, the findings from the KD neurons were recapitulated in CMTR1-KO cortices. Therefore, CMTR1-catalyzed methylation regulates CaMK2α expression for dendritic morphogenesis but is dispensable for masking cellular mRNAs from RIG-I detection.

RESULTS

Nuclear MTase Activity of CMTR1 Is Required for Dendritic Development

The three cap structures shown the methylated positions with corresponding MTases are illustrated in Figure 1A. In agreement with cap1 as the dominant cap structure in mammalian cells and tissues (Akichika et al., 2019; Furuichi et al., 1975; Wang et al., 2019), CMTR1 is expressed in various tissues (Figure S1A) and localized predominately in the nucleus of neurons and in microtubule-associated protein 2 (MAP2)-negative non-neuronal cells (Figure S1B). Notably, the expression of CMTR1 in neurons remained high during the first 6 days in vitro (DIV6), the critical period for axonal and dendritic outgrowth, and declined thereafter to a steady level (Figure 1B). Thus, we examined whether CMTR1 is involved in neuromorphogenesis by infecting DIV2 neurons with lentivirus expressing control short hairpin RNA (shRNA) (siCTL) or rat CMTR1-targeted shRNA (siCM1#1 or siCM1#2) along with mCherry red fluorescent protein (RFP) to mark infected cells. The neurons at DIV7 were harvested for immunoblotting to confirm the diminished CMTR1 level (Figure S2A) or fixed for MAP2 immunostaining. Because CMTR1-KD did not affect MAP2 expression (Figure S2B), we used an MAP2-immuno stained signal to outline dendritic processes (Figure S2C). RFP-positive (i.e., lentivirus-infected) and MAP2-positive pyramidal neurons were selected for Sholl analysis (Sholl, 1953). Both siCM1#1 and siCM1#2 neurons exhibited defective dendritic arborization, as indicated by the decreased number of dendritic intersections (Figure S2D), thereby leading to reduced total dendritic number and length (Figure S2E).

The K239R-D236A-K404 triad in the catalytic domain of human CMTR1 (hCMTR1) is important for MTase activity (Smietanski et al., 2014). We used hCMTR1 for the rescue experiment because its nucleotide sequence is resistant to siCM1#1 (hereafter called siCM1)-mediated cleavage and its protein sequence shares ~93% identity with that of the rat homolog. CMTR1 contains a nuclear localization signal (NLS) at the N terminus, followed by Q patch and MTase domains. The C-terminal region contains guanylyltransferase-like and WW domains (Figure 1C). The WW domain is important for the association with RNA polymerase II for co-transcriptional N1 2′-O-Me (Galloway and Cowling, 2019). For simultaneous KD of rat CMTR1 and expression of wild-type (WT) or mutant hCMTR1, the GFP reporter in the pLL3.7-Syn plasmid (siCTL and siCM1) was replaced with an RFP or RFP-hCMTR1 construct. We generated K239A and ∆NLS (∆3–18 amino acid [aa] mutants, which render catalytic dead MTase and cytoplasm-localized CMTR1, respectively (Figure 1D)). DIV2 cortical neurons were infected with the designated lentivirus and harvested at DIV7 for examining the KD of endogenous CMTR1 and the expression of RFP-hCMTR1 WT or mutants (Figure 1C). Similarly, the infected neurons on coverslips were processed for MAP2 immunostaining (Figure 1E) for Sholl analysis. CMTR1-KD reduced dendritic complexity (Figure 1F) and total dendritic number and length (Figure 1G), which could be rescued by ectopic expression of WT but not K239A or ∆NLS mutant. Therefore, CMTR1-mediated 2′-O-Me in the nucleus is critical to support dendritic outgrowth.

Defective Dendritic Ramification in CMTR1-KD Neurons Is Independent of Secretory Factors and RIG-I-Mediated Signaling

Abnormal dendritic arborization could be due to secretory factors such as a lack of brain-derived neurotrophic factor (Moya-Alvarado et al., 2018) or presence of inflammatory cytokines (O’Neill et al., 2016). KD of CMTR1 in AS49 cells induces type I IFN signaling by RIG-I activation (Schuberth-Wagner et al., 2015), so abnormal dendritic development could be caused by inflammatory cytokines secreted from CMTR1-KD neurons. However, we detected no evident changes in mRNA levels of many IFN-signaling-related genes (Figure 2F). Moreover, the qRT-PCR signals of some transcripts, such as IFN-β, IFIT1, and tumor necrosis factor α (TNF-α), were too low to confidently claim the activation of innate immunity, so we performed co-culture experiments (Figure 2A) and found that WT neurons cocultured with siCM1 neurons exhibited normal dendritic number and length (Figure 2B). Thus, secretory factors by autocrine or paracrine action are not the culprits of defective dendritic outgrowth in CMTR1-KD neurons.

RIG-I and melanoma-differentiation-associated protein 5 (MDA5) are pattern recognition receptors that sense viral RNAs with loosely defined features. Once activated by RNA molecules, both RIG-I and MDA5 convey their signaling through MAVS to induce type I IFN synthesis and innate immune responses (Reikine et al., 2014). Because RIG-I-mediated IFN responses exist in virus-infected neurons (Nazmi et al., 2011), it was unexpected that secretory factors were not involved to impair dendritic...
We wondered whether RIG-I, presumably activated by elevated cap1-deficient mRNAs in the CMTR1-KD condition (Schuberth-Wagner et al., 2015), may affect dendritic growth independent of IFN secretion. If so, such morphological defects should be rescued in RIG-I-KO or MAVS-KO neurons. WT and KO embryos from RIG-I heterozygous matings were used for neuronal cultures (Figure 2C). KD of CMTR1 in RIG-I-KO neurons still impaired dendritic complexity, which could be rescued by RFP-hCMTR1 expression (Figures 2D and 2E). Similar results were also found in MAVS-KO neurons (Figure 2F).
Figure 3. Knockdown of CMTR1 Decreases CaMK2α Expression to Affect Dendrite Development

(A) Gene Ontology (GO) analysis of enrichment clusters from duplicate microarrays. The statistical significance of each cluster is expressed as $-\log_{10} P$.

(B) The list of genes from the dendrite GO cluster with most transcriptomic changes in siCM1 neurons. **Ltbp1**, latent transforming growth factor β binding protein 1; **Cdkn1a**, cyclin-dependent kinase inhibitor 1; **Pdyn**, prodynorphin; **Sort1**, sortilin1; **Atp7a**, copper-transporting P-type ATPase; **Camk2a**, calcium/calmodulin-dependent protein kinase 2α; **Ptk2b**, protein tyrosine kinase 2b; **Twf1**, twinfilin-1; **Pafah1b1**, platelet-activating factor acetylhydrolase 1b subunit 1; **Prkcg**, protein kinase C gamma type; **Kif5a**, kinesin family member 5a.

(C) The normalized RNA levels relative to Gapdh were determined by qRT-PCR.

(D) The CaMK2α level relative to GAPDH in siCTL and siCM1 neurons ± ectopic expression of denoted mutant.

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so RIG-I/MAVS-mediated signaling does not contribute to dendritic maldevelopment in CMTR1-KD neurons.

**CMTR1 Deficiency Alters Camk2α mRNA Expression to Affect Dendritic Morphogenesis**

We used microarray and Gene Ontology (GO) analyses of genes with a corresponding mRNA level showing more than ±1.2-fold change in siCM1 neurons relative to siCTL neurons (Table S1). The GO: 0030425 dendrite category scored the highest among different biological processes (Figure 3A). Among 48 genes in this cluster (Table S1), the top 5 to 6 genes showing the most up- or downregulation (Figure 3B) were validated by qRT-PCR (Figure 3C). Only Camk2α and Pafah1b1 levels showed a consistent reduction. We focused on Camk2α because it is the most downregulated gene and Camk2α-haploinsufficient mice also show reduced dendritic branches and length (Yamasaki et al., 1999), so cap1 as a molecular signature for abnormally frequent cap1-free nascent mRNA molecules carry the cap1 structure (Akichika et al., 2019; Furuichi et al., 1975), so cap1 as a molecular signature for Abnormal Dendritic Morphology in the Absence of Innate Immunity

To assess the physiological functions of CMTR1, we generated mice carrying a targeted KO-first Cmtr1 allele (zCmtr1) and then produced mice carrying the floxed Cmtr1 allele (fCmtr1). In the zCmtr1 allele, the splicing of exon 8 to LacZ reporter resulted in a truncated transcript without a functional catalytic domain (zKO; Figure S4A). Crossing of Cmtr1f1/2 mice produced no live Cmtr1−/zKO (Cmtr1f1/2) mice (Figure S4B), as Cmtr1f2/2 embryos at the embryonic day 8.5 (E8.5) gastrulation stage were mostly absorbed (Figure S4C). Thus, we crossed tCmtr1 mice with Emx1-Cre or Nestin-Cre mice to generate cKOEmx1 and cKO Nes mice (Figure 4A). The expression of Cre in Emx1-Cre mice begins at E9.5 and is limited to the EMX1-expressing progenitors (Gorski et al., 2002). The expression of Cre in Nestin-Cre mice begins at E10.5 in pan neuron progenitors (Tronche et al., 1999), so Cmtr1 in cKO Nes cortices was ablated in all neurons and glia. We used cKOEmx1 cortices for anatomical and morphological analyses and cKO Nes cortices for molecular studies and culture. CMTR1-cKOEmx1 mice showed reduced cortical and hippocampal areas (Figure 4B). The poly(A) RNAs isolated from cKO Nes cortices did not contain N1 2′-O-Me (Figure 4C), and CaMK2α was also reduced in cKO Nes cortices (Figure 4D). Although the amounts of 4S-labeled transcripts in conditional wild-type (cWT) and cKO Nes neurons were comparable, the level of nascent Camk2α mRNA was significantly reduced in cKO Nes neurons (Figure 4E). In addition, DKO-KD could not restore the expression of Camk2α mRNA (Figure S5A) and protein (Figure S5B), so DKO does not degrade cap1-free Camk2α mRNA. Together with the findings in CMTR1-KD neurons (Figure S3), these results show that CMTR1 deficiency impairs Camk2α transcription rather than stability.

To address whether CMTR1 affects dendritic maturation in vivo, we used Thy1-YFP-H transgenic mice, which express yellow fluorescent protein (YFP) in the subsets of cortical (layer 5) and hippocampal (CA1) pyramidal neurons (Feng et al., 2000), to produce cWT:: or cKOEmx1::Thy1-YFP mice. Immunochemistry confirmed the absence of CMTR1 in cortical and hippocampal pyramidal neurons (Figure S6A), so we acquired images of YFP-expressing cortical layer 5 neurons (Figure 4F) and found impaired dendritic morphology in cKOEmx1 mice (Figure 4G). Moreover, no inflammation was found in the cKOEmx1 brain because the number and morphology of microglia (Iba1-positive) appeared at the resting state (Figure S6B) and the mRNA levels of IFN-β1, TNF-α, IFIT1, and RIG-I were not upregulated in cKOEmx1 cortices (Figure 4H). In contrast, these transcripts were highly elevated in RNA-virus-infected spinal cords and brains (Figure 4H). Thus, the loss of CMTR1 did not trigger inflammatory responses to affect dendrite morphogenesis in vivo.

**DISCUSSION**

This study demonstrates that CMTR1 insufficiency affects Camk2α expression to impair dendritic arborization and cortical development. Ectopic expression of CaMK2α but not depletion of RIG-I or MAVS rescued dendritic maldevelopment caused by CMTR1 deficiency, which supports that CMTR1-catalyzed N1 2′-O-Me regulates gene expression to control neuronal development.

The conserved histidine 830 in the RIG-I RNA binding pocket is important for its steric exclusion binding to cap1-methylated RNA (Devarkar et al., 2016), so cap1-deficient cellular transcripts in CMTR1-KD cells were recognized by RIG-I to activate IFN signaling (Schuberth-Wagner et al., 2015). Almost all mammalian mRNA molecules carry the cap1 structure (Akichika et al., 2019; Furuichi et al., 1975), so cap1 as a molecular signature for discriminating self and foreign transcripts seems to make perfect sense. Surprisingly, CMTR1 deficiency in neurons did not induce type-I-IFN-signaling-related gene expression (Figures 3A, 4H, and S2F) or release cytokines to affect co-cultured neurons (Figure 2A) nor resulted in any inflammatory signs in the KO brain (Figure S6B). Moreover, neurons do not appear to express other MTases to catalyze N1 2′-O-Me in the absence of CMTR1 (Figure 4C) or lack innate immunity because the expression of type I IFN genes is robustly elevated in the Japanese-encephalitis-virus-infected brain (Figure 4H), which is mediated by RIG-I.
activation (Kato et al., 2006). MDA5 could be another cap1-free RNA sensor (Roth-Cross et al., 2008; Züst et al., 2011). However, KD of CMTR1 in MAVS-KO neurons did not ameliorate dendritic defects (Figure 2F), so neither RIG-I nor MDA5 can detect cap1-free cellular RNA in CMTR1-deficient neurons. We reason that in the life of the mRNA after being transcribed, cap1-free mRNAs are assembled in various messenger ribonucleoproteins to escape from RIG-I surveillance. Alternatively, cap1-free mRNAs may simply not be the substrate for RIG-I. A previous study using the in vitro binding assay showed that RIG-I does not recognize cap0 single-stranded RNA even though its binding affinity for cap0 double-stranded RNA is ~2 nM (Devarkar et al., 2016). Together with our results, N1 2'-O-Me of cellular mRNAs is not meant for escaping RIG-I surveillance, at least, in uninfected cells. Notably, CMTR1 expression was remarkably elevated in virus-infected neurons (Figure 4H). CMTR1-KO in A549 cells

Figure 4. Defective Dendritic Arborization of Cortical Pyramidal Neurons in CMTR1-cKOEmx1 Mice without Evident Inflammation
(A) The fCmtr1 mice were used to generate cKOEmx1 or cKOEmx mice.
(B) The dorsal view of postnatal day 22 brains with medial sagittal sections stained with hematoxylin and eosin. The outlined cortical areas are denoted.
(C) The poly(A) RNAs isolated from cortices were used to detect N1 2'-O-Me (pAm and pCm, circled by dotted line) by thin-layer chromatography.
(D) CalMk2α level in P7 cortices (n = 3 mice) were determined as mean ± SEM.
(E) 4-Thiouridine (4sU)-labeled transcripts were biotinylated, examined by dot blotting, and isolated by streptavidin beads for qRT-PCR. The nascent mRNA level of Calm2α relative to that of β-actin is expressed as mean ± SEM (n = 3 experiments).
(F) Representative z stack images of YFP-expressing cortical layer 5 neurons. Scales, 100 μm.
(G) Total dendritic intersections, number, and length from both apical and basal dendrites are mean ± SEM from 20 cWT neurons and 16 cKO neurons (three mice per group).
(H) qRT-PCR. The levels of denoted mRNAs relative to Gapdh mRNA in cortices are mean ± SEM (four mice per group). Total RNAs isolated from enterovirus-71-infected spinal cord (SC) and Japanese-encephalitis-infected cortex (brain) were positive controls of innate immunity. "p < 0.05, "p < 0.01, ""p < 0.001; Student’s t test in (D), (E), and (H) and two-way ANOVA in (G). See also Figures S4–S6.
inhibited replication of influenza A virus (Li et al., 2020), which
snatches the 5’ end of host mRNAs for priming its own transcription
(Bouloy et al., 1980; Wakai et al., 2011). In contrast, CMTR1-KD
enhanced replication of Zika and dengue viruses in Huh7
cells due to IFIT-1-mediated translational repression to attenuate
IFN responses (Williams et al., 2020). Depending on the
type of virus, CMTR1 can have proviral or antiviral effects.

The cap1 structure is implicated in posttranscriptional gene
regulation (Kuge et al., 1998; Picard-Jean et al., 2018), but
decreased Camk2α mRNA in CMTR1-deficient neurons re
sulted from reduced transcription instead of a stability change
(Figures 4E and S3) or DXO-mediated degradation (Figure S5).
Two possible scenarios are that Camk2α transcription is regu
lated by a yet-to-be-uncovered transcription factor whose post
transcriptional expression depends on cap1 modification in a
DXO-independent manner or that Camk2α transcription de
pends on cap1 methylation because the nuclear cap-binding
complex (i.e., CBP80 and CBP20) is known to promote tran
scription elongation (Lenasi et al., 2011). Nuclear pre-mRNA
capping recruits the nuclear cap-binding complex, which then
associates with various factors to regulate transcription elonga
tion, pre-mRNA 3’-end processing, splicing, RNA decay, and
nuclear export. Because these interactions are conserved
from yeast to mammals, the cap0 structure alone is believed
to be sufficient to regulate gene expression at these stages
(Bentley, 2014; Gonatopoulos-Pournatzis and Cowling, 2014;
Jiao et al., 2013; Lenasi et al., 2011; Ramanathan et al., 2016).
Because cap1 is the dominant cap structure in mammals (Aki
chika et al., 2019; Furuichi et al., 1975; Wang et al., 2019),
previous studies in mammalian cells could not exclude the
contribution of cap1 in these nuclear processes to affect gene
expression.

Why the ubiquitous cap1 modification affects selective gene
expression is unclear. To our knowledge, only IFIT1 and DXO
exhibit preferential recognition for cap0 RNA instead of cap1
RNA in vitro binding and cleavage assays, respectively (Hab
jan et al., 2013; Kumar et al., 2014; Picard-Jean et al., 2018),
and RIG-I specifically binds to double-stranded cap0 RNA
in vitro (Devarkar et al., 2016). However, the expression of
IFIT1 in our microarray data is too low in uninfected neurons
(accumulation: GSE145223) to possibly repress translation of
cap0 mRNAs. We suspect that most cap-binding proteins,
such as eIF4E, with similar binding affinity to cap0 and cap1
in vitro (Haghjhat and Sonenberg, 1997; Niedzwiecka et al.,
2002), may exhibit a binding preference for one rather than the
other cap structure, which likely depends on its associated part
ners and perhaps the 5’-end sequence of transcripts. Other
players act in concert with the cap1 moiety to regulate Camk2α
expression requires further investigation.

CMTR1 binds RNA helicase DHX15 through its G-patch
domain. One study showed that such an interaction facilitates
cap1 methylation on oligoribonucleotides with structural 5’-
termini in vitro (Toczydlowska-Socha et al., 2018), but the other
study contradictorily suggested that DHX15 inhibits CMTR1’s
MTase activity in vitro (Inesta-Vaquera et al., 2018). Moreover,
overexpression of CMTR1 in HCC1806 cells enhanced transla
tion of a small subset of mRNAs involved in proliferation and
DNA damage response (Inesta-Vaquera et al., 2018). However,
this study did not measure whether CMTR1 overexpression
can further increase N1 2’-O-Me in poly(A) RNAs. Interestingly,
the CapQuant study detected ~10% and 3% transcripts
carrying the cap0 structure in CCRF-SB cells and mouse
liver, respectively (Wang et al., 2019), so the notion that
mammalian mRNAs are 100% cap1 modified needs to be
closely examined.

In summary, our data indicate that CMTR1-catalyzed N1 2’-O-
Me is an important epitranscriptomic signal for gene regulation,
dendritic morphogenesis, and brain development.

STAR METHODS

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NMDAR signaling facilitates the IPO5-mediated nuclear import of CPEB3. Chao, H.W., Lai, Y.T., Lu, Y.L., Lin, C.L., Mai, W., and Huang, Y.S. (2012). Daffis, S., Szretter, K.J., Schriewer, J., Li, J., Youn, S., Errett, J., Lin, T.Y., Chang, Y.W., and Huang, Y.S. (2014). Arsenite-activated JNK signaling enhances CPEB4-Vinexin interaction to facilitate stress granule assembly and cell survival. PLoS One 9, e107961. Haghighat, A., and Sonenberg, N. (1997). elf4G dramatically enhances the binding of elf4E to the mRNA 5’-cap structure. J. Biol. Chem. 272, 21677–21680. Hernández, G., Altmann, M., and Lasko, P. (2010). Origins and evolution of the mechanisms regulating translation initiation in eukaryotes. Trends Biochem. Sci. 35, 63–73.

REFERENCES

Akichika, S., Hirano, S., Shichino, Y., Suzuki, T., Nishimasu, H., Ishtiani, R., Suzuki, A., Hirose, Y., Iwashasi, S., Nureki, O., and Suzuki, T. (2019). Cap-specific terminal N'-methylation of RNA by an RNA polymerase II-associated methyltransferase. Science 363, eaav0080. Belanger, F., Stepinski, J., Darzykiewicz, E., and Pelletier, J. (2010). Characterization of MTR1, a human Cap1 2'-O-ribose methyltransferase. J. Biol. Chem. 285, 33037–33044.

Bentley, D.L. (2014). Coupling mRNA processing with transcription in time and space. Nat. Rev. Genet. 15, 163–175. Bouloy, M., Plotch, S.J., and Krug, R.M. (1989). Both the 7-methyl and the 2'-O-Methyl groups in the cap of mRNA strongly influence its ability to act as primer for influenza virus RNA transcription. Proc. Natl. Acad. Sci. USA 77, 3952–3956. Chang, Y.W., and Huang, Y.S. (2014). Arsenite-activated JNK signaling enhances CPEB4-Vinexin interaction to facilitate stress granule assembly and cell survival. PLoS One 9, e107961. Chao, H., Lai, Y.T., Lu, Y.L., Lin, C.L., Mai, W., and Huang, Y.S. (2012). NMDAR signaling facilitates the Ipo5-mediated nuclear import of CPEB3. Nucleic Acids Res. 40, 8484–8498. Cheng, H., Dufu, K., Lee, C.S., Hsu, J.L., Dias, A., and Reed, R. (2006). Human mRNA export machinery recruited to the 5’ end of mRNA. Cell 127, 1389–1400. Daffis, S., Szretter, K.J., Schriewer, J., Li, J., Youn, S., Errett, J., Lin, T.Y., Schneller, S., Züst, R., Dong, H., et al. (2010). 2'-O-Methylation of the viral mRNA cap evades host restriction by IFIT family members. Nature 468, 452–456.

Devarkar, S.C., Wang, C., Miller, M.T., Ramanathan, A., Jiang, F., Khan, A.G., Lenasi, T., Peterlin, B.M., and Barboric, M. (2011). Cap-binding protein complex in the regulation of arbor subregions. Cytometry A 85, 231–242.

Feng, G., Mellor, R.H., Bernstein, M., Keller, O.V., Nguyen, Q.T., Wallace, M., Nerbonne, J.M., Lichtman, J.W., and Sanes, J.R. (2000). Imaging neuronal morphology at multiple scales: Whole cell Sholl analysis versus Sholl analysis of arbor subregions. Cytometry A 40, 1160–1168.

Geiss, G.K., Carter, V.S., He, Y., Kwieciszewski, B.K., Holzman, T., Korth, M.J., Lazaro, C.A., Fausto, N., Bumgarner, R.E., and Katze, M.G. (2003). Gene expression profiling of the cellular transcriptional network regulated by alpha/beta interferon and its partial attenuation by the hepatitis C virus nonstructural 5A protein. J. Virol. 77, 6367–6375.

Gonatopoulos-Pournatzis, T., and Cowling, V.H. (2014). Cap-binding complex (CBC). Biochem. J. 457, 231–242.
CRISPR screen identifies host dependency factors for influenza A virus infection. Nat. Commun. 17, 164.

Loo, Y.M., Fornek, J., Crochet, N., Bajwa, G., Perwitasari, O., Martinez-Sobrido, L., Akira, S., Gill, M.A., Garcia-Sastre, A., Katze, M.G., and Gale, M., Jr. (2008). Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J. Virol. 82, 335–345.

Lu, W.H., Yeh, N.H., and Huang, Y.S. (2017). CPEB2 Activates GRASP1 mRNA Translation and Promotes AMPA Receptor Surface Expression, Long-Term Potentiation, and Memory. Cell Rep. 21, 1783–1794.

Moya-Alvarado, G., Gonzalez, A., Stuardo, N., and Bronfman, F.C. (2018). Brain-Derived Neurotrophic Factor (BDNF) Regulates Rab5-Positive Early Endosomes in Hippocampal Neurons to Induce Dendritic Branching. Front. Cell. Neurosci. 12, 493.

Nazmi, A., Dutta, K., and Basu, A. (2011). RIG-I mediates innate immune response in mouse neurons following Japanese encephalitis virus infection. PLoS One 6, e21761.

Niedzwiecka, A., Marcotrigiano, J., Stepinski, J., Jankowska-Anyszka, M., Wyslouch-Cieszynska, A., Dadlez, M., Gingras, A.C., Mak, P., Darzyynkiewicz, E., Sonenberg, N., et al. (2002). Biophysical studies of eIF4E cap-binding protein: recognition of mRNA 5’ cap structure and synthetic fragments of eIF4G and 4E-BP1 proteins. J. Mol. Biol. 319, 615–635.

Noack, F., and Calegari, F. (2018). Epitranscriptomics: A New Regulatory Mechanism of Brain Development and Function. Front. Neurosci. 12, 85.

O’Neill, E., Kwock, B., Day, J.S., Connor, T.J., and Harkin, A. (2016). Amritpallyne protects against TNF-alpha-induced atrophy and reduction in synaptic markers via a Trk-dependent mechanism. Pharmacol. Res. Perspect. 4, e00195.

Picard-Jean, F., Brand, C., Tremblay-Létourneau, M., Allaire, A., Beaudoin, M.C., Boudreault, S., Duval, C., Rainville-Sirois, J., Robert, F., Pelletier, J., et al. (2018). 2’-O-Methylation of the mRNA cap protects RNAs from decapping and degradation by DKO. PLoS One 13, e0193804.

Ramanathan, A., Robb, G.B., and Chan, S.H. (2016). mRNA capping: biological functions and applications. Nucleic Acids Res. 44, 7511–7526.

Reikine, S., Nguyen, J.B., and Modis, Y. (2014). Pattern Recognition and Signaling Mechanisms of RIG-I and MDA5. Front. Immunol. 5, 342.

Rodriguez, A., Ehlenberger, D.B., Dickstein, D.L., Hof, P.R., and Wearne, S.L. (2008). Automated three-dimensional detection and shape classification of dendritic spines from fluorescence microscopy images. PLoS One 3, e19977.

Roth-Cross, J.K., Bender, S.J., and Weiss, S.R. (2008). Ribose 2’-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. Nat. Immunol. 12, 137–143.

Sholl, D.A. (1953). Dendritic organization in the neurons of the visual and motor cortices of the cat. J. Anat. 87, 387–406.

Shuman, S. (2002). What messenger RNA capping tells us about eukaryotic evolution. Nat. Rev. Mol. Cell Biol. 3, 619–625.

Smietanski, M., Werner, M., Purta, E., Kamasinska, K.H., Stepinski, J., Darzyynkiewicz, E., Nowotny, M., and Bujnicki, J.M. (2014). Structural analysis of human 2’-O-ribose methyltransferases involved in mRNA cap structure formation. Nat. Commun. 5, 3004.

Su, A.I., Pezacki, J.P., Wedicka, L., Brideau, A.D., Supekoa, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R.H., Schultz, P.G., and Chisari, F.V. (2002). Genomic analysis of the host response to hepatitis C virus infection. Proc. Natl. Acad. Sci. USA 99, 15669–15674.

Toczyldowska-Socha, D., Zielinska, M.M., Kurkowska, M., Astha, Almeida, C.F., Stefaniak, F., Purta, E., and Bujnicki, J.M. (2018). Human RNA cap 1 methyltransferase CMTR1 cooperates with RNA helicase DHX15 to modify RNAs with highly structured 5’ termini. Philos. Trans. R. Soc. Lond. B Biol. Sci. 373, 20180161.

Tronche, F., Kellenndonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R., and Schütz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. 23, 99–103.

Wakai, C., Iwama, M., Mizumoto, K., and Nagata, K. (2011). Recognition of cap structure by influenza B virus RNA polymerase is less dependent on the methyl residue than recognition by influenza A virus polymerase. J. Virol. 85, 7504–7512.

Wang, J., Alvin Chew, B.L., Lai, Y., Dong, H., Xu, L., Balamkundu, S., Cai, W.M., Cui, L., Liu, C.F., Fu, X.Y., et al. (2019). Quantifying the RNA cap epitranscriptome reveals novel caps in cellular and viral RNA. Nucleic Acids Res. 47, e130.

Werner, M., Purta, E., Kamasinska, K.H., Cymerman, I.A., Campbell, D.A., Mittra, B., Zamudio, J.R., Sturm, N.R., Jaworski, J., and Bujnicki, J.M. (2011). 2’-O-ribose methylation of cap2 in human: function and evolution in a horizontally mobile family. Nucleic Acids Res. 39, 4756–4768.

Williams, G.D., Gokhale, N.S., Snider, D.L., and Horner, S.M. (2020). The mRNA Cap 2’-O-Methyltransferase CMTR1 Regulates the Expression of Certain Interferon-Stimulated Genes. MSphere 5, e00202-20.

Yamashaki, N., Maekawa, M., Kobayashi, K., Kajii, Y., Maeda, S., Somai, T., Takao, K., Tanda, K., Ohira, K., Toyama, K., et al. (2008). Alpha-CaMKII deficiency causes immature dentate gyrus, a novel candidate endophenotype of psychiatric disorders. Mol. Brain 1, 6.

Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A.H., Tanaseichuk, O., Benner, C., and Chanda, S.K. (2019). Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat. Commun. 10, 1523.
## STARS METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-α-tubulin (clone DM1A) | Sigma-Aldrich | Cat# T9026, RRID:AB_477593 |
| Mouse monoclonal anti-β-Actin (clone AC15) | Sigma-Aldrich | Cat# A5441, RRID:AB_476744 |
| Mouse monoclonal anti-CaMKII alpha (clone 6G9) | Thermo Fisher Scientific | Cat# MA1-048, RRID:AB_325403 |
| Rabbit polyclonal anti-KIAA0082 (CMTR1) | Bethyl | Cat# A300-304A, RRID:AB_309477 |
| Mouse monoclonal anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (clone 6C3) | Millipore | Cat# MAB374, RRID:AB_2107445 |
| Mouse monoclonal anti-Glia Fibrillary Acidic Protein (GFAP) (clone GA5) | Millipore | Cat# MAB360, RRID:AB_11212597 |
| Goat polyclonal anti-Histone H3 | Santa Cruz Biotechnology | Cat# sc-8654, RRID:AB_2118303 |
| Mouse monoclonal anti-lba1 (clone GT10312) | Thermo Fisher Scientific | Cat# MA5-27726, RRID:AB_2735228 |
| Mouse monoclonal anti-MAP2A,2B (clone AP20) | Millipore | Cat# MAB378, RRID:AB_11214935 |
| Chicken polyclonal anti-MAP2 | Novus | Cat# NB300-213, RRID:AB_2138178 |
| Mouse monoclonal anti-NeuN (clone A60) | Millipore | Cat# MAB377, RRID:AB_2298772 |
| Rabbit polyclonal anti-mCherry | Abcam | Cat# ab167453, RRID:AB_2571870 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| 4-Thiouridine | Sigma-Aldrich | T4509 |
| Actinomycin D | Sigma-Aldrich | A1410 |
| Cycloheximide | Sigma-Aldrich | 01810 |
| EZ-Link HPDP-Biotin | Thermo Scientific | 21341 |
| Puromycin dihydrochloride | Sigma-Aldrich | P8833 |
| **Critical Commercial Assays** | | |
| KAPA mouse genotyping kit | KAPA Biosystems | KR0385 |
| ViraPower Lentiviral Expression Systems | Invitrogen | K4950-00 |
| Light Cycler 480 Probes Master | Roche | 0487301001 |
| VECTASTAIN Elite ABC-Peroxidase Kit | Vector Laboratories | Cat# PK-7100, RRID:AB_2336827 |
| PolyATtract mRNA Isolation Systems | Promega | Z5210 |
| **Deposited Data** | | |
| Microarray data for transcriptomic analyses | This paper | NCBI GEO: GSE145223 |
| **Experimental Models: Cell Lines** | | |
| HEK293T | ATCC | CRL3216 |
| **Experimental Models: Organisms/Strains** | | |
| Mouse CMTR1f/f | This paper | N/A |
| Mouse RIG-I+/− | Dr. Shizuo Akira | N/A |
| Mouse MAVS+/− | Dr. Michael Gale | N/A |
| Mouse Thy1-YFP-H | Jackson Laboratory | #003782 |
| Mouse Tg-ActFLPe | Jackson Laboratory | #003800 |
| Mouse Nestin-Cre | Jackson Laboratory | #003771 |
| Mouse Emx1-IRES-Cre | Jackson Laboratory | #005628 |
| Pregnant Rat (E18.5) | Biosasco | N/A |
| **Oligonucleotides** | | |
| Primers for qPCR, see Table S2 | This paper | N/A |
| Primers for shRNA constructs, see Table S2 | This paper | N/A |
| Primers for genotyping, see Table S2 | This paper | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Yi-Shuian Huang (yishuian@bms.sinica.edu.tw).

Materials Availability
All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability
The accession number for the microarray data reported in this paper is NCBI GEO: GSE145223.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals and Genotyping
Experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Utilization Committee. Mice were housed under a 12-h light-dark cycle (lights on from 8 a.m. to 8 p.m.) in a temperature- and humidity-controlled room with ad libitum access to food and water. All efforts were made to minimize the number of animals used and their suffering. To produce CMTR1-conditional KO (cKO) mice, littermates from mating Cmtr1f/f, Emx1-Cre/+ or Cmtr1f/+, Nestin-Cre/+ females and Cmtr1f/f, +/+ male mice were used. RIG-I−/− mice were obtained from Dr. Fang Liao with permission from Dr. Shizuo Akira (Osaka University, Japan) (Kato et al., 2005) and MAVS−/− mice were from Dr. Sue-Jane Lin with permission from Dr. Michael Gale (University of Washington, USA) (Loo et al., 2008). Thy1-YFP-H (#003782), Tg-ActFLPe (#003800), Nestin-Cre (#003771) and Emx1-IRESCre (#005628) mice were obtained from the Jackson Laboratory. The genotypes were determined by PCR of tail biopsies and the KAPA mouse genotyping kit (KR0385, KAPA Biosystems) following the manufacturer’s protocol. The primer sequences are in Table S2.

Generation of Mice Carrying Floxed Cmtr1 (fCmtr1) Allele
For producing chimeric mice, 4-week-old C57BL/6 female mice were super-ovulated with an intraperitoneal injection of gonadotropin from pregnant mare serum (G4877, Sigma-Aldrich), then human chorionic gonadotropin (CG1063, Sigma-Aldrich) 46 h later.
Super-ovulated female mice were mated with C57BL/6 male mice to collect blastocysts. Two embryonic stem (ES) cell clones carrying KO-first with conditional potential of Cmtr1 allele were obtained from the European mouse mutant cell repository (EUMMCR) and microinjected into the cavity of blastocysts, which were recovered in M2 medium and then transferred into the uterus of pseudo-pregnant ICR female mice. Only one ES clone-derived founder produced germline-transmitted progenies. The mouse carrying the targeted Cmtr1 allele was first crossed with the Frt-LacZ-Neo-Frt cassette and generate the floxed Cmtr1 allele (fCmtr1). The resulting line was maintained as fCmtr1 mice and then crossed with Nestin-Cre or Emx1-Cre transgenic mice to derive conditional KO (cKO) mice.

**Primary Neuron Culture**

Rat cortices isolated from embryonic day (E) 18.5 brains were cut into small pieces, washed with Hank’s balanced salt solution (HBSS) to remove debris and then digested in papain solution (0.6 mg/ml papain, 0.6 mg/ml DNase I, 0.2 mg/ml L-cysteine, 1.5 mM CaCl₂ and 0.5 mM EDTA in HBSS) at 37°C for 20 min, followed by the addition of 10% horse serum in Neurobasal medium to stop the enzymatic reaction and 25-times trituration to obtain a cell suspension (Chao et al., 2012; Huang and Richter, 2007). To culture RIG-I (or MAVS) wild-type (WT) and KO neurons, cortices of E17.5 embryos from heterozygous matings were isolated and maintained individually in HBSS for ~2-3 h on ice before determining genotypes on tail biopsies (Lu et al., 2017). The WT and KO cerebral cortices were pooled and processed similarly for neuronal cultures (Chao et al., 2012; Huang and Richter, 2007). CMTR1-cWT and -cKO_KO cortical neurons were prepared in the same way by using E17.5 embryos isolated from Cmtr1<sup>f/f</sup>, Nestin-Cre/+ females crossed with Cmtr1<sup>1<sup>f</sup>/f<sup>, +/+ males. The cell density was 6 × 10<sup>4</sup> cells/well in a 12-well containing an 18-mm glass coverslip for immunostaining and morphological analysis and 10<sup>5</sup> cells/well in a 6-well plate for biochemical and molecular analysis. For coculture experiments, each well in a 12-well plate was deposited with 4-5 wax spots, coated with poly-L-lysine and then seeded with 2 × 10<sup>5</sup> cells. Cortical neurons cultured in Neurobasal medium with 0.5 mM glutamine, 12.5 μM glutamate, 1X antibiotic-antimycotic and B27 supplement until the designated days in vitro (DIV) were used for experiments.

**METHOD DETAILS**

**Lentivirus Production and Deltiviral Infection**

HEK293T cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, Lentivirus particles were generated by using the Virapower packaging system (Invitrogen). The mixture of 3 μg pLL3.7-Syn or pLKO plasmid and 9 μg Virapower DNA was mixed with 30 μl Lipofectamine 2000 (Invitrogen) and the DNA–liposome complex was transfected into 6 × 10<sup>6</sup> HEK293T cells overnight, then replaced with new medium, which was collected 2 days after and centrifuged at 120,000 g for 2 h at 4°C to pellet viral particles. The virues were resuspended in Neurobasal medium, aliquoted and stored at ~80°C. For the knockdown experiment, cortical neurons at DIV2 were incubated with the designated lentiviruses overnight and harvested at DIV7 for experiments. HEK293T cells were incubated with the lentivirus in the presence of polybreve overnight, replaced with new medium for one day, then selected with puromycin to obtain stably transformed CMTR1-KD cells as described previously (Chang and Huang, 2014).

**Plasmid Construction**

The oligonucleotides containing short hairpin RNA (shRNA) sequences, targeted against rat CMTR1 or mouse DXO mRNA as well as a non-target control (Table S2), were cloned into Hpal and Xhol linearized pLL3.7-Syn (Huang and Richter, 2007). The TRCN0000297447 (CGTTAAGTGGTCACCTCCCATT) shRNA clone against human CMTR1 was purchased from the RNAi Core Facility. To generate RFP-hCMTR1 fusion, the mCherry red fluorescent protein (RFP) plasmid (Chang and Huang, 2014) and human CMTR1 plasmid (OHu06786, GenScript) were PCRamplified in the presence of 4 primers, RFP_F, 5’-ATGCTGGCTAGCCACCATGGTGAAGCAAGG-3’, RFP-hTR1_R, 5’-TTCTGGGTCAGCTTCTCTTCATGTACCCTTGTGAAGCCTGCATGCGCC-3’, RFP-hTR1_F, 5’-GGAATTCGGGACGAGCTTCTACAAAGGTGACACCAGAAGAGGAGAAGCTGACCCAGAA-3’ and hTR1_R, 5’-ATTCCAGTTTTAACCTCGAGCCTCGATCTGGA-3’. The amplified fragment was digested with Nhel and Pmel, then cloned into pLL3.7-Syn in which the green fluorescent protein (GFP) coding region was replaced with the RFP-hCMTR1 sequence. The K239A and deletion mutants of CMTR1 were generated by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s protocol.

**Quantitative RT-PCR (RT-qPCR)**

Total RNA from cortical tissue and cells was extracted by using TRIzol reagent (Invitrogen) and analyzed by using the NanoDrop 1000 spectrophotometer (Thermo Fisher). Approximately 2 μg total RNA was incubated with RNase-free DNase for 30 min at 37°C, followed by phenol/chloroform extraction and ethanol precipitation. The purified DNA-free RNA samples were reverse-transcribed by using random primers and GoScript reverse transcriptase (Promega). The resulting cDNAs were analyzed by qPCR with the Universal Probe Library (UPL) reagent in the LightCycler 480 system (Roche). The relative expression of targets was calculated by the comparative threshold cycle value with Gapdh mRNA as the reference. The primer sequences and UPL probes designed at the Roche Assay Design Center are in Table S2.
Nucleocytoplasmic Fractionation and Western Blot Analysis
Cultured neurons were harvested in lysis buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM EDTA and 0.5% NP-40), incubated on ice for 10 min, then centrifuged at 800 xg for 5 min at 4°C. The supernatant was collected as the cytoplasmic fraction and the pellet was washed twice with the lysis buffer, then lysed in the buffer containing 20 mM HEPES, pH 7.5 and 400 mM KCl for 10 min on ice to collect the nuclear fraction. Tissues and cells were harvested in the lysis buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 5% glycerol, 0.5% Triton X-100, 0.1% SDS, 1 mM dithiothreitol and 1X protease inhibitor cocktail (Roche) and sonicated on ice for 20 s to break up chromosomal DNA (Misonix sonicator 3000), followed by centrifugation at 12,000 xg for 5 min at 4°C to collect supernatant. The protein concentration of collected supernatants, nuclear and cytosolic fractions was determined by use of the Pierce BCA Protein Assay Kit (Pierce), then diluted in Laemmli sample buffer. The protein samples were denatured at 95°C for 5 min, separated on 10% Tris-glycine SDS-polyacrylamide gels and transferred to 0.45 µm nitrocellulose membrane (GE Healthcare Life Science), which was incubated with the designated primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and detection with the Immobilon Western ECL system (Millipore). The chemiluminescence signals were captured by an ECL imaging system (LAS-4000, FUJINON).

Immunofluorescence Staining, Immunohistochemistry and Image Acquisition
All solutions were prepared in 1X phosphate buffered saline (PBS) and all procedures were conducted at room temperature except for primary antibody incubation at 4°C. Cultured neurons were fixed with 4% formaldehyde for 10 min, washed with PBS for 3 times and permeabilized in 0.2% Triton X-100 for 30 min. After 3 washes of PBS, permeabilized neurons were blocked in 10% horse serum for 10 min, then centrifuged at 800 xg for 5 min at 4°C to collect supernatant. The protein concentration of collected supernatants, nuclear and cytosolic fractions was determined by use of the Pierce BCA Protein Assay Kit (Pierce), then diluted in Laemmli sample buffer. The protein samples were denatured at 95°C for 5 min, separated on 10% Tris-glycine SDS-polyacrylamide gels and transferred to 0.45 µm nitrocellulose membrane (GE Healthcare Life Science), which was incubated with the designated primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and detection with the Immobilon Western ECL system (Millipore). The chemiluminescence signals were captured by an ECL imaging system (LAS-4000, FUJINON).

Sholl Analysis
Dendrite tracking and process connection were performed by using Neuro J (a plugin of ImageJ) (Schindelin et al., 2012) and Neuro-nStudio (Rodriguez et al., 2008), respectively. The data were then compiled and analyzed by using Bonfire (Langhammer et al., 2010) to count the number of dendritic intersections in every 10-µm segment away from the soma in concentric circles.

Microarray and Gene Ontology Analysis
Total RNA isolated from control and CMTR1-KD neurons at DIV7 (2 samples per group) were submitted to a commercial service (Wel-Microarray and Gene Ontology Analysis to count the number of dendritic intersections in every 10-µm segment away from the soma in concentric circles.

Thin Layer Chromatography (TLC) Detection of N1 2’-O-Me
Total RNA extracted from HEK293T cells and cortical tissues was purified by using the PolyATract mRNA Isolation System (Promega) to isolate poly(A) RNA, which was decapped and dephosphorylated by using Cap-Clip acid pyrophosphatase (CELLSCRIPT) and FastAP alkaline phosphatase (Thermo Fisher), respectively. The resulting 5’-OH RNA was radiolabeled with T4 polynucleotide kinase (Thermo Fisher) in the presence of [γ-<sup>32</sup>P] ATP, digested to mononucleotides with Nuclease P1 (Sigma Aldrich), then separated on a PEI cellulose F-coated TLC plate (Millipore) with isobutyric acid: 0.5M ammonium hydroxide (67:33) in the first dimension.
and isopropanol: HCl: H₂O (68:18:14) in the second dimension as modified from the previous protocol (Kruse et al., 2011). The radioactive image was acquired by using Typhoon 9410 Imager (GE Healthcare Life Science).

4-Thiouridine (4sU)-Labeling and Isolation of Nascent RNAs
Cultured neurons at DIV7 were incubated with 50 μM 4sU (T4509, Sigma-Aldrich) for 12 h prior to isolating total RNA with TRIzol (Invitrogen). The 4sU-incorporated nascent transcripts were biotinylated with 20 fmol EZ-LINK HPDP-Biotin (Thermo Fisher) per μg total RNA in 10 mM Tris pH 7.5 and 1 mM EDTA at 60°C for 3 h. The biotinylated samples were denatured at 65°C for 10 min and chilled on ice for 5 min. A part of reaction containing 1 μg total RNA was applied to nitrocellulose membrane. The membrane was UV (120,000 J/cm²)-crosslinked for 2 min, blocked with 3% BSA in PBS for 30 min and washed with PBS for 3 times, followed by 1-h incubation of horse peroxidase-conjugated streptavidin (PK6102, Vestastain) at room temperature. After 3 washes of PBS, the membrane was developed with the Immobilon Western ECL system (Millipore). The remained biotinylated samples were incubated with streptavidin paramagnetic beads (Z5481, Promega), which were pre-treated with 1% polyvinylpyrrolidone (P5288, Sigma-Aldrich) for 10 min to block non-specific binding. After 30-min incubation at room temperature, the beads were washed 5 times with 10 mM Tris pH 7.5, 1 mM EDTA and 1 M NaCl and then eluted with 100 mM dithiothreitol. The eluted RNAs were extracted with phenol/chloroform and precipitated with ethanol, followed by reverse transcription with oligo-dT primers and GoScript reverse transcriptase (Promega) and qPCR.

QUANTIFICATION AND STATISTICAL ANALYSIS
Data are expressed as mean ± SEM. GraphPad Prism software was used to evaluate statistical differences between groups. Single-factor comparisons were determined by two-tailed Student’s t test. Multivariate data were analyzed by two-way ANOVA with Tukey post hoc comparisons. Sample sizes and statistical methods are described in figure legends.
CMTR1-Catalyzed 2′-O-Ribose Methylation
Controls Neuronal Development by Regulating
Camk2α Expression Independent of RIG-I Signaling

Yen-Lurk Lee, Fan-Che Kung, Chia-Hsuan Lin, and Yi-Shuian Huang
Figure S1, related to Figure 1. CMTR1 Expression Pattern in Tissues and Neurons. (A) Western blot analysis of CMTR1 in tissues from 2-month-old C57BL/6 male mice. (B) Subcellular distribution of CMTR1. Cortical neurons at 7 days in vitro (DIV) were fractionated to collect cytoplasmic and nuclear lysates for immunoblotting of denoted proteins or fixed for labeling DAPI, CMTR1 and MAP2. Scale, 20 μm. Arrows denote CMTR1-positive non-neuronal cells (MAP2-negative).
Figure S2, related to Figures 1 and 2. Knockdown of CMTR1 in Cultured Neurons Affects Dendritic Ramification But Not the Expression of IFN Signaling-Related Genes. (A, B) Rat cortical neurons at DIV2 were infected with lentivirus without (siCTL) or with shRNA against rat CMTR1, siCM1#1 or siCM1#2 and harvested at DIV7 for immunoblotting. The protein levels of CMTR1 and MAP2 are expressed as a relative ratio to GAPDH in siCTL and siCM1 neurons. Data are mean ± SEM from 3 independent experiments. **P < 0.01, Student's t test. (C) Cortical neurons cultured on coverslips were processed similarly for immunostaining of MAP2 to outline dendritic processes. Scales, 50 μm. (D) Sholl analysis to count dendritic intersections in every 10-μm segment away from the soma. (E) The number of dendrites and total dendritic length in siCTL, siCM1-1 and siCM1-2 neurons was calculated and expressed as mean ± SEM (n = 60 neurons per group from 4 independent cultures). **P < 0.01, ***P < 0.001, two-way ANOVA. (F) RT-qPCR. The levels of denoted mRNAs relative to Gapdh mRNA in cortices are mean ± SEM from 3 independent experiments. ISG20, interferon stimulated gene 20; IRF3, interferon regulatory factor 3; Mx1, MX dynamin-like GTPase 1; CH25H, cholesterol 25 hydroxylase. ns, not significant, *P < 0.05, Student's t test.
Figure S3, related to Figure 3. CMTR1 Deficiency Affects Transcription but not Stability of *Camk2a* mRNA in Neurons. DIV2 neurons were infected with the lentivirus expressing siCTL or siCM1 ± RFP-tagged human CMTR1 (hCM1). (A) siCTL and siCM1 neurons at DIV7 were treated with 2 μg/ml actinomycin D (ActD) for the indicated time and then harvested for total RNA isolation. The RNA samples were used for RT-qPCR detection of *Camk2a* mRNA and 18S ribosomal RNA (rRNA). The normalized *Camk2a* levels relative to 18S rRNA expressed as a relative ratio to time 0 are mean ± SEM from 3 experiments. ns, not significant, two-way ANOVA. (B) Schematic diagram for nascent transcript measurement. siCTL and siCM1 neurons at DIV7 were treated ± 4-thiouridine (4sU) for 12 h and then harvested for total RNA isolation. The newly transcribed RNAs were biotinylated, captured by streptavidin-coated magnetic beads, eluted with dithiothreitol (DTT) and then used for RT-qPCR. (C) The normalized nascent *Camk2a* levels relative to nascent *Gapdh* are mean ± SEM from 3 experiments. *P < 0.05, Student's t test. No detectable RT-qPCR signal from the negative controls (i.e., siCTL and siCM1 neurons without 4sU labeling).
Figure S4, related to Figure 4. CMTR1-KO Mice Die before Gastrulation. (A) The Cmtr1 gene consists of 24 exons (numbered boxes) and spans a region of 42.6 kb. The MTase domain spans from exon 7 to exon 13. The critical catalytic residue, K239, is located in exon 9. The mice carrying the KO-first targeted Cmtr1 allele (zCmtr1, zKO) in which two loxP sites flank exon 9 of Cmtr1 gene and two Flp recombinase target (Frt) flank the LacZ reporter and neomycin-resistant gene (NeoR) cassette. The presence of this cassette results in splicing of exon 8 to the LacZ reporter instead of exon 9, so the zCmtr1 allele is a type of KO allele (zKO). This cassette was removed by crossing with a C57BL/6 mouse line expressing Flp recombinase driven by a ubiquitous β-actin promoter (actin-Flp). The resulting female progenies carrying the floxed Cmtr1 allele (fCmtr1 = cWT allele) were mated with C57BL/6 males containing a Cre recombinase transgene under the control of Nestin or Emx1 promoter to excise exon 9 in pan neuron progenitors or EMX1-expressing progenitor lineage, respectively. The asterisk denotes the premature termination codon in exon 10 of the KO/cKO allele. (B) No Cmtr1-zKO embryos were found in P18 and P20 litters. (C) Cmtr1-zKO embryos were mostly absorbed as early as at E8.5 and E9.5 stages, so only the remaining yolk sacs were collected for genotyping.
Figure S5, related to Figure 4. Knockdown of DXO Does Not Restore Camk2α mRNA Expression in CMTR1-KO Neurons. (A) Knockdown of DXO failed to restore CaMK2α expression in CMTR1-cKO<sup>Nes</sup> neurons. DIV2 neurons prepared from CMTR1-cWT and -cKO<sup>Nes</sup> mouse cortices were infected with the lentivirus expressing siCTL, siDOX#1, siDOX#2 or RFP-hCM1 and then harvested at DIV7 for RT-qPCR analysis. Camk2α and DXO mRNA levels relative to Gapdh are mean ± SEM from 3 independent experiments. *P < 0.05, ***P < 0.001, Student's t test. (B) Similar to (A), except the infected DIV7 neurons were harvested for western blotting. The quantified CaMK2α levels relative to GAPDH were presented in the bar graph.
Figure S6. related to Figure 4. Reduced Cortical and Hippocampal Areas in CMTR1-cKO<sup>Emx1</sup> Mice in the Absence of Microglial Activation. (A) Immunohistochemistry of CMTR1 in the brain (cortex and hippocampus) of P22 cWT and cKO<sup>Emx1</sup> mice. Scales, 500 μm. (B) Immunofluorescence staining of CMTR1 and Iba1 in the hippocampus of P22 cWT and cKO<sup>Emx1</sup> mice. The resting morphology of Iba1-positive microglia is shown in the magnified images. Iba1, ionized calcium-binding adapter molecule 1. Scales, 100 μm.