Geminin-Deficient Neural Stem Cells Exhibit Normal Cell Division and Normal Neurogenesis

Kathryn M. Schultz¹,², Ghazal Banisadr³,⁴, Ruben O. Lastra¹,²,⁴, Tammy McGuire⁵, John A. Kessler⁵, Richard J. Miller⁶, Thomas J. McGarry¹,²,³*¹

1 Feinberg Cardiovascular Research Institute, Northwestern University Feinberg School of Medicine, Chicago, Illinois, United States of America, 2 Department of Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, United States of America, 3 Robert H. Lurie Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois, United States of America, 4 Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Feinberg School of Medicine, Chicago, Illinois, United States of America, 5 Department of Neurology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, United States of America

Abstract

Neural stem cells (NSCs) are the progenitors of neurons and glial cells during both embryonic development and adult life. The unstable regulatory protein Geminin (Gmnn) is thought to maintain neural stem cells in an undifferentiated state while they proliferate. Geminin inhibits neuronal differentiation in cultured cells by antagonizing interactions between the chromatin remodeling protein Brg1 and the neural-specific transcription factors Neurogenin and NeuroD. Geminin is widely expressed in the CNS during throughout embryonic development, and Geminin expression is down-regulated when neuronal precursor cells undergo terminal differentiation. Over-expression of Geminin in gastrula-stage Xenopus embryos can expand the size of the neural plate. The role of Geminin in regulating vertebrate neurogenesis in vivo has not been rigorously examined. To address this question, we created a strain of Nestin-Cre/Gmnn²/² mice in which the Geminin gene was specifically deleted from NSCs. Interestingly, we found no major defects in the development or function of the central nervous system. Neural-specific Gmnn²/² mice are viable and fertile and display no obvious neurological or neuroanatomical abnormalities. They have normal numbers of BrdU⁺ NSCs in the subgranular zone of the dentate gyrus, and Gmnn²/² NSCs give rise to normal numbers of mature neurons in pulse-chase experiments. Gmnn²/² neurosphere cells differentiate normally into both neurons and glial cells when grown in growth factor-deficient medium. Both the growth rate and the cell cycle distribution of cultured Gmnn²/² neurosphere cells are indistinguishable from controls. We conclude that Geminin is largely dispensable for most of embryonic and adult mammalian neurogenesis.

Introduction

All neurons and glial cells in the brain are derived from neural stem cells (NSCs). NSCs maintain their own numbers by self-renewal and also give rise to daughter cells that terminally differentiate into neurons, astrocytes, and oligodendrocytes [1,2]. NSCs have been found to persist in the adult brain and generate new neurons throughout adult life, particularly in the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles [3]. This raises the exciting possibility that NSCs may be useful for the therapy of neurodegenerative diseases. The factors that control the division and differentiation of NSCs are of tremendous scientific and medical importance.

Geminin (Gmnn) is an unstable regulatory protein that is thought to maintain neural progenitor cells in an undifferentiated state while they proliferate [4]. Geminin is expressed in both embryonic and adult mouse neural progenitor cells, and in the Xenopus central nervous system throughout embryonic development [5,6]. Geminin is preferentially expressed in neural precursor cells, and expression is down-regulated before neural differentiation [7] [6]. Geminin binds to Brf1, the catalytic (ATPase) subunit of a SWI/SNF chromatin remodeling complex, and inhibits its recruitment to neuron-specific promoters by the basic helix-loop-helix (bHLH) transcription factors Neurogenin (Ngn) and NeuroD [7]. A complex between Geminin and the transcription factor AP4 represses the transcription of neuronal genes in non-neuronal cell types [8]. In addition to its effects on the nervous system, Geminin inhibits tissue differentiation in a variety of other organs by binding and inhibiting various transcription factors and chromatin remodeling proteins, including sine oculis (Six) and Homeobox (Hox) transcription factors and the Polycomb protein Scmh1 [7,9,10].
In addition to regulating cell differentiation, Geminin also limits the extent of DNA replication to one round per S phase by binding and inhibiting the essential replication factor Cdt1 [11]. The concentration of Geminin is cell-cycle regulated; the protein begins to accumulate at the G1/S transition and persists throughout S and G2 phase. Geminin is destroyed by ubiquitin-dependent proteolysis during M phase, which allows a new round of replication in the next cell cycle [12]. This expression pattern has been documented extensively in developing mouse brains [6]. Six and Hox transcription factors can compete with Cdt1 for binding to Geminin [9,10], raising the possibility that Geminin links exit from the cell cycle with cell differentiation. According to this model, the destruction of Geminin when cells enter G0 phase would relieve the repression of Brd1 and other transcription proteins and trigger terminal differentiation [4,13,14].

In early embryos Geminin can also act as an inducer of nervous tissue. In an unbiased expression-cloning screen, Geminin was identified as a molecule that expands the size of neural plate in Xenopus embryos [5]. These effects are correlated with increased expression of the proneural gene Neurogenin-related 1 (Ngr1) and decreased expression of BMP4, an epidermis-inducing growth factor. Over-expression of Geminin in Drosophila embryos induces ectopic neural cells in the epidermis [15].

The role of Geminin in regulating neural development has been examined by deleting its gene from model organisms. C. elegans embryos treated with Geminin siRNA show gonadal abnormalities and ~20% of the worms are infertile, but no neural phenotype has been described [16]. *Geminin* knockout mice were obtained by targeting a floxed STOP cassette into the *Gmnn* gene [17,18]. Their cells contain more nuclear DNA than normal, and none express markers of the embryonic stem cells that form the epidermis [15].

To address this question, we constructed a strain of mice in which a floxed Geminin allele (*Gemfl/fl*) is deleted from the neural stem cells. To test whether Geminin is essential for neural development we used the Nestin-Cre transgenic mouse line (Figure 1A). The brains of e14.5 and e16.5 Nestin-Cre/Gmnnfl/fl mice had virtually complete recombination of reporter and Geminin message, and none of the brains showed obvious neuroanatomical defects upon sectioning (Figure 1A and 1C).

| Table 1. Viability of Nestin-Cre/Gemfl/fl Mice. |
|------------------------------------------------|
| Parents | Offspring         |                          |                          |                          |                          |                        |
|         | Nestin-Cre Gem(+/loxP) | Gem(+/loxP) | Gem(loxP/loxP) | Nestin-Cre Gem(loxP/loxP) | P Value (χ²) |
| Nes-Cre/Gem+/- X Gem+/-       | 22          | 21          | 27           | 28           | 0.82                     |
| Nes-Cre/Gemfl/fl X Gemfl/fl   | --          | --          | 42           | 33           | 0.75                     |

doi:10.1371/journal.pone.0017736.t001
neurons while the brains of littermate Gmmnfl/fl/R26R-LacZ mice showed no β-galactosidase expression (Figure 2B). Taken together, our results indicate that deletion of the Geminin gene is mostly complete by e14.5 and virtually complete by birth, consistent with what has been previously reported for other mice that express Nestin-Cre [24,25]. The persistence of β-galactosidase-expressing neurons in the brains of adult Nestin-Cre/Gmnnfl/fl/R26R-LacZ mice excludes the possibility that neurogenesis is rescued by rare cells that escape Cre-mediated Geminin deletion.

Next we examined the process of neurogenesis in more detail to see if we could detect quantitative defects in neuron formation. First we compared the number of NSCs present in the SGZ of the dentate gyrus of 2-month old Nestin-Cre/Gmnnfl/fl and control mice. The mice were injected with bromodeoxyuridine (BrdU) and 24 hours later the brains were fixed, sectioned, and stained with anti-BrdU antibodies. There was no significant difference in the number of BrdU+ NSCs in the dentate gyri of Nestin-Cre/Gmnnfl/fl and control mice (Figure 3A and 3B). We also performed a pulse-chase experiment to monitor the differentiation of NSCs into neurons. Adult 7 week-old mice were injected daily with BrdU for 12 days, then 9 weeks later their brains were fixed, sectioned, and stained with antibodies against BrdU and the neuronal marker NeuN (Figure 3C). We counted the number of newly born neurons (NeuN+BrdU+ cells) in the dentate gyrus using confocal microscopy. We found no difference in the number of BrdU”NeuN” cells in the dentate gyri of Nestin-Cre/Gmnnfl/fl or control mice. These results indicate that in Nestin-Cre/Gemnnfl/fl brains the number of NSCs is normal and their rate of differentiation into neurons is normal.

Table 2. Fertility of Nestin-Cre/Gmnnfl/fl Mice.

| Genotype          | Sex | Litter size (mean ± SD) | # of Litters | # of Animals | P value |
|-------------------|-----|-------------------------|--------------|--------------|---------|
| Control           | M or F | 7.8±2.0             | 19           | 6            | --      |
| Nestin-Cre/Gmnnfl/+ | F    | 7.4±1.3               | 5            | 3            | 0.700   |
| Nestin-Cre/Gmnnfl/+ | M    | 5.3±2.0               | 9            | 4            | 0.007   |
| Nestin-Cre/Gmnnfl/fl | F    | 5.2±2.9               | 15           | 4            | 0.002   |
| Nestin-Cre/Gmnnfl/fl | M    | 5.3±2.6               | 19           | 3            | 0.006   |

doi:10.1371/journal.pone.0017736.t002
To test whether Geminin regulates the differentiation of NSCs in vitro, we used primary neurosphere cultures from neonatal Nestin-Cre/Gmnnfl/fl and control brains. Neurosphere cultures could easily be established from both genotypes. To induce differentiation, neurospheres were cultured in a low concentration of growth factors then stained with specific antibodies to identify differentiated neurons (TuJ1+ cells) or astrocytes (GFAP+ cells). We found that Nestin-Cre/Gmnnfl/fl and control neurospheres produced equal numbers of GFAP+ and TuJ1+ cells (Figure 4).

Finally, we examined the effect of Geminin deletion on the cell cycle in neurospheres. Both Nestin-Cre/Gmnnfl/fl and control neurospheres grew at identical rates (Figure 5A), and the distribution of cells in different phases of the cell cycle was indistinguishable between the two types of cells (Figure 5B). In particular, the percentage of Nestin-Cre/Gmnnfl/fl cells that had excessive DNA contents >4n was the same as in Gmnnfl/fl littermate controls, and both genotypes had the same proportion of cells with a G2/M DNA content (Figure 5C). These results indicate that Gmnnfl/fl neurosphere cells do not over-replicate their DNA and display no obvious cell cycle defects.

Discussion

Geminin is widely expressed in the developing brain and is thought to have two functions in controlling the development of the central nervous system. Early in development, Geminin acts as a neural inducer: over-expression of the protein causes expansion of the neural plate at the expense of the epidermis [5]. Later in development, Geminin inhibits neuronal differentiation by inhibiting the recruitment of a SWI/SNF chromatin-remodeling complex to neuron-specific promoters [7]. Geminin also limits the extent of DNA replication during the cell cycle by binding and inhibiting the essential replication factor Cdt1 [27]. Because of its effects on both the cell cycle and on gene expression, it has been proposed that Geminin maintains neural stem cells in an undifferentiated state while they proliferate [4,13,14].

In this study we rigorously examined the role of Geminin in vertebrate neurogenesis by constructing a strain of Nestin-Cre/Gmnnfl/fl mice in which Geminin was deleted from neural stem cells early in embryonic development. To our surprise, we could detect no defects in neurogenesis in Gemininfl/fl brains. Nestin-Cre/Gmnnfl/fl mice had normal viability and displayed no obvious neurological or neuroanatomical abnormalities. Quantitative assays of neural stem cell division and differentiation patterns also revealed no abnormalities. Previous studies have shown that mice with defects in adult neurogenesis have well defined and unmistakable phenotypes. For example, mice that carry a NSC-specific deletion of the chromatin remodeling factor Mll1 develop growth retardation and ataxia during the second week of life and die around 4 weeks of age [28]. Mice with a postnatal deletion of both Numb and Numb-like show defects in lateral ventricle integrity and SVZ neuroblast survival [29]. We conclude that Geminin is largely dispensable for adult neurogenesis and for embryonic neurogenesis after e14.5. Our results are consistent with what has
Figure 3. Normal Hippocampal Neurogenesis in Nes-Cre/Gmnnfl/fl Mice. (A) Adult mice were injected with BrdU 24 hours before sacrifice. Adjacent sections of the hippocampus were stained with either anti-BrdU antibodies or with DAPI. Scale bar, 50 μM. (B) Quantification of the number of BrdU(+) cells in the subgranular zone of the hippocampus in Nes-Cre/Gmnnfl/fl mice and control littermates. n = 4 for controls; n = 3 for Nes-Cre/Gmnnfl/fl mice. (C) 7 week-old mice were injected with BrdU for 12 days then sacrificed 9 weeks later. Sections of the hippocampus were stained with anti-BrdU antibodies and with anti-NeuN antibodies. The Z-stack image on the right shows BrdU(+), NeuN(+), and BrdU+NeuN+ cells. Scale bar, 50 μM. (D) Quantification of the number of BrdU(+)NeuN(+) cells in the hippocampus of Nes-Cre/Gmnnfl/fl mice and control littermates. n = 2 for both genotypes.
doi:10.1371/journal.pone.0017736.g003

Figure 4. Normal Differentiation of Nes-Cre/Gmnnfl/fl Neurosphere Cells. (A) Representative images of TuJ1(+) neurons and GFAP(+) astrocytes derived from Nes-Cre/Gmnnfl/fl and control neurosphere cells. Scale bar, 50 μM. (B) Quantification of the number of TuJ1(+) neurons and GFAP(+) astrocytes derived from Nes-Cre/Gmnnfl/fl and control neurosphere cells. HPF, High Powered Field.
doi:10.1371/journal.pone.0017736.g004
been reported for Geminin<sup>+/−</sup> Drosophila larvae, most of which have normal neural architecture [15].

It is unlikely that the mouse genome encodes a protein that can substitute for Geminin, which is a single copy gene. The 41 kD GEMC1 protein (GEMinin Coiled-coil domain containing protein 1) includes a 49 amino-acid sequence that is ∼30% identical to Geminin’s coiled coil, but GEMC1 serves a different function than Geminin and is actually required for DNA replication [30]. Mice carrying a homozygous deletion of the Geminin gene die at the early blastula stage, as soon as the maternal supply of Geminin is exhausted [19,20]. We have also observed strong phenotypes when Geminin is deleted from other types of cells using other types of Cre drivers. For example, deleting Geminin profoundly affects the differentiation pattern of bone marrow cells; the production of red blood cells is virtually abolished while the production of megakaryocytes is greatly expanded [22].

Geminin’s role in controlling the extent of DNA replication has been well documented by many different laboratories using many different experimental systems [11,27]. It is therefore somewhat surprising that DNA replication and cell division appear completely normal in Gmnn<sup>+/−</sup> neurosphere cells. These cells probably cycle normally in the absence of Geminin because of redundant Geminin-independent mechanisms that limit the extent of DNA replication. For example, in addition to being inhibited by Geminin, Cdt1 is also destroyed by ubiquitin-dependent proteolysis during S phase, and the degradation is coupled to the initiation of DNA replication[31]. In order to induce over-replication in Xenopus egg extracts, it is necessary to both remove Geminin and inhibit the proteolysis of Cdt1 [17,31,32]. The requirement for Geminin to prevent re-replication seems to vary depending upon the type of cell and upon conditions. For example, Gmnn<sup>+/−</sup> white blood cells over-replicate their DNA when stimulated with growth factors [22], and Gmnn<sup>+/−</sup> T lymphocytes show cell cycle defects when stimulated to proliferate in vitro [33].

Some of the previous studies that implicated Geminin in neurogenesis were based on over-expressing the protein, which may cause non-physiological effects [5,15]. Others employed cultured cell lines, which may not reproduce all the characteristics of in vivo NSCs [7]. Some of the previously observed effects on neurogenesis may have been caused by cell cycle abnormalities rather than a specific effect of Geminin on neural differentiation.

It remains possible that Geminin is required for very early neural induction. The neural plate becomes visible as distinct anatomical structure at e7.5 and the neural tube becomes completely closed by e10 [34]. Both these events occur several days before Nestin-Cre mediated recombination is complete. Even so, Nestin-Cre-mediated recombination has been detected as early as e7.5, and extensive recombination has occurred in the midbrain and hindbrain by e9.5 [24,25]. Our targeting construct did not delete the sequences encoding the small “neuralizing domain” found near Geminin’s amino terminus. Expression of this fragment in Xenopus embryos induces neural tissue as efficiently as full-length Geminin [5]. Although we could detect transcripts encoding this domain in Nestin-Cre/Gmnn<sup>fl/fl</sup> mice, we could not detect a new fusion protein in Gmnn<sup>fl/fl</sup> brains (Figs. S2 and S3). Geminin deficiency might cause defective neurogenesis in strains with a different genetic background. It is also possible that Nestin-Cre/Gemfl/fl mice will prove to have a subtle neurological or neuroanatomical defect than could not be detected by the methods employed here. Such a defect might explain the mild decrease in fertility we observed in Nestin-Cre/Gmnn<sup>fl/fl</sup> mice. Nevertheless, our results clearly indicate that Geminin is largely dispensable for mammalian neurogenesis.

Materials and Methods

Ethics Statement

All animal work was performed according to the protocol approved by the Northwestern University Animal Care and Use Committee (Protocol # 2009-0911).

Mouse Breeding

The construction of the Gmnn<sup>−/−</sup> allele was described previously [22]. Nestin-Cre mice [B6.Cg-Tg(Nes-Cre)1Kln/J, Jackson Labs Strain 3771] were kindly provided by Dr. Anjen Chenn.
(Northwestern University). For most experiments, Nes-Cre/Gmnntfl/fl mice were mated to Gmnntflo/flo mice to produce experimental Nes-Cre/Gmnntflo/flo mice and control Gmnntflo/flo littermates. To obtain embryos at defined embryonic stages, timed matings were performed in which male and female mice were caged together overnight then separated immediately after the mating plug appeared. Genotypes were determined by PCR of tail DNA. The following primers were used for amplification: Nes-Cre forward (5'-gccctggatcaggctgtgcatg-3'), Nes-Cre reverse (5'-ggtccggagtgggaggcaacattcc-3'), Geminin forward (5'-gctacagctcagttctcagttctg-3'), GemininWT reverse (5'-cateagctcagttctcagttctg-3'), and Gemininα reverse (5'-gctacagctcagttctcagttctg-3').

Antibodies
Affinity-purified anti-mouse Geminin antibody was obtained by immunizing rabbits with recombinant his-Geminin and purifying antibodies from serum over a column of recombinant his-Geminin coupled to cyanogen bromide sepharose beads [22,35].

Real Time PCR
RNA was isolated from fresh tissue using Trizol reagent (Invitrogen). cDNA synthesis was carried using a standard kit (Ambion) and RT-PCR was performed using an Applied Biosystems 7500 Fast Real Time PCR System. Primers and fluorescently labeled probes for RT-PCR were designed using Primer Design software (Applied Biosystems). All RNA levels were normalized to the amount of 18S ribosomal RNA in each sample. For Figure 2, Geminin RNA primer sequences were 5'-acggatgctaggccgtgtac-3' (forward), 5'-gtgaagaatagtcctgtccc-3' (reverse), and 5'-aagaactgccgtgctcc-3' (probe). 18S RNA primer sequences were 5'-aagagctctcttgctgctact-3' (forward), 5'-gctacagctcagttctcagttctg-3' (reverse), and 5'-aagaactgccgtgctcc-3' (probe). For PCR of exons 3 and 4 (Figure S3), Geminin primers were 5'-ggaagataatgtgccctcct-3' (forward) and 5'-ccacagctgctgctgctc-3' (reverse). SYBR green was used instead of a fluorophore and SYBR Green I was used as a melting curve indicator. SYBR green was used instead of a fluorophore and SYBR Green I was used as a melting curve indicator.

Histology and Immunohistochemistry
For pulse labeling, mice were injected intra-peritoneally with three doses of bromodeoxyuridine (Invitrogen B23151, 10 mg/ml in PBS, 50 mg/kg) separated by two hours. Brains were fixed 24 hours after the last BrdU dose by transcardial perfusion with a medium containing 1 mg/ml BrdU, 2% B27 supplement, penicillin, streptomycin, and 2% NGS (all from Invitrogen), and 20 ng/ml Epidermal Growth Factor (EGF, BD Biosciences). After 5 days the brains were fixed 9 weeks after the final dose. Free-floating sections were blocked in 1X PBS, 4% NDS, and 0.25% Triton X-100 (TX-100) and stained overnight at 4°C. For Nissl staining, paraformaldehyde-fixed brains were handled differently. Genotypes were determined by PCR of tail DNA. The following primers were used for amplification: Nes-Cre forward (5'-gccctggatcaggctgtgcatg-3'), Nes-Cre reverse (5'-ggtccggagtgggaggcaacattcc-3'), Geminin forward (5'-gctacagctcagttctcagttctg-3'), GemininWT reverse (5'-cateagctcagttctcagttctg-3'), and Gemininα reverse (5'-gctacagctcagttctcagttctg-3').

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Neurosphere Culture and Differentiation
Primary neurosphere cultures were obtained from newborn (P0) mice as described [37,38,39]. Neurospheres were grown in DMEM/F-12 medium containing N2 supplement, B27 supplement, penicillin, streptomycin, and 2% NGS (all from Invitrogen), and 20 ng/ml Epidermal Growth Factor (EGF, BD Biosciences). After 5 days the cells were dissociated with trypsin, trittrated, and re-seeded at a density of 5 X 10^5 cells/ml. To calculate the doubling time, cells were trypsinized and re-counted three days later. For cell cycle analysis, the cells were stained with propidium iodide using standard procedures[40]. To induce differentiation, dissociated neurosphere cells were seeded onto poly-D-lysine coated coverslips (Sigma, 20 μg/ml for >1 hour) in medium containing 1 ng/ml EGF. The medium was changed after 3 days, and after 7 days the coverslips were washed with PBS and fixed in 1X PBS/4% paraformaldehyde. For antibody staining, coverslips were washed with 1X PBS/0.1% TX-100 (PBT) then incubated overnight at 4°C in PBS/0.5% NGS containing either anti-Tuj1 antibody (Mouse IgG2b, clone SDL3D10, Sigma #T8660) or anti-GFAP antibody (Mouse IgG1, clone GA5, Sigma #G3893). The secondary antibodies were Alexa 488-conjugated goat anti-mouse IgG2b (Invitrogen # A21141) and Cy3-conjugated goat anti-rabbit IgG (Jackson Immuno Research #111-165-144) respectively.

Supporting Information
Figure S1 Protein Domains Deleted in Gmnntflo/flo Mice. (Top) Map of the Geminin locus with color-coded exons. Deleted exons are enclosed by the rectangle. (Bottom) Exon boundaries mapped onto the domains of the Geminin protein. D-box, destruction box; NLS, bipartite Nuclear Localization Signal; Neural, neutralizing domain (underlined) which overlaps the D-box, respectively.

Figure S2 Geminin Deletion does not Generate a Detectable New Fusion Protein. The full immunoblot from Figure 2A. Biotinylated MW markers are shown on the left. (TIF)

Figure S3 Geminin Deletion does not Cause Over-Expression of Geminin RNA. RNA was isolated from undifferentiated or differentiated neurospheres from control or
Nes-Cre/Gmmnt(−/−) mice. The amount of exon 3/4-containing RNA was determined by RT-PCR. The location of the amplified fragment is indicated in Figure S1.

TIF

Acknowledgments

We thank Dr. Anjen Chen for the gift of Nestin-Cre mice.

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Author Contributions

Conceived and designed the experiments: TJM RJM. Performed the experiments: KMS ROL GB TM. Analyzed the data: TJM KMS ROL GB. Contributed reagents/materials/analysis tools: TJM JAK RJM. Wrote the paper: TJM.

Geminin Is Dispensable for Neurogenesis

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