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Among γ-secretase substrates Notch1 alone is sufficient to block neurogenesis but does not confer self-renewal properties to neural stem cells

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ABSTRACT

Notch signaling pathway enhances neural stem cell characters and regulates cell fate decisions during neural development. Interestingly, besides Notch, other γ-secretase substrates such as APP, LRP2, and ErbB4 have also proven to have biological functions in neural development. We designed a unique experimental setting, combining gain-of- (expression of Notch intracellular domain, NICD) and loss-of-function (γ-secretase inhibition) methods, and were able to examine the function of Notch alone by excluding the activity of other γ-secretase substrates. Here, we show that the frequency and size of neurospheres generated from embryonic neural stem cells (NSCs) significantly decreased by 62.7% and 37.2%, respectively, in the presence of γ-secretase inhibitor even when NICD was expressed. Under the condition of differentiation, however, the γ-secretase inhibitor treatment did not influence the promotion of astrogenesis at the expense of neurogenesis by NICD. These results indicate that other γ-secretase substrate(s) along with Notch are important in the maintenance of the stemness of NSCs, but that Notch alone can sufficiently inhibit neurogenesis without the action of the other γ-secretase substrates during differentiation.

1. Introduction

In the mammalian nervous system, the Notch signaling pathway is thought to regulate cell fate specification during neural development [1]. Notch activation also maintains the neural progenitor state and expands the neural progenitor pool while preventing progenitors from differentiating into neurons [2]. Consistently, deletion of the Notch gene leads to precocious expression of early neuronal markers [3].

Most of the Notch-related gain-of-function studies have used the truncated form of the intracellular region of Notch receptors (Notch intracellular domain, NICD) to induce Notch signaling without binding of ligands [1,2,4–6]. Endogenously, NICD is generated from cleavage of the Notch receptor by γ-secretase activity upon ligand binding. NICD then translocates into the nucleus, associated with CBF1 and activates expression of its target genes [7].

γ-Secretase complex consists of presenilin (PS), nicastrin (NCT), anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN-2) [8]. The substrates of this enzyme that have so far been identified are amyloid precursor protein (APP), low-density lipoprotein receptor-related protein (LRP)-2, E-cadherin, and ErbB-4 in addition to the Notch family genes. Interestingly, besides the ICD of Notch, the ICDs of APP, LRP2, and ErbB4 are also known to have biological functions in neural development by exerting influence on neurogenesis and astrogenesis [9–12].

The experimental method that has been commonly used to investigate the effect of Notch on progenitor cells including neural and hematopoietic stem cells, as well as cancer cells in a loss-of-function manner is the use of γ-secretase inhibitors (e.g., *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester, DAPT). However, as treatment with the γ-secretase inhibitor hampers the processing not only of Notch but also of other γ-secretase substrates, it cannot be concluded that Notch solely exerts influence on these cells. In this study, NICD in its fully processed form was expressed in the presence of γ-secretase inhibitors to investigate the function only of Notch among the γ-secretase substrates (Fig. 1A). In this unique experimental setting, combination of gain-of- (forced expression of constitutively active form of Notch) and loss-of-function (γ-secretase inhibitor treatment) methods, it was possible to exclude the activity of other γ-secretase substrates. Here, we show that the frequency of generation and size of neurospheres considerably decreased in the presence of γ-secretase

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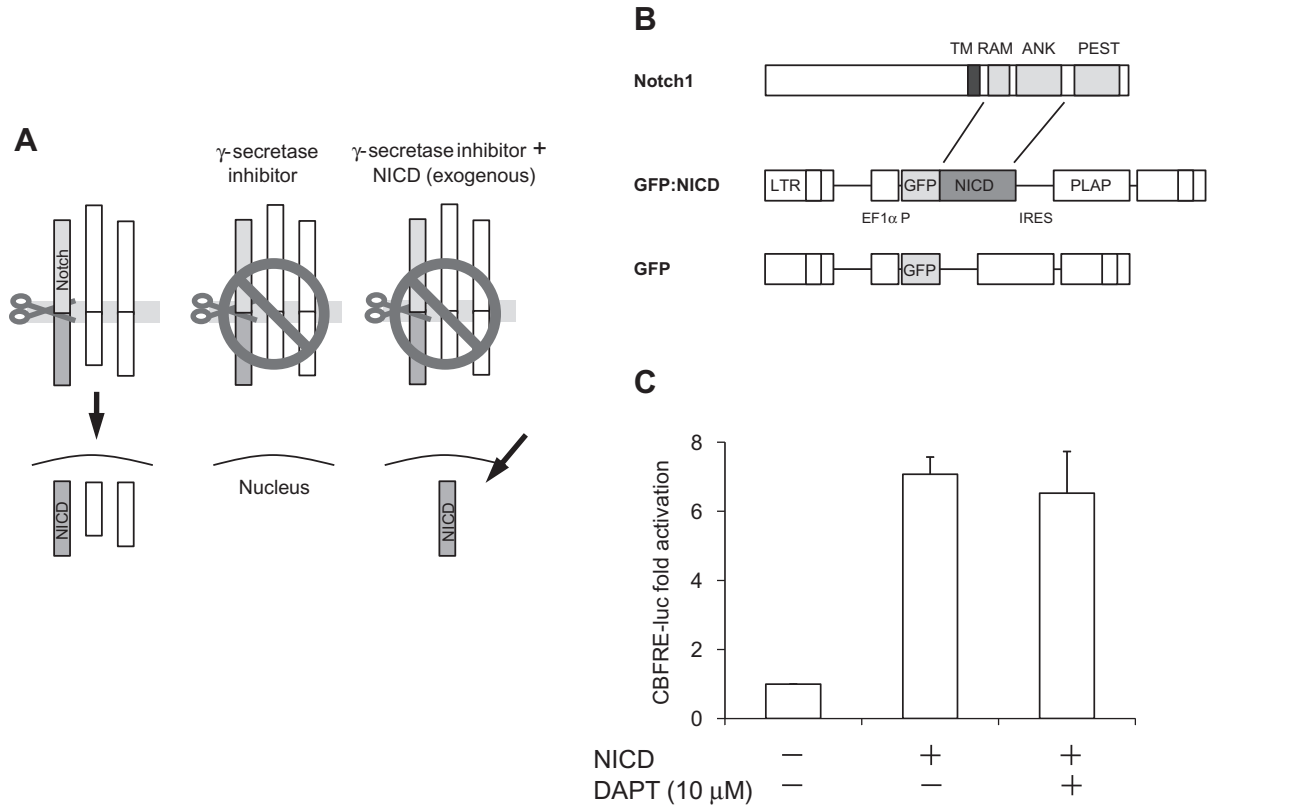

Fig. 1. Experimental settings used in this study. (A) Forced expression of NICD in the presence of γ-secretase inhibitor would make it possible to exclude activities of other γ-secretase substrates and determine the effect of Notch alone. (B) Retroviral vectors expressing NICD as a form of GFP fusion protein or GFP alone. NICD from Notch1 lacking PEST domain fused to GFP at the C-terminus. (C) CBFRE-luc construct designed to express firefly luciferase in response to Notch activity was transfected into NIH3T3 cells together with NICD in the presence or absence of DAPT. No inhibitory effect of DAPT on reporter gene expression by NICD was observed. TM, transmembrane domain; RAM, ram domain; ANK, ankyrin repeat; PEST, PEST domain; LTR, long terminal repeat; EF1α P, Xenopus EF1α enhancer-promoter; IRES, internal ribosome entry site; PLAP, placenta alkaline phosphatase. Error bars represent SD.

inhibitors even when NICD was expressed. During differentiation, however, the promotion of astrogenesis at the expense of neurogenesis by NICD was not influenced by the γ-secretase inhibitor. These results suggest that other γ-secretase substrate(s) along with Notch are important in the maintenance of the stemness of NSCs, but that Notch alone can sufficiently inhibit neurogenesis without the action of the other γ-secretase substrates during differentiation.

2. Materials and methods

2.1. Animals and neural progenitor cell preparation

Female mice (CD1; 8 week) were purchased from Orient Bio Inc. (Osan, Korea) and maintained in specific pathogen-free condition before sacrifice. All animal protocols were approved by the Institutional Review Board and conducted in the Laboratory Animal Research Center of Sungkyunkwan University. Neurosphere cultures were prepared from the lateral and medial ganglionic eminences of embryonic day (E) 14.5 embryos (the day of vaginal plug was considered E0.5.). Dissected brain tissue was minced, washed three times with PBS, and incubated in 0.25% trypsin (Invitrogen) at 37 °C for 5 min. DNase and ovomucoid trypsin inhibitor (both from Worthington, Freehold, NJ) were added, and samples were triturated using a fire-polished Pasteur pipette and washed in PBS.

2.2. Retroviral vectors

The method of retroviral vector production has been described previously [13]. Briefly, retroviral constructs were transfected to 293T cells by a three-plasmid transfection method [14]. Cells were transfected at roughly 90% confluence using Lipofectamine 2000 (Invitrogen) in 15-cm dishes. The supernatant of transfectant was collected and stored at −80 °C roughly 36, 48 and 60 h after the start of transfection. For concentration, the harvests were pooled, filtered through a 0.45-μm filter, and pelleted for 1.5 h in an SW28 rotor at 25,000 rpm at 4 °C. Pellets were dissolved in PBS and aliquots of virus were stored at −80 °C.

2.3. Luciferase assay

Reporter plasmid containing the firefly luciferase gene driven by a CBF1-responsive element [15] was introduced into NIH3T3 cells together with GFP or NICD expression vector. pRL-TK plasmid was used to control transfection efficiency. Ten micromolar DAPT was added to culture medium after transfection. Forty-eight hours after transfection, the cells were lysed with lysis buffer (Promega) and incubated for 15 min at room temperature. Luciferase activities were measured with a Dual-Luciferase reporter assay system (Promega) and a luminometer (model 2020-000; Turner Designs, Sunnyvale, CA), according to the manufacturer's protocol.

2.4. Retroviral transduction and neurosphere assay

The concentrated retroviral vector supplemented with polybrene (8 μg/ml) was added to the dissociated neural progenitor cells and incubated for 1 h in a 37 °C, 5% CO_2 incubator. Twenty-four hours after the start of infection, cells expressing NICD together with GFP were isolated using a FACS Calibur (Becton Dickinson, San Jose, CA). Sorted cells were transferred to 6-well dishes and

cultured at clonal density (2000–3000 cells/well) in Dulbecco's modified Eagle's medium (DMEM, Invitrogen)/F12 with serum-free supplements B-27 and N2 (both from Invitrogen, San Diego, CA), 2 mM glutamine, and 20 ng/ml fibroblast growth factor 2 (FGF2, Peprotech Rocky Hill, NJ) in the presence or absence of DAPT (Sigma). Frequency and size of neurospheres were scored after 1 week. Fluorescent images were obtained with an inverted microscope (Nikon Eclipse Ti, Tokyo, Japan) equipped with a GFP filter set.

2.5. In vitro differentiation and triple immunostaining

Murine immortalized neural stem cell line C17.2 was maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 5% horse serum (Invitrogen) and 2 mM glutamine (Invitrogen) in a humidified 5% CO_2 incubator at 37 °C. For differentiation, the medium was replaced with DMEM/2% FBS and cultured in the same medium for another 7 days. For immunostaining, differentiated cells were fixed and processed for immunostaining as described earlier [1]. As primary antibodies, we used rabbit antibody to GFP (Molecular Probes), mouse antibody to βIII-tubulin (TuJ1, Covance) and rat antibody to GFAP (Invitrogen). Alexa-405, 488 and -555 conjugated secondary antibodies (Invitrogen) were used for triple fluorescence. Antibodies were diluted in PBS plus 2% normal donkey serum plus 0.2% Triton X-100.

3. Results

3.1. Inhibition of γ-secretase does not block NICD transactivation of its target gene

We designed an experimental setting by which the sole effect of NICD, not of the ICDs of the other γ-secretase substrates, could be investigated. First, we tested whether DAPT could alter the level of Notch signaling induced by forced expression of the fully processed form of Notch, NICD. The NICD that was used in the study was derived from Notch1 and about 45% of its C-terminal region including the PEST domain was removed (Fig. 1B). This truncated form of NICD showed constitutive signaling activity; it effectively promoted the radial glial identity, and increased the frequency of the neurosphere formation [1,16]. To efficiently visualize the cells to which the NICD gene was transferred, GFP was fused to NICD. This GFP-NICD fusion or GFP alone was expressed by a retroviral vector. The luciferase reporter construct driven by a CBF1-responsive element (CBFRE, four CBF1-binding sites and the basal SV40 promoter) [15] was introduced into NIH3T3 cells together with or without the NICD expression vector. As expected, NICD significantly increased the expression of the reporter gene and this activation was not influenced by DAPT (Fig 1C). This proof-of-principle experiment indicates that activation of Notch target genes by exogenously expressed constitutively active form of Notch, NICD, would not be altered by inhibition of γ-secretase and this condition would help us to study the effect of Notch alone among γ-secretase substrates.

3.2. NICD does not confer resistance to a γ-secretase inhibitor during neurosphere formation

To determine whether Notch alone is sufficient to increase neural stem cell (NSC) character among γ-secretase substrates, we performed neurosphere assay. The formation of neurosphere has been used to measure the self-renewing activity of NSC quantitatively [17–19]. Embryonic day (E) 14.5 ventral telencephalic progenitors were infected with NICD virus and plated into serum-free stem cell media including FGF2 and/or DAPT. The DAPT concentration that was used in this experiment was 10 μM, a concentration widely used in most studies.

Surprisingly, the frequency of formation and size of neurospheres in which NICD was expressed also considerably decreased in the presence of DAPT, as in the case of the control GFP-neurospheres (Fig. 2A–D). In the presence of 10 μM DAPT, the frequency of formation of NICD-neurospheres decreased by 62.7%, whereas that of the GFP control neurospheres was reduced by 82% (Fig. 2B). In addition, the hampered formation of neurospheres was also very remarkable in the presence of 1 μM, one-tenth of the working concentration, of DAPT. The NICD-neurospheres decreased by 51.1%, and the GFP-neurospheres decreased by 80.9%. Relatively less severe reduction in NICD-neurospheres is considered attributable to the expression of NICD. These results suggest that inhibition of γ-secretase by DAPT, even at a low concentration, can very effectively hamper the formation of neurospheres even expressing NICD.

The size of neurospheres also differed greatly. Under the concentration of 1 μM, the size of the NICD-neurospheres decreased by 37.6%, and that of the GFP control neurospheres decreased by 50.4% (Fig. 2C). Besides the average size, neurospheres larger than 150 μm in diameter were not observed, regardless of the presence of NICD, when treated with DAPT (Fig. 2D). Neurospheres ranging from 100 to 150 μm were also very rare in the presence of DAPT even in the NICD-neurospheres. These results imply that the expression of NICD cannot effectively rescue the inhibitory effect of DAPT on NSCs, and that other substrates of the γ-secretase besides NICD might be involved.

3.3. L685,458 shows a similar level of inhibitory effect on NICD-neurosphere formation to DAPT

Although the inhibition on neurosphere generation was very obvious even with 1/10 of the commonly used concentration, we could not completely exclude the possibility that cells were influenced by the unexpected effects of DAPT other than γ-secretase inhibition. To answer this question, the same experiment was conducted using L-685,458, a γ-secretase inhibitor that is used as often as DAPT is. L-685,458 contains a hydroxyethylene dipeptide isostere, which may function as an aspartyl protease transition state mimic [20]. DAPT is known to be distinct from such transition-state analogues; its binding site is in the presenilin C-terminal fragment that is distinct from the catalytic site or the substrate binding site [21]. The working concentration of L-685,458 is 1 μM. At this concentration, the result of L-685,458 treatment was almost the same as that of DAPT treatment; the formation of neurospheres decreased 59.6% even in NICD-neurospheres, consistent with the results when treated with DAPT (60.6%) (Fig. 3). Moreover, at the concentration of 0.1 μM, 1/10 of the working concentration, it also strongly exerted negative influence on the formation of neurospheres.

3.4. Inhibition of γ-secretase does not block astrogenesis induced by NICD

Next we tested the impact of DAPT on another major function of NICD, regulation of neurogenesis and astrogenesis during differentiation of NSCs. We induced C17.2 cells, an immortalized mouse neural stem cell line derived from neonatal cerebellum [22], to differentiate by exposing them to DMEM/2% FBS media before performing triple-labeling studies. Under this condition, most of the control C17.2 cells gave rise to βIII-tubulin$^+$ neurons (Fig. 4B) and the addition of DAPT in this culture appeared to further increase the amount of beta III tubulin protein (Fig. 4F). Consistent with many previous reports, cells expressing NICD generated mostly astrocytes [positive for glial fibrillary acidic protein (GFAP)]

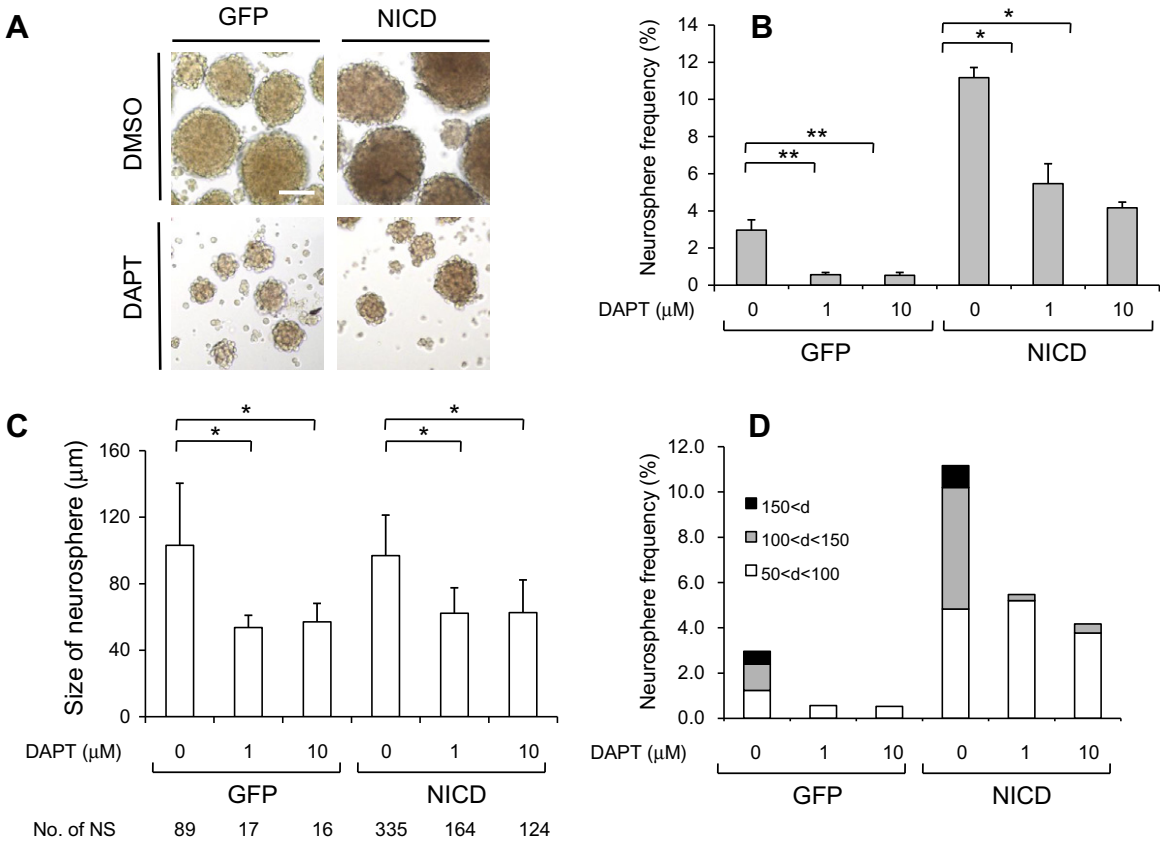

Fig. 2. Treatment with a γ-secretase inhibitor leads to decreased frequency as well as size of neurospheres regardless of NICD expression. (A) Example images of neurospheres. These images do not reflect frequency of neurosphere formation. Cells from E14.5 ganglionic eminence were infected with NICD virus. GFP$^+$ cells were isolated by FACS the next day and cultured with or without DAPT. Frequency (B) and size (C) of neurospheres were measured 1 week later. (D) NICD-neurospheres larger than 100 μm significantly reduced even in 1 mM of DAPT. In all cases $n \geqslant 3$ independent populations scored. Error bars show SD $^*P < 0.001$, $^{**}P < 0.002$. Scale bar, 100 μm.

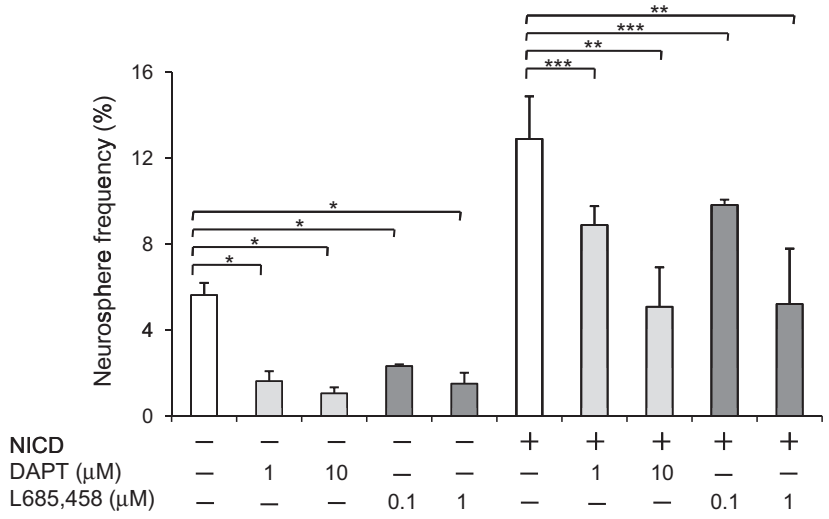

Fig. 3. Inhibitory effect on the formation of neurosphere was not due to unexpected effects of DAPT other than γ-secretase inhibition. L-685,458, inhibiting γ-secretase with a different mechanism from DAPT, caused negative effects on neural stem cells. It greatly impacted on the formation of NICD-neurospheres even at 1/10 of working concentration and showed very similar level of inhibition to that of DAPT. All error bars represent SD $^*P < 0.001$, $^{**}P < 0.02$, $^{***}P < 0.05$; $n = 3$.

(Fig. 4K) at the expense of neurogenesis (Fig. 4J). However, treatment with DAPT had no effect on the increased astrogenesis induced by NICD (Fig. 4O). Taken together, these findings suggest that for the maintenance of the stemness of NSCs, other products processed by γ-secretase are required in addition to NICD, whereas the functions that block the differentiation into neurons and promote astrogenesis are solely performed by NICD among γ-secretase substrate genes.

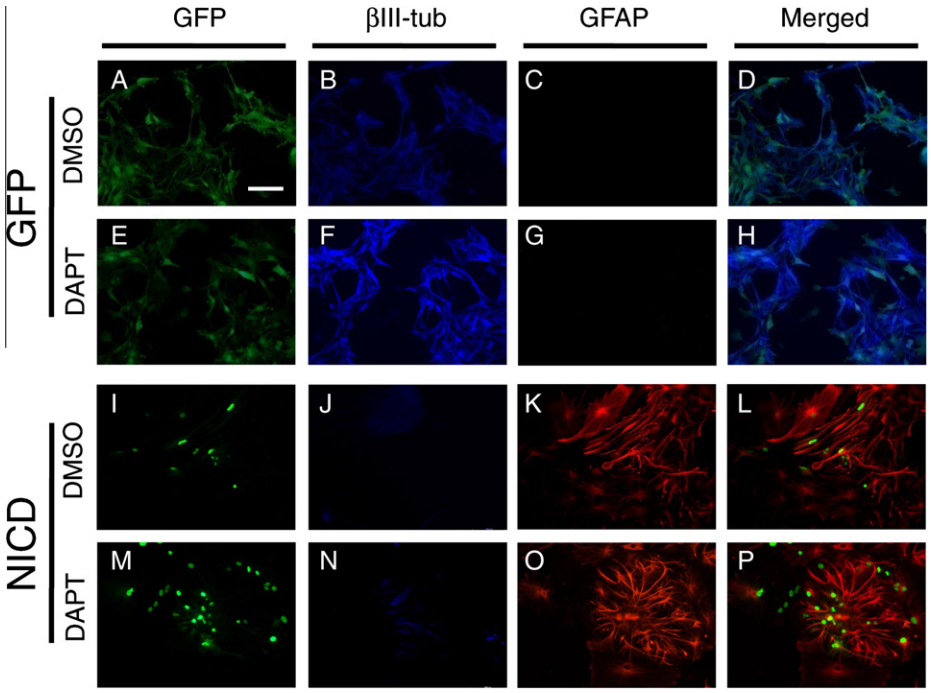

Fig. 4. The ability of NICD to promote astrogliogenesis at the expense of neurogenesis was not affected by DAPT. Representative images show GFP (green), βIII-tubulin (neuron, blue) and GFAP (astrocyte, red)-triple stainings after in vitro differentiation of the C17.2 murine neural stem cell line. GFP expression in the cells infected with NICD virus was detected in the nucleus due to nuclear localization signals in NICD (I, L, M, P). Most NICD$^+$ cells generated GFAP$^+$ astrocytes (K) while control cells produced βIII-tubulin$^+$ neurons (B). These functions of NICD during differentiation of neural stem cells were not compromised by DAPT (O). Scale bar, 100 μm.

4. Discussion

There are two major effects of Notch on NSCs; the promotion of NSC character (e.g., self-renewal capability) and the inhibition of neurogenesis/concurrent promotion of astrogenesis. Various genes, including Notch as well as Shh and Wnt, are known to be involved in the maintenance of NSCs. Among the γ-secretase substrates, however, only Notch has been known to be important in this function and extensive studies are under way. In this study, attempts were made to quantify the contribution of Notch to the maintenance of NSC character. It was expected that the γ-secretase inhibitors would not influence NICD-transferred NSCs because NICD itself was in a fully processed form by γ-secretase.

Despite the presence of NICD, the frequency of formation and size of neurospheres were greatly influenced by DAPT. These observations suggest that the stemness of the neural progenitor cells cannot be maintained by Notch alone and the substrates of other γ-secretase are required. There are three facts that indicate that the above-mentioned results were not caused by the unexpected side effects of DAPT that had nothing to do with the γ-secretase inhibition. First, at 1/10 of the commonly used concentration, NICD-neurosphere formation was significantly influenced, indicating that it was not due to an adverse toxic effect of DAPT. Second, inhibitory effects of DAPT at 1 and 10 μM concentrations were very similar. If DAPT was toxic, reduction in the number of NICD-neurospheres at 10 μM must have been far greater than that at 1 μM. Third, L-685,458, a γ-secretase inhibitor with a different mechanism of inhibiting this enzyme, resulted in the very similar level of reduction in neurosphere formation to DAPT. If it were due to the effect of only the DAPT chemical rather than of γ-secretase inhibition, a different level of reduction would have been obtained.

The inhibition of the differentiation of NSCs into neurons and promotion into astrocytes, were not influenced by DAPT. This means that the substrate(s) of other γ-secretase were not involved in the regulation of differentiation of NSCs by Notch but were specifically required in the self-renewal of NSCs.

It would be interesting to examine which γ-secretase substrate other than Notch would be responsible for the self-renewal property of NSCs that accounts for the remaining 60%, which Notch cannot rescue. The first candidate gene would be that among other Notch family members. However, if we consider the results of the following studies, it is not easy to speculate which Notch receptor would be a good candidate. Notch3 or Notch4 knockout mice do not have significant defect [23,24] and Notch3 is functionally redundant with Notch1 in the developing telencephalon [25]. Although deletion of Notch2 causes early embryonic lethality like Notch1, it does not show alterations in somitogenesis and Hes5 expression in the central nervous system [26]. However, if neurosphere formation is promoted additionally through the coexpression of ICDs of Notch1 and another Notch family member in the presence of the γ-secretase inhibitor, it would mean that some of the Notch family genes play a distinct role in which Notch1 cannot promote the self-renewal of NSCs. It has so far not been possible to answer this question via conventional assay.

We used the truncated but constitutively active form of NICD1 containing the deletion in the C-terminal region. Therefore, we cannot exclude the possibility that the unidentified C-terminal domain which is not relevant to transactivation of Notch's target genes might be important for enhancing the stemness of NSCs to the maximum.

One of the most well-known proteins among γ-secretase substrates other than Notch is the amyloid precursor protein (APP) whose cleavage product, Aβ, is a major causative agent responsible for Alzheimer's disease. The role of APP intracellular domain (AICD) in the central nervous system has been known to negatively modulate neurogenesis [10,11]. However, it is not yet clear whether the inhibition of neurogenesis can be directly related with the maintenance and proliferation of NSCs. Low-density lipoprotein receptor-related protein (LRP)-2 also needs to be considered.

LRP-2 knock-out results in severe forebrain abnormalities [27] and impaired proliferation of neural precursor cells in the subependymal zone in adult mice [9]. However, its role in the self-renewal of NSCs was not elucidated. E-cadherin is also relevant to the regulation of NSCs. This gene is expressed in the adult and embryonic forebrain germinal zones [28] and conditional deletion showed defects in the self-renewal of NSCs. However, generation of its ICD did not appear to be responsible for these self-renewal effects because a mutated E-cadherin that lacks the intracellular region could also increase number of NSC colonies. Cytoplasmic domain of ErbB4 is also cleaved by presinilin/γ-secretase, releasing its ICD into the cytosol [29]. So far, the ICD of ErbB4 has only been shown to regulate the timing of astrogenesis by binding promoters of astrocytic genes and repressing their expression [12].

In summary, the findings presented here suggest that the level of contribution of Notch to its two well-defined functions, regulation of self-renewal property and cell fate choice of NSCs are very different; Notch alone can induce astrogliogenesis at the expense of neurogenesis but co-existence with another γ-secretase substrate is required for stemness of neural stem cells. Further studies are required to search for genes that minimize the negative effect of the γ-secretase inhibitor on the self-renewal and proliferation of NSCs when co-expressed with the ICD of Notch1. Our observations also have important implications for stem cell manipulation techniques such as expansion and differentiation into a correct cell type of interests.

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