Presenilin-1 regulates neuronal differentiation during neurogenesis

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SUMMARY

Mutations in Presenilin-1 (PS1) are a major cause of familial Alzheimer’s disease. Our previous studies showed that PS1 is required for murine neural development. Here we report that lack of PS1 leads to premature differentiation of neural progenitor cells, indicating a role for PS1 in a cell fate decision between postmitotic neurons and neural progenitor cells. Neural proliferation and apoptotic cell death during neurogenesis are unaltered in PS1−/− mice, suggesting that the reduction in the neural progenitor cells observed in the PS1−/− brain is due to premature differentiation of progenitor cells, rather than to increased apoptotic cell death or decreased cell proliferation. In addition, the premature neuronal differentiation in the PS1−/− brain is associated with aberrant neuronal migration and disorganization of the laminar architecture of the developing cerebral hemisphere. In the ventricular zone of PS1−/− mice, expression of the Notch1 downstream effector gene Hes5 is reduced and expression of the Notch1 ligand Dll1 is elevated, whereas expression of Notch1 is unchanged. The level of Dll1 transcripts is also increased in the presomitic mesoderm of PS1−/− embryos, while the level of Notch1 transcripts is unchanged, in contrast to a previous report (Wong et al., 1997, Nature 387, 288-292). These results provide direct evidence that PS1 controls neuronal differentiation in association with the downregulation of Notch signalling during neurogenesis.

Key words: Alzheimer’s disease, Hes5, Dll1, Notch1, Cell fate determination, Brain

INTRODUCTION

PS1 is a major gene responsible for familial Alzheimer’s disease (FAD), and mutations in PS1 account for approximately 50% of early-onset FAD cases (Selkoe, 1998). Understanding the normal physiological functions of PS1 may shed light on the pathogenic mechanism of FAD-linked PS1 mutations. Identification of the PS1 homologue in C. elegans, sel-12, which facilitates signalling mediated by the Notch homologue LIN-12, provided the first evidence that PS1 interacts with the Notch signalling pathway (Levitan and Greenwald, 1995). The LIN-12/Notch family of receptors mediates cell-cell interactions that specify cell fate during development. SEL-12 affects LIN-12 activity by regulating its processing or trafficking (Levitan and Greenwald, 1998). PS1 shares functional homology with SEL-12, based on the finding that the wild-type human PS1 cDNA complements the sel-12 mutant phenotype (Baumeister et al., 1997; Levitan et al., 1996). However, PS1 containing FAD-linked mutations exhibited reduced ability to rescue sel-12 mutations, suggesting that mutant PS1 has reduced biological activity (Baumeister et al., 1997; Levitan et al., 1996).

Recent identification of the Drosophila Presenilin (PS) provided further evidence for the interaction between PS1 and the Notch signalling pathway (Struhl and Greenwald, 1999; Ye et al., 1999). Fly mutants lacking both maternal and zygotic PS exhibit a neurogenic phenotype and are virtually indistinguishable from the Notch-null mutant, suggesting that PS function is required for normal Notch signalling in Drosophila (Struhl and Greenwald, 1999; Ye et al., 1999). PS is also required for the proteolytic cleavage of Notch to release its intracellular effector domain (ICD) (Struhl and Greenwald, 1999). The involvement of Presenilins in Notch processing is further supported by in vitro studies using truncated Notch1 and primary cell cultures derived from PS1−/− mice (De Strooper et al., 1997; Song et al., 1999). Levels of the ICD fragment were reduced in cultured PS1−/− neurons and fibroblasts, indicating that PS1 is important for the efficient proteolytic release of the ICD. PS1 bearing FAD-linked mutations exhibits reduced ability to rescue this Notch processing defect in cultured PS1−/− cells, suggesting that mutant PS1 has reduced activity in Notch processing (Song et al., 1999).

To characterize the normal physiological role of PS1 in mice, we previously generated mice with a targeted germ-line disruption of the PS1 gene (Shen et al., 1997). Mutant mice homozygous for the resulting null allele exhibited similar defects in somitogenesis to those observed in Notch1-null mutant mice (Conlon et al., 1995; Swiatek et al., 1994), as well as severe malformation of the axial skeleton and cerebral hemorrhage (Shen et al., 1997; Wong et al., 1997). Furthermore, we showed that lack of PS1 results in a reduction...
in the neural progenitor population, indicating a subsequent reduction in the neuronal population, indicating a critical role for PS1 in murine neurogenesis (Shen et al., 1997). We also observed symmetric, region-specific loss of neural progenitor cells in the diencephalon and telencephalon of the PS1−/− brain beginning at E12.5 and 14.5, respectively. Loss of Cajal-Retzius neurons in late embryonic development has also been reported (Hartmann et al., 1999).

Here, we report that the reduction in the neural progenitor population in PS1−/− mice is caused by premature differentiation of neural progenitor cells. To investigate further the mechanism by which PS1 regulates neuronal differentiation, we examined the expression of genes involved in the Notch signalling pathway. We found a reduction in the level of HES-5 transcripts as well as an increase in the level of Dll1 transcripts in the PS1−/− brain, indicating that absence of PS1 function leads to reduced Notch signalling during neural development. We also found that expression of Dll1 is increased in the presomitic mesoderm of PS1−/− embryos, whereas Notch1 expression is unchanged, in contrast to a previous publication reporting that expression of both Notch1 and Dll1 is markedly reduced in the presomitic mesoderm of PS1−/− embryos (Wong et al., 1997). Taken together, our findings provide the first evidence that PS1 controls neuronal differentiation and the expression of the downstream target genes regulated by Notch signalling.

MATERIALS AND METHODS

Preparation of brain sections

Timed matings between heterozygous PS1 knockout mice were set up, and the morning of the day when a vaginal plug is seen is designated as embryonic day 0.5 (E0.5). Embryos at the desired developmental stages were dissected from pregnant females, and the tail and limbs were removed from each embryo for genotyping. Embryonic heads were then removed and fixed in 4% paraformaldehyde for 2-3 hours at 4°C, cryoprotected overnight at 4°C in 30% sucrose, embedded in OCT medium (Tissue-Tek) and frozen on dry ice. Serial transverse sections (7-10 μm) were cut on a cryostat and collected on coated slides. Embryos with cerebral hemorrhages, regardless of the severity, were discarded.

Immunohistochemical analysis

Brain sections were blocked for 3 hours at room temperature with 3% nonfat milk, rinsed briefly in PBS and incubated overnight at 4°C with an anti-microtubule-associated protein 2 (MAP2) monoclonal antibody (1:1000, kindly provided by Dr Ken Kosik) or an anti-Nestin monoclonal antibody (1:1000, PharMingen). For Notch1 immunostaining, sections were incubated with a blocking solution containing 5% goat serum, 1% BSA, 0.1% glycine, 0.1% L-lysine and 0.4% Triton X-100 in PBS for 1 hour at room temperature followed by incubation with a polyclonal antibody (IC) against mouse Notch1 amino acid residues 1759-2306 (1:250, kindly provided by Dr Alan Israel) overnight at 4°C. Sections were then rinsed twice in PBS and incubated at room temperature with a rhodamine-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch) for MAP2 (1:400) and Nestin (1:100). For Notch1 immunostaining, the sections were rinsed three times in PBS and incubated with a fluorescein-conjugated goat anti-rabbit secondary antibody (1:500, Alexa) for 1 hour at room temperature. Images were viewed and collected on a Zeiss Axioscop equipped with a reticular eyepiece and a BioRad MRC 1024 confocal laser scanning microscope.

BrdU labelling, immunodetection and quantification

Pregnant females were injected intraperitoneally with BrdU (10 mg/ml) at 100 μg/g body mass. After 30 minutes or 96 hours of labelling, embryos were removed, processed and serially sectioned. The brain sections were incubated in 2 N HCl for 1 hour at 37°C, neutralized in borate buffer (pH 8.5) for 1 hour at room temperature, rinsed twice in PBS and blocked with 3% nonfat milk for 3 hours at room temperature. The sections were then incubated with an anti-BrdU monoclonal antibody (1:10, Becton Dickinson) overnight at 4°C, rinsed twice in PBS, followed by incubation with a rhodamine-conjugated goat anti-mouse secondary antibody (1:100, Jackson Immunoresearch) for 1 hour at room temperature.

The percentage of BrdU-labelled cells was determined by dividing the number of BrdU+ cells by the total number of progenitor cells. For both PS1+/− and littermate control brains, the number of BrdU+ cells was counted in a comparable area of the anterior telencephalon and in half of the diencephalon. The total number of progenitor cells in the telencephalon was determined by counting the number of nuclei stained with Sytox Green in the comparable area on the adjacent sections, since there are few postmitotic neurons in the telencephalon at E10.5. Due to the presence of postmitotic neurons in the diencephalon at this stage, the total number of progenitor cells in the diencephalon was determined by subtracting the number of MAP2-positive cells from the total number of Sytox Green-stained cells in half of the diencephalon on the adjacent sections. A Zeiss Axioscop equipped with a reticular eyepiece was used to count the number of cells in an area of 5x 104 μm2 in the telencephalon and half of the diencephalon under 63× magnification.

Detection and quantification of apoptotic cells

The TUNEL (terminal-deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick-end labelling) assay was used to label cells that are undergoing apoptosis. Brain sections were rinsed in PBS for 5 minutes and then 50-75 μl of TUNEL reaction mixture (In situ cell death detection kit, Boehringer Mannheim) was applied to each slide. The sections were incubated at 37°C for 1 hour and 15 minutes, rinsed twice in PBS and then incubated in bisbenzimide (2 μg/ml) for 10 minutes at room temperature. Apoptotic cells were identified by their TUNEL-reactivity as well as their fragmented and condensed nuclei stained by bisbenzimide. The number of apoptotic cells in each brain was quantified by counting three serial sections under 40× magnification. Statistical significance was determined by Student’s t-test.

In situ hybridization

Sense and antisense riboprobes were synthesized using an in vitro transcription kit (Boehringer Mannheim) following the manufacturer’s instructions and then hydrolyzed to approximately 100-200 nucleotides (nt) fragments. In situ hybridization was carried out as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993) with minor modifications. Brain sections were incubated with the prehybridization solution (50% formamide, 5x SSC, 0.02% SDS, 0.1% N-lauroylsarcosine and 2% blocking reagent) at room temperature (RT) for 6 hours, then hybridized together in hydrolyzed riboprobes (40 ng/ml) at 70°C overnight.

For color detection, the sections were placed upside down on NBT/BCIP reaction mixture containing 2 mM freshly prepared levamisole. The slides containing brain sections were elevated with two spacers (0.2 mm) to allow sufficient and equal contact with the reaction mixture. Precautions were taken to ensure identical conditions for all procedures of in situ hybridization involving the PS1−/− and littermate control brain sections. All images were collected with a cooled CCD camera with identical exposure time between the
of neural progenitor cells could occur via several possible mechanisms: premature differentiation of progenitor cells, decreased proliferation, or increased apoptotic cell death. To distinguish among these possibilities, we compared the PS1−/− and control brains for differences in neuronal differentiation and migration, cell proliferation and survival. Due to severe cerebral hemorrhage often associated with PS1−/− mice at later embryonic stages, our analysis is focused on the examination of the PS1−/− and control brains that are free of hemorrhages between E10.5 and 13.5.

**Premature differentiation of neural progenitor cells in PS1−/− mice**

To assess neuronal differentiation in the PS1−/− and control brains, we performed immunostaining for MAP2, a marker specific for postmitotic neurons (Crandall et al., 1986). Serial transverse sections of the PS1−/− and littermate control brains at E10.5, 11.5, 12.5 and 13.5 were stained with anti-MAP2 antibody, and corresponding sections of the PS1−/− and control brains were compared. Although few MAP2-immunoreactive neurons are present in the telencephalon at E10.5 (data not shown), as many as 4-6 layers of MAP2+ neurons were detected in the PS1−/− diencephalon, whereas only 1-2 layers of MAP2+ neurons were detected in the control (Fig. 1A). The ratio of MAP2+ cells to total cell population in the PS1−/− diencephalon is two- to threefold higher than in the littermate control (data not shown).

At E11.5, increases in MAP2 immunoreactivity in the PS1−/− brain relative to the control are found in the anterior telencephalon (Fig. 1Ba,b), and more substantially in the posterior telencephalon (Fig. 1Bc,d). In the anterior telencephalon, 2-4 layers of MAP2+ neurons were detected in the PS1−/− brain in comparison to a single layer of MAP2 immunoreactive neurons in the littermate control (Fig. 1Ba,b). The ventricular zone in this region contains similar numbers of MAP2-negative progenitor cell layers in the PS1−/− brain and the control. The posterior telencephalon of the PS1−/− brain contains more layers of MAP2+ neurons and a smaller ventricular zone in comparison to the control (Fig. 1Bc,d). The diencephalon exhibits the most substantial differences in the populations of MAP2-immunoreactive neurons and MAP2-negative progenitor cells. In the diencephalon of the PS1−/− brain, the MAP2-immunoreactive neurons encompass numerous cell layers and have largely replaced the MAP2-negative progenitor cells in the ventricular zone (Fig. 1Be-h).

At E12.5, these differences are still prominent in the telencephalon and diencephalon. More layers of MAP2+ neurons are present in the anterior telencephalon in the PS1−/− brain (Fig. 1Ca,b), and the diencephalic ventricular zone is largely replaced by the MAP2+ neurons (Fig. 1Ce,f). By E13.5, however, MAP2 immunoreactive neurons comprise a similar number of cell layers in the anterior telencephalon of the PS1−/− brain and littermate control brains (Fig. 1Da,b).

In summary, markedly increased numbers of postmitotic neurons identified by MAP2 immunoreactivity accumulate in the telencephalon and diencephalon of the PS1−/− brain during early neural development, accompanied by a progressive reduction in size of the ventricular zone. These observations indicate that neural progenitor cells differentiate into postmitotic neurons prematurely in the absence of PS1, resulting in early depletion of the neural progenitor population.
Interestingly, the severity of this phenotype varies in the brain subregions examined. The diencephalon exhibits the most striking difference in MAP2 immunoreactivity, whereas the anterior telencephalon shows the least difference. Thus, during neurogenesis, PS1 controls neuronal differentiation in a region-specific manner by regulating the cell-fate choice between neural progenitor cells and postmitotic neurons.

The role of PS1 in proliferation of neural progenitor cells

Although premature neuronal differentiation provides an explanation for the reduction in the number of neural progenitor cells observed in the PS1<sup>−/−</sup> brain, it remained possible that a decrease in cell proliferation and/or an increase in apoptotic cell death could be contributing factors as well. Cell proliferation in the neuroepithelium was examined by BrdU labelling. The fraction of cells labelled with BrdU during a short pulse reflects the fraction of cells in S phase during that period, permitting an assessment of the proliferation rate of a population of cells (Gratzner, 1982). After 30 minutes of BrdU pulse-labelling, serial transverse sections of the PS1<sup>−/−</sup> and littermate control brains were stained with a monoclonal antibody directed against MAP2. Schematic diagrams of transverse views of the telencephalon and diencephalon are included to indicate the positions of brain subregions shown in the panels. At E10.5 (A) more MAP2 immunoreactive neurons are detected in the PS1<sup>−/−</sup> diencephalon compared to the littermate control (arrowheads in a and b). At E11.5 (B), an increased number of MAP2<sup>+</sup> neurons are present in the telencephalon (small arrows in a and b), and more substantially in the ganglionic eminence (ge; large arrows in c and d) and diencephalon, where the difference is most striking (arrowheads in e and f). The ventricular zone (vz), which consists of MAP2 negative cells and is indicated by bars, is similar in size in the anterior telencephalon (a and b) but considerably thinner in the diencephalon (e and f) of the PS1<sup>−/−</sup> brain. BrdU-labelled cells are shown residing within the ventricular zone in the diencephalon (g and h). At E12.5 (C), there are more MAP2<sup>+</sup> neurons in the anterior telencephalon of the PS1<sup>−/−</sup> brain (a and b). Nestin immunostaining is used to label neural progenitor cells in the neuroepithelium (c and d). A significant increase in the number of MAP2<sup>+</sup> cells and a reduction in the ventricular zone are detected in the PS1<sup>−/−</sup> diencephalon (e and f). By E13.5 (D), the number of MAP2<sup>+</sup> cell layers in the anterior telencephalon of the PS1<sup>−/−</sup> and control brains becomes similar (a and b), which could be explained by reduced neurogenesis in the PS1<sup>−/−</sup> brain due to a reduction in the progenitor population at this embryonic stage. More postmitotic neurons are generated in the control brain between E12.5 and 13.5, compensating for the smaller neuronal population at E12.5. lv, lateral ventricles; v3, third ventricle. Bars, 100 μm.
We did, however, observe differences in BrdU labelling in the diencephalon at E11.5. The BrdU labelling pattern in the telencephalon of the $PS1^{-/-}$ brain appears similar to that of the control (Fig. 2Ba,b), but fewer BrdU-labelled cells are detected in the diencephalon of the $PS1^{-/-}$ brain (Fig. 2Bc-f). The location of these BrdU-labelled cells in the $PS1^{-/-}$ brain corresponds to the thin remnant of the diencephalic ventricular zone revealed by the absence of MAP2 immunostaining at this age (Fig. 2Be,f; compare with unstained cells in Fig. 1Be,f). The BrdU labelling patterns of the anterior telencephalon are indistinguishable at E12.5 (Fig. 2Ca,b) and E13.5 (Fig. 2Da,b), but fewer BrdU-labelled cells appear to be present in the ganglionic eminence of the $PS1^{-/-}$ brain (Fig. 2Cc,d). The simplest explanation for the reduced BrdU labelling in the ganglionic eminence and diencephalon of the $PS1^{-/-}$ brain is that it is a secondary effect, reflecting early and progressive depletion of progenitor population in the ventricular zone of these brain regions due to premature differentiation of neural progenitor cells. The corresponding presence of increased numbers of postmitotic neurons in these particular regions supports this explanation.

**Table 1. Comparison of BrdU labelling in the $PS1^{-/-}$ and control brains at E10.5**

<table>
<thead>
<tr>
<th></th>
<th>Percentage of BrdU-labelled cells</th>
<th>Percentage of BrdU-labelled cells</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>$PS1^{-/-}$</td>
</tr>
<tr>
<td>Telencephalon</td>
<td>83.6±12.4</td>
<td>82.3±12.6</td>
</tr>
<tr>
<td>Diencephalon</td>
<td>80.4±7.1</td>
<td>75.3±1.1</td>
</tr>
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The percentage of BrdU-labelled cells was determined by dividing the number of BrdU-positive cells by the total number of neural progenitor cells. The data shown represent an average of 1-2 sections from 2-3 brains per genotype. Values are means ± s.d.

The role of $PS1$ in apoptotic cell death during early neurogenesis

To determine whether increased apoptotic cell death might contribute to the reduction in the neural progenitor population observed in the $PS1^{-/-}$ brain, we assessed the number of apoptotic cells in the $PS1^{-/-}$ and control brains at E11.5 and E12.5. Apoptotic cells were detected by the TUNEL assay, which labels the 3'-OH termini of DNA strand breaks generated during DNA fragmentation (Gavrieli et al., 1992). Bisbenzimide staining was also used to confirm the identity of apoptotic cells, which contain condensed, clumped and/or fragmented nuclei (Deckwerth and Johnson, 1993). Similar numbers of apoptotic cells labelled by the TUNEL assay were detected in the telencephalon (Fig. 3) and diencephalon (data not shown) of the $PS1^{-/-}$ and littermate control brains at E11.5 (Fig. 3a,b) and E12.5 (Fig. 3c,d).

Quantitative comparison of the number of apoptotic cells in multiple comparable sections of the $PS1^{-/-}$ and littermate control brains at E11.5 and E12.5 revealed no significant differences (Table 2). A total of 42-50 apoptotic cells were detected in transverse sections of the entire telencephalon and diencephalon of both $PS1^{-/-}$ and control brains. In addition, the small percentages of apoptotic cells (approx. 0.6% for E11.5 and 0.3% for E12.5) cannot account for the marked reduction of the progenitor population in the $PS1^{-/-}$ brain. Likewise, no significant differences in the number of apoptotic cells were seen in cultured primary neural progenitor cells derived from the $PS1^{-/-}$ and littermate control telencephalons at E12.5 (data not shown). These results indicate that increased apoptosis is not a primary cause of the defective neurogenesis in $PS1^{-/-}$ brain, and that $PS1$ is not involved in the regulation of apoptosis during early neurogenesis.

**The role of $PS1$ in neuronal migration during early neural development**

The laminar architecture of the developing cerebral hemisphere in the $PS1^{-/-}$ brain is indistinguishable from that in the control brain. Likewise, no significant differences in the laminar architecture were detected in transverse sections of the entire telencephalon and diencephalon of both $PS1^{-/-}$ and control brains at E10.5 and E12.5. These results indicate that increased apoptosis is not a primary cause of the defective neurogenesis in $PS1^{-/-}$ brain, and that $PS1$ is not involved in the regulation of apoptosis during early neurogenesis.

**Table 2. Comparison of apoptosis in the $PS1^{-/-}$ and control brains**

<table>
<thead>
<tr>
<th>Embryonic stage</th>
<th>Genotype</th>
<th>Number of apoptotic cells</th>
</tr>
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<tbody>
<tr>
<td>E11.5</td>
<td>+/-</td>
<td>50±9</td>
</tr>
<tr>
<td></td>
<td>-/-</td>
<td>47±5</td>
</tr>
<tr>
<td>E12.5</td>
<td>+/-</td>
<td>42±3</td>
</tr>
<tr>
<td></td>
<td>-/-</td>
<td>44±6</td>
</tr>
</tbody>
</table>

For each genotype, the number of apoptotic cells represents an average of TUNEL-positive cells in the telencephalon and diencephalon of 2-3 adjacent sections per brain ($n=3-5$ brains). Values are means ± s.d.

There is no significant difference in the numbers of apoptotic cells in the $PS1^{-/-}$ and control brains at E11.5 ($P=0.68$) and E12.5 ($P=0.54$).
is disrupted in the $PS1^{-/-}$ brain, as indicated by the absence of the characteristic demarcations among the developing cortical plate, the intermediate zone, the ventricular and subventricular zones in the $PS1^{-/-}$ brain at E14.5 (Fig. 4b). The cortical plate in particular appears poorly defined and generally thinner in the $PS1^{-/-}$ brain (Fig. 4b), compared to wild type (Fig. 4a) (Shen et al., 1997). In addition, occasional neuronal heterotopias are present in the marginal zone of the developing cortex in the $PS1^{-/-}$ brain at E17.5 (data not shown), as previously reported (Hartmann et al., 1999). These results indicate that cortical neuronal migration is abnormal in the $PS1^{-/-}$ brain.

To investigate further the pattern of cortical neuronal migration in $PS1^{-/-}$ mice, we pulse-labelled newly generated progenitor cells and neurons with BrdU at E10.5 and analyzed their migration pattern at E14.5. Comparison of corresponding transverse sections of the $PS1^{-/-}$ brain (Fig. 4d) and the littermate control (Fig. 4c) revealed an increased number of brightly stained cells in the $PS1^{-/-}$ telencephalon (Fig. 4d). These brightly stained cells represent differentiated neurons that exited the cell cycle immediately following the pulse, thereby retaining all of the incorporated BrdU. In contrast, progenitor cells in the ventricular zone that have undergone multiple rounds of S and M phase following the pulse stain rather faintly due to dilution of the incorporated BrdU (Fig. 4c,d). This result indicates that greater numbers of neuronal cells in the $PS1^{-/-}$ brain exit the progenitor pool early to become postmitotic neurons, which is consistent with our results obtained with MAP2 immunostaining (Fig. 1). Furthermore, the BrdU-labelled neurons in the control telencephalon are localized appropriately within a well-defined cortical plate (Fig. 4c),
whereas the labelled neurons in the $PS1^{-/-}$ telencephalon are scattered diffusely across multiple cell layers (Fig. 4d), forming a disorganized cortical plate lacking a clear boundary with the adjacent intermediate and ventricular zones. These results support a role for PS1 in cortical layer formation and neuronal migration.

**Molecular basis of premature neuronal differentiation in $PS1^{-/-}$ mice**

To understand the mechanism underlying the premature neuronal differentiation observed in the $PS1^{-/-}$ brain, we examined the expression of genes in the Notch signalling pathway, which is known to be involved in cell-fate determination during neurogenesis in *Drosophila* (Artavanis-Tsakonas et al., 1999). The role of Notch receptors in murine neurogenesis is poorly understood. Mice lacking Notch1 or Notch2 function die at approximately E9 or E11, respectively, before cortical neurogenesis begins (Conlon et al., 1995; Hamada et al., 1999; Swiatek et al., 1994). An increased number of cells expressing proneural transcription factors, however, was identified in the midbrain and hindbrain of Notch1$^{-/-}$ mice (de la Pompa et al., 1997). The basic helix-loop-helix transcription factors Hes1 and Hes5 (mammalian hairy and Enhancer-of-split homolog), which are downstream effectors of the Notch signalling pathway, have been shown to regulate murine neuronal differentiation (Ishibashi et al., 1995, 1994; Kageyama and Nakanishi, 1997; Ohtsuka et al., 1999; Tomita et al., 1996). We therefore first examined
expression of Hes1 and Hes5 transcripts by in situ hybridization and northern analyses to determine whether their expression patterns and levels are affected in PS1−/− mice.

In situ hybridization analysis of comparable sections of PS1−/− and littermate control brains at E11.5 showed that expression of Hes5 is reduced in the PS1−/− brain (Fig. 5A). Consistent with previous studies (Akazawa et al., 1992), Hes5 expression is localized to the ventricular zone within each brain region. In the control brain, the expression level of Hes5 transcripts is higher in the ganglionic eminence (Fig. 5Ac) and diencephalon (Fig. 5Ad) than in the anterior telencephalon (Fig. 5Ad′) in the PS1−/− brain. Therefore, the reduction in the level of Hes5 transcripts is greater in the ganglionic eminence and diencephalon than in the anterior telencephalon. These regional differences in reduced Hes5 expression parallel the regional differences in premature neuronal differentiation observed at the same developmental stage, with a more severe phenotype in the ganglionic eminence and diencephalon than in the anterior telencephalon of the PS1−/− brain. In addition, the region of Hes5 expression is thinner, with fewer cell layers

in the PS1−/− diencephalon (Fig. 5Ad′), corresponding to the thinned ventricular zone in this region of the PS1−/− brain (Fig. 1Bf,h).

Despite the precautions we took to perform in situ hybridization of PS1−/− and littermate control brain sections simultaneously and under identical conditions, it is still a less quantitative method than northern analysis for comparing the expression level of mRNA transcripts in PS1−/− and control mice. Therefore, we also performed northern analysis using poly(A)+ RNA derived from the PS1−/− and littermate control heads at E11.5. The northern result showed an approximately 25% reduction in the level of the Hes5 transcript in the PS1−/− brain, confirming the downregulation of Hes5 expression in the absence of PS1 (Fig. 5B).

In situ hybridization analysis of Hes1 expression revealed a similar pattern and level of expression in the PS1−/− and control brains at E11.5 (data not shown). Consistent with a previous report (Sasai et al., 1992), Hes1 appears to be expressed in all cells of the embryonic brain with the highest expression in the progenitor cells of the ventricular zone. We also performed Northern analysis using poly(A)+ RNA derived from the PS1−/− and littermate control heads at E11.5, and the result showed similar levels of Hes1 transcripts in the PS1−/− and control brains, providing further confirmation of the expression levels.
that expression of Hes1 is unaffected in the PSI-/- brain (Fig. 5B).

In summary, the reduced expression of the Notch1 downstream target gene Hes5 suggests a downregulation of Notch signalling in the absence of PS1. These results also provide a molecular basis for the premature neuronal differentiation observed in the PSI-/- brain, as suggested by the finding that neural progenitor cells prematurely differentiate into postmitotic neurons in Hes5-/- mice (Ohtsuka et al., 1999). The fact that expression of Hes1 is unaltered in the PSI-/- mouse suggests that Hes1 and Hes5 expression might be regulated via different mechanisms.

It has been postulated in Drosophila that Notch and its ligand Delta are linked by a regulatory negative feedback loop under the transcriptional control of the Enhancer-of-split and achaete-scute complex gene products (Heitzler et al., 1996; Heitzler and Simpson, 1993, 1991). Expression of Dll1, which encodes a ligand of Notch1, is upregulated in the Notch1-/- mouse embryo (de la Pompa et al., 1997). We therefore examined expression of Dll1 to determine whether it is upregulated in the PSI-/- brain. In situ hybridization analysis revealed that Dll1 expression is confined to isolated cells in the ventricular zone, consistent with previous reports (Bettenhausen et al., 1995; Henrique et al., 1995). At E11.5, the number of Dll1-expressing cells is increased in the PSI-/- brain in all brain regions examined, including the anterior telencephalon (Fig. 6Aa,b) and diencephalon (Fig. 6Ac.d). Quantitative comparison of Dll1-expressing cells in the telencephalon revealed a 40% increase in the density of Dll1-expressing cells in the PSI-/- neuroepithelium (Fig. 6B).

Northern analysis of total RNA derived from the PSI-/- and control heads at E11.5 showed a 30% increase in the level of Dll1 transcripts, providing further evidence for the downregulation of Notch signalling.

To determine whether PSI regulates Notch signalling at the level of transcription, translation and/or post-translational activation, we examined the PSI-/- and control brains for differences in the levels of Notch1 expression and subcellular localization (Fig. 7). In situ hybridization analysis of comparable brain sections of the PSI-/- and littermate control at E11.5 revealed no significant differences in the expression pattern and the level of Notch1 transcripts in the anterior telencephalon (Fig. 7Aa,b), ganglionic eminence (Fig. 7Ac.d) and diencephalon (Fig. 7Ac.f). Northern analysis confirmed that the level of the Notch1 transcript is unaltered in the PSI-/- brain (Fig. 7B). Immunohistochemical analysis using a polyclonal antiserum (Logeat et al., 1998) raised against the ICD of mouse Notch1 showed intense Notch1 immunoreactivity in the ventricular zone, particularly in the ependymal cell layer and the adjacent progenitor cell layers (Fig. 7C). Notch1 immunoreactivity is localized predominantly to the cytoplasm and associated with the plasma membrane, although very low levels of nuclear Notch1 immunoreactivity could be detected in some progenitor cells (Fig. 7C). Comparison of corresponding sections of the PSI-/- and control brains revealed...
no differences in the intensity of cytoplasmic and plasma membrane associated Notch1 immunoreactivity in the telencephalon and diencephalon (Fig. 7C). These results indicate that Notch signalling is unlikely to be regulated by PS1 at the levels of transcription and translation, thus most likely to be regulated at the level of post-translational activation. These findings are consistent with previous studies by us and other groups using cultured $PS1^{-/-}$ cells, which showed a requirement for PS1 in the normal production of NICD (De Strooper et al., 1999; Song et al., 1999).

Expression of Notch1 and Dll1 transcripts in the presomitic mesoderm of $PS1^{-/-}$ embryos

Previously, Wong et al. (1997) reported that expression of Notch1 and Dll1 transcripts is markedly reduced in the presomitic mesoderm of $PS1^{-/-}$ embryos. We observed increased Dll1 expression and unchanged Notch1 expression in the $PS1^{-/-}$ brain, however, raising the possibility that expression of Notch1 and Dll1 is regulated differently in the developing CNS and mesoderm. To address this issue, we examined the expression of Notch1 and Dll1 in the presomitic mesoderm in the $PS1^{-/-}$ and littermate control embryos by whole-mount in situ hybridization and northern analyses.

Whole-mount in situ hybridization analysis of $PS1^{-/-}$ and littermate control embryos at day 9.5 showed that Notch1 and Dll1 transcripts are expressed at highest levels in the presomitic mesoderm (Fig. 8A), consistent with previous reports (Bettenhausen et al., 1995; Franco Del Amo et al., 1992; Reaume et al., 1992). As we previously described (Shen et al., 1997), the somites in the caudal region of the $PS1^{-/-}$ embryo are disorganized and the boundaries between these somites are blurred (Fig. 8Ab,d). However, similar levels of Notch1 and Dll1 transcripts were detected in the presomitic mesoderm, which appears to be at its appropriate position (Fig. 8A). Because whole-mount in situ hybridization does not permit quantitative comparisons of mRNA expression levels, we carried out northern analysis to examine the levels of Notch1 and Dll1 transcripts. Northern analysis using total RNA derived from the tail buds of $PS1^{-/-}$ and control embryos at day 11.5, where Notch1 and Dll1 are expressed at their highest level, revealed similar levels of Notch1 transcripts and increased levels of Dll1 transcripts in the $PS1^{-/-}$ embryo relative to the control (Fig. 8B). These results indicate that regulation of Notch signalling does not differ in the CNS and paraxial mesoderm.

Fig. 7. Expression of Notch1 in the $PS1^{-/-}$ and littermate control brains. (A) In situ hybridization analysis of the expression level and pattern of Notch1 using comparable transverse sections (10 μm) of the $PS1^{-/-}$ (b,d,f) and littermate control (a,c,e) brains at E11.5. The levels of the Notch1 transcript in the $PS1^{-/-}$ brain are similar to those of the control in the telencephalon (a,b), ganglionic eminence (c,d) and diencephalon (e,f). Abbreviations as in Fig. 1. (B) Northern analysis of Notch1 expression. Total RNA was prepared from $PS1^{-/-}$ and littermate control (+/+,-/-) brains at E12.5, and hybridized with a 260 bp Notch1 cDNA probe, which was generated by PCR amplification. The same blot was then hybridized with a control probe, G3PDH, to normalize the amounts of mRNA in each lane. Similar levels of Notch1 transcripts are observed in $PS1^{-/-}$ and littermate control brains. (C) Confocal microscopic images of Notch1 immunostaining in the telencephalon (a,b) and diencephalon (c,d). Comparable transverse sections (10 μm) of the $PS1^{-/-}$ and littermate control brains at E11.5 were stained with a polyclonal antiserum raised against the mouse Notch1 ICD. The Notch1 immunoreactivity is similar in the $PS1^{-/-}$ and control brains, and is strongest in the ependymal cell layer and deep layers of the ventricular zone, with gradually weaker reactivity in the more superficial layers of the ventricular zone. The intense Notch1 immunostaining is localized to the cytoplasm and plasma membrane. Bars, 400 μm (A) and 50 μm (C).
DISCUSSION

Our previous characterization of PS1−/− mice documented specific defects in central nervous system development, revealing a function for PS1 in the mammalian brain (Shen et al., 1997). Here we characterize the mechanisms underlying the progressive reduction in neural progenitor cells that we observed in the PS1−/− brain, as depicted schematically in Fig. 9. Analysis of the PS1−/− brain between E10.5 and 13.5 revealed increased numbers of postmitotic neurons detected by MAP2 (Fig. 1) and TuJ1 (data not shown) immunoreactivity in the telencephalon and diencephalon. The premature neuronal differentiation in the PS1−/− brain leads to a reduction in the neural progenitor population and thinning of the ventricular zone. This is particularly evident in the diencephalon, where increased MAP2 immunoreactivity at E10.5 (Fig. 1Ab) is followed by decreased BrdU immunoreactivity at E11.5 (Fig. 2Bf). These results demonstrate that lack of PS1 function leads to an increase in the number of differentiatated postmitotic neurons at the expense of progenitor cells during early neurogenesis, resulting in a partial depletion of the neural progenitor population. These observations provide an explanation for the progressive reduction of the neural progenitor population and the subsequent reduction of neuronal population previously observed in the PS1−/− brain (Shen et al., 1997). Taken together, these findings support a novel role for PS1 in the regulation of neuronal differentiation and a cell-fate decision between neural progenitor cells and postmitotic neurons in the developing brain.

We further investigated the role of PS1 in neuronal proliferation, survival and migration during early neural development. The similar proliferation rates of neural progenitor cells in the PS1−/− and control brains, as measured by the percentage of BrdU-labelled cells to total progenitor cells, suggest that PS1 is not involved in the regulation of neural progenitor proliferation (Fig. 2 and Table 1). Our analysis of the PS1−/− brain at E11.5 and 12.5 also shows that lack of PS1 does not result in increased apoptotic cell death at these early neural developmental stages (Fig. 3 and Table 2). Severe neuronal loss was, however, observed in specific regions of the PS1−/− telencephalon beginning at E16.5 (Shen et al., 1997). Thus, it remains possible that PS1 influences neuronal survival during later stages of neurogenesis.

An involvement of wild-type and FAD-linked mutant presenilins in regulation of apoptosis has been suggested by previous investigations in various cell culture systems (Deng et al., 1996; Guo et al., 1996; Kim et al., 1997; Loetscher et al., 1997; Roperch et al., 1998; Vito et al., 1996; Wolozin et al., 1996; Zhang et al., 1998). The Notch signaling pathway has also been implicated in the regulation of apoptosis, with Notch activity appearing to promote cell survival. Loss of Notch activity in Drosophila is spatiotemporally correlated with increased levels of apoptosis (Kim et al., 1996; Van Doren et al., 1992). In mice lacking Notch2 function, increased apoptosis was observed in neural tissues (Hamada et al., 1999). Finally, a connection between Presenilin and Notch activity in apoptosis was suggested by the finding that mutant flies either overexpressing or lacking PS exhibited increased levels of apoptotic cell death; this phenotype was suppressed in both cases by expression of constitutively active Notch (Ye and Fortini, 1999). It is not yet clear whether the loss of neural progenitor cells and neurons observed in the PS1−/− brain is
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Notch ICD, reduced pool through continued proliferation. In the absence of PS1, Notch signalling activity is diminished, leading to decreased levels of nuclear postmitotic neurons, which migrate superficially to the intermediate zone, while the remainder produce a steady expansion in the progenitor activation of the Notch signalling pathway. A small proportion of neural progenitor cells residing in the ventricular zone gives rise to Thick arrows and bold type represent relative increases. In the presence of PS1, the proliferation of neural progenitor cells is favored by neuronal loss by generating conditional PS1 function specifically in neuronal cell types.

Investigating the mechanism underlying this region-specific neuronal loss by generating conditional PS1−/− mice lacking PS1 function specifically in neuronal cell types.

The results of our neuroanatomical and BrdU birthdating analysis support a role for PS1 in the regulation of neuronal migration during cortical neurogenesis. Examination of the PS1+/− brain stained with Hematoxylin and Eosin suggested disruption of the normal architecture of the developing forebrain, with an indistinct boundary between the ventricular and intermediate zones and thinning of the cortical plate (Fig. 4). The BrdU birthdating study revealed disorganization of postmitotic neurons in the developing cortical plate (Fig. 4). However, our birthdating analysis was limited to PS1+/− brains at E14.5, due to severe cerebral hemorrhage often associated with PS1−/− brains at later developmental stages. Therefore, whether cortical lamination defects in the PS1−/− brain are similar to those seen in the reeler mouse is unclear. Extensive BrdU birthdating analysis on PS1 conditional knockout mice, in which PS1 inactivation is restricted to the neuronal population, should address this question. The mechanism by which PS1 regulates cortical layer formation and neuronal migration is unknown. The perturbed laminar organization may be associated with the progressive loss of Cajal-Retzius neurons observed in the PS1−/− brain; however, the cause of the progressive loss of these neurons is unclear. It is also possible that PS1 is an upstream regulator or a downstream target of Cdk5/p35 or mDab1, both of which play an important role in neuronal migration (Chae et al., 1997; Howell et al., 1997; Ohshima et al., 1996). Reduced Notch signalling in PS1−/− mice may also contribute to the neuronal migration defects, as suggested by the findings in Drosophila that Notch genetically interacts with Abl and its intracellular domain binds directly to Dab in vitro (Giniger, 1988).

To understand the mechanism by which PS1 controls neuronal differentiation during neurogenesis, we examined the expression of genes involved in the Notch signalling pathway: Notch1, Dll1 and the Notch downstream effector genes Hes1 and Hes5. Previous studies have suggested a connection between Notch signalling and the regulation of neuronal differentiation. Premature neuronal differentiation has been observed at E10.5 in mutant mice lacking Hes1, Hes5, or both Hes1 and Hes5 (Ishibashi et al., 1995; Ohtsuka et al., 1999). Here we have shown that expression of Hes5 transcripts is downregulated in the PS1−/− brain, particularly in the ganglionic eminence and diencephalon, whereas expression of Hes1 is unaffected (Fig. 5). These results suggest that Notch signalling is compromised in the absence of PS1, as evidenced by the downregulation of Hes5 expression. In addition, regulation of Notch downstream target genes appears to be complex, since PS1 regulates specifically the expression of Hes5 but not Hes1. It is not yet clear whether Hes5 is the only downstream target of PS1 in the regulation of neuronal differentiation during neurogenesis. The extent of the premature differentiation of neural progenitor cells observed in the PS1−/− and Hes5−/−
brains at E10.5 appears to be similar (Fig. 1; Ohtsuka et al., 1999). Examination of the PS1+/- and Hes5+/- brains at later embryonic stages for differences in neuronal differentiation and neurogenesis might reveal whether PS1 controls neuronal differentiation and cell fate decision between progenitor cells and postmitotic neurons through the regulation of Hes5.

Further supporting evidence for the downregulation of Notch signalling includes the upregulation of Dll1 expression in PS1+/- mice. Our results demonstrated that the level of Dll1 transcripts is elevated in the brain and the presomitic mesoderm of PS1+/- mice and more Dll1-expressing cells are present in the PS1+/- brain (Figs 6, 8). Studies in Xenopus and the chick embryo have shown that Dll1 homologues are expressed in the prospective neurons and their expression precedes expression of early neuronal markers (Chitnis et al., 1995; Henrique et al., 1995). An increase in Dll1-expressing cells is consistent with the presence of more differentiated neurons identified in the PS1+/- brain.

Lack of PS1 function was previously reported to result in reduced transcription of Notch1 and Dll1 in the presomitic mesoderm of the PS1+/- embryo, suggesting a role for PS1 in the regulation of Notch1 and Dll1 at the transcriptional level (Wong et al., 1997). We detected unaltered levels of Notch1 transcripts (Figs 7, 8) and elevated levels of Dll1 transcripts (Figs 6, 8) both in the PS1+/- embryonic brain and presomitic mesoderm. Furthermore, we observed no differences in the intensity of cytoplasmic and plasma membrane associated Notch1 immunoreactivity in the PS1+/- and control brains (Fig. 7). Therefore, PS1 is unlikely to be involved in the regulation of Notch1 transcription and translation in the developing mammalian brain and paraxial mesoderm. However, PS1 is involved in the activation of Notch signalling, as indicated by the alterations of Hes5 and Dll1 expression in PS1+/- mice (Figs 5, 6, 8). These results are consistent with recent studies showing that similar levels of furin-cleaved Notch1 fragment were detected in the PS1+/- and control mouse brains by immunoprecipitation-western analysis (De Strooper et al., 1999). Analysis of the proteolytic processing of truncated Notch1 proteins in cultured cells derived from PS1+/- mice has shown that PS1 is required for efficient release of the Notch1 ICD (De Strooper et al., 1999; Song et al., 1999). The present study supports such a role for PS1 in the regulation of Notch signalling, and further provides evidence that the reduced Notch processing observed in PS1+/- cells is functionally significant in neural development.

Finally, our results indicate an important difference in the consequences of reduced Notch signalling during neurogenesis in Drosophila and mice. In Drosophila neurogenesis, Notch controls a cell-fate decision between two cell types produced from a multipotent common precursor, promoting epidermal production at the expense of neuronal production. Loss of function mutations in Notch thereby lead to excessive neuronal production (Artavanis-Tsakonas et al., 1999). Our findings suggest that Notch1 regulates a cell-fate choice between neural progenitor cells and differentiated neurons early in murine neurogenesis, promoting regeneration of neuronal precursor cells at the expense of differentiation of postmitotic neurons. Therefore, although Notch functions to suppress the production of postmitotic neurons in both mice and Drosophila, downregulation of Notch activity in mice and Drosophila results in a reduction in neuronal population and a neurogenic phenotype, respectively.

In summary, we demonstrate that lack of PS1 during neural development results in a reduction in Notch signalling activity, as indicated by reduced Hes5 expression and increased Dll1 expression, which alters the cell-fate decision of neural progenitor cells in favor of differentiation into postmitotic neurons (Fig. 9). The roles of PS1 and the Notch signalling pathway in the adult brain are currently unknown. A recent report documented the existence of Notch1-expressing neural stem cells residing in the ependymal cell layer of the adult rat brain (Johansson et al., 1999), which suggests that Notch signalling may be involved in the maintenance of a neural stem cell population in the adult brain. Understanding the roles of PS1 and Notch signalling in the adult brain may therefore lead to novel therapeutic strategies for combating the neuronal and synaptic loss that occurs in neurodegenerative illnesses such as Alzheimer’s disease.

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REFERENCES


Franco Del Amo, E., Smith, D., Swiatek, P., Gendron-maguire, M.,
Genes of the Enhancer of split and achaete-scute complexes are required for spatial regulation of proneural gene activity: auto- and cross- signalling by Notch and Delta during lateral signalling in Drosophila [see comments].

Structure and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Identification of the neural precursor cell-specific enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix transcription factors, premature neurogenesis, and severe neural tube defects. 

Helix-loop-helix factors provide evidence for a role of Notch as a receptor in cell fate decisions. Development 117, 1113-1123.


Proteolytic release and nuclear translocation of notch-1 are induced by presenilin-1 and impaired by pathogenic presenilin-1 mutations. Proc. Natl. Acad. Sci. USA 96, 6959-6963.

Skeletal and CNS defects in presenilin-1 deficient mice. Cell 89, 629-639.


Notch1 is essential for postimplantation development in mice. Genes Dev. 8, 707-719.

The cell cycle of the pax2a-expressing ventricular epithelium of the embryonic murine cerebral wall. J. Neurosci. 15, 6046-6057.

Spatial regulation of proneural gene activity: auto- and cross-activation of achaete is antagonized by extramicrocactaeae. Genes Dev. 6, 2929-2605.


Neurogenic phenotypes and altered notch processing in Drosophila Presenilin mutant. Genes 98, 525-529.

Nature 395, 698-702.