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G9a is Required for Normal Cerebral Cortical Development

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G9a is Required for the Normal Cerebral Cortical Development

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And of-course thanks to my mom, without whom I would not be finishing my second Master’s. I feel very lucky to have you all in my life.

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INTRODUCTION/BACKGROUND

Mammalian cortical development

The cerebral cortex is a highly organized structure comprised of different types of neurons and diverse range of glia. Layer-specific neurons are produced earlier during development in an inside-out manner followed by the glial cells, as the complexity of the mammalian brain is sequentially established through a multistep process. The development of this complex brain architecture however begins from a common pool of neural precursor cells, where the differentiation potential of the cells progressively reduces or the cell acquires different fate potential during various stages of development (Molyneaux et al., 2007). This is accomplished by maintaining the expression of certain genes in an active state and/or by maintaining other genes in a repressed state, in the presence or absence of extracellular signaling molecules. Recent studies has shown that this could be achieved by some modifications, affecting either DNA or histone proteins, altering the state of chromatin and thereby making the DNA more or less accessible to the various transcription factors (Hirabayashi et al., 2010). Scientists have coined a term for these modifications as “epigenetic modifications”.

Epigenetic modifications

Epigenetic modifications are of three types: DNA methylation, Histone modifications and non-coding RNA mediated regulations, where the former two modifications has been widely studied in relation to cell fate determination and cell differentiation. Genome-wide profiling studies of pluripotent stem cells and differentiated adult cells shows that chromatin remodeling occurs throughout differentiation and plays a major role in determining the state of a cell at any given time during differentiation (Chen et al., 2014). This is mainly accomplished by establishing a transient repression or a long-term repression on the developmental genes, depending on the stages of development (Hirabayashi et al., 2010). Earlier during development, transient/reversible repression helps to keep several genes in a poised state until appropriate signals initiate their differentiation towards different lineages. However, long-term/permanent repression of developmental genes comes into play once the cell lineage has been defined and restricts their differentiation potential to alternative lineages by permanently repressing a set of genes, which are generally expressed in alternative lineages. These repressions are normally epigenetic and the epigenetic mechanisms that are mainly associated with repression are DNA methylation and histone methylation (Kouzarides, 2007).

Histone methylation

Nucleosomes are the fundamental unit of chromatin, consisting of an octamer of histone core proteins around which the DNA is wrapped. The part of these core proteins, which is of great importance, is the N-terminal “tail” as they possess

large number of residues that can be modified in eight distinct ways (Kouzarides, 2007). These modifications dictate the assembly and package of chromatin, which in turn determines their interactions with other non-histone proteins and thereby influencing different biological processes such as DNA repair, transcription, replication and gene expressions (Kouzarides, 2007). Of these modifications, methylation is considered to be the most characterized modification and methyltransferase enzyme is the most specific along with the kinases, amongst all the histone modifying enzymes. Histone methyltransferases catalyze the transfer of a methyl group/groups to either an arginine or a lysine residue, thereby activating or repressing the transcription depending on the residues being methylated. The first mammalian histone lysine methyltransferase(HKMT) to be identified was Suv39h1, known to target H3K9, contained the Su(var)3,9-Enhancer of zeste-Trithorax (SET) domain, the catalytic domain for lysine methylation (Rea et al., 2000). This discovery led to the identification of several other HKMTs in various species. Among the other methyltransferases, identification of G9a and GLP is considered to be of biological significance.

Role of HKMTs in neurogenesis

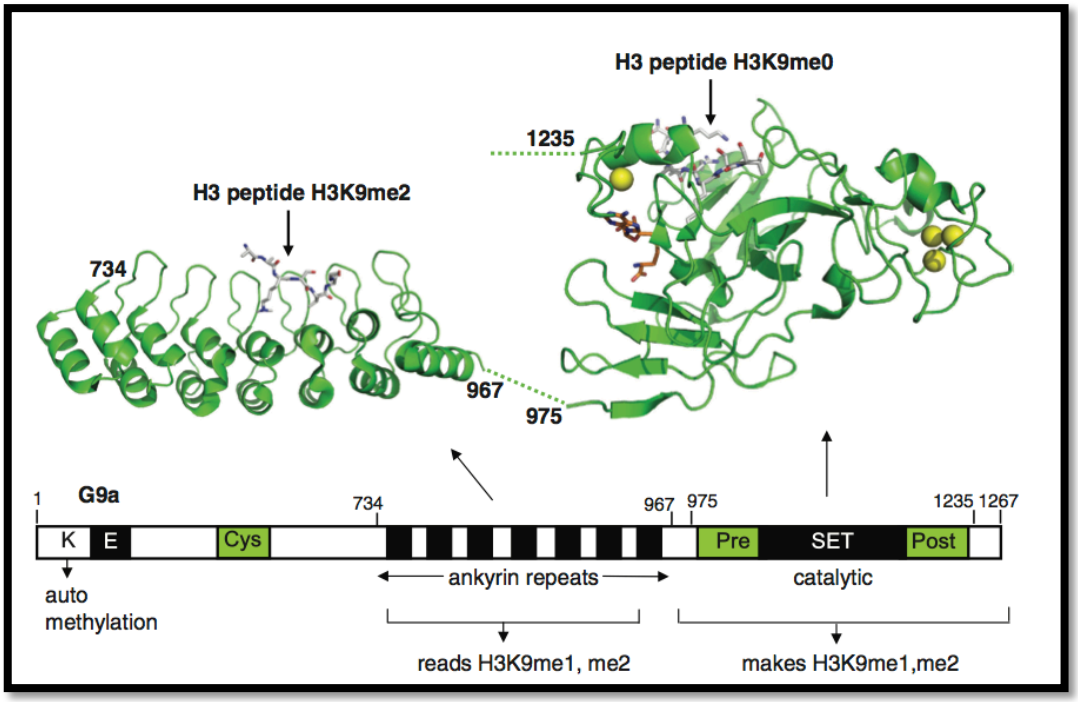
Studies in mice have demonstrated the significant role played by the HKMTs in the process of neurogenesis. Ezh2, enhancer of zeste homolog 2, is associated with gene repression by catalyzing the trimethylation of lysine residue 27 in the tail of histone H3. Deletion of Ezh2 before the onset of cortical neurogenesis

resulted in disrupting the balance between self-renewal and differentiation, by switching the balance towards differentiation, where neurons and basal progenitors were produced at the expense of self-renewal. The deletion also affected the switch between gliogenesis and neurogenesis by reducing the time window in which the neurons were generated and resulted in reduced production of neurons (Pereira et al., 2010). Similarly another study showed that, deletion of Setdb1, a histone trimethyltransferase, methylating lysine 9 of histone H3, in the mouse forebrain resulted in smaller cortex and increased production of deeper layer neurons at the expense of upper layer neurons. This proved that deletion of Setdb1 is essential for early neurogenesis and it regulates neurogenesis by repressing the expression of non-neuronal genes during development (Tan et al., 2012).

G9a/GLP

G9a/ Eu-HMTase2 and GLP/Eu-HMTase1 are members of the Suv39h subgroup of SET-domain containing molecules, which are known to form a heteromeric complex via their SET domain that methylates the H3K9 residue in vivo (Collins et al., 2010). Studies demonstrate that the mammalian Suv39h1 acts as a crucial lysine methyltransferase for H3K9me3 on pericentromeric heterochromatin region (Rea et al.,2000), however G9a is the only mammalian H3K9me1 and H3K9me2 HKMT known to establish transcriptional silencing in the euchromatic regions and is also known to methylate H1 histone protein and other non-histone proteins (Tachibana et al., 2001). G9a is an enzyme, which has separate

domains for “reading” and “writing” the methylation marks. It “reads” via its ankyrin repeat domain, through which it can interact and bind to the H3K9me1 and H3k9me2 marks and “writes” through it SET domain (Collins et al., 2008) (Fig i).



(i) **Domain organization of G9a components.** G9a consists of an automethylation site in the N-terminal domain, the ankyrin repeat domain (binds to H3K9me1 and H3K9me2) and a catalytic SET domain involved in mono and dimethylation of H3K9 histone residue and binds to GLP protein (adapted from Collins et al., 2010)

Recent studies have demonstrated the pivotal role played by G9a in diverse mammalian biological processes. G9a plays an essential role in the early embryogenesis of mice as deletion of G9a results in lethality of embryos around day E9.5 due to growth retardation (Tachibana et al., 2001). G9a also plays a

role in maintaining the differentiation status of postmitotic neurons (Katoh et al., 2012; Schaefer et al., 2009), as deletion of G9a resulted in derepression of non-neuronal and neural progenitor genes causing complex behavioral changes in these mice and most recent studies implicate G9a has a role in the specification of neuronal subtypes in striatum (Maze et al., 2014). However the exact role of G9a during brain development has not been studied before, partly because of the reason that G9a deletion resulted in early embryonic lethality (Tachibana et al., 2001). Hence we sought to study the role of G9a in cerebral cortical development by restricting its deletion to progenitors originating in Emx-1 lineage. This was possible as we obtained a G9a floxed animal, where the exons 26, 27/28 responsible for the HMTase activity can be targeted for a cre recombinase-mediated deletion (Tachibana et al., 2007). We thus took advantage of the cre-loxP system in generating our desired G9a conditional knockout by crossing this G9a flox animal with Emx-1-cre transgenic mice as shown in the Fig ii-A. Ablating G9a during cortical development resulted in reduced cortical growth. However the laminar organization was preserved in these animals. These animals also exhibited signs of aggression and hyper-excitability. Overall, our data suggests that G9a is necessary for proper development of cerebral cortex and could also have a potential role in regulating the social behavior of animals.

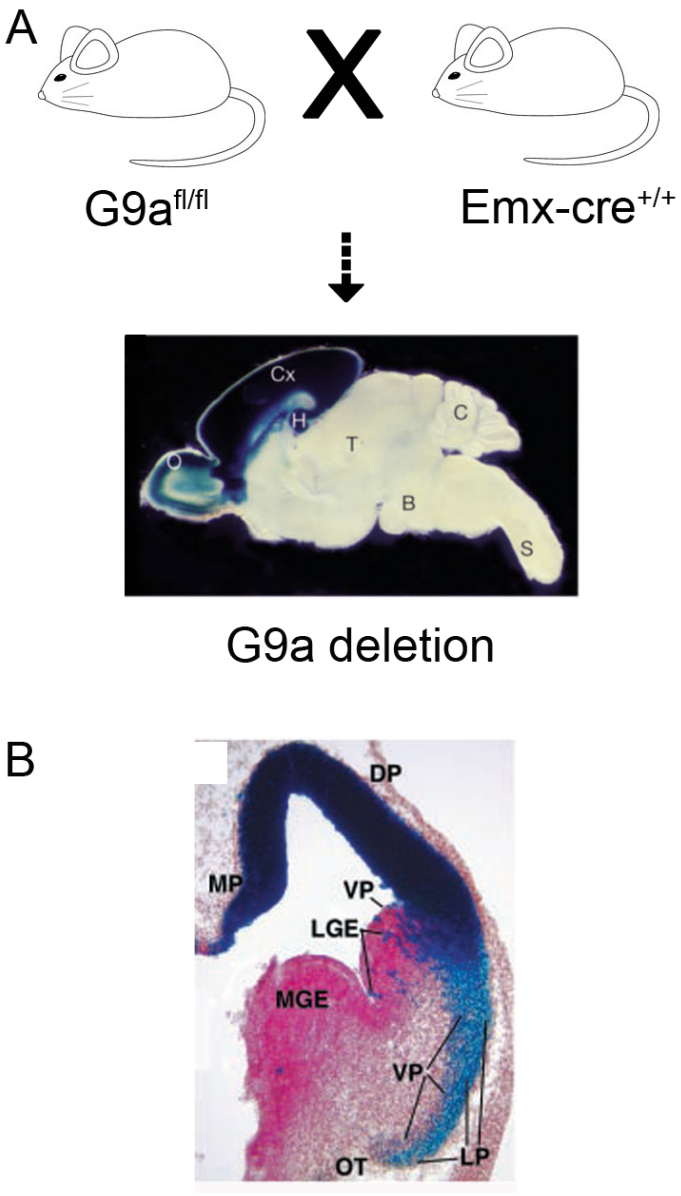


Figure (ii) Strategy for generating G9a conditional knockout. (A) We crossed G9a floxed animal with an animal expressing cre-recombinase under the Emx-1 promoter. This would result in the deletion of G9a in brain regions expressing the X-gal staining, olfactory bulb (O), neocortex (Cx) and Hippocampus (H) while the G9a expression in other regions such as thalamus (T), cerebellum (C), brain stem (B) and spinal cord (S) would remain unaffected. The image adapted from Iwasato et al., 2000, shows the X-gal staining in a sagittal brain section from a P7 mouse expressing LacZ under Emx-1 promoter. (B) The image adapted from Gorski et al., 2002, shows the X-gal staining in a coronal brain section from a E12.5 mouse expressing LacZ under Emx-1 promoter. MP, medial pallium, DP, dorsal pallium, VP, ventral pallium, LP, lateral pallium, OT, olfactory tuberculum, LGE, lateral ganglionic eminence, MGE, medial ganglionic eminence.

MATERIALS AND METHODS

I. Generation of G9a CKO mice

G9a^{flox}/^{flox} animal used in this study was obtained from Yoichi Shinkai lab, Kyoto University, Japan. The strategy for generating G9a flox conditional mutant allele mice was previously described as shown in Figure iii. This animal was crossed with Emx-cre transgenic mice obtained from Jackson laboratories. Genotyping was performed using tail biopsy and PCR using the following primers: mGE28R (GCTCCAGGGCGATGGCCTCCGCTGAATGC); G3 (GGGCCAGCTCATTCCTC CACTC) and mGI27-2F (CGGGACAGGGTTTCTCTGTGTAGTCC) as shown in Figure 1.

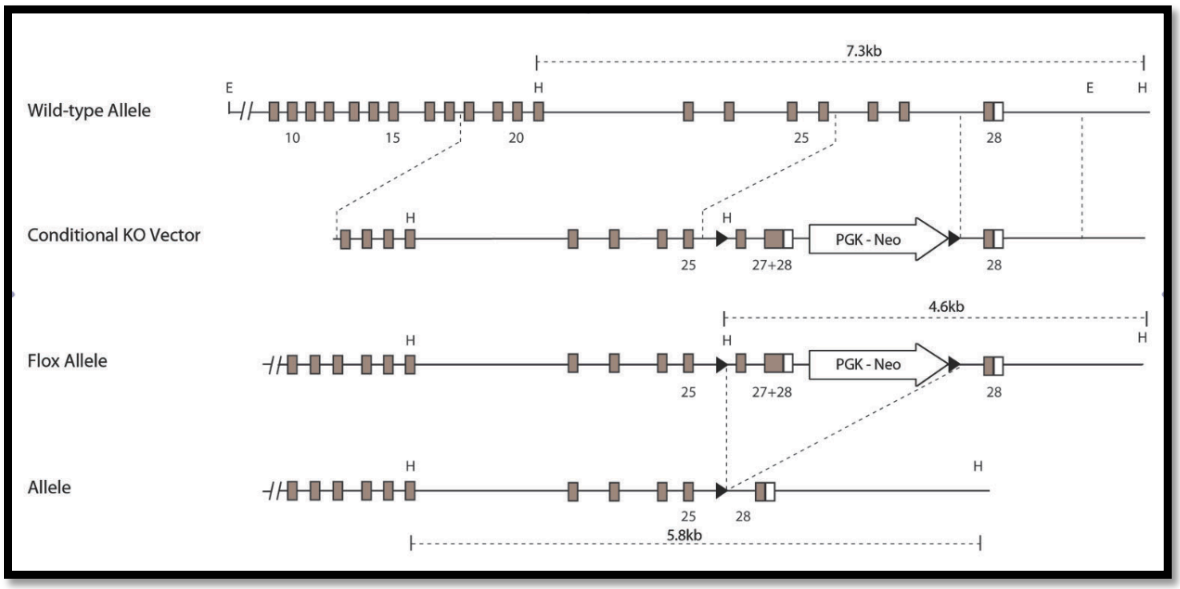


Figure iii: Strategy for generating G9a conditional KO mice. The partial map of G9a targeting vectors is shown. The strategy involved flanking exons 26 and fused 27/28 exons with loxP sites, followed by the generation of G9a floxed animal after introducing the targeting vector into ES cells. Exons, filled rectangles, loxP sites, filled triangles, H, Hind III; E, EcoRI. (Adapted from Tachibana et al., 2007)

II. Animal Use

All animal procedures were performed in accordance with protocols approved by the University of Connecticut Institutional Animal Care and Use Committee (IACUC) and conform to National Institutes of Health guidelines. The animals used in this study were euthanized using isofluorane.

III. Western Blot Analysis

Protein extracts from the cerebral cortex were collected from P2 WT and G9a CKO mice. Tissues were homogenized in a lysis buffer (50mM Tris, pH 7.5, 0.5M NaCl, 1% NP40, 1%DOC, 0.1% SDS, 2mM SDS, 2mM EDTA and complete Protease inhibitor cocktail (Sigma, 8340)). Lysates were centrifuged at 13,000rpm for 15 min at 40^{o}C and the supernatants collected. The protein concentrations were determined using BCA Assay kit (Thermo Scientific, 23227). Equivalent amounts of protein (40 µg) were run on 7.5% Tris-glycine gel and the proteins were transferred onto polyvinylidene difluoride Immoblilon Transfer membranes (Millipore, Bedford, MA) at 350 mA for 1.5 hr. Membranes were blocked overnight at 4^{o}C in 1% casein in Tris-buffered saline solution. Immunoblotting was performed with the following antibodes: rabbit anti-G9a (1:500; Abcam Cat# ab31874) and mouse anti-GAPDH (1:1000; Sigma Cat# G8795) dissolved in casein blocker and incubated overnight at 4^{o}C. The membrane was washed several times with TBS-Tween. The secondary antibodies used were IRDye anti-mouse and anti-rabbit antibodies (1:10000; LI-

COR Biosciences) diluted in casein blocker and incubated at room temperature for 2 hours. The membranes were again washed several times with TBS-Tween, followed by TBS solution. The proteins were detected by LI-COR Odyssey Imaging system.

IV. Immunohistochemistry analysis

Forebrains from WT and G9a cKO animals were examined at the following ages: E14, E19, P2 and P30. All embryonic brains and P2 brains were collected into cold HBSS (Invitrogen) and fixed with 4% paraformaldehyde in 0.1M phosphate buffer using the immersion fixation technique, whereas postnatal brains beyond P2 were perfused with 4% PFA and stored in PBS until further processing. Brains were sectioned using both vibratome and cryostat. The samples were cryoprotected, embedded, frozen, and sectioned at 20µm thickness on a cryostat in coronal sections, which were slide mounted. Similarly 60µm thick coronal free-floating sections were obtained from postnatal brains using Leica vibratome. The sections were subjected to heat-induced epitope retrieval before the immunostaining procedures, where the sections were cooked at 90^{o}C for 5-10 mins in a steamer using an antigen retrieval buffer (Tris/EDTA pH 9.0, sodium citrate pH 6.0). The slides were allowed to cool down and washed with PBS before incubation in a blocking buffer (4% normal goat serum and 0.1% Triton X-100 in PBS). The primary antibodies used on sections were as follows: rabbit anti-G9a (1:500; Abcam Cat# ab31874), mouse anti-H3K9me2 (1:500; Millipore Cat# 07-212), rabbit anti-H3K9me (1:500; Abcam Cat# ab9045), rabbit anti-TBR1

(1:500; Abcam Cat# ab31940), mouse anti-Satb2 (1:400; Abcam Cat# ab51502), and mouse anti-Ctip2 (1:1000; Abcam Cat# ab28448), rabbit anti-Cux1 (1:300; Santa cruz Cat# sc-13024). The primary antibodies were diluted in blocking solution and the sections were incubated overnight at 4^{o}C. Sections were washed with PBS three times for 10 minutes and incubated with secondary antibodies for 1 hour at room temperature. Secondary antibodies used were as follows: Alexa Fluor goat anti-rabbit IgG (H+L) (Molecular Probes) and Alexa Fluor goat anti-mouse IgG (H+L) (Molecular Probes). DAPI (Molecular Probes) were used for labeling nuclei. Sections were observed and imaged under a laser confocal microscope (Leica TCS SP2). Montage images were obtained using Carl Zeiss Axio Imager microscope with Apotome and processed using Adobe Photoshop CS2.

RESULTS

I. Validation of G9a conditional knockout mice

To investigate the *in vivo* epigenetic function of G9a in the developing cerebral cortex, we restricted the ablation of G9a to cerebral cortex using a Cre-loxP system. G9a floxed animal was crossed with Emx1-cre transgenic mice in which Cre-mediated recombination occurs mainly in progenitors generated in the dorsal pallium. We obtained $G9a^{loxP/loxP}$Emx-$cre^{+/-}$ mice as our G9a cKO mice in the F2 generation. Frequency of different genotypes obtained from this crossing was consistent with the expected Mendelian ratios, indicating that none of the G9a cKO mice were dying embryonically or perinatally. The actual loss of G9a was confirmed using the below mentioned techniques:

(i) Genotyping

The initial genotyping was performed by PCR analysis of tail DNA, using the following pair of primers (Figure 1A). The first pair of primers (mGI27-2F and mGE28R, Fig 1A, upper panel) was used for wild-type G9a allele detection. The second pair of primers (G3 and mGE28R, Fig 1A, bottom panel) was used for flox G9a allele detection. Both the primer pairs help in distinguishing the homozygous, heterozygous mutants and wild type animals. Every experimental animal used in this study was genotyped using these two sets of primers and using Emx1-

cre genotyping primers recommended by Jackson Laboratory. Figure 1B illustrates that the PCR amplification product of size 500bp denotes a G9a wildtype mouse, band size of 450 bp denotes a G9a^{flox} homozygous mouse and presence of both the bands represents a heterozygous mouse. The presence of Cre transgene was indicated by a band size of 100bp. The absence of Cre can be tested using the 324bp band size following PCR analysis, while the heterozygous animals are detected using band sizes of 100bp and 324bp. A PCR sample with water was used as a negative control to prevent any false positive amplification.

(ii) Western Blot

Western blot analysis using a G9a antibody demonstrated the partial loss of G9a in the cerebral cortices of G9a cKO animals compared to their wild type littermates (Figure 1C). The reason for us not seeing a complete loss of G9a is discussed later in this section.

(iii)Immunohistochemistry

To confirm the loss of G9a protein expression in the G9a cKO animals, we performed immunohistochemical analysis of coronal brain sections of control P2 animals and compared them with G9a cKO animals. At postnatal day 2, G9a is expressed nearly in all the cells, including

neurons and glial cells present in the cerebral cortex (Figure 1D, upper panel). In G9a cKO animals, we observed a cell-type specific deletion of G9a, where 75% of cells showed loss of G9a protein. However 25% of cerebral cortical cells still expressed G9a (Figure 1D, lower panel). This could be due to the incomplete recombination of the floxed allele, as previous studies shows that Emx1 gene is not expressed ubiquitously in all cortical neurons and the deletion efficiency using the Emx1-cre transgenic mice has been around 85-90% (Chan et al., 1999, Guo et al., 2000). The other 10-15% cells showing immunopositivity for G9a could be due to the fact that these neurons failed to undergo proper cre-mediated recombination due to low levels of Cre protein expression (Tsien et al., 1996). These results help us in validating the deletion of G9a in the neocortex of G9a cKO animals.

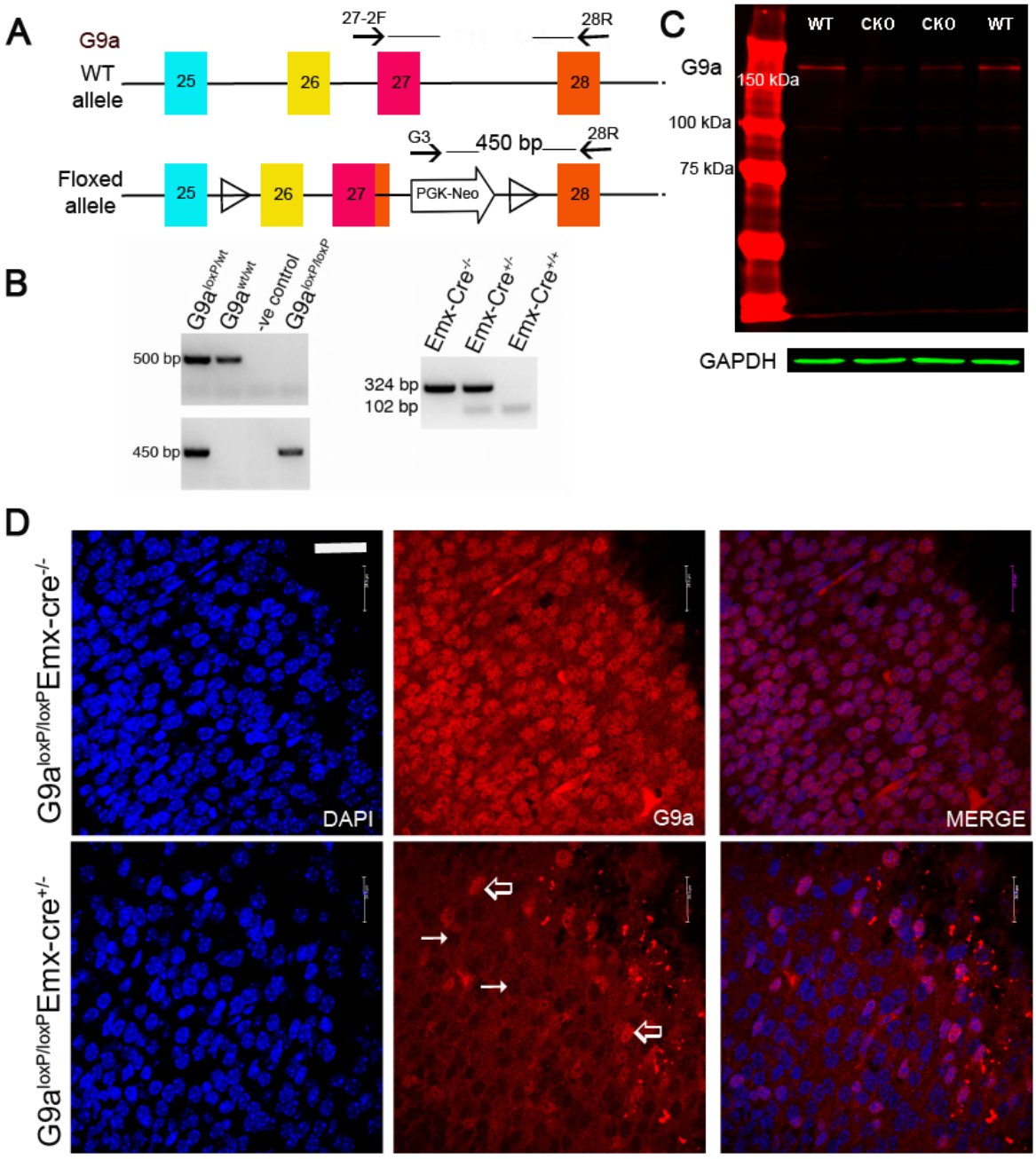

A
G9a
WT allele
27-2F
28R
25
26
27
28
Floxed allele
G3
450 bp
PGK-Neo
B
G9a loxP/wt
G9a wt/wt
-ve control
G9a loxP/loxP
500 bp
450 bp
Emx-Cre -/-
Emx-Cre +/-
Emx-Cre +/+
324 bp
102 bp
C
WT
CKO
CKO
WT
G9a
150 kDa
100 kDa
75 kDa
GAPDH
D
G9a loxP/loxP Emx-cre -/-
G9a loxP/loxP Emx-cre +/-
DAPI
G9a
MERGE

Figure 1: Generation of G9a Conditional Knockout mice: A) The partial map of G9a locus, showing the G9a WT and Floxed allele, in which exons 26, fused 27/28 and PGK-neo cassette are flanked by two loxP sequences. Exons, filled rectangles, lox-P sequences, unfilled triangles. Arrows indicates the forward and reverse primers, denoting their approximate hybridization position along the alleles and the expected band size for the corresponding PCR reactions. B) PCR genotyping carried out using the above mentioned primer pairs. Genotyping results shown for different genotype combinations for G9a and Emx-cre animals. C) Western blot analysis of P2 cerebral cortical lysates obtained from P2 control and G9a cKO animals show the major G9a band at 160 kDa in the wildtype brain and partial loss of immunoreactivity in the KO mouse brain (n=2). D) Conditional cell-specific deletion of G9a in cerebral cortex. Expression of G9a in the cerebral cortex of control (upper panel) and G9a cKO mice (lower panel) at P2. G9a was expressed ubiquitously in the control animals as shown by the red nuclear signal. G9a expression was lost in most of the cells in G9a cKO animals (arrow), however some cells were still expressing G9a (block arrow). Blue staining indicates nuclear dye. Scale bar, 20 μm.

II. Loss of G9a resulted in reduced H3K9me2 levels in G9a-deficient cells

Studies on histone lysine methyl transferases (HKMTases) started gaining biological significance since the identification of the first mammalian HKMT, Suv39h. This also led to the identification of G9a, which was associated with euchromatic gene silencing in mammals and had a stronger HMTase activity compared to Suv39h (Tachibana et al., 2002). Its loss resulted in a global loss of H3K9 methyl levels in euchromatic regions, however H3K9me3 levels in heterochromatic regions were unaffected by the loss of G9a (Rice et al., 2003). G9a possess a SET domain flanked by two cysteine rich regions in its carboxyl terminal, through which it exerts its HMTase activity. In our study, the exons responsible for encoding this catalytic SET domain are flanked by loxP sequences. Hence Cre-mediated deletion of these exons would result in the loss of HKMTase activity in the $G9a^{flox}$ animal before the onset of neurogenesis. Thus the H3K9me2 levels were analyzed using coronal brain sections from both control (Figure 2A) and G9a cKO animals. Consistent with the previous studies, deletion of G9a resulted in a loss of H3K9me2 levels in the cerebral cortex of G9a cKO animals (Figure 2B). However a small percentage of cells expressing G9a are also positive for H3k9me2 suggesting that G9a is the major histone dimethytransferase in the cerebral cortex and SET domain is essential for its HMTase activity. In addition to this, surprisingly deletion of G9a also resulted in small reduction in the levels of H3K9me in G9a cKO animals (Figure 2D). This

could be indicative of a possible role of G9a in monomethylation of the H3 lysine residue along with other HMTases, which could compensate for the loss of G9a.

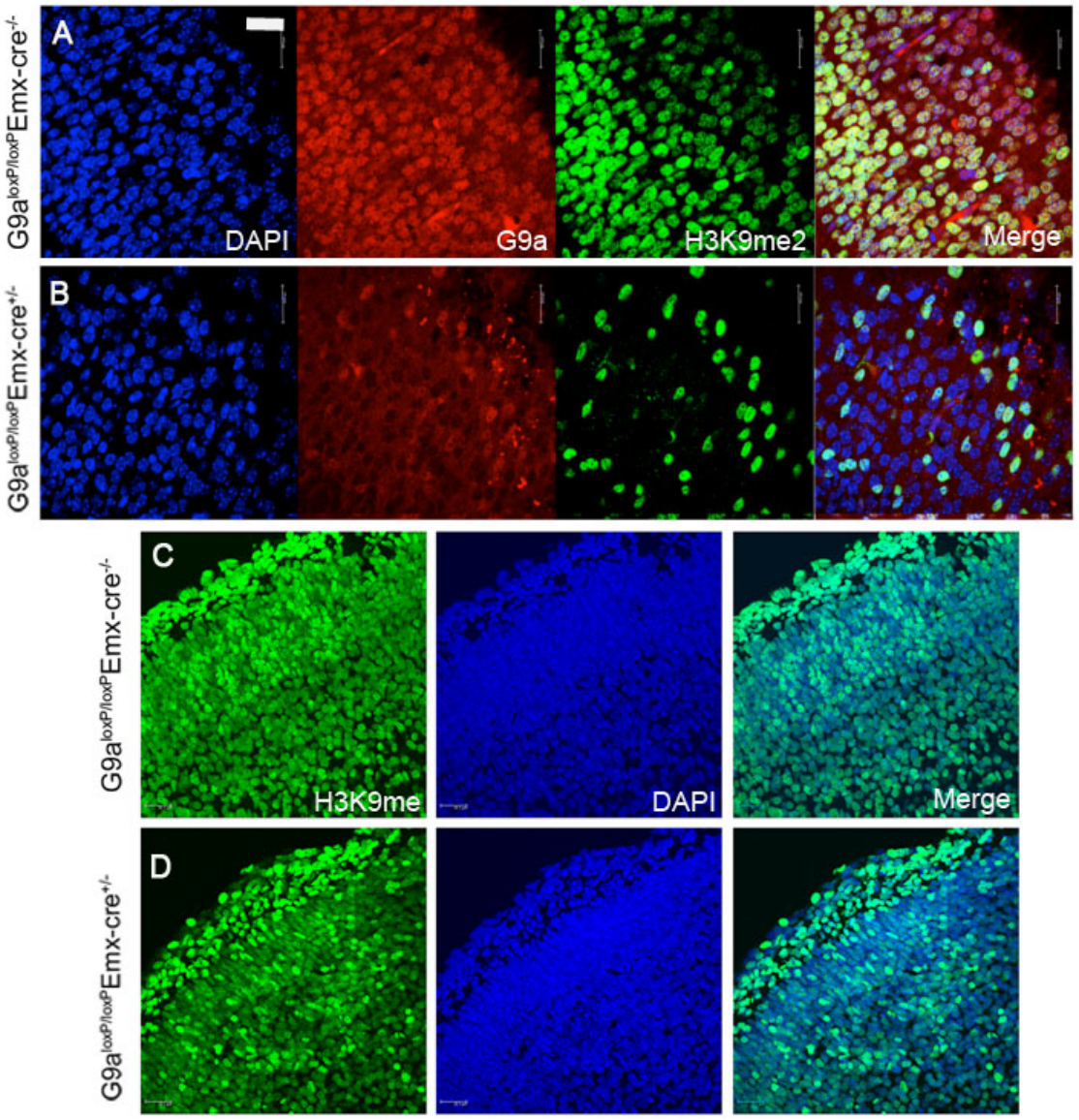


Figure 2: Conditional ablation of G9a results in loss of H3K9 dimethylation and small reduction in H3k9 monomethylation levels. A) Expression of G9a (red signal) and H3K9me2 (green signal) in the P2 WT cerebral cortex. B) Loss of H3K9me2 (green signal) only in cells lacking G9a (red signal) indicating that G9a is the major dimethyltransferase in the cerebral cortex. C) Expression of H3K9me (green signal) in P2 WT cerebral cortex. D) H3K9me levels show a slight reduction in cortex of G9a cKO mice indicated by the green signal. Blue staining indicates nuclear dye. Scale bar, 20 μm.

III. Region specific deletion in G9a cKO animals

Vertebrate cerebral cortex primordium, which is otherwise called as pallium, is divided into four primary progenitor zones, medial, ventral dorsal and lateral. Each of these zones gave rise to different regions in a developed brain, hippocampus, amygdala, neocortex and piriform cortex respectively (Puelles et al., 2000). It has also been established that these regions give rise to glumatergic projection neurons and glial cells while the interneurons originating and tangentially migrate from the subpallial regions into the cortex during development (Marin and Rubenstein, 2001). In this strategy, we have used a transgenic mouse strain in which the Emx1 promoter drives the Cre recombinase. Emx1 expression is restricted to medial, dorsal and lateral pallial progenitor cells and postmitotic neurons originating from these zones, and is not expressed in the ventral pallium. By E12.5, all cells present in these zones should have undergone successful recombination and similarly we achieved deletion of G9a, which was confirmed by immunocytochemistry for both G9a and H3K9me2 at day E12.5 (Fig 2 A-D). The pattern of deletion observed in our G9a cKO is consistent to the expression pattern of a reporter under Emx-1 promoter as shown in a study before (Fig ii-B). Moreover this study also indicates that interneurons originate outside the Emx-1 lineage and account for 20% of the cortical population. This is consistent to the number of cells still expressing G9a in our cKO cerebral cortex and sections also show some scattered cells around the LGE region, which appear to be migrating from these regions into the cortex (Figure 3 A-D). The

same pattern exists in adult animals following development, as G9a deletion is restricted to the cortex. G9a expressions in cells present in the striatum, which originate from the subpallial regions, remain unaffected following recombination in G9a cKO (Figure 3E-H).

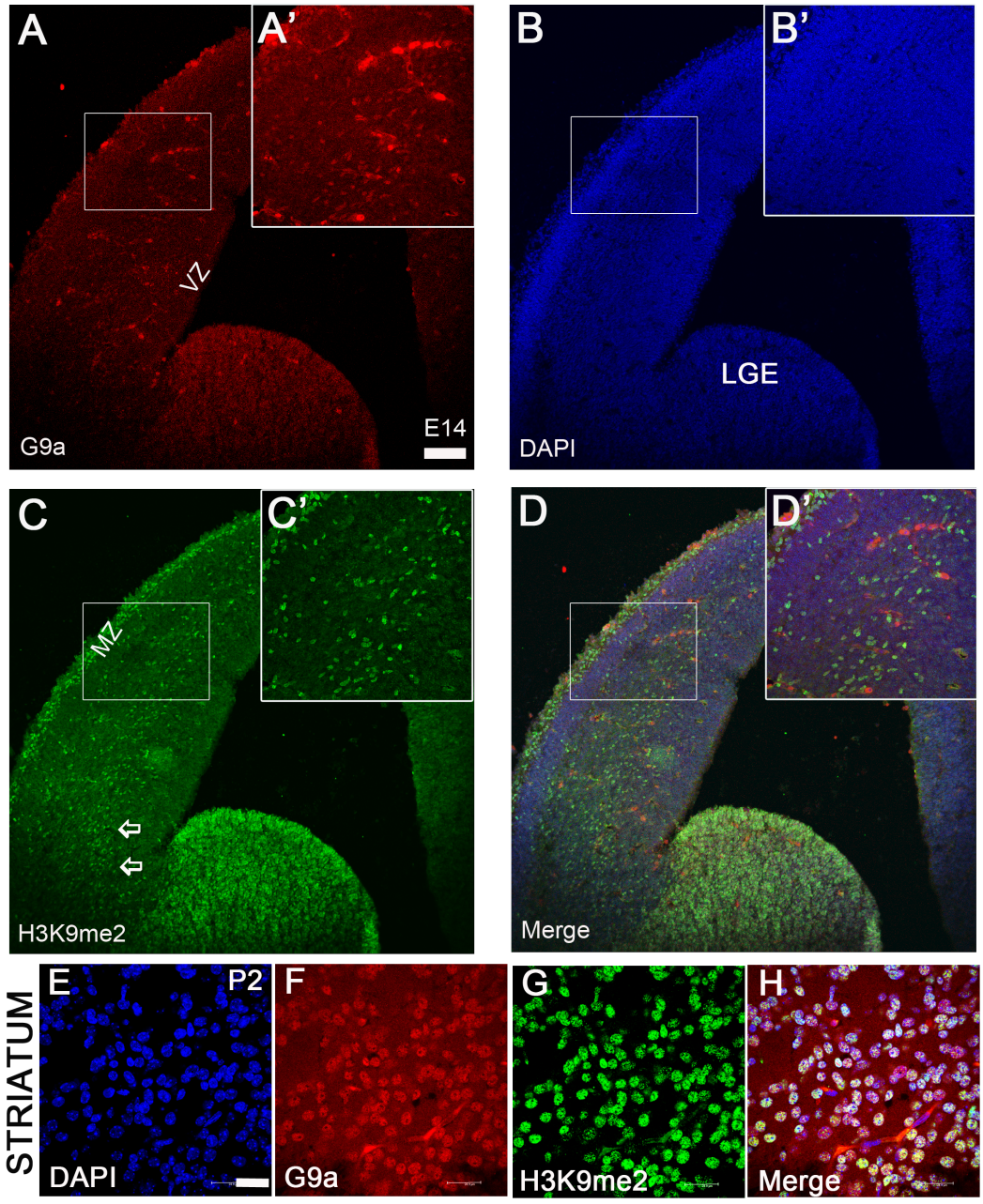

A
A'
VZ
G9a
E14
B
B'
LGE
DAPI
C
C'
MZ
H3K9me2
D
D'
Merge
STRIATUM
E
P2
DAPI
F
G9a
G
H3K9me2
H
Merge

Figure 3: Region-specific deletion of G9a in cKO: (A-D) Coronal sections from G9a cKO showing double immunostaining for G9a (red signal) and H3K9me2 (green signal) at embryonic ages E14. This confirms the region-specific deletion of G9a, only in cells originating from Emx1-lineage. The cells that are still positive for G9a/H3k9me2 might be interneurons and cells populating layer 1(MZ), as they originate outside the progenitor zones that do not fall under Emx1-lineage. Unfilled arrows indicate the scattered cells, which could be possibly migrating from subpallial regions into cortex. (E-H) Conservation of this pattern in the striatum of postnatal animals. This was confirmed by immunostaining for G9a (red signal) and H3K9me2 (green signal) at postnatal day 2. (A'-D') Inset shows the cells at higher magnification. Scale bar, 20 μm (A-D); 25 μm (E-H).

IV. Phenotypic characterization of G9a cKO animals

Following the generation of a G9a conditional knockout where we successfully deleted the G9a in the cerebral cortex, we wanted to see if these animals exhibited any gross phenotype. The animals were fertile; survive to adulthood and mate successfully giving rise to progeny except for the male mice as they exhibited aggressive and self-mutilating behavior. This resulted in them being housed separately or together with the same female as they caused self-inflicting injuries or serious injuries to other animals housed along with them. Eventhough these animals were easily excitable and hyperactive, they did not show any signs of spontaneous seizures or hyper-excitability, based on the electrophysiological data recorded using G9a cKO animals and control littermates (data not shown). Hyper-exicitability, spontaneous seizures and epilepsy have been mainly associated to loss or presence of abnormal interneurons (Sarkisian et al., 2001). Absence of these phenotype suggests that interneuron population are not affected by the loss of G9a in our conditional knockout animals, as they are generated from subpallium from where they migrate into the overlying cortex during development and do not fall under the Emx-1 lineage (Gorski et al., 2002).

V. Cerebral-cortex specific G9a deficient animals exhibit cortical thinning

The cerebral cortices of P2 G9a$^{loxP/loxP}$Emx-cre$^{+/-}$ animals were considerably thinner compared to their wildtype littermates, G9a$^{loxP/wt}$Emx-cre$^{+/-}$ (Fig 4, Upper panel). Quantification of G9a $^{loxp/loxp}$ / Emx1-Cre and G9a $^{loxp/loxp}$ / Emx1-Cre cortical thickness at P2 (n=7) indicated a significant reduction in radial thickness in somatosensory barrel region of cortex by approximately 20% (601 $\pm$ 17 μm *vs* 497 $\pm$ 19 μm: two tailed t test p=0.00175). The cortical thinning increased in adult G9a cKO animals, P30 (Fig 4, Bottom panel). These results were consistent with our previous studies where it was shown that loss of Citron kinase in rats, a Rho-GTPase activated kinase, known to have a role in cytokinesis, resulted in microcephaly (Di cunto et al., 2000). It has also been shown that CitK interacts with G9a for maintaining the epigenetic status during the neuronal lineage formation by repressing several genes involved in the neuronal development and differentiation (unpublished data). This cortical thinning phenotype observed in G9a cKO animals is consistent with the microcephaly phenotype seen in Cit-K mutants. However G9a-cKO animals did not replicate all the phenotype characteristics of Cit-K mutants. This could be due to that the fact that CitK mutants completely lacked CitK expression and G9a deletion has been restricted to the cerebral cortex in our model.

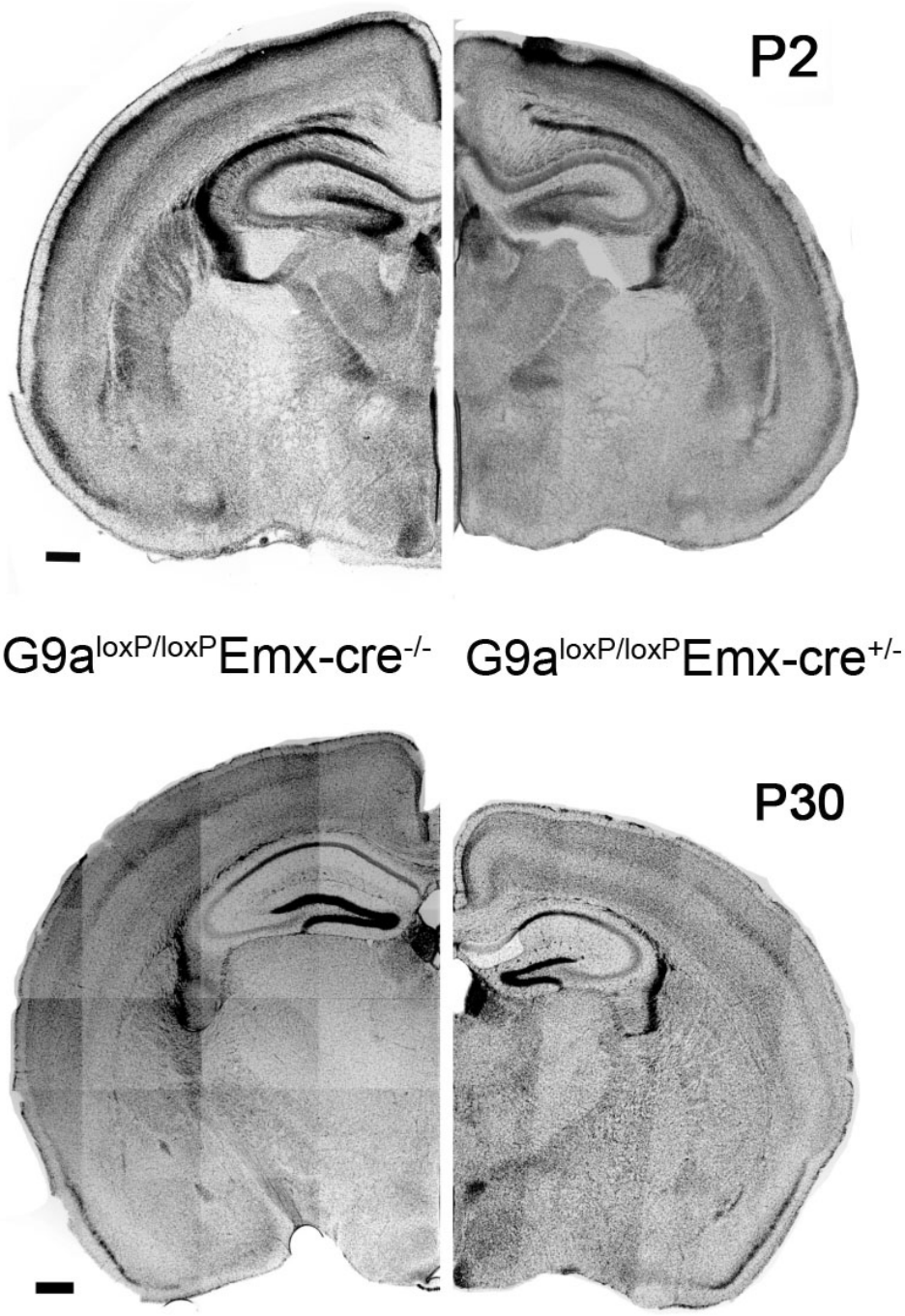


Figure 4: Reduced cortical growth in G9a cKO mice: Coronal brain hemisphere DAPI- stained sections from P2 and P30 a G9a$^{loxP/loxP}$Emx-cre$^{+/-}$ animals showing thinner cortex when compared to a G9a$^{loxP/loxP}$Emx-cre$^{-/-}$ littermates. Scale bar 150µm in upper panel, 500µm in bottom panel.

VI. Laminar organization was partially preserved in G9a conditional knockout postnatal animals

Cortical thinning in G9a cKO animals persuaded us to study the laminar organization in these animals, as we wanted to see if deletion of G9a altered the process of neurogenesis. In spite of cortical thinning, the G9a cKO animals did not exhibit any abnormal laminar pattern and the relative positions of different laminar markers such as Cux1, Tbr1 were partially conserved in postnatal animals (Fig 5). This laminar patterning is again consistent to the Cit-K mutant models, as partial conservation was observed in these mutants. Even though the G9a cKO and WT animals exhibited similar laminar organization; there was a slight decrease in the number of neurons occupying layer 2/3 (Fig 5 A, B) and layer 6(Fig 5 C), based on the Cux1 and Tbr1 immunopositivity, corresponding to their smaller brain sizes.

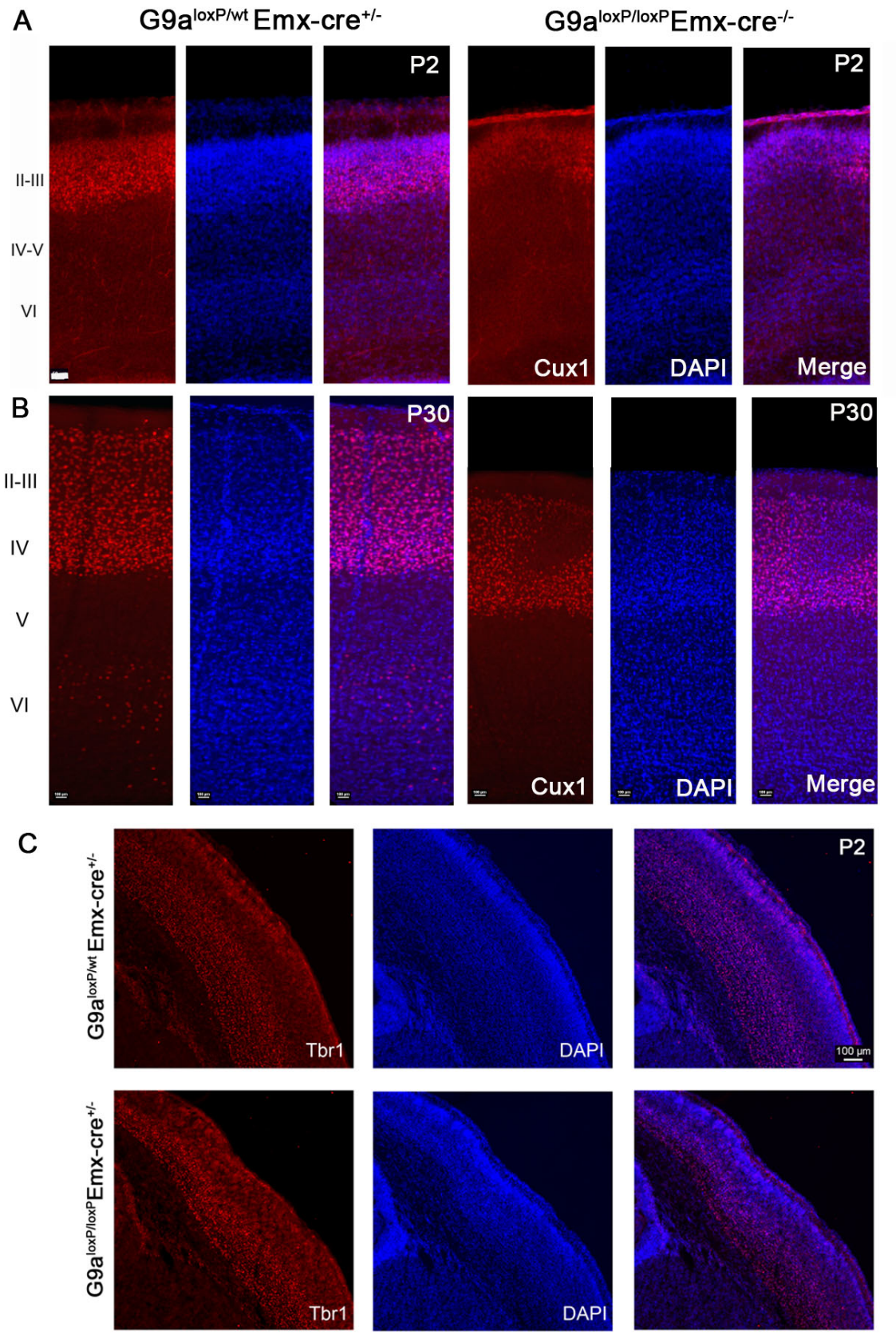

A
G9a loxP/wt Emx-cre +/-
G9a loxP/loxP Emx-cre -/-
P2
P2
II-III
IV-V
VI
Cux1
DAPI
Merge
B
P30
P30
II-III
IV
V
VI
Cux1
DAPI
Merge
C
G9a loxP/wt Emx-cre +/-
Tbr1
DAPI
P2
100 µm
G9a loxP/loxP Emx-cre +/-
Tbr1
DAPI

Figure 5: Preservation of laminar organization in G9a cKO animals: (A) Immunofluorescence for upper layer marker Cux1 (red) on 60μm thick sections of P2 and (B) P30 knock out (right panels) and wild type (left panels) (C) Immunofluorescence for deep layer marker Tbr1 (red) on 20μm thick sections of P2 knock out (right panels) and wild type (left panels. Scale bar, 100 μm

VII. Cortical thinning and preservation of laminar markers was also observed during embryonic ages in G9a cKO

The major aim of this study was to identify the role of G9a in early and late cortical neurogenesis. We were also interested in capturing the time window during which the cortical thinning might set in the G9a cKO animals. For this purpose, we analyzed the brains of G9a cKO at embryonic time points E14 and E19. Cortex-specific deletion of G9a was observed as early as embryonic age E12 (Fig 2 A, A'), which is consistent with the other deletion studies performed using Emx1-cre transgenic system (Fig ii-B). The cortical thinning was visible at age as early as E14 (Fig 6A) and it started becoming more apparent around age E19 (Fig 6B). In spite of the reduced cortical growth, the laminar organization was preserved in both the ages. Corresponding to the smaller cortices, the neuronal population occupying upper and deeper Satb2, Tbr1, Ctip2 positive layers were proportionately reduced in G9a cKO animals (Fig 6A, B).

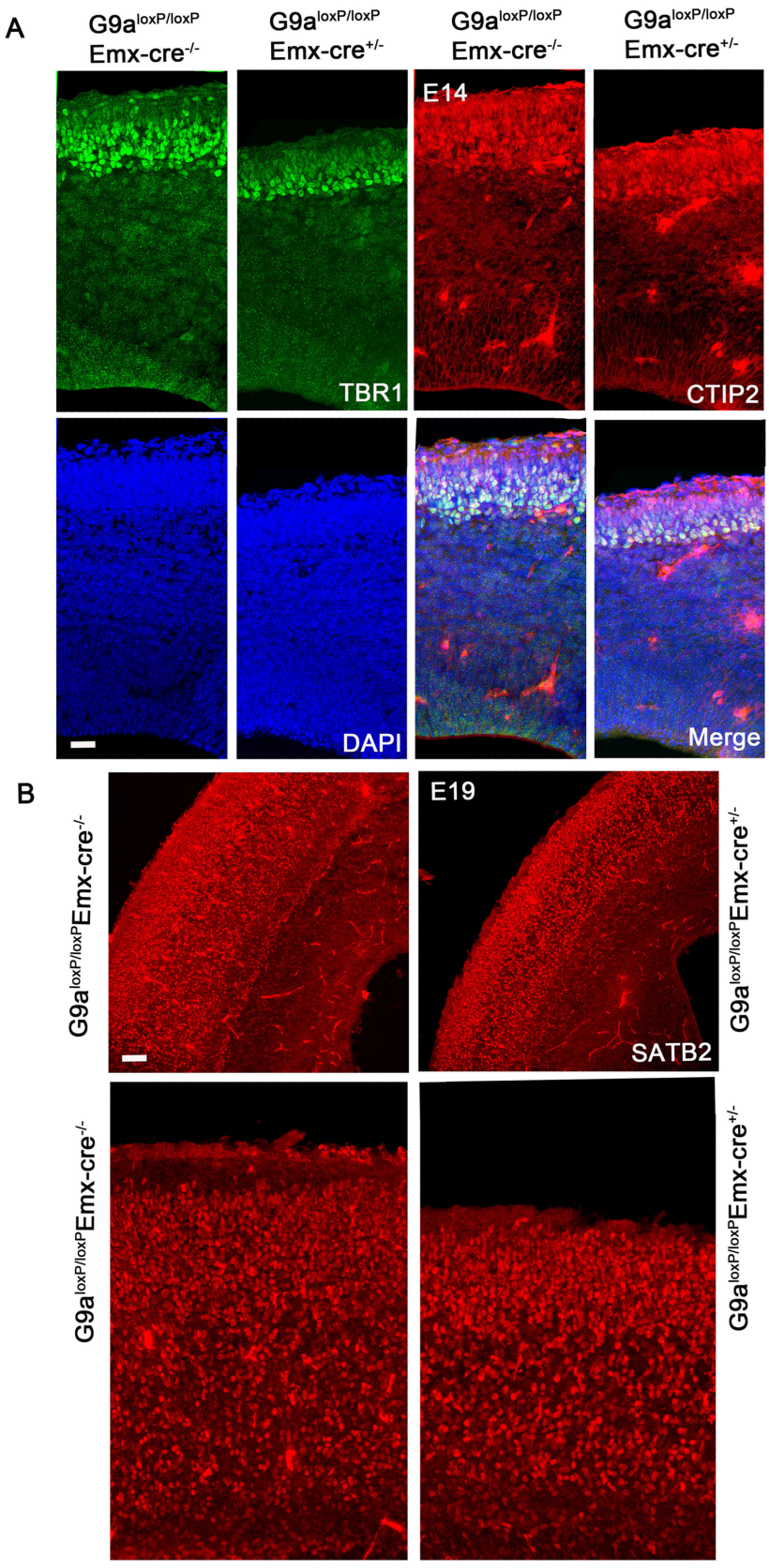

A
G9a loxP/loxP Emx-cre -/-
G9a loxP/loxP Emx-cre +/-
G9a loxP/loxP Emx-cre -/-
G9a loxP/loxP Emx-cre +/-
E14
TBR1
CTIP2
DAPI
Merge
B
G9a loxP/loxP Emx-cre -/-
E19
SATB2
G9a loxP/loxP Emx-cre +/-
G9a loxP/loxP Emx-cre -/-
G9a loxP/loxP Emx-cre +/-

Figure 6. Cortical thinning and laminar preservation in embryonic ages: (A) Reduced cortical thickness at E14 in G9a cKO. Immunofluorescence for deep layer maker Tbr1 (green) in wild type (left panel) and cKO (right panel) and immunofluorescence was performed for deep layer marker Ctip2 (red) on wild type (left panels) and cKO (right panels). (B) Immunofluorescence of Satb2 (red) on 20μm sections of E19 knock out (right panel) and wild type (left panels). Scale bar, 25 μm (A), (B) bottom panel, 100 μm (B) top panel.

DISCUSSION

In the current study, we analyzed the consequence of deleting G9a in the cerebral cortex. Analyzing the in vivo role of G9a using G9a-deficient mice was hindered as complete deletion of G9a resulted in embryonic lethality (Tachibana et al., 2001). However, the gene-targeting strategy devised in previous studies (Tachibana et al., 2007) helped in eliminating the histone methyltransferase activity of G9a using cre-mediated deletion. Using this strategy, we generated a conditional knockout mouse in which the G9a expression was deleted in cells originating from the dorsal, medial and lateral pallium. We achieved both region-specific and cell-specific deletion of G9a, and G9a expression was unaffected in other subcortical regions. However 25% of cells present in G9a cKO cerebral cortex were still expressing G9a. These cells might have originated from ganglionic eminence, which falls outside the Emx-1 specific progenitor zones, and would have migrated into the cortex later during development. Also inefficient recombination of the floxed allele could also be a reason for these to be still positive for G9a. Cre-mediated deletion studies using Emx-1-IRES-Cre transgenic mice shows only 85-90% recombination efficiency and an additional 10% reduction in efficiency of neurons has been attributed to low level expression of cre proteins in these neurons (Gua et al., 2000). Analyzing the percentage of cells that are double-positive for G9a and a interneuronal marker such as parvalbumin, somatostatin, calretinin, in G9a conditional knockout animals would help us better understand if these cells showing G9a

immunopositivity are from different lineage or a product of inefficient recombination or both.

G9a, a histone methyltransferase found in mammals, associated with H3K9 methylation-mediated silencing of genes present in the euchromatic regions (Tachibana et al., 2001). This repressive mark of G9a has shown to be essential during the process of cellular differentiation and for maintaining the post-mitotic status of cells. G9a deletion in a distinct set of striatal neurons led to the up-regulation of transcriptional programs observed in a different set of neurons, along with the acquisition of their electrophysiological properties, thus proving the role of G9a in specifying neuronal subtype identity (Maze et al., 2014). G9a is also associated with silencing of neuronal genes in non-neuronal regions and deletion of G9a in postnatal neurons has resulted in ectopic expression of non-neuronal genes and neural progenitor genes in post-mitotic neurons. G9a carries out all these functions through its HMTase domain. Thus loss of G9a is mostly accompanied by loss of H3K9me2-mediated silencing of these genes. Our study also demonstrated that deletion of G9a resulted in a loss of H3K9me2 levels in cerebral cortex. This suggests the fact that G9a could be the major histone dimethyltransferase in cerebral cortex. We also observed that H3K9 monomethylation levels were slightly affected by the loss of G9a. This might suggest that G9a is not the major histone monomethyltransferase enzyme in the neocortex and there could be other SET domain containing lysine methytransferases involved in maintenance of H3K9me levels in the brain.

Study in our lab has reported that citron kinase, a small Rho-GTPase, known to have an important role in both cytokinesis and neurogenesis, establishes the repressive marks on developmental genes involved in neuronal development through G9a (Sarkisian et al., 2001). Previous study demonstrates that loss of citron kinase in rat results in defective neurogenesis and smaller brain size in mutants (Di Cunto et al., 2000). Thus, we hypothesized an in vivo role of G9a in cortical development and deletion of G9a would affect the brain architecture. We observed cortical thinning in our G9a conditional knockout animals, which correlated well with the phenotype of CitK-/- mutants reported in the study by Di Cunto et al. Cortical thinning was observed as early as E14 in G9a cKO animals which became more pronounced as age progressed. Partial preservation of laminar organization was another feature found to be common between both the mutant models. In spite of these common phenotypes, our G9a cKO neocortex did not have any binucleate cells, a hallmark of CitK mutants. This is not surprising, as G9a, unlike CitK, has not been implicated in the process of cytokinesis regulation. We also did not observe the characteristic neurological phenotype in our G9a cKO, epilepsy and lethal seizures, which were present in CitK mutants. Abnormal interneuron development was attributed to these characteristics in CitK mutants. Lack of this phenotype suggests that the interneuron development was unaffected by G9a deletion, thereby further validating the fact that we achieved region- or cell type- specific deletion of G9a in our cKO. Even though the animals did not show any signs of spontaneous

seizures or epilepsy, the G9a cKO male animals exhibited hyper-aggressiveness and excitability. The male animals displayed aggression towards intruders and caused self-inflicting wounds or injured the intruder, be it male or female. This suggests a possible role of G9a in regulating the social behavior or adaptive behavior. A previous study suggests that deletion of G9a/GLP in postnatal neurons resulted in abnormalities including cognitive impairment and defective behavior (Schaefer et al., 2009). A copy of EHMT1/GLP gene is deleted in chromosome 9q34 in subtelomeric deletion syndrome patients resulting in severe mental retardation. In addition to mental retardation, 9q34 syndrome patients also exhibit aggressive behavior and hyper-excitability (Kleefstra et al., 2009). These results suggest a possible role of G9a in controlling the social behavior of individuals along with cognitive and adaptive behavior. This aggressive nature might be due to the global loss of G9a in cerebral cortex or could be restricted to the loss of G9a in specific neuronal population, for e.g. neurons present in the olfactory bulb of these animals, could have resulted in this aggressive phenotype. Olfaction is an important requirement for social recognition and social cognition in mice. G9a cKO animals might have neurons, which are impaired and are unable to respond to appropriate olfactory cues due to loss of G9a. This ultimately would have resulted in this behavior, which could be an interesting topic for future. This would help us in elucidating a possible role of G9a in regulating the social behavior of animals. Also these results could be extrapolated to human mental retardation syndrome, if we know the fact that deficiency of G9a/GLP in specific

brain regions during development causes complex behavioral abnormalities in mice.

The major objective of this study was to identify the role of G9a in cerebral cortical development. G9a cKO mimics Ezh2-cKO in terms of cortical growth and neuronal layer production in postnatal animals. Both G9a and Ezh2 are methyltransferases, known to silence genes by methylating different lysine residues of histone core protein H3 and has shown to have a similar role in many cellular processes. Ezh2 is responsible for the enzymatic activity of the PRC2 complex, and study has shown that G9a and Ezh2 control common set of genes, which are mainly involved in neuronal differentiation processes. Also G9a methylation activity is required for the recruitment of PRC2 complex to these genes (Mozetta et al., 2014). These results along with the similarities in phenotype between G9a-cKO and Ezh2-cKO animals shed some light on the possible role of G9a in cortical development. Thus I would like to hypothesize the potential role of G9a in cerebral cortical development: G9a-mediated histone methylation is necessary for the initial repression of several neuronal genes that are turned on later during the initial stages of neuronal development. However, loss of G9a in our cKO could remove these repressive marks, possibly leading to up-regulation of several genes including several transcription factors and genes that are normally expressed in differentiating neurons. This might result in two conditions: (1) the cells instead of self-renewal are directed towards differentiation and (2) the G9a cKO animals start making differentiated neurons at

an earlier time point compared to the wildtype, thereby accelerating the neurogenesis, which might also reduce the neurogenic output. Deletion of Ezh2 resulted in the up-regulation of these genes at E12.5: NeuroD6, Myt1L, Bcl1 1b, Reelin, Tbr1, Foxp2, Brn2, Lmo4, Satb2 (Pereira et al., 2010). Thus similar up-regulation could be expected in our G9a cKO and depending on which transcription factor genes are upregulated, it could result in the production of those neuronal subtypes at an earlier timepoint during development, thereby producing less number of neurons, however the temporal order in which these neurons are produced might not be affected by loss of G9a. Thus microarray analysis and quantitative analysis of neurons populating different layers would give us more insight on the exact role of G9a in cerebral cortical development. Lack of repressive marks especially on genes coding for different transcription factors could direct the neurogenesis more towards differentiation than self-renewal, thereby reducing the number of neurons produced, but conserving the pattern in which they might populate different layers during development.

Thus our study has demonstrated that deficiency of G9a in neural progenitors during cerebral cortical development has resulted in reduced cortical outgrowth and minor behavioral anomalies, indicating that G9a is essential for normal cortical development and for regulating social behavior in mice.

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