BRIEF REPORT

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Lineage tracing identifies in vitro microglia-to-neuron conversion by NeuroD1 expression

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Abstract

Neuronal regeneration to replenish lost neurons after injury is critical for brain repair. Microglia, brain-resident macrophages that have the propensity to accumulate at the site of injury, can be a potential source for replenishing lost neurons through fate conversion into neurons, induced by forced expression of neuronal lineage-specific transcription factors. However, it has not been strictly demonstrated that microglia, rather than central nervous system-associated macrophages, such as meningeal macrophages, convert into neurons. Here, we show that NeuroD1-transduced microglia can be successfully converted into neurons in vitro using lineage-mapping strategies. We also found that a chemical cocktail treatment further promoted NeuroD1-induced microglia-to-neuron conversion. NeuroD1 with loss-of-function mutation, on the other hand, failed to induce the neuronal conversion. Our results indicate that microglia are indeed reprogrammed into neurons by NeuroD1 with neurogenic transcriptional activity.

KEYWORDS
direct reprogramming, microglia, NeuroD1, neurogenesis

1 | INTRODUCTION

Lineage-specific transcription factors, which strongly influence cell fate decisions during embryonic development, can induce epigenetic rewriting of somatic cells and convert them into other cell types, such as neurons, without transition through a pluripotent stem cell state, by a process referred to as direct reprogramming.

Takashi Irie and Kanae Matsuda-Ito contributed equally to this study.

The first successful conversion of mouse embryonic fibroblasts into functional induced neuronal (iN) cells was revealed through the combined expression of Ascl1, Brn2, and Myt1l (Vierbuchen et al., 2010). More recent studies have shown the generation of specific subtypes of iN cells, such as sensory and motor neurons, by utilizing combinations of factors for reprogramming (Masserdotti et al., 2016). Many groups have also reported in vitro and in vivo conversion of glial cells such as astrocytes to neurons, which are responsive after brain injury and ultimately contribute to glial scar formation. For example, ectopic expression of NeuroD1 converts brain-resident astrocytes to neurons both in the intact and the injured mouse brain (Brulet et al., 2017; Chen et al., 2020; Guo et al., 2014; Liu et al., 2020; Wu et al., 2020).

Microglia, another type of glial cell, are resident immune cells in the brain parenchyma that originate from primitive macrophages (Ginhoux et al., 2010). In addition to parenchymal microglia, central nervous system-associated macrophages that include perivascular macrophages and subdural leptomeningeal macrophages are the organ-specific macrophages of the adult brain involved in functional homeostasis (Mrdjen et al., 2018). Microglia and macrophages, which accumulate at tissue damage sites to remove dead cells and debris after a brain injury such as stroke (Fumagalli et al., 2015), can be target cells for neuronal reprogramming. We previously showed that mouse microglia are converted into neurons both in vitro and in vivo by the ectopic expression of lentivirus-encoded NeuroD1 (Matsuda et al., 2019; Matsuda-Ito et al., 2022). Microglia and macrophages are known to share similar gene expression patterns in the healthy CNS, although they reside in different locations (Grassivaro et al., 2020). As a cell source for neuronal conversion, we had not strictly separated microglia and macrophages in our previous studies, leaving open the possibility that neurons are derived by conversion from macrophages rather than microglia. Moreover, recent studies suggested that the presumed astrocyte- and microglia-converted neurons are actually endogenous neurons labeled by the leaky expression of AAV- and lentivirus-encoded reporter genes (Rao et al., 2021; Wang et al., 2021). Thus, further studies using rigorous lineage-tracing strategies are needed to prove the microglia-to-neuron conversion.

Here, we re-examined NeuroD1-induced microglia-to-neuron conversion in vitro with microglia-specific labeling. We were able to isolate specifically labeled microglia of hexosaminidase subunit beta (Hexb)-Cre^{ERT2} mice (Masuda et al., 2020) and found that NeuroD1-converted iN cells originated from these microglia. The addition of a chemical cocktail increased the efficiency of neuronal conversion. In contrast, NeuroD1

with loss-of-function mutations failed to induce microglia-to-neuron conversion. These results indicate that neither contaminating neurons nor macrophages, but microglia, are the cell source of converted neurons induced by NeuroD1 possessing transcriptional activity.

2 | RESULTS

2.1 | Microglia can be specifically labeled in Hexb-Cre^{ERT2} mice

To investigate microglia-to-neuron conversion using lineage-tracing strategies, we employed Hexb-Cre^{ERT2}::Stop-YFP mice (Masuda et al., 2020). We administered tamoxifen intraperitoneally to Hexb-Cre^{ERT2}::Stop-YFP mice at postnatal days P1 and P3 (Figure 1a). In the cortex at P4, almost all YFP-positive cells were Iba1-positive microglia (99%) (Figure 1b–d,g). Meningeal macrophages were barely labeled by YFP (Figure 1e; Figure S1). The percentage of YFP-positive cells among the Iba1-positive cells in the cortex at P4 was approximately 70% (Figure S1), although the percentage was not 100% probably because YFP is not fully expressed in all cortical microglia at 1 day after tamoxifen administration. Furthermore, ectopic leakage in NeuN-positive neurons was not observed (Figure 1f,g), in agreement with our previous paper (Masuda et al., 2020).

2.2 | NeuroD1-converted iN cells originated from microglia

To examine whether NeuroD1-converted iN cells in our study originated from microglia, we isolated microglia from the cortex of 4-day-old Hexb-Cre^{ERT2}::Stop-YFP mice whose meninges were removed by dissection after tamoxifen administration (Figure 2a). We then investigated the purity of the isolated cells by whole-well imaging after staining with antibodies against Iba1, Map2ab, and YFP. Almost all of the isolated cells were Iba1-positive (>98%) and Map2ab-negative cells (Figure 2b,c,e). Furthermore, all YFP-positive cells we detected were Iba1 positive (Figure 2e). Careful inspection of the whole-well images revealed that YFP-positive microglia might engulf Map2ab-positive debris, although in small numbers (<0.2%) (Figure 2d,e). These data indicate that primary cultured microglia in our experimental setting contained neither macrophages nor neurons. To convert microglia to neurons, we used a lentivirus expressing NeuroD1 under the control of the doxycycline (Dox)-inducible tetracycline response element promoter. At 7 days after NeuroD1-viral infection, YFP-positive
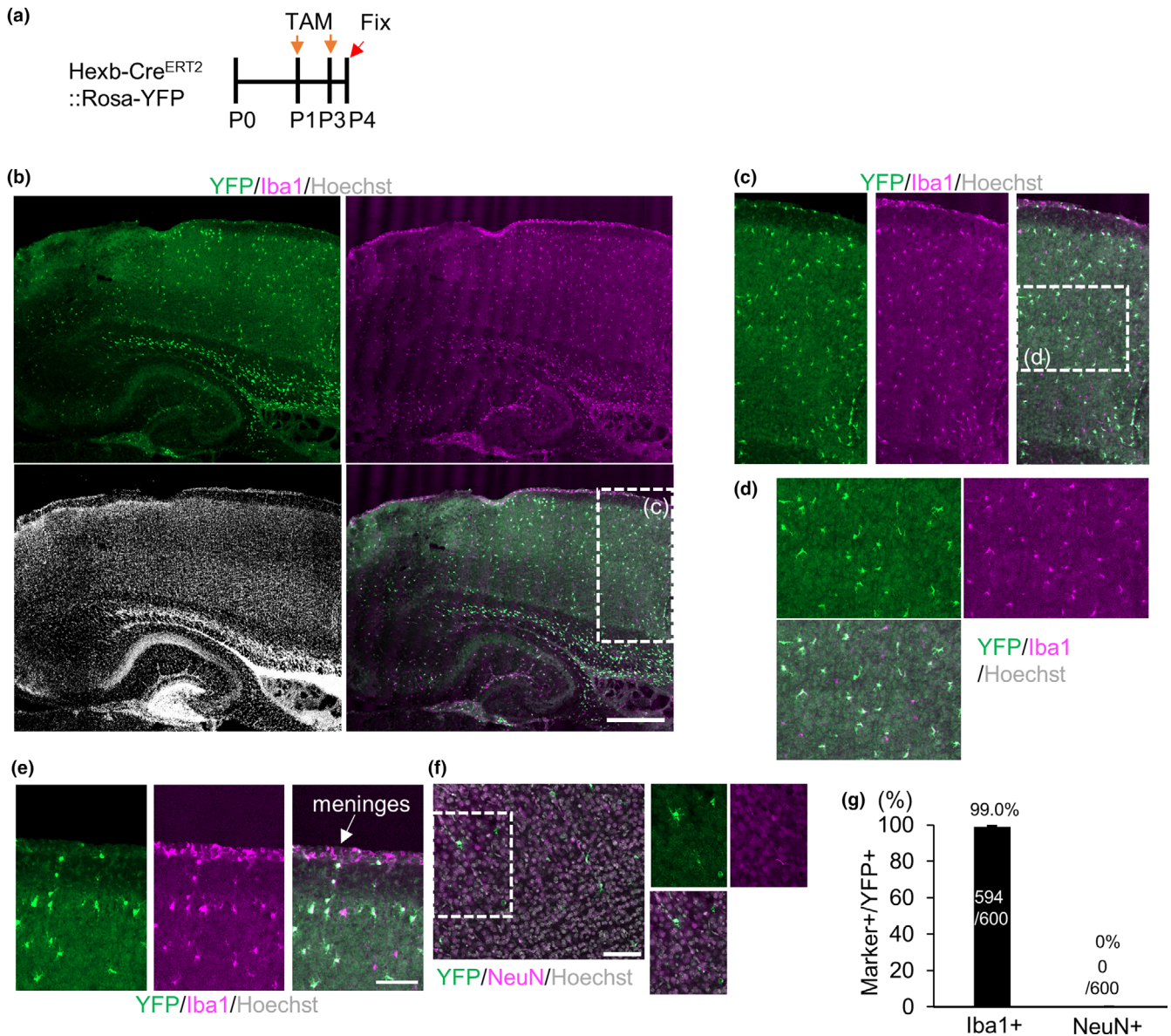
FIGURE 1 Microglia rather than macrophages are specifically labeled in Hexb-Cre^ERT2::Stop-YFP mice without ectopic leakage of marker expression into neurons. (a) Schematic representation of tamoxifen (TAM)-triggered labeling of Hexb-expressing cells for fate mapping. Hexb-Cre^ERT2::Stop-YFP mice were given tamoxifen intraperitoneally at P1 and P3, to permanently label Hexb-expressing cells. (b–d) Representative images of staining for YFP (green) and Iba1 (magenta) in the sagittal brain section at P4. Nuclei were stained with Hoechst. The panels in (c) show enlargements of the white dashed boxed region in (b) (cortex), and the panels in (d) show enlargements of the white dashed boxed region in (c). Scale bar, 500 μm (b). (e) Representative images of staining for YFP (green) and Iba1 (magenta) in the meninges at P4. Scale bar, 100 μm. (f) Representative images of staining for YFP (green) and NeuN (magenta) in the cortex at P4. The right panels show enlargements of the white dashed boxed region in the left panel. Scale bar, 100 μm. (g) Percentage of the indicated marker-positive cells among YFP-positive cells in the cortex excluding meninges at P4 ($n = 3$).

cells expressed βIII-tubulin and Map2ab (Figure 2f), suggesting that microglia could be converted into iN cells. Furthermore, we observed that the addition of a cocktail of three chemicals: valproic acid (VPA), forskolin and Y-27632 to NeuroD1-transduced microglia dramatically increased neuronal conversion efficiency (Figure 2g,h). These data indicate that NeuroD1-converted iN cells originated from microglia in vitro.

2.3 | NeuroD1 with loss-of-function mutations failed to induce microglia-to-neuron conversion

To investigate whether the neurogenic activity of NeuroD1 is essential for neuronal conversion, two NeuroD1 mutants were created and used for in vitro neuronal conversion: ΔbHLH, a mutant with complete lack of the
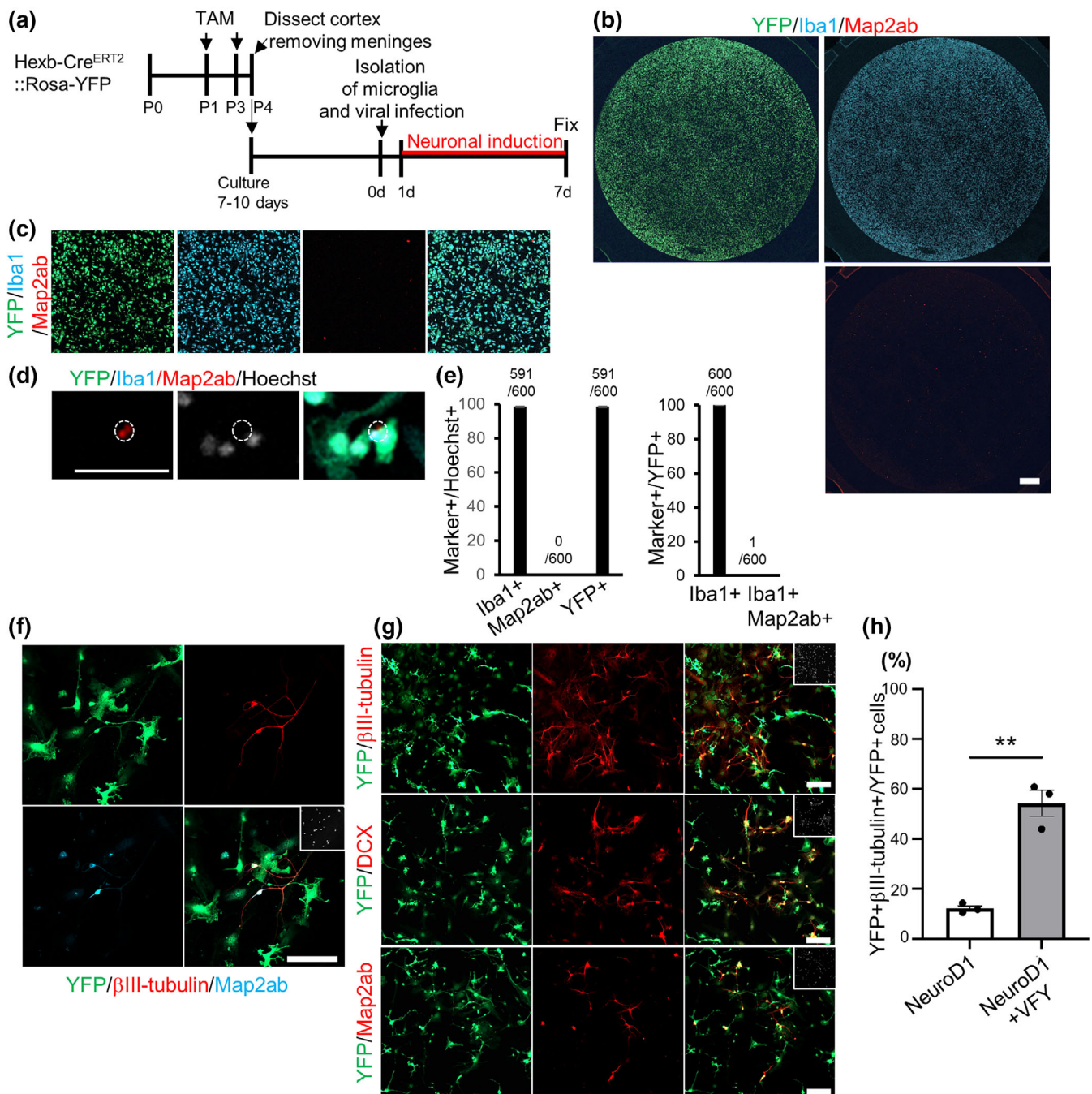
FIGURE 2 A chemical cocktail treatment can facilitate NeuroD1-induced microglia-to-neuron conversion. (a) Schematic representation of the conversion of Hexb-positive microglia to neurons. (b) Representative images of staining for YFP (green), Iba1 (cyan), and Map2ab (red) after isolation of microglia. (c) Enlargements of a representative part in (b). Scale bar, 1 mm (b). (d) Enlarged image of a region in (c). Dotted circles indicate Map2ab-positive debris. Scale bar, 50 μm. (e) Quantification of indicated marker-positive cells with staining for YFP (green), Iba1 (cyan), and Map2ab (red) after isolation of microglia ($n = 3$). (f) Representative images of staining for YFP (green), βIII-tubulin (red), and Map2ab (cyan) at 7 days after NeuroD1-viral infection. Scale bar, 100 μm. (g) Representative images of staining for YFP (green) and the indicated neuronal marker (red) at 7 days after NeuroD1-viral infection and treatment with valproic acid, forskolin and Y27632. Scale bars, 100 μm. (h) Quantification of βIII-tubulin-positive cells at 7 days after NeuroD1-viral infection with or without treatment with valproic acid, forskolin and Y27632 ($n = 3$ per group). $**p < .005$ by unpaired Student's t test.

basic helix-loop-helix (bHLH) domain, and R111L, a mutant with arginine to leucine single amino acid substitution in the bHLH domain to abolish the ability of DNA binding (Wang et al., 2021). These mutants were cloned into a doxycycline-inducible lentiviral vector, and their neurogenic activity was then investigated in cultured
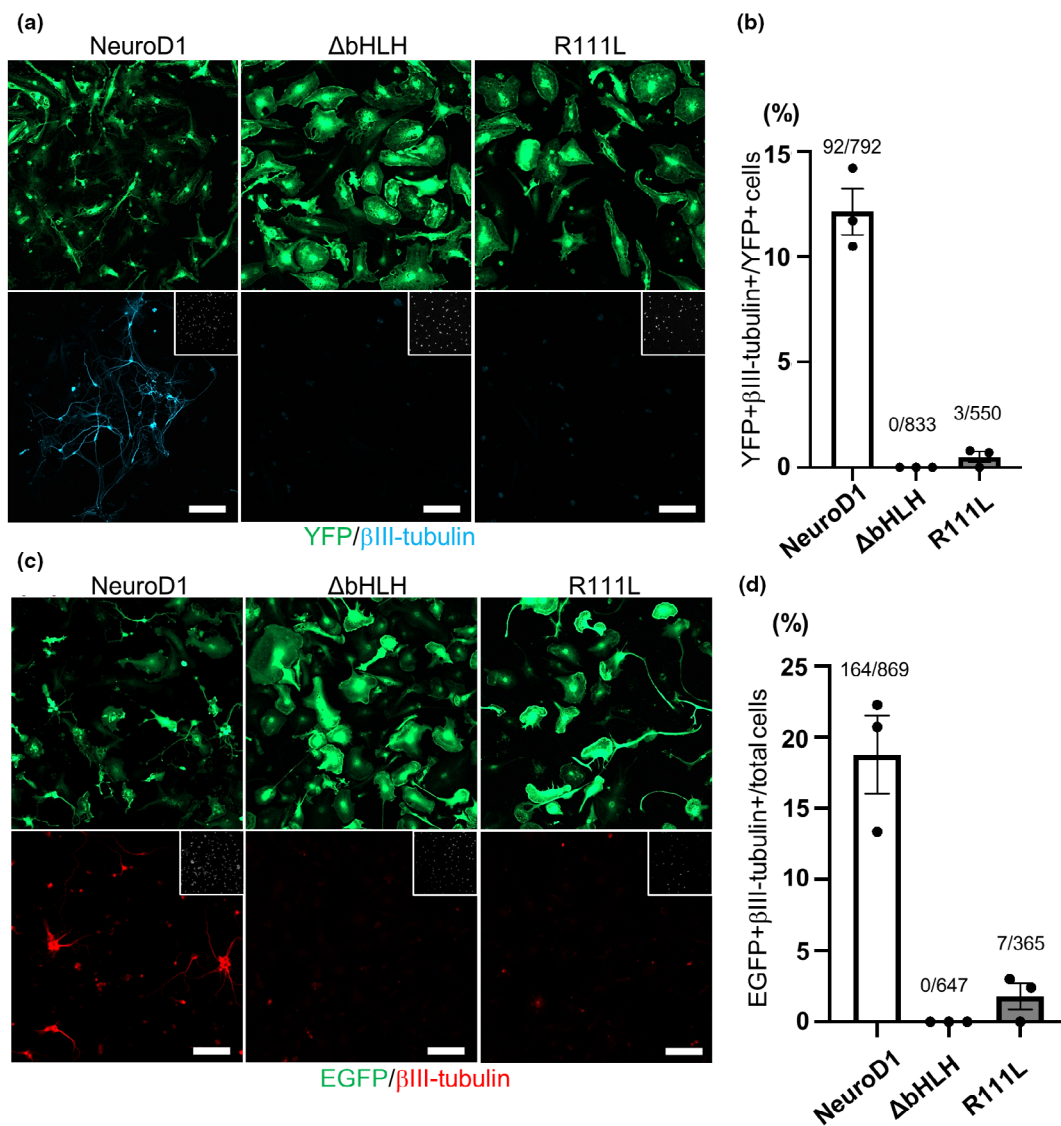
FIGURE 3 NeuroD1 mutants fail to convert microglia into neurons. (a) Representative images of staining for YFP (green) and βIII-tubulin (cyan) at 7 days after NeuroD1-, bHLH- or R111L-viral infection with Hexb-positive microglia. Scale bars, 100 μm. (b) Percentage of YFP/βIII-tubulin-double-positive cells among YFP-positive cells in (a) ($n = 3$ per group). (c) Representative images of staining for EGFP (green) and βIII-tubulin (red) at 7 days after NeuroD1-, bHLH- or R111L-viral and EGFP-viral infection with microglia isolated from P1 wild-type mice cortex. Scale bars, 100 μm. (d) Percentage of EGFP/βIII-tubulin-double-positive cells among total cells in (c) ($n = 3$ per group).

mouse neural stem cells (NSCs) (Figure S2a). As expected, wild-type NeuroD1 efficiently induced βIII-tubulin-positive neuronal differentiation of NSCs with doxycycline treatment, whereas the point mutants exhibited extremely poor neurogenic activity (Figure S2b–d). Microglia isolated from tamoxifen-administered Hexb-Cre^ERT2::Stop-YFP mice were infected with these point mutants and intact NeuroD1-expressing lentiviruses. Few YFP-positive iN cells were observed in these mutant NeuroD1-transduced microglia compared to the intact NeuroD1-transduced microglia (Figure 3a,b). Similar results were obtained when expression of these mutants'

NeuroD1 was induced in microglia isolated from P1 wild-type mouse cortices (Figure 3c,d). These results indicate that NeuroD1 with loss-of-function mutations failed to induce neuronal conversion and further support our conclusion, that is, NeuroD1-transduced microglia convert to iN cells.

3 | DISCUSSION

In this study, we have shown that microglia, but neither endogenous neurons nor macrophages, are the cell

source of NeuroD1-converted iN cells, as revealed by using strict lineage-tracing strategies. The failure of NeuroD1 mutants to generate neurons indicates that NeuroD1's transcriptional activity is essential for microglia-to-neuron conversion, further excluding the possibility that leaky marker expression in endogenous neurons is the reason for NeuroD1-indued microglia-to-neuron conversion. Although the present results have clearly indicated that microglia can be a cellular source for iN cells, we did not examine whether macrophages can be converted into neurons by NeuroD1 expression, warranting future investigations in this regard. It has been shown that under conditions of CNS inflammation or injury, macrophages infiltrate into the brain parenchyma and acquire a highly similar gene expression pattern to that of brain-resident microglia and act similarly to microglia (Grassivaro et al., 2020; Rajan et al., 2019). Thus, macrophage-to-neuron conversion may well be expected, however, this needs to be confirmed in the future.

We also observed that the addition of a chemical cocktail to NeuroD1-transduced microglia drastically increased neuronal conversion efficiency. The use of small molecules, including VPA, forskolin, and Y27632, has been reported to increase reprogramming efficiency (Biswas & Jiang, 2016). VPA directly inhibits histone deacetylase and promotes neuronal differentiation but suppresses astrocytic and oligodendrocytic differentiation of NSCs through the induction of neurogenic transcription factors, including NeuroD (Cheng et al., 2015; Hsieh et al., 2004). Forskolin activates adenylate cyclase to produce cyclic adenosine monophosphate (cAMP), and this cAMP activates protein kinase A, which improves the survivability and maturation after neuronal conversion (Liu et al., 2013). Forskolin is also known to induce Brn2 expression (Liu et al., 2021), which has been shown to promote NeuroD1-mediated neuronal reprogramming (Matsuda-Ito et al., 2022). These facts suggest that VPA and Forskolin enhance neuronal conversion, possibly through the induction of endogenous gene expression associated with reprogramming. The Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Li et al., 2015) is used to promote cell survival during neuronal lineage conversion and may also promote the NeuroD1-induced iN cell survival.

As we have shown in the present study, microglia can be efficiently converted into iN cells by NeuroD1 expression together with a chemical cocktail treatment. Microglia accumulate at the core of lesions after injuries such as stroke and spinal cord injury, where endogenous neurons are lost. Therefore, if microglia in these pathogenic conditions can be converted into neurons, this could provide a new treatment for intractable neurological diseases.

4 | EXPERIMENTAL PROCEDURES

4.1 | Isolation and culture of primary microglia

We prepared primary microglia from mouse using a previously reported protocol (Matsuda et al., 2019; Matsuda-Ito et al., 2022). We dissected cortexes of BL6 mice at postnatal day 1 (P1) or Hexb-Cre^{ERT2}::Stop-YFP mice at P4 (Masuda et al., 2020) after peeling of meninges to obtain microglia from glial cell mixtures. Before dissection, Hexb-Cre^{ERT2}::Stop-YFP mice were treated with 0.1 mg of tamoxifen/5 µL of oil intraperitoneally at P1 and P3. Dissected tissues were digested with papain (22.5 U/mL, Sigma) at 37°C for 30 min and treated with DNase (200 U/mL, Sigma). After centrifugation (200 × g, 5 min), the cell pellet was suspended in alpha minimum essential medium (MEM) with 5% fetal bovine serum (FBS) and 0.6% glucose and filtered with a 40-µm cell strainer (BD Falcon). After centrifugation (200 × g, 5 min), the cell pellet was again suspended in alpha MEM and re-centrifuged (200 × g, 5 min). The cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (Nacalai Tesque) containing 20% FBS, 1 mM L-sodium pyruvate, and MEM nonessential amino acids solution. This isolated glial mixture was incubated in T75 tissue culture flasks (BD Falcon) and the medium was changed every 2–3 days. Subsequently, we collected microglia by strong shaking for 1 h after 7–10 days in culture. The microglia were then plated onto an uncoated 35-mm culture dish and oligodendrocyte precursor cells were removed by changing the medium 30 min after plating. We used the cells attached to the dish as primary cultured microglia and maintained them in DMEM/Ham's F-12 containing 20% FBS, 1 mM L-sodium pyruvate, and MEM nonessential amino acids solution.

All experimental procedures were approved by the Kyushu University Animal Experiment Committee, and the care and use of the animals were performed in accordance with institutional guidelines. In addition, all experimental protocols were approved by the Animal Care Committee of Kyushu University. The study was carried out in compliance with the ARRIVE guidelines.

4.2 | Virus production

Lentiviruses were produced by transfecting HEK293T cells in a 10-cm dish with the constructs pCMV-VSV-G-RSV-Rev and pCAG-HIVgp using polyethylenimine. Since lot-to-lot variation in FBS preparations added to the culture medium critically influences the resultant

viral tropism, we avoided using FBS for virus preparation (Torashima et al., 2007). After transfection, we cultured the cells with 5 mL of serum-free N2 medium (DMEM/F12 supplemented with insulin (25 μg/mL), apo-transferrin (100 μg/mL), progesterone (20 nM), putrescine (60 μM), and sodium selenite (30 nM)) for 2 days. The supernatant was collected and used for virus infection experiments after filtration through a 0.22 μm filter to remove cell debris. HEK293T cells were kindly provided by Dr. Tetsuya Taga at Tokyo Medical and Dental University. The titer of recombinant viruses was checked using Lenti-X GoStix Plus (Takara Bio) according to the manufacturer's procedures and the viruses were used at 1.0×10^6 IFU/mL in this study.

4.3 | Induction of neuronal conversion

To induce neurons from glial cells, we used lentiviral vectors (derived from the Tet-O-FUW vector) in which gene expression of NeuroD1 and EGFP is controlled by the tetracycline operator. The vector backbone of the lentiviral plasmid expressing M2rtta is FUW. Plasmids used in this study are similar to those described in our previous report (Matsuda et al., 2019; Matsuda-Ito et al., 2022). For cells to be infected efficiently with the lentivirus, the virus must be added as soon as possible after plating the microglia. Therefore, virus suspensions were added at the time of medium exchange 30 min after isolation of primary microglia, and infection was performed overnight. The medium was then replaced with a neuronal medium (Neurobasal Medium [Gibco] supplemented with B27 [Gibco], GlutaMAX [2 mM, Gibco], BDNF, GDNF, NT3 [10 ng/mL each, Peprotech], and penicillin/streptomycin/fungizone [Hyclone]), and Dox induction was performed for 7 days to convert microglia into neuronal cells. Dox was added only once to the medium to activate NeuroD1 expression. A cocktail of three chemicals: VPA (Sigma), forskolin (Cayman), and Y-27632 (Wako) were added only once at the same time as Dox induction. The medium was changed every 2–3 days for the duration of the culture period.

4.4 | Mutagenesis

Site-directed mutagenesis was performed using a PrimeSTAR Mutagenesis Basal Kit (Takara) according to the protocol of the manufacturer. cDNAs encoding the FLAG-tagged mouse NeuroD1 (R111L and ΔbHLH) were subcloned into the Tet-O-FUW vector. To introduce desired mutations, the following primers were used:

5′-CGCGAGCTCAACCGCATGCACGGGCTG-3′ and 5′-GCGGTTGAGCTCGCGGGCGTTGGCCTT-3′ (mouse NeuroD1 R111L); 5′-AGAACGTATCCTGCGCTCAGGCAAA-3′ and 5′-CGCAGGATACGTTCTAGGCGCGCCTT-3′ (mouse NeuroD1 ΔbHLH). In each case, the mutation was confirmed by DNA sequencing analysis.

4.5 | Immunochemistry

Cells were fixed in 4% paraformaldehyde for 10 min and blocked for 1 h at room temperature (RT) with blocking buffer (5% FBS and 0.3% Triton X-100). After blocking, the cells were incubated with the following primary antibodies for 2 h at RT: anti-GFP (1:500, Aves), anti-Iba1 (1:500, Abcam), anti-Map2ab (1:500, Sigma), anti-βIII-tubulin (1:500, Covance), or anti-DCX (1:500, Merck Millipore). Nuclei were stained using Hoechst 33258 (Nacalai Tesque). Stained cells were visualized with a fluorescence microscope (Axiovert 200 M, Zeiss) and a confocal microscope (LSM800, Zeiss).

4.6 | Immunohistochemistry

Mouse brains were fixed in 4% paraformaldehyde, and 40-μm sections were cut with a cryostat (Leica). Cryosections were washed with PBS and blocked for 1 h at RT with blocking solution (5% FBS, 0.3% Triton X-100), and incubated overnight at 4°C with primary antibodies diluted in blocking solution. The following primary antibodies were used in this study: anti-GFP (1:500, Aves), anti-Iba1 (1:500, Abcam), and anti-NeuN (1:500, Merck Millipore). Nuclei were stained using Hoechst 33258 (Nacalai Tesque). Stained sections were visualized with a confocal microscope (LSM800, Zeiss).

4.7 | Statistical analysis

Data were analyzed using Prism 9 ver.9.1.2. Unpaired Student's t tests or ANOVA with Tukey post hoc tests were used to calculate the p value. Data represent mean ± SEM. We considered probabilities of $p < .05$ to be significant.

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SUPPORTING INFORMATION

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