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The pharmacological stimulation of Nurr1 improves cognitive functions via enhancement of adult hippocampal neurogenesis

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ABSTRACT

The nuclear receptor related-1 (Nurr1) protein plays an important role in both the development of neural precursor cells (NPCs) and cognitive functions. Despite its relevance, the effects of Nurr1 on adult hippocampal neurogenesis have not been thoroughly investigated. Here we used RT-PCR, western blot, and immunocytochemistry to show that adult hippocampal NPCs abundantly express Nurr1. We then examined the effect of Nurr1 activation on adult hippocampal NPCs using amodiaquine (AQ), an anti-malarial drug that was recently discovered to be a Nurr1 agonist. Cell proliferation assay showed that AQ significantly increased cell proliferation. AQ-treated NPCs showed increased levels of phosphorylation of Akt and ERK1/2 whereas AQ-treated *Nurr1* siRNA-transfected NPCs showed no changes in those levels. Further immunocytochemical and immunohistochemical analyses confirmed the stimulating effect of Nurr1 agonist on the proliferation and differentiation of adult hippocampal NPCs both *in vivo* and *in vitro*. In addition to its effects on proliferation and differentiation of NPCs, AQ-treated mice showed a significant enhancement of both short- and long-term memory in the Y-maze and the novel object recognition test. These data suggest that activation of Nurr1 may enhance cognitive functions by increasing adult hippocampal neurogenesis and also indicate that Nurr1 may be used as a therapeutic target for the treatment of memory disorders and cognitive impairment observed in neurodegenerative diseases.

1. Introduction

Neurogenesis is a process characterized by the generation of neurons from neural precursor cells (NPCs) (Kintner, 2002; Kaslin et al., 2008). Two main brain regions where the neurogenesis persistently takes place throughout adulthood have been described (Ming and Song, 2005; Zhao et al., 2008). Many studies have shown adult neurogenesis, generation and differentiation of neurons from NPCs occurring in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Goritz and Frisen, 2012). Interestingly, adult hippocampal neurogenesis is responsible for the regulation of cognitive functions, including learning and memory. Increasing evidence supports the hypothesis that NPCs-derived newborn neurons may play a key role in long- and short-term spatial memory as well as in object recognition memory (Deng et al., 2010; Jessberger et al., 2009; Piatti et al., 2013). Thus, increasing adult hippocampal neurogenesis by stimulating NPCs has been suggested as a viable strategy for the enhancement of cognitive functions.

Nurr1 (or Nr4a2) is a nuclear receptor acting as an intracellular transcription factor and it is considered to be important for the development of NPCs (Kim et al., 2002, 2007; Bae et al., 2009; Hong et al., 2014; Rodriguez-Traver et al., 2015; Zetterstrom et al., 1997; Vergano-Vera et al., 2015; Wagner et al., 1999; Park et al., 2008; Shim et al., 2007; Saucedo-Cardenas et al., 1998; Castillo et al., 1998). Previous studies manipulating the *Nurr1* gene have demonstrated that Nurr1 plays key roles in both the proliferation and differentiation of mouse embryonic stem cells (Kim et al., 2002; Hong et al., 2014), mouse embryonic olfactory bulb stem cells (Vergano-Vera et al., 2015) and rat NPCs of the ventral midbrain, striatum, cortex, lateral ganglionic eminence, SVZ and white matter (Kim et al., 2007; Bae et al., 2009; Wagner et al., 1999; Park et al., 2008; Shim et al., 2007). Consistent with *in vitro* studies, Nurr1 was shown to be required for maintaining the development of

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NPCs *in vivo*. Indeed, *Nurr1* knockout mice showed reduced differentiation of NPCs into neurons in the ventral midbrain, including the substantia nigra and the ventral tegmental area (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Castillo et al., 1998), while transplanted *Nurr1*-engineered NPCs exhibited an enhanced differentiation of neurons in the striatum (Kim et al., 2002; Shim et al., 2007). In particular, several studies have focused on the role played by Nurr1 in promoting neurogenesis from dopaminergic precursors. However, to date, the role of Nurr1 in the generation of neurons from adult hippocampal NPCs has not been thoroughly examined.

In addition to the role played by Nurr1 on the development of NPCs in the Central Nervous System (CNS), recent studies suggest that Nurr1 may be involved in the regulation of cognitive functions (Hawk et al., 2012; Hawk and Abel, 2011; Colón-Cesario et al., 2006). Indeed, both *Nurr1* knock-down and heterozygous mice showed an impairment of hippocampus-dependent memory (Colón-Cesario et al., 2006; Rojas et al., 2007) and *Nurr1* mRNA expression was increased in the hippocampus following acquisition of hippocampal-dependent learning task (Pena de Ortiz et al., 2000). Despite the fact that the underlying mechanism was not directly examined, it has been suggested that Nurr1 may play an important role in both neurogenesis and the deficits of spatial memory observed in juvenile mice (Ibi et al., 2008). Nurr1 was considered as an orphan nuclear receptor (Wang et al., 2003; Law et al., 1992) until a very recent study identified a selective and potent agonist for Nurr1: an anti-malarial drug amodiaquine (AQ) which stimulates Nurr1's transcriptional function via a direct interaction with its ligand-binding domain (Moon et al., 2015). To date, effects of AQ on adult hippocampal neurogenesis have not been investigated. Taking advantage of AQ's agonistic effect on the activation of Nurr1, we examined whether Nurr1 can regulate cognitive functions via the induction of adult hippocampal neurogenesis.

In the present study, we aimed to address two main questions. First, using a Nurr1-specific agonist, we studied the effect of Nurr1 activation on adult hippocampal neurogenesis. Second, we examined the effect of the neurogenic Nurr1 agonist on cognitive functions in mice. Here we show for the first time that agonist-mediated Nurr1 activation enhances adult hippocampal neurogenesis and increases learning and memory processing via a direct neurogenic action of Nurr1.

2. Materials and methods

2.1. Rat adult hippocampal NPCs culture

Rat adult hippocampal NPCs were purchased from Chemicon (Catalog no. SCR022, Billerica, MA). These are ready-to-use primary NPCs isolated from the DG of adult Fisher 344 rats. These self-renewing NPCs have a potential to differentiate into neurons and express markers representing the stages of adult hippocampal neurogenesis such as Ki67, doublecortin (DCX), neuronal nuclear antigen (NeuN) likewise NPCs of mice (Marschallinger et al., 2015; Jarvinen et al., 2010; Jin et al., 2011; Rabenstein et al., 2015). They were grown in a neural stem cells expansion medium containing Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco/Invitrogen, Carlsbad, CA) with B27 supplement, L-glutamine, 1× solution of penicillin, fungizone, streptomycin, and basic FGF (bFGF, 20 ng/mL). Tissue culture glass or plastic wares that were used to culture hippocampal NPCs were coated with laminin (5 μg/mL) and poly-L-ornithine (10 μg/mL). These cells were maintained at 37 °C in a 5% CO_2 humidified incubator and passaged once every 3–4 days. All the *in vitro* experiments were performed with passage 6–8 rat adult hippocampal NPCs, and those passaged cells did not show any features in issues of aging/maturity/differentiation.

2.2. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from cortical neuronal cells was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and was reverse transcribed using the Superscript II reverse transcriptase (Life Technologies, Rockville, MD). An RNA control tube containing all RT reagents except reverse transcriptase was included as a negative control to monitor genomic DNA contamination. To confirm the Nurr1 expression in rat adult hippocampal NPCs, the resultant cDNA was amplified using primers specific for Nurr1 (sense: 5′-GAC ACT TCA CAA CTT CCA CCA GAA CT-3′ and antisense: 5′-ACT GCG ATG CGT GGC CGA TCT GC-3′) and the GeneAmp PCR System StepOnePlus (Applied Biosystems, Helios, Singapore). The thermal cycling profile was as follows: 95 °C for 5 min, 38 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min.

2.3. Western blot

For the determination of Nurr1 protein levels, hippocampus was dissected and homogenized in lysis buffer (150 mM NaCl, 20 mM Tris–HCl, 10% glycerol, 5 mM EDTA, and 1% Nonidet P-40) supplemented with protease and phosphatase inhibitors (50 μg/mL phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 25 μg/mL Leupeptin, and 100 nM orthovanadate). Rat adult hippocampal NPCs were lysed in a buffer containing 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 140 mM NaCl, 1% (w/v) Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 10 μg/mL aprotinin. Protein samples were separated by 12% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membranes were soaked in blocking buffer (1× Tris-buffered saline, 1% BSA, 1% nonfat dry milk) for 1 h and incubated overnight at 4 °C with the primary antibody against Nurr1 (Santa Cruz Biotechnology, Dallas, TX; 1:500). Blots were developed using a peroxidase-conjugated anti-rabbit IgG and a chemiluminescent detection system (Santa Cruz Biotechnology, Dallas, TX). The bands were visualized using a ChemicDoc XRS system (Bio-Rad, Hercules, CA) and quantified using Quantity One imaging software (Bio-Rad, Hercules, CA).

2.4. Immunocytochemistry

Rat adult hippocampal NPCs were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS for 10 min at room temperature. After blocking with 3% normal goat serum (Vector Laboratories, Burlingame, CA) and 1% BSA (Sigma-Aldrich, St. Louis, MO), the slides were incubated with primary antibodies to Nurr1 (Santa Cruz Biotechnology, Dallas, TX, 1:200) overnight at 4 °C. After washes, the slides were incubated with a secondary Cy3-goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA; 1:400) at room temperature for 4 h. Cells were counterstained with 4–6-diamidino-2-phenylindole (DAPI) before mounting and images were acquired by the Carl Zeiss LSM 700 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.5. Cell proliferation assay

For the evaluation of effect of Nurr1 agonist AQ (Amodiaquin dihydrochloride dehydrate, Sigma-Aldrich, St. Louis, MO) on cell proliferation, the proliferation index of each group was determined using the cell counting kit-8 (CCK-8) method (Enzo Life Science Inc., Lausen, Switzerland) according to the manufacturer's instructions. Cells in the exponential phase of growth were seeded in 24-well plates at a density of 3×10^5 cells/well, and cultured in F12/DMEM (500 μL) supplemented with 0.1% B27 (24 wells per group). Cells were treated with AQ (10, 100 and 1000 nM) for 24, 48 and 48 h. In brief, 20 μL of CCK-8 solution was added into each well (containing 200 μL of medium), and further cultured for 2 h at 37 °C. The absorbance of each group at 450 nm was detected ($n = 3$) using an absorbance microplate reader (Molecular Devices, Sunnyvale, CA) and it was directly proportional to the number of living cells.

2.6. Evaluation of cell proliferation and differentiation

Cell proliferation and differentiation were assessed by performing immunocytochemical staining for BrdU and counting the number of BrdU and DCX-positive cells, respectively. For immunocytochemical detection of BrdU in hippocampal NSCs, the fixed cells were incubated in 2 M HCl and 0.3% Triton X-100 for 30 min followed by incubation in 0.1 M boric acid (pH 8.0) for 10 min, after which cells were treated with AQ 10 nM for 24 h, 48 h and 48 h. Cells were incubated in a blocking solution (0.3% Triton X-100, 1% BSA, and 3% normal goat serum in PBS) for 2 h. After overnight incubation with a primary antibody (mouse anti-BrdU, 1:400; Santa Cruz Biotechnology, Dallas, TX) at 4 °C, cells were rinsed with PBS, followed by incubation with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, 1:400, Gibco/Invitrogen, Carlsbad, CA) for 4 h at room temperature. BrdU-labeled cells were visualized by the Carl Zeiss LSM 700 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.7. RNA interference

Rat *Nurr1* small interfering RNA (siRNA duplexes (SR513154)) was purchased from Origene (Rockville, MD), three different strands of siRNA were pooled in this siRNA reagent in order to target different parts of the *Nurr1* mRNA to improve knockdown efficiency. The siRNA sequences targeting Nurr1, as the manufacturer provided, were the following. SR513154A – rGrCrArGrUrUrArArGrArCrArArArUrGrUrArArGrGrCrAAA, SR513154B – rGrGrArArGrArUrUrGrCrArArArUrGrUrArUrGrArUrGrGGA, and SR513154C – rArGrArUrGrArUrArCrUrCrArArCrArUrArUrCrCrArGrCAG. Scrambled siRNA (Origene, Rockville, MD) was used as a control. Adult rat hippocampal NPCs were transfected using Lipofectamine® 2000 siRNA Transfection kit (Life Technologies, Rockville, MD), after which these cells were utilized for the functional studies 24 h later then treated with AQ 10 nM for 0 h, 2 h, 4 h, 8 h, 12 h and 24 h.

2.8. Animals and administration

Adult (8 weeks) male C57BL/6 mice were purchased from Koatech (Pyeongtaek, South Korea) and acclimatized for 1 week before the experiment. All animals were housed in accordance with the Guide to Care and Use of Experimental Animals. Experimental procedures were approved and reviewed by the regulation of the Institutional Animal Care and Use Committee in Konyang University. For activation of Nurr1, mice injected intraperitoneally with AQ at concentration of 20 mg/kg, 2 times per day at 12 h intervals, for 14 days. The AQ dose (20 mg/kg) used in this study was referred from previous report regarding the activating effect of AQ on Nurr1 in rodent (Moon et al., 2015). AQ was diluted in 0.9% saline and prepared before administration. Two weeks after the last AQ injection, mice were sacrificed and analyzed.

2.9. Immunohistochemistry and quantification

For immunohistochemical analysis, brain sections were rinsed briefly in phosphate buffered saline and treated with 1% hydrogen peroxide for 15 min. The sections were incubated with mouse anti-Ki67 antibody (1:500; Abcam, Cambridge, UK) or goat anti-doublecortin (DCX) antibody (1:1000; Santa Cruz Biotechnology, Dallas, TX) overnight at 4 °C. The sections were then incubated with biotinylated horse anti-mouse IgG or biotinylated horse anti-goat IgG (1:200; VECTOR, Burlingame, CA) and avidin-biotin-peroxidase complex solution, and then visualized with a SIGMA *FAST*™ 3.3′-Diaminobenzidine tablet (Sigma-Aldrich, St. Louis, MO) as a chromogen. For the double-labeling of Ki67 and DCX, brain sections were incubated with mouse anti-Ki67 antibody and goat anti-DCX 16 h at room temperature in the presence of 0.3% Triton X-100. After rinsing in PBS buffer, the sections were then incubated with Alexa 488-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA) and Alexa 594-conjugated donkey anti-goat IgG (1:200; Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. Stained sections were mounted on gelatin coated slides and coverslipped using fluorescent mounting medium with DAPI. To quantify numbers of Ki67 and DCX-positive cells in the DG, the images were processed and analyzed using Image-Pro Plus 6.0 program (Media Cybernetics, Rockville, MD). The analysis was performed blindly in both hemispheres of six brain sections per animal.

2.10. Y maze

The Y-maze apparatus has three arms separated by 120° angles (30 cm long and 8 cm wide with 15 cm high) extending from a central space (8 × 8 cm). Each mouse was placed in one arm and allowed to explore freely for 5 min to assess their rates of spontaneous alternation. Spontaneous alternation is defined as successive entries into three different arms consecutively without repetition (i.e. ABC, BCA but not ABA). Spontaneous alternation percentage was calculated by equation [successive entries / (total arm entries − 2) × 100].

2.11. Novel object recognition test

The novel object recognition test (NORT) was performed in an open field box (45 × 45 × 45 cm). Prior to the test, mice were allowed a habituation period of 5 min in the test box without any objects for three consecutive days. After habituation, mice were placed into the test box and allowed to explore two identical objects for 3 min. The objects used in this study were wooden blocks of the same size but of different shape (defined as a familiarization session). 24 h after the familiarization session, mice were allowed to explore with one familiar object and one novel object for 3 min (defined as a test session). All sessions were recorded and analyzed using a video tracking system (EthoVision XT 10.0, Noldus Information Technology, Wageningen, Netherlands). The time that the mice spent exploring each of the objects, or object recognition time, was measured for each session. The object recognition time was defined as the time when mice were facing, sniffing, and biting the object or staying within 2 cm of it. Results were expressed as percentage of novel object recognition time [discrimination index = ${}_t$novel/(${}_t$novel + ${}_t$familiar) × 100].

2.12. Statistical analysis

The experiments were repeated three times and were performed in triplicate. The results are shown as mean ± SEM. Statistical significance between groups were analyzed by Student's unpaired *t*-test using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). A p-value <0.05 was considered statistically significant.

3. Results

3.1. Expression of Nurr1 in adult rat hippocampal precursor cells

The expression of Nurr1 has been characterized in several NPCs and regions of the rodent brain (Bae et al., 2009; Moon et al., 2015; Xiao et al., 1996). However, there is no evidence of the presence of Nurr1 in adult hippocampal NPCs. Therefore, to detect the expression of Nurr1 in adult hippocampal NPCs, we measured the mRNA and protein levels of Nurr1 using RT-PCR, western blot and visualized the Nurr1 using immunocytochemistry. Since the rodent brain exhibits abundant expression of Nurr1 (Zetterstrom et al., 1996), we used the rat brain as a positive control to detect Nurr1 expression. The result of the RT-PCR analysis indicated that *Nurr1* mRNA is expressed in adult hippocampal NPCs, adult rat hippocampus, and whole rat brain (Fig. 1A and Supplementary Fig. 1A). The results of the western blot analysis also revealed the presence of Nurr1 protein in adult hippocampal NPCs and the rat brain (Fig. 1B). Furthermore, both immunocytochemistry and western blot analysis confirmed the expression of Nurr1 proteins in adult rat

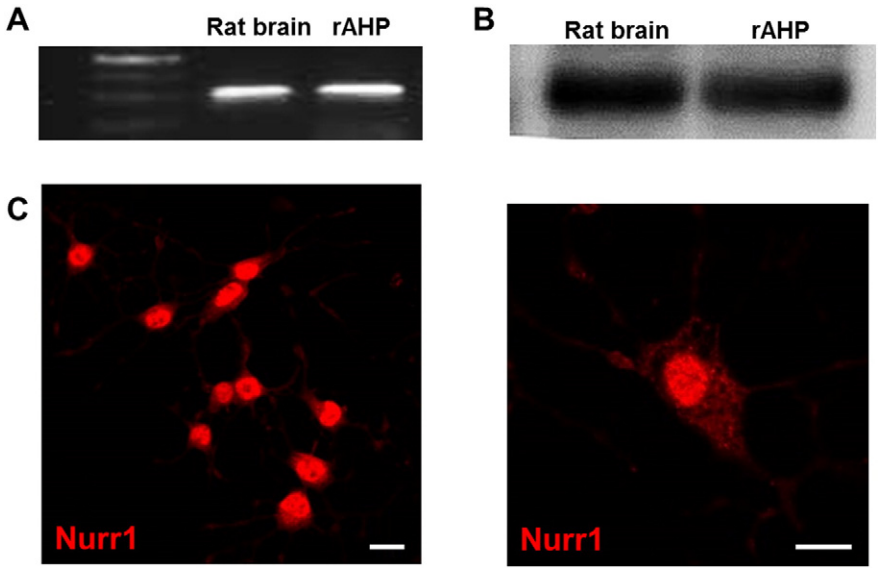

Fig. 1. Expression of Nurr1 in adult rat hippocampal NPCs. (A) RT-PCR was performed to detect the mRNA expression of Nurr1 in rat hippocampal NPCs. Result showed comparative level of *Nurr1* mRNA in both rat brain and adult hippocampal NPCs. (B) Western blot was carried out to compare the protein level of Nurr1 in the rat brain and adult hippocampal NPCs. Data showed similar expression level of Nurr1 protein in both samples. (C) Immunocytochemistry was conducted to confirm the Nurr1 protein expression in rat hippocampal NPCs. We observed abundant expression of Nurr1 in nucleus of adult hippocampal NPCs. Scale bar = 10 μm.

hippocampal NPCs (Fig. 1C, Supplementary Fig. 1B and Supplementary Fig. 2). Thus, our data clearly show that adult rat hippocampal NPCs express the nuclear receptor Nurr1.

3.2. Nurr1 agonist stimulates proliferation and differentiation of adult rat hippocampal NPCs

It has been shown that Nurr1 plays a critical role in the development of neural precursor/stem cells. (Bae et al., 2009; Park et al., 2006; Kim et al., 2003; Chung et al., 2002). In addition, a recent study has shown that AQ stimulates the generation of dopaminergic neurons from neural progenitors isolated from E14.5 rat cortex (Moon et al., 2015). To investigate the effect of Nurr1 on the proliferation of adult hippocampal NPCs we treated the cells with the Nurr1 agonist AQ and performed CCK-8 as a cell proliferation assay. AQ significantly increased cell proliferation in adult rat hippocampal NPCs, showing a time-, but not dose-dependent increase (Fig. 2A). In addition to CCK-8 assay, we further performed a double-staining immunocytochemistry to evaluate the effect of AQ on adult hippocampal NPCs. An increased number of DCX/BrdU co-labeled cells demonstrate that AQ stimulates both proliferation and differentiation of adult hippocampal NPCs, and BrdU-positive newborn cells are differentiated to DCX-positive neuroblasts (Fig. 2B). AQ-treated NPCs showed the phenotype of differentiating neuroblast stained with DCX (Supplementary Fig. 2). The present study is the first to examine whether AQ stimulates the proliferation and differentiation of adult rat hippocampal NPCs.

3.3. The proliferative effect of Nurr1 stimulation is possibly mediated via activation of ERK1/2 and Akt signaling pathways

In order to further characterize the molecular mechanisms underlying the proliferative effects of Nurr1 activation, we investigated the molecular signaling pathways activated during Nurr1 agonist-dependent proliferation of NPCs. It has been shown by previous studies that MAPK/ERK1/2 and PI3K/Akt signaling pathways are very important for both the proliferation and neurogenesis of neural progenitor/stem cells (Hao et al., 2004; Shioda et al., 2009; Le Belle et al., 2011). We confirmed the activation of ERK1/2 and Akt signaling pathways using western blot analysis. As shown in Fig. 3, we observed increased band intensity of p-ERK1/2 and p-Akt after AQ treatment in adult hippocampal NPCs. These data indicate that the phosphorylation of ERK1/2 and Akt signaling molecules occurs during AQ-induced proliferation of adult hippocampal NPCs. To more clarify the significance of Nurr1 in proliferation of adult hippocampal NPCs by activating ERK1/2 and Akt signaling pathway, we studied the effects of decreased Nurr1 expression on activation of signaling molecules by using siRNA treatment. A pharmacological inhibition of Nurr1 using Nurr1 antagonists that were yet to be developed was not available, and therefore RNA interference was used to evaluate the contributions of Nurr1 in proliferation of NPCs. Phosphorylation of ERK1/2 and Akt were not changed after AQ treatment in *Nurr1* siRNA-transfected NPCs (Fig. 3). The result indicates that limited levels of Nurr1 expression were not sufficient to activate ERK1/2 and Akt signaling pathways in spite of treatment of AQ, a known agonist of Nurr1 receptor. Taken together, our data suggest that the Nurr1 agonist AQ significantly promotes the proliferation of rat adult hippocampal NPCs via, in part, activation of ERK1/2 and Akt signaling pathways.

3.4. Administration of Nurr1 agonist enhances adult hippocampal neurogenesis in mice

To confirm the proliferative effect of Nurr1 activation on adult hippocampal NPCs *in vivo*, we conducted immunohistochemistry with a proliferation marker in the mouse hippocampus. Ki67 has been a generally used as a proliferation marker of adult neurogenesis (Kee et al., 2002). The result of the immunohistochemistry for Ki67 revealed that AQ-injected mice have a significantly increased number of Ki67-positive cells in the DG of the hippocampus compared to vehicle-injected mice (Fig. 4). Thus, these findings confirm the proliferative effect of Nurr1 activation induced by AQ administration on adult rat hippocampal NPCs. In addition, to examine the neurogenic effect of Nurr1 activation in adult hippocampal cells, we performed immunohistochemistry with a marker of neuronal differentiation. Since DCX is a marker of neuronal fate specification, it reflects the differentiation step of adult neurogenesis, and can be a marker for adult neurogenesis (Couillard-Despres et al., 2005). The DCX immunohistochemical analysis revealed that AQ-injected mice have a significantly increased number of DCX-positive cells in the DG of hippocampus compared to saline-injected control mice (Fig. 5). In addition to single-staining of two separate markers representing different stages of neurogenesis,

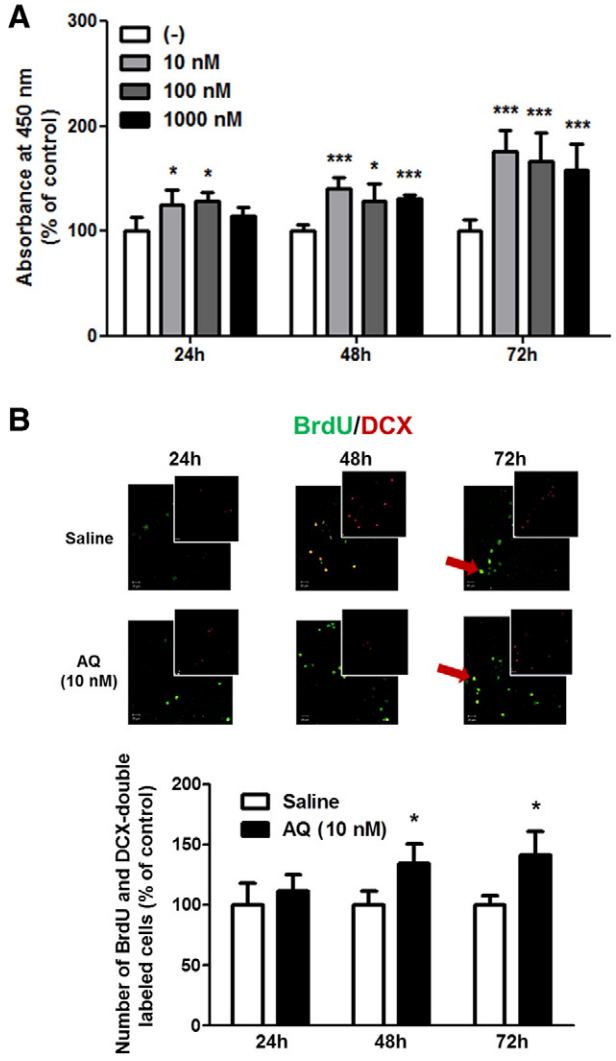

Fig. 2. (A) Increased proliferation of adult rat hippocampal NPCs after treatment with amodiaquine (AQ), Nurr1 agonist. The CCK-8 method was used to analyze the proliferation of cells. The treatment with AQ (10, 100, and 1000 nM) resulted in time-dependent increase of proliferation of hippocampal NPCs. (B) Quantitative analysis showed that the number of BrdU and DCX-double labeled cells was increased by AQ treatment at concentrations of 10 nM when compared with the control. All values are indicated as mean ± SEM. $^{***}p < 0.001$ and $^{*}p < 0.05$ compared to the saline-treated control group.

double-staining immunohistochemistry was done to further confirm the increment of neurogenesis in DG (Jin et al., 2006; Wojtowicz and Kee, 2006; Luzzati et al., 2006; Zonis et al., 2015). AQ-administered mice showed increased number of cells expressing both Ki67 in the nucleus and DCX in the cytoplasm (Fig. 6). Moreover, these double stained cells exhibited a migratory morphology from SGZ to granular cell layer (Fig. 6). Thus, it could be interpreted that AQ administration may result in neuronal differentiation of newborn progenitors in DG. Taken together, these immunohistochemical data suggest that the administration of the Nurr1 agonist AQ significantly enhances adult hippocampal neurogenesis *in vivo*.

3.5. Enhanced adult hippocampal neurogenesis by Nurr1 activation results in cognitive improvement in mice

Several lines of evidence suggest that enhanced neurogenesis promotes cognitive improvement in animals (Deng et al., 2010; Sahay et al., 2011). Therefore, to examine the impact of Nurr1 neurogenic activity on cognitive performance, AQ and saline-treated mice were subjected to two behavioral tests. AQ-treated mice showed a significant increase in spontaneous alteration in the Y maze test but no significant differences in total arm entry (Fig. 7A). This suggests that AQ-induced Nurr1 activation improves cognitive memory without affecting motor functions. We then tested AQ- and saline treated mice in the NORT. AQ-treated mice spent more time exploring the novel object than the familiar object while saline-treated mice spent an equal amount of time exploring the novel and the familiar objects during the test session (Fig. 7B). Mice from both groups showed no significant differences in object exploration during the habituation session (Fig. 7B). Taken together, the present findings suggest that the neurogenic Nurr1 agonist AQ improves learning and memory in mice.

4. Discussion

The role played by Nurr1 in the regulation of NPCs across several brain regions has been extensively investigated. It has been suggested that Nurr1 may exert a strong control on both the proliferation and differentiation of NPCs into neurons. These results prompted us to further investigate the role played by Nurr1 in adult hippocampal neurogenesis. The findings reported here suggest that Nurr1 activation may induce the proliferation and differentiation of adult hippocampal NPCs both *in vitro* and *in vivo*. Moreover, we have identified two signaling pathways, MAPK/ERK1/2 and PI3K/Akt involved in the proliferation of adult hippocampal NPCs. We also showed in mice that the administration of Nurr1 agonist significantly increases the expression of neurogenesis markers in the hippocampus. Our data clearly indicate that Nurr1 can regulate adult hippocampal neurogenesis.

Nurr1 expression has been well characterized in several NPCs but not in adult hippocampal NPCs (Kim et al., 2002, 2007; Bae et al., 2009; Hong et al., 2014; Rodriguez-Traver et al., 2015; Zetterstrom et al., 1997; Vergano-Vera et al., 2015; Wagner et al., 1999; Park et al., 2008; Shim et al., 2007; Saucedo-Cardenas et al., 1998; Castillo et al., 1998). Thus, we investigated the expression of the nuclear receptor Nurr1 in adult hippocampal NPCs using three independent methods: RT-PCR, western blotting and immunohistochemistry, because effects of an agonist that activates specific receptor should be examined under presence of receptor (Chung et al., 2013). We found that Nurr1 is abundantly expressed in adult hippocampal NPCs (Fig. 1). To our knowledge, this is the first study to report Nurr1 expression in hippocampal NPCs. Although Nurr1 has been considered an orphan nuclear receptor, many studies have tried to identify the ligands that bind and activate Nurr1 (Moon et al., 2015; Dubois et al., 2006). The Nurr1 agonist (AQ) used in this study is an anti-malaria drug that has the potential to activate the nuclear receptor Nurr1. One recent study showed that AQ physically binds to the ligand-binding domain of Nurr1 and it activates the *Nurr1* transcriptional pathways. This result was corroborated by five independent approaches: [^{3}H]-CQ radioligand-binding assay, Biacore S51 SPR sensor, fluorescence quenching analysis, nuclear magnetic resonance analysis and site-directed mutagenesis (Moon et al., 2015). These experimental evidences strongly support the hypothesis that AQ is a selective and potent Nurr1 agonist.

To our knowledge, this is the first study to investigate the effect of AQ on adult hippocampal neurogenesis. A recent study reported that the Nurr1 agonist AQ promotes the proliferation of dopaminergic precursor cells *in vitro* (Moon et al., 2015). Moreover, it has been shown that several CNS-derived NPCs can be efficiently differentiated into neurons by overexpressing Nurr1 (Bae et al., 2009; Park et al., 2006; Kim et al., 2003; Chung et al., 2002). As we predicted, AQ significantly enhances both the proliferation and differentiation of neurons in the hippocampal dentate gyrus. Thus, the data presented in this study suggest that AQ treatment is sufficient to enhance adult hippocampal neurogenesis. Interestingly, there are reports that microRNA (miRNA) regulates self-renewal of neural stem cells and neurogenesis (Shi et al., 2010). Recent study identified that miR-124, one of miRNAs enriched in the brain, regulates adult neurogenesis in the subventricular

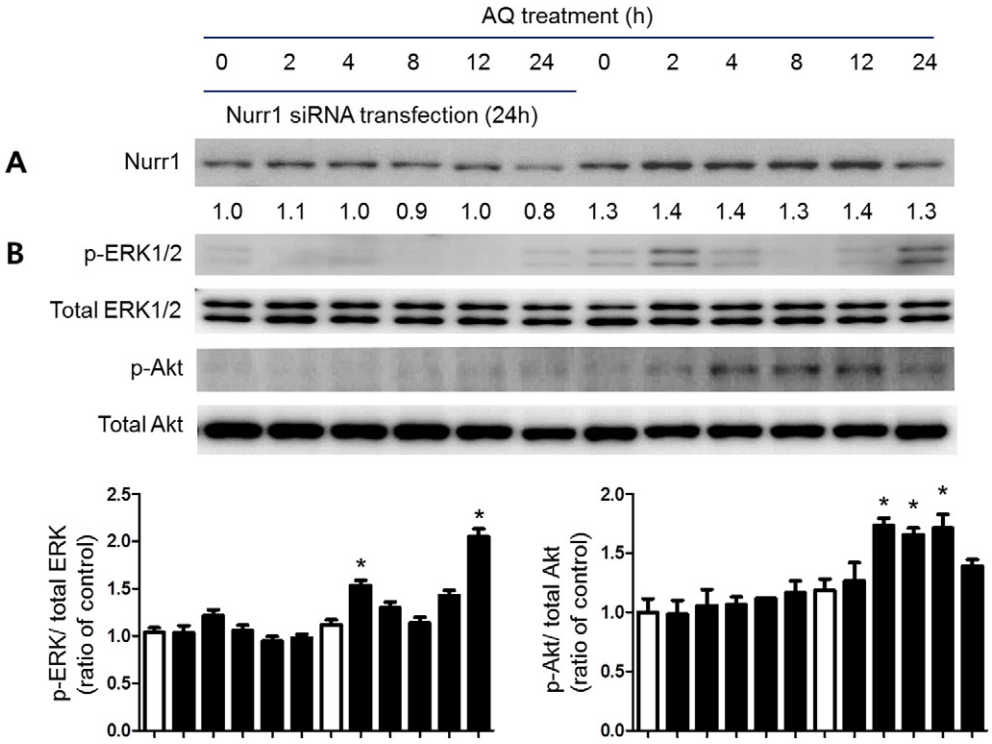

Fig. 3. Changes of phosphorylation of ERK1/2 and Akt signaling pathways after AQ treatment or siRNA transfection in adult rat hippocampal NPCs. (A) Efficiency of siRNA silencing was determined by western blot. In adult rat hippocampal NPCs, the Nurr1 receptor was knocked-down by transfection of siRNA. (B) Phosphorylation of both ERK1/2 and Akt were increased by treatment with 10 nM of AQ in adult rat hippocampal NPCs while those in *Nurr1* siRNA transfected showing decreased expression of Nurr1 receptor were not changed. All values are indicated as mean ± SEM. $^{*}p < 0.05$ compared to the control group.

zone by downregulation of Sox9 translation (Shen and Temple, 2009). It could be applicable to use such miRNAs as markers of neurogenesis to support the data from analysis of neurogenic markers expression, especially in *in vitro* studies. Further studies are needed to identify miRNAs regulating the adult hippocampal neurogenesis.

In addition to the direct evidence suggesting that AQ may promote proliferation of adult hippocampal NPCs *in vitro*, phosphorylation of Akt and ERK1/2 were found to be increased after AQ treatment. Moreover, RNA interference test showed that phosphorylation of ERK1/2 and Akt were not changed after AQ treatment that is certain levels of Nurr1 expression are required for activation of ERK1/2 and Akt signaling molecules. Consistently, it is reported that phosphorylation of signaling pathways such as MEK1/2-ERK1/2 and PI3K-Akt pathways is affected by Nurr1 expression level (Jacobsen et al., 2008; Do, 2014). Both PI3K/Akt and MAPK/ERK pathways are major signaling pathways responsible for adult hippocampal neurogenesis (Chung et al., 2013; Garcia-Yague et al., 2013; Fan et al., 2009; Sacchetti et al., 2006; Jiang et al., 2015; Peltier et al., 2007; Vithayathil et al., 2015). Although it is not examined the role of other pathways except two pathways, PI3K/Akt pathway and MAPK/ERK, these data propose important intimations. Previous studies

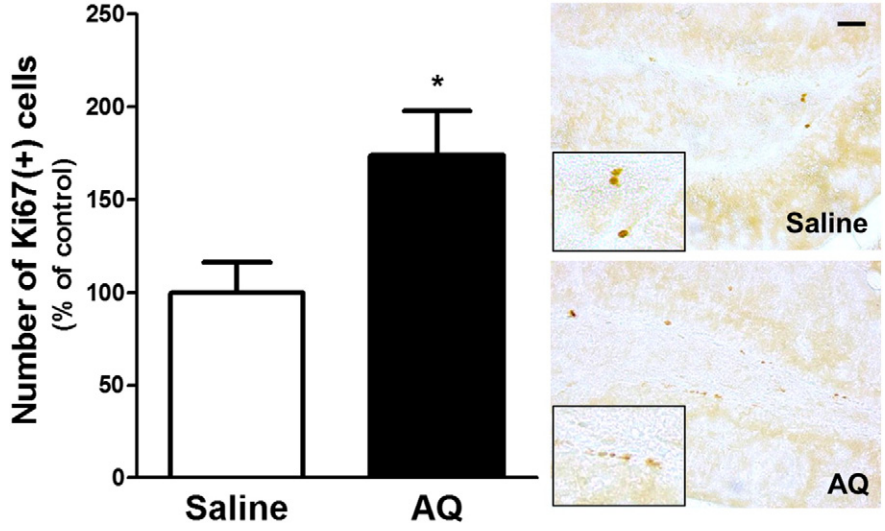

Fig. 4. Proliferative effect of Nurr1 activation induced by AQ on NPCs of dentate gyrus of hippocampus. C57BL/6 mice were administered with AQ for 2 weeks, and then 2 weeks of withdrawal period was taken. Immunohistochemistry with Ki67, a proliferation marker, was conducted in the sacrificed brain tissue, especially including hippocampal formation. The administration of AQ significantly increased the number of Ki67-positive cells of dentate gyrus in C57BL/6 mice. All values are indicated as mean ± SEM. $^{*}p < 0.05$ compared to the saline-treated control group.

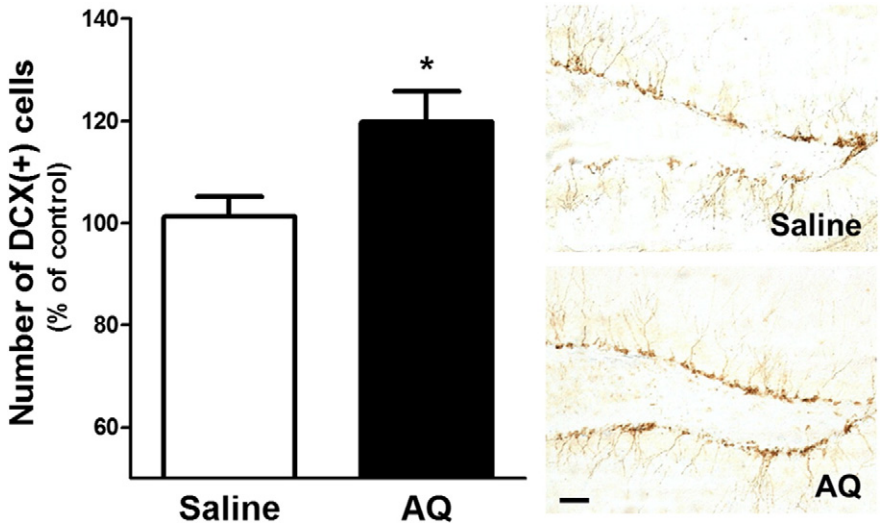

Fig. 5. Neurogenic effect of Nurr1 activation induced by AQ on hippocampal NPCs. AQ-administered C57BL/6 mice were sacrificed after two weeks of treatment and withdrawal each. Immunohistochemistry with doublecortin (DCX), a marker of neuronal fate specification in adult neurogenesis, was conducted in the sacrificed brain tissue, especially hippocampus part. The immunoreactivity of DCX showed that AQ significantly increased the number of DCX-positive cells of dentate gyrus in C57BL/6 mice. All values are indicated as mean ± SEM. $^{*}p < 0.05$ compared to the saline-treated control group.

reported that the proliferation of adult hippocampal NPCs requires multiple signaling pathways such as ERK1/2, PI3K/Akt and STAT3 signaling pathways. These signaling molecules were found to be necessary for the increase in neurogenesis induced by the treatment with valproic acid (Hao et al., 2004), ghrelin (Chung et al., 2013), hexarelin (Johansson et al., 2008), basic fibroblast growth factor-2 and sonic hedgehog (Peltier et al., 2007). In addition, little is established on the related pathways involving Nurr1 activation induced by Nurr1 agonist. Therefore, future study should be aimed at unraveling these signaling pathways. Nevertheless, our findings indicate that AQ treatment may promote adult hippocampal neurogenesis and also suggest that this effect may be mediated by Nurr1 activation and the phosphorylation of Akt and ERK1/2 signaling pathways.

It has been hypothesized that neurogenesis in DG may play an important role in learning and memory and especially in spatial memory (Deng et al., 2010; Jessberger et al., 2009; Piatti et al., 2013). In the present study, mice with AQ treatment showed an increased percentage of spontaneous alternation in Y-maze task and an improved discrimination index in the test session of the novel object recognition task, suggesting that AQ administration enhances both short- and long-term learning and memory in mice. A previous study has shown that both faster acquisition and longer retention in the Morris water maze are associated with exercise-induced increase in adult hippocampal neurogenesis in mice (van Praag et al., 2005). Other studies also observed that mice with decreased adult hippocampal neurogenesis show impaired cognitive behaviors (Li et al., 2013; Lee et al., 2015). Interestingly, one placebo-controlled study reported that the intermittent preventive treatment of AQ improves the cognitive ability of semi-immune schoolchildren (Clarke et al., 2008). Therefore, we can speculate that AQ-induced cognitive enhancement may occur via a Nurr1-dependent modulation of adult hippocampal neurogenesis.

While neuronal stem cells including neural stem cells (NSCs) in CNS and neural crest stem cells (NCSCs) in peripheral nervous system (PNS) have been successfully achieved to study developmental or degenerative neuronal disease with adequate cell types (Lee et al., 2009; Lafaille et al., 2012), still it is challenging to control transplanted cells in the specific region without various risks. Since adult neural stem cells were first identified in the mammalian brain (Ming and Song, 2005), the characterization of their functional properties allowed significant insights into the biology of stem cells and the mechanistic studies of brain disease (Tabar et al., 2005). This population of cells has been described as a reserve of endogenous stem cells with limited differentiation potential in comparison to other types of neuronal stem cells derived from embryonic stem cells (Lee et al., 2007; Kim et al., 2014).

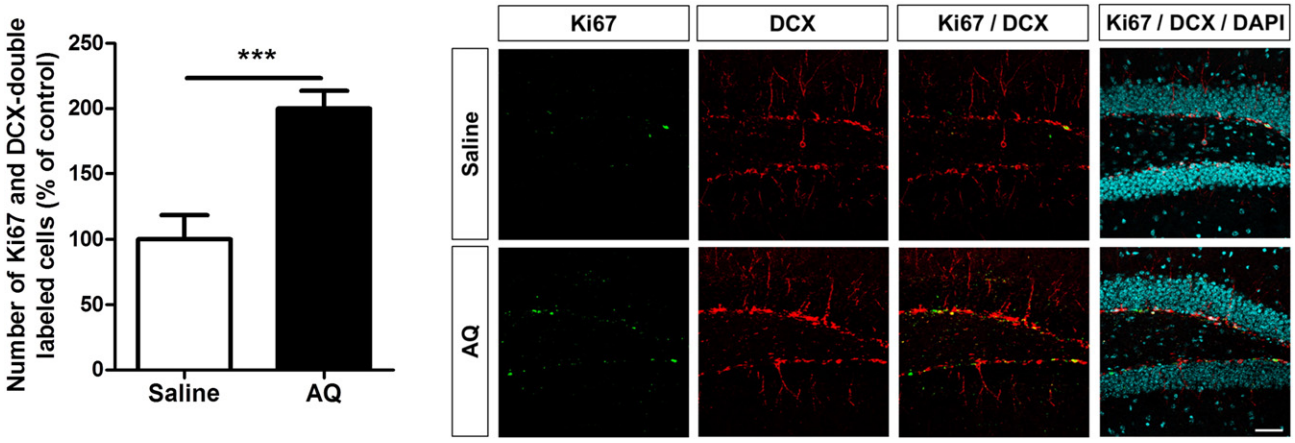

Fig. 6. Immunoreactivity of Ki67 and DCX in the DG of adult hippocampus. Sections were double-stained with Ki67 and DCX and co-stained cells were counted. AQ-administered mice showed significantly increased numbers of Ki67-positive cells, DCX-positive cells and DCX/Ki67 co-labeled cells in the DG. Co-labeled cells exhibited a migratory morphology from subgranular zone to granular cell layer. All values are indicated as mean ± SEM. $^{*}p < 0.001$ compared to the saline-treated control group.

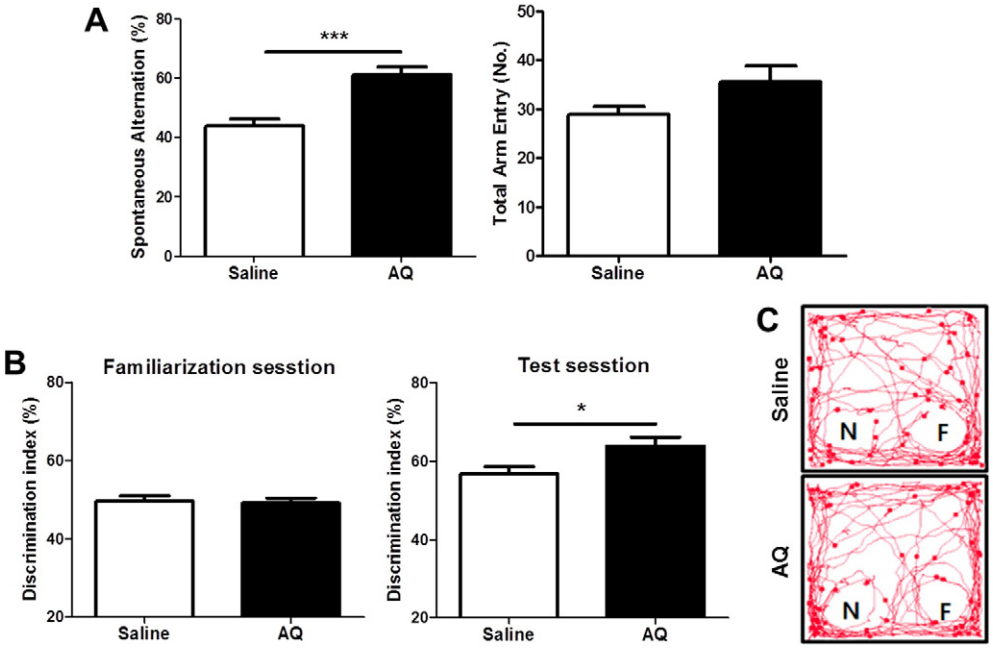

Fig. 7. Effects of Nurr1 activation induced by AQ on cognitive ability of learning and memory. After two weeks of AQ treatment and withdrawal each, the AQ-administered mice had two behavioral tests: Y-maze test and novel object recognition test (NORT). (A) In the Y-maze test, AQ-treated group showed significant increase in spontaneous alteration compared to the saline-treated control group. However, there was no significant difference between the saline and AQ-treated groups. (B) In the NORT, AQ-treated group showed significant preference for the novel object than the familiar object in the test session compared to the saline-treated control group. During the familiarization session, there was no significant preference. (C) Representative tracing of mouse center point during test session of NORT was detected with Ethovision software. Novel and familiar object is shown as a symbol 'N' and 'F', respectively. Values are expressed as the mean with SEM. $^{*}p < 0.05$ as compared to the saline-injected control group in test session.

Current studies have shown that the direct regulation of cellular fate is possible through the manipulation of environmental conditions (Najm et al., 2015). In addition, more advanced technologies, including pharmacological approaches, have been reported to increase our ability to control cell activity, including its differentiation and cellular functions (Kim and Lee, 2013) both *in vitro* and after *in vivo* transplantation. Moreover, it has been suggested that promoting the differentiation of endogenous neural stem cells, rather than introducing exogenous cells, might be a more effective strategy to modulate adult hippocampal neurogenesis.

Here we showed that activation of Nurr1 induces hippocampal neurogenesis in the mouse brain by stimulating neural stem cells. Furthermore, the direct stimulation of neural stem cells exerted by AQ is a viable pharmacological approach to regulate gene activation and to control the differentiation of NPCs into neuronal cells. Indeed, the observation that this endogenous stem cell activation enhances cognitive functions suggests that novel concept for pharmacological regulation of endogenous NSC control for future studies related to stem cell regulation and memory failure diseases. However, there were reports that the discrepancies in the duration of cell maturation and the amount of new granule cells after stimulation of NPCs in DG between two different species; rat and mice (Snyder et al., 2009; Ray and Gage, 2006). Since we compared the *in vivo* results using C57BL/6 mice with *in vitro* results using NPCs derived from fisher 344 rats, there may be a discrepancy in effectiveness of AQ on neuronal maturation between two different species. Thus, conclusion based on comparison between two different species should be limited to adult hippocampal neurogenesis. Further *in vitro* studies using mice adult hippocampal NPCs are needed to further elucidate the effects of pharmacological stimulation of Nurr1 on adult hippocampal neurogenesis and cognitive functions.

In summary, using the Nurr1 agonist AQ we first showed that Nurr1 plays an important role in the regulation of adult hippocampal neurogenesis. AQ may increase adult hippocampal neurogenesis *via*, in part, an up-regulated phosphorylation of Akt and ERK1/2. Moreover, the Nurr1 agonist enhances both short- and long-term memory, the cognitive processes strongly associated with adult hippocampal neurogenesis. Taken together, these findings suggest that Nurr1 can be used as a therapeutic target for the treatment of memory disorders or neurodegenerative diseases associated with impaired adult neurogenesis. In conclusion, AQ is a viable candidate as a pharmacological compound for treating the impaired memory functions described in many cognitive diseases.

Supplementary data to this article can be found online at doi:10.1016/j.scr.2016.09.027.

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