Age-related neurodegeneration and cognitive impairments of NRMT1 knockout mice are preceded by misregulation of RB and abnormal neural stem cell development

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INTRODUCTION

N-terminal methylation is an important posttranslational modification that regulates protein/DNA interactions and plays a role in many cellular processes, including DNA damage repair, mitosis, and transcriptional regulation. Our generation of a constitutive knockout mouse for the N-terminal methyltransferase NRMT1 demonstrated its loss results in severe developmental abnormalities and premature aging phenotypes. As premature aging is often accompanied by neurodegeneration, we more specifically examined how NRMT1 loss affects neural pathology and cognitive behaviors. Here we find that Nrmt1−/− mice exhibit postnatal enlargement of the lateral ventricles, age-dependent striatal and hippocampal neurodegeneration, memory impairments, and hyperactivity. These morphological and behavior abnormalities are preceded by alterations in neural stem cell (NSC) development. Early expansion and differentiation of the quiescent NSC pool in Nrmt1−/− mice is followed by its subsequent depletion and many of the resulting neurons remain in the cell cycle and ultimately undergo apoptosis. These cell cycle phenotypes are reminiscent to those seen with loss of the NRMT1 target retinoblastoma protein (RB). Accordingly, we find misregulation of RB phosphorylation and degradation in Nrmt1−/− mice, and significant de-repression of RB target genes involved in cell cycle. We also identify novel de-repression of Noxa, an RB target gene that promotes apoptosis. These data identify Na-methylation as a novel regulatory modification of RB transcriptional repression during neurogenesis and indicate that NRMT1 and RB work together to promote NSC quiescence and prevent neuronal apoptosis.

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Those that survive past 6 months exhibit phenotypes associated with premature aging [9]. As premature aging is often accompanied by neurodegeneration [10], we looked more specifically at how NRMT1 loss affects age-related neural pathology and cognitive behaviors. Here we find significant enlargement of the lateral ventricles in Nrmt1−/− mice, striatal and hippocampal neurodegeneration, and corresponding behavioral impairments. As the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus contain the postnatal neural stem cell (NSC) niches [11], we wanted to next determine whether Nrmt1−/− mice exhibit abnormal NSC development. We find that after an early expansion, Nrmt1−/− mice show premature depletion of the quiescent NSC pool, which is concurrent with an expansion of both the intermediate progenitor cell (IPC) and neuroblast pools, indicating the NSCs are undergoing differentiation. We also see an increase in the number of differentiated neurons remaining in the cell cycle and undergoing apoptosis. These NSC phenotypes are reminiscent of those seen with loss of the NRMT1 target RB protein [4, 12]. During normal...
neurogenesis, hypophosphorylated RB inhibits E2F-mediated transcription of cell cycle genes and enables both NSC quiescence and exit from the cell cycle upon terminal differentiation [13]. Phosphorylation of RB during the proliferative phases of neurogenesis releases this inhibition and promotes RB protein degradation and cell cycle entry [14]. Loss of RB results in an expansion of IPCs and neuroblasts within the niches [12, 15]. The resultant IPCs and neuroblasts have time- and context-specific cell cycle and/or differentiation defects, and many ultimately undergo apoptosis [15, 16].

To confirm misregulation of RB in Nrmt1−/− mice, we measured its overall protein and phosphorylation levels, as well as expression of its target genes. Nrmt1−/− mice exhibit an increase in RB phosphorylation and decrease in overall RB protein, similar to what is seen with RB inactivation [14]. We also see transcriptional de-repression of RB/E2F1 target genes that promote cell cycle entry and identify de-repression of Noxa, a target gene that promotes apoptosis [17]. We propose a model where Na-methylation of RB by NRMT1 promotes its activity as a transcriptional repressor. Loss of NRMT1 promotes RB phosphorylation and release from E2F1, resulting in premature activation of NSC proliferation and neuronal apoptosis. Combined, the depletion of the NSC pool and high rates of neuronal apoptosis would lead to an inability of the niches to replace neurons as they age or become damaged, leading to the observed neurodegenerative and behavioral phenotypes. Taken together, our data identify a new role for NRMT1 in NSC development and suggest its regulation of RB contributes to the maintenance of NSC quiescence and neuronal viability.

METHODS

Maintenance of Nrmt1−/− mice

Homozygous C57BL/6J- Nrmt1−/− mice used in the described experiments are generated and genotyped as previously described [9]. Both sexes of mice were used in the molecular studies, their results combined and averaged. The behavioral studies were done with only females, due to the high number of animals required and the need for most Nrmt1−/− males to maintain the breeding colony. A priori power analysis was used to predict that eight to ten mice/group will allow for a 20% change between measured variables to be statistically significant in behavioral studies. Mice not needed for experiments were killed by inhalation of carbon dioxide in a clear container followed by cervical dislocation. Mice needed for histological brain sections were killed by perfusion with 4% paraformaldehyde followed by 10% neutral buffered formalin for 72 h and then cryoprotected in a 30% sucrose solution for 1 week. Serial sections (30 µm) were cut on a Microm HM 525 cryostat (Thermo Fisher Scientific, Waltham, MA) and mounted on glass slides for immunohistochemistry. Sections were stored in 50% ethanol for 2 weeks before immunohistochemistry.

Barnes maze

The Barnes maze test was performed with minor modifications [18]. Briefly, animals were habituated for 5 min on a circular platform that contained eight equally spaced holes. After habituation, the animals were placed back on the platform for either two or ten training (learning) acquisition trials. During the training phase, an escape hole was present for the animal to enter. A bright light above the platform was used as a weak aversive stimulus to encourage the animal to escape during each training trial. Each animal had up to 3 min to navigate the platform and find the escape hole. Unique shapes and colors on four sides of the maze were used as spatial cues to help the animal locate the escape hole. If the animal was unable to locate the correct escape hole in the 3 min training trial, the animal was gently guided to the correct hole. The learning interval between each animal was 5 min. After two or ten training trials, a test trial was administered 15 min or 24 h after the last training trial, respectively. During the test trial, animals were placed back on the platform for 3 min where correct (T1) and incorrect (T2) hole exploration was measured. Spatial memory index was calculated by: (T1 − T2)/(T1 + T2).

Open-field and rotarod assays

For open-field assays, animals were placed in a transparent plastic cage (40 × 40 × 30 cm) where they were allowed to freely move around for 30 min. ANYMAZE tracking software (Stoelting, Wood Dale, IL) was used to measure total distance traveled and average speed. Quantification of 5 min bins was used in the analysis. For rotarod assays, animals were placed on an accelerating rotarod (SD Instruments, San Diego, CA) as previously described with minor adaptations [19]. The rotarod machine was programmed to accelerate from 4 revolutions per minute (RPM) to 40 RPMs over a 5 min trial. To prevent injury after falling, thick foam was used to cushion the rotarod arms. Two trials were run for each animal. The mouse was gently guided to the animal to the test. Ten minutes after the last practice session, two test trials were administered and latency to fall was measured. The average latency of fall between the two test trials was used for analysis.

QuPath volume analysis

To assess brain volume of specific regions, two sets of slides were created to represent the whole brain (eight sections per set, each spaced 300 µm apart). As a result, the first set of slides contained the rostral half and the second set covered the caudal half of the brain. One full set of cresyl violet stained slides for each animal was scanned using the Aperio ScanScope whole slide scanner (Leica Biosystems, Wetzlar, Germany) with the ×20 objective. Each digitized file was uploaded onto QuPath (version 0.1.2) where they were further processed. Each section containing the brain region of interest was manually traced using the polygon tool. The mouse brain atlas reference of the Allen Brain Atlas was used as a guide for a more accurate trace of the different brain regions. After each trace was completed, an area (µm²) given by QuPath was used to calculate the volume of each tissue section. To calculate the volume of a traced region, the formula: area of trace (µm²) × thickness of section (30 µm) = volume of traced area (µm³) was determined for each section and brain region. To calculate the total volume of a given region, the sum of all volumes calculated for that region were added to give the final volume.

Immunohistochemistry

Mice were anesthetized and transcardially perfused with 1% phosphate-buffered saline (PBS) following by 4% PFA before brain removal. Brains were post-fixed in 4% PFA for 72 h and then cryoprotected in a 30% sucrose solution for 1 week. Coronal slices (30 µm) were cut on a Microm HM 525 cryostat (Thermo Fisher Scientific, Waltham, MA) and attached to HM 525 cryostat slides (Thermo Fisher Scientific). All tissue was stored in −20°C until required. Sections were mounted in vectashield mounting media (Vector Laboratories, Burlington, Ontario). Slides were visualized using an Aperio ScanScope whole slide scanner (Leica Biosystems), a Cytation 5 Multi-Mode reader (BioTek, Winooksi, VT), a Leica DMi8 fluorescence microscope, or a Leica TCS SP8 spectral confocal. All images captured were at identical regions and exposures between genotypes. Quantification of pixel intensity was performed through ImageJ (NIH) software, where the region or cells of interest were traced and analyzed for mean pixel intensity. Cell counting was also done through ImageJ using the cell counter plugin. Data from medial levels of coronal sections is shown and used in quantifications.
RESULTS

Nrmt1−/− mice exhibit postnatal enlargement of the lateral ventricles and neurodegeneration

Nrmt1−/− mice present phenotypes associated with premature aging and phenocopy mouse models deficient in DNA damage repair [9]. As an impaired DNA damage response in neurons has been connected to the onset of age-related neurodegenerative disorders such as Alzheimer’s disease (AD) and amyotrophic lateral sclerosis [20], we sought to determine whether Nrmt1−/− mice exhibit any age-related neurological pathologies. We first stained whole-brain mounted sections of WT C57BL/6J mice and C57BL/6J-Nrmt1−/− mice with cresyl violet to look for gross morphological defects. In 14-day-old postnatal mice (P14), there were no apparent morphological differences between WT andNrmt1−/− brains (Fig. 1a). However, by 6 weeks, Nrmt1−/− mice showed a significant enlargement of the lateral ventricles (Fig. 1b, e). This increase became larger at 3 months (Fig. 1c, e) and persisted through 6 months (Fig. 1d, e).

Ventricle enlargement can be due to global changes in brain volume or come at the expense of neighboring regions of gray matter [21]. To determine which is occurring in Nrmt1−/− mice, volumes of the striatum, cortex, and total brain were calculated. Nrmt1−/− mice did not display global increases in brain volume at any age and overall volume actually decreased with age (Fig. 1f). At 6 weeks, when the ventricle volume increased significantly, overall brain volume did not differ and cortical volume also remained similar (Fig. 1f, g). However, the proportional volume of the striatum to total brain volume was significantly decreased (Fig. 1h), indicating the lateral ventricle enlargement seen in Nrmt1−/− mice corresponds to reduced striatal volumn. At both 3 and 6 months, Nrmt1−/− brains had significantly decreased overall volume (Fig. 1f). The proportional volume of theNrmt1−/− cortex at both these ages showed no difference from WT (Fig. 1g), but the proportional volume of the Nrmt1−/− striatum remained significantly different from WT at both 3 and 6 months (Fig. 1h). These data indicate that the lateral ventricle enlargement seen in Nrmt1−/− mice occurs concurrently with a decrease in striatal volume.

We next used whole-brain mounted sections to examine the general morphology of the hippocampus at P14, 6 weeks, 3 months, and 6 months. The hippocampal circuit comprises four main regions, the three Cornu Ammonis subfields (CA1, CA2, and CA3) and the dentate gyrus (DG). At P14 and 6 weeks, no obvious morphological differences were observed between Nrmt1−/− and WT mice (Fig. 2a, b). However, at 3 months, the volumes of the CA3 and DG are significantly decreased in Nrmt1−/− mice, indicating neuronal loss (Fig. 2c, e, f). By 6 months, the volumes of the CA3 and DG are even further decreased, indicating that significant neurodegeneration has occurred by this age (Fig. 2d, e, f). Together, these data show that Nrmt1−/− mice exhibit both abnormal developmental morphologies and age-related neurodegeneration.

Behavioral analysis of Nrmt1−/− mice

Enlargement of the lateral ventricles and/or neurodegeneration of specific brain regions are common pathologies seen in many types of brain disorders, including schizophrenia, AD, Parkinson’s disease, and Huntington’s disease [22–25]. Many of these disorders are associated with memory or motor impairments. As the hippocampus contributes to many aspects of short-term, long-term, and spatial memory [26–28], and the striatum is the major input for voluntary motor control [29], we wanted to see whether Nrmt1−/− mice similarly exhibited memory or motor impairments. First, we used a two-trial Barnes maze test to assess short-term spatial memory. At 6 weeks and 3 months, Nrmt1−/− mice spent significantly less time exploring the correct hole than WT controls during the test trials (Fig. 3a, b). Furthermore, by 6 months, Nrmt1−/− mice spent significantly more time at the incorrect holes than the correct hole (Fig. 3c). To summarize the test trial data, spatial memory indices were calculated for all ages. A negative spatial memory index indicates the animal did not remember the escape hatch during the test trial. At all ages, Nrmt1−/− mice exhibited negative spatial memory indices that were significantly decreased from WT controls (Fig. 3d), indicating Nrmt1−/− mice have short-term spatial memory deficits.

To rule out the possibility that these animals have learning impairments rather than memory deficits, a ten-training trial Barnes maze test was performed and this test also showed significantly decreased spatial memory indices in Nrmt1−/− mice at all ages (Fig. 3e). First, mice were given ten 3 min training periods to learn the correct escape hatch from the maze. Although the Nrmt1−/− mice progressed somewhat slower, by the tenth trial they showed no significant difference from control animals in their ability to locate the escape hole at any age (Fig. 3f–h), indicating
they could learn the task. Twenty-four hours after the last training trial, the escape hatch was removed and exploration time at the correct and incorrect holes was measured. At 6 weeks and 3 months, Nrmt1−/− mice spent significantly less time exploring the correct hole than WT controls (Fig. 3i, j), and at 6 months, Nrmt1−/− mice spent significantly more time at the incorrect holes than the correct hole (Fig. 3k), indicating Nrmt1−/− mice also have impaired long-term memory.

The inability of Nrmt1−/− mice to locate and explore the correct hole in the Barnes maze could also result from impaired motor activity. To test motor performance in Nrmt1−/− mice, rotarod tests were performed. Mice were placed on an accelerating rotarod for three training trials, followed by two test trials. The amount of time each animal stayed on the rotarod before falling (latency to fall) was measured. There was no significant difference in the latency to fall between Nrmt1−/− and WT mice at 6 weeks or 3 months (Fig. 3l). At 6 months, there was a small, yet significant, decrease in motor performance in the Nrmt1−/− mice (Fig. 3l).

However, this small difference at 6 months on the rotarod cannot account for the impaired performance of Nrmt1−/− mice at earlier ages in the Barnes maze. To confirm these results, we also performed open-field assays to test the motor activity of Nrmt1−/− mice. Surprisingly, at all ages, Nrmt1−/− mice moved at a significantly higher average speed and traveled significantly longer distances than WT controls (Fig. 3m–p), indicating that Nrmt1−/− mice do not have any significant motor impairments, although they do exhibit locomotor hyperactivity. This hyperactive phenotype could result from selective death of dopamine D2 receptors in the striatum, which are responsible for inhibition of movement [30, 31].

Molecular analysis of the NSC niches

The morphological changes seen in the lateral ventricles and hippocampus, and the corresponding behavioral phenotypes, suggested a potential misregulation of adult NSC neurogenesis, as the two postnatal NSC niches are found in the SVZ of the lateral
ventricles and SGZ of the DG [11]. The SVZ contains radial glia-like stem cells (RGLs) that produce both the ependymal cells (E1 cells) that line the ventricle and NSCs [32]. Increased proliferation of RGLs has been shown to sustain ventricle enlargement in mouse model of hydrocephalus [33]. To determine whether there was an expansion of the RGL population in Nrmt1−/− mice, we stained ventricular sections of P14 mice with antibodies against GFAP (a marker of both RGLs and astrocytes). Surprisingly, GFAP staining in ventricular sections of P14 mice with antibodies against GFAP (a marker of both RGLs and astrocytes) in Nrmt1−/− mice is significantly different (Fig. 4c, d), indicating abnormal proliferation is not occurring at the IPC stage. As the RGLs in the Nrmt1−/− mice could have prematurely begun differentiation, we also stained ventricle sections of P14 mice with antibodies against SOX2, a marker of both RGLs and IPCs. We found a significant increase in SOX2-positive (SOX2+) cells in and around the SVZ in Nrmt1−/− mice as compared to WT (Fig. 4c, d). As GFAP is a marker for RGLs and SOX2 marks both RGLs and IPCs [34], the decrease in GFAP staining and increase in SOX2+ cells in Nrmt1−/− mice indicate a shift from normal basal neurogenic activity to an aberrant expansion of IPCs. However, <2% of the SOX2+ cells co-stained with the proliferative marker Ki-67 and this was not significantly different from WT mice, indicating abnormal proliferation is not occurring at the IPC stage (Fig. 4c, d). To determine whether the differentiation of these NSCs is proceeding past the IPC stage to form neuroblasts, we performed immunostaining for the neuroblast marker DCX. There was a significant increase in DCX staining in the ventricle of Nrmt1−/− mice (Fig. 4e, f), but the Ki-67/DCX ratio was not significantly different (Fig. 4f), indicating the neuroblast population is also not abnormally proliferative. Taken together, these data indicate expansion of both the IPC and neuroblast pools in the SVZ of Nrmt1−/− mice without increased proliferation at these stages. Similar NSC phenotypes were seen in the SGZ (Supplemental Fig. 1).

The lack of increased proliferation in the SOX2+ and DCX+ populations suggests that proliferative expansion of the NSCs came before these stages of differentiation. At P14, we see a decrease in the GFAP staining in the SVZ of Nrmt1−/− mice (Fig. 4b), indicating the RGLs have already begun differentiation and an earlier expansion of GFAP+ cells must have occurred. We immunostained brain sections from WT and Nrmt1−/− mice from 0-day-old postnatal mice (P0) with GFAP and found a significant increase in GFAP intensity in Nrmt1−/− mice (Fig. 5a, c), indicating abnormal expansion of the RGL pool. However, despite the high GFAP staining and increased width of the SVZ (Fig. 5d), the Ki-67/GFAP ratio is significantly lower in Nrmt1−/− mice (Fig. 5e), indicating the proliferative expansion of GFAP+ cells must be occurring embryonically.

To see whether the RGL depletion at P14 is maintained as animals age, we also immunostained brain sections from 3- and 6-month-old mice. At 3 months, there is still a significant decrease in GFAP intensity in Nrmt1−/− mice (Fig. 5b, c), but by 6 months the GFAP intensity in WT mice decreases to that seen in the knockouts (Fig. 5c and Supplemental Fig. 2), indicating the GFAP+ population normally decreases with age but does so prematurely in Nrmt1−/− mice. There is also very little overlap between GFAP and Ki-67 staining in the SVZ of either WT or Nrmt1−/− mice at 3 or 6 months, indicating proliferation of this population has mostly ceased by these ages (Fig. 5b and Supplemental Fig. 2). Taken together, these data indicate expansion...
Fig. 3  

Nrmt1−/− mice exhibit short- and long-term memory impairment and hyperactivity. Two-trial Barnes maze spatial memory tasks show that Nrmt1−/− mice spend significantly less time exploring the correct hole than WT mice at a 6 weeks and b 3 months. c At 6 months, Nrmt1−/− mice spend significantly more time at the incorrect holes. d The spatial memory indexes (SMI) calculated from the two-trial tasks indicate short-term memory loss at all three ages. SMI = (time exploring correct hole – time exploring incorrect hole)/total exploration time). e The SMIs calculated from the ten-trial tasks also indicate long-term memory loss at all three ages. Although Nrmt1−/− mice are able to learn the correct hole when given ten training trials at f 6 weeks, g 3 months, and h 6 months, the ten-trial Barnes maze also shows Nrmt1−/− mice spend significantly less time exploring the correct hole at i 6 weeks and j 3 months, and significantly more time at incorrect holes at k 6 months. l Rotarod assays show a small motor impairment in Nrmt1−/− mice at 6 months. Open-field assays show Nrmt1−/− mice travel at m higher average speeds for n–p longer distances than WT mice. *p < 0.05, **p < 0.005, ***p < 0.0005, and ****p < 0.0001 as determined by a–c, i–k two-way ANOVA or d–h, l–p unpaired t-test, n = 6–10. Error bars represent mean ± SEM.
Fig. 4  At P14, Nrmt1−/− mice exhibit an expansion of the IPC and neuroblasts pools in the SVZ. a, b GFAP staining is significantly decreased in the SVZ (arrows) of Nrmt1−/− mice. However, there is a significant increase in c, d SOX2-positive IPCs and e, f Doublecortin (DCX)-positive neuroblasts in Nrmt1−/− mice. Neither the d percentage of SOX2, Ki-67 double-positive cells, or the f ratio of Ki-67/DCX intensity in Nrmt1−/− mice is significantly different from WT, indicating proliferation is not occurring at these stages. *p < 0.05 and **p < 0.005 as determined by unpaired t-test, n = 3. Error bars represent mean ± SEM. Scale bar = 1000 μm.
Fig. 5 An early expansion of the RGL pool in the SVZ of Nrmt1−/− mice is followed by premature depletion. a, c At P0, there is a significant increase in GFAP staining in the SVZ (brackets) of Nrmt1−/− mice. b, c At 3 months, GFAP staining is significantly decreased in the SVZ (arrows) of Nrmt1−/− mice, indicating the early expanded GFAP+ pool has become depleted. d, e Although the width of the SVZ and intensity of GFAP staining are significantly higher in Nrmt1−/− mice at P0, the ratio of Ki-67/GFAP is lower than WT, indicating the GFAP+ cells are not undergoing higher rates of proliferation in Nrmt1−/− mice. **p < 0.005, ***p < 0.0005, and ****p < 0.0001 as determined by unpaired t-test, n = 3–4. Error bars represent mean ± SEM. Scale bar = 1000 μm.
abnormal embryonic proliferation of the GFAP+ population in \( N_{rmt1}^{-/-} \) mice, followed by premature entry into the differentiation pathway before P14. This results in a postnatal expansion of both the IPC and neuroblast pools, and a depletion of the GFAP+ RGL population that persists throughout aging.

**Functional analysis of RB protein in \( N_{rmt1}^{-/-} \) mice**

The expansion of IPC and neuroblast populations seen in \( N_{rmt1}^{-/-} \) mice was extremely reminiscent of phenotypes seen in mice deficient for the NRMT1 target protein RB [12–16]. Also seen with RB loss in neuronal lineages is abnormal cell cycle regulation and apoptosis [13, 15]. To determine whether neurons in \( N_{rmt1}^{-/-} \) mice exhibited abnormal cell cycle activity, we co-stained ventricle/striatal sections from P14 mice with NeuN (a marker of mature neurons) and Ki-67. At P14, Ki-67 staining was restricted to the SVZ in WT mice and no mature neurons in the striatum co-stained with Ki-67 (Fig. 6a). However, in \( N_{rmt1}^{-/-} \) mice, Ki-67+ cells were also present in the striatum and there were significantly more NeuN/Ki-67 double-positive cells (Fig. 6a). In total, over 10% of NeuN+ cells co-stained with Ki-67 in \( N_{rmt1}^{-/-} \) mice (Fig. 6a). These data indicate loss of NRMT1, similar to loss of RB, results in mature neurons that have abnormal cell cycle regulation. To see whether there is also increased apoptosis in the striatum of \( N_{rmt1}^{-/-} \) mice, we stained ventricle/striatal sections with cleaved caspase-3, a marker of apoptosis. Punctate cleaved caspase-3 staining (green) appears in \( N_{rmt1}^{-/-} \) mice by 6 weeks and this coincides with the loss of NeuN (gray) and Ki-67 (red) double-positive neurons (pink) in the striatum. **** \( p < 0.0001 \) as determined by unpaired \( t \)-test, \( n = 4 \). Error bars represent mean \( \pm \) SEM. Scale bar = 1000 \( \mu \)m.

**Fig. 6** Neurons in \( N_{rmt1}^{-/-} \) mice remain in the cell cycle and ultimately undergo apoptosis. a At P14, Ki-67 staining (red) is restricted to the SVZ (arrow) in WT mice. In \( N_{rmt1}^{-/-} \) mice, Ki-67+ cells are also found in the neighboring striatum and significantly more of these Ki-67+ cells co-stain (yellow) with NeuN (green), a marker of mature neurons. Over 10% of NeuN-positive cells co-stain with Ki-67. b Punctate cleaved caspase-3 staining (green) appears in \( N_{rmt1}^{-/-} \) mice by 6 weeks and this coincides with the loss of NeuN (gray) and Ki-67 (red) double-positive neurons (pink) in the striatum. **** \( p < 0.0001 \) as determined by unpaired \( t \)-test, \( n = 4 \). Error bars represent mean \( \pm \) SEM. Scale bar = 1000 \( \mu \)m.
sections for the apoptotic marker cleaved caspase-3. Very little cleaved caspase-3 staining is visible at P14, 3 weeks, or 4 weeks in WT or Nrmt1−/− mice (data not shown). However, at 6 weeks, although cleaved caspase-3 staining remains low in WT mice, it increases in the Nrmt1−/− mouse and this increase corresponds to a decrease in Ki-67/NeuN double-positive cells in the striatum (Fig. 6b). These data indicate that, as with RB deficiency, neurons in Nrmt1−/− mice abnormally remain in the cell cycle and ultimately undergo a delayed apoptosis.

RB normally acts to inhibit the cell cycle through its repression of E2F-mediated transcription of cell cycle regulatory genes, including many cyclins, cell division genes, and even E2f1 itself [35–37]. To monitor whether RB function is disrupted in Nrmt1−/− mice, we looked at mRNA expression of cyclin A2, cyclin E2, and E2f1. Consistent with expression patterns found with RB deficiency [13, 38], we found that expression of all three cell cycle genes is increased in Nrmt1−/− mice (Fig. 7a). We then used Western blots to determine whether this transcriptional de-repression of RB target genes corresponded to changes in RB phosphorylation or protein levels. In Nrmt1−/− mice, phosphorylation of RB at Ser795 significantly increases, whereas overall RB protein levels significantly decrease (Fig. 7b), indicating that loss of N- methyltransferase triggers the release of RB from E2F proteins and its subsequent degradation [14, 35].

It has been hypothesized that the neuronal apoptosis seen with RB loss is triggered by transcriptional de-repression of the RB/E2F target genes Puma and Apaf1, but increased expression of these genes was not detected in RB-deficient neurons grown in culture [13]. Similarly, there was no detectable difference in Puma levels between WT and Nrmt1−/− ventricle/striatal samples (Fig. 7c), and Apaf1 transcripts were not detectable in either genotype (data not shown). However, RB/E2F also transcriptionally regulate expression of the apoptotic driver Noxa [39]. Although p53 upregulated modulator of apoptosis (PUMA) is a direct activator of BAX-induced apoptosis and NOXA is an inhibitor of the anti-apoptotic protein MCL1 [40], they have both been shown to differentially participate in dual pathways of p53-mediated apoptosis induction [41]. Interestingly, we do see a significant increase in Noxa expression in Nrmt1−/− mice (Fig. 7c), indicating PUMA and NOXA may also play differential roles in RB-mediated apoptosis induction. Taken together, our data suggest that misregulation of RB-dependent cell cycle arrest in NSCs contributes to the morphological and behavior phenotypes seen in Nrmt1−/− mice.

**DISCUSSION**

Postnatal neurogenesis promotes plasticity and provides a source for replacement of cells lost to turnover or injury [42–44]. Here we show for the first time that loss of NRMT1 results in early expansion and differentiation of quiescent NSCs, which ultimately depletes the postnatal NSC pool. This is followed by morphological and behavioral abnormalities, and ultimately striatal and hippocampal neurodegeneration. We propose that misregulation of RB in Nrmt1−/− mice contributes to these phenotypes by promoting both abnormal entry of quiescent NSCs into the cell cycle and apoptosis of terminally differentiated neurons that cannot completely withdraw from the cell cycle. We also propose that other brain regions, such as the cortex, are less affected by loss of NRMT1, because they are less reliant on NSCs from the SVZ for postnatal neurogenesis [45, 46].

NRMT1 (also known as METTL11A) is a member of the Methyltransferase like (METTL) family of methyltransferases, and although we show the first identified role for NRMT1 in NSC fate determination, there is an abundance of recent data showing roles for different members of the METTL family in stem cell development. The N6-methyladenosine (m6A) methyltransferases METTL3 and METTL14 regulate the self-renewal of adult hematopoietic stem cells by promoting the expression of genes that

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**Fig. 7** Nrmt1−/− mice show misregulation of RB function. a mRNA levels of RB target genes cyclin A2, cyclin E2, and E2f1 are significantly increased in Nrmt1−/− mice. b In addition, RB phosphorylation at serine 795 (p-RB Ser795) significantly increases but overall RB protein levels significantly decrease. Quantification of Western blots shown at the right. All samples normalized to tubulin loading control. c qRT-PCR analysis indicates apoptosis is not driven through increased expression of the RB target Puma but instead by the RB target Noxa. **p < 0.005 and ***p < 0.0005 as determined by unpaired t-test, n = 3–4. Error bars represent mean ± SEM.
maintain quiescence [47]. The more recently discovered m^A methyltransferase METTL5 is needed for embryonic stem cell pluripotency [48]. Its loss results in decreased global translation rates and lowered differentiation potential [48]. METTL17, a mitochondrial RNA methyltransferase, regulates the stability of 125 nt-rRNA and its associated proteins, and is required for differentiation of mouse embryonic stem cells [49]. The closest sequence homolog to NRM1, NRM2 (also known as METTL11B), is an N-naomonomethylase, and its transcript levels have been shown to increase during osteocytic and myogenic differentiation [50]. We have seen a similar upregulation of NRM1 transcript levels during myogenic differentiation [51].

Similar to other METTL family members, our data indicate that NRM1 normally acts to constrain cell cycle activity. We propose it does this through its activation of RB transcriptional repression. As Na-methylation regulates DNA/protein interactions, our model posits that loss of NRM1 disrupts the interaction of the N-terminal RB tail with DNA at target promoters and alters the conformation of the RB N-terminal domain (RbN). When RbN interacts with the phosphorylated RB interdomain linker (RbIDL), they are able to bind the RB pocket domain and displace E2F [52]. We propose loss of Na-methylation of the RB tail promotes interaction between RbN and RbIDL, and displacement of RB from E2F. This leads to increased phosphorylation and degradation of RB and subsequent activation of E2F target genes that promote cell cycle.

We have shown the first role for NRM1 in neural development, neurodegeneration, and NSC regulation. However, the phenotypes described here result from constitutive NRM1 loss and subsequent restoration, respectively, stimulate NSC proliferation and allow withdrawal of neurons from the cell cycle. During both neurodegenerative diseases and stroke, the burden of neuronal loss and subsequent activation of E2F target genes that promote cell cycle.

DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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AUTHOR CONTRIBUTIONS

JC performed the majority of experiments and analyzed the data. LM assisted with the immunofluorescence studies and BR assisted with the behavioral assays. MLF oversaw LM, ZY oversaw BR, and CST oversaw JC. JC and CST conceptualized the study and wrote the paper.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Animal experiments in these studies were approved by the SUNY Buffalo Animal Care and Use Committee.

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