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Deficiency of Mesencephalic Astrocyte-derived Neurotrophic Factor Affects Neurogenesis in Mouse Brain

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Abstract

The mechanisms underlying the regulation of neurogenesis in the adult brain remain unclear. Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a neurotrophic factor that has been implicated in various neuropathological processes and endoplasmic reticulum stress. However, the role of MANF in neurogenesis has not been investigated. Using a central nervous system (CNS)-specific *Manf* knock-out mouse model, we examined the role of MANF in mouse neurogenesis. We demonstrated that MANF deficiency increased BrdU labeling and Ki-67 positive cells in the subgranular zone and subventricular zone. MANF knock-out-induced upregulation of proliferative activity was accompanied by a decrease of cell cycle inhibitors (p15 and p27), an increase of G2/M marker (phospho-histone H3), as well as an increase of neural progenitor markers (Sox2 and NeuroD1) in the brain. *In vitro* studies using N2A neuroblastoma cells showed that the gain-of-function of MANF inhibited cell cycle progression, whereas the loss-of-function of MANF promoted cell cycle progression. Collectively, our findings indicate MANF deficiency affects cell proliferation and suggest a role of MANF in the neurogenesis of the adult brain.

Keywords

Cell cycle; development; differentiation; neural stem cells; subgranular zone; subventricular zone

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Conflicts of Interest

The authors declare no conflict of interest.

All authors have read and approved the latest version. We have no competing interests. This manuscript has never been submitted to *Brain Research Bulletin*. It has not been and will not be submitted or published in other scientific journals in whole or in part while it is under the consideration of *Brain Research Bulletin*.

Introduction

Neurogenesis is a process in which neural stem cells (NSCs) give rise to various types of neurons [1, 2]. The first discovery of neurogenesis in the adult rodent brain was made in the hippocampal dentate gyrus in the 1960s [3]. Subsequent studies revealed that neurogenesis exists throughout life in two brain regions: the subgranular zone (SGZ) of hippocampal dentate gyrus and the subventricular zone (SVZ) of anterior lateral ventricles [4–6]. Neurons born in the adult SVZ migrate through the rostral migratory system (RMS) and eventually evolve into granule neurons and periglomerular neurons in the olfactory bulb [1]. Neurons born in the adult SGZ migrate to the granule layer of the dentate gyrus and differentiate into granule cells [1, 7, 8]. The newly formed neurons need to integrate into local circuitry and receive input from other neurons to become functional [1, 2, 8].

Neurogenesis is a complex process that involves proliferation, differentiation, migration, survival, and maturation of newly born neurons [1]. Numerous factors have been implicated in the regulation of neurogenesis at distinct stages of brain development [2]. Neurotrophic factors are a group of extracellular signaling proteins that constitute part of the regulatory network that enables neuronal growth and maturation. For example, brain-derived neurotrophic factor (BDNF), one of the most important neurotrophic factors in the brain, regulates various aspects of neurogenesis in the SVZ and SGZ and actively engages in the stimulation of post-stroke neurogenesis [9]. Mechanistically, BDNF exerts its effect through binding and activating receptor tyrosine kinase known as Trk receptors and their co-receptor p75NTR [9]. Glial cell line-derived neurotrophic factor (GDNF) is another well-studied neurotrophic factor that has been shown to promote differentiation and tangential migration of cortical GABAergic neurons during the mouse brain development [10]. Recent evidence shows that GDNF is required for adult-born neurons to properly integrate into local circuitry in the hippocampal dentate gyrus [11]. In addition, GDNF can act as a chemoattractant to modulate the movement of precursor neurons along RMS [12]. In a mouse stroke model, GDNF has been shown to promote the proliferation of neural stem cells (NSCs) in the SVZ and their migration toward the damaged striatum [13].

The mesencephalic astrocyte-derived neurotrophic factor (MANF) together with the cerebral dopamine neurotrophic factor (CDNF) form a unique family of neurotrophic factors (NTFs) that are structurally and functionally different from other proteins with neurotrophic activity. Neurotrophic factors exert effects by binding to cognate receptors in the plasma membrane; however, no cell surface receptors have been identified for MANF and CDNF. MANF and CDNF are endoplasmic reticulum (ER) luminal proteins that confer trophic activities in a wide range of tissues under diverse pathological conditions. Both can act as unfolded protein response (UPR) proteins that modulate the UPR and inflammatory processes [14–16]. MANF responds to ER stress and is upregulated in various pathological conditions in which ER stress is a sharing trait [17, 18]. MANF confers neuroprotection, particularly to dopaminergic neurons *in vivo* and *in vitro* [15, 19, 20]. During retinal injuries, MANF is induced and participates in the regeneration and restoration of the retina [21]. In addition, MANF is also involved in neuronal migration and neurite outgrowth in the developing cortex and cultured neuronal cells [22, 23]. With an oxygen-glucose-deprivation (OGD)-induced stoke model, MANF has been shown to protect NSCs from OGD-induced injury [13].

Furthermore, the administration of MANF promotes differentiation and migration of neural progenitor cells in a rat model of cortical stroke [13]. We have previously demonstrated that the expression of MANF is developmentally regulated [24]. Although MANF is implicated in the regulation of various developmental events, its role in neurogenesis has never been explored. In this study, we sought to investigate the effect of MANF on the neurogenesis in adult mouse brain using a CNS-specific MANF knock-out animal model. We also have investigated potential mechanisms by examining the effects of gain- and loss-of-function of MANF on cell cycle progression *in vitro*.

Materials and Methods

Materials

Antibodies against Ki-67 (12202, RRID:AB_2620142), cyclinE1 (20808, RRID:AB_2783554), sox2 (23064, RRID:AB_2714146), HA (3724, RRID:AB_1549585), p-histone H3 (s10) (pHH3) (3377, RRID:AB_1549592), calbindin (13176, RRID:AB_2687400), PCNA (13110, RRID:AB_2636979), p27 (3686, RRID:AB_2077850) and NeuroD1 (7019, RRID:AB_10859914) were from Cell Signaling Technology (Danvers, MA). Antibodies against doublecortin (DCX) (ab18723, RRID: AB_732011), Bromodeoxyuridine (BrdU) (AB8152, RRID:AB_308713), cyclin A2 (ab181591, RRID:AB_2890136), p15 (ab53034, RRID:AB_2078578) and MANF (ab67203, RRID:AB_1267729) were from Abcam (Cambridge, MA). NeuN antibody (MAB377, RRID:AB_2298772) was from EMD Millipore (Burlington, MA). HRP-conjugated antibi-rabbit IgG (NA934, RRID:AB_772206) and anti-mouse IgG (NA931, RRID:AB_772210) secondary antibodies were from GE Healthcare Life Sciences (Piscataway, NJ). Mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (Cat No. H-1200-10) were from Vector Laboratories (Burlingame, CA). Alexa-488 conjugated anti-rabbit IgG (A-11008, RRID:AB_143165) and Alexa-594 conjugated anti-mouse IgG (A-11005, 2534073) antibodies were from Life Technologies (Grand Island, NY). Other chemicals were purchased either from Sigma-Aldrich or Life Technologies (Frederick, MD).

CNS-specific MANF knock-out animal model

CNS-specific *Manf* KO mice were created as described before [25]. Briefly, *Manf*$^{flox/flox}$ mice were generated by the knock-out mouse project (KOMP) repository and the mouse biology program at the University of California Davis. Then *Manf*$^{flox/flox}$ mice (CTL) were crossed with Nestin-Cre$^{+/}$ mice (obtained from Jackson Laboratory) to generate Nestin-Cre$^{+/-}$::*Manf*$^{flox/+}$ mice. Their offspring were then crossed with *Manf*$^{flox/flox}$ mice again to generate Nestin-Cre$^{+/-}$::*Manf*$^{flox/flox}$ mice (cKO). In all studies that involve comparing cKO and CTL, sex- and age-matched siblings were achieved by cross-breeding of *Manf*$^{flox/flox}$ and Nestin-Cre$^{+/-}$::*Manf*$^{flox/flox}$ mice (cKO). All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and performed following the rules set by the National Institutes of Health Guide. Mice were maintained in a clean facility with a 12-hour shift of light/dark cycle and access to food and water.

Immunoblotting

Briefly, samples were prepared from the mouse brain or cell culture followed by lysis in RIPA buffer (50Mm Tris pH 7.4, 150mM sodium chloride, 1% NP-40, 0.1% SDS, 2mM EDTA, 1X complete mini protease inhibitor, and 1X complete mini phosphatase inhibitor) for proteins used for the immunoblotting analysis of neuronal progenitor and cell cycle markers. Protein-specific signals were then detected with enhanced chemiluminescence substrate (GE Healthcare, Chalfont, Buckinghamshire, UK) using a Chemi™Doc imaging system (Bio-Rad 215 Laboratories, Hercules, CA) and then quantified with the software of Image lab 5.2 (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry and immunofluorescence

Briefly, brain samples were fixed in 4% paraformaldehyde (PFA) overnight and then dehydrated in sucrose solution. Next, brain samples were sagittally sectioned (15 μm), treated with 0.3% H_2O_2 to quench endogenous peroxidase, permeabilized by 1% Triton X-100 and then subject to immunohistochemistry (IHC) or Immunofluorescence (IF) analyses as described previously [26, 27]. Primary antibodies used in the study are anti-DCX (1:500 dilution), anti-Calbindin (1:200 dilution), anti-Ki-67 (1:500 dilution), anti-BrdU (1:50 dilution), anti-MANF (1:200 dilution), and anti-NeuN (1:200 dilution). Secondary antibodies used in IF analyses are Alexa Fluor 488-conjugated anti-rabbit IgG (1:200 dilution) or Alexa Fluor 594-conjugated anti-mouse IgG (1:200 dilution). Secondary antibodies used in IHC analyses are biotin-conjugated anti-mouse IgG (1:200 dilution) or biotin-conjugated anti-rabbit IgG (1:200 dilution). Avidin biotin complex (ABC) kits and 3,3'-Diaminobenzidine (DAB) were used in IHC to amplify and visualize signals.

In vivo labeling with BrdU

Briefly, BrdU was constituted in PBS to make a sterile solution of 10 mg/ml. BrdU solution was administered to postnatal day 7 (PD7) pups (CTL and cKO) through intraperitoneal injection at a dose of 100 mg/kg [28]. At 4 hours after BrdU treatment, the animals were sacrificed and the brains were removed, dissected, fixed, paraffin-embedded, sectioned, and then subject to IF analysis with antibodies against BrdU and Calbindin according to the standard immunofluorescence protocol (IF). The same or similar tissue areas were chosen by staining across 5–10 consecutive sections for each animal. BrdU positive staining was quantified by ImageJ software across these 5–10 sections and the staining results from 3–5 animals in each group were used for statistical analysis.

Cell culture

N2A cells (mouse neuroblastoma) were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin. *Manf*-knock-out stable cell lines were established using the CRISPR/Cas9 gene-editing method. In brief, N2A cells were transfected with *Manf* CRISPR/Cas9 KO plasmid (Cat No. sc-428989-NIC) or the control plasmid (Cat No. sc-418922) according to the protocol from manufacture (Santa Cruz Biotechnology). Colonies with successful transfection of the KO plasmids or control plasmids were visually confirmed by the detection of the green fluorescent protein (GFP) and immunoblot of MANF. Three colonies with complete *Manf* knock-out (validated by

immunoblot of MANF) were selected for the following immunochemical or flow cytometric analysis with colonies transfected with control plasmids as the control.

MANF adenovirus and transduction

MANF adenoviruses (AD-MANF-HA) were prepared in Applied Biological Materials (ABM). In brief, a DNA sequence encoding MANF was inserted into pAdenoG-HA vector and then packaged into adenoviruses (Cat No. 210179A). An empty vector virus, AD-vector (Cat. No. 000047A) was used as the control virus. Both adenoviruses were prepared at titer more than 1×10^6pfu/ml and amplified in our lab by transducing HEK293 cells. To obtain MANF overexpression in N2A cells, N2A cells were infected with AD-MANF-HA or AD-vector for 48 hours following the protocol from manufacture (ABM). In brief, N2A cells were seeded in 6-well plates or 6-well chambers at 50% confluency one day before transduction. In transduction, N2A cells were covered with 200 μl viral culture ($\sim1\times10^8$pfu/ml) for 2 hours and then incubated with fresh media. After transduction for 48 hours, N2A cells were collected for the subsequent immunochemical or flow cytometric analysis.

Cell cycle analysis

Briefly, N2A cells with loss- or gain-of-function of MANF were trypsinized and fixed in 70% ethanol for 30 minutes. Next, following RNA removal by ribonuclease I (5 μg/ml), these samples were incubated with PI for at least 30 minutes and then subject to flow cytometric analysis of PI content. ModFit LT V3.3.11 (software) was used to quantitate the percentage of cells in each cell cycle phase.

MTT assay

Briefly, to determine the effects of gain-of-function of MANF on cell viability, N2A cells were infected with AD-MANF-HA or AD-vector for 48 hours. Cell viability/proliferation was assessed and compared by MTT assay according to the protocol from manufacture (Roche, Cat No. 11465007001). To determine the effects of loss-of-function of MANF on cell viability, N2A cells of MANF KO and control cells were seeded (D0) and then grew for 24 hours (D1) or 48 hours (D2). Cell viability/proliferation was assessed and compared by MTT assay fowling the same protocol.

Statistical analysis

The data were expressed as mean ± SEM, and statistical significance was determined using student unpaired t-test (GraphPad Prism version 7). A p-value of less than 0.5 was considered statistically significant.

Results

MANF knock-out enhances neurogenesis in the SGZ of the hippocampal dentate gyrus

Neurons born in the SGZ migrate to the granule cell layer and differentiate into granule cells in the hippocampal dentate gyrus [1]. We evaluated neurogenesis/proliferation level in the SGZ by immunostaining of DCX and BrdU incorporation. DCX is a microtubule-

associated protein expressed in neuronal precursor cells and immature neurons [29]. BrdU is an analog of the nucleoside thymidine that can be incorporated into the genome during DNA replication; BrdU labeling is a classical method used for labeling dividing neurons [30]. We first assessed the neurogenesis level in SGZ at the postnatal stage and compared the level between the CTL group and the cKO group. As shown in Figure 1A and B, there was a significant increase in DCX-positive cells in the SGZ of cKO mice compared to that in their CTL littermates. Moreover, the increase in DCX-positive cells in cKO mice extended to the adult stage, e.g., in three-month- or six-month-old mice (Figure 1A and 1B). However, we did not observe a significant change in DCX-positive cells in the SGZ of ten-month-old cKO mice compared to that of CTL mice (Figure 1A and 1B). Consistently, the number of BrdU positive cells in the hippocampal dentate gyrus of PD7 cKO mice was significantly more than that in CTL mice (Figure 1C and 1D). Therefore, it appears MANF deficiency alters neurogenesis, and these results suggest a role of MANF is neurogenesis, which aligns with the pattern of developmental MANF expression in the brain [24].

MANF knock-out enhances cell proliferation in the SVZ of the anterior lateral ventricles

We evaluated the proliferation of progenitor cells in the SVZ by determining Ki-67 expression and BrdU incorporation. Ki-67 is a general cell proliferating marker and has been widely used as a proliferating marker in tumorigenesis and neurogenesis [30]. Our data showed that the number of Ki-67-positive cells was increased in the SVZ of the anterior lateral ventricles of cKO mice of early postnatal days (PD7) and early adult stage (three- and six-month-old) but not late adult stage (ten-month-old) (Figure 2A and 2B). Consistently, the number of BrdU positive cells in the SVZ of PD7 cKO mice was significantly higher than that of CTL mice, supporting an increased proliferation of progenitor cells in the SVZ of MANF cKO mice (Figure 2C and 2D).

MANF knock-out promotes NeuroD1 and Sox2 expression and cell cycle progression

A number of transcriptional factors have been shown to control lineage progression and proliferation during the neurogenesis [2]. Among them, the SRY-related high-mobility group box (Sox) family member, Sox2 and NeuroD1 are the most predominant transcriptional factors that maintain the multipotency and proliferative capacities of NSCs, and are frequently used as markers for proliferative neural precursors [2]. We examined the expression of NeuroD1 and Sox2 along with other regulators of cell cycle progression in the *Manf* cKO mouse brain to that in the CTL mouse brain. Our results showed that MANF knock-out resulted in a significant increase in the expression of NeuroD1 and Sox2 (Figure 3A). We have compared the expression levels of several activators and inhibitors of cell cycle progression. As shown in Figure 3A, cell-cycle inhibitors (p15 and p27) were downregulated in the brain of *Manf* cKO mice. Consistent with these findings, mitotic markers present in the G2/M phase (cyclin A2 and pHH3) were increased, whereas the quiescent phase marker (cyclin E1) was decreased. Collectively, these findings on cell cycle-associated proteins correlate with the results obtained from the IHC of DCX and Ki-67 as well as BrdU incorporation, suggesting that MANF knock-out increases neurogenesis or cell proliferation in the mouse brain.

Over-expression of MANF inhibits cell cycle progression *in vitro*

To confirm our findings *in vivo*, we first examined the effect of over-expression of MANF on cell proliferation *in vitro*. Mouse neuroblastoma cells (N2A) cells were infected with AD-MANF-HA to obtain MANF over-expression in comparison with cells infected with AD-CTL (AD-vector). First, we confirmed the over-expression of MANF in these cells by immunoblots and immunofluorescence staining of MANF and HA 48 hours after the viral infection (Figure 4A and 4B). Cell cycle analysis by flow cytometric measurements of propidium iodide (PI) incorporation revealed a decreased percentage of proliferative cells (the summation of S phase and G2/M phase) in AD-MANF-HA infected cells when compared to that of AD-CTL infected cells (Figure 4C). The results were supported by a reduction of the S phase or G2/M phase marker (cyclin A2, PCNA, and pHH3) and an increase of cell cycle inhibitors (p15 and p27) in AD-MANF-HA infected cells (Figure 4D). MTT assay was used to determine the number of viable cells. The results of MTT assay showed that over-expression of MANF decreased cell number, suggesting an inhibition of cell proliferation (Figure 4E).

Knock-down of MANF expression promotes cell cycle progression *in vitro*

To further investigate the effect of MANF down-regulation on cell proliferation *in vitro*, we have developed several colonies in which *Manf* was knocked down in N2A cells using the CRISPR/Cas9 method. First, we confirmed that the expression MANF was significantly decreased in N2A cells by immunoblotting and IHC of MANF (Figure 5A and 5B). In contrast to MANF over-expression, *Manf* knock-down in N2A cells increased the percentage of proliferative cells (the summation of S phase and G2/M phase) (Figure 5C). Likewise, this increase in cell proliferation was supported by an increase of S phase or G2/M phase marker (cyclin A2, PCNA, and p-HH3) and a decrease of cell cycle inhibitors (p15 and p27) in *Manf* knock-out N2a cells (Figure 5D). Moreover, as expected, MANF down-regulation augmented cell proliferation as shown by MTT assay (Figure 5E). Taken together, the results indicated that up-regulation of MANF inhibited cell cycle progression, whereas down-regulation of MANF promoted cell proliferation.

Discussion

We have demonstrated that MANF knock-out promotes cell proliferation in the SVZ and SGZ of the mouse brain. Besides, we have shown that two crucial transcriptional factors (NeuroD1 and Sox2) that are associated with NSC multipotent and proliferative capacities are increased in the brain of *Manf* cKO mice. *In vitro* studies confirm that over-expression of MANF inhibits cell proliferation and cell cycle progression, while MANF deficiency enhances cell proliferation and cell cycle progression.

MANF was first identified in an astrocyte cell line in search of astrocyte-derived neurotrophic factor [19]. MANF is neuroprotective, especially for dopaminergic neurons *in vivo* and *in vitro* [19, 20]. It has also been shown that MANF is involved in neurite outgrowth and neuronal migration [22, 23]. MANF promotes cell survival by interacting with proapoptotic proteins such as Bax and Bak to prevent their pro-apoptotic properties or by promoting mTOR signaling to increase the transcription of pro-survival genes [13,

31, 32]. The expression of MANF in the brain is developmentally regulated, implicating its involvement in the regulation of brain development. However, the role of MNAF in neurogenesis is unknown. In our study, MANF deficiency results in an increase in cell proliferation in both the SGZ and SVZ of *Manf* cKO mice, suggesting that MANF may negatively regulate the proliferative activity of neural precursors. This is consistent with the finding that MANF may increase neurite-outgrowth/differentiation and migration of neural progenitor [22, 23], because usually the promotion of neuronal differentiation is accompanied with the inhibition of proliferative activity.

Neurogenesis is a complex process involving multiple developmental steps or stages characterized by cell proliferation, differentiation and lineage commitment prior to integration into local neural circuitry. For example, in the hippocampal dentate gyrus, mature granule neurons undergo at least three developmental stages before they are functionally integrated into the hippocampal circuitry. Type1 radial glia-like cells (RGLs) are thought to represent the NSCs population that possess self-renewal and multipotent capabilities. Intermediate progenitor cells (IPCs, or type 2 cells) are believed to stem from RGL differentiation and possess transient amplifying activities. IPC can differentiate into neuroblasts (type 3) that give rise to mature granule dentate granule neurons [2]. It has been reported that DCX is expressed in neuroblasts but not in RGL or IPC cells [1]. Ki-67 is a general cell proliferation maker that can label any cells that are undergoing cell division regardless of the specific stage of lineage differentiation. Although MANF is implicated in cell cycle progression and proliferation, it is unclear whether it affects other neurogenesis steps, such as the differentiation in the hippocampal dentate gyrus. To decipher the regulatory role of MANF in neurogenesis, it is important to investigate the effect of MANF on various stages of neurogenesis. Studies using molecular markers that are expressed at specific stages of hippocampal neurogenesis and SVZ may provide further insight.

Our results suggest that MANF may impact cell cycle progression. Our *in vivo* studies indicate that Sox2 and NeuroD1 are upregulated in the brain of *Manf* cKO mice. Sox2 has been shown to regulate the expression of several genes that controls cell proliferation; among them, the nuclear orphan receptor, Tlx, is positively regulated by Sox2 [2]. Tlx promotes proliferation and self-renewal of NSCs either by activating the canonical Wnt pathway or by inhibiting pathways that maintain cell quiescence [2, 33]. Additionally, Sox2 promotes neurogenesis by repressing pathways that promote cell quiescence such as p53 pathway and cell-cycle inhibitor p21 [2]. NeuroD1 is involved in the proliferation of neuronal progenitors and promoting hippocampal neurogenesis [2]. Mechanistic studies show that NeuroD1 promotes cell proliferation by suppressing the p53/p21 axis [34]. In this study, the whole brain was used for IB analysis; It is desirable to use hippocampal tissues or brain tissues surrounding the anterior ventricle for IB analysis to obtain a more accurate assessment in future studies. In addition, double labeling of Ki67/Sox2 or Ki67/Nestin would provide further verification. However, due to technical difficulty, we were not able to double-label these markers in the brain tissues using all available antibodies. In our studies, MANF deficiency in the mouse brain upregulates mitotic markers (p-HH3 and cyclin A2) and meantime downregulates cell cycle inhibitors (p15 and p27). The effects of MANF on the expression of the abovementioned cell cycle-associated proteins are consistent with

the result of *in vivo* BrdU labeling (Figs 1 and 2) and *in vitro* cell proliferation/cell cycle analysis (Figs. 4 and 5). Our *in vitro* studies show that MANF over-expression inhibits the proliferation of N2A cells, increases the expression of cell cycle inhibitors, but decreases the expression of cell cycle activators. In contrast, MANF knock-down stimulates the proliferation of N2A cells, decreases the expression of cell cycle inhibitors, but increases the expression of cell cycle activators. N2A is a mouse neuroblastoma cell line, it would be better to verify these findings in N2A cells in primary NPC cultures in future studies, since NA2 cells may differentiate *in vitro*. Our results are also consistent with the findings in cancer biology, showing that MANF over-expression inhibits tumor cell proliferation and thus reduces tumor aggressive properties [35].

In addition to its impact on cell cycle progression, MANF may affect neurogenesis through other mechanisms such as the metabolic pathways. Metabolic status has been implicated in stem cell behavior or activity alteration [2]. Inhibition of fatty acid synthesis through genetic or pharmacological approaches can result in a decrease in stem cell proliferation [2]. A recent study shows that MANF is involved in energy metabolism by inducing insulin resistance in the hypothalamus [36]. Therefore, MANF could affect neurogenesis by altering metabolic status. A better understanding of the mechanisms underlying MANF regulation of adult neurogenesis would have an important implication for its potential therapeutic value in neurodegenerative and psychiatric diseases.

In our study, while MANF knock-out overall promotes neurogenesis in the mouse brain, it shows no significant impact on neurogenesis in aged mice. It has been reported that aging negatively regulates neurogenesis manifested by a sharp and continuous decrease in cell population in both the SGZ and SVZ of the aged mouse brain [37, 38]. With aging, NSCs lose their proliferative capability and become more quiescent even though some reports indicate that neurogenesis can be reactivated to a certain extent in aged mice upon stimulation such as exercise or calorie restriction [39, 40]. In humans, using postmortem and fresh tissues, scientists have shown that neurogenesis is drastically diminished with aging and neurogenesis in human after adolescence is arguably detectable [41]. In summary, recent findings point to the conclusion that neurogenesis is decreased with aging both in humans and rodents. Our data appear consistent with these reports, showing that MANF knock-out cannot rejuvenate the quiescent NSCs in aged mice. Alternatively, the role of MANF in neurogenesis may phase out because MANF expression declines over time after birth as shown in the rat brain [24].

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Highlights

- MANF deletion in the CNS increases proliferation of neurol precursors
- Neuronal MANF deficiency alters cell cycle regulating proteins in the brain
- MANF regulates cell cycle progression of neuronal cells *in vitro*

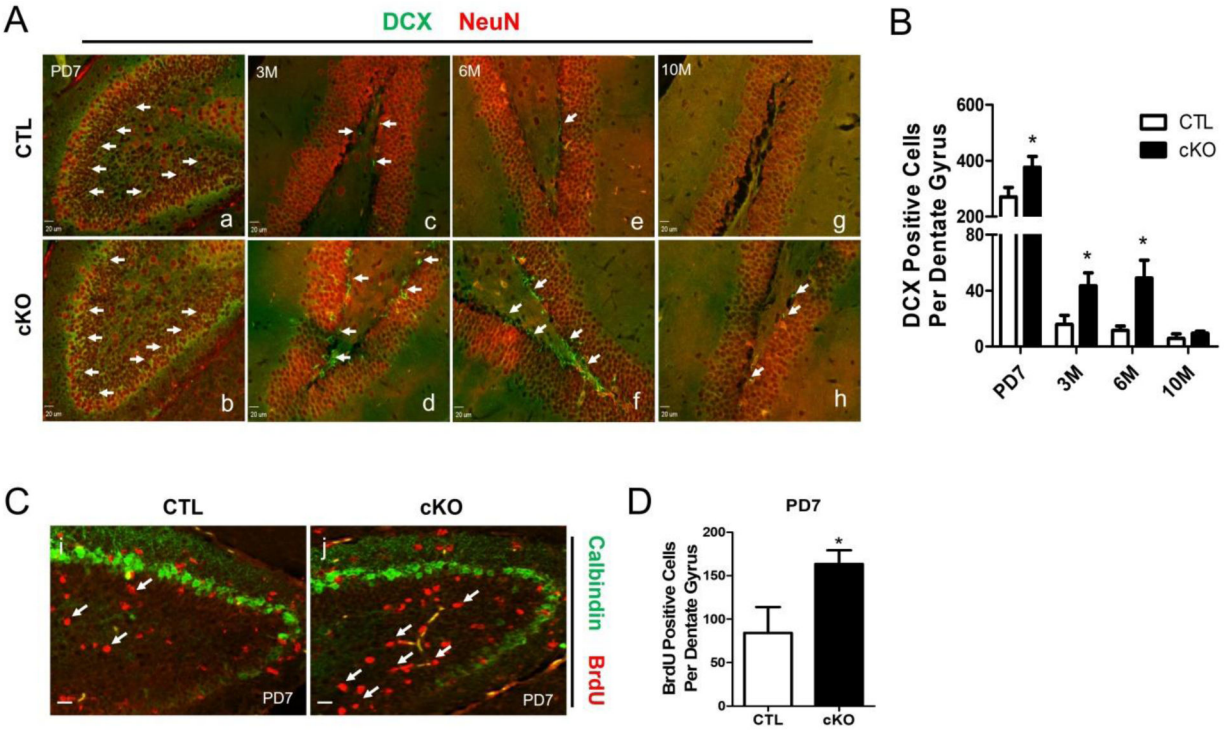

Figure 1. MANF knock-out enhances neurogenesis in the SGZ of the hippocampal dentate gyrus.

A: Representative co-immunostaining of DCX (green) and NeuN (red) in the hippocampal dentate gyrus from the control (CTL) mice (a, c, e and g) and *Manf* knock out (cKO) mice (b, d, f and h). PD: postnatal day. M: postnatal month. Arrows indicate DCX-staining positive neurons. **B**: The same or similar tissue areas by staining across 5–10 sections were used to quantify DCX positive cells in the SGZ of CTL and cKO mice. **C**: Representative images of co-labeling with calbindin (green) and BrdU (red) in the hippocampal dentate gyrus of PD7 mouse pups from CTL (i) and cKO (j) groups. Arrows indicate BrdU-staining positive neurons. **D**: The same brain area of 5–10 sections was used to quantify BrdU positive cells in the SGZ of PD7 pups from CTL and cKO mice. Scale bar = 20 μm. Each data point is mean ± SEM. n=3–5 mice, *P<0.05 vs. CTL.

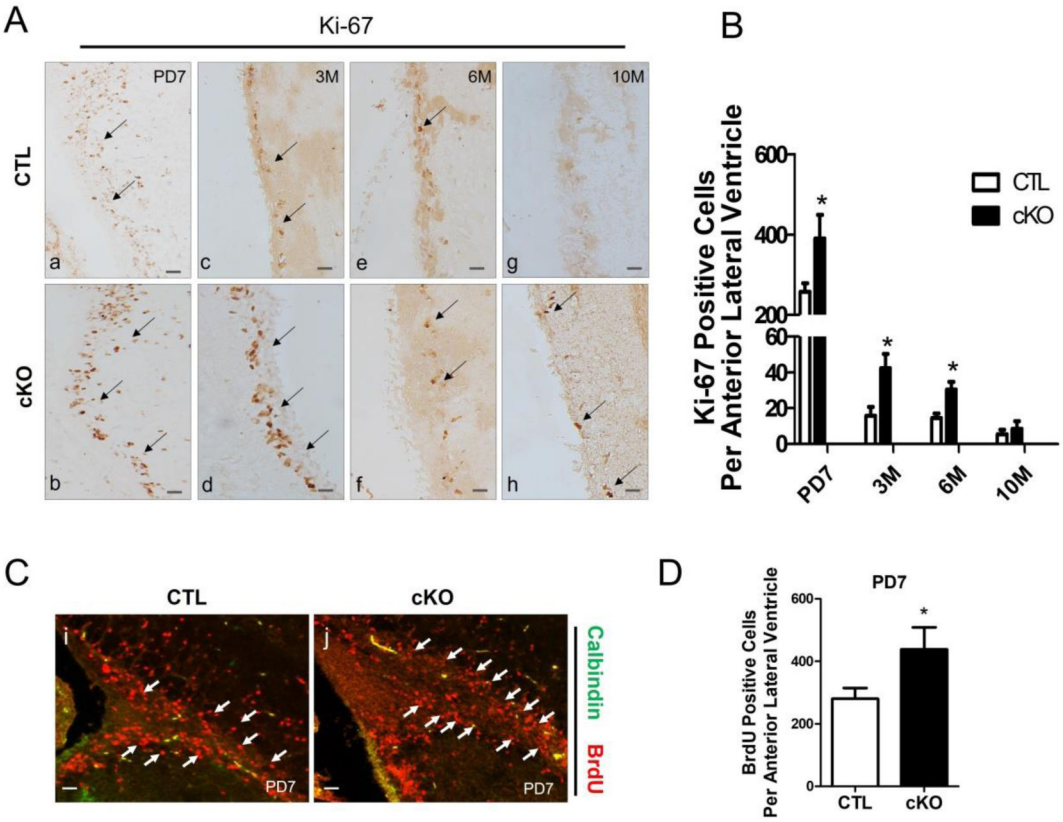

Figure 2. MANF knock-out enhances cell proliferation in the SVZ of the anterior lateral ventricles.

A. Representative images of immunohistochemical staining of Ki-67 in the lateral ventricle from the CTL (a, c, e and g) and cKO (b, d, f and h) mice. PD: postnatal day. M: postnatal month. Arrows indicate Ki-67-staining positive cells. **B.** The same or similar tissue areas by staining across 5–10 sections were used to quantify Ki-67 positive cells in the SVZ of CTL and cKO mice. **C.** Representative images of co-labeling with calbindin (green) and BrdU (red) in lateral ventricles of PD7 mouse pups from the CTL (i) and cKO (j) groups. Arrows indicate BrdU-staining positive cells. **D.** The same brain area of 5–10 sections was used to quantify BrdU positive cells in the SVZ of PD7 mouse pups from CTL and cKO mice. Scale bar = 20 μm. Data are mean ± SEM. n=3–5 mice, *P<0.05 vs. CTL.

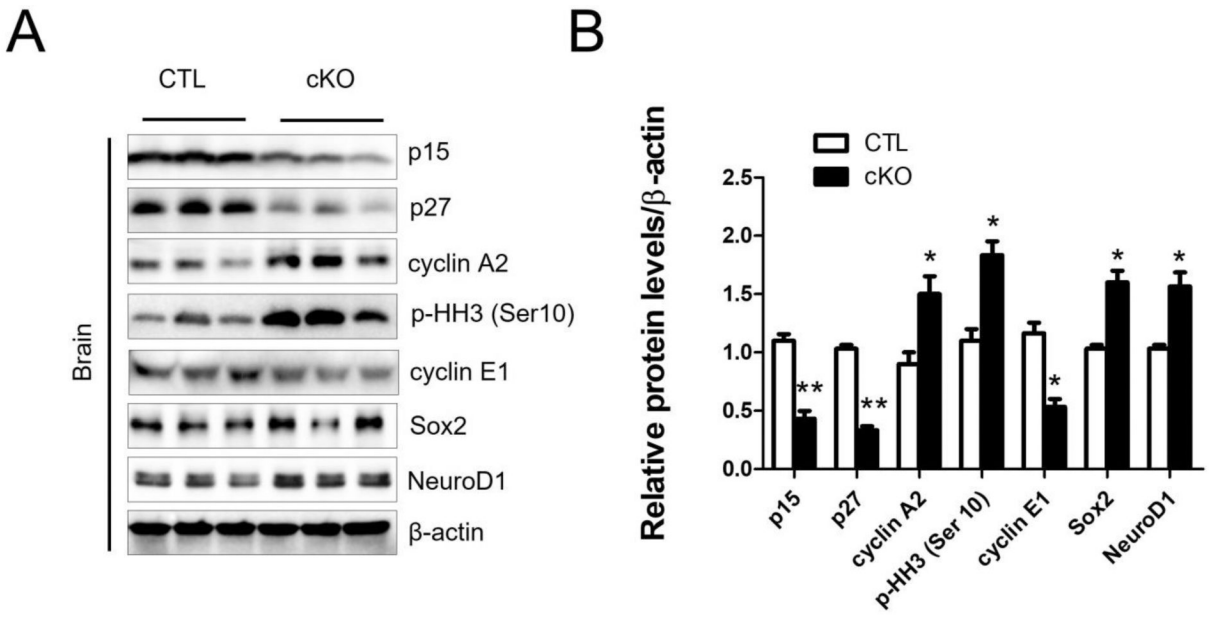

Figure 3. MANF knock-out promotes NeuroD1 and Sox2 expression and cell cycle progression.

The half brain of PD7 mouse pups from CTL and cKO groups was processed for immunoblotting (IB) analysis. **A.** Representative IB images of the expression of p15, p27, cyclin A2, p-HH3 (ser10), cyclin E1, Sox2, and NeuroD1 are presented. **B.** The expression of specific proteins was quantified and normalized to β-actin. Each data point is mean ± SEM. n=3–5 mice, *P<0.05 or **P<0.01 vs. CTL.

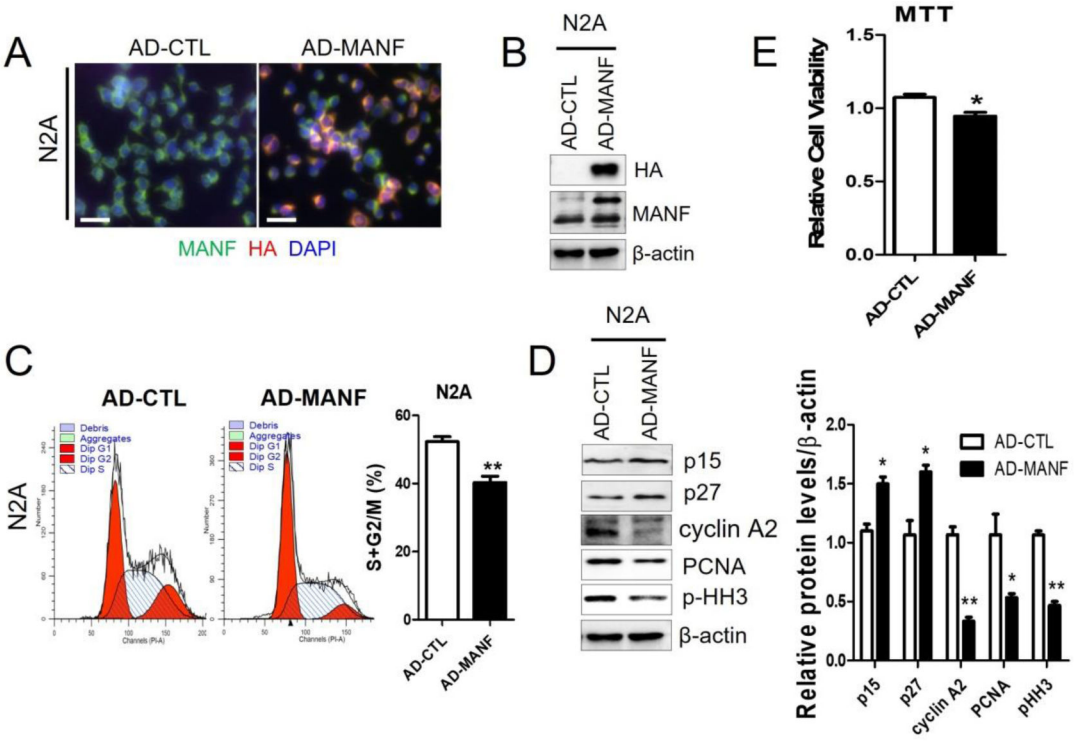

Figure 4. Over-expression of MANF inhibits cell cycle progression in cultured neuroblastoma cells.

N2A neuroblastoma cells were infected with AD-MANF-HA or AD-CTL viral particles as described in “Materials and Methods”. **A** and **B**. 48 hours after the infection, the over-expression of MANF was confirmed by immunofluorescence staining of MANF (green) and HA (red) as well as DAPI (blue). The expression of MANF and HA was analyzed by IB. Scale bar = 20 μm. Upper band: HA-tagged MANF; Lower band: endogenous MANF. **C**. Flow cytometric analysis of PI content was performed for cell cycle analysis and the proliferative phases (S+G2/M) were quantitated. **D**. Representative IB images show the expression of cell cycle regulators. Their expression was quantified and normalized to β-actin. **E**. 48 hours after the infection, the number of viable cells was determined by MTT assay and expressed relative to the control (AD-CTL). Three independent experiments (n = 3) were performed to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. AD-CTL.

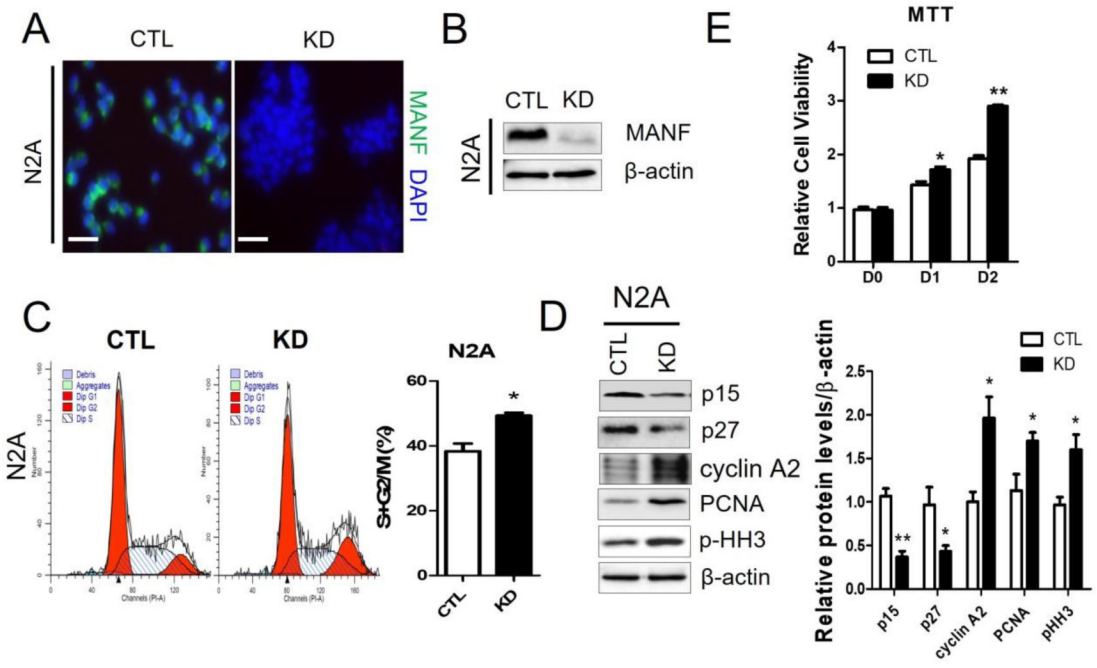

Figure 5. Knock-down of MANF expression promotes cell cycle progression.

Several colonies of N2A cells in which *Manf* was knocked down by CRISPR/Cas9 were established as described in "Materials and Methods". **A** and **B**. Representative images of immunofluorescence staining of MANF (green) and DAPI (blue) are shown. Representative IB images of MANF in control (CTL) and MANF knock-down (KD) colonies are presented. Scale bar = 20 μm. **C.** Flow cytometric analysis of PI content was performed for cell cycle analysis and the proliferative phases (S+G2/M) were quantified in these cells. **D**: Representative IB images show the expression of cell cycle regulators. Their expression was quantified and normalized to β-actin. **E.** Equal amounts of CTL and KD cells were seeded on day 0 (D0) and cultured for 1 day (D1) or two days (D2). The number of viable cells was determined by MTT assay and expressed relative to CTL. Three independent experiments (n = 3) were performed to calculate the mean ± SEM. *P<0.05 or **P<0.01 vs. CTL.