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The Alzheimer-related gene presenilin 1 facilitates notch 1 in primary mammalian neurons

O. Berezovska [a], M. Frosch [b,c], P. McLean [a], R. Knowles [a], E. Koo [d], D. Kang [d], J. Shen [c], F.M. Lu [e], S.E. Lux [e], S. Tonegawa [f], B.T. Hyman [a,*]

[a] *Alzheimer Research Unit, Massachusetts General Hospital, Neurology Service, Rm. 6405, 149 13th Street, Charlestown, MA 02129, USA*
[b] *Division of Neuropathology, Brigham and Women's Hospital, Boston, MA 02115, USA*
[c] *Center for Neurological Diseases, Brigham and Women's Hospital, Boston, MA 02115, USA*
[d] *Department of Neuroscience, University of California, San Diego, La Jolla, CA 92093, USA*
[e] *Division of Hematology / Oncology, Children's Hospital, Boston, MA 02115, USA*
[f] *Department of Biology, Howard Hughes Medical Institute, Center for Learning and Memory, Center for Cancer Research, Massachusetts Institute of Technology, Boston, MA 02139, USA*

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Abstract

The normal functional neurobiology of the Alzheimer's disease (AD) related gene presenilin 1 (PS1) is unknown. One clue comes from a genetic screen of *Caenorhabditis elegans*, which reveals that the presenilin homologue sel-12 facilitates lin-12 function [D. Levitan, I. Greenwald, Facilitation of lin-12-mediated signalling by sel-12, a *Caenorhabditis elegans* S182 Alzheimer's disease gene, Nature 377 (1995) 351–355]. The mammalian homologue of lin-12, Notch1, is a transmembrane receptor that plays an important role in cell fate decisions during development, including neurogenesis, but does not have a known function in fully differentiated cells. To better understand the potential role of Notch1 in mammalian postmitotic neurons and to test the hypothesis that Notch and PS 1 interact, we studied the effect of Notch1 transfection on neurite outgrowth in primary cultures of hippocampal/cortical neurons. We demonstrate that Notch1 inhibits neurite extension, and thus has a function in postmitotic mature neurons in the mammalian CNS. Furthermore, we present evidence demonstrating that there is a functional interaction between PS1 and Notch1 in mammalian neurons, analogous to the sel-12/lin-12 interaction in vulval development in *C. elegans* [D. Levitan, T. Doyle, D. Brousseau, M. Lee, G. Thinakaran, H. Slunt, S. Sisodia, I. Greenwald, Assessment of normal and mutant human presenilin function in Caenorhabditis elegans, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 14940–14944; D. Levitan, I. Greenwald, Effect of Sel-12 presenilin on Lin-12 localization and function in *Caenorhabditis elegans*, Development, 125 (1998) 3599–3606]. The inhibitory effect of Notch1 on neurite outgrowth is markedly attenuated in neurons from PS1 knockout mice, and enhanced in neurons from transgenic mice overexpressing wild type PS1, but not mutant PS1. These data suggest that PS1 facilitates Notch1 function in mammalian neurons, and support the hypothesis that a functional interaction exists between PS1 and Notch1 in postmitotic mammalian neurons. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Presenilin 1; Notch1; PS1 knockout mice; PS1 transgenics; Primary neurons

1. Introduction

Mutations in presenilins (PS1 and PS2) cause an aggressive form of early onset AD. The normal physiological function of presenilins or mechanism(s) by which the mutations in PS can cause such severe neuropathology is unknown. Mice lacking PS1 have been recently generated [31,36]. The null mutation of the PS1 gene causes significant skeletal defects, brain hemorrhages and impaired neurogenesis, that leads to embryonic or early postnatal lethality. Interestingly, the phenotype of PS1 knockout mice resembles that of Notch1 knockout mice [33]. In addition, the expression of Notch1 and Delta-like mRNA is diminished in the paraxial mesoderm of PS1 knockout embryos [36].

Initial data suggesting a possible relationship between PS1 and Notch derives from a genetic screen for lin-12/Notch interactors in Caenorhabditis elegans, which

Abbreviations: N1(FL), Full length Notch1; N1(IC) and N1(ΔEC), Truncated Notch1; PS1, presenilin 1; WT PS1, Wild type presenilin 1; M146L PS1, Mutated presenilin 1

* Corresponding author. Fax: +1-617-726-5677; E-mail: b_hyman@helix.mgh.harvard.edu

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revealed that sel-12, a homologue of PS1, facilitates lin-12/Notch mediated vulval development [26]. In addition, genetic analysis has established that reducing or eliminating sel-12 activity reduces the activity of lin-12 and causes an egg-laying defective phenotype, and that this defective phenotype in *C. elegans* can be rescued by human wild type PS1, and to a lesser extent by mutant PS1 [6,24].

Signaling via the Notch/lin-12 family of receptors plays a critical role during development in regulating cell fate decisions, including neurogenesis, and possibly axon guidance. [2–4,12,13,17–20,28,32,35]. It is known that Notch1 (both mRNA and protein) is highly expressed during brain development. Recently, it has been shown that Notch1 continues to be expressed in some neuronal populations in the CNS in adults, however, the function of Notch1 in postmitotic neurons in mammals remains unknown [1,8,9,34]. There is regional and cellular coexpression of Notch1 with PS1 during development, as well as in adult mouse and human brain [8,9], supporting the plausibility of PS1/Notch1 interaction in mammals. However, the question of whether a PS1-Notch1 functional interaction exists in mammalian neurons remains unanswered.

To study the potential physiological consequences of PS1-Notch1 interactions in neurons, we transfected Notch1 into primary neurons derived from transgenic (TG) animals overexpressing human PS1 (wild type or M146L mutation), or lacking PS1 (PS1 knockout mice). Our results suggest that normal, but not mutant PS1 facilitates Notch1 function in this system, extending evidence for PS1-Notch1 interaction to a mammalian neuronal model.

2. Materials and methods

2.1. Primary neuronal cultures

Primary neuronal cultures were prepared from combined neocortex and hippocampi of embryonic day 16–18 mice (E16-18). The neurons were maintained in Neurobasal™ medium (Gibco BRL) with 2% B27 supplement (Gibco BRL) at 37°C with 5% CO_2. In the case of transgenic or knockout mice, the cultures were prepared separately from individual embryos, and tails of the embryos were dissected to establish the genotype of each culture, i.e., TG (expressing human mutant or wild type PS1), PS1 knockout (−/− or −/+), or non-TG. Under our culture conditions, only rare non-neuronal cells are present.

2.2. Transfection

Co-transfection of 2 days in vitro (DIV) primary neurons with green fluorescent protein as a transfection marker (EGFP, Clontech) and pSG5/Notch1 plasmids (at 1:3 ratio) using the method of calcium phosphate co-precipitation was performed as described previously (total cDNA concentration was 2 μg per 200 μl of DMEM) [11,37]. An empty vector pSG5 (Stratagene), or one of the following Notch1 constructs were used for the transfection: (1) expression pSG5 vector containing full length Notch1, N1(FL), (2) a truncated constitutively activated form of Notch1 containing the transmembrane region and intracellular signaling domain, N1(ΔEC) or (3) the intracellular signaling domain only, N1(IC), which is also constitutively active [27] (Fig. 1b). The low efficiency of transfection using this technique (1–2%), was beneficial in this particular experimental design because we were able to identify the morphology of every transfected neuron. The efficiency of co-transfection (i.e., co-expression of two plasmids, EGFP and Notch1, in the same cell) was assessed earlier [7] and was about 93%. The cultures were examined 48 h after transfection, and approximately 150–200 green fluorescent cells (transfected cells) were counted in each chamber. We used neuron-specific MAP2 antibody (Sigma) to insure that transfected cells counted in this study were neurons (Fig. 2). Neuron morphology was assessed in three groups: I (round cells), II (cells with single neurite or bipolar cells with processes no longer than approximately 4–5 diameters of the cell body), and III (cells with well developed ramified short or long processes) (Fig. 1a). The percentage of cells in every group (I to III) was calculated for each chamber transfected with different expression vectors. The data were analyzed by using StatView software. The kind of transfection as well as the genotype of the culture where neurons were counted remained masked to the observer. Data were collected from 26 transfection experiments, representing over 30,000 individual observations.

2.3. Western blot analysis

2.3.1. Homogenization of mouse brains

Half brains (excluding cerebellum) were homogenized by > 60 strokes in a micro-dounce homogenizer in CSK buffer (0.5% Triton X-100, 50 mM NaCl, 10 mM HEPES pH 6.8, 3 mM $MgCl_2$, 300 mM sucrose, 100 mg/ml AEBSF, 10 mg/ml leupeptin). Homogenates were cleared twice by centrifugation at 14,000 rpm in the cold room, and protein concentrations were determined by the micro-BCA method (Pierce).

2.3.2. Immunoprecipitation and Western blotting

For detection of endogenous and transgenic PS1 N-terminal fragment (NTF), 150 mg of brain homogenate was precleared with normal rabbit serum, immunoprecipitated with J27 antibody, washed 3 times in CSK buffer, and the precipitates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The NTF was then detected by incubation with human specific PSN2 monoclonal antibody (gift from Dr. Hiroshi Mori) fol-

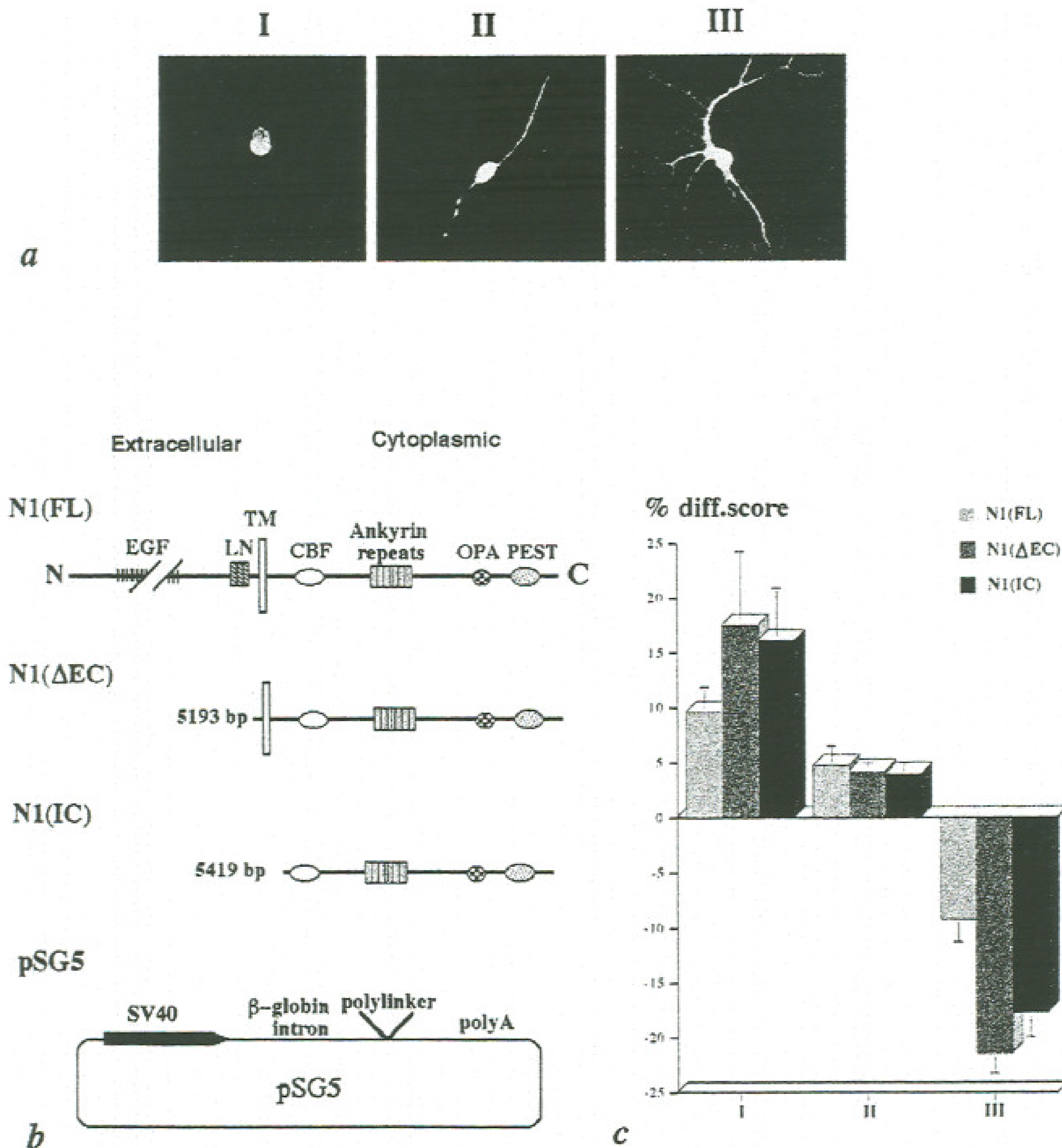

Fig. 1. Transfection of 2 DIV primary neurons with different Notch1 constructs. (a) The semiquantitative scale reflects three stages of morphological differentiation: round cells (group I), cells with minor processes (group II), and cells with long processes (group III); (b) Notch1 constructs used in Notch1-EGFP co-transfection experiments: N1(FL)—full length Notch1 cDNA; N1(ΔEC)—truncated constitutively active form of Notch1 cDNA containing intracellular and transmembrane (TM) domains; and N1(IC)—constitutively active intracellular domain of the Notch1 molecule only. All Notch1 constructs were cloned into the pSG5 mammalian expression vector [27]. (c) The graph represents the difference score in the percentage (% diff. score) of group I, group II, and group III cells between the neurons transfected with the Notch1 constructs (FL, ΔEC, IC) and the control transfection with an empty pSG5 vector. Neurons were derived from non-transgenic (non-TG) mouse embryos. % diff. score for N1(FL), N1(IC), or N1(ΔEC) equals the percentage of neurons in each morphological group in cultures transfected with N1(FL), N1(IC), or N1(ΔEC) minus the percentage of neurons in each morphological group in pSG5 transfected cultures. Transfection with Notch1 constructs leads to an increase in the percentage of round cells (% diff. score is positive) and a decrease in the percentage of fully elaborated group III neurons (% diff. score is negative).

lowed by detection with an HRP-conjugated secondary antibody, and enhanced chemiluminescence [38]. A Bio-Rad phosphorimager and the Molecular Analyst software BioRad were utilized for quantitation of band intensity.

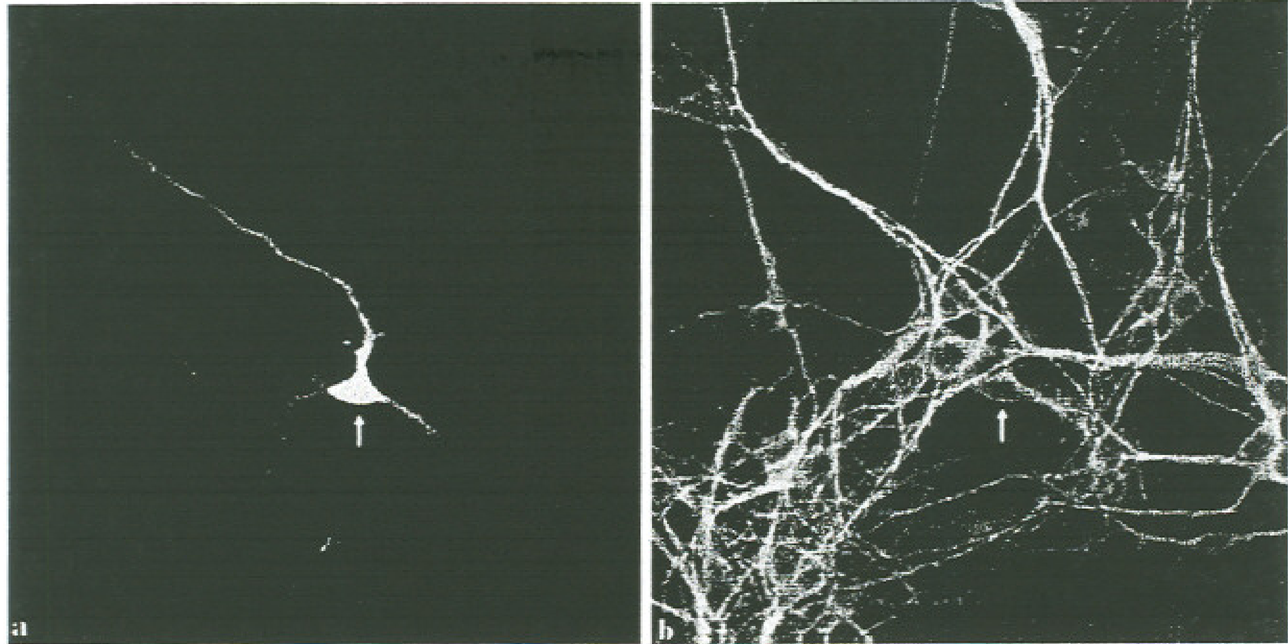

Fig. 2. Immunostaining of the primary neurons transfected with EGFP-Notch1 (FL, IC or ΔEC) using a neuron specific marker (MAP2 antibody) show that all transfected cells counted in the experiments were neurons (MAP2 immunoreactive). (a) EGFP-N1(FL) transfected neuron; (b) MAP2 immunostaining. Arrow indicates transfected/MAP2 immunoreactive neuron.

2.4. PS1 knockout and transgenic animals

PS1 knockout mice were generated by replacement of ATG containing exon 3 in presenilin 1 with a *pkg-neo* cassette. The mice were characterized previously [31].

To generate PS1 transgenic animals, the PDGF B-chain promoter was mobilized as an *XbaI* fragment from previously-prepared constructs [30] and cloned 5′ to a synthetic intron (derived from the vector pCI (Promega, Madison, WI). PCR with high fidelity polymerase was used to generate a PS1 cDNA, including the entire coding region with a re-engineered Kozak sequence 5′ to the start codon. PCR-based mutagenesis was performed in this vector to introduce the M146L PS1 mutation, and confirmed by sequencing. The cDNA was subcloned 3′ to the intron and a 3.4 kb DNA fragment containing the promoter, cDNA and other required sequences was used for pronuclear injection.

Genomic DNA was prepared using standard methods [22]. Genotype was determined by 3-primer PCR using one forward primer (anneals to the endogenous mouse PDGF B-chain gene and the human PDGF B-chain promoter of the transgene) and two reverse primers (one annealing to the PS1 cDNA and the other to mouse genomic DNA in the PDGF B-chain gene).

For tissue culture experiments, non-transgenic females (> 8 weeks of age) were housed with heterozygous transgenic males (3–9 months of age). The day after mating was taken as embryonal day 0.5. Embryos were genotyped by the methods described above, with DNA prepared from somatic tissue (usually hind limbs) obtained at the time of neuronal culturing.

3. Results

Transfection of primary neurons with full length Notch1 significantly inhibited neurite outgrowth (Fig. 1c). As expected, this effect was much more pronounced when constitutively active forms of Notch1 were used. Both N1(ΔEC), which contains a portion of the transmembrane region, and N1(IC), which contains only the intracellular domain of the Notch1 molecule, markedly impaired neurite outgrowth (Fig. 1). The neuronal identity of the transfected cells, and the morphology of the dendrites were confirmed with MAP2 immunostaining (Fig. 2).

To test the hypothesis that Notch1 interacts with PS1 in mammals analogous to lin-12/sel-12 interaction in *C. elegans* [26], we studied the effect of Notch1 transfection on neurite outgrowth in neurons lacking either both copies (PS1 −/−) or one copy of PS1 (PS1 +/−), as well as in neurons overexpressing either human wild type PS1 (WT PS1) or human mutant PS1 (M146L PS1). Primary neuronal cultures were prepared separately from transgenic and non-transgenic embryos, because in each experiment transfected neurons were compared directly to littermate non-transgenic neurons (non-TG).

Previously we demonstrated that in 2 DIV neuronal cultures prepared from control non-TG embryos and transfected with an empty vector on average 20% of all neurons

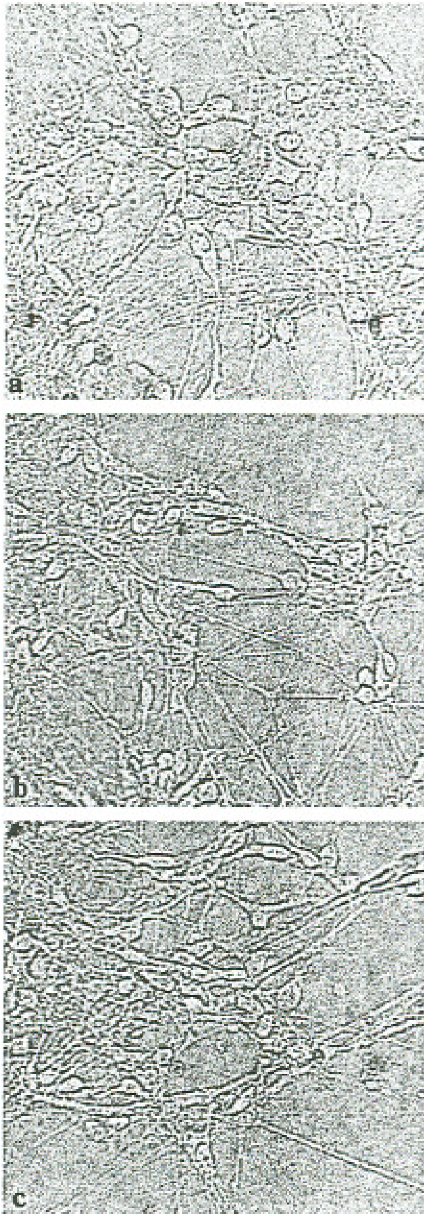

Fig. 3. Neuronal cultures transfected with an empty vector (a), full length Notch1 (b) or constitutively active N1(ΔEC) (c), were stained with trypan blue. There was no significant difference in the number of dead cells (dark, trypan blue positive cells) between the cultures transfected with various Notch1 constructs and an empty pSG5 vector.

were round (group I) and about 60% had long processes (group III), 48 h post-transfection [7]. Here, a similar relationship between these groups of neurons was observed in neuronal cultures prepared from transgenic (WT PS1, or M146L PS1) and PS1 knockout (PS1 $-/-$, or PS1 $+/-$) embryos. There were no obvious differences in the morphological appearance of the cells or the transfection efficiency between the neuronal cultures prepared from transgenic, knock-out, and non-transgenic embryos during the observed period of time from 0 DIV to 10 DIV. Furthermore, no excess neuronal death was observed in any of the cultures following transfection with Notch1 constructs when compared to an empty vector transfected cultures (Fig. 3).

The difference score in the percentage of cells of each morphological group (I–III) following the transfection with different Notch1 constructs vs. an empty pSG5 vector was calculated (Fig. 1c). Transfection with full length Notch1, N1(FL), had a modest (compared with constitutively active Notch1), but statistically significant effect on neuronal maturation, decreasing the percentage of cells with long processes ($P < 0.01$), and concomitantly increasing the percentage of round cells when compared to transfection with an empty vector alone. This effect of N1(FL) can be explained by the presence of Notch ligands in the culture [7] that could activate N1(FL) in transfected cells and cause inhibition of neurite extension.

The inhibitory effect of Notch1 on neurite outgrowth was even more profound after transfection of primary neurons with truncated constitutively active Notch1 constructs, N1(ΔEC) or N1(IC), ($P < 0.0001$). No significant difference was observed between transfections with these two truncated forms of Notch1, indicating that membrane spanning Notch1 (ΔEC) could be cleaved and/or signal similarly to N1(IC), which contains only the signaling cytoplasmic domain of the Notch1 molecule.

To test the idea that PS1 interacts with Notch1, we transfected primary neurons prepared from PS1 knockout animals with these Notch1 constructs. The PS1 knockout neurons had normal viability in culture and matured with long processes to the same extent as normal controls (data not shown). The efficiency of transfection was also comparable. However, the magnitude of the Notch1 effects were significantly different between the groups of animals. For example, the inhibitory effect of Notch1 on neurite outgrowth, as indicated by an increased percentage of round cells and a decreased percentage of group III neurons with established processes, was nearly abolished in both heterozygous and homozygous PS1 knockout mice, in comparison to non-TG mice (Fig. 4), ($P < 0.001$). We asked whether the PS1 facilitation of Notch1 was based on an effect on Notch1 trafficking to the cell surface by using both the N1(IC), which is constitutively active but not membrane associated, and N1(ΔEC), which has a truncated extracellular domain, but is nevertheless inserted into membrane prior to activation. If PS1 facilitated Notch1 trafficking to the membrane one would expect an effect on N1(ΔEC) but not N1(IC). Instead, we observe an equivalent effect of both constructs.

We next transfected neurons derived from transgenic mouse embryos overexpressing wild type (WT) or mutant (M146L) PS1 with the Notch1 constructs. To compare the level of expression of human PS1 in transgenic mouse brain, semi-quantitative immunoprecipitation followed by Western blot analysis of brain homogenates from PS1 transgenic mice overexpressing human wild type or mutant

PS1 was performed (Fig. 5a). This demonstrated that the M146L PS1 transgenics had similar or slightly higher levels of expression of the transgene than the wild type PS1 transgenics.

To test the hypothesis that PS1 facilitates the Notch1 effect, we next examined Notch1 transfections in neurons overexpressing wild type PS1. Forty-eight hours after transfection there were significantly fewer cells with long ramified processes (group III) in WT PS1 overexpressing cultures in comparison to the corresponding transfection in littermate non-TG cultures. Thus, the inhibitory effect of the Notch1 constructs on neurite outgrowth was significantly facilitated by WT PS1 overexpressing neurons compared to non-TG ($P < 0.01$) (Fig. 5b). However, in neurons overexpressing mutant (M146L) PS1 the effect of Notch1 was not significantly different from that in non-transgenic neuronal cultures ($P = 0.78$), arguing against a dominant negative interaction.

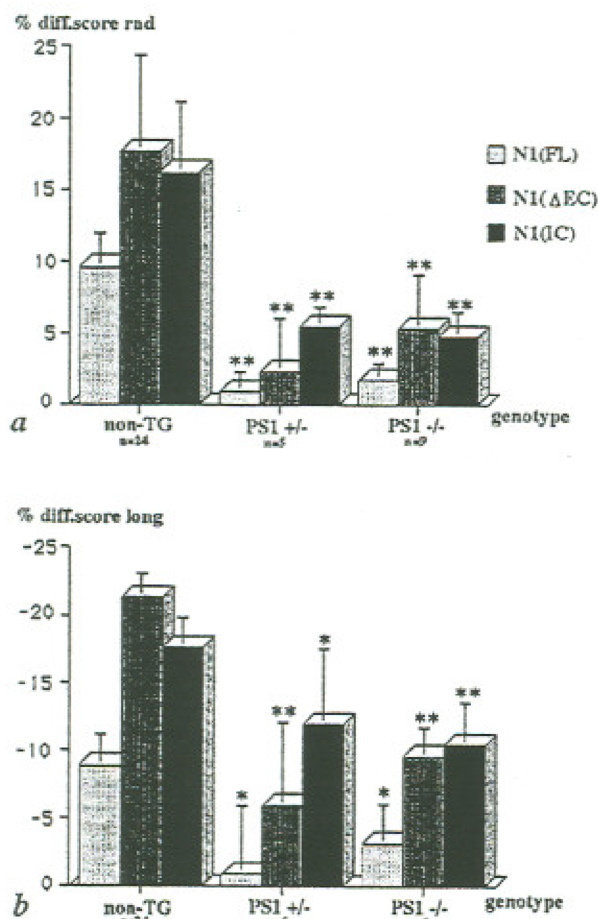

Fig. 4. Difference score in the percentage of (a) round cells (group I) and (b) cells with long processes (group III) between various Notch1 constructs (FL, ΔEC, IC) and empty pSG5 vector transfected neurons derived from non-transgenic (non-TG), PS1 knockout homozygous (PS1 −/−), and PS1 knockout heterozygous (PS1 +/−) mice. The inhibitory effect of Notch1 on neurite outgrowth was significantly reduced in PS1 knockout neurons when compared to the control non-TG neurons ($P < 0.0001$. ANOVA and Fisher's PLSD analyses, n = number of embryos).

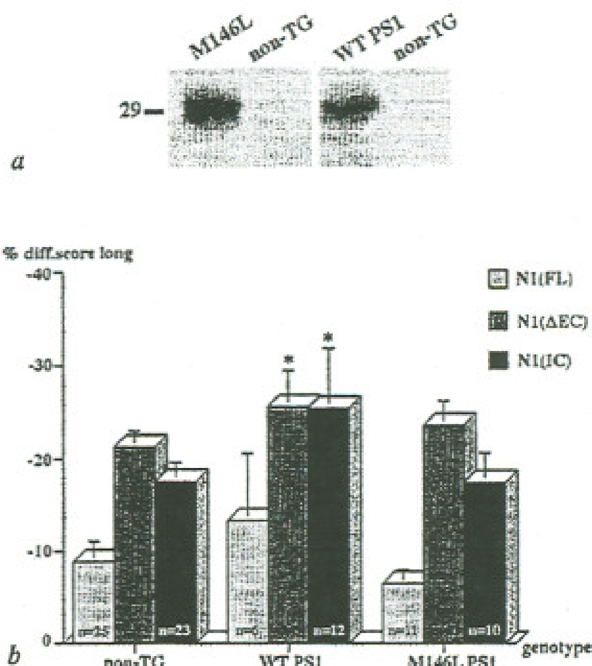

Fig. 5. Effect of Notch1 on neurite outgrowth in PS1 transgenic mice. (a) Semi-quantitative Western blot analysis of human PS1 content in PS1 transgenic mice overexpressing WT PS1 or mutant PS1. Brain homogenates were immunoprecipitated with J27 antibody and blotted with N-terminal PSN2 antibody. (b) Difference score in the percentage of cells with long processes (group III) between various Notch1 constructs (FL, ΔEC, IC) and empty pSG5 vector transfected neurons derived from non-transgenic (non-TG), human wild type PS1 (WT PS1) or mutant PS1 (M146L PS1) overexpressing mice. The difference score was significantly higher in WT PS1 mice compared to that in non-TG ($P < 0.01$), while the difference score in mutant M146L PS1 overexpressing mice was not significantly different from that in non-TG mice ($P = 0.78$), (ANOVA and Fisher's PLSD analyses, n = number of embryos).

4. Discussion

A presenilin–Notch interaction was initially suggested in a study of vulval morphology in *C. elegans* [26]. To examine if this interaction is relevant from the perspective of Alzheimer's disease, we studied whether there is a functional interaction between PS1 and Notch1 in mammalian CNS neurons. We observed that Notch1 affects morphological differentiation in postmitotic mammalian primary neurons by inhibiting neurite outgrowth. Other studies in neuronal and mesodermal cell lineages [15,21] and in developing rat retina [5] also showed that Notch1 can block differentiation, although, Notch's effect may be cell specific [28]. We found that lack or overexpression of PS1 in primary neurons affected Notch1 function in terms of neurite morphology. The Notch1 effect was attenuated in neurons lacking PS1 and enhanced in neurons over-expressing WT PS1. These results clearly indicate that there

is a biological interaction between Notch1 and presenilin 1 in mammalian neurons, and is consistent with the hypothesis that presenilin 1 facilitates Notch1 function.

In primary neurons prepared from WT PS1 overexpressing transgenic mice the effect of Notch1 on neurite outgrowth was significantly more robust when compared to that of non-transgenic neuronal cultures. Mutant PS1 over-expression in transgenic mice did not preclude Notch1 function, although unlike normal PS1, mutant PS1 did not appear to facilitate Notch1 function either. These results indicate that the human M146L PS1 familial Alzheimer disease (FAD) linked mutation has reduced PS1 function as assessed by interaction with Notch1 in differentiated neurons. This conclusion is in accord with data that normal human PS1 can substitute for *C. elegans* sel-12 and rescue a mutant phenotype, but six tested FAD-linked mutant human presenilins had reduced ability to rescue the sel-12 mutant phenotype [24]. Similar results were shown in *C. elegans* by Baumeister et al. [6]. Thus, in terms of Notch1-PS1 interactions in both mammalian neurons and in development in *C. elegans*, it appears that FAD-associated mutations lead to diminished PS1 functional ability.

By contrast, a discrepancy exists between our data and studies in *C. elegans* on the effects of constitutively active Notch1. We observed that the PS1 null mutation interfered with neurite inhibition due to both N1(IC), containing the cytoplasmic portion of Notch1 molecule, and N1(ΔEC), containing a truncated transmembrane domain. This result differs from the data of Levitan and Greenwald [25] in vulval precursor cells, where a reduction of sel-12 activity suppressed the effect of lin-12 activated by a missense mutation, but not when lin-12 was activated by removal of extracellular and transmembrane domains (similarly to N1(IC)). Although the Notch signaling pathway is evolutionary conserved, this discrepancy could be due to species differences, or to differences in the cell type studied.

FAD-associated PS1 mutations have been suggested to show either a presumed loss-of-function in genetic complementation studies and in this work, or a presumed gain-of-function with an alteration of Aβ metabolism and enhanced Aβ deposition [10,14,23]. In addition, it has been reported recently that both wild type PS1 and mutant (A246E) PS1 rescue the PS1 knockout phenotype in mice [16,29]. These apparently dichotomous results are not necessarily mutually exclusive if PS1 facilitation of Notch1 and a new Aβ-related PS1 'gain-of-function' represent separate biological effects of PS1. Indeed, we have recently found that in the hippocampus of patients with sporadic Alzheimer disease (AD), Notch1 is significantly elevated when compared to that in control brain [8]. We suggest that the increase in Notch1 can 'facilitate' the function of the normal PS1 present in these individuals. Our data in AD do not address whether the increased Notch1 expression is a failed compensation for diminished PS1 function or contributes to increased Notch1/PS1 function, perhaps in relationship to plasticity and sprouting events. Thus, it is possible that one of the outcomes of Notch1 action in the adult may be to impact the only 'cell fate' decisions possible in postmitotic mature neurons—synaptic remodelling or neurite extension/retraction, and hence influence neuronal plasticity mechanisms in disease as well.

Acknowledgements

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