Investigating the effect of in vivo reprogramming using OCT4 and SOX2 on neurogenesis and motor function preservation in a Huntington's disease mouse model

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ABSTRACT

Investigating the effect of in vivo reprogramming using OCT4 and SOX2 on neurogenesis and motor function preservation in a Huntington's disease mouse model

Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by motor dysfunction, cognitive decline, and psychiatric symptoms. Despite advancements in understanding its molecular mechanisms, no disease-modifying treatments are currently available. In vivo reprogramming using transcription factors like OCT4 and SOX2 has shown promise for neurodegenerative diseases by promoting neuronal regeneration. This study investigates the effects of in vivo reprogramming using OCT4 and SOX2 in the R6/2 mouse model of HD. Mice received stereotaxic injections of AAV vectors encoding OCT4 or SOX2, and their motor function, neuronal differentiation, and safety were evaluated.

In vivo reprogramming with OCT4 and SOX2 led to better-preserved brain weight, suggesting protection against HD-related atrophy. Motor function was significantly improved, with AAV4-OCT4 and AAV4-SOX2 treated mice showing slower declines in rotarod performance and higher grip strength compared to PBS and AAV4-Null control groups.

AAV4 vectors, which localize to ependymal cells, primarily target neural progenitors within the subventricular zone, driving neuronal differentiation, while moderate interaction with astrocytes suggests a supplementary role. Immunohistochemistry and qRT-PCR analyses revealed a significant increase in Nestin+/BrdU+ cells in the subventricular zone and elevated DARPP32+/BrdU+ cells in the striatum of the AAV4-OCT4 and AAV4-SOX2 groups, suggesting enhanced neurogenesis and neuronal differentiation. qRT-PCR further showed a significant upregulation of neuronal markers, such as Nestin, GAD67, Tuj-1, and GABAAα1, indicating that OCT4 and SOX2 play a role in promoting neuronal differentiation and the proliferation of neural progenitor cells. Interestingly, the significant reduction in GFAP combined with in vitro evidence, suggests that OCT4 and SOX2 can induce astrocyte-to-neuron conversion. No tumor formation was observed, confirming the safety of this therapeutic approach.

These findings suggest that in vivo reprogramming using OCT4 and SOX2 holds potential as a therapeutic strategy for HD, with the ability to enhance neurogenesis and preserve motor function.

Key words : Huntington’s disease, Reprogramming

I. INTRODUCTION

1.1. Overview of Huntington's Disease

Huntington's disease (HD) is a devastating autosomal dominant neurodegenerative disorder caused by a mutation in the huntingtin (HTT) gene, specifically an expanded CAG trinucleotide repeat that encodes an abnormal polyglutamine stretch in the huntingtin protein[1,2]. This mutation leads to the production of a toxic protein that progressively damages neurons, particularly in the striatum and cerebral cortex, resulting in widespread cellular degeneration and brain atrophy [1,3]. The disease is characterized by a triad of symptoms, motor dysfunction, cognitive decline, and psychiatric disturbances. Symptoms typically begin between the ages of 30 and 50 and worsen over time, ultimately leading to severe physical and mental disability.[2,4] The life expectancy of individuals with HD is significantly reduced. On average, patients live about 15 to 20 years after the onset of symptoms. [2,5] The progressive nature of the disease leads to a gradual loss of independence and requires long-term care, placing a significant emotional and financial burden on caregivers and healthcare systems.[6]

Despite extensive research and advances in understanding the genetic and molecular mechanisms underlying HD, no disease-modifying treatments have been approved, and current therapies are primarily palliative, focusing on symptom management to improve quality of life[3 6]. The lack of effective treatments to halt or reverse neuronal loss in HD underscores the urgent need for innovative therapeutic strategies.

1.2. Emergence of Induced Pluripotent Stem Cells

Shinya Yamanaka's 2006 discovery that adult differentiated cells could be reprogrammed into induced pluripotent stem cells (iPSCs) using the transcription factors OCT4, SOX2, Klf4, and c-Myc (collectively known as Yamanaka factors) has indeed revolutionized regenerative medicine, particularly in the context of neurodegenerative diseases.[7] This breakthrough has enabled the generation of iPSCs that can differentiate into various cell types, offering immense potential for disease modeling, drug discovery, and cell therapy. [8,9]

The ability to generate iPSCs from adult cells has several significant implications. First, iPSCs provide a source of patient-specific pluripotent cells, which can differentiate into any cell type, including neurons[10]. This makes iPSCs an invaluable tool for modeling diseases, screening drugs, and potentially developing personalized therapies. Moreover, the use of patient-derived iPSCs circumvents ethical concerns associated with the use of embryonic stem cells and reduces the risk of immune rejection in cell transplantation therapies.[11]

Since the advent of iPSC technology, there has been a surge in research focused on utilizing iPSCs for neural differentiation. Researchers have developed various protocols to efficiently differentiate iPSCs into neural progenitors and mature neurons. These iPSC-derived neurons have been used to model a wide range of neurodegenerative diseases, such as Alzheimer's disease[10], amyotrophic

lateral sclerosis[12], Parkinson's disease[13], and HD[14], allowing scientists to study disease mechanisms and test potential treatments in vitro.

In addition to differentiating iPSCs into neurons for transplantation, researchers have also explored the direct injection of iPSCs or iPSCs derived neural cells into the brain, aiming to induce neuronal differentiation in situ[15,16]. This approach seeks to leverage the pluripotent nature of iPSCs to generate new neurons directly within the affected regions of the brain. However, this method presents several challenges, including the risk of tumorigenesis, as iPSCs can potentially form teratomas if not properly controlled[17]. Additionally, ensuring the precise differentiation and integration of these cells into existing neural networks remains a significant hurdle.

The development of iPSC technology has thus provided a powerful platform for understanding and potentially treating neurodegenerative diseases. Building on this foundation, subsequent research has explored more direct methods of cellular reprogramming, such as in vivo reprogramming, which aim to convert one cell type directly into another within the body, bypassing the pluripotent state and potentially offering a faster and safer approach to cell replacement therapies[18].

1.3. In Vivo Reprogramming for Brain Injury

In vivo reprogramming refers to a technique where transcription factors are introduced or expressed in the target site within a subject's body to induce the desired cell type directly at the site of need. This technique has been particularly promising in research focused on the brain. Studies have shown that in vivo reprogramming can effectively induce the formation of new neurons within the brain, addressing neuronal loss caused by injuries or neurodegenerative diseases. By directly converting or reprogramming cells in their native environment, researchers aim to promote functional recovery and enhance neural plasticity in the brain. This innovative approach includes both direct and indirect methods[19,20].

Direct methods involve converting specific cells, such as astrocytes, directly into neurons. This is achieved by introducing specific transcription factors such as NeuroD1 [21-24] and Neurogenin 2 [25-27] that trigger the transformation of these supporting cells into functional neurons. This method bypasses the need for intermediate stem cell stages, potentially providing a quicker route to neuronal replacement. The rapid generation of neurons through direct reprogramming can quickly replenish lost neurons and restore some degree of neural function. However, it also presents challenges, such as ensuring the long-term survival and proper integration of these newly formed neurons into existing neural circuits.

Indirect methods involve inducing neural stem cells (NSCs) or progenitor cells within the target area, which can then differentiate into neurons. This approach typically employs Yamanaka factors (OCT4, SOX2, Klf4, and c-Myc) to create a pool of neural progenitors. These progenitors can give rise to neurons, astrocytes, and oligodendrocytes, potentially providing a more versatile and sustained regenerative effect. While this method may take longer to produce mature neurons, it can result in a more stable and integrated neural network over time.

Several studies have demonstrated the efficacy of Yamanaka factors in in vivo reprogramming. For instance, introducing these factors into the brain has been shown to induce the formation of neural progenitor cells (NPCs), which can subsequently differentiate into neurons[28,29]. One of the notable advantages of using Yamanaka factors is their ability to induce a pluripotent state, which can then be directed towards neuronal differentiation. This method can potentially produce a broader range of cell types needed for comprehensive neural repair. Moreover, the use of fewer factors, such as only OCT4 and SOX2, has been explored to reduce the risk of tumorigenesis while still promoting significant neural regeneration[30]. Therefore, comparing the effects of these two factors is crucial to understanding their unique and combined potential in neuronal regeneration. This comparison could provide valuable insights into optimizing in vivo reprogramming strategies for treating neurodegenerative diseases.

1.4. Aims of this Study

The primary aim of this study is to investigate the potential of in vivo reprogramming using Yamanaka factors, specifically SOX2 and OCT4, in a mouse model of HD. We aim to compare the individual effects of SOX2 and OCT4 in inducing neuronal regeneration, assess their ability to generate and integrate new neurons, evaluate improvements in motor functions, and ensure the safety of these reprogramming methods by monitoring for tumorigenesis.

II. MATERIALS AND METHODS

2.1. Animal Model

For this study, we employed the R6/2 transgenic mouse model of HD, which carries approximately 160 ± 5 CAG repeats. These mice were sourced from the Jackson Laboratory (B6CBA-Tg(HDexon1)62Gpb/1J, Stock No: 002810) and exhibit neurological symptoms similar to those observed in human HD, including choreiform movements, involuntary stereotypic behaviors, tremors, seizures, and abnormal vocalizations[31-33]. Symptoms typically begin to manifest between 6 and 8 weeks of age, with an average lifespan extending to around 13 weeks. To support the animals during the terminal stages, we provided water-soaked food pellets daily to prevent dehydration and malnutrition.

All experimental animals were housed in an AAALAC-accredited facility, with all procedures approved by the Institutional Animal Care and Use Committee (IACUC 2016-0298, 2020-0007). The mice were maintained in a temperature-controlled environment on a 12-hour light/dark cycle, with ad libitum access to food and water.

2.2. Genotyping HD Mice

Genotyping of mice was performed based on a protocol from Jackson Laboratories. Genomic DNA (gDNA) was extracted from a 2-mm piece of each mouse ear using the standard procedure from a KAPA Express Extract Kit (EXPEXTKB, Roche). The ear tissue was incubated with 10 μL of 10X KAPA, 2 μL of Enzyme, and 88 μL of autoclaved deionized distilled water at 75°C for 10 min Lysis and 95°C for 5 min Enzyme inactivation. The following primers were used for PCR: positive control forward, 5′-GTA GGC CAC AGA ATT GAA AGT TCT -3′; positive control reverse, 5′-GTA GGT GGA AAT TCT AGC ATC ATC C-3′ (length of WT product, 217 bp); Transgene forward, 5′-CCG CTC AGG TTC TGC TTT TA-3′; Transgene reverse, 5′-TGG AAG GAC TTG AGG GAC TC-3′ (length of mutant product, 117 bp). Electrophoresis was performed after loading 10 μL of each PCR product on a 2% agarose gel.

2.3. Experimental Timeline of the Study

The mice were randomly assigned to one of four groups: phosphate-buffered saline (PBS) (N =10), AAV4-Null (N = 3), AAV4-SOX2 (N = 7), or AAV4-OCT4 (N =8). At week 3, pre-behavioral tests were conducted, including the rotarod test, clasping test, grip strength test, and open field test. The viral vectors were administered at week 4. Following the injection, the mice were monitored for a total of 8 weeks. During this follow-up period, the rotarod test and clasping test were conducted weekly. The grip strength test and open field test were performed at week 12. At the end of the 12-week period, the mice were sacrificed, and qRT-PCR was performed to analyze gene expression. (PBS (N = 10), AAV4-OCT4 (N = 8), AAV9-SOX2 (N = 7))

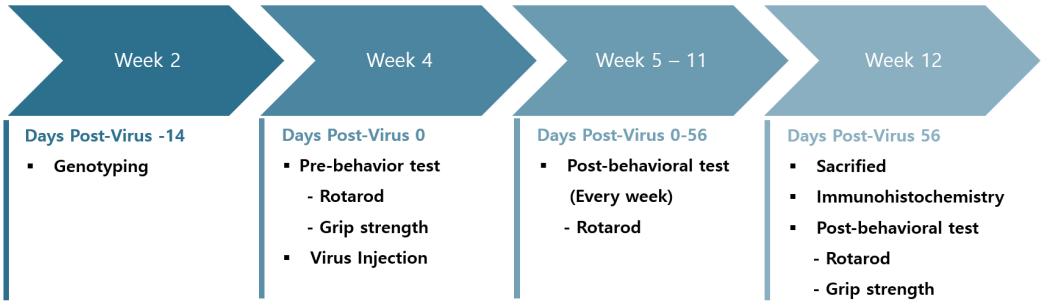

Figure 1. Timeline of the study

2.4. Stereotaxic Injection of AAV Vector with OCT4 and SOX2

At 4 weeks of age, mice were anesthetized with intraperitoneal injections of ketamine (100 mg/kg; Huons, Gyeonggi-do, Korea) and xylazine (10 mg/kg; Bayer Korea, Seoul, Korea). A stereotaxic procedure was performed, during which the mice received injections into both lateral ventricles (LV) with a viral load of 1×10^9 vg/mL, 1 μL each. The injection regions were targeted to the ventricles, specifically at coordinates AP +0.3, ML +0.7, DV -2.0 for the left side, and AP +0.3, ML -0.7, DV -2.0 for the right side. AAV4-NESTIN-OCT4-HA and AAV4-NESTIN-SOX2-FLAG were expressed using the Nestin promoter.

2.5. Immunohistochemistry (IHC)

Mice were daily given an IP injection of 5-bromo-2′-deoxyuridine (BrdU; 50 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) for 12 days, beginning after stereotaxic surgery[34]. R6/2 mice euthanasia performed at 12 weeks of age, slowly perfused with cold 1X PBS and thereafter the same procedure with 4% paraformaldehyde (PFA). The harvested brain tissues were freezed in frozen section compound (Leica, Wetzlar, Germany) using isopentane and cryosectioned at 16-μm thickness using cryomicrotome (Cryostat Leica 1860, Leica biosystem, MI, Italy), and immunohistochemical staining was performed on four sections, representing a range of more than 128 μm. The tissue sections were stained with the following antibodies: cell proliferation marker; BrdU (1:200, abcam, ab6326); OCT4 (1:100, santacruz, sc-5279); neuron-specific class III β-tubulin (βIII-tubulin, 1:400, abcam, ab18207) and mature neuronal marker NeuN (1:400, Millipore, MAB377); glial fibrillary acidic protein (GFAP, 1:400, Neuronomics, RA-22101); Nestin (1:400, abcam, ab6142); and dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32, 1:400, cell signaling technology, 2306) and GABA (1:400, Sigma, A2052). The stained sections were observed by confocal microscopy (LSM700, Zeiss, Gottingen, Germany) and analyzed using ZEN black and blue edition (Zeiss, Gottingen, Germany).

2.6. Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

Table 1. List of primers used for qRT-PCR

Genes	Primer Type	Sequence	Annealing
mOCT4	Forward Primer	5'-CAGCAGATCACTCACATCGCCA-3'	60°C, 60s
	Reverse Primer	5'-GCCTCATACTCTTCTCGTTGGG-3'	
mSOX2	Forward Primer	5'-AACGGCAGCTACAGCATGATGC-3'	60°C, 60s
	Reverse Primer	5'-CGAGCTGGTCATGGAGTTGTAC-3'	
Nestin	Forward Primer	5'-CCCTGAAGTCGAGGAGCTG-3'	57.4°C, 20s
	Reverse Primer	5'-CTGCTGCACCTCTAAGCGA-3'	
NeuN	Forward Primer	5'-GAAACCGCAAGCCCTCATTTC-3'	62°C, 20s
	Reverse Primer	5'-TTGGATGCCTCTTGGTTTGGT-3'	
GFAP	Forward Primer	5'-TTGCTGGAGGGCGAAGAAAA-3'	62°C, 20s
	Reverse Primer	5'-CATCCCGCATCTCCACAGTC-3'	
PPP1r1b	Forward Primer	5'-AGATTCAGTTCTCTGTGCCCG-3'	62°C, 20s
	Reverse Primer	5'-TGGGTCTCTTCGACTTTGGG-3'	
βIII-tubulin	Forward Primer	5'-TAGACCCCAGCGGCAACTAT-3'	58.2°C, 20s
	Reverse Primer	5'-GTTCCAGGTTCCAAGTCCACC-3'	
GAD67	Forward Primer	5'-CAAGTTCTGGCTGATGTGGA-3'	62°C, 20s
	Reverse Primer	5'-GCCACCCTGTGTAGCTTTTC-3'	
GABAA α1	Forward Primer	5'-CGGCTAAACAACCTTATGG-3'	60°C, 45s
	Reverse Primer	5'-ATTATGCACGGCAGATATGT-3'	
GAPDH	Forward Primer	5'-GTGGAGCCAAAAGGGTCATCA-3'	62°C, 20s
	Reverse Primer	5'-CCCTTCCACAATGCCAAAGTT-3'	

Twelve-week-old R6/2 mice were euthanized for biochemical studies and cardially perfused with cold 1X PBS. The subventricular zone was collected, and total RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). After assessing the quality and purity of the extracted RNA, 1 μg of purified total RNA was used as a template for cDNA synthesis with the ReverTra Ace® qPCR RT master mix with gDNA remover (TOYOBO, Japan), following the manufacturer's instructions. qRT-PCR reactions were performed using SYBR Green dye according to Roche Applied Science guidelines. The reactions were carried out on a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany) with the LightCycler 480 SYBR Green master mix. Each reaction had a total volume of 20 μL, including 1 μL of synthesized cDNA. All reactions were performed in triplicate to ensure reproducibility. Relative expression levels were quantified using

the 2^−ΔΔCt method, where ΔCT represents the difference in cycle threshold (Ct) values between the target gene (e.g., OCT4) and the reference gene (GAPDH)[35,36]. The ΔΔCt value was calculated as the difference in ΔCt between treated and control groups. The primer sequences used are listed in Table 1. All results are expressed as means ± standard error of the mean (SEM) from at least three independent experiments.

2.7. Primary Cell Isolation

Neonatal mice (3-5 days old) were euthanized, and both cortices were aseptically dissected and placed in HBSS. The cortical tissue was then dissociated into a single-cell suspension by repeated pipetting in DMEM/Ham's F-12 medium supplemented with 10% FBS, 1% Penicillin/Streptomycin (P/S), 1X MEM NEAA, Sodium Pyruvate, and 1X 200 mM L-Glutamine. The suspension was homogenized, strained, and rinsed with DPBS before being seeded into a T-75 flask and incubated at 37°C with medium changes every 3-4 days. After 7 days, the culture was shaken for at least 6 hours, and the supernatant was removed. The cells were then treated with 0.25% Trypsin-EDTA for 10 minutes at 37°C to detach them and used for subsequent experiments.

2.8. Primary Astrocyte Transfection

Once the adherent cells in the T-75 flask have grown sufficiently, wash them with PBS and then treat with 0.05% trypsin at 37°C for 5 minutes to detach the cells. Collect the detached cells into growth medium, prepare a cell suspension, and adjust the cell concentration to 1x10^5 cells/mL. Add 1 mL of the cell suspension to each well of a 4-well plate (SPL, 30104) and incubate at 37°C with 5% CO2 for 24 hours. For transfection, prepare 200 μL of Opti-MEM™ and let it sit at room temperature for 5 minutes. Then add MOI 1 concentration of AAV4-Virus, 7 μL of Lipofectamine® 2000 reagent, and F-12 media (containing only FBS) to the mixture, and incubate at room temperature for 20 minutes. Add this mixture dropwise to the cells in the 6-well plate and gently rock the plate to ensure uniform distribution. After 24 hours, replace the culture medium with fresh medium and continue to incubate the cells for an additional 24 hours.

2.9. Immunocytochemistry (ICC)

Immunocytochemistry was performed as described previously by Lasič et al.[37] with some modification. Briefly, cell cultures were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. After blocking 1h with 5% albumin bovine, the cells were incubated overnight anti-GFAP, -GABA, -HA, -FLAG. Antibodies were incubated at a concentration of 1: 400. After a 3-stage washing with PBS, specific secondary antibodies conjugated with Alexa Flour 488 (green staining) or 594 (red staining) were incubated for 1h at room temperature. Cell nuclei were stained with Hoechst 33342 (trihydrochloride, trihydrate) 10 mg/mL solution in PBS. The cells were visualized using confocal microscopy (LSM700, Zeiss, Gottingen, Germany).

2.10. Neurobehavioral Test

To evaluate the progression of HD in the mouse model, we conducted four behavioral tests: the Rotarod test, Grip Strength test.

2.10.1. Rotarod Test

HD is characterized by progressive deterioration in motor function and coordination. To assess these impairments, we used a Rotarod apparatus (Model 47600, Ugo Basile, Comerio, Italy) to evaluate motor performance weekly from Week 4 to Week 12. Two protocols were employed: an accelerating speed ranging from 4 to 40 rpm seconds and a constant speed of 12 rpm, measuring the time until the mouse fell off, with a maximum trial duration of 60 seconds.

2.10.2. Grip Strength Test

As HD progresses, muscle strength diminishes. The Grip Strength test quantifies the force exerted by a mouse while gripping an object, providing a direct measure of muscle strength. This test was conducted using the SDI Grip Strength System (San Diego Instruments, Inc., San Diego, CA, USA) at weeks 3 and 12. Mice gripped a 2-mm diameter triangular metal wire, and the apparatus automatically recorded the peak grip force in kilogram-force (kgf). The average of peak forces from three trials was used for analysis.

2.11. Statistical Analysis

All data were expressed as means ± S.E.M. Group comparisons were made using an independent t-test for two groups, and one-way analysis of variance (ANOVA) with post hoc Bonferroni or Tukey comparisons for multiple groups.

For the weekly evaluations of the rotarod test, a Linear Mixed Model (LMM) was used to examine the interaction between Group (PBS, AAV4-Null, AAV4-OCT4, AAV4-SOX2) and Week, focusing on performance changes over time. Week was treated as both a numeric variable to calculate the rate of decline and as a factor for week-to-week comparisons. A random intercept was included to account for between-subject variability, and model fitting was performed using the restricted maximum likelihood (REML) approach. The significance of the Group * Week interaction was tested using Type III ANOVA with Kenward-Roger degrees of freedom adjustment. Post-hoc comparisons were conducted for each week using Tukey's Honest Significant Difference (HSD) test.

For in vitro experiments, results were confirmed by performing each experiment at least three times to ensure reproducibility. Both female and male mice were used and randomly assigned to experimental groups for in vivo studies. Molecular studies utilized data from three independent experiments.

The statistical significance threshold was set at $p < 0.05$. All analyses were performed using R software version 3.5.3 (R Foundation for Statistical Computing).

III. RESULTS

3.1. In Vivo OCT4 and SOX2 Reprogramming Prevents Brain Atrophy

In vivo reprogramming using OCT4 and SOX2 in the R6/2 mouse model of HD resulted in a significant increase in brain weight compared to the PBS, and AAV4-Null groups. Statistical analysis confirmed that the brain weights in the AAV4-OCT4 and AAV4-SOX2 treated groups were significantly higher, indicating a potential protective effect against brain atrophy commonly associated with HD. This suggests that OCT4 and SOX2 may contribute to the preservation of brain mass in this HD model. (Figure 2)

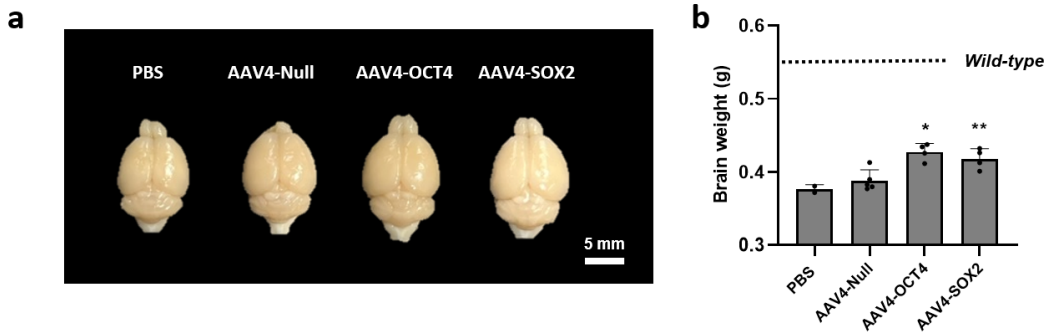

Figure 2. Effects of in vivo reprogramming of OCT4 and SOX2 on brain weight in the R6/2 mouse model of Huntington's disease

(a) Images of brain morphology from different treatment groups at 12 weeks of age

(b) Quantitative analysis of brain weight across treatment groups, showing increases in the AAV4-OCT4 and AAV4-SOX2 treated groups compared to PBS, and AAV4-Null controls.

** $p < 0.01$ between AAV-OCT4 group and PBS ($p=0.005$) / AAV4-Null ($p=0.006$)*

*** $p<0.05$ between AAV- SOX2 group and PBS ($p=0.021$) / AAV4-Null ($p=0.037$)*

3.2. In Vivo Reprogramming Using OCT4 and SOX2 Preserves Functional Performance in HD Mice

3.2.1. Rotarod Performance Using Accelerating Speed (4-40rpm)

A linear mixed model (LMM) indicated a significant Group * Week interaction ($F(3, 390) = 15.12$, $p < 0.001$), suggesting that the rate of decline varied significantly across the groups over time. The AAV4-Null group showed a decline of 5.05 seconds per week, while the PBS group declined at 4.13 seconds per week. In contrast, the AAV4-OCT4 group exhibited a significantly slower decline rate of 1.97 seconds per week, and the AAV4-SOX2 group declined by 2.40 seconds per week.

Post-hoc analysis revealed significant differences between the PBS group and both the AAV4-OCT4 and AAV4-SOX2 groups starting from Week 10. Additionally, the AAV4-Null group

displayed significant differences in performance compared to the AAV4-OCT4 and AAV4-SOX2 groups at Week 12. By Week 12, the AAV4-OCT4 group outperformed the PBS group by 19.10 seconds ($p = 0.0007$), and the AAV4-SOX2 group outperformed the PBS group by 18.93 seconds ($p = 0.0004$). Additionally, the AAV4-OCT4 group outperformed the AAV4-Null group by 16.30 seconds ($p = 0.0179$), and the AAV4-SOX2 group outperformed the AAV4-Null group by 16.13 seconds ($p = 0.0139$). No significant differences were observed between the AAV4-OCT4 and AAV4-SOX2 groups ($p = 1.0000$) at Week 12. (Figure 3a)

3.2.2. Rotarod Performance Using Constant Speed (12rpm)

A linear mixed model (LMM) was applied to assess differences in rotarod performance at a constant speed of 12 rpm across the groups over time. A significant Group * Week interaction was observed ($F(3, 264) = 14.80$, $p < 0.001$), indicating that the rate of decline in performance varied significantly between the groups. The PBS group exhibited a decline of 5.06 seconds per week, while the AAV4-Null group showed a similar decline rate of 4.03 seconds per week, which was not significantly different from PBS. In contrast, the AAV4-OCT4 group exhibited a significantly slower decline rate of 0.85 seconds per week, and the AAV4-SOX2 group declined by 1.64 seconds per week.

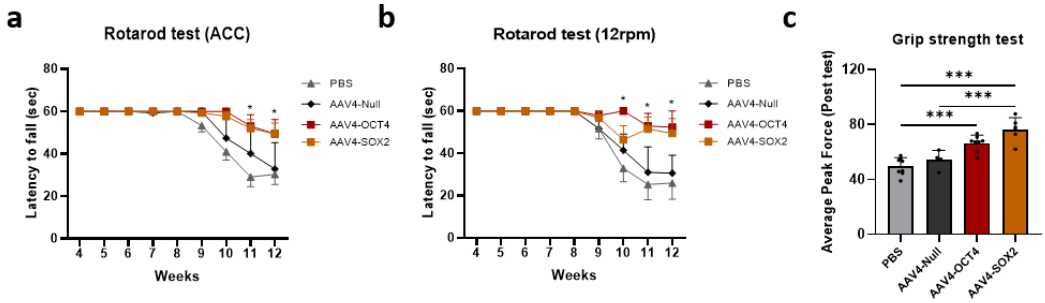

Figure 3. In vivo reprogramming with OCT4 and SOX2 Prolongs Motor Function and Strength in R6/2 Mouse Model of Huntington's Disease

(a) Accelerating rotarod test (4–40 rpm) shows slower performance declines in AAV4-OCT4 (red) and AAV4-SOX2 (orange) groups compared to controls (PBS, AAV4-Null), with significant differences from Week 10.

(b) Constant speed rotarod test (12 rpm) reveals slower declines in OCT4 and SOX2 groups, with significant differences from Week 11.

(c) Grip strength test demonstrates improved muscle strength in OCT4 and SOX2 groups compared to controls.

*Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p<0.001*

Post-hoc analysis revealed that significant differences between the PBS group and both the AAV4-OCT4 and AAV4-SOX2 groups emerged at Week 11. Additionally, the AAV4-Null group showed significant differences compared to the AAV4-OCT4 and AAV4-SOX2 groups also at Week 11. By Week 12, the AAV4-OCT4 group outperformed the PBS group by 26.59 seconds ($p = 0.0001$), and the AAV4-SOX2 group outperformed the PBS group by 23.56 seconds ($p = 0.0004$). Furthermore, the AAV4-OCT4 group outperformed the AAV4-Null group by 23.47 seconds ($p = 0.0050$), and the AAV4-SOX2 group outperformed the AAV4-Null group by 20.44 seconds ($p = 0.0275$). No significant difference was found between the AAV4-OCT4 and AAV4-SOX2 groups at Week 12 ($p = 0.9721$). (Figure 3b)

3.2.3. Grip Strength

In the grip strength test (Figure 3c), the AAV4-OCT4 and AAV4-SOX2 treated mice exhibited significantly higher peak force values compared to the PBS ($p = 0.0003$ for AAV4-OCT4, $p < 0.0001$ for AAV4-SOX2) and AAV4-Null groups ($p = 0.0397$ for AAV4-OCT4, $p = 0.0003$ for AAV4-SOX2). This indicates that in vivo reprogramming with OCT4 and SOX2 helps preserve muscle strength in HD mice. These findings highlight the potential of OCT4 and SOX2 to mitigate the motor deficits typically associated with HD.

3.3. Localization of AAV4 Vector in the Lateral Ventricle Ependyma and its Potential to Target NSCs/NPCs

3.3.1. AAV4 Vector Localized to Periventricular Lesions

To evaluate the distribution and cellular specificity of the AAV4 vector, mCherry-labeled AAV4 was injected into the lateral ventricle of 4-week-old mice, and brain tissues were analyzed 12 weeks post-injection. The results showed that the AAV4 vector predominantly localized to the ependymal layer surrounding the lateral ventricle, with robust mCherry fluorescence observed in this region (Figure 4). Quantitative analysis revealed that the percentage of mCherry-positive cells was highest in the SVZ (~6%), followed by lower percentages in the striatum and septum. Minimal mCherry-positive cells were detected in the corpus callosum, indicating that the AAV4 vector is primarily confined to the periventricular regions.

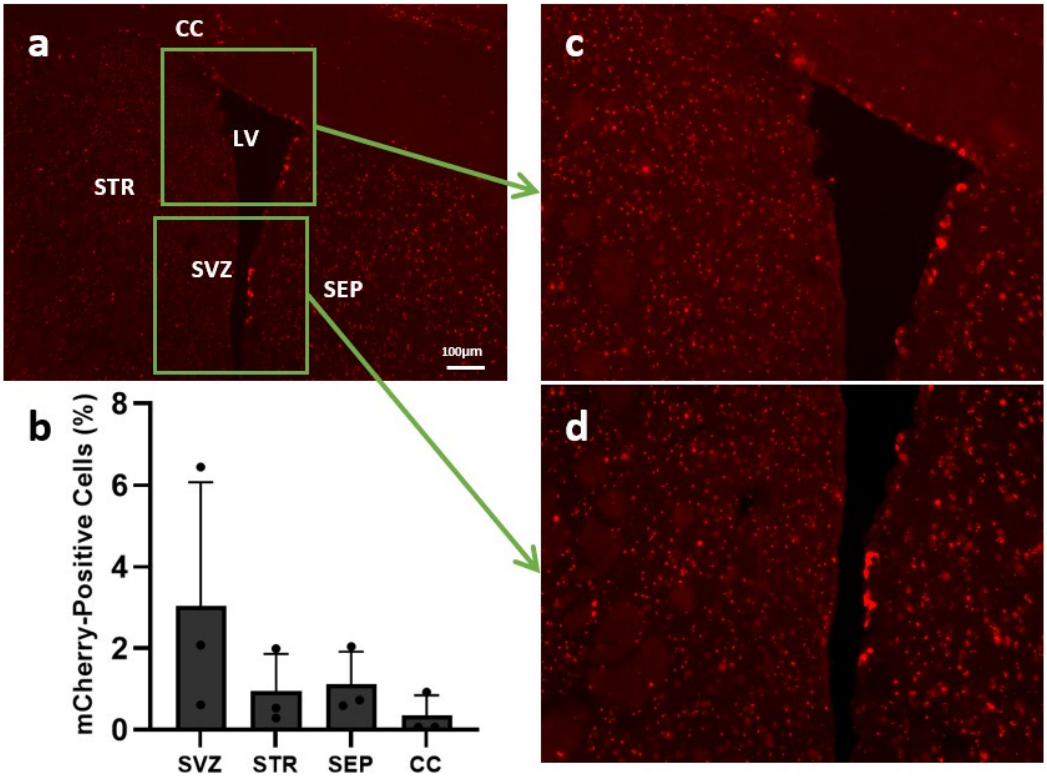

Figure 4. Localization and quantification of mCherry-positive cells in the periventricular regions following AAV4 Injection

*** CC, colpus callosum; LV, lateral ventricle; STR, striatum; SEP, septum*

3.3.2. Cellular Targeting of AAV4 Vector in the Ependymal Layer

To investigate the specific cell types targeted by the AAV4 vector, mCherry fluorescence was analyzed alongside various cellular markers, including Vimentin, β-Catenin, AQP4, GFAP, and S100β (Figure 5). Significant co-localization of mCherry with Vimentin and β-Catenin, markers of NSCs/NPCs, demonstrated that the AAV4 vector primarily targets NSCs/NPCs within the ependymal layer. Moderate co-localization with AQP4 and GFAP suggests some interaction with astrocytes, while minimal overlap with S100β indicates that AAV4 does not significantly target mature glial cells. These findings highlight the selectivity of AAV4 for progenitor-like cells, with limited astrocyte interaction, supporting its potential for neuroregenerative therapies.

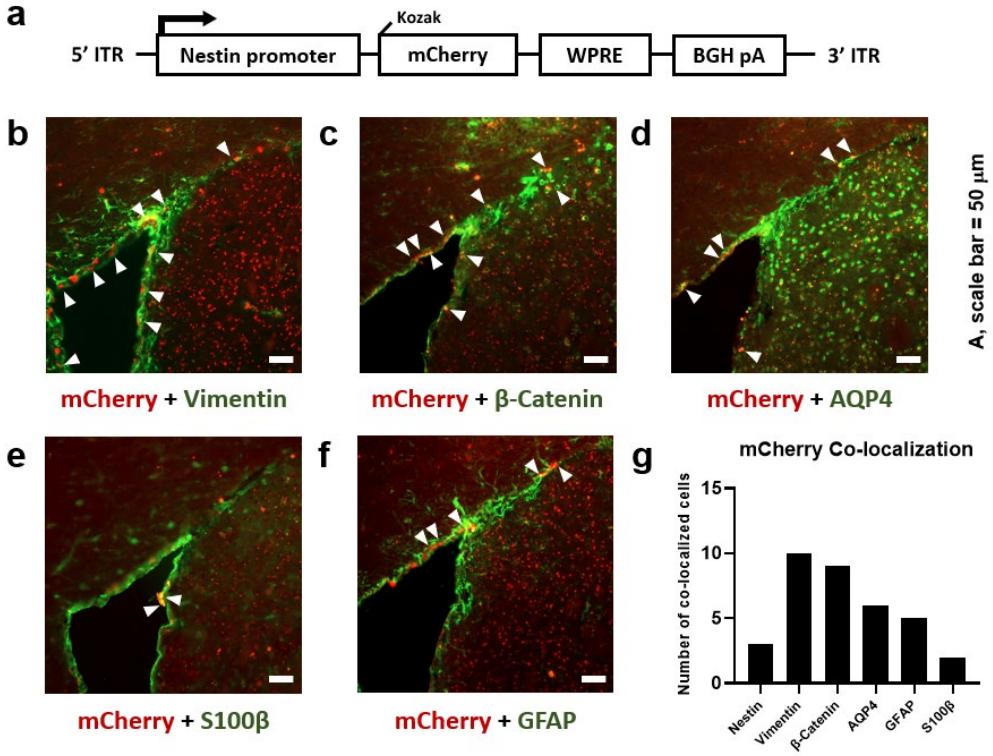

Figure 5. Cellular targeting of the AAV4 vector in the ependymal layer
(a) mCherry fluorescence co-localized with various cellular markers to identify cell types targeted by the AAV4 vector. Strong co-localization with Vimentin (b) and β-Catenin (c) indicates primary targeting of NSCs/NPCs. Moderate co-localization with AQP4 (d) and GFAP (f) suggests partial interaction with astrocytes, while minimal overlap with S100β (e) shows limited targeting of mature glial cells. (g) Quantification of mCherry co-localization highlights the selectivity of AAV4 for NSCs/NPCs over astrocytic or glial populations. Scale bar = 50 µm.

3.4. Immunohistochemistry Shows Enhanced Neurogenesis and Proliferation of New Neurons in the SVZ and Striatum Following In Vivo Reprogramming with OCT4 and SOX2

Immunohistochemical analysis was performed to evaluate the effects of in vivo overexpression of OCT4 and SOX2 on neuronal marker expression in the SVZ and striatum of R6/2 mice. As shown in Figure 6a the expression of Nestin, a marker for NSCs, was increased in the SVZ of both AAV4-OCT4 and AAV4-SOX2 groups compared to the PBS group. This indicates that OCT4 and SOX2 overexpression may enhance the proliferation or maintenance of NSCs in the SVZ. Furthermore, in the striatum, there was a significant increase in the expression of GABA and DARPP-32, which are markers for GABAergic neurons and medium spiny neurons, respectively. This suggests that the

NSCs in the SVZ may have migrated to the striatum and differentiated into these specific neuron types under the influence of OCT4 and SOX2.

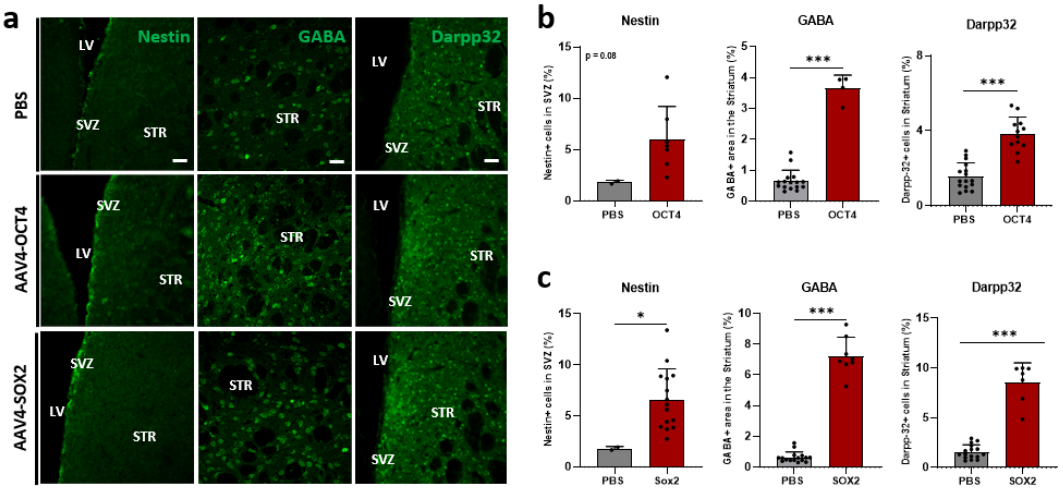

Figure 6. Overexpression of OCT4 and SOX2 enhances neurogenesis in the SVZ and striatum of R6/2 mice

(a) Immunohistochemical analysis showing the expression of Nestin in the SVZ, and GABA and DARPP32 in the striatum of R6/2 mice treated with AAV4-OCT4 or AAV4-SOX2 compared to the PBS group. Increased Nestin+ cells were observed in the SVZ, and elevated levels of GABA+ and DARPP32+ cells were detected in the striatum following OCT4 and SOX2 treatment.

(b) Quantification of Nestin+ cells in the SVZ and GABA+ and DARPP32+ cells in the striatum of OCT4-treated mice.

(c) Quantification of Nestin+ cells in the SVZ and GABA+ and DARPP32+ cells in the striatum of SOX2-treated mice.

*Data are presented as mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.*

Quantitative analysis (Figure 6b and 6c) further confirmed these observations. The number of Nestin+ cells in the SVZ was higher in both AAV4-OCT4 and AAV4-SOX2 groups, with AAV4-SOX2 showing a statistically significant increase. In the striatum, both GABA+ and DARPP-32+ cells were significantly more abundant in the AAV4-OCT4 and AAV4-SOX2 groups compared to the PBS group, further supporting the role of these factors in promoting neuronal differentiation.

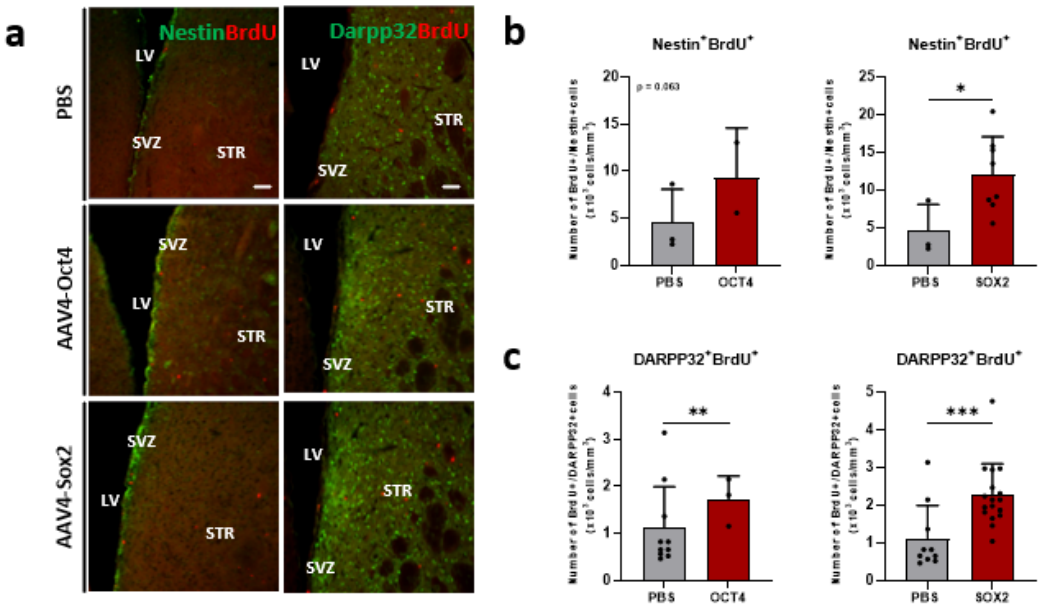

Figure 7. BrdU labeling indicates new neural progenitors and neurons in the SVZ and striatum following OCT4 and SOX2 overexpression

(a) BrdU+ cells co-expressing Nestin in the SVZ and DARPP32 in the striatum after AAV4-*OCT4 and* AAV4-*SOX2 treatment.*

(b) Quantification of Nestin+/BrdU+ cells in the SVZ shows a significant increase in the AAV4-*SOX2 group.*

(c) DARPP32+/BrdU+ cells in the striatum were significantly higher in both AAV4-*OCT4 and* AAV4-*SOX2 groups.*

*Data are mean ± SEM. *p < 0.05, **p < 0.01, **p < 0.001*

To assess whether the increases in neuronal markers were due to newly generated cells, we used BrdU labeling to track proliferating cells. Immunohistochemical analysis (Figure 7a) showed that BrdU+ cells co-expressed Nestin in the SVZ and DARPP32 in the striatum, indicating these cells were newly proliferated as a result of OCT4 and SOX2 overexpression.

Quantitative analysis (Figure 7b) revealed an increase in Nestin+/BrdU+ cells in the SVZ, particularly in the AAV4-SOX2 group, which was statistically significant. In the striatum, the number of DARPP32+/BrdU+ cells was significantly higher in both the AAV4-OCT4 and AAV4-SOX2 groups compared to the PBS group (Figure 7c). These findings suggest that OCT4 and SOX2 enhance the proliferation and differentiation of neural progenitors into neurons in the SVZ and striatum.

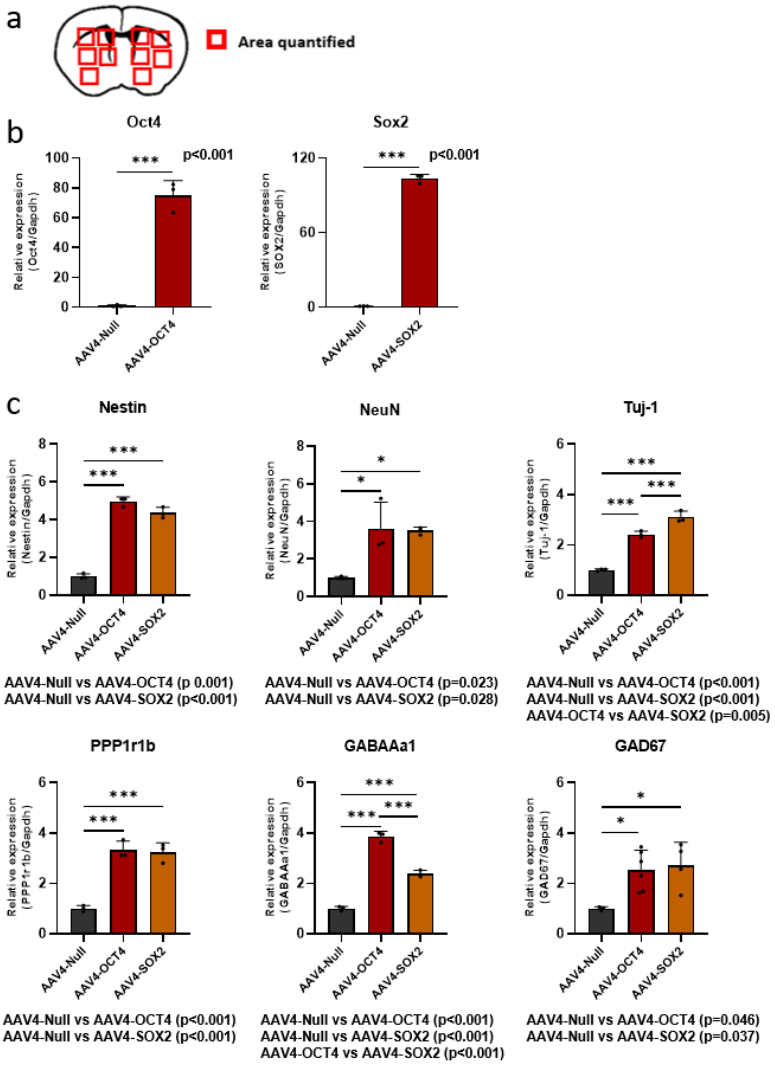

Figure 8. qRT-PCR analysis of neuronal markers in AAV4-OCT4 and AAV4-SOX2 groups compared to AAV4-Null controls.

(a) Quantified regions of the brain used for analysis.

(b) qRT-PCR analysis demonstrated that both OCT4 and SOX2 expression remained significantly elevated at 12 weeks post-treatment.

(c) Relative expression of neuronal markers (Nestin, NeuN, Tuj-1, PPP1r1b, GABAAα1, GAD67) showed a significant increase in both the AAV4-OCT4 and AAV4-SOX2 groups compared to the AAV4-Null controls. However, the expression of GFAP, an astrocyte marker, was significantly decreased in both treatment groups.

*Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.*

3.5. In Vivo Reprogramming with OCT4 and SOX2 Shows Increased Neuronal Marker Expression in qRT-PCR Analysis

qRT-PCR analysis was conducted on specific brain regions, as shown in Figure 8a, to evaluate the expression of neuronal markers following in vivo reprogramming with OCT4 and SOX2. The results showed that both SOX2 and OCT4 expression remained significantly elevated at 12 weeks post-treatment (Figure 8b). Additionally, there was a significant increase in the expression of neuronal markers, including Nestin, NeuN, Tuj-1, PPP1R1B, GABAAα1, and GAD67 in both the AAV4-OCT4 and AAV4-SOX2 groups compared to the AAV4-Null control group (Figure 8c).

Nestin expression, a marker of NPCs, was significantly higher in the AAV4-OCT4 and AAV4-SOX2 groups ($p < 0.001$), and NeuN and Tuj-1 expression, markers for mature neurons, also showed significant increases in both treatment groups compared to controls ($p < 0.05$ for NeuN, $p < 0.001$ for Tuj-1). PPP1R1B, GABAAα1, and GAD67 expression levels were also significantly upregulated in both treatment groups ($p < 0.05$ for all comparisons).

These findings indicate that neuronal markers were consistently upregulated following in vivo reprogramming with OCT4 and SOX2, as confirmed by qRT-PCR analysis.

3.6. In Vivo Reprogramming with OCT4 and SOX2 Reduces Astrocyte Marker GFAP in the SVZ

In the R6/2 mouse model, in vivo reprogramming with OCT4 and SOX2 led to a significant reduction in GFAP expression, a marker for astrocytes, in the SVZ. Immunohistochemical and quantitative analyses (Figure 9a, 9b) showed that GFAP+ cells significantly decreased in both the AAV4-OCT4 and AAV4-SOX2 groups compared to the PBS group. qRT-PCR analysis further confirmed the downregulation of GFAP in the AAV4-OCT4 and AAV4-SOX2 groups compared to the AAV4-Null group (Figure 9c).

This reduction in GFAP expressions can be explained by two possible mechanisms. First, astrocytes in the SVZ may have been reprogrammed into NPCs following OCT4 and SOX2 treatment, leading to a shift in their marker expression profile. Alternatively, the observed decrease in GFAP+ cells could reflect a suppression of gliosis, which is characterized by astrocyte activation and proliferation in response to neurodegeneration. By mitigating gliosis, OCT4 and SOX2 may have created a more permissive environment for neurogenesis and functional recovery. Together, these mechanisms may contribute to the therapeutic potential of in vivo reprogramming for neural regeneration in HD.

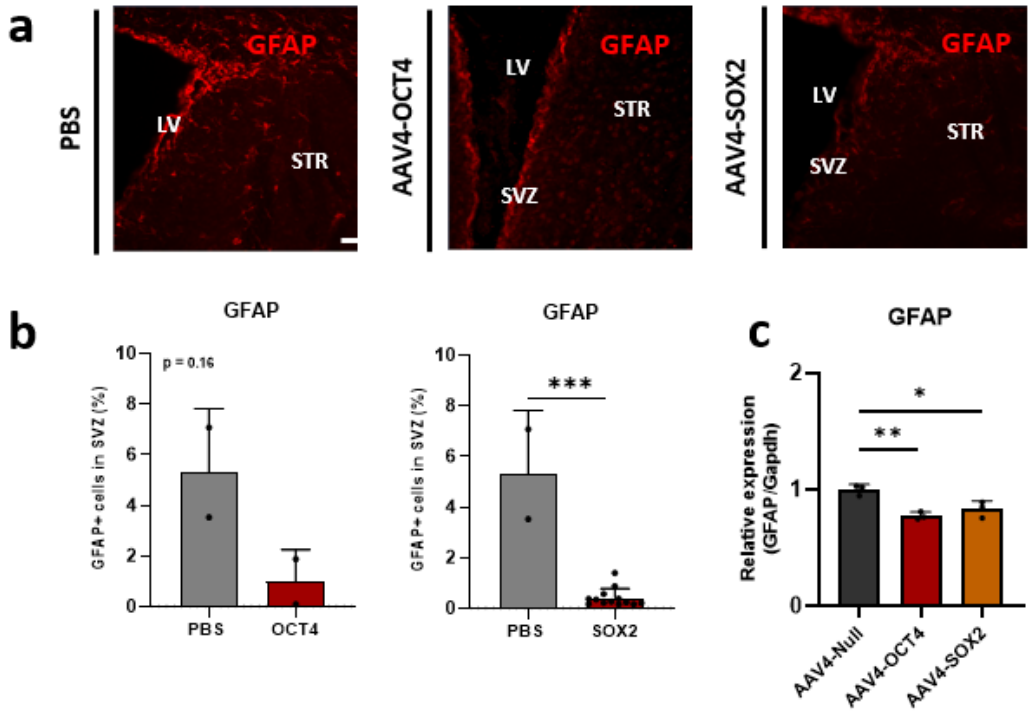

Figure 9. Reduction of GFAP expression in the SVZ following in vivo reprogramming with AAV4-OCT4 and AAV4-SOX2 in R6/2 mice

(a) Immunohistochemical analysis showing GFAP (red) expression in the SVZ of R6/2 mice treated with AAV4-OCT4 or AAV4-SOX2, compared to the PBS group. A reduction in GFAP expression is observed compared to the PBS group.

(b) Quantification of GFAP+ cells in the SVZ from immunohistochemical analysis, showing a significant reduction in the AAV4-OCT4 group ($p = 0.016$) and AAV4-SOX2 group ($p < 0.001$) compared to the PBS group.

*(c) qRT-PCR analysis of GFAP expression in the SVZ, demonstrating a significant reduction in the AAV4-OCT4 group ($p = 0.001$) and AAV4-SOX2 group ($p = 0.012$) relative to the AAV4-Null group. Data are presented as mean ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.*

3.7. In Vitro Experiments Suggest OCT4 and SOX2 May Convert Astrocytes into NPCs

To investigate whether astrocytes could be reprogrammed into NPCs through the expression of OCT4 and SOX2, primary astrocytes were isolated and transfected with AAV4-OCT4 and AAV4-SOX2. ICC was performed to assess the co-expression of GFAP and Nestin in these cells.

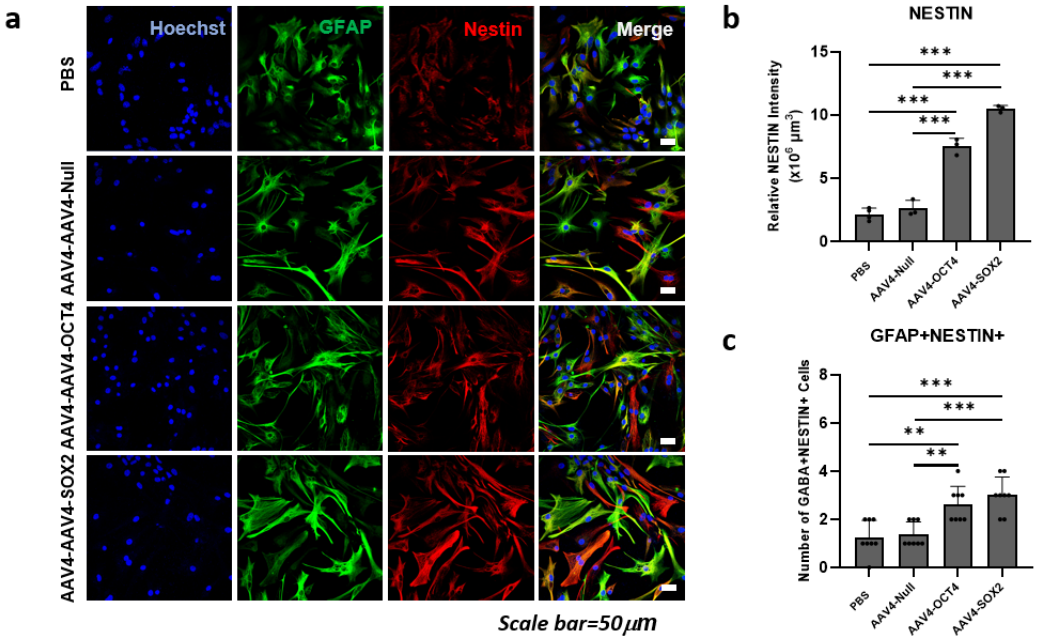

Figure 10. Induction of neural stem cell markers in astrocytes following in vitro treatment with OCT4 and SOX2

(a) Immunocytochemical staining showing the expression of GFAP (green), a marker for astrocytes, and Nestin (red), a marker for NPCs, in primary astrocytes treated with PBS, AAV4-Null, AAV4-OCT4, and AAV4-SOX2. Hoechst (blue) was used to stain the nuclei. The merge images demonstrate significant co-localization of GFAP and Nestin, particularly in the AAV4-OCT4 and AAV4-SOX2 treated groups, indicating enhanced conversion of astrocytes into NPCs compared to the PBS and AAV4-Null groups. (b) Quantitative Analysis of Nestin Expression. Both AAV4- OCT4, and AAV4-SOX2 significantly increased Nestin expression compared to PBS and AAV4- Null group.
(c) Quantitative Analysis of GFAP+Nestin+ Cells. The number of cells co-expressing both GFAP and Nestin was significantly increased in both the AAV4- OCT4 and AAV4 -SOX2 groups compared to the PBS and AAV4-null groups.
*Data are presented as mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.*

As shown in Figure 10a, PBS-treated astrocytes and those transfected with the control vector AAV4-Null exhibited minimal to no Nestin expression, indicating that the cells largely retained their astrocytic characteristics. In contrast, astrocytes transfected with AAV4-OCT4 or AAV4-SOX2 showed a marked increase in Nestin expression, suggesting a successful conversion into NPCs.

The quantitative analysis of Nestin expression is illustrated in Figure 10b. Both OCT4 and SOX2 significantly increased Nestin expression compared to the control groups. Statistical analysis revealed that AAV4-OCT4 significantly increased Nestin expression compared to the PBS group ($p < 0.001$) and AAV4-Null group ($p < 0.001$). Similarly, AAV4-SOX2 significantly increased Nestin expression compared to the PBS group ($p < 0.001$) and AAV4-Null group ($p < 0.001$).

Additionally, Figure 10c quantifies the number of cells co-expressing both GFAP and Nestin (GFAP+Nestin+), further supporting the reprogramming of astrocytes. The AAV4-OCT4 treated group showed a significant increase in GFAP+Nestin+ cells compared to the PBS group ($p = 0.003$) and AAV4-Null group ($p = 0.007$). The AAV4-SOX2 group also showed a significant increase compared to the PBS group ($p < 0.001$) and AAV4-Null group ($p < 0.001$).

These findings suggest that both OCT4 and SOX2 are capable of inducing astrocytes to transition into a progenitor-like state, as evidenced by increased Nestin expression and GFAP+Nestin+ cell populations. This reprogramming likely contributes to the neurogenic potential observed in vivo and supports the role of OCT4 and SOX2 in promoting neural regeneration.

3.8. No Tumor Formation Observed Following OCT4 and SOX2 Reprogramming

To assess the safety of in vivo reprogramming, we monitored AAV4-OCT4 and AAV4-SOX2 treated groups for 12 weeks for potential tumor formation. Both AAV4-OCT4 and AAV4-SOX2 treated groups showed no signs of tumorigenesis during the observation period. Histological analysis of brain sections confirmed the absence of any abnormal tissue growth or tumor formation, further supporting the safety of this reprogramming approach in vivo.

IV. DISCUSSION

4.1. Summary of the Research Findings

HD results in devastating consequences and, unfortunately, the CNS lacks inherent regenerative capacity. The induction of differentiated cells into pluripotent stem cells presents a novel approach for treating CNS diseases, as these cells possess the unique ability to differentiate into various cell types including neurons. Reprogramming is typically achieved by introducing specific transcription factors (such as OCT4, SOX2, Klf4, and c-Myc) into adult cells, resetting the cells to an embryonic-like state[38]. A variety of in vitro studies have demonstrated the potential for various cell types to be converted into iPSCs, with a specific focus on their differentiation into neurons[10]. The use of cell transplantation with these induced cells has garnered significant interest as a potential treatment approach for CNS diseases. However, concerns regarding the ethical implications[39] and the risk of immune rejection associated with human fetal tissues have been addressed through the development of iPSCs derived from a patient's own somatic cells[40]. Furthermore, persistent concerns about teratoma formation due to iPSCs remain a consideration[41].

In this study, we investigated the efficacy of in vivo reprogramming using OCT4 and SOX2 in promoting neuronal regeneration in a mouse model of HD. At 13 weeks, brain weight was significantly better preserved in the OCT4 and SOX2 groups compared to the controls, and motor function preservation was also observed. Our results demonstrate that both OCT4 and SOX2 significantly enhance neuronal differentiation, as evidenced by the increased expression of neural markers. The AAV4 vector, which predominantly targets ependymal cells, was used for precise delivery of SOX2 and OCT4 to progenitor cell populations in the SVZ, facilitating their proliferation and differentiation into neurons. In addition, moderate co-localization with astrocytic markers suggests that some astrocytes were partially reprogrammed into NPC-like cells, as supported by in vitro findings of increased Nestin expression and decreased GFAP expression in astrocytes treated with AAV4-OCT4 or AAV4-SOX2.

4.2. Review of Past In Vivo Reprogramming Studies Using OCT4/SOX2 in the Brain

In vivo reprogramming using Yamanaka factors, particularly OCT4 and SOX2, has shown significant promise in the field of neurodegenerative disease research. Several studies have demonstrated that these factors can induce neuronal regeneration within the brain, providing a potential avenue for treating disorders such as HD. To accurately interpret the effects of in vivo reprogramming using SOX2 and OCT4 in HD, we searched for studies that utilized these two transcription factors in the brain. The detailed methodology is provided in the Appendix. The process of reviewing articles and extracting data followed the structure outlined in Figure 11, as represented in the PRISMA-ScR flow diagram. Initially, a total of 313 articles were identified across the four selected databases using the designated search strategy. In addition, three articles were obtained and included through manual searching. Excluding duplicates, there were initially 164 papers. After a

review of titles and abstracts, 127 articles were further removed. Subsequent full-text screening led to the exclusion of 37 more articles, leaving a total of 15 articles.

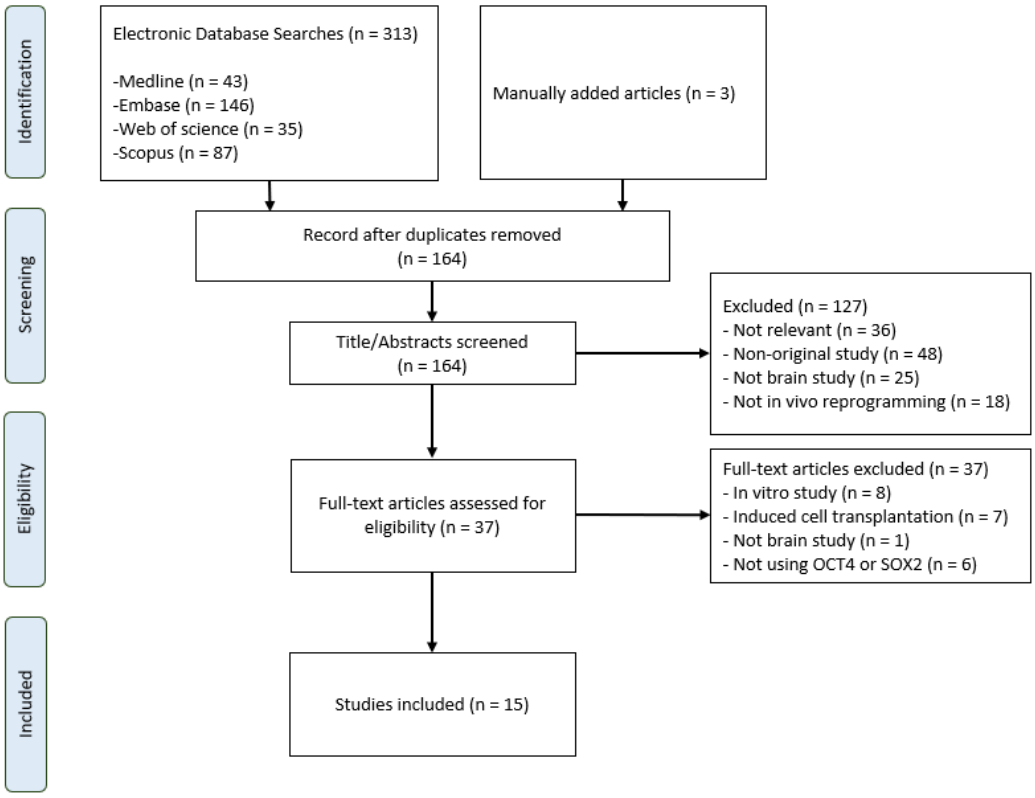

Figure 11. Flowchart of search strategy used in scoping review

4.2.1. In Vivo Reprogramming Study Using OCT4 in the Brain

A total of five studies utilized OCT4 as the sole transcription factor (Table 2). Sim S. et al. overexpressed OCT4 to investigate changes in the dentate gyrus and behavioral alterations, but the results did not yield significant findings [18]. Javan M.'s team conducted several studies on OCT4-driven reprogramming [19,20,21]. In their 2015 paper published in Cell Journal [19] and Life Science [20], they investigated the effectiveness of a combination therapy involving OCT4 and small molecules. Valproic acid (VPA), BIX-01294, Bay K8644, and RG-108 are chemicals that have been identified as influential in enhancing reprogramming efficiency or substituting certain reprogramming factors in in vitro research [33,34]. Upon administering exogenous OCT4 to the right cerebral ventricle, they observed an increase in markers such as NanoG, Klf4, c-Myc, Pax6, and Sox1, which became significantly enhanced when combined with VPA [19,20]. However, the co-administration of BIX-01294, Bay K8644, and RG-108 did not yield a synergistic effect, and when added to OCT4 + VPA, these compounds even reduced the expression of the earlier markers [19]. Interestingly, the simultaneous administration of VPA and OCT4 from 7 days before exogenous OCT4 significantly increased markers of neural stem cells such as Pax6 and Sox1, along with pluripotent indicators like endogenous OCT4, Nanog, Klf4, and c-Myc. This combinational treatment of VPA and OCT4 led to the reprogramming of endogenous somatic cells in the brain rather than inducing the proliferation of endogenous neural stem cells [19,20]. Moreover, through immunohistochemical analysis, it was confirmed that astrocytes were the main type of transfected cells, leading to the inference that astrocytes were the cell of origin.

One study showed the in vivo reprogramming effects of OCT4 in mice with optic chiasm demyelination. In this study, mice received oral administration of VPA for a week, followed by the injection of lentiviral particles capable of inducing OCT4 expression into the lateral ventricle. Subsequently, one week post-OCT4 induction, LPC was administered into the optic chiasm to induce demyelination. At 7 days post-injury, the group that received pre-VPA + OCT4 treatment showed a significantly reduced extent of demyelination. Furthermore, the expression of OCT4 enhanced myelination by converting transduced cells into myelinating oligodendrocytes. When assessing the recovery of the optic chiasm through visual evoked potentials, it was confirmed that the pre-VPA + OCT4 group exhibited the restoration of visual evoked potentials [21].

In a 2021 study by Yu et al., the reprogramming effects of OCT4 were investigated using R6/2 mice, a HD model [22]. Two weeks after OCT4 injection, an increase in Nestin+ cells, a marker of neural stem cells, was observed. Furthermore, there was an increase in NG2+ cells, a marker of oligodendrocyte precursor cells. By the 13th week, the AAV9-OCT4 group exhibited a substantial upregulation of markers related to oligodendrocyte precursor cells, including NG2, Olig2, PDGFRα, Wnt3, MYRF, and GDNF. When assessed using transmission electron microscopy and magnetic resonance imaging, a reduction in myelination defects was observed. Additionally, increased expression of markers associated with neurons (b3 tubulin, Neun) and GABAergic neurons (GAD67, and DARPP32) was confirmed. The study also involved a serial assessment of behavioral performance in mice. Notably, the OCT4 group showed significantly improved motor function between weeks 8 and 13. In conclusion, this research demonstrated that OCT4

Table 2. In vivo reprogramming study using OCT4

Reprogramming Factors	Expression Location	Animal Model /Lesion Model	Animal Age (Time of Reprogramming *)	Delivery Methods		Target Cell (Markers)	Functional Outcome	References
OCT4	Dentate gyrus	C57BL/6 male mice	8 weeks old	Lentivirus	Stereotactic injection	-	Behavioral test (open field test, elevated plus maze, Y-maze test, contextual fear conditioning paradigm)	42
OCT4 + VPA	Lateral ventricle	C57BL/6 mice	8~9 weeks old	Lentivirus	Stereotactic injection	Neural stem cell (Pax6, Sox1) Pluripotency marker (OCT4, Nanog, c-Myc, Klf4 and SOX2)	-	43
OCT4 + VPA	Lateral ventricle	C57BL/6 mice	8~9 weeks old	Lentivirus	Stereotactic injection	Neural progenitor and pluripotency markers (OCT4, Nanog, Klf4, c-Myc, Pax6 and Sox1, SSEA1,Nanog)		44
OCT4 + VPA	Lateral ventricle	C57BL/6 mice Optic chiasm demyelination by 1% lysolecithin	not mentioned (1 week before inducing demyelination)	Lentivirus	Stereotactic injection	Myelinating oligodendrocytes	Visual evoked potential	45
OCT4	Lateral ventricle	R6/2 mice HD model	4 weeks old	Adenovirus	Stereotactic injection	Neuron (NeuN (cortex) GAD67, Darpp32 (striatum))	Behavioral test (Rotarod test, Grip strength test)	46

VPA, valproic acid. * if applicable, any additional specific time points.

overexpression in a HD mouse model increased neural stem cells in the subventricular zone, expanded the oligodendrocyte lineage, promoted GABAergic neuron formation, reduced myelin defects, and positively impacted functional outcomes.

In the entire study, no specific safety issues were reported. One study disclosed that no teratoma formation was observed even 100 days post-infection, thereby confirming the safety of OCT4 injection [20].

4.2.2. In Vivo Reprogramming Study Using SOX2 in the Brain

There were six in vivo reprogramming studies about the effects of SOX2 alone in the brain. According to studies, SOX2 alone can induce the transformation of non-neuronal cells into DCX+ neurons (Table 3).

Zhang, C. L. and colleagues conducted research on reprogramming in the brain. In their 2013 study, they confirmed that DCX+ induced adult neuroblasts could be induced in the striatum using SOX2 alone[47]. In their 2015 study, they demonstrated that the use of SOX2 transformed striatal astrocytes into ASCL1+ neural progenitors, which subsequently progressed into DCX+ induced adult neuroblasts[48]. These induced adult neuroblasts showed proliferative activity after reprogramming[47]. Furthermore, they demonstrated that SOX2 alone was insufficient for the formation of mature neurons, particularly Neun+ neurons [47,48]. However, when combined with additional factors such as the neurotrophin Bdnf and the bone morphogenetic protein inhibitor Noggin[47] or with the histone deacetylase inhibitor VPA[48], these cells could overcome the apparent barrier preventing further neuronal maturation in the brain. These induced neurons were identified as calretinin+ and Neun+ neurons, and they were detected for up to 36 weeks. Moreover, these induced neurons displayed electrophysiological functionality and integrated into local circuits, allowing them to receive inputs from presynaptic neurons[46]. Regarding the cell of origin, Zhang C. L.'s group suggested astrocytes as the source. However, Heinrich's study proposed that NG2 glial cells were the origin of induced neurons.

There have been studies exploring the factors involved in SOX2-induced in vivo reprogramming. In their study, Islam, M. M. and colleagues provided evidence that Tlx expression in astrocytes significantly reduced the detection of SOX2-induced DCX+ cells in the adult striatum, which implies that SOX2-regulated Tlx expression is required for the in vivo reprogramming process[49]. Niu et al. demonstrated that the deletion of ASCL1 significantly reduced the number of DCX+ cells induced by SOX2 reprogramming[48]. While ASCL1 plays a critical role in SOX2-driven reprogramming, it is not sufficient on its own to trigger a complete cell fate switch.

Niu et al. used SOX2 in a different reprogramming pathway while aiming to generate dopaminergic neuron-like cells[50]. When they added FOXA2, LMX1A, or NURR along with VPA to SOX2, they observed the expression of TH+ cells. Notably, these induced dopaminergic neuron-like cells did not originate from NG2 glia, astrocytes, resident glial cells, or neurogenic neural progenitors in the subventricular zone. Instead, they were derived from endogenous (local) striatal neurons. The induced dopaminergic neuron-like cells expressed DARPP32 and CTIP2 and exhibited electrophysiological properties and firing patterns similar to dopaminergic neurons. They were

Table 3. In vivo reprogramming study using SOX2

Reprogramming Factors	Expression Location	Animal Model /Lesion Model	Animal Age (Time of Reprogramming *)	Delivery Methods		Target Cell (Markers)	Functional Outcome	References
SOX2 + BNDF/noggin or VPA	Striatum	C57BL/6J and ICR mice hGFAP–Cre, mGfap–Cre line 77.6, Nes–CreERTM, NG2–Cre, PrP–CreERT, Rosa–YFP, Rosa–tdTomato (Ai14)	Between 6 weeks and 24 months	Lentivirus	Stereotactic injection	Neuron (NeuN)	Functional electrophysiology	47
SOX2	Striatum	C57BL/6 and ICR mice Tlxflox/flox mice transgenic pGFAP-Cre mice	Not mentioned	Lentivirus	Stereotactic injection	Neuron (DCX)		49
SOX2+VPA	Striatum	Cst3-CreERT2, Nes-CreERTM, Ascl1-CreERT2, Ascl1neoflox/neoflox, Rosa-YFP, and Rosa-tdTomato	2–6 months of age	Lentivirus	Stereotactic injection	Neuron (NeuN, Calretrin)		48
SOX2 + Nurr1 + Lmx1a + Foxa2 + VPA	Striatum	C57BL/6J mice mGfap-Cre line 77.6, PrP-CreERT, Pdgfra-CreERT, Dat-Cre, and Rosa-tdTomato (Ai14)	6 weeks to 24 months	Lentivirus	Stereotactic injection	Dopaminergic neuron	Electrophysiological Properties and firing patterns, network connectivity	50
SOX2 ± ASCL1	Cerebral cortex	C57BL/6J mice Sox10-iCreERT2/GFP or GLASTCreERT2/GFP mice Stab Wound Lesion	8–10 weeks old (3 days after stab wound injury)	Retrovirus Lentivirus	Stereotactic injection	Neuron (DCX, NeuN)		51
SOX2	Corpus callosum (left)	C57BL/6J mice Demyelination induced by 0.2% Cuprizone in diet chow	12 weeks old	Lentivirus	Stereotactic injection	Oligodendrocyte precursor cells (PDGFRα+) oligodendrocytes		52

* if applicable, any additional specific time points.

functionally connected to other neurons, indicating their similarity to dopaminergic neurons in terms of functional properties.

Heinrich's 2014 study showed that SOX2-induced immature neurons were formed in a stab wound injury of the cortex[51] from NG 2 glial cells. These induced neurons also exhibited immature neuronal activity as evidenced by electrophysiological analysis.

In the study conducted by Farhangi et al. in 2019, they demonstrated that SOX2 could facilitate the conversion of astrocytes into oligodendrocyte precursor cells, ultimately leading to myelinating cells (PDGFRa+) in a multiple sclerosis model[52].

Regarding tumor formation, two studies conducted follow-ups for up to 50 weeks after SOX2 injection but neither observed tumor formation [47,48].

4.2.3. In Vivo Reprogramming Study Using SOX2 and OCT4 Simultaneously in the Brain

While several studies have explored the use of SOX2 and OCT4 in in vivo reprogramming, most have employed all four Yamanaka factors (OCT4, Klf4, SOX2, and c-Myc, collectively known as OKSM). A summary of these studies is presented in Table 4.

In a 2020 study conducted by Rodriguez et al. [14], researchers used reprogrammable mice to investigate whether the expression of Yamanaka factors is associated with the induction of aging markers in the dentate gyrus. Continuous expression of OKSM led to an increase in premature death, prompting the researchers to test a cyclic protocol (active for 3 days, followed by a 4-day rest, over 15 cycles) from 6 months to 10 months of age. Cyclic expression of OKSM increased migrating cells containing the neurogenic markers doublecortin (DCX, marker for immature neurons) and calretinin. Furthermore, H3K9me3, which typically decreases in the dentate gyrus with age, showed a smaller reduction, and there was an increase in the GluN2b subunit within NMDA receptors. Notably, after five days of treatment, the OKSM group displayed a significant improvement in memory index, proportional to the duration of exposure.

In Wi et al.'s 2016 study [16], high concentrations of OKSM were directly injected into the lateral ventricles of HBI mice. The treatment group exhibited increased proliferative cells, with a 3.1-fold increase in βIII-tubulin (early neuronal marker)-positive cells and a 6.2-fold increase in Neun (mature neuronal marker)-positive cells. Additionally, there was a 4.3-fold increase in Nestin -positive cells and a 2.9-fold increase in GFAP-positive cells observed in the subventricular zone. Hippocampal synaptic plasticity was enhanced, and treated mice showed functional improvements, including better long-term memory, reduced anxiety, and overall enhanced functionality.

In another 2016 study by the same group, experiments were conducted using a cerebral ischemia model in mice [17]. Reprogrammable mice expressed the four pluripotency factors OKSM in the presence of doxycycline. An infusion cannula was precisely positioned in the lateral ventricle using stereotaxic methods. Different concentrations were assessed by infusing either low (1 μg/mL; DOX-L) or high (100 μg/mL; DOX-H) concentrations of doxycycline or PBS (as a solvent control) into zone and striatum without increasing glial scar formation. There was also an increase in the lateral ventricle via a micro-osmotic pump. High expression of OKSM led to increased neural progenitor

Table 4. In vivo reprogramming using all four Yamanaka factors

Reprogramming Factors	Expression Location	Animal Model/Lesion Model	Animal Age (Time of Reprogramming *)	Delivery Methods		Target Cell (Markers)	Functional Outcome	References
OKSM	Dentate gyrus	Reprogrammable i4F-B mice (with a C57BL/6 genetic background)	6 months old (6 to 10 months of age)	Doxycycline-inducible	3 days on doxycycline, then 4 days off for 15 weeks	Levels of migrating cells	Object Recognition Test	53
OKSM	Cerebral cortex	C57BL/6 mice Controlled cortical impact TBI)	12 weeks old (3 days after TBI)	Retrovirus	Stereotactic injection	Neuron (NeuN, Map2)	Functional electrophysiology	54
OKSM	Lateral ventricle	ICR mice Chronic Hypoxic–Ischemic Brain Injury model (unilaterally carotid artery ligation at 1 week of age)	6 weeks old	Adenovirus	Stereotactic injection	Neuron (NeuN)	Behavioral test (Passive Avoidance Task, open field test)	28
OKSM	Lateral ventricle	Reprogrammable i4F-B mice (with a C57BL/6 genetic background) Cerebral ischemia model (bilateral common carotid artery occlusion for 20 min)	8–16 weeks (immediately after cerebral ischemia)	Doxycycline-inducible	Infused doxycycline into the lateral ventricle for 7 days using an osmotic pump	Neuron (NeuN)	Behavioral test (Rotarod test, ladder walking test)	29

TBI, traumatic brain injury; i4F-B, doxycycline-inducible polycistronic cassette encoding the four murine factors OCT4, SOX2, Klf4, and c-Myc. * if applicable, any additional specific time points.

cells in the SVZ and promoted astrocyte proliferation in the subventricular neovascularization in the striatum, and the high-expression group showed an increase in Neun+ cells and PSD95 (a synaptic marker) expression, resulting in improved motor function.

In the 2016 study by Gao et al. [15], researchers focused on in vivo reprogramming in the brain cortex of traumatic brain injury mice. Using retroviruses, they induced the expression of OKSM in reactive glial cells, resulting in the expansion of cell clusters that transformed into NanoG (a marker for embryonic stem cells)- or SSEA4 (cell surface marker expressed in embryonic stem cells)-positive embryonic stem cell-like cells, which further differentiated into various cell types. At 4 weeks, they observed the formation of neural tube-like structures, with the presence of Nestin+ neural stem cells and DCX+ cells. By 6 weeks, Neun+ and Map2 (mature neuron markers)-positive mature neurons were detected, exhibiting electrophysiological activity, indicating functionality. The

reprogramming also led to the conversion of cells into astrocytes and oligodendrocytes, but not into microglia.

When all OKSM factors were used, potential side effects were more easily observed. In Gao's study, teratomas emerged after 8 weeks [15], while in Rodriguez's research, continuous expression of OKSM led to premature death [14]. These results suggest that the tumorigenic potential is likely due to the presence of Klf4 and c-Myc, rather than OCT4 and SOX2 alone.

4.3. Interpretation of Current Study Results Based on Previous Findings

The collective findings from these studies highlight the significant potential of in vivo reprogramming using OCT4 and SOX2 as therapeutic strategies for neurodegenerative diseases, including HD. By inducing neuronal regeneration and promoting the formation of functional neurons, these factors offer a promising avenue for mitigating the progression of HD. Moreover, the absence of severe safety concerns, such as tumorigenesis associated with the use of OCT4 and SOX2 alone, further supports their potential application in clinical settings.

4.3.1. Neuroprotection Effects of OCT4 and SOX2 by In Vivo Reprogramming

Our results demonstrate that both OCT4 and SOX2 significantly enhance neural reprogramming and promote neuronal differentiation, as evidenced by the increased expression of Nestin+ cells, GABA+ cells, and DARPP32+ cells in the IHC analysis, as well as the upregulation of neuronal markers such as Nestin, GAD67, Tuj-1, PPP1R1B, and GABAAα1 in the qRT-PCR analysis. Additionally, brain weight was significantly better preserved in both the AAV4-OCT4 and AAV4-SOX2 groups compared to controls Notably, our functional assessments revealed statistically significant improvements in motor performance, such as enhanced rotarod and grip strength test results, particularly in the AAV4-OCT4 and AAV4-SOX2 groups compared to controls. These findings suggest that in vivo reprogramming with OCT4 and SOX2 not only supports neuronal regeneration but also contributes to functional recovery.

Previous studies have shown that using additional factors such as VPA or BDNF can facilitate the final differentiation of immature neurons into mature neurons. For example, both SOX2 and OCT4 displayed significantly increased reprogramming efficiency when used in conjunction with VPA, especially in promoting the transformation of cells into mature neurons[43-45,48]. VPA, a histone deacetylase inhibitor known for its roles in triggering BDNF expression and encouraging neural differentiation[55], played a vital role in enhancing this process. The heightened efficacy of VPA may be partly due to the greater activity of histone deacetylases in mouse cells. Additionally, maturation can be further enhanced using BDNF-Noggin[47].

Although we did not use VPA or BDNF in our study, we still observed significant improvements in motor function and neuronal differentiation, as well as preserving brain weight. This suggests that OCT4 and SOX2 alone can confer substantial neuroprotection and functional benefits, even without

additional differentiation factors. However, future studies should explore the addition of factors like VPA and BDNF to further amplify the therapeutic effects of OCT4 and SOX2, potentially optimizing their use in treating neurodegenerative conditions such as HD.

4.3.2. Safety of In Vivo Reprogramming

Regarding the safety concerns, both OCT4 and SOX2 treatments were found to be safe, with no tumor formation observed in either group throughout the 13-week study period. Although previous studies have shown that the use of the full OKSM cocktail, which includes SOX2 and OCT4, can lead to adverse effects, it is the other two factors, c-Myc and Klf4, that are known to be oncogenic and may promote tumor development[56,57]. In cases where only SOX2[47,48] and OCT4[44] are used for reprogramming, no tumor formation was reported during extended monitoring periods. This suggests that in vivo reprogramming using SOX2 and OCT4 is a safer approach, further supporting their use in clinical settings. The absence of tumorigenesis in our relatively long-term study underscores the potential of these factors as safe therapeutic agents for neurodegenerative diseases.

4.3.3. Origin of Induced Neurons

Understanding the origin of neurons induced by in vivo reprogramming with OCT4 and SOX2 is crucial to elucidating the mechanisms underlying neurogenesis. In this study, mCherry fluorescence and marker analysis revealed that the AAV4 vector primarily transduced NSCs/NPCs in the SVZ, as evidenced by strong co-localization with markers such as Vimentin and β-Catenin. These findings indicate that the dominant mechanism involves the direct activation of NSCs/NPCs, promoting their proliferation and differentiation into neurons.

We used the AAV4 vector, which predominantly targets ependymal cells.[58] This specificity ensures that SOX2 and OCT4 are delivered primarily to progenitor cell populations capable of contributing to neurogenesis. Importantly, this targeted delivery minimizes the risk of gliosis or astrocyte-mediated inhibitory responses, which are commonly associated with neurodegenerative conditions like HD.

Interestingly, moderate co-localization of mCherry with astrocytic markers, such as GFAP, suggests that astrocytes were also partially targeted by AAV4. This raises the possibility that some astrocytes may undergo reprogramming into NPC-like cells, contributing to neurogenesis. Supporting this hypothesis, in vitro experiments using primary astrocytes treated with AAV4-SOX2 or AAV4-OCT4 demonstrated increased Nestin expression, an NPC marker, and decreased GFAP expression. These results confirm the ability of astrocytes to transition into progenitor-like cells under the influence of OCT4 and SOX2.

Previous studies, particularly those involving SOX2, have similarly identified astrocytes as the cell of origin. For example, Gao's work postulates the involvement of reactive glial cells[54], while Dehghan's study points directly to astrocytes as the initial source of reprogrammed neurons[44]. Although there are fewer studies showing that OCT4 has the same effect, our findings suggest that OCT4 may also contribute to this reprogramming process. Further studies are needed to explore the second hypothesis regarding the effect of neuroinflammation reduction on NPC proliferation.

4.3.4. Comparative Dynamics of Neuronal Induction by Transcription Factors

Some transcription factors, such as NeuroD1[21-24] and Neurogenin2[25-27] are known for their ability to induce the direct conversion of astrocytes into neurons, leading to comparatively rapid neuronal expression. For instance, research findings from a study using NeuroD1 revealed the emergence of neuronal markers as early as 11 days[24]. In another study using NeuroD1, markers indicative of mature neurons, such as Neun+, were observed within a week [21]. The differentiation of neural progenitors facilitated by Neurogenin 2 occurs rapidly, with observable neurons appearing as soon as 3 days after injection; however, the majority of these generated neurons were unable to persist beyond 56 days, even with additional BDNF[27].

Compared to these factors, our study using SOX2 and OCT4 showed that the transcription factors were still detectable at 12 weeks, with increased expression of nestin, GAD67, Tuj1, and GABAAα1 observed. Unlike the aforementioned transcription factors that directly induce astrocyte-to-neuron conversion, in vivo reprogramming using SOX2 and OCT4 suggests a comparatively slower but longer-lasting process. This finding aligns with previous studies. For example, Niu et al. demonstrated that induced adult neuroblasts were detectable from 1 to 3 weeks, peaked at 7 weeks, and persisted until 14 weeks after using SOX2[47] Similarly, Gao et al. showed that when OKSM was used in a traumatic brain injury model, DCX+ cells were found around 4 weeks and Neun+ cells at 6 weeks [54].

We hypothesize that these differences might stem from varying stages of transformation. The processes involving SOX2 or OCT4 potentially facilitate the proliferation and differentiation of NPCs within the brain into neurons. This approach, which involves the expansion and differentiation of NPCs rather than the direct conversion of glial cells into neurons, could result in a slower but more sustained reprogramming process. Further comparative studies with clearer time-series analyses will be necessary to delineate the differences among these transcription factors.

V. CONCLUSION

In conclusion, our study supports the therapeutic potential of in vivo reprogramming using OCT4 and SOX2 for neurodegenerative diseases including HD. Both factors significantly enhanced neuronal differentiation and preserved brain weight and functional ability indicating their neuroprotective effects. The safety of OCT4 and SOX2 treatments further highlights their promise as therapeutic agents. Future research should focus on optimizing these reprogramming techniques and exploring combinations with other factors to enhance efficacy and ensure long-term safety.

APPENDIX

A scoping review about in vivo reprogramming using yamanaka factors in the brain

1. Methods

This review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for scoping reviews (PRISMA-ScR) statement[59].

1.1. Literature Search

We conducted an extensive search of published scientific literature in databases including MEDLINE, EMBASE, Web of Science, and SCOPUS using the following search strategy until 1 July 2024: [((in vivo) OR (in situ)) AND (reprogramming) AND ((brain))] AND ((SOX2) or (OCT4))]. Only the titles, abstracts, or keywords were searched in SCOPUS. We applied no language restrictions in our search. To identify duplicate entries, we considered factors such as the author, publication year, article title, and the source's volume, issue, and page numbers. Our search included studies of all types, including descriptive studies and case reports. Additionally, we manually reviewed the bibliographies of selected articles.

1.2. Study Selection and Eligibility Criteria

In vivo reprogramming studies that introduced SOX2 and OCT4 directly into the brain to regenerate cells were the primary focus of this research. To identify relevant studies, a comprehensive two-stage screening process was implemented.

1.2.1. Stage 1: Title and Abstract Screening

Two independent reviewers initially screened the titles and abstracts of all retrieved articles. Any discrepancies in assessments were resolved by a third reviewer. Articles deemed relevant based on initial screening proceeded to full-text evaluation.

1.2.2. Stage 2: Full-Text Evaluation

During the full-text evaluation phase, the reviewers critically assessed the eligibility of each study against a set of pre-established inclusion and exclusion criteria:

Inclusion Criteria:

- ✓ Articles reporting the use of one or more of the reprogramming factors, SOX2 and OCT4 for inducing in vivo reprogramming in brain.
- ✓ Peer-reviewed articles written in English.

Exclusion Criteria:

- ✓ Articles focused on in vitro exams.
- ✓ Articles involving the transplantation of cells induced through in vitro reprogramming.
- ✓ Articles related to reprogramming other than brain

- ✓ Non-original articles (such as reviews), editorials, letters from editors, book chapters, unpublished or non-peer-reviewed studies, abstracts, and PhD theses.
- ✓ Articles for which the full text was not accessible.

Any discrepancies between reviewers during full-text evaluation were resolved through discussion and consensus. This rigorous two-stage screening process ensured the selection of high-quality, relevant studies that aligned with the research objectives.

1.3. Data Extraction

The reviewers conducted an in-depth analysis of the full-text articles, extracting key information from relevant studies. These details included the first author, publication year, title, journal, the transcription factors used, animal models, the method of transcription factor delivery, the cell of origin, the target-induced fate, a description of the main findings, and the study's conclusions.

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Abstract in Korean

헌팅턴병 마우스 모델에서 OCT4 와 SOX2 를 이용한 생체 내 리프로그래밍이 신경 생성 및 기능 보존에 미치는 영향

헌팅턴병(Huntington's disease)은 운동 기능 장애, 인지 기능 저하, 그리고 정신과적 증상을 특징으로 하는 진행성 신경퇴행성 질환이다. 분자 메커니즘에 대한 이해가 발전했음에도 불구하고, 현재까지 질병을 근본적으로 치료할 수 있는 방법은 없다. OCT4와 SOX2와 같은 전사 인자를 활용한 생체 내 리프로그래밍은 신경 퇴행성 질환에서 신경 재생을 촉진하는 잠재력을 보이고 있다. 본 연구에서는 헌팅턴병 마우스 모델(R6/2)을 사용하여 OCT4와 SOX2를 이용한 생체 내 리프로그래밍의 효과를 조사하였다. AAV 벡터를 사용하여 OCT4 또는 SOX2를 발현시켰고, 이를 통해 운동 기능, 신경 분화, 그리고 안전성을 평가하였다.

연구 결과, OCT4와 SOX2를 이용한 생체 내 리프로그래밍은 헌팅턴병과 관련된 뇌 위축을 방지하고, 뇌 무게를 보존하는 데 기여함을 확인하였다. 또한, OCT4와 SOX2 처리 그룹에서 회전 막대 성능 저하 속도가 느려지고, 그립 강도가 증가하여, 운동 기능이 유의미하게 개선되었다.

AAV4 벡터는 주로 뇌실막세포(ependymal cells)에 국소화되며, 측실하부 내 신경 전구세포를 주로 표적으로 삼아 신경 분화를 유도하였다. 면역조직화학 및 실시간 정량적 역전사 중합효소 연쇄반응(qRT-PCR) 분석 결과, 측실하부에서 Nestin+/BrdU+ 세포가 유의미하게 증가하였고, 선조체에서는 DARPP32+/BrdU+ 세포가 AAV4-OCT4 및 AAV4-SOX2 그룹에서 유의미하게 증가한 것을 확인하였다. 또한 qRT-PCR 분석을 통해 Nestin, GAD67, Tuj-1, GABAAα1과 같은 신경 세포 마커들의 발현이 증가함을 확인할 수 있었다. 이는 OCT4와 SOX2가 신경 재생과 신경 전구 세포의 뉴런으로의 분화를 촉진함을 시사한다. 흥미롭게도, 성상세포 마커인 GFAP의 발현은 감소하였다. 이는 OCT4와 SOX2가 성상세포를 부분적으로 신경 전구세포로 리프로그래밍하는 보조적인 역할을 할 수 있음을 시사하며 이를 in vitro 실험을 통해 확인하였다. 또한 본 연구에서는 종양 형성이 관찰되지 않아 이 방법의 안전성 또한 입증되었다.

이러한 결과는 OCT4와 SOX2를 이용한 생체 내 리프로그래밍이 헌팅턴병 치료에서 신경 재생을 촉진하고 운동 기능을 보존할 수 있는 잠재적인 치료 전략임을 시사한다.

핵심되는 말: 헌팅턴병, 리프로그래밍

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