

Normal Adult Hippocampal Neurogenesis in SRG3-overexpressing Transgenic Mice

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ABSTRACT

SRG3 (SWI3-related gene) is a core subunit of mouse SWI/SNF complex and is known to play a critical role in stabilizing the SWI/SNF complex by attenuating its proteasomal degradation. SWI/SNF chromatin remodeling complex is reported to act as an important endogenous regulator in the proliferation and differentiation of mammalian neural stem cells. Because limited expression of SRG3 occurs in the brain and thymus during mouse embryogenesis, it was hypothesized that the altered SRG3 expression level might affect the process of adult hippocampal neurogenesis. Due to the embryonic lethality of homozygous knockout mice, this study focuses on dissecting the effect of overexpressed SRG3 on adult hippocampal neurogenesis. The BrdU incorporation assay, immunostaining with neuronal markers for each differentiation stage, and immunoblotting analysis with intracellular molecules involved in survival in adult hippocampal neurogenesis found no alteration, suggesting that the overexpression of SRG3 protein in mature neurons had no effect on the entire process of adult hippocampal neurogenesis including proliferation, differentiation, and survival.

Key words: SRG3 (SWI3-related gene), SWI/SNF complex, adult hippocampal neurogenesis, proliferation, differentiation, survival

INTRODUCTION

Mammalian SWI/SNF complex, also known as BAF (Brg/Brm-associated factor) complex, is a >2 MDa multiprotein chromatin remodeling complex composed of at least 10 elements and contain BRG or BRM subunit as a central core ATPase subunit.

All subunits of SWI/SNF are well conserved from yeast to human, reflecting the functional significance of the SWI/SNF complex (Tsukiyama, 2002). SWI/SNF complex is involved in various biological events such as tumor suppression, regulation of immune system, neural development and plasticity (Roberts and Orkin, 2004). A diverse composition of SWI/SNF complex subunits determines which transcription factors or coactivators/corepressors activate or repress target genes. For example, transcription factors including C/EBP, c-Myc and c-Fos/c-Jun activate target genes by cooperating

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with the SWI/SNF complex (Cheng et al., 1999). Likewise, the SWI/SNF complex represses neuron-specific genes in nonneuronal cells through the association with neuron restrictive silencer factor (NRSF) and its corepressors, mSin3A and CoREST (Battaglioli et al., 2002; Watanabe et al., 2006).

SRG3 (SWI3-related gene) is a recently found subunit of mouse SWI/SNF complex and has a high homology to a yeast transcriptional activator SWI3 and human BAF155 proteins (Jeon et al., 1997). Although SRG3 is widely expressed during early mouse embryogenesis, its main expression is limited to thymus and brain around E18.5, suggesting a role for SRG3 in the immune and the nervous system (Kim et al., 2001). SRG3 heterozygote mice frequently show exencephaly (Kim et al., 2001). Interestingly, the expression pattern of SRG3 overlaps with that of Brg1, and Brg1 heterozygote mice also have high incidence of exencephaly (Bultman et al., 2000). Brg1 is constantly expressed during differentiation of neural progenitor cells (NPC) and P19 embryonic carcinoma cells (Machida et al., 2001). It is also required for the proneural activities of *Ngnr1*, *NeuroD* and for neuronal differentiation in both *Xenopus* and P19 (Seo et al., 2005). In zebrafish, Brg1 deficiency impairs neural crest induction and has defects in neurogenesis (Eroglu et al., 2006). Moreover, SRG3 interacts directly with BRG1 through SANT domain (Sohn et al., 2007), suggesting that SRG3 might presumably be functional in neural development.

The whole step of adult neurogenesis is subdivided into proliferation, cell cycle exit, differentiation and functional maturity (Ehninger and Kempermann, 2008). Each step is tightly controlled by a variety of extrinsic and intrinsic factors, most of which remain uncharacterized (Zhao et al., 2008). Intriguingly, SWI/SNF chromatin remodeling complex acts as an important endogenous regulator in the proliferation and differentiation of mammalian neural stem cells (Lessard et al., 2007). The switch of subunits from BAF45a and BAF53a to BAF45b, BAF45c, and BAF53b is essential for the transition from neural stem/progenitors to postmitotic neurons, suggesting that the genetic disturbance of only one subunit might disorganize the entire process of adult hippocampal neurogenesis. Therefore, in this study, the functional significance of SRG3 and how

chromatin remodeling complex contributes to maintain the integrity of adult hippocampal neurogenesis was approached by analyzing the effect of overexpressed SRG3 on adult hippocampal neurogenesis.

MATERIALS AND METHODS

Animals

The knock-in transgenic (TG) mice overexpressing SRG3 were provided by Dr. R.H. Seong (Seoul National University). These mice are heterozygous for overexpressed SRG3 and produced and maintained in FVB background. The mouse SRG3 gene is designed to be overexpressed in neurons of forebrain regions by mouse CaMKII (calcium/calmodulin-dependent protein kinase II) α promoter (11.5 kb) (Benson et al., 1992; Jacobs et al., 1993; Mayford et al., 1996). The transgenic offspring was generated by crossing heterozygous transgenic mice with wild type FVB mice and genotyped by PCR using sense primer 5'-GACTA GACCA AACAT CTACC TC-3', antisense primer 5'-GTCAA CTGAG CGACT TGGAT C-3'. Mice were bred and maintained under pathogen-free conditions, and experiments were performed in accordance with institutional guidelines of Hanyang University Veterinary Committee. To evaluate the proliferation in subgranular zone (SGZ) of adult hippocampus, 5-bromo-2'-deoxyuridine (BrdU) (100 mg/kg bodyweight, Sigma-Aldrich) was daily administered intraperitoneally to twenty week-old male TG mice for 3 days and then animals were sacrificed 2 h after the last injection.

Western blotting

Immunoblotting followed conventional method. Briefly, hippocampal and cortical tissues were immediately collected from an isolated brain on ice after mice were sacrificed by cervical translocation. Tissues were homogenized using a Dounce homogenizer and lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1% Deoxycholate) including protease inhibitor cocktail (Sigma-Aldrich)/phosphatase inhibitor cocktail (Sigma-Aldrich). The kinds and dilutions of primary antibodies are as follows; anti-BAF155 (1 : 500, Santa Cruz Biotechnology Inc), anti-phospho-

CaMKII (Thr286) (1 : 1,000; Cell Signaling Technology), anti-CaMKII (1 : 500, Cell Signaling Technology), anti- β -actin (GeneTex), anti-p-Trk (1 : 400, Cell Signaling Technology), anti-TrkB (1 : 600, Cell Signaling Technology), anti-p-ERK1/2 (1 : 1,000, Cell Signaling Technology), anti-ERK1/2 (1 : 1,000, Cell signaling Technology), anti-p-CREB (Ser133) (Abcam Inc, Cambridge, MA, USA), anti-CREB (1 : 500, Cell Signaling Technology), anti-BDNF (1 : 500, SantaCruz Biotechnology), anti-VEGF (1 : 200, SantaCruz Biotechnology), anti-Angiopoietin-1 (1 : 200, SantaCruz Biotechnology), anti-Tuj1 (1 : 1,000, Covance, Berkeley, CA, USA), anti-Brg1 (1 : 1,000, Sigma-Aldrich).

Semiquantitative RT-PCR and real time RT-PCR

Sample tissues were collected in the same way as mentioned in immunoblotting and homogenized in Trizol reagent (Life Technologies Inc, Rockville, MD, USA). One μ g of total RNA was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen). The following PCR primers were employed; sense 5'-CTGCATCTTCGATCACC TCA-3', antisense 5'-CTCCCTTCCAGCACTAGCAC-3' for mouse SRG3; sense 5'-CGGAACCGC TCATTGCC-3', antisense 5'-ACCCACACTGTGCCCATCTA-3' for β -actin sense.

Immunohistochemistry

After anesthetized with Ketamine/Xylazine cocktail, animals were intracardially perfused with cold normal saline and, in turn, ice-cold 4% paraformaldehyde in PBS. Isolated brains were postfixed in 4% paraformaldehyde in PBS overnight at 4°C, and then fully dehydrated in 30% sucrose in PBS. Slices were prepared as 30- μ m-thick. For immunoperoxidase staining, free-floating sections were first treated with 1% H₂O₂ for 15 min and washed three times with PBS. After incubated in blocking buffer (0.3% Triton X-100/5% normal serum in PBS) for 1 h, they were placed in primary antibody solution overnight at 4°C. Primary antibodies used are goat anti-BAF155 antibody (1 : 200, Santa Cruz Biotechnology), anti-p-CREB (Ser133) (1 : 200, Abcam Inc, Cambridge, MA, USA), and anti-BDNF (1 : 500, SantaCruz Biotechnology). Biotinylated anti-goat IgG antibody (Vector Laboratories, Burlingame, CA, USA)

was used as a secondary antibody. Immunoreactivity was detected by Vectastain ABC Kit (Vector Laboratories) and DAB peroxidase substrate kit (Vector Laboratories). For BrdU immunofluorescent staining, sections were incubated in 50% formamide/50% 2X SSC buffer (0.3 M NaCl and 0.03 M sodium citrate) at 65°C for 2 h and rinsed with 2X SSC buffer for 10 min. They were then incubated for 30 min in 2 N HCl at 37°C and rinsed in 0.1 M boric acid (pH 8.5) for 15 min. After PBS wash, they were incubated in blocking buffer for 1 h and rat anti-BrdU antibody (1 : 200, Abcam) solution overnight at 4°C. For Tuj-1 (1 : 400, Covance), PSA-NCAM (1 : 100, Chemicon International), NeuN (1 : 500, Chemicon International), MAP2 (1 : 400, Sigma-Aldrich) and Dcx (1 : 200, Santa Cruz Biotechnology), the incubation in formamide/2X SSC buffer and 2 N HCl was skipped. Cy3-conjugated anti-IgG (Jackson ImmunoResearch, West Grove, PA, USA) and Alexa Fluor 488-conjugated anti-IgG (Molecular Probes, Eugene, OR, USA) were used as secondary antibody. The samples were mounted with Vectashield (Vector Laboratories).

RESULTS

SRG3 gene is overexpressed specifically in the forebrain neurons of SRG3 TG mice

To confirm whether SRG3 overexpression transgenic mice are working as genetically designed to be driven by CaMKII α promoter, which expresses in postmitotic neurons in forebrain regions like neocortex, hippocampus and amygdala (Benson et al., 1992; Jacobs et al., 1993), SRG3 TG mice were examined to see forebrain-specific overexpression of SRG3. The gross brain morphology and size of 20-week-old male SRG3 TG was not different from that of wild type (Fig. 1A), suggesting that overall development of brain is not affected by the overexpression of SRG3. Both hemispheres were symmetrical, and the size of cerebellum and olfactory bulb where CaMKII α promoter is not activated also looks similar in SRG3 TG and wild type (wt) animals. The normal development of neocortex and the hippocampal subregions like dentate gyrus (DG), CA3 and CA1 of SRG3 TG mouse was confirmed by DAPI labeling on a coronal section at postbregma -2.0 mm (Fig. 1A).

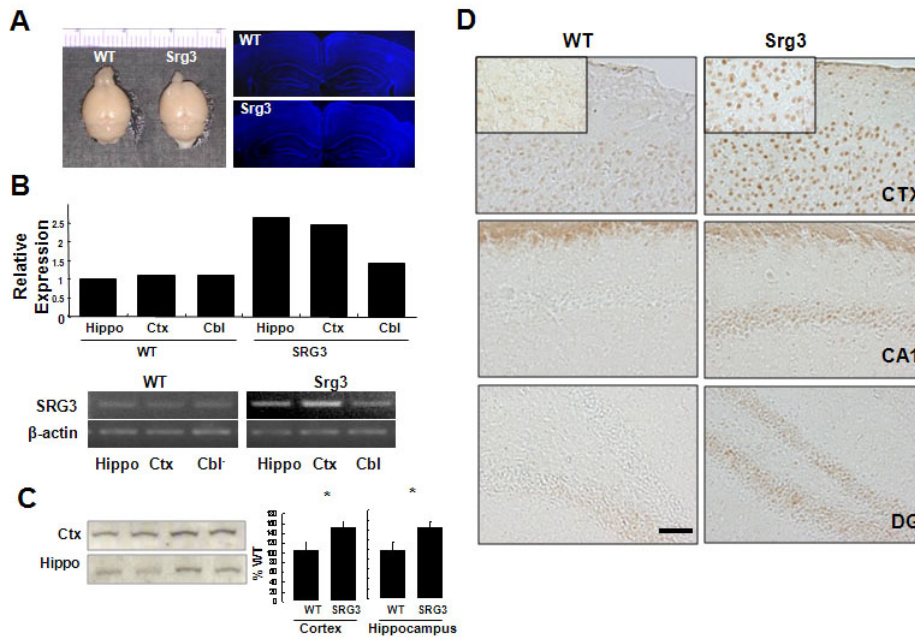


Fig. 1. *SRG3* gene is overexpressed in the forebrain of *SRG3* TG mice. (A) Gross morphology and DAPI-stained coronal section at postbregma -2.0 mm of wt and *SRG3* TG mice. There was no detectable difference between *SRG3* and wt mice brain. (B) The expression of *SRG3* mRNA increased more than twice in hippocampus and cortex, but not in cerebellum, of *SRG3* TG mice, when normalized to β -actin mRNA ($n=1$ per group). (C) Western blotting using antibody against BAF, a human homologue of *SRG3*, showed that the *SRG3* protein level also significantly increased in forebrains of *SRG3* TG. Values are expressed as a percentage of expression in wt mice. The bars represent average *SRG3* protein expression \pm SEM ($n=3$ per group). $*p < 0.05$, Student's *t* test. (D) *SRG3* was detected in nuclei (inset) and *SRG3* TG mice displayed more intense signals in forebrain. Scale bar = $100 \mu\text{m}$.

The expression of *SRG3* was evaluated at the levels of mRNA and protein. Real-time RT-PCR showed the forebrain specific overexpression of *SRG3* gene (Fig. 1B, hippocampus, 2.6-fold increase; cortex, 2.4-fold increase; cerebellum, 1.3-fold increase; respectively compared with wt, $n=1$). The *SRG3* protein level revealed by western blotting using antiserum against BAF155, a human homologue of *SRG3*, also significantly increased in *SRG3* TG mice compared to in wild type (mean \pm SEM; hippocampus, $143.7 \pm 5\%$ vs wild; cortex, $137.9 \pm 5.6\%$ vs wild, $*p < 0.05$ respectively, $n=3$ per group) (Fig. 1C). In order to compare the expression level of *SRG3* protein in individual neurons, immunoperoxidase activity against *SRG3* was examined in brain slices from both groups. The *SRG3* signal was detected in the nuclei (Fig. 1D inset) and it displayed more intense signals in *SRG3* TG mice forebrain region than in wt (Fig. 1D). These results consistently demonstrate that the genetic manipulation for a *SRG3* overexpression TG mouse is reliably working, showing an increased expression pattern for *SRG3* mRNA and

protein in forebrain neurons.

Proliferation of neural progenitor cells in SGZ is not affected by the overexpression of SRG3

To determine whether the overexpression of *SRG3* causes an altered pattern in the proliferation of neural progenitor cells, the number of newly generated progenitor cells in SGZ was examined by the incorporation of BrdU. Although *CaMKII α* promoter does not operate in proliferating cells, the neural progenitor cells in the DG of adult hippocampus can be affected indirectly by post-mitotic neurons (Chen et al., 2007) overexpressing *SRG3* gene by *CaMKII α* promoter. BrdU immunopositive (BrdU+) cells were counted within SGZ throughout the DG of both genotypes (Fig. 2A). Some BrdU+ cells were found in clusters like those in the previous reports (Yagita et al., 2001; Zhu et al., 2005; Hodge et al., 2008). The total number of BrdU+ cells located in the SGZ (mean \pm SEM; wt, $1,099 \pm 63/\text{mm}^3$ vs. *SRG3* TG, $1,024 \pm 59/\text{mm}^3$, $p > 0.1$, $n=4$ per group) was not statistically different between wt and *SRG3* TG mice (Fig. 2B),

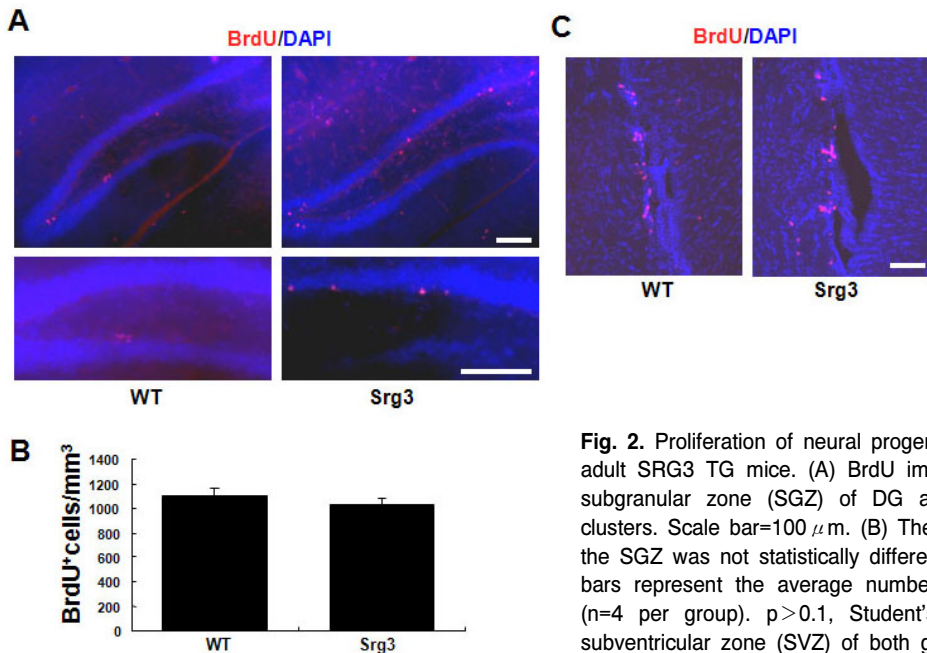


Fig. 2. Proliferation of neural progenitor cells in SGZ/SVZ is not changed in adult SRG3 TG mice. (A) BrdU immunopositive cells were detected in the subgranular zone (SGZ) of DG and some BrdU+ cells were found in clusters. Scale bar=100 μ m. (B) The total number of BrdU+ cells located in the SGZ was not statistically different between wt and SRG3 TG mice. The bars represent the average number of BrdU+ cells/mm³ of granule layer (n=4 per group). $p > 0.1$, Student's *t* test. (C) BrdU immunostaining on subventricular zone (SVZ) of both genotypes. Scale bar=100 μ m.

indicating that the proliferation was not affected by the overexpression of SRG3.

Cell proliferation in the subventricular zone (SVZ) of a lateral ventricle was also examined (Gould, 2007). Though CaMKII α promoter operates only in matured neurons, a possible effect of the overexpression of SRG3 on the proliferation pattern of SVZ was needed to be checked when considering of SVZ as another important niche for adult neurogenesis. However, no difference in the proliferation rate in SVZ was found in both genotypes (Fig. 2C).

Neuronal development looks normal in the hippocampus and cortex of SRG3 TG mice

To determine whether early neuronal development is normally organized in SRG3 TG mice, postmitotic differentiation in a hippocampal region was immunostained with early neuronal markers. During postmitotic and neuronal differentiation stage in adult hippocampal neurogenesis, most newborn neurons are destined to die, but only survived neurons mature to integrate into a functional neural circuit (Tashiro et al., 2007). Previous studies reported that cells committed to a neuronal lineage start to express doublecortin (Dcx) (Francis et al., 1999) and the polysialylated embryonic form of the neural cell adhesion molecule (PSA-NCAM), right after they exit out of the cell cycle (Fukuda et al.,

2003). The total number of Dcx immunoreactive (Dcx+) cells in the hippocampi of SRG3 TG mice was not significantly changed compared to that of wt (mean number \pm SEM: wt, 9,974 \pm 450/mm³; SRG3 TG, 9,133 \pm 701/mm³, $p > 0.1$, n=4 per group), suggesting that neuronal differentiation of neural progenitor cells in the hippocampus of adult SRG3 TG mice seems normal during early postmitotic period (Fig. 3A). As Dcx expression is temporally overlapped with PSA-NCAM expression on the time course of development, the immunoreactivity of PSA-NCAM+ cells on randomly chosen brain slices from both genotypes cells was compared (Nacher et al., 2001). It turns out that the number and the signal intensity of PSA-NCAM+ cells was apparently similar to result of Dcx immunostaining and there is no difference between SRG3 TG and wt groups.

In order to know what effect the overexpressed SRG3 on mature neurons has on the maturation of the hippocampi and cortex, Tuj-1 was used as a neuroblast marker as well as a fully differentiated neuronal marker to examine SRG3 TG mice. Neuron-specific class III β -tubulin (Tuj-1) has been found to be expressed in newly generated immature postmitotic neurons (Menezes and Luskin, 1994) and in mature neurons (Ambrogini et al., 2004) (Fig. 3C). Tuj-1 was detected in somata and neurites of neurons within cortex, CA1 and DG.

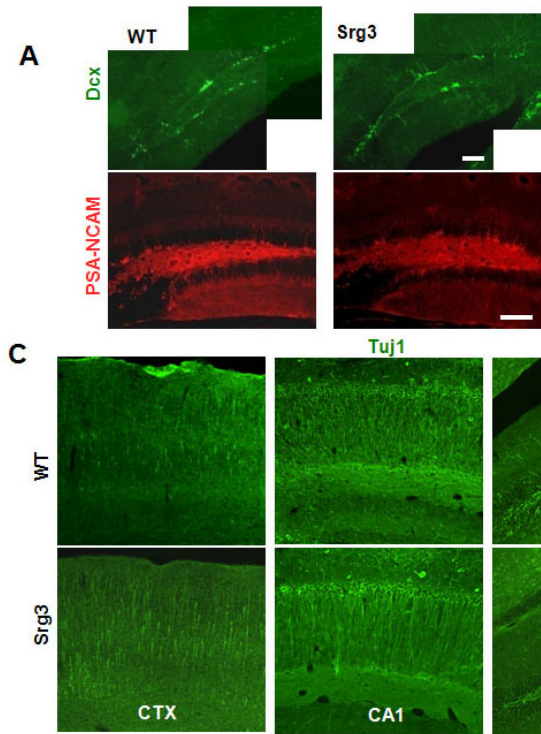


Fig. 3. Early neuronal development is normal in adult SRG3 TG mice. (A) Dcx and PSA-NCAM immunolabeling on the DG of both genotypes. Scale bar=100 μ m. (B) The total number of Dcx immunopositive (Dcx⁺) cells in the DG of SRG3 TG mice was not significantly different from that of wt mice. Each bar represents the average number of Dcx⁺ cells/mm³ of granule layer (n=4 per group). $p > 0.1$, Student's *t* test. (C) Immunostaining of Tuj-1, another early neuronal marker, in forebrain regions showed no observable difference between both genotypes. Mature neurons within each forebrain regions investigated were also immunopositive for Tuj-1, for Tuj-1 is still expressed in mature neurons. Scale bar=100 μ m.

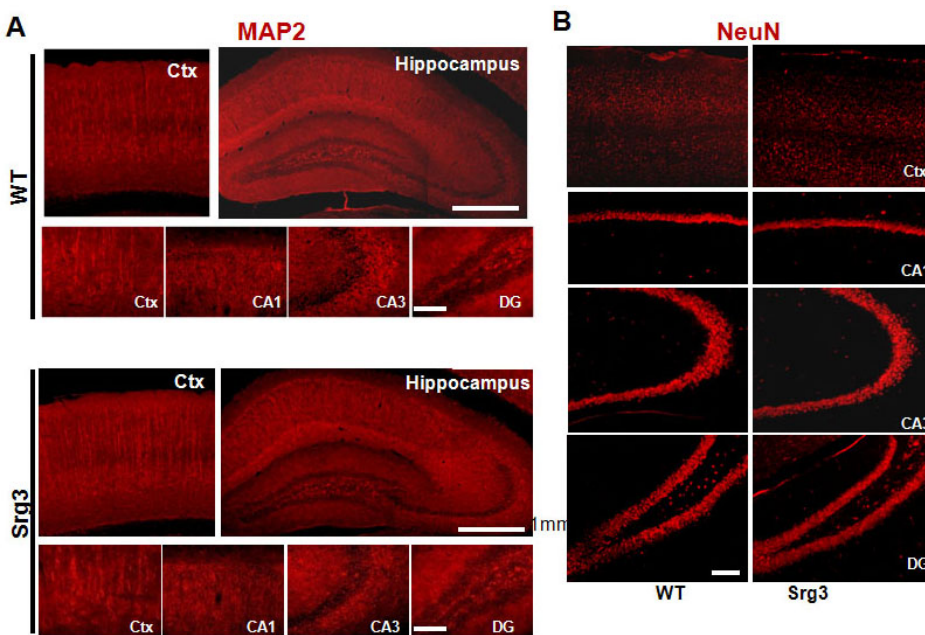


Fig. 4. Late neuronal development is normal in adult SRG3 TG mice. (A) MAP2 was detected on dendrites and somata of mature neurons in cortex and hippocampal subregions, and there was no apparent difference between both genotypes. (B) Nuclei were labeled by NeuN, a mature neuronal marker. The distribution and immunoreactivity in cortex and hippocampal regions showed a similar pattern between two groups. Scale bar=100 μ m.

Especially, in DG, Tuj-1 was detected in the somata of almost all granule cells and found at the neurites of some granule cells reaching the hippocampal fissure. The distribution and immunoreactivity of Tuj-1 in cortex, CA1 and DG was indistinguishable between SRG3 TG and wt animals, suggesting that the neuronal maturation in adult hippocampal

neurogenesis as well as cortical neuronal development appear normal in SRG3 TG mice.

To see whether overexpressed SRG3 affects the late neuronal differentiation process and the survival of neuronal lineage-committed cells during the late stage of adult hippocampal neurogenesis, microtubule-associated protein 2 (MAP2) and NeuN were

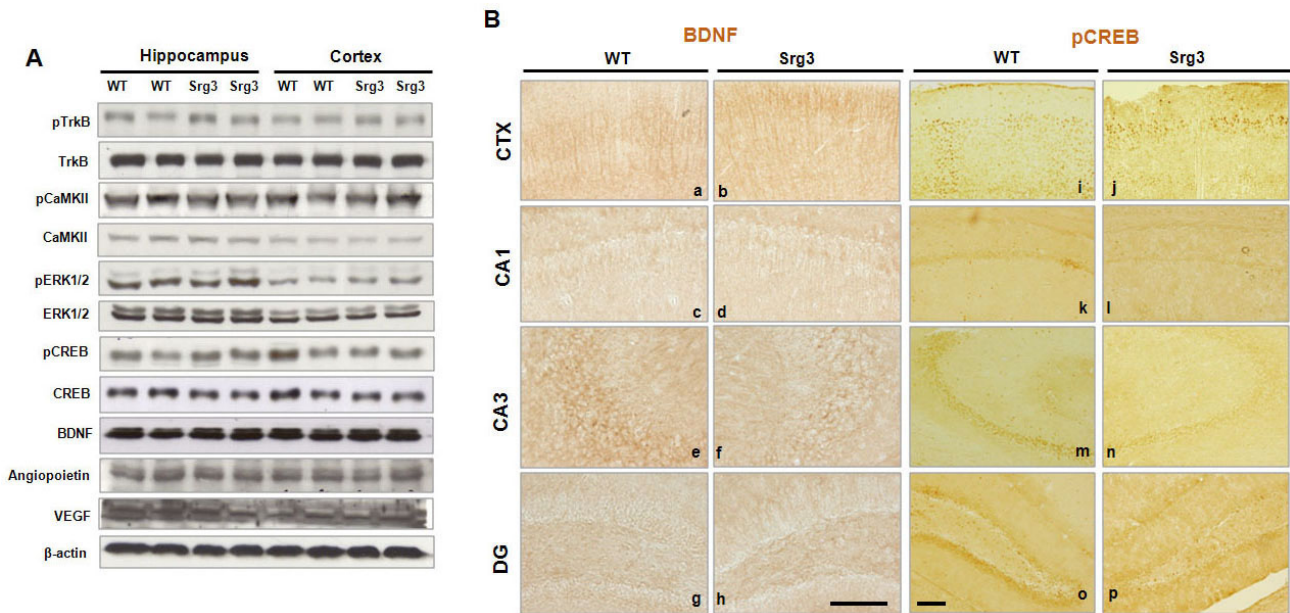


Fig. 5. Immunoblotting and immunostaining images for key signaling molecules involved in survival of newborn hippocampal neurons in both animals. (A) Western blots for signaling molecules involved in survival of newborn hippocampal neurons. Expression level of angiogenesis-related molecules such as angiopoietin1 and VEGF as well as phosphorylation level of TrkB, CaMKII, ERK1/2, and CREB were similar between both genotypes. Hippocampus and cortex were separately examined. (B) no observable difference in immunolabeling for pCREB between wt and TG mice. BDNF was weakly observed at somata and dendritic arbor of hippocampal neurons (c~h) and cortical neurons (a, b). Phosphorylated CREB (pCREB) was detected in nuclei of cells within SGZ (o, p) and across cortical layers (i, j). Scale bar=100 μ m.

immunolabeled, as MAP2 and NeuN have been known as mature neuronal markers (Menezes and Luskin, 1994). The signal was detected at dendrites and somata of neurons (Fig. 4A) as reported previously (Biranowska et al., 2000; Jalava et al., 2007). Though there were some cells in SGZ missing MAP2 immunoreactivity, they might be immature or proliferating cells. The distribution and immunoreactivity of MAP2 immunopositive (MAP2+) cells in the cortex and the hippocampus was not significantly different in SRG3 TG and wt animals (Fig. 4A). In addition, the immunolabeling of NeuN, a nuclear protein for labeling the nuclei of mature neurons (Lind et al., 2005), displayed a similar pattern in SRG3 TG and wt mice (Fig. 4B), suggesting that the late neuronal differentiation process during adult hippocampal neurogenesis and the neuronal survival remains normal in SRG3 TG mice.

The activation of intracellular signaling molecules involved in the survival of adult hippocampal neurons looks similar between SRG3 TG and wild type mice

Though the immunostaining with MAP2 and Tuj-1 results in no impaired pattern of the survival of newly generated hippocampal neurons by the overexpressed SRG3, this might not mean that the neuronal maturation is completely normal. To determine whether the neuronal survival is not altered in a subtle manner by the overexpression of SRG3, CREB activation, which is a critical step for survival of newborn neurons in a hippocampus (Nakagawa et al., 2002), was examined using western blotting and immunolabeling. ERK1/2 and CaMKII, critical mediators in activating CREB, were expressed and activated to a similar degree (Fig. 5A, pERK1/2, $104 \pm 6\%$ vs wt, $p > 0.05$; pCaMKII, $97 \pm 2\%$ vs wt, $p > 0.05$), and similarly CREB activation was not different between two groups (Fig. 5A, pCREB, $106 \pm 3\%$ vs wt, $p > 0.05$). Moreover, the expression of BDNF regulated by phosphorylated CREB and the activation of TrkB, a receptor for BDNF, were not significantly different in both

groups (Fig. 5A: BDNF, $103\pm 3\%$ vs. wt, $p > 0.05$; pTrkB, $106\pm 5\%$ vs wt, $p > 0.05$), which is consistent with the immunolabeling of each marker on tissues, implying that neuronal survival and the survival signaling is not affected by the overexpression of SRG3 in mature neurons.

To see whether the expression of VEGF and angiopoietin1 was altered by the overexpressed SRG3, the western blotting was performed with using hippocampal homogenates from each genotype, because vascular endothelial growth factor (VEGF) and angiopoietin1 are all angiogenesis-related and were reported to be linked to adult neurogenesis (During and Cao, 2006; Warner-Schmidt et al., 2008). Recently, SRG3 has been reported to be required for angiogenesis and visceral endoderm development in the yolk sac, and the expression of angiogenesis-related genes, including Angiopoietin1, Tie2, EphrinB2, Ihh and Notch1, was markedly reduced in SRG3^{-/-}Tg⁺ yolk sacs (Han et al., 2008). The expression of VEGF and angiopoietin1 was not different in both genotypes (Fig. 5A: VEGF, $104\pm 3\%$ vs wt, $p > 0.05$; angiopoietin1, $102\pm 3\%$ vs wt, $p > 0.05$), unlike the reduced expression of angiogenesis related genes by the depletion of SRG3.

To overcome the possible limitation of the western blotting missing the subtle change of the altered expression level of specific molecules which are very small portion of the whole hippocampus, the activation or expression of BDNF and CREB was examined by immunofluorescent and immunoperoxidase staining at a single cell level (Fig. 5B, C). BDNF signal was not strongly detected at somata and dendritic arbors of hippocampal neurons from both groups and rather more intensely observed in neurites of cortical neurons (Fig. 5B). The phosphorylated CREB was detected in nuclei of cells within SGZ and across cortical layers (Fig. 6C). Taken together, a line of evidence from western blotting and immunostaining with phosphorylated-CREB and BDNF clearly demonstrate that overexpression of SRG3 makes no significant impact on the expression and activation of intracellular signaling molecules involved in the survival of newborn neurons in adult hippocampus.

DISCUSSION

TG mice overexpressing SRG3 by CaMKII α promoter only in neurons of forebrain regions such as hippocampus were used to explore the role of SRG3 protein, a core subunit of SWI/SNF chromatin remodeling complex, in adult hippocampal neurogenesis *in vivo*. The overall results demonstrated that overexpression of SRG3 in mature neurons makes no significant influence on the entire process of adult hippocampal neurogenesis including proliferation, differentiation, and survival.

BrdU incorporation assay demonstrated no alteration in the proliferation within DG of SRG3 TG mice. This is consistently supported by the western blotting analysis showing that there is no difference in the expression level of neurogenic factors such as VEGF and BDNF. SRG3 is overexpressed specifically in postmitotic neurons so that its direct influence on proliferating cells within hippocampus might not be accessed. However, this might not be simply unchanged pattern caused by SRG3 overexpression. The transient increase in the number of neuronal cells in DG of SRG3 TG mice might be compensated by subsequently increased cell death and, as a consequence, the total number of early neuronal marker-positive cells went down to a normal level. However, TUNEL assay showed that cell death level was also not changed by overexpressed SRG3 (data not shown), and gross brain morphology and DAPI-coronal section staining showed neither brain atrophy nor neuronal loss, indicating there is no abnormal ratio in cell death in SRG3 TG mice.

With respect to early neuronal differentiation during adult hippocampal neurogenesis, the total numbers and expression patterns of Dcx, PSA-NCAM and Tuj-1 molecules were similar between wt and SRG3 TG mice. This might be because the expression of early neuronal marker proteins such as Dcx, PSA-NCAM precedes the CaMKII α expression (Plumpe et al., 2006; Earnheart et al., 2007). Immunolabeling with MAP2 and NeuN, late neuronal differentiation markers, also showed no difference between wt and SRG3 TG mice even though NeuN⁺ or MAP2⁺ cells would overexpress SRG3 protein by the control of CaMKII α promoter. It might imply that SRG3 overexpression in mature

neurons do not necessarily affect the expression of late differentiation markers. In this point, the down-regulated level of SRG3 might answer the issue how SRG3 might contribute to adult hippocampal neurogenesis.

It may be argued that it was an improper choice for studying the role of SRG3 in newly generated neurons, because CaMKII α promoter operates only in postmitotic cells committed to neurons. However, some reports that neural stem/progenitor cells communicate with neighboring cells like endothelial cells, microglia or astrocytes in a neurogenic niche (Ma et al., 2005) and that neurotrophic or angiogenic factors act in a paracrine manner (Emanuelli et al., 2003) suggest a possibility that the overexpression of SRG3 might affect the generation of neurons in adult hippocampus. Therefore, it might be premature to conclude that SRG3 overexpression has no significant effect on adult hippocampal neurogenesis. Rather, it might need an electrophysiological approach or exploration at the synaptic level to detect subtle change of structural plasticity possibly induced by the overexpression of SRG3.

In summary, the present study demonstrates that overexpressed SRG3 in hippocampal neurons has no influence on the entire process of adult hippocampal neurogenesis including proliferation, differentiation, and survival *in vivo*. However, this might not mean that the expression level of SRG3 has nothing to do with the adult hippocampal neurogenesis. Though the overexpression of SRG3 has no effect on the generation of neurons in an adult hippocampus, the study with the reduced level of SRG3 or conditional knock-out mice would provide interesting information to understand the role of SRG3 regarding adult hippocampal neurogenesis.

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