Sigma-1 Receptor Knockout Impairs Neurogenesis in Dentate Gyrus of Adult Hippocampus *Via* Down-Regulation of NMDA Receptors

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Keywords

Hippocampus; Neurogenesis; *N*-methyl-D-aspartate receptor; Sigma-1 receptor; γ -Aminobutyric acid.

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Received 23 February 2013; revision 24 April 2013; accepted 27 April 2013

SUMMARY

Aims: This study investigated the influence of sigma-1 receptor (σ_1R) deficiency on adult neurogenesis. **Methods:** We employed 8-week-old male σ_1R knockout ($\sigma_1R^{-/-}$) mice to examine the proliferation and differentiation of progenitor cells, and the survival and neurite growth of newborn neurons in hippocampal dentate gyrus (DG). **Results:** In comparison with wild-type (WT) littermates, the numbers of 24-h-old BrdU⁺ cells and Ki67⁺ cells in $\sigma_1R^{-/-}$ mice increased, while the number of 28-day-old BrdU⁺ cells decreased without changes in proportion of BrdU⁺/NeuN⁺ cells and BrdU⁺/GFAP⁺ cells. The neurite density of newborn neurons was slightly reduced in $\sigma_1R^{-/-}$ mice. In DG granular cells, *N*-methyl-D-aspartate (NMDA)-activated current (I_{NMDA}) and phosphorylation of NMDA receptor (NMDAR) NR2B were reduced in $\sigma_1R^{-/-}$ mice without the alteration of NR2B expression and membrane properties compared to WT mice. The NR2B antagonist abolished the difference in I_{NMDA} between $\sigma_1R^{-/-}$ mice and WT mice. The application of NMDAR agonist in $\sigma_1R^{-/-}$ mice prevented the over-proliferation of cells and reduction in newborn neurons, but it had no effects on the hypoplastic neurite. The administration of NMDAR antagonist in WT mice enhanced the cell proliferation and depressed the survival of newborn neurons. **Conclusion:** The σ_1R deficiency impairs neurogenesis in DG through down-regulation of NMDARs.

doi: 10.1111/cns.12129

Introduction

Sigma-1 receptor (σ_1R) has been implicated to be involved in learning and memory processes. It is reported that several σ_1R agonists possess anti-amnesic effects in rodents. In particular, the anti-amnesic effects induced by the novel σ_1R agonists, such as (+)-pentazocine, SA4503, and PRE-084, have been shown in β -amyloid peptide-induced, basal forebrain-lesioned and carbon monoxide-induced amnesia models and senescence-accelerated mouse [1,2]. In addition, the involvement of σ_1R in the improving cognitive effects of neurosteroids, particularly pregnenolone sulfate (PREGS) and dehydroepiandrosterone (DHEA), has been documented [3–8].

Recent studies have demonstrated that stem cells and neural progenitor cells in the hippocampal dentate gyrus (DG) retain an ability to proliferate and develop into neurons in adult mammalian species [9]. There are several reports concerning the potential contributions of newborn neurons to cognitive processes. For example, some aged rats with preserved spatial memory exhibit a higher number of newborn neurons in comparison with the aged rats with impaired spatial memory [10]. Reduced neurogenesis in

the DG is considered to be a major cause for abolishing the memory-enhancing effect of environmental enrichment [11]. Hayashi and Su [12] found the expression of σ_1R in neuronal and oligodendrocyte progenitors at moderate and high levels. The σ_1R agonists enhance the differentiation (e.g., neurite sprouting) of PC12 cells through nerve growth factor [13]. A large body of evidence has established the effects of PREGS and DHEA on the adult neurogenesis [14–16]. DHEA antagonizes the suppressive effect of corticosterone on neuronal precursor cells proliferation [17]. However, *in vivo* σ_1R -mediated actions in the processes of neurogenesis including mitosis of progenitor cells, neurite growth, and survival of newborn neurons remain largely unclear.

The connection between σ_1R and *N*-methyl-D-aspartate receptor (NMDAR) has been widely explored, mainly in studies of the NMDA-induced firing activity in the dorsal hippocampus [18]. The activation of σ_1R is reported to increase Ca²⁺ influx across NMDAR [19]. Selective σ_1R ligands enhance NMDA-induced [3H] norepinephrine release [8]. Influx of Ca²⁺ through the NMDAR channels activates a small conductance Ca²⁺-induced K⁺ current (SK channels), which in turn shunts the NMDAR responses [20]. Selective σ_1R agonists prevent SK channel opening, and

consequently increase the NMDA response [20]. Nuwayhid and Werling [21] have demonstrated that σ_1 R agonists regulate the NMDA-induced dopamine release *via* protein kinase C. In addition, σ_1 R is able to negatively modulate the GABAergic system [22]. Low concentration of PREGS inhibits presynaptic γ -aminobutyric acid (GABA) release, which is abolished by the blockade of σ_1 R [22].

To determine the σ_1 R-mediated effects on adult neurogenesis, we employed the established σ_1 R knockout (σ_1 R^{-/-}) mice [23,24] to investigate the influence of σ_1 R deficiency on the proliferation and differentiation of progenitor cells, and the survival of newborn neurons in hippocampal DG. In addition, we explored the molecular mechanisms underlying changes in the neurogenesis in σ_1 R^{-/-} mice using patch-clamp and pharmacological methods.

Materials and Methods

Animals

All animal handling procedures followed the guidelines for Laboratory Animal Research of the Nanjing Medical University. The use of animals was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. The σ_1 R knockout (σ_1 R^{-/-}) mice were generated and characterized as described previously [23]. The genotype of mice was identified by PCR using genomic DNA from mice tail. The animals were maintained in a constant environmental condition (temperature $23 \pm 2^\circ\text{C}$, humidity $55 \pm 5\%$, and 12:12 h light/dark cycle) in the Animal Research Center of Nanjing University. Eight-week-old male σ_1 R^{-/-} mice and wild-type (WT) littermates were used at the beginning of all experiments.

Immunocytochemistry Examination

Three intraperitoneal injections (i.p.) of bromodeoxyuridine (BrdU, 50 mg/kg; Sigma Aldrich, St. Louis, MO, USA) were given with the interval of 8 h. The animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and transcardially perfused with 4% phosphate-buffered paraformaldehyde. Brain coronal sections (40 μm) were cut using a vibrating microtome. Every 5th free-floating sections were treated with 2 M HCl at 37°C for 30 min. For BrdU immunostaining, the sections were treated with 3% normal goat serum for 45 min, and then incubated in mouse monoclonal anti-BrdU antibody (1:1000; Millipore, Billerica, MA, USA) at 4°C overnight. After several PBS rinses, the sections were incubated in biotin-labeled goat anti-mouse IgG antibody (1:500; Bioworld Technology, Inc., St. Louis Park, MN, USA) under agitation for 2 h. Immunoreactivities were visualized by avidin–biotin–horseradish peroxidase complex (ABC Elite; Vector Laboratories, Inc., Burlingame, CA, USA) using 3,3'-diaminobenzidine (DAB; Vector Laboratories). For BrdU and NeuN or antigenial fibrillary acidic protein (GFAP) double immunostaining, the sections were incubated with rat monoclonal anti-BrdU antibody (1:200; Millipore), which was revealed using CY3-labeled anti-rat IgG antibody (1:200; Millipore); mouse monoclonal antineuronal nuclei (NeuN) and GFAP antibodies (1:200; Millipore), which were revealed using fluorescein-labeled anti-mouse antibody (1:50; Millipore). The Sections were mounted onto subbed slides and

stored at 4°C . For Ki67 immunostaining, the sections were incubated with rabbit monoclonal anti-Ki67 antibody (1:500; Abcam, Cambridge, UK) with 3% normal goat serum at 4°C overnight, and then incubated in fluorescein-labeled Cy3 goat anti-rabbit antibody (1:200; Jackson ImmunoResearch Labs, West Grove, PA, USA) for 2 h. For doublecortin (DCX) immunostaining, the sections were incubated with goat polyclonal DCX antibody (1:500; Santa Cruz, CA, USA) with 2% normal rabbit serum at 4°C overnight, and then incubated in biotin-labeled rabbit anti-goat IgG antibody (1:500; Bioworld) for 2 h.

In order to avoid any bias, specimen analyzers were blinded to the examination. (1) Immuno-positive BrdU (BrdU⁺) and Ki67 (Ki67⁺) cells were observed on every 5th section (200 μm apart) using a conventional light microscope (Olympus DP70, Tokyo, Japan) with a 60 $\times$ objective. Total number of BrdU⁺ or Ki67⁺ cells per section were counted and multiplied by 5 to obtain the total number of cells in DG. (2) BrdU⁺/NeuN⁺ and BrdU⁺/GFAP⁺ cells were assessed by a confocal laser-scanning microscope (Leica, Heidelberg, Germany). (3) DCX immuno-positive (DCX⁺) neurites in molecular layer were assessed by a conventional light microscope with 100 $\times$ objective using image analysis software (Image J; NIH Image, Bethesda, MD, USA) as previously described [25]. Density of DCX⁺ neurites was expressed as the number of DCX⁺ dendrites per mm in central third of molecular layer.

Electrophysiological Analysis

Mice were decapitated under deep anesthesia with ethyl ether. The brains were rapidly removed and the coronal brain slices (400 μm) were cut using a vibrating microtome (Microslicer DTK 1500; Dousaka EMCo, Kyoto, Japan) in ice-cold modified artificial cerebrospinal fluid (mACSF) composed of (in mM) NaCl 126, CaCl₂ 1, KCl 2.5, MgCl₂ 1, NaHCO₃ 26, KH₂PO₄ 1.25, and D-glucose 20 oxygenated with a gas mixture of 95%O₂/5% CO₂. After 1 h recovery, hippocampal slices were transferred to a recording chamber.

The granular cells in hippocampal DG were perfused continually with oxygenated mACSF at room temperature ($22\text{--}24^\circ\text{C}$). NMDA-activated current (I_{NMDA}), GABA-activated current (I_{GABA}), and whole-cell current (I_{whole}) were recorded using an EPC-10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Data were acquired at a sampling rate of 10 kHz and filter (Bessel) of 2.9 kHz. The capacitance and series resistance were compensated more than 90%. Data obtained from neurons in which uncompensated series resistance resulted in voltage-clamp errors >5 mV were not taken in further analysis. Cell membrane capacitance and resistance were determined online after membrane rupture by using PatchMaster software (HEKA Elektronik). For recording I_{NMDA} and I_{GABA} , the holding potential was -60 mV and the glass pipettes resistance was 4–5 M Ω filled with (in mM) CsCl 140, MgCl₂ 2, CaCl₂ 1, Tris-ATP 2, HEPES 10, EGTA 10 at pH 7.2. I_{NMDA} was induced by NMDA (30 μM) and glycine (10 μM) which were dissolved in Mg-free mACSF and applied using a rapid drug delivery system. One micromolar strychnine, 10 μM bicuculline, 10 μM NBQX, and 0.1 μM TTX were added extracellularly. I_{GABA} was activated by GABA (10 μM) which was dissolved in mACSF containing 1 μM strychnine and 0.1 μM TTX and applied using a rapid drug delivery system. For recording I_{whole} , the pipette solution was composed of (in mM) KCl 140, MgCl₂ 2,

CaCl₂ 1, EGTA 11, HEPES 10, and Tris-ATP 5 with pH 7.2. I_{whole} was elicited by a series of depolarizing pulses (200 ms) from -80 to $+60$ mV stepping by 10 mV with interval time of 5 second.

Quantitative Real-Time Reverse Transcription PCR (Q-RT-PCR) Analysis

The hippocampal DG regions were microdissected from coronal brain slices (1 mm) and stored at -80°C until assayed. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (1 μg) was used to reverse transcribe using high-capacity cDNA of the reverse transcription kit RT (Takara Biotechnology Co., Ltd., Dalian, China) in accordance with the instructions. The primer sequences of *NR2A* and *NR2B* mRNA were designed according to the previous publication [26]. Q-RT-PCR was performed using a Light Cycler Fast Start DNA Master SYBR Green I kit and an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative expression of genes was determined using the $2^{-\Delta\Delta\text{Ct}}$ method with normalization to *GAPDH* expression. Results were averaged from four sets of independent experiments. The levels of *NR2A* and *NR2B* mRNA were expressed as percent of WT mice.

Western Blotting Analysis

Protein was extracted from hippocampal DG regions. The amount of protein was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein (50 μg) was separated by SDS-PAGE and transferred to membranes. The membranes were incubated with rab-

bit monoclonal anti-phospho-NR2B or NR2A antibodies (1:1000; Abcam) at 4°C overnight. After several PBS rinses, the membranes were incubated with horse radish peroxidase-labeled goat anti-rabbit antibody (1:10,000; Millipore) for 2 h, and then were developed using an enhanced chemiluminescence detection Kit (Millipore). Following visualization, the blots were stripped by stripping buffer (Restore, Pierce) for 15 min, and then were incubated with anti-NR2B or NR2A antibodies (1:1000; Millipore). Western blotting bands were scanned and analyzed with image analysis software (Image J; NIH). The summarized data represent the average of three experimental sets.

Drug Treatment

N-methyl-D-aspartate receptor agonist NMDA and NMDAR antagonist MK801 (Sigma, St. Louis, MO, USA) were dissolved in 0.9% saline. NMDA (30 mg/kg/day) and MK801 (3 mg/kg/day) were injected (i.p.) as described elsewhere [27,28]. GABA receptor agonist GABA, glycine receptor antagonist strychnine, GABA_A receptor antagonist bicuculline, AMPA receptor antagonist NBQX, voltage-gated sodium channel blocker TTX, NMDAR NR2A antagonist NVP-AMM077, NMDAR NR2B antagonist Ro25-6981, and SK channel blocker apamin were purchased from Sigma. These drugs were dissolved according to the instructions and were diluted to the mACSF just prior usage.

Data Analysis/Statistics

Data were retrieved and processed with the software PulseFit (HEKA Elektronik) and Micro cal Origin 6.1. The group data were

Figure 1 The σ_1 R deficiency enhances the proliferation of progenitor cells in hippocampal dentate gyrus (DG). **(A)** Left panels represent representative images of 24-h-old BrdU⁺ cells (black arrows) in $\sigma_1\text{R}^{-/-}$ mice and wild-type (WT) mice. Scale bar = 100 μm . Bar graph shows mean number of 24-h-old BrdU⁺ cells in $\sigma_1\text{R}^{-/-}$ mice ($n = 8$) and WT mice ($n = 8$). ** $P < 0.01$. **(B)** Representative images of Ki67⁺ cells (white arrowheads) in $\sigma_1\text{R}^{-/-}$ mice and WT mice. Scale bar = 50 μm . Bars indicate mean number of Ki67⁺ cells in $\sigma_1\text{R}^{-/-}$ mice ($n = 8$) and WT mice ($n = 8$). * $P < 0.05$. BrdU, bromodeoxyuridine.

Figure 2 The σ_1 R deficiency decreases survival of newborn neurons in hippocampal dentate gyrus (DG). **(A)** Left panels represent images of 28-day-old BrdU⁺ cells (black arrows) in $\sigma_1R^{-/-}$ mice and wild-type (WT) mice. Scale bar = 100 μ m. Bar graph shows mean number of 28-day-old BrdU⁺ cells in $\sigma_1R^{-/-}$ mice ($n = 8$) and WT mice ($n = 8$). ** $P < 0.01$. **(B, C)** BrdU and NeuN or GFAP were double-stained on 28th day after injection of BrdU in $\sigma_1R^{-/-}$ mice and WT mice (left panel). Neurons (immunoreactive for NeuN) and glial cells (immunoreactive for GFAP) are shown in green, and newborn neurons (immunoreactive for both BrdU and NeuN or GFAP) are shown in yellow (white arrowheads). Scale bar = 50 μ m. Bar graph shows the mean number of 28-day-old BrdU⁺/NeuN⁺ cells and BrdU⁺/GFAP⁺ cells in $\sigma_1R^{-/-}$ mice ($n = 8$) and WT mice ($n = 8$). ** $P < 0.01$. BrdU, bromodeoxyuridine; GFAP, antigen fibrillary acidic protein.

expressed as the means $\pm$ standard error (SE). Difference between two groups was evaluated using Student's t test. When analyzing one-variable experiments with more than two groups, significance of difference among means was evaluated using ANOVA followed by Bonferroni's *post hoc* tests. Statistical analysis was performed using Stata 7 software (STATA Corporation, College Station, TX, USA). Differences at $P < 0.05$ were considered statistically significant.

Results

Enhanced Proliferation of Progenitor Cells in $\sigma_1R^{-/-}$ Mice

As proliferating cells complete one mitotic division within 24 h [29], the cell proliferation was examined at 24 h after the last BrdU injection (24-h-old BrdU⁺ cells). In comparison with WT mice, the number of 24-h-old BrdU⁺ cells was significantly

increased in $\sigma_1R^{-/-}$ mice ($P < 0.01$; Figure 1A). The cell proliferation marker Ki67 is expressed during the active phases of cell cycle (G1, S, G2, and mitosis) [30]. We further examined the cells labeled with Ki67 (Ki67⁺ cells) in the subgranular zone (SGZ) of hippocampal DG [31] to investigate the status of cell proliferation (Figure 1B). As expected, the number of Ki67⁺ cells was higher in $\sigma_1R^{-/-}$ mice than that in WT mice ($P < 0.05$).

Reduced Survival of Newborn Neurons Without Alternation of Differentiation in $\sigma_1R^{-/-}$ Mice

To evaluate the survival of newborn neurons, BrdU⁺ cells were examined on 28 day after the last BrdU injection (28-day-old BrdU⁺ cells). Data showed that the number of 28-day-old BrdU⁺ cells in $\sigma_1R^{-/-}$ mice was reduced by approximately 30% compared to WT mice ($P < 0.01$; Figure 2A). Similarly, the number of 28-day-old BrdU⁺ cells expressing neuron-specific nuclear protein (BrdU⁺/NeuN⁺) in $\sigma_1R^{-/-}$ mice was lower than that in WT mice

Figure 3 The σ_1 R deficiency decreases neurite growth of newborn neurons in hippocampal dentate gyrus (DG). (A) Representative pictures of doublecortin (DCX) immunostaining in $\sigma_1R^{-/-}$ mice and wild-type (WT) mice. Scale bar = 50 μm . Bars indicate the mean density (B) and length (C) of DCX+ neurites in $\sigma_1R^{-/-}$ mice ($n = 8$) and WT mice ($n = 8$). * $P < 0.05$.

($P < 0.01$; Figure 2B), but the ratio of BrdU⁺/NeuN⁺ cells against total BrdU⁺ cells was not different between two groups ($74.6 \pm 4.61\%$ vs. $81.1 \pm 4.32\%$, $P > 0.05$). In contrast, the number of 28-day-old BrdU⁺ cells expressing the glial marker GFAP (BrdU⁺/GFAP⁺) in $\sigma_1R^{-/-}$ mice was nearly the same as WT mice ($P > 0.05$; Figure 2C).

Reduced Neurite Growth of Newborn Neurons in $\sigma_1R^{-/-}$ Mice

Doublecortin, a microtubule-associated protein, is expressed specifically in newly generated healthy neurons, reaching a peak during the second week, and is down-regulated concomitantly with the appearance of the mature neuronal marker NeuN [32]. As shown in Figure 3A, the DCX-positive (DCX⁺) cells located in SGZ. In comparison with WT mice, the density of DCX⁺ neurites ($P < 0.05$; Figure 3B), but not their length ($P > 0.05$; Figure 3C), reduced approximately 15% in $\sigma_1R^{-/-}$ mice.

Down-Regulated NMDAR NR2B Function in DG Granular Cells of $\sigma_1R^{-/-}$ Mice

As the activation of σ_1 R positively regulates NMDAR [33] and negatively modulates GABA_A receptor [22], we measured the NMDA-activated current (I_{NMDA}) and the GABA-activated current (I_{GABA}) in DG granular cells using whole-cell patch-clamp technique. The results showed that I_{NMDA} was less in $\sigma_1R^{-/-}$ mice than that in WT mice ($P < 0.01$; Figure 4A), however, I_{GABA} had no difference between both ($P > 0.05$; Figure 4B).

Figure 4 The σ_1 R deficiency decreases the N-methyl-D-aspartate receptor (NMDAR) function in granular cells of hippocampal dentate gyrus (DG). (A) Typical traces of I_{NMDA} in $\sigma_1R^{-/-}$ mice and wild-type (WT) mice (up panel). Bar graph shows mean density of I_{NMDA} in $\sigma_1R^{-/-}$ mice ($n = 12$) and WT mice ($n = 12$). ** $P < 0.01$. (B) Typical traces of I_{GABA} in $\sigma_1R^{-/-}$ mice and WT mice (up panel). Bar graph shows mean density of I_{GABA} in $\sigma_1R^{-/-}$ mice ($n = 12$) and WT mice ($n = 12$). (C) Typical traces of I_{whole} in $\sigma_1R^{-/-}$ mice and WT mice (up panel). Bar graph shows the maximal amplitudes of outward (Max_{outward}) and inward (Max_{inward}) current in $\sigma_1R^{-/-}$ mice ($n = 12$) and WT mice ($n = 12$).

To explore the mechanisms underlying the reduction in I_{NMDA} induced by σ_1 R deficiency, we further analyzed the membrane properties of the granular cells. The membrane capacitance and resistance in $\sigma_1R^{-/-}$ mice (213.71 ± 8.06 pF; 275 ± 10.14 M Ω , $n = 94$) had no significant difference from WT mice (209.35 ± 10.14 pF; 280.40 ± 9.75 M Ω , $n = 88$, $P > 0.05$). Voltage-dependent I_{whole} includes an inward and an outward component. The maximal amplitudes of outward (Max_{outward}) and inward (Max_{inward}) current in $\sigma_1R^{-/-}$ mice were not significantly different from those in WT mice ($P > 0.05$ in each case; Figure 4C).

The levels of NR2A and NR2B mRNA in hippocampal DG had no difference between $\sigma_1R^{-/-}$ mice and WT mice ($P > 0.05$ in each case; Figure 5A). Figure 5B, C illustrates representative gel patterns of NR2A and NR2B phosphorylation (phospho-NR2A/2B) in WT mice and $\sigma_1R^{-/-}$ mice. A densitometry analysis revealed that the level of phospho-NR2B was significantly reduced in $\sigma_1R^{-/-}$ mice without the change in the protein level of NR2B compared to WT mice ($P < 0.01$; Figure 5B). In contrast, the level of phospho-NR2A and the protein level of NR2A in $\sigma_1R^{-/-}$ mice showed no significant alteration ($P > 0.05$; Figure 5C). Notably, the addition of NR2A antagonist NVP-AMM077 (50 nM) could

Figure 5 The σ_1 R deficiency reduces phosphorylation of N-methyl-D-aspartate receptor (NMDAR) NR2B. **(A)** The levels of NR2A and NR2B mRNA in hippocampal dentate gyrus (DG) of σ_1 R^{-/-} mice ($n = 12$) and wild-type (WT) mice ($n = 12$). **(B, C)** Western blots of phospho-NR2A/2B and NR2A/2B in hippocampal DG of σ_1 R^{-/-} mice and WT mice. The densitometric values for pNR2A and pNR2B were first normalized by the protein amounts of NR2A and NR2B, respectively, and then were normalized again by the basal values in WT mice, respectively. **(D)** Bar graph shows mean density of I_{NMDA} in σ_1 R^{-/-} mice and WT mice in the presence of NR2A antagonist NVP-AMM077, NR2B antagonist Ro25-6981, or SK channels blocker apamin, respectively ($n = 12$ mice in each group). ** $P < 0.01$; # $P < 0.05$, and ## $P < 0.01$ versus vehicle-treated slices of WT mice or σ_1 R^{-/-} mice.

reduce the amplitude of I_{NMDA} in WT mice or σ_1 R^{-/-} mice ($P < 0.01$ in each case; Figure 5D), whereas it did not alter the reduced I_{NMDA} in σ_1 R^{-/-} mice compared to WT mice ($P < 0.01$). In contrast, the application of NR2B antagonist Ro25-6981 (3 μ M) not only reduced the amplitude of I_{NMDA} in WT mice ($P < 0.01$) and σ_1 R^{-/-} mice ($P < 0.05$) but also it abolished the difference in I_{NMDA} between both groups ($P > 0.05$). To test whether the σ_1 R deficiency depressed I_{NMDA} through increasing SK channels, the influence of SK channel blocker apamin on the reduction in I_{NMDA} was examined in σ_1 R^{-/-} mice. As shown in Figure 5D, the addition of apamin (0.1 μ M) could not rescue the reduction in I_{NMDA} in σ_1 R^{-/-} mice compared to WT mice ($P > 0.05$ in each case), although it caused a slight, but no significant difference, increase in I_{NMDA} of both groups. These results suggest that σ_1 R deficiency depresses the function of NMDAR through reducing the phosphorylation level of NR2B subunit.

Effects of NMDA Treatment on Abnormal Neurogenesis in σ_1 R^{-/-} Mice

To determine whether the impaired neurogenesis in σ_1 R^{-/-} mice is related with the dysfunction of NMDAR, we examined the effects of the NMDAR agonist NMDA on abnormal neurogenesis in

σ_1 R^{-/-} mice. Notably, the administration of NMDA for 7 days before the initial injection of BrdU (pre-BrdU) prevented the increase of 24-h-old BrdU⁺ cells in σ_1 R^{-/-} mice ($P < 0.01$; Figure 6A). Although WT mice treated with NMDA tended to reduce the number of 24-h-old BrdU⁺ cells, the group comparison failed to reach significance (vs. vehicle-treated WT mice, $P > 0.05$). Additionally, NMDA when administered on day 14–21 after the last BrdU injection (post-BrdU) could attenuate the loss of 28-day-old BrdU⁺ cells in σ_1 R^{-/-} mice ($P < 0.01$; Figure 6B), although it did not affect on the number of newborn neurons in WT mice ($P > 0.05$). However, the administration of NMDA for 7 days failed to affect the density of DCX⁺ neurites in σ_1 R^{-/-} mice and WT mice ($P > 0.05$ in each case; Figure 6C). In WT mice, the administration of NMDAR antagonist MK801 for 7 days pre-BrdU increased the number of 24-h-old BrdU⁺ cells ($P < 0.05$; Figure 6D), and on day 14–21, post-BrdU reduced the number of 28-h-old BrdU⁺ cells ($P < 0.05$; Figure 6E).

Discussion

In this study, we employed σ_1 R^{-/-} mice to establish for the first time *in vivo* that the σ_1 R deficiency enhances the proliferation of progenitor cells, but depresses the neurite growth and survival of

Figure 6 The N-methyl-D-aspartate receptor (NMDAR) agonist corrects the abnormal neurogenesis in σ_1 R^{-/-} mice. (A, B) Bar graphs show mean numbers of 24-h-old BrdU⁺ cells and 28-day-old BrdU⁺ cells in σ_1 R^{-/-} mice or wild-type (WT) mice treated with vehicle or NMDA ($n = 8$ mice in each group). ** $P < 0.01$; ## $P < 0.01$ versus vehicle-treated σ_1 R^{-/-} mice. (C) Bars indicate the density of DCX⁺ neurites in σ_1 R^{-/-} mice or WT mice treated with NMDA ($n = 8$ mice in each group). * $P < 0.05$ versus vehicle-treated WT mice and σ_1 R^{-/-} mice. (D, E) Bar graphs show the numbers of 24-h-old BrdU⁺ cells and 28-day-old BrdU⁺ cells in WT mice treated with vehicle or MK801 ($n = 8$ mice in each group). * $P < 0.05$. BrdU, bromodeoxyuridine; DCX, doublecortin.

newborn neuronal cells without affecting the differentiation of progenitor cells in the adult hippocampal DG. The deficit in neurogenesis of σ_1 R^{-/-} mice is highly likely due to the dysfunction of NMDAR.

The findings in the present study give a clear indication that the σ_1 R deficiency arrests the NMDAR function probably through reducing the phosphorylation of NR2B. This is supported by the major findings as follows: (1) the amplitude of I_{NMDA} was smaller in σ_1 R^{-/-} mice than that in WT mice; (2) the phosphorylation of NR2B, but not NR2A, was reduced in σ_1 R^{-/-} mice without the alteration of NR2B and NR2A expression; (3) the NR2A antagonist reduced the amplitude of I_{NMDA} in σ_1 R^{-/-} mice and WT mice, whereas it could not alter the reduced I_{NMDA} in σ_1 R^{-/-} mice compared to in WT mice; (4) in the presence of NR2B antagonist, the

amplitude of I_{NMDA} had no significant difference between WT mice and σ_1 R^{-/-} mice, although the I_{NMDA} was reduced in both; (5) the voltage-dependent I_{whole} , membrane resistance, and capacitance were not altered in σ_1 R^{-/-} mice compared to WT mice; (6) the blockade of SK channels did not affect the reduction in I_{NMDA} in σ_1 R^{-/-} mice, indicating that the NMDAR dysfunction is unlikely due to opening SK channels. It is also reported that antagonism of σ_1 R can suppress phosphorylation of NMDAR NR1 subunit [34]. Whether the σ_1 R deficiency causes the down-regulation of NMDAR NR1 leading to deficits in NMDAR function should be an interesting subject for future work.

Precursor cells in the adult hippocampal DG express functional NMDARs [35] and can respond to excitatory stimuli such as glutamate and depolarization [36]. Although the NMDAR activity in newly generated neurons of σ_1 R^{-/-} mice remains to be further examined, our results indicate that the dysfunction of NMDAR is responsible for the over-proliferation of progenitor cells. In line with this hypothesis, the NMDAR agonist prevented the over-proliferation of progenitor cells in σ_1 R^{-/-} mice. By contrast, in WT mice, the NMDAR agonist did not affect their cell proliferation, while the blockade of NMDAR could increase the number of 24-h-old BrdU⁺ cells. This discrepancy further supports the idea that the NMDAR-mediated action in cell proliferation is weaker in σ_1 R^{-/-} mice than that in WT mice. Previous studies reported that the NMDAR agonist inhibits the cell proliferation in the SGZ of DG [27], while the competitive and noncompetitive NMDAR antagonists increase the rate of cell proliferation [37]. The Ca²⁺ influx of NMDAR channels had been reported to inhibit DNA synthesis and cell cycle [38] through inducing transient AP1 expression [39].

Another important finding in this study is that the σ_1 R deficiency impaired the survival of newly generated neurons, which could be rescued by the treatment with NMDA on day 14–21 post-BrdU. Several studies have provided evidence to suggest that the activation of NMDAR is required for the survival of newborn neuronal cells after spine formation in an input–output-correlated way [40]. The period of NMDAR activation that can contribute to the survival of newborn neurons [41] is restricted to the third week after neuronal birth [42]. This short critical time window corresponds to the period of morphological synapse formation in newborn neurons [43]. Indeed, our results showed that the blockade of NMDAR on day 14–21 post-BrdU reduced the number of mature newborn neurons in WT mice. Thus, it is highly likely that the dysfunction of NMDAR induced by the σ_1 R deficiency impairs the survival of newborn neuronal cells. Our recent study [14] has reported that the activation of σ_1 R increases the nNOS expression by promoting the Ca²⁺ influx through NMDAR and subsequently enhances NO release from postsynaptic neurons, which in turn increases the presynaptic glutamate release toward newborn neurons to facilitate NMDAR-dependent survival of newborn neurons in the hippocampal DG.

The density of DCX⁺ neurites was decreased in hippocampal DG of σ_1 R^{-/-} mice without changes in the number of 14-day-old BrdU compared to WT mice (data not shown), indicating that the σ_1 R deficiency depressed the neurite growth of newborn neuronal cells. Compagnone and Mellon [44] reported that the activation of σ_1 R increases the length of neurites containing the axonal marker Tau-1 and the dendritic marker microtubule-associated protein-2 in a dose-dependent fashion. The activation of σ_1 R enhances the

growth of neuronal projections through a modulation of cytoskeleton-related signaling [45]. The administration of DHEA increases the expression of growth factors IGF-1 to promote axonal and dendritic growth [46], and spine synapse formation [47,48]. In addition, DHEA regulates neurogenesis by triggering the Akt signal [49]. We recently reported the σ_1 R-mediated protection against $A\beta_{25-35}$ -impaired dendritic growth of newborn neurons through up-regulation of phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) [16]. On the other hand, σ_1 R enhances the brain-derived neurotrophic factor (BDNF) signaling and increases BDNF mRNA in the rat brain [50]. BDNF activates a variety of signal molecules, including Ras/ERK, PI3K/Akt, and PLC- γ , all of which are required for neurite growth and survival of newborn neuronal cells. Our recent work has established that the activation of σ_1 R can increase the level of BDNF in the hippocampus of APPswe/PS1dE9 transgenic mice [51]; thus, it would be interesting to examine whether the σ_1 R deficiency reduces the BDNF expression in hippocampus.

Continual neurogenesis in adult animals is required to maintain the full range of hippocampus-dependent functions [52]. The hippocampus-dependent spatial memory has been demonstrated to depend on the degree of neurogenesis in the DG [53]. The newly generated neurons are functionally integrated into the local circuitry [54], during which dendritic spines contacting perforant

path synapses and axons projecting into the CA3 area [43]. Particularly, the newborn neurons in the DG have lower activation thresholds and higher resting potentials, leading to a facilitated long-term potentiation induction in comparison with mature neurons [55,56]. Neurogenesis is an extremely dynamic process, as the number of cells in each "stage" of neurogenesis can be changed by pharmacological, environmental, and physiological stimuli. The present study, for the first time, provides *in vivo* evidence that the σ_1 R deficiency interrupts the adult neurogenesis, which would open a new way to prevent and treat cognitive deterioration.

Acknowledgments

We thank Dr. Zorrilla (Neurosciences Graduate Program, University of California) for providing us the sigma-1 receptor knockout mice. This work was supported by grants for 973 (2009CB941701), NSFC (81071027; 31271206), BK2011029 to Chen L.

Conflict of Interest

The authors declare no conflict of interest.

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