suggest that this demethylation process is executed by a collaboration between HIF1α and Notch signal activation (Figs. 4, 5), probably through the formation of a complex between HIF1α and NICD, to effectively induce the expression of Notch-target genes [15]. The augmentation of Notch signal activation under hypoxia led to higher expression of *Nfia* (Supporting Information Fig. S4A), which has been shown to induce dissociation of DNMT1 from astrocytic gene promoters, resulting in demethylation of the promoters [7]. It has been suggested that HIFs act cooperatively with other signaling molecules, thereby influencing a wide range of biological processes including NPC maintenance and differentiation [11, 18-20]. Nevertheless, we report for the first time, to the best of our knowledge, that oxygen levels not only modify the behavior of transcription factors but also affect the epigenetic status of genes. The promotion of astrocytic gene demethylation by the hypoxic condition contributes to specifying the appropriate timing of the neural-to-glial cell fate switch of NPCs, by ensuring a proper balance between neurons and astrocytes that are generated during brain development.

We also found in this study that oxygen tension affects the DNA methylation status of astrocytic genes in mgNPCs via HIF-Notch signaling. Strikingly, hypoxia, HIF1α, Notch signaling and DNA methylation are all known to participate in the onset and/or progression of glioblastoma [12, 13, 15, 37], the most common and malignant type of brain tumor. Thus, a deeper understanding of glial cell-generating mechanisms, including astrocyte differentiation, may be of therapeutic interest.

As has been shown in a previous study [5] and the present work, DNA demethylation in the astrocytic gene promoters of NPCs is crucial for astrocyte differentiation, and the efficacy of astrocyte differentiation of NPCs is influenced by oxygen levels in the brain throughout development. Premature infants in neonatal intensive care units (NICUs) are often incubated under hyperoxic conditions to support their immature respiration. Oxygen concentration is strictly controlled in NICUs, since it has been shown that excess oxygen administration causes retinopathy of prematurity [38]. Furthermore, a few studies report other risks of hyperoxia to the central nervous system of extremely premature infants, including defects in mental and psychomotor development [39, 40]. The present study suggests that such effects may be attributable to an imbalance in NPC differentiation caused by a high level of oxygen in the incubator, and identifying their underlying mechanism in future work could provide new approaches for clinical applications that address developmental abnormalities in the nervous system, particularly in the context of neonatal intensive care.

Conclusion

We showed that local oxygen concentration can affect the fate of NPCs through an epigenetic mechanism. Unraveling how actual microenvironmental oxygen levels in the embryo influence epigenetic gene regulation in NPCs will provide new aspects for the study of NPC regulation.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Induction of superficial cortical layer neurons from mouse embryonic stem cells by valproic acid

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ABSTRACT

Within the developing mammalian cortex, neural progenitors first generate deep-layer neurons and subsequently more superficial-layer neurons, in an inside-out manner. It has been reported recently that mouse embryonic stem cells (mESCs) can, to some extent, recapitulate cortical development *in vitro*, with the sequential appearance of neurogenesis markers resembling that in the developing cortex. However, mESCs can only recapitulate early corticogenesis; superficial-layer neurons, which are normally produced in later developmental periods *in vivo*, are under-represented. This failure of mESCs to reproduce later corticogenesis *in vitro* implies the existence of crucial factor(s) that are absent or uninduced in existing culture systems. Here we show that mESCs can give rise to superficial-layer neurons efficiently when treated with valproic acid (VPA), a histone deacetylase inhibitor. VPA treatment increased the production of Cux1-positive superficial-layer neurons, and decreased that of Ctip2-positive deep-layer neurons. These results shed new light on the mechanisms of later corticogenesis.

1. Introduction

The mammalian cerebral cortex displays a complex structure with a high diversity of neuronal subtypes (Molyneaux et al., 2007). Within this structure, approximately 80% of the neurons are pyramidal excitatory cells which are derived from radial glial progenitors in the ventricular zone (VZ), and are generated in a well defined spatio-temporal manner (Guillemot et al., 2006; Leone et al., 2008). The first neurons generated are Cajal-Retzius cells, which will reside in the most superficial cortical layer (layer I). Subsequent neurogenesis proceeds in an inside-out fashion, by producing deep-layer neurons (layers V and VI) and then progressively more superficial-layer neurons (layers II–IV).

We have devised an adherent monolayer culture of mouse embryonic stem cells (mESCs) for the efficient generation of neuroectodermal precursors and neural stem cells (Ying et al., 2003; Conti et al., 2005). This system reduces the limitation and complexity of neural induction that are characteristic of multicellular aggregation (Bain et al., 1995; Wiles and Johansson, 1999) and/or co-culture methods (Kawasaki et al., 2000), and permits direct observation and manipulation of the cells under study. Recently, a better understanding of cortical neurogenesis was attained using our monolayer culture system. Gaspard et al. (2008) showed that, in the presence of sonic hedgehog (Shh) inhibitor, mESCs generate cortical neurons in a sequential fashion similar to that observed in the developing cortex. Despite this breakthrough result, the experiment failed to completely recapitulate later aspects of cortical development. Gaspard et al. (2008) had better success in generating early-born or deep-layer neurons than in generating superficial-layer neurons. Earlier, Shen et al. (2006) also reported that fewer superficial-layer neurons than deep-layer neurons were generated when they cultured cortical neural stem cells isolated at different gestational time points. This inefficiency in reproducing later events of cortical neurogenesis *in vitro* implies the existence of crucial factors which are not present or induced in current experimental systems.

Epigenetic mechanisms such as DNA methylation and histone modification including acetylation are now known to be critical intrinsic programs that dictate fate specification and differentiation of stem cells. Histone acetylation and deacetylation are mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. In general, an increase of histone acetylation

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by HATs causes the remodeling of chromatin from a tightly to a loosely packed configuration, leading to transcriptional activation. Conversely, a decrease of histone acetylation by HDACs results in a condensed chromatin structure and thus suppresses transcription (Juliandi et al., 2010). Inhibition of HDAC activity by valproic acid (VPA), a widely used anticonvulsant and mood-stabilizing drug, has been shown to drive mESCs to differentiate into the ectodermal lineage at the expense of mesodermal and endodermal lineages (Murabe et al., 2007). This ectodermal lineage differentiation is further biased in favor of neuronal rather than glial fates by the VPA treatment (Murabe et al., 2007). Prior to this finding in mESCs, we and others (Hsieh et al., 2004; Balasubramaniyan et al., 2006) had also found a similar tendency for neuronal over glial fate preference when we cultured neural progenitor cells (NPCs) in the presence of HDAC inhibitors such as VPA and trichostatin A. The types of neurons produced in these studies were not examined in detail, however, and the effects of HDAC inhibition on the differentiation of mESC-derived NPCs have not yet been studied.

Here we report that HDAC inhibition in mESC-derived NPCs treated with VPA resulted in a recapitulation of later stages of corticogenesis. VPA treatment increased the production of cut-like homeobox 1 (Cux1)-positive superficial-layer neurons and decreased that of B-cell leukemia/lymphoma 11B (Bcl11b; also called Ctip2)-positive deep-layer neurons. These results suggest an important role of histone acetylation for the specification of superficial-layer neurons in late corticogenesis.

2. Materials and methods

2.1. Maintenance of mESCs

The mESC line 46C (*Sox1*-GFP-IRES-pac knock-in) was routinely propagated without feeder cells as described previously (Ying et al., 2003; Conti et al., 2005). mESCs were grown at 37 °C in a 5% (v/v) CO_2 incubator in ESC medium (ESM) containing Glasgow Minimum Essential Medium (Invitrogen), supplemented with 10% (v/v) fetal bovine serum (Biowest), 1 mM sodium pyruvate (Invitrogen), 0.1 mM MEM non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), and 1000 U/ml murine leukemia inhibitory factor (Millipore), on 0.1% (v/v) gelatin-coated (Sigma) 9-cm dishes (Nunc). Medium was changed every day, and when the cells reached 60–70% confluence they were passaged onto new dishes at a plating density of 1×10^6 cells per 9-cm dish.

2.2. Neural differentiation

mESCs were induced to differentiate to the neural lineage as described previously (Ying et al., 2003; Conti et al., 2005; Gaspard et al., 2008, 2009). In brief, mESCs were trypsinized, dissociated and plated on 0.1% (v/v) gelatin-coated (Sigma) dishes at a density of 0.3×10^6 cells per 9-cm dish (Nunc) in ESM. One day later, the medium was replaced with DDM, which is composed of DMEM/F12 (Invitrogen) supplemented with freshly prepared modified N2-supplement (Ying and Smith, 2003), 1 mM sodium pyruvate (Invitrogen), 0.1 mM MEM non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), 0.5 mg/ml bovine serum albumin fraction V (Invitrogen), 1× GlutaMAX (Invitrogen) and 0.5× antibiotic–antimycotic (Invitrogen). This day was designated as differentiation day 0. Cyclopamine (Calbiochem) was added to a final concentration of 1 μM from differentiation day 2 to day 10. On differentiation day 10, the medium was replaced with DDM only (without cyclopamine). For selection of Sox1-expressing neural progenitor cells, from differentiation day 8 to day 10, puromycin (Sigma) was added to a final concentration of 0.5 μg/ml. The culture was maintained until differentiation day 12 and the medium was changed every 2 days during day 0 to day 12.

At differentiation day 12, mESC-derived NPCs were trypsinized, dissociated and plated on poly-L-lysine/laminin-coated (Sigma, Becton Dickinson) dishes at a density of 0.5×10^6 cells per 3.5-cm dish (Nunc) in N2/B27 medium, which consists of a 1:1 mixture of DDM (without sodium pyruvate and MEM non-essential amino acids) and Neurobasal/B27 medium (Neurobasal, 1× B27 supplement without vitamin A, 1× GlutaMAX and 0.5× antibiotic–antimycotic (all from Invitrogen)). Valproic acid (Sigma) was added one time to the culture medium to a final concentration of 0.5 mM at differentiation day 12 where appropriate. Culture was maintained until differentiation day 14 or 21, and the medium was changed every 2 days during day 12 to day 21.

2.3. Immunocytochemistry

Medium was removed and cells were washed with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde in PBS for 15 min. After 3 washes with PBS, the cells were incubated for 1 h at room temperature (RT) in blocking solution (PBS containing 3% FBS and 0.1% Triton X-100). They were then incubated overnight at 4 °C with the appropriate primary antibodies. The following primary antibodies were used: chick anti-GFP (1:500, Aves Labs), rabbit anti-β-tubulin isotype III (Tuj1; 1:1000, Covance), mouse anti-nestin (1:250, Millipore), rabbit anti-Pax6 (1:500, Covance), mouse anti-Map2ab (1:1000, Sigma), rat anti-Ctip2 (1:1000, Abcam), mouse anti-reelin (1:1000, MBL), and rabbit anti-Cux1 (1:500, Santa Cruz). After 3 washes with PBS, the cells were incubated for 2 h at RT with the appropriate secondary antibodies. The following secondary antibodies were used: FITC-conjugated donkey anti-chick, Cy5-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-rabbit, Cy5-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-mouse (all 1:500, Jackson ImmunoResearch), Alexa Fluor 488-conjugated donkey anti-mouse, Alexa Fluor 488-conjugated donkey anti-rabbit, and Alexa Fluor 488-conjugated donkey anti-rat (all 1:500, Invitrogen). After 3 washes with PBS, nuclei were stained for 15 min at RT with Hoechst 33258 (Nacalai Tesque). Cells were washed with PBS, mounted on cover slips with Immu-Mount (Thermo Scientific), and examined and photographed using a fluorescence microscope (Axiovert 200M, Zeiss) equipped with a camera and appropriate epifluorescence filters.

3. Results

3.1. 46C mESCs differentiate into the neuroectodermal lineage under Shh inhibition

We used 46C mESCs, one of whose characteristic features is the replacement of the *Sox1* open reading frame with that encoding green fluorescent protein (GFP) (Ying et al., 2003). Since *Sox1* is the earliest known neuroectodermal marker in the mouse embryo (Pevny et al., 1998; Wood and Episkopou, 1999), we could follow neural commitment of 46C mESCs in culture by monitoring their GFP expression.

46C mESCs can differentiate efficiently into the neural lineage in feeder-free adherent monolayer culture supplemented with serum-free medium (Ying et al., 2003; Conti et al., 2005; Abranches et al., 2009). *Sox1*-GFP-expressing NPCs can be detected from differentiation day 2, and composed more than 75% of the total cell population at differentiation day 4 in N2/B27 medium (Ying et al., 2003). To assess the neural commitment of 46C mESCs in the adherent monolayer culture system proposed by Gaspard et al. (2008), we cultured these cells at low density in a chemically defined

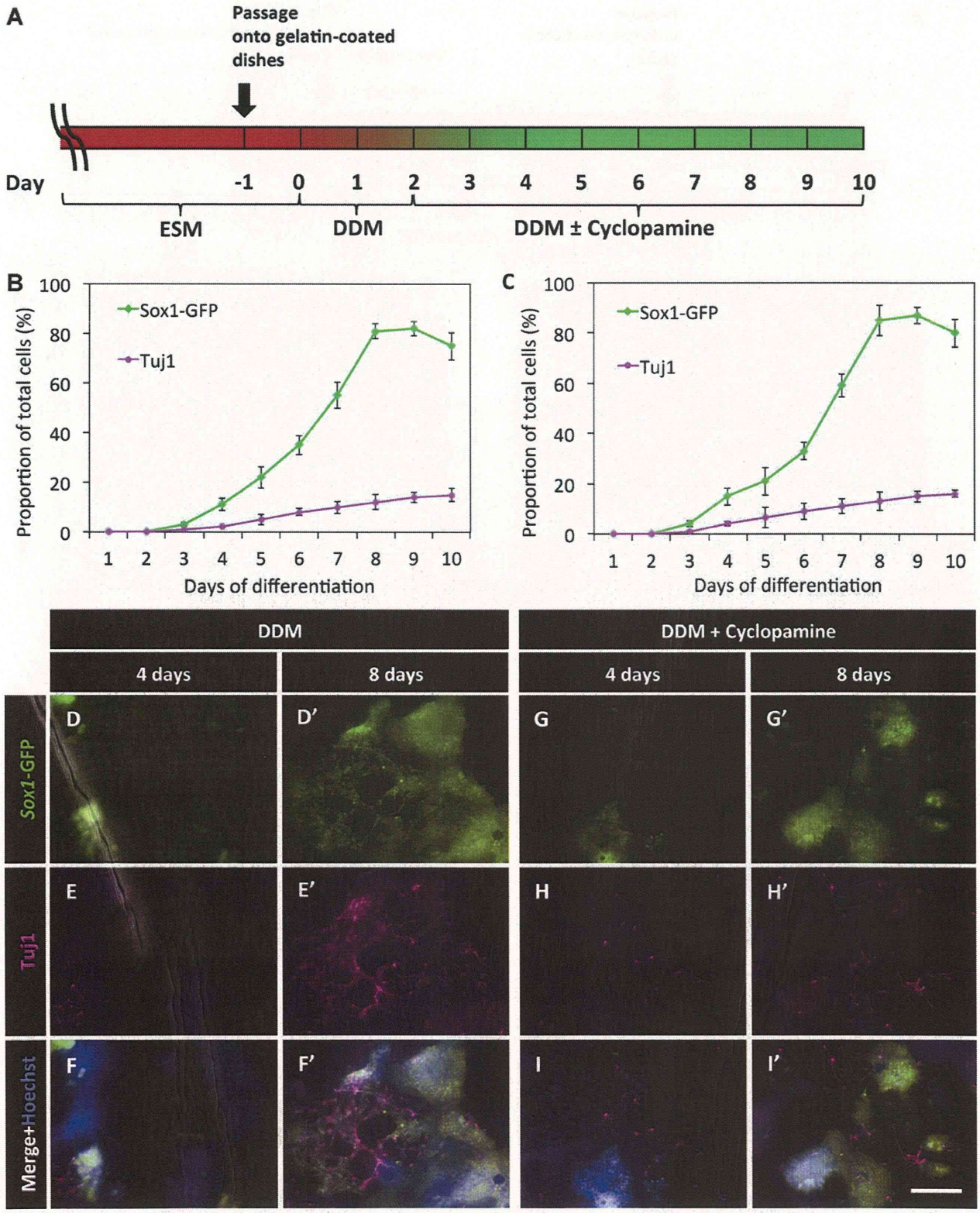
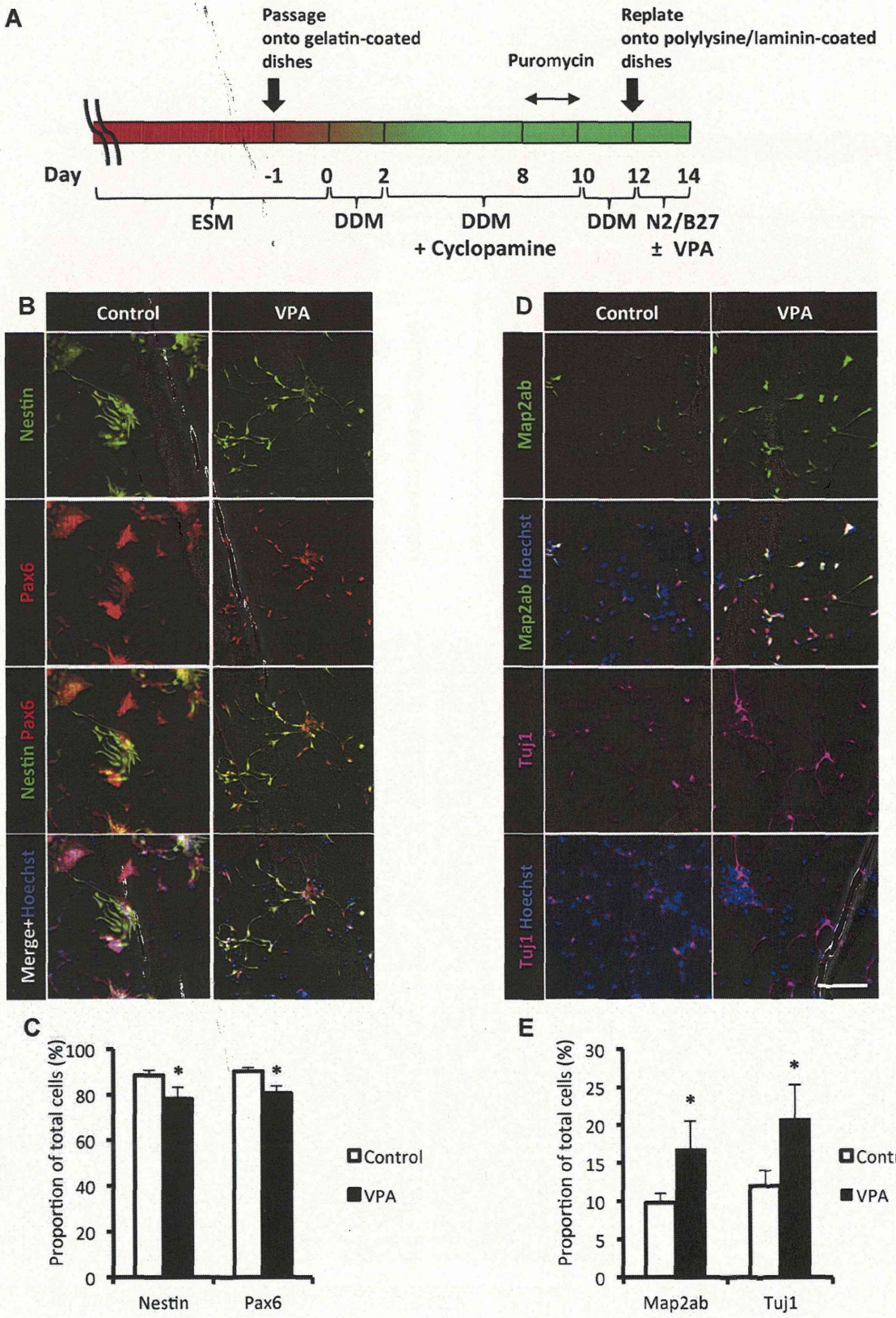


Fig. 1. 46C mESCs differentiate to the neuroectodermal lineage. (A) Timeline of the neural induction protocol using chemically defined default medium (DDM). mESCs were routinely propagated and then passaged to gelatin-coated dishes in embryonic stem cell medium (ESM) 1 day before neural induction (day −1). The next day (day 0), the medium was changed to DDM, and cyclopamine was added where appropriate from differentiation day 2 to day 10. The proportions of *Sox1*-GFP+ NPCs and Tuj1+ neurons did not differ in DDM (B) and in DDM with cyclopamine (C) during the culture period. Data are mean ± SD from at least three independent experiments. (D–I) Representative immunostaining images from differentiation day 4 and day 8 of *Sox1*-GFP-expressing (green in D, D′, G, and G′) and Tuj1-expressing (magenta in E, E′, H, and H′) cells, used for the quantitative data shown in B and C. Merged images with Hoechst (blue in F, F′, I, and I′) are also shown. Scale bar is 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)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default medium (DDM) for 10 days (Fig. 1A). We monitored *Sox1*-GFP expression and neuronal differentiation (as judged by Tuj1 immunostaining) each day during this period. We found that 46C mESCs differentiated more slowly into the neural lineage in DDM than in N2/B27. Although *Sox1*-GFP-positive NPCs could already be observed from differentiation day 3, they reached 80% of the total cell population only after differentiation day 8 (Fig. 1B, D, D′, F and F′). We also observed that starting from differentiation day 4, some of the NPCs had already differentiated into neurons (Fig. 1B, E, E′, F and F′).

We next examined the effects of the Shh inhibitor cyclopamine on 46C mESC survival and neural commitment. mESC-derived NPCs in DDM reportedly tend to possess ventral forebrain-like identity, and the addition of cyclopamine converts most of them into dorsal forebrain-like cells without affecting their proliferative pattern (Gaspard et al., 2008). We observed a similar pattern of neural commitment when we cultured 46C mESCs in DDM with or without cyclopamine, and found no significant difference in terms of cell survival (Fig. 1C, G–I and G′–I′). Thus, we conclude that 46C mESCs can survive and differentiate into the neural lineage in DDM with cyclopamine.

3.2. VPA enhances neurogenesis of 46C mESC-derived NPCs

Another advantage of using 46C mESCs is the existence of an internal ribosome entry site (IRES)-linked puromycin resistance gene which was also knocked-in together with GFP-encoding sequence to replace the *Sox1* gene's open reading frame (Ying et al., 2003). Hence, we can enrich the resulting NPCs by addition of puromycin to the medium. Because we found that the majority of 46C mESCs (>80%) had already differentiated into NPCs by differentiation day 8 (Fig. 1B and C), we added puromycin from differentiation day 8 to day 10 for our subsequent analysis.

Next, we examined the differentiation potential of enriched NPCs which were derived from 46C mESCs. We adopted the differentiation protocol of Gaspard et al. (2008, 2009) for making cortical neurons (Figs. 2A and 3A). At differentiation day 14 (Fig. 2A), we found that more than 80% of the cells were nestin- and Pax6-positive, indicating that they were mostly still NPCs (Fig. 2B and C). VPA treatment for 2 days reduced the proportion of cells positive for both markers (Fig. 2B and C). This reduction was accompanied by an increase of Map2ab- and Tuj1-positive neuronal cells (Fig. 2D and E). We then conducted the same analysis for a longer culture period, up to differentiation day 21 (Fig. 3A). We still found higher proportion of neuronal marker-positive (Map2ab+, Tuj1+) cells in the VPA-treated dishes compared with control (Fig. 3B and C). These results indicate that the enrichment of NPCs by puromycin was successful and that VPA treatment enhances neuronal differentiation of these NPCs.

3.3. VPA induces the generation of superficial-layer neurons

Given that neurogenesis was enhanced by VPA treatment, we then looked at the cortical types of these neurons at differentiation day 14 (after two days exposure to VPA; Fig. 2A). We first found a decreased proportion of early born and deep-layer neuron markers. The proportions of reelin- and Ctip2-positive cells among Tuj1-positive cells were significantly decreased by VPA treatment (Fig. 4A and C). Next, we found an increased proportion of superficial-layer marker (Cux1)-positive cells among Map2ab-positive cells after VPA treatment (Fig. 4B and D). When we prolonged the culture until differentiation day 21 (Fig. 3A), we obtained similar results. The proportions of cells positive for early born or deep-layer markers (reelin+, Ctip2+) among Tuj1-positive neuronal cells were still lower (Fig. 5A and C), while that for cells expressing the superficial-layer marker (Cux1+) was also higher, after VPA treatment (Fig. 5B and D).

When we compared the proportions of neurons in control and VPA-treated cell populations at day 14 with those at day 21, we saw a 3-fold increase during this period under both conditions (Figs. 2E and 3C). In control cells, we observed an increased proportion of Ctip2-positive cells among Tuj1-positive cells during this period (Figs. 4C and 5C). In contrast, the proportions of reelin- or Cux1-positive cells among neuronal marker-positive cells remained unchanged (Figs. 4C and D and 5C and D). These results indicate that in control cells, the majority of neurons produced during this period were Ctip2-positive, even though some of the NPCs still generated reelin- and Cux1-positive neurons.

On the other hand, the proportion of cells positive for the cortical markers we tested among neuronal marker-positive cells after VPA treatment did not change during the extended culture period (Figs. 4C and D and 5C and D). This indicates that VPA enhanced the production of Cux1-positive neurons only while it was being applied to the culture (differentiation day 12 to day 14). Nevertheless, the production of Ctip2-positive neurons in VPA treatment diminished compared to that in control cells during the extended culture period. These results suggest that transient VPA treatment enhances the temporal progression of some deep-layer-producing NPCs into superficial-layer-producing types during the treatment period, whereafter the residual NPCs retain this temporal progression even when VPA has been withdrawn from the culture.

4. Discussion

The brains of mammals differ in many aspects from those of other vertebrates. Most striking among the features that mammals have acquired during evolution are the increased size and complexity of the cerebral cortex, the largest brain structure where many of the higher cognitive functions reside (Finlay and Darlington, 1995; Hill and Walsh, 2005). The mammalian cerebral cortex is highly organized, with a six-layered structure that contains early-born or deep-layer (layers I, V and VI) and superficial-layer neurons (layers II–IV) that are produced in an orderly inside-out fashion (Molyneaux et al., 2007). The increased size and complexity partly reflect the overrepresentation of superficial-layer neurons, which are very abundant in primates, and especially so in human (Marin-Padilla, 1992). These cortical neurons are mainly of two types: pyramidal projection neurons, which mostly originate from NPCs of the dorsolateral wall of the telencephalon (Molyneaux et al., 2007), and interneurons, which originate from the ventral telencephalon during embryonic development (Wonders and Anderson, 2006). Although several studies have reported that ESCs can recapitulate

Fig. 2. VPA enhances neurogenesis of 46C mESC-derived NPCs. (A) Timeline of the corticogenesis protocol used for early differentiation analysis. mESCs were routinely propagated and passaged to gelatin-coated dishes in embryonic stem cells medium (ESM) 1 day before neural induction (day −1). The next day (day 0), medium was changed to chemically defined default medium (DDM) and cyclopamine was added from differentiation day 2 to day 10. At differentiation day 12, mESC-derived NPCs were replated to polylysine/laminin-coated dishes in N2/B27 (1:1 mixture of DDM and Neurobasal + B27). Valproic acid (VPA) was added where appropriate at differentiation day 12 and cells were harvested at differentiation day 14. (B–E) Representative immunostaining images from differentiation day 14 of nestin- and Pax6-expressing (green and red, respectively, in B), or Map2ab- and Tuj1-expressing (green and magenta, respectively, in D) cells and their quantification (C and E). Merged images with and without Hoechst (blue in B and D) are also shown. Tuj1 images in D were derived from the triple immunostaining data of Fig. 4A. Data are mean ± SD from at least three independent experiments. *$P < 0.05$ (Student's *t*-test). Scale bar is 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

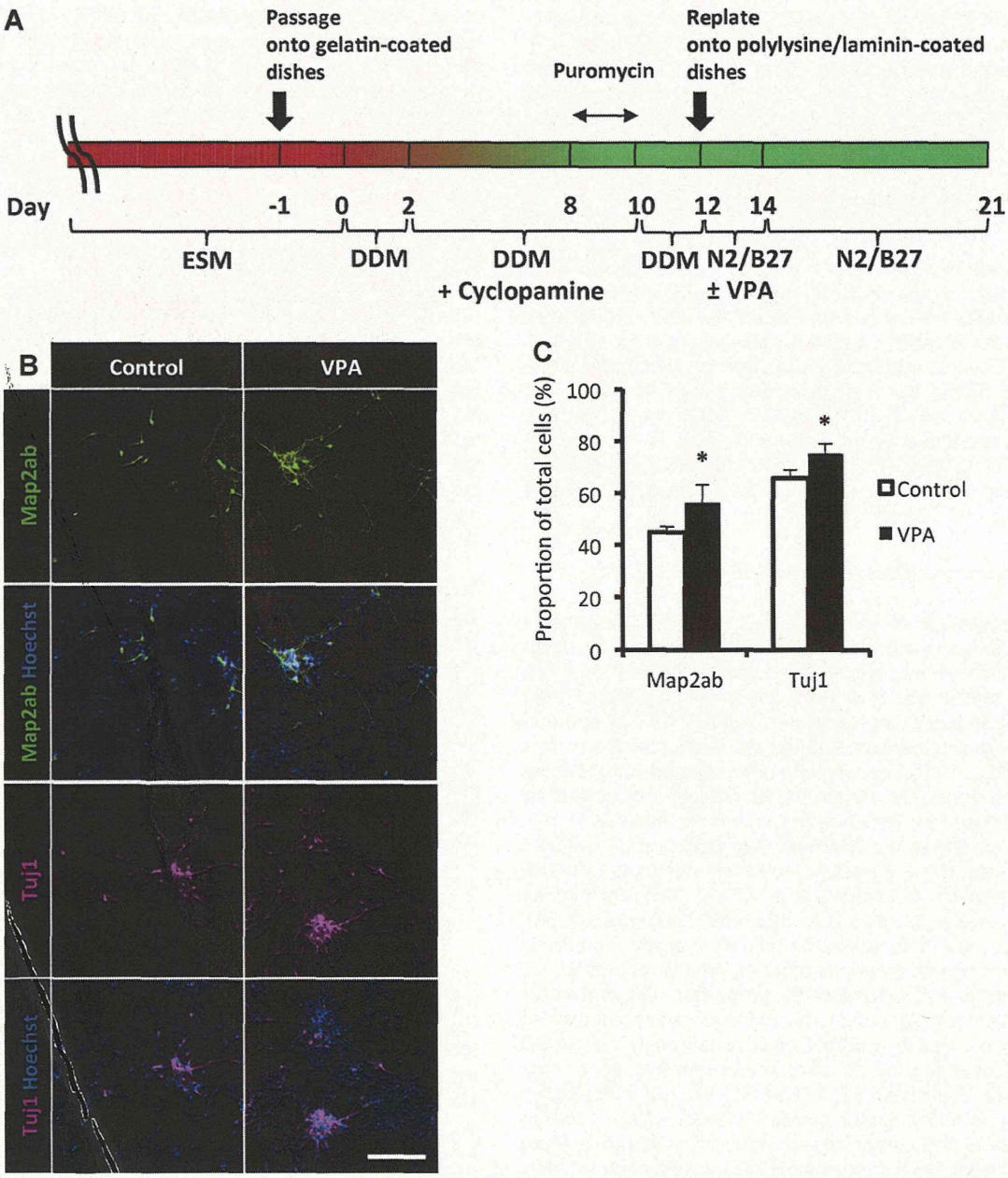

Fig. 3. Increased neurogenesis by VPA is observed even in prolonged culture. (A) Timeline of corticogenesis protocol used for late differentiation analysis. mESCs were routinely propagated and passaged to gelatin-coated dishes in embryonic stem cells medium (ESM) 1 day before neural induction (day −1). The next day (day 0), medium was changed to chemically defined default medium (DDM) and cyclopamine was added from differentiation day 2 to day 10. At differentiation day 12, mESC-derived NPCs were replated to polylysine/laminin-coated dishes in N2/B27 (1:1 mixture of DDM and Neurobasal + B27). Valproic acid (VPA) was added where appropriate at differentiation day 12 and cells were harvested at differentiation day 21. (B) Representative immunostaining images from differentiation day 21 of Map2ab- and Tuj1-expressing (green and magenta, respectively) cells. Merged images with Hoechst (blue) are also shown. Map2ab and Tuj1 images were derived from double- and triple-immunostaining data of Fig. 5A and B, respectively. (C) Quantification of neuronal marker-positive cells found in B. Data are mean ± SD from at least three independent experiments. $^*P < 0.05$ (Student's *t*-test). Scale bar is 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

certain aspects of cortical neurogenesis *in vitro* (Eiraku et al., 2008; Gaspard et al., 2008), the generation of superficial-layer neurons in those studies was very limited.

In the present study, we have demonstrated that transient HDAC inhibition by VPA in 46C mESC-derived NPCs enhances their neuronal differentiation (Fig. 2D and E). This has been reported previously in several studies using different types of NPCs (Hsieh et al., 2004; Murabe et al., 2007; Yu et al., 2009). Using an mESC culture system that can specifically produce and recapitulate the generation of cortical-layer neurons (Gaspard et al., 2008, 2009), we further showed that the increasing neuronal population after VPA treatment includes a higher proportion of superficial-layer neurons (Cux1+) (Fig. 4B and D), accompanied by a decreasing proportion of early-born or deep-layer neurons (reelin+, Ctip2+) (Fig. 4A and C). Therefore, it is conceivable that VPA enhances the temporal progression of some of deep-layer-producing NPCs into superficial-layer-producing NPCs during the time when VPA was being supplied to the culture. The residual NPCs retained this temporal progression even when VPA was withdrawn from the culture, because we still observed the same proportion of all cortical

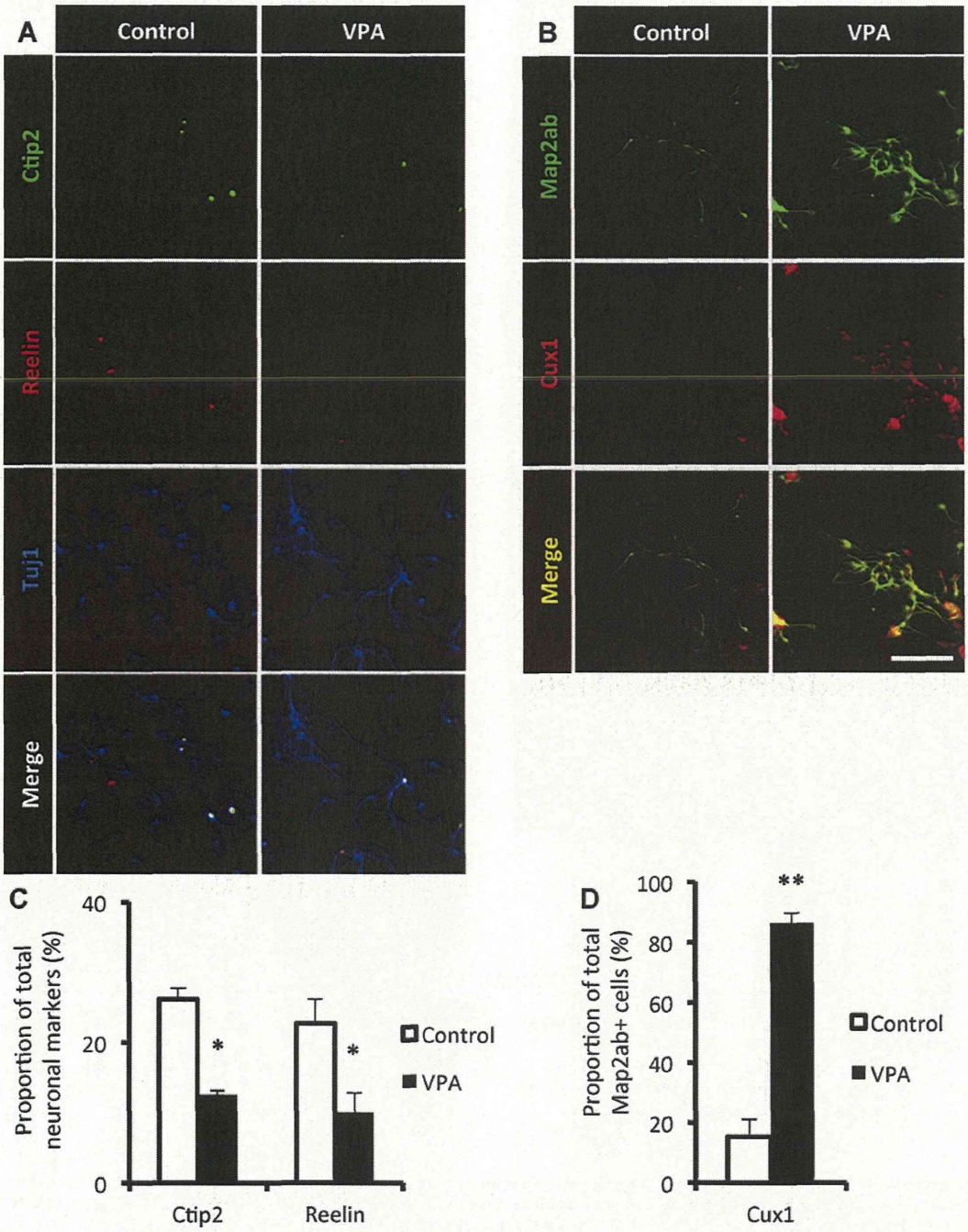

Fig. 4. VPA induces the production of superficial-layer neurons. (A and B) Representative immunostaining images from differentiation day 14, as in Fig. 2A, for Ctip2-, reelin- and Tuj1-expressing (green, red, and blue, respectively, in A), and Map2ab- and Cux1-expressing (green and red, respectively, in B) cells. Merged images are also shown. Hoechst staining can be found in Fig. 2D. Quantification of cortical layer marker-positive cells among neuronal marker-expressing cells for early-born or deep-layer (C) and superficial-layer neurons (D). Data are mean ± SD from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ (Student's *t*-test). Scale bar is 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

markers even at differentiation day 21, 7 days after VPA treatment was terminated (Fig. 5A–D).

Elucidating the mechanisms that lead to the enhanced progression of NPCs from producing deep-layer neurons to producing superficial-layer neurons after transient VPA treatment is an important challenge for future study. It has been proposed that the zinc-finger transcription factor *Fezf2*, which acts upstream of *Ctip2*, plays an important role in the specification of deep-layer neurons (Chen et al., 2008; Leone et al., 2008). VPA might repress *Fezf2* directly or indirectly in our culture system, which could in turn decrease the generation of deep-layer neurons and release the inhibition of upper-layer neuron production. This scenario is plausible, since VPA treatment of mouse embryos reduces levels of *Fezf2* mRNA in the forebrain (B.J. and K.N., unpublished data), and since there was an increase of Tbr1-positive cells in our culture after VPA treatment (data not shown). It has been reported recently that Tbr1 can act as a direct transcriptional repressor for *Fezf2* (Han et al., 2011).

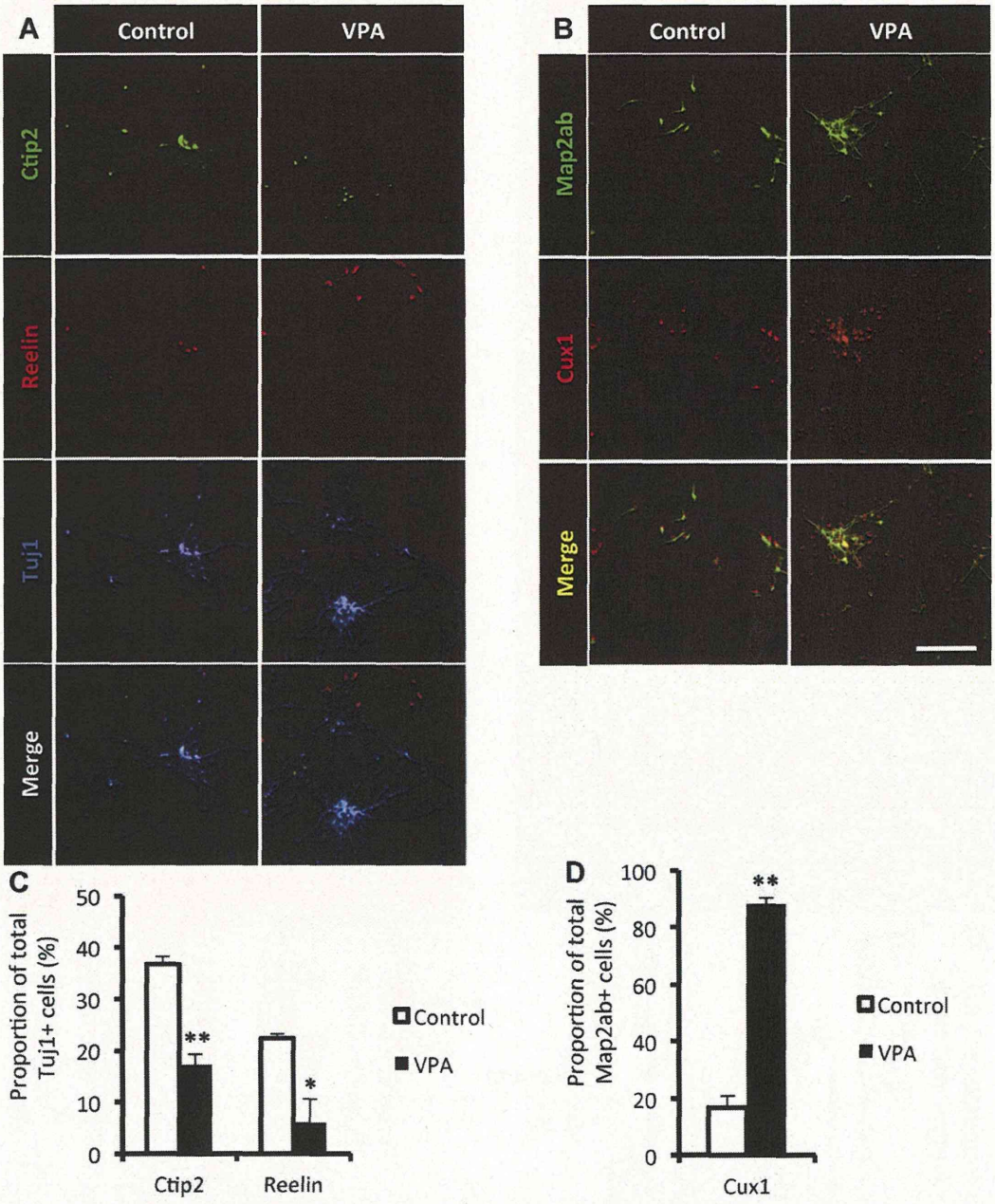

Fig. 5. Increased generation of superficial-layer neurons by VPA is observed even in prolonged culture. (A and B) Representative immunostaining images from differentiation day 21, as in Fig. 3A, for Ctip2-, reelin- and Tuj1-expressing (green, red, and blue, respectively, in A), and Map2ab- and Cux1-expressing (green and red, respectively, in B) cells. Merged images are also shown. Hoechst staining can be found in Fig. 3B. Quantification of cortical layer marker-positive cells among neuronal marker-expressing cells for early-born or deep-layer (C) and superficial-layer neurons (D). Data are mean ± SD from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ (Student's *t*-test). Scale bar is 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Our results suggest that histone acetylation plays important roles in the production of superficial-layer neurons in an adherent monolayer system. Similar efficient production of superficial-layer neurons from mESCs was also recently reported in a study of modified embryoid body (EB) formation (Eiraku et al., 2008). It is tempting to hypothesize that in the EB, high levels of histone acetylation occur and persist until late differentiation to help ensure the generation of superficial layer neurons. Nevertheless, generation of cortical neurons from human (h)ESCs using the same EB method was skewed toward deep-layer identity (Eiraku et al., 2008), and both mESCs and hESCs failed to recapitulate the same inside-out pattern of cortical neurogenesis observed in the developing cortex (Au and Fishell, 2008; Gaspard and Vanderhaeghen, 2010). It will be of interest to explore the effects of increasing histone acetylation by VPA treatment on the production of superficial-layer neurons in hESCs, and whether histone acetylation plays a role in the inside-out mode of cortical neurogenesis.

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