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ORIGINAL ARTICLE

APPL2 Negatively Regulates Olfactory Functions by Switching Fate Commitments of Neural Stem Cells in Adult Olfactory Bulb *via* Interaction with Notch1 Signaling

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Abstract Adult olfactory neurogenesis plays critical roles in maintaining olfactory functions. Newly-generated neurons in the subventricular zone migrate to the olfactory bulb (OB) and determine olfactory discrimination, but the mechanisms underlying the regulation of olfactory neurogenesis remain unclear. Our previous study indicated the potential of APPL2 (adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 2) as a modulating factor for neurogenesis in the adult olfactory system. In the present study, we report how APPL2 affects neurogenesis in the OB and thereby mediates olfactory discrimination by using both *in vitro* neural stem cells (NSCs) and an *in vivo* animal model—APPL2 transgenic (Tg) mice. In the *in vitro* study, we found that APPL2 overexpression resulted in NSCs switching from neuronal differentiation to gliogenesis while APPL2 knockdown promoted neurogenesis. In the *in vivo* study, APPL2 Tg mice had a higher population of glial cells and dampened neuronal production in the olfactory system, including the corpus callosum, OB, and rostral migratory stream. Adult APPL2 Tg mice displayed impaired performance in olfactory discrimination tests compared with wild-type mice. Furthermore, we found that an interaction of APPL2 with Notch1 contributed to the roles of APPL2 in modulating the neurogenic lineage-switching and olfactory behaviors. In conclusion, APPL2 controls olfactory discrimination by switching the fate choice of NSCs *via* interaction with Notch1 signaling.

Keywords APPL2 · Neurogenesis · Olfactory bulb · Notch1 signaling

Introduction

Olfactory functions like olfactory sensitivity and smell memory are important factors affecting cognitive and mental functions [1, 2]. Disrupted olfactory function is considered to be an indicator of multiple neurological disorders including head trauma, depression, and neurodegeneration [3, 4]. Neurogenesis in the olfactory bulb (OB) provides new neurons to facilitate olfactory functions such as odor information processing, learning, and discrimination [5–8]. Neuroblasts committed from adult neural stem cells (NSCs) in the subventricular zone (SVZ), migrate along the rostral migratory stream (RMS), mature into neurons, and integrate into local OB circuits [9]. The balance of lineage commitment between neurogenesis and gliogenesis determines the production of neurons in the olfactory system [10], and thereby regulates the ability of animals to encode odor information [11–13]. The NSCs in the SVZ decline with age and their re-activation can be

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harnessed as regenerative power against neurodegeneration [14, 15]. Olfactory dysfunction with impaired olfactory neurogenesis may be one of the characteristics in neurodegenerative diseases [16, 17]. Thus, elucidating the factors that modulate olfactory neurogenesis may provide new therapeutic targets for drug discovery in the treatment of neurodegenerative disorders.

By affecting adult olfactory neurogenesis, neurogenic mediators may regulate olfactory behaviors. Among multiple neurogenic regulators, Notch signaling may be a key regulator in controlling olfactory behaviors and adult neurogenesis [18, 19]. Notch signaling is responsible for cell fate choice during the development of NSCs [20, 21]. Activation of Notch signaling initiates NSCs switching from neurogenesis to gliogenesis [22]. In *Drosophila* and mice, Notch signaling plays critical roles in regulating olfactory plasticity and turnover of the olfactory epithelium progenitor/stem cells [23, 24]. Loss of Notch1 in mitral cells affects the neuronal response to olfactory stimuli and Notch1cKOKln mice display reduced olfactory aversion to propionic acid compared to wild-type littermates [25]. Therefore, uncovering the underlying mechanisms regulating Notch signaling would help to identify new therapeutic targets for promoting adult olfactory functions.

APPLs (adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper) are critical signaling molecules in regulating cellular behaviors. APPLs bind to multiple cellular molecules such as membrane receptors, nuclear factors, and signaling proteins linked to cell proliferation, chromatin remodeling, endosomal trafficking, cell survival, cell metabolism, and apoptosis [26]. There are two proteins in the APPL family: APPL1 and APPL2. APPL1 was the first member identified, with the functions of interacting with adiponectin receptors and adiponectin signaling and having crosstalk with insulin signaling pathways for metabolic regulation [26, 27]. As a downstream effector of Adipo-R1 and -R2, APPL1 mediates adiponectin-evoked endothelial nitric oxide production and endothelium-dependent vasodilation [28]. APPL1-deficient mice have impaired glucose-stimulated insulin secretion through inhibition of mitochondrial function in pancreatic beta cells [29]. APPL1 has also been implicated in regulating the Akt and/or MAPK signaling pathways and affects cell growth [30–32]. APPL1 participates in the pathological process of diabetic diseases *via* modulating the insulin, adiponectin, and epidermal growth factor (EGF) signaling pathways [32–35]. However, whether APPL1 modulates the growth of NSCs remains unknown.

APPL2 is an isoform with 54% identity in sequence and co-localizes with APPL1 in cells [32]. By interacting with TBC1D1, APPL2 also regulates insulin signaling for glucose metabolic regulation. It has been reported that the APPL2-inhibited insulin-stimulated glucose uptake is mediated by the membrane recruitment of GLUT4 in skeletal muscle [36]. Our previous study has identified APPL2 as a key neurogenic regulator contributing to the depression-induced disruption of olfactory sensitivity [37, 38]. By regulating the glucocorticoid receptor, APPL2 acts as a cellular signaling molecule to affect adult neurogenesis and its related symptoms include depressive/anxiety-like behaviors and impaired olfactory sensitivity [37, 38]. However, how APPL2 regulates olfactory functions, particularly olfactory discrimination, at the physiological level remains unknown. Like the functions of Notch signaling, an *in vivo* clone study revealed that overexpressed APPL2 induces hippocampal NSCs to switch from neurogenesis to gliogenesis [38]. Thus, in the present study, we tested the hypothesis that APPL2 regulates olfactory neurogenesis and affects olfactory discrimination, and the underlying mechanisms are associated with affecting the Notch signaling for cell-fate decision in NSCs.

Materials and Methods

Cell Culture

Mouse neural progenitor C17.2 cells were obtained from the American Type Culture Collection and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Hyclone, Omaha, NE, USA) supplemented with 8% fetal bovine serum (FBS; Gibco, Waltham, MA, USA). Primary NSCs were prepared from fetal Sprague–Dawley rats (embryonic days E14–15) as previously described [39]. Briefly, cells were dissociated from the cerebral cortex and suspended (at 2×10^5 cells/mL) in DMEM/F12 medium supplemented with 2% B27, recombinant human basic fibroblast growth factor (20 ng/mL), and EGF (20 ng/mL). Neurospheres formed during culture were dissociated into single-cell suspensions in the culture medium for subculture. NSCs at 2–5 passages were used in the experiments.

Cell Culture for NSC Differentiation

Cultured NSCs at 2–5 passages were mechanically dissociated into single cells and directly plated onto poly-*L*-lysine-coated coverslips in different culture media. DMEM/F12 supplemented with 2% B27 was used to induce normal differentiation in the dissociated cells. Neurobasal medium supplemented with 2% B27 was applied to induce neuronal differentiation in the dissociated cells, whereas DMEM/F12 with 10% FBS was used to induce astrocytic differentiation.

Western Blot

Cells were harvested in ice-cold RIPA lysis buffer containing protease inhibitor and phosphatase inhibitor cocktail (Sigma, USA). Protein concentrations were determined with a protein assay kit (Bio-Rad, USA). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA). After blocking, the membranes were probed with the appropriate primary antibodies overnight at 4°C, including β-tubulin III (Tuj-1, 1:1000; Covance), glial fibrillary acidic protein (GFAP, 1:1000; Sigma-Aldrich, St. Louis, MO, USA), Notch1 (1:300; Cell signaling, Danvers, MA, USA), Hes1 (1:200; Santa Cruz, CA, USA), notch intracellular domain (NICD, 1:400; Cell Signaling, Danvers, MA, USA), β-actin (1:2000; Sigma-Aldrich, St. Louis, MO, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:2000; Millipore, Burlington, MA, USA). The blots were subjected to HRP-conjugated corresponding secondary antibodies. Protein bands were visualized by adding ECL Advance (GE Healthcare Bio-Sciences, Wauwatosa, WI, USA) according to the manufacturer's instructions. Results were analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

RNAi Treatment and Adenovirus Infection

We applied short-interfering RNA (siRNA) transfection to knock down APPL2 in cells. Commercial APPL2 siRNA or control siRNA (Life Technologies, Waltham, MA, USA) was transfected into NSCs with Lipofectamine RNAiMAX. To maintain the knockdown efficiency, we transfected the differentiated NSCs on days 1 and 4 post-differentiation and harvested them on day 7. To construct adenoviral vectors for overexpression of APPL2, cDNA encoding the APPL2 gene was cloned into the pAdeasy-1 adenoviral backbone vector (Stratagene, Bellingham, WA, USA) as described previously [33, 36]. Adenovirus encoding 3*flag-tagged APPL2 and luciferase control was generated and titered. NSCs were infected with adenovirus at a multiplicity of infection (MOI) of 10 for 2 h, followed by incubation with fresh medium for 7 days. The MOI was assessed with bioluminescence assays.

Animals, Drug Treatment, and Tissue Processing

APPL2 transgenic (Tg) mice were gifts from Prof. Xu Aimin's group. Mice were mated and genotyped. Wild-type C57BL/6 mice at the same age were obtained from the Laboratory Animal Unit at the University of Hong Kong. We measured the level of APPL2 expression in brain tissue and the genotyping protocol was used as in our previous report [38]. The experimental protocol was approved by the institutional Animal Care and Ethics Committee at the University of Hong Kong (CULATR No. 2969-13). Every effort was made to minimize the number of animals used and their suffering. DAPT (20 mg/kg; N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester, GSI-IX; Sigma-Aldrich, St. Louis, MO, USA) was injected i.p. into APPL2 Tg mice daily for 2 weeks. PBS was daily administered i.p. into mice in the vehicle control group. The mouse brain was perfused with 4% paraformaldehyde for fixation, penetrated with 30% sucrose solution, and frozen-sectioned at 20 μm. One out of every 6 consecutive sections (at 100-μm intervals) was selected to avoid repeated counting of cells.

Bromodeoxyuridine (BrdU) Incorporation

To monitor cellular proliferation, we injected BrdU (50 mg/kg per day, i.p.) to mice for 5 days and sacrificed them 2 h after the final injection. To determine newborn neuron maturation and cell survival, we injected BrdU at the same dose and route for 3 days and sacrificed the mice 11 days later. Positive staining with BrdU in sections from the OB and SVZ were identified as actively-proliferating cells.

Immunofluorescence

Cells and frozen brain sections were fixed in 4% paraformaldehyde, blocked in PBS with 5% goat serum and 0.1% Triton X-100, and then incubated with primary antibodies against Tuj1 (1:300; Covance, Beijing, China), glial fibrillary acidic protein (GFAP, 1:500; Millipore, Burlington, MA, USA), microtubule-associated protein 2 (MAP2, 1:500; Millipore, Burlington, MA, USA), APPL1 (1:500; provided by Prof. Aimin Xu), APPL2 (1:500; provided by Prof. Aimin Xu), Doublecortin (DCX, 1:300; Cell Signaling, Danvers, MA, USA), and Notch1 (1:300; Santa Cruz, Santa Cruz, CA, USA) overnight in a cold room and then incubated with the secondary antibodies Alexa 488 or 568 (Life Technologies, Waltham, MA, USA) at room temperature for 2 h. For BrdU staining, tissue was incubated with 2 N HCl for 1 h at room temperature before blocking and primary antibody incubation (BrdU, 1:400; Abcam, Cambridge, UK). DAPI was used to stain nuclei. Ten random fields from each experiment were selected and > 1000 cells were counted.

For *in vitro* tests, cell images were captured with a confocal fluorescence microscope (LSM700, LSM780, Carl Zeiss, Jena, Germany). Tissue images were observed with the 20× objective of a confocal fluorescent microscope (LSM800, Carl Zeiss, Jena, Germany). Each image was obtained projection of Z-stack with range of 20 μm.

The tissue images of whole RMS-OB region were obtained by tiles scanning. dSTORM (direct stochastic optical reconstruction microscopy) images were processed following our previous protocol [37]. Cell counts and fluorescence intensity were recorded with ImageJ. Super-resolution images were obtained by dSTORM microscopy (NBI, Los Angeles, CA, USA) [37]. Molecular distances in dSTORM were obtained with the 'spot profile' model and exported with ImageJ (https://imagej.nih.gov/ij/).

Olfactory Discrimination Test (ODT)

A 60-min locomotion test was used to assess the differences in total distance covered by the APPL2 transgenic and wild-type mice (n = 10 per group). Then, olfactory performance was evaluated by conducting odor discrimination experiments in which 8–12 week-old mice were familiarized with a first odor (habituation odor) in four sessions and exposed once to a novel odor (dishabituation odor). The tests were run during the light phase of the animals' 12-h dark/light cycle. The odor solutions butanol, pentanol, and limonene were diluted 1×10^{-3} in mineral oil and applied to a cotton stick. The odors were presented by inserting the stick into the animal's home cage (habituation) in 4 successive sessions for 50 s per session, separated by 2-min intervals. In the fifth presentation, a different odor was delivered (dishabituation). If a mouse spent more time exploring in the fifth dishabituation session than in the fourth habituation session, the mouse was recognized being able to discriminate a novel from a familiar odor.

Statistical Analysis

Data are presented as the mean ± SEM. For statistical analysis, we used Student's t-test for two independent groups and ANOVA (one-way for single factor and two-way for two factors) followed by Tukey's *post-hoc* test for multiple group analysis. Statistical significance was defined as $P < 0.05$.

Results

Increased APPL2 Expression is Associated with NSC Differentiation *in vitro*

To assess the correlation between APPL proteins and adult neurogenesis, we measured the dynamic changes in the expression levels of APPL1 during NSC differentiation *in vitro* (n = 6 batches of cultures per group). Western blot was used to assess the expression of APPL1 and APPL2 in the cultured NSCs at days 1, 3, 5, and 7. The expression of Tuj1 and GFAP was used as references for neurogenesis and gliogenesis, respectively. We found that the expression of APPL1 was little changed in the process of neurogenesis or glia formation (Fig. 1A–D). We further used immunofluorescence to check the distributions of APPL1 and APPL2 in the cell types. Dual positive staining of APPL1 and Tuj1 was found in the cultured NSCs at day 7 rather than day 1, indicating that APPL1 is mainly present in differentiated cells committed into neural progenitors (Fig. 1E). Interestingly, APPL2 differed from APPL1 during the process of NSC differentiation. Western blot analysis revealed that the expression of APPL2 gradually increased with culture time (Fig. 1A–D). The increased expression of APPL2 coincided with the enhanced levels of Tuj1 and GFAP expression. Importantly, the expression of APPL2 was co-localized with $GFAP^+$ rather than $Tuj1^+$ cells in both the cultured NSCs *in vitro* and the wild-type mice *in vivo* (Fig. 1F, G). Taken together, these results indicated that APPL1 is not associated with the process of neurogenesis whereas APPL2 is a potential modulating factor to promote gliogenesis and affects the cell-fate decision of NSCs.

APPL2 Regulates Cell-Fate Choice During NSC Differentiation

To elucidate the roles of APPL2 in regulating the lineage commitments of NSCs, we manipulated the APPL2 levels in cultured NSCs using adenovirus-mediated overexpression and siRNA-induced knockdown. Western blots showed that the transfection approach successfully resulted in overexpression and knockdown of APPL2 (Fig. 2D, I). The overexpressed APPL2 reduced the percentages of $Tuj1^+$ and $MAP2^+$ neuronal cells but increased the $GFAP^+$ astrocytic cells (Fig. 2A–C, H). To exclude the possibility of artificial effects of APPL2 siRNA on NSC differentiation, we applied two APPL2 siRNAs targeting different domains. Knockdown of APPL2 remarkably increased $Tuj1^+$ and $MAP2^+$ cells along with decreased $GFAP^+$ cells (Fig. 2E–G, J). These results provided evidence supporting the role of APPL2 in regulating the fate choice of NSCs towards the glial direction.

APPL2 Overexpression Suppresses Neurogenesis but Elevates Gliogenesis in the Olfactory Bulb

To further confirm the roles of APPL2 in regulating neurogenesis and gliogenesis in the olfactory system, we conducted an immunofluorescence study of APPL2 Tg mice. The fluorescence intensity of doublecortin (DCX) was lower in the granule cell layer of the OB in the APPL2 Tg mice (Fig. 3A), suggesting a negative role of APPL2 in neuronal differentiation. We then injected BrdU daily for

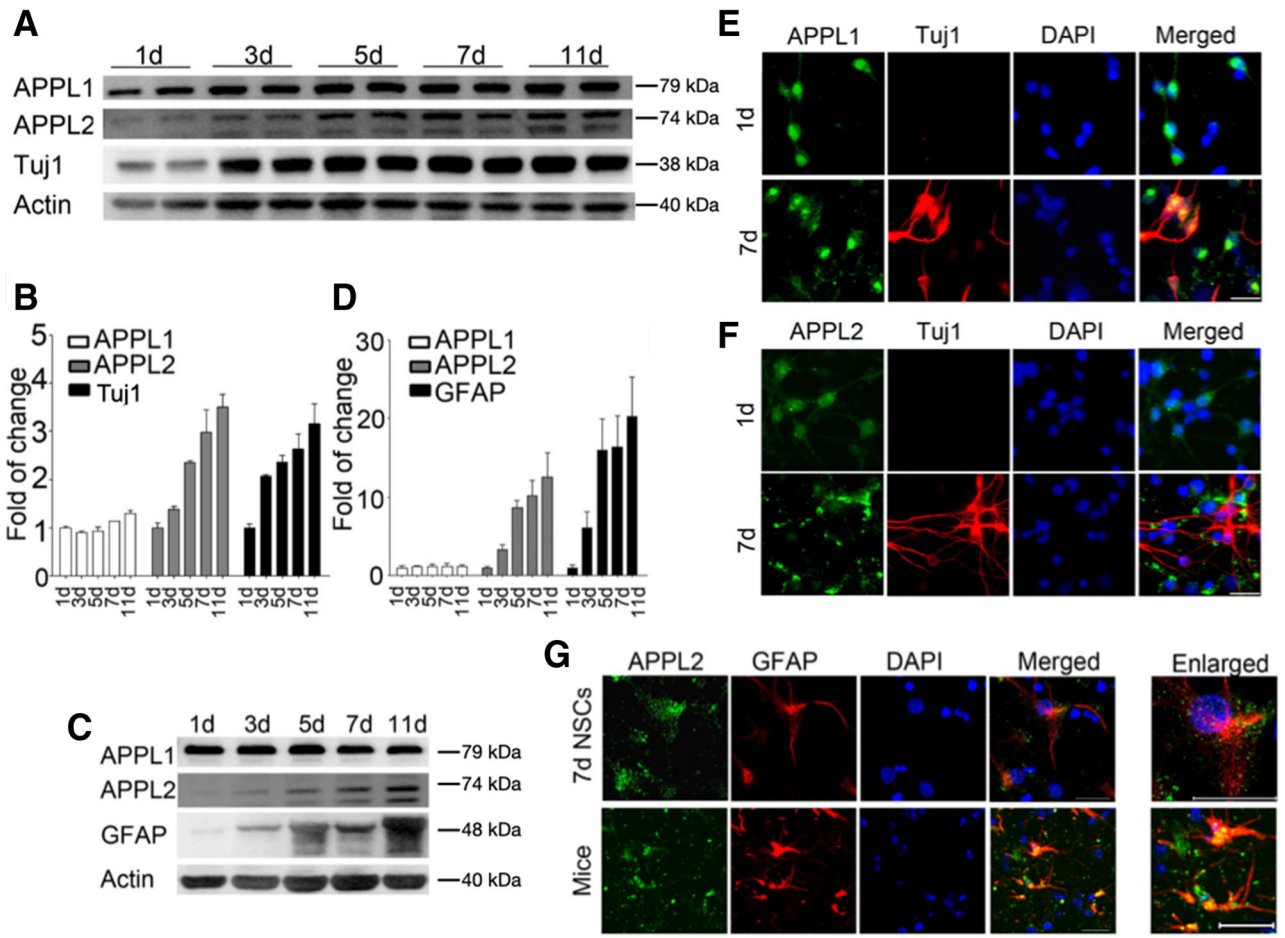

Fig. 1 APPL2 increases during NSC differentiation *in vitro*. **A** Representative western blots for APPL1, APPL2, and Tuj1 when NSCs are cultured for neuronal differentiation. **B** Quantitative analysis of the expression of APPL1, APPL2, and Tuj1. Data are presented as the mean ± SEM, $n = 6$ per group. **C** Western blots of APPL1, APPL2, and GFAP when NSCs are cultured to induce astrocytic differentiation. **D** Quantitative analysis of the expression of APPL1, APPL2 and GFAP. Data are presented as the mean ± SEM, $n = 6$ per group. **E**, **F** Representative immunofluorescence images revealing the co-localization of APPL1 and Tuj1 (**E**) and of APPL2 and Tuj1 (**F**) in cultured NSCs at days 1 and 7 of neuronal differentiation (scale bars, 20 μm). **G** Representative immunofluorescence images showing the expression of APPL2 and GFAP in differentiated NSCs at day 7 and in wild-type C57BL/6 mouse brain tissue (scale bar, 20 μm. $n = 6$ batches of culture per group).

5 days to label actively proliferating cells. The APPL2 Tg mice displayed fewer $BrdU^+/DCX^+$ cells than WT mice, indicating reduced neural differentiation (Fig. 3B). BrdU was then injected daily for 3 days to examine the effects of APPL2 on the survival of newborn neurons, and the mice were sacrificed 11 days later. APPL2 Tg mice showed fewer $BrdU^+/NeuN^+$ cells than WT mice (Fig. 3C). Meanwhile, APPL2 Tg mice had a lower DCX fluorescence intensity and higher GFAP/Iba1 fluorescence in the OB region than WT mice, indicating reduced neurogenesis but enhanced gliogenesis in the APPL2 Tg mice (Fig. 3D). Consistent with this, APPL2 Tg mice showed fewer $BrdU^+/DCX^+$ co-stained cells in the SVZ (Fig. 3G). Western blot analysis also showed the APPL2 Tg mice established the increased expression of astrocytic and microglial markers GFAP and Iba1, indicating the enhanced gliogenesis (Fig. 3E, F). Notably, APPL2 Tg mice had more $GFAP^+$-staining cells around the SVZ, especially in the CC (Fig. 3H). Collectively, these results suggested that APPL2 participates in modulating fate choice to induce gliogenesis but inhibits neurogenesis in the olfactory system.

APPL2 Overexpression Decreases Neurogenesis but Elevates Gliogenesis along the Rostral Migratory Stream

To clarify the effects of APPL2 on the migration of NPCs along the RMS, we injected BrdU daily for 3 days and sacrificed the mice 11 days later. $BrdU^+$-staining cells were labelled in the SVZ and distributed along the RMS (Fig. 4A–C). In APPL2 Tg mice, the $BrdU^+$ staining was

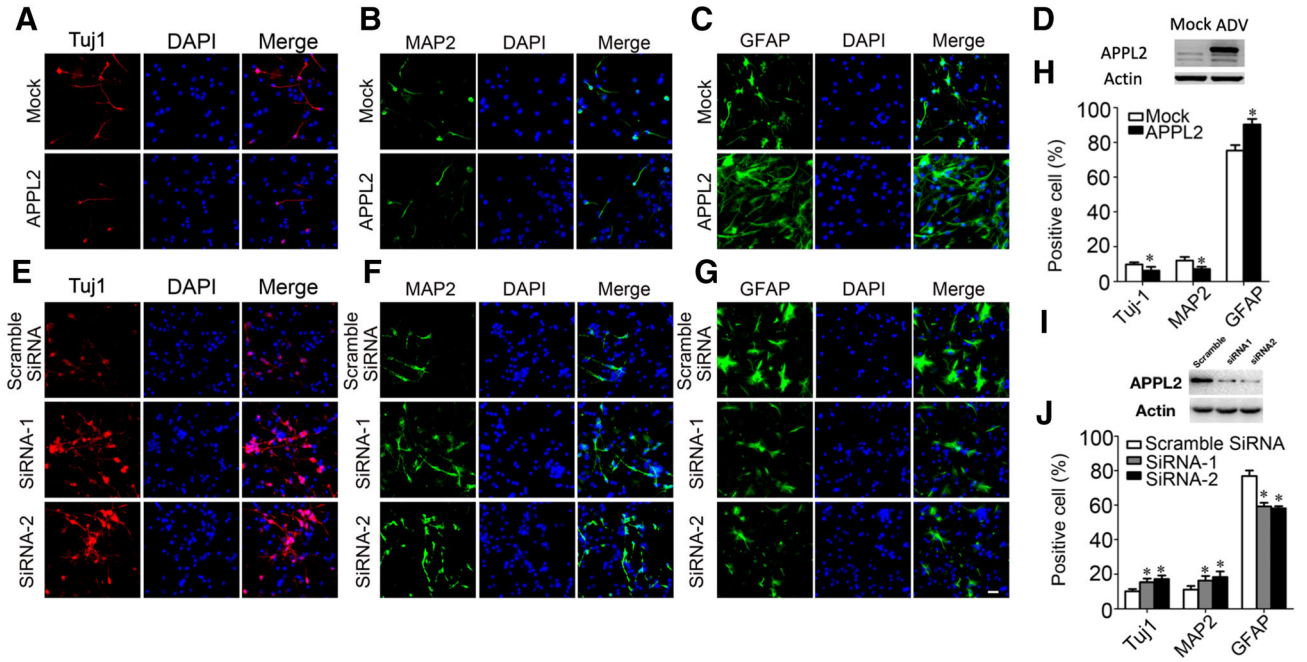

Fig. 2 APPL2 regulates cell-fate decision in NSC differentiation. **A–C** Images of immunofluorescence staining for Tuj1, MAP2, and GFAP in cells treated with adenovirus overexpressing luciferase (Mock) or APPL2 adenovirus. **D, I** Western blots showing the APPL2 levels in adenovirus- and siRNA-induced overexpression or downregulation. **H** Statistics for experiments as in **A–C** (*$P < 0.05$ *vs* Mock). **E–G** Images as in **A–C**, but in cells treated with scrambled siRNA or two APPL2 siRNAs (scale bar, 20 μm). **J** Statistics for experiments as in **E–G** (*$P < 0.05$, *vs* scrambled siRNA; $n = 6$ batches of culture per group).

decreased along the RMS and became sparse at its OB end (Fig. 4A, D). Quantitative analysis revealed a decreased distribution of $BrdU^+/DCX^+$ cells from the SVZ to the anterior RMS in the APPL2 Tg mice (Fig. 4E). We also detected $GFAP^+$ signaling at the CC and RMS regions from the start and end points, respectively. Elevated gliogenesis was found along the RMS in the APPL2 Tg mice (Fig. 4B, E, F). Collectively, these results suggested that the overexpression of APPL2 reduces neuronal survival but promotes gliogenesis along the RMS.

APPL2 Tg Mice Display Impaired Odor Discrimination

The renewal of integrated interneurons is important for maintaining normal olfactory functions. With decreased newborn neurons in the OB of APPL2 Tg mice (Figs. 3, 4), we conjectured that they might have impaired olfactory functions. To test this hypothesis, we compared performance in the ODT in APPL2 Tg mice and WT littermates. When presented with pentanol after 4 sessions of butanol exposure, the WT mice spent much more time sniffing the stick, indicating normal olfactory discrimination (Fig. 5). Interestingly, APPL2 Tg mice showed unchanged time sniffing the stick when butanol was replaced with pentanol, indicating impaired olfactory discrimination (Fig. 5A). The same results were obtained in the limonene + *vs* limonene– tests (Fig. 5B). To exclude the potential influence of preferences for different odors on sniffing behavior, all odors were presented separately for a 2-min session, and the sniffing durations were similar in the APPL2 and WT mice (Fig. 5C, D). These results suggested that APPL2 overexpression remarkably impairs olfactory discrimination.

APPL2 Mediates Neurogenesis/gliogenesis by Interacting with Notch1

The Notch1 signaling pathway is important in regulating the fate choice of NSCs [40]; activated Notch signaling leads NSCs to differentiate to a glial fate commitment [41]. We then addressed the question whether Notch1 signaling contributes to the switching of neurogenesis to gliogenesis in the olfactory system of APPL2 Tg mice. To test this, we investigated the spatial correlation between APPL2 and Notch1 using the dSTORM super-resolution system. The results showed an apparent co-localization of APPL2 and Notch1 in cultured C17.2 NSCs (Fig. 6A, B). In addition, APPL2 Tg mice had significantly increased expression of Notch1 and its downstream NICD and Hes1 in the OB (Fig. 6C). Consistent with this, the knockdown of APPL2 suppressed NICD expression and reduced GFAP expression in the cultured NSCs (Fig. 6D). Suppression of APPL2 subsequently down-regulated the expression of

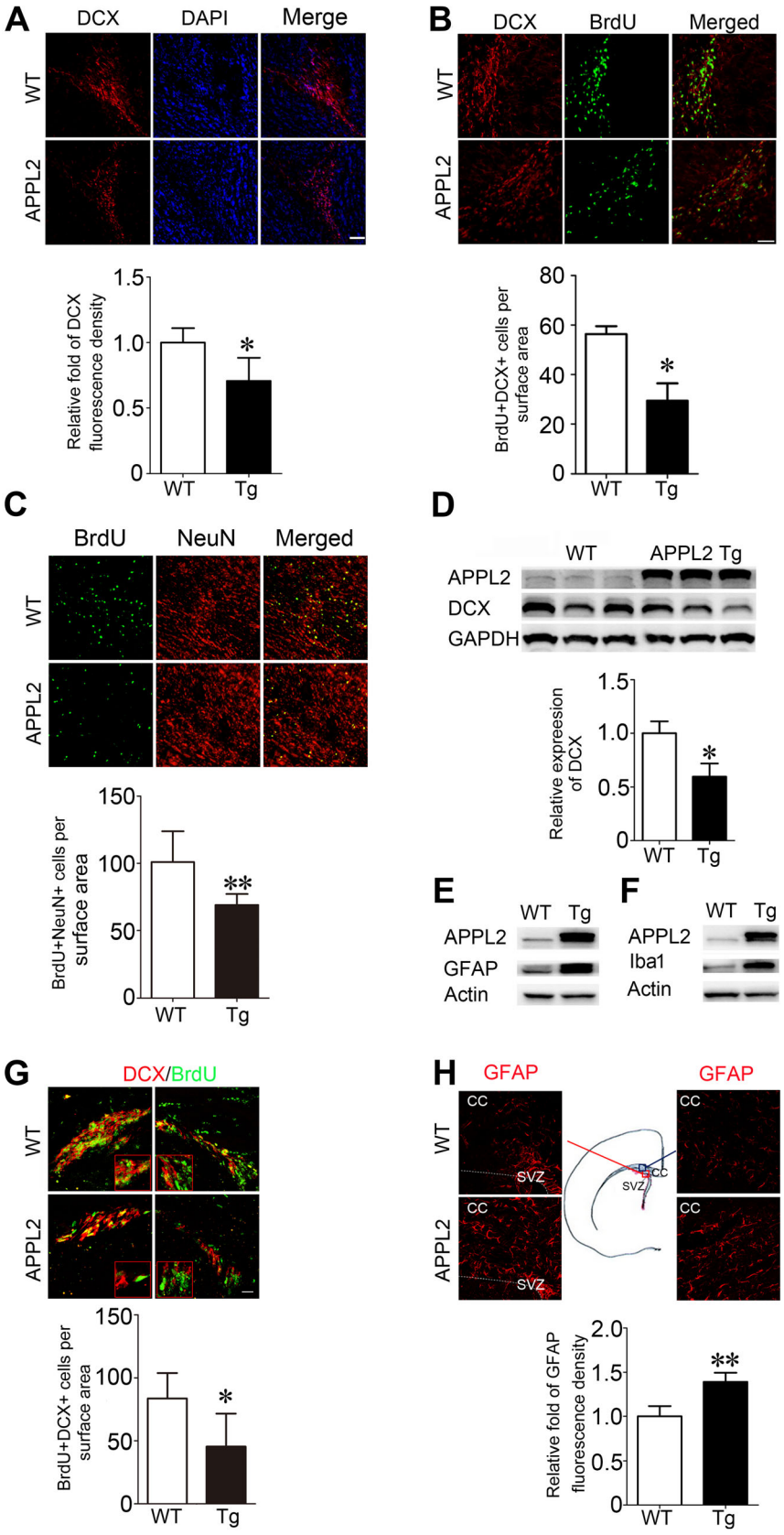

Fig. 3 APPL2 overexpression attenuates neurogenesis in the olfactory bulb. **A, B** Immunofluorescence images and statistics of $DCX^+/DAPI^+$ and $DCX^+/BrdU^+$ identifying newborn neurons in the granule cell layers of the OB in WT and APPL2 Tg mice. DAPI staining identifies nuclei and overall structure (scale bar, 20 μm; $^*P < 0.05$ *vs* WT). **C** Immunofluorescent images and statistics of $BrdU^+/NeuN^+$ identifying mature newborn neurons in the granule cell layers of the OB in WT mice and APPL2 Tg mice (data are presented as the mean ± SEM; $^{**}P < 0.01$ *vs* WT group). **D** Western blots and analysis for protein expression of APPL2 and DCX in the OB of WT and APPL2 Tg mice (data are presented as the mean ± SEM; $^*P < 0.05$ vs WT group). **E, F** Western blots of protein expression of APPL2/GFAP and APPL2/Iba1 in the OB of WT and APPL2 Tg mice. **G** Immunofluorescence images and statistics of $DCX^+/BrdU^+$ identifying immature newborn neurons in the SVZ of WT and APPL2 Tg mice (data are presented as the mean ± SEM; $^*P < 0.05$ *vs* WT group; scale bar, 20 μm). **H** Immunofluorescent images and statistics of $GFAP^+$ staining in the SVZ and CC of WT and APPL2 Tg mice (dashed line, CC border; scale bar, 20 μm; data are presented as the mean ± SEM; $^{**}P < 0.01$ *vs* WT group; $n = 6$ animals per group).

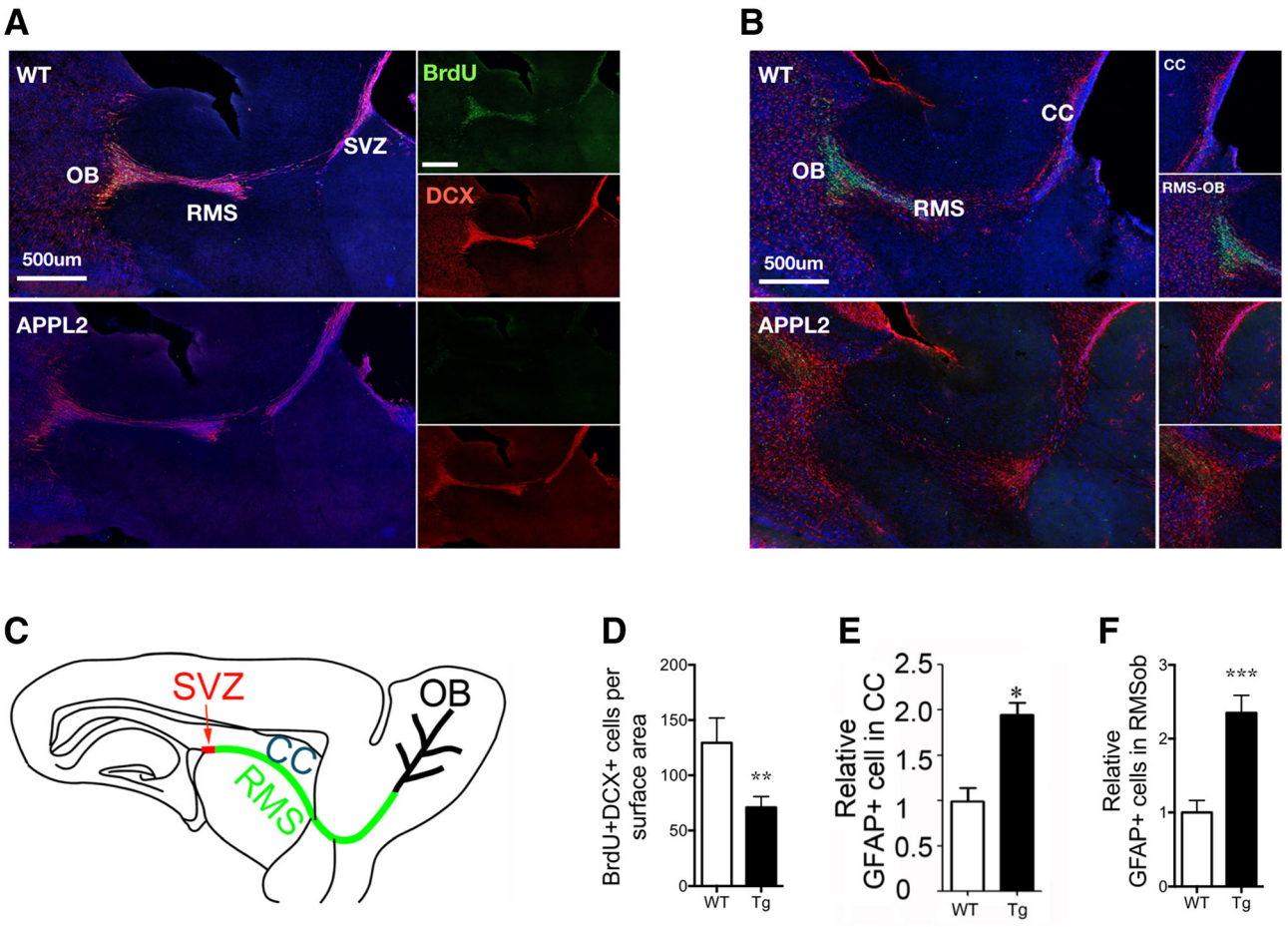

Fig. 4 APPL2 overexpression attenuates survival of progenitor cells in the rostral migratory stream. **A, B** Immunofluorescence images of $DCX^+/BrdU^+$ and $GFAP^+/BrdU^+$ identifying the lineage choice of newborn NSCs in the RMS of WT and APPL2 Tg mice. **C** Diagram of the anterior SVZ–RMS. **D** Cell density of $BrdU^+/DCX^+$ cells in the APPL2 RMS (**$P < 0.01$ *vs* WT group). **E, F** GFAP intensity in the CC and RMS-olfactory bulb (RMSob) regions of WT and APPL2 Tg mice (*$P < 0.05$, ***$P < 0.001$ *vs* WT group; $n = 6$ animals per group).

NICD and Hes1, downstream from Notch (Fig. 6E). To further confirm the causal relationship between APPL2 and Notch signaling, we treated APPL2 Tg mice with the Notch inhibitor DAPT and found that this significantly improved their olfactory discrimination (Fig. 6F). Taken together, Notch1 is a critical cellular signal linking the impaired APPL2-mediated olfactory neurogenesis and olfactory discrimination behavior.

Discussion

The continuous generation of neurons provides structural plasticity in the olfactory system, and olfactory neurogenesis is rigorously modulated by multiple signaling systems [42–44]. Our previous studies showed that APPL2 serves as a negative regulator of adult neurogenesis in the hippocampus and olfactory system, contributing to depressive behaviors [38, 45]. In the current study, we found that APPL2 regulates the cell-fate choice of NSCs with reduced neurogenic and increased glial formation in the OB, leading to impaired olfactory discrimination. By interacting with Notch1, APPL2 mediates the gliogenic switching of olfactory NSCs and olfactory behaviors.

APPL2 is an isoform with 54% identity in sequence and co-localizes with APPL1 [32]. APPL1 participates in metabolic regulation [33–35] and mediates cell survival in vertebrate development [46]. Our previous study revealed that APPL2 is expressed in $nestin^+$ NSCs in the hippocampus [38]. In the current study, we found unchanged APPL1 expression in NSC differentiation but a gradual increase in APPL2 with culture time, accompanied by increased $Tuj1^+$ and $GFAP^+$ populations (Fig. 1). Although APPL2 and Tuj1 simultaneously increased in cultured NSCs (Fig. 1A), only the $GFAP^+$ astrocytic population occurred with $APPL2^+$ staining in both NSCs at day 7 and brain tissue of WT mice (Fig. 1F, G). Given its ubiquitous expression in $GFAP^+$ cells rather than $Tuj1^+$

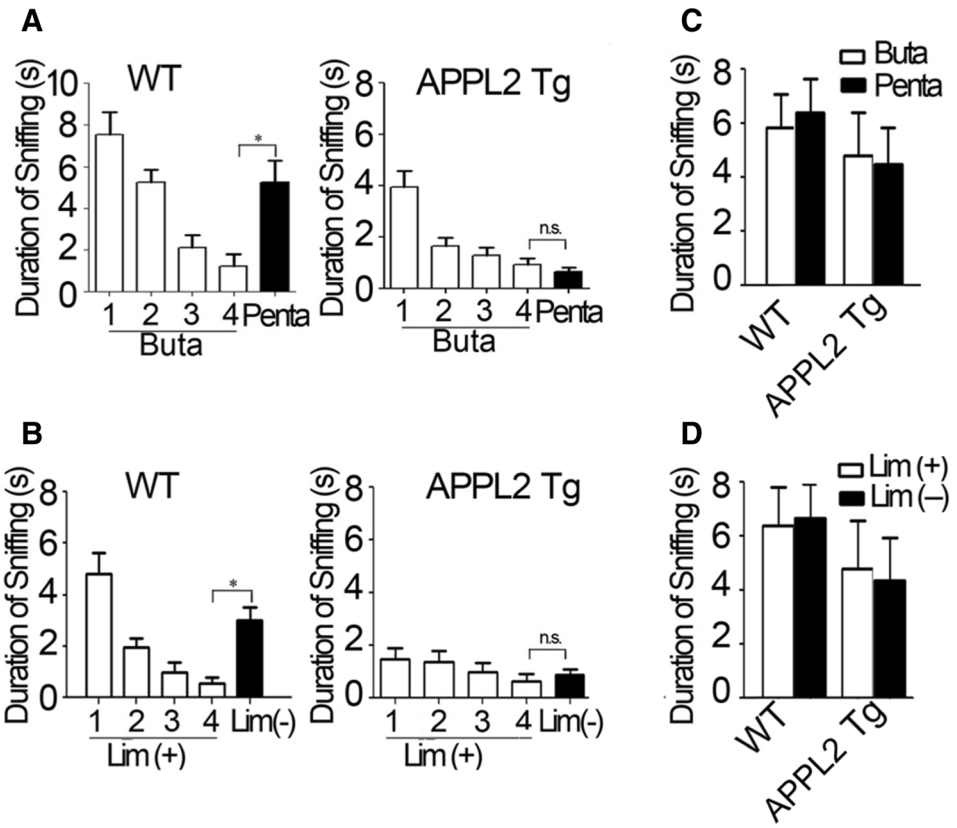

Fig. 5 APPL2 Tg mice are deficient in the discrimination of odor in a cotton stick presentation-based task. **A** Duration of sniffing in 10-week-old mice exposed to 4 sessions of butanol (Buta) followed by pentanol (Penta) (*$P < 0.05$). **B** Duration of sniffing in mice exposed to 4 sessions of limonene [Lim(+)] followed by mineral oil [Lim(–)] (data presented as the mean ± SEM; *$P < 0.05$). **C, D** Durations of sniffing showing no preference for the odors presented in the experiments. $n = 10$ animals per group.

cells, APPL2 may be a player in gliogenesis. The presence of APPL2 in differentiated NSCs or progenitor cells could specifically promote gliogenesis.

We then conducted the siRNA and adenovirus transfer experiments to overexpress and knock down APPL2, respectively, and used WT and APPL Tg mice to further confirm the roles of APPL2 in the modulation of gliogenesis and the cell-fate decision for lineage commitment. In the cultured NSCs, APPL2 overexpression decreased the $Tuj1^+$ and $MAP2^+$ neuronal cells but increased the $GFAP^+$ astrocytes (Fig. 2A–D). In contrast, APPL2 knockdown remarkably increased the $Tuj1^+$ and $MAP2^+$ cells but decreased the $GFAP^+$ cells (Fig. 2E–H). Consistent with this, APPL2 Tg mice had fewer $BrdU^+/DCX^+$ and $BrdU^+/NeuN^+$ cells but more $GFAP^+$ cells in both the OB and SVZ than WT mice. The $BrdU^+/DCX^+$ and $BrdU^+/NeuN^+$ staining marked newly-generated immature and mature neurons, respectively. With these *in vitro* and *in vivo* experiments, we demonstrated that APPL2 functions to modulate fate choice by inducing gliogenesis but inhibiting neurogenesis in the olfactory system (Figs. 3, 4). Similarly, our recent study revealed that APPL2 Tg mice have elevated gliogenesis and suppressed neurogenesis in the hippocampus [38]. Normally, neuronal stem/progenitor cells migrating along the lateral ventricle give rise to different lineages [19, 47]. These NSCs enter into rapid proliferation to generate neuroblasts (type-A cells) that migrate along the RMS towards the OB and differentiate into interneurons [19, 47]. Here, we found that APPL2 Tg mice had decreased neurogenesis in the OB and SVZ (Fig. 3). Along the RMS, overexpressed APPL2 suppressed neuronal generation but elevated astrocyte production (Fig. 4). Individual neuroblasts in the OB migrate radially to the outer layer and differentiate into olfactory interneurons, granule cells, or periglomerular cells that integrate into the olfactory neuronal network [48]. These newly-generated neurons determine the capacity for olfactory discrimination [49]. As expected, the APPL2 Tg mice showed impaired olfactory discrimination under normal conditions (Fig. 5). Our previous study suggested roles of APPL2 in impaired olfactory sensitivity in an animal model of depression [37]. Here, we further addressed how APPL2 regulated olfactory neurogenesis and olfactory functions at the physiological level. Thus, we can draw the conclusion that APPL2 is an important factor in inhibiting olfactory functions and determines olfactory memory and discrimination.

The strength of neurogenesis is orchestrated by a combination of intrinsic genetic programs and environmental niches [47, 50, 51]. The Notch signaling pathway is now widely recognized as a critical mechanism that determines the fate choice of NSCs at an early stage

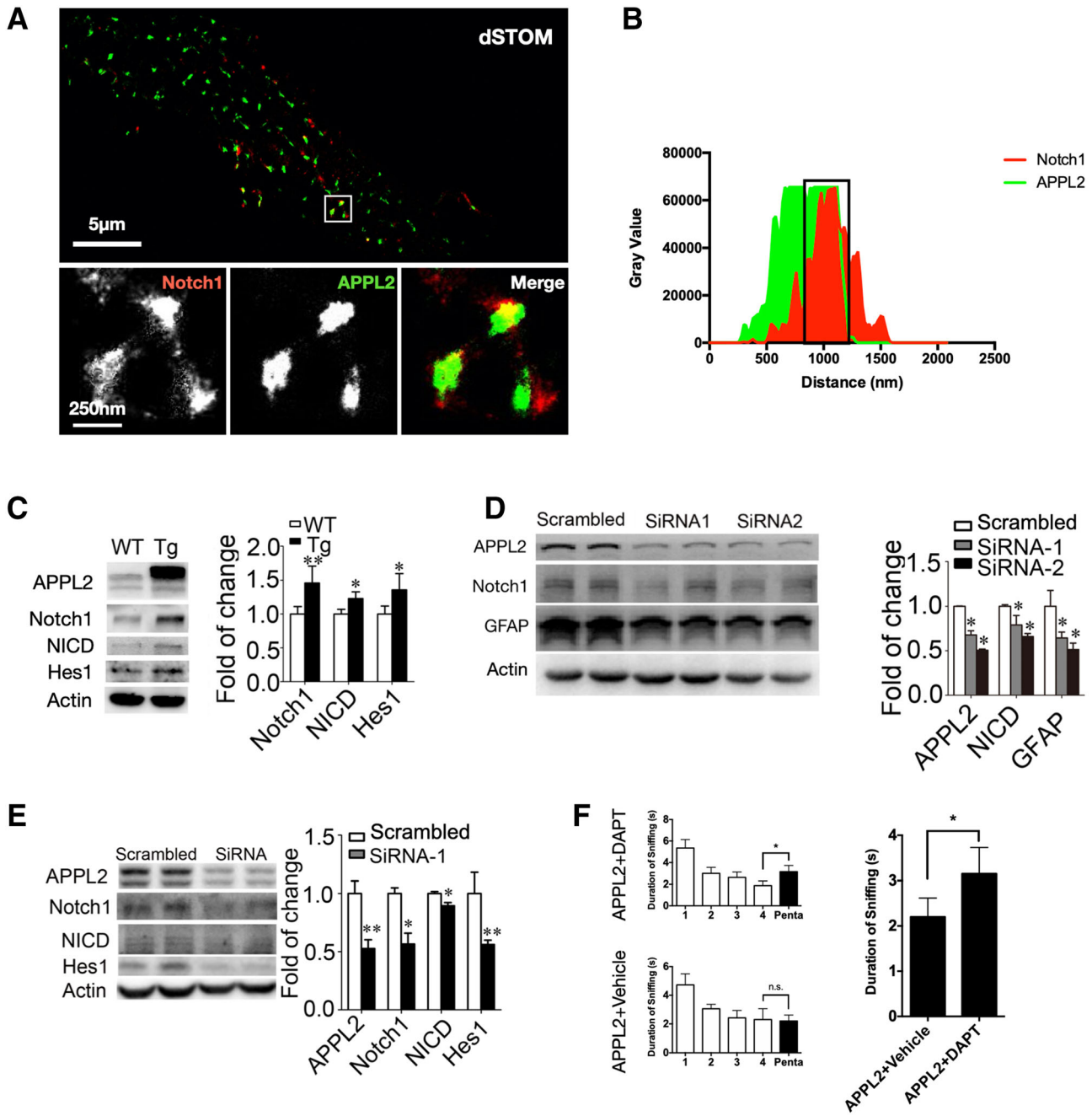

Fig. 6 APPL2 modulates the Notch signaling pathway. **A** Reconstructed dSTORM images showing co-localization of Notch1 and APPL2 in C17.2 cells (100 × oil immersion lens). **B** Spot intensity analysis of the selected region (intensity of the two channels show the same distribution with distance and intensity). **C** Western blots and analysis of targeted proteins Notch1, NICD, and Hes1 in the OB of APPL2 Tg and WT mice (*$P < 0.05$ *vs* WT group). **D** APPL2 siRNA decreases NICD and GFAP expression in primary cultured NSCs (*$P < 0.05$ *vs* scrambled siRNA group). **E** Decreased Notch1, NICD, and Hes1 induced by down-regulated APPL2 (**$P < 0.01$, *$P < 0.05$ *vs* scrambled siRNA group). **F** Effects of Notch inhibitor DAPT on olfactory function in APPL2 Tg mice (*$P < 0.05$ *vs* APPL2 Tg vehicle; $n = 6$ animals or batches of culture per group).

[19, 52–55]. This pathway promotes the switching of NSCs to gliogenic differentiation [56]. Notch1 cKOKln mice display reduced olfactory aversion to propionic acid and Notch1 is involved in olfactory processing and affects olfactory behavior [25]. These lines of evidence indicate critical roles of Notch signaling in regulating olfactory neurogenesis and olfactory behaviors. By regulating downstream Hes1/5, molecules like Tis21 act as essential regulators of olfactory neurogenesis and olfactory behaviors [57]. Consistent with this finding, our study suggests

that APPL2 performs as a regulator of the fate choice of NSCs by inducing gliogenesis but inhibiting neurogenesis *via* interaction with Notch1, subsequently affecting olfactory functions and olfactory discrimination.

In conclusion, we uncover APPL2 as a novel cell lineage regulator for adult neurogenesis. By interacting with Notch1, APPL2 can affect neurogenesis in the adult SVZ-OB system and subsequently regulate olfactory behaviors, and APPL2 may be a new therapeutic target to regulate olfactory behaviors and promote neural regeneration.

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Conflict of interest The authors have no conflict interest.

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