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Notch Signaling Is Required to Maintain All Neural Stem Cell Populations – Irrespective of Spatial or Temporal Niche

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Presenilin · Retinal stem cell · Self-renewal · Symmetric division · Asymmetric division · Cell cycle time

Abstract
Recently, Notch signaling has been reported to underscore the ability of neural stem cells (NSCs) to self-renew. Utilizing mice deficient in *presenilin-1 (PS1),* we asked whether the function of Notch signaling in NSC maintenance was conserved. At embryonic day 14.5, all NSCs – both similar (cortex-, ganglionic eminence- and hindbrain-derived) and distinct (retinal stem cell) – require Notch signaling in a gene-dosage-sensitive manner to undergo expansionary symmetric divisions, as assessed by the clonal, in vitro neurosphere assay. Within the adult, however, Notch signaling modulates cell cycle time in order to ensure brain-derived NSCs retain their self-renewal property. At face value, the effects in the embryo and adult appear different. We propose potential hypotheses, including the ability of cell cycle to modify the mode of division, in order to resolve this discrepancy. Regardless, these findings demonstrate that *PS1,* and presumably Notch signaling, is required to maintain all NSCs.

Introduction

The ultimate precursor cell of the brain is considered to be the neural stem cell (NSC) [1, 2]. Although all NSCs are multipotent and can self-renew, they differ along the neural axes in their molecular profile, suggesting that they are regionally specified [3]. NSCs have been demonstrated to retain their regional identity in vitro; this 'address', however, is labile [4]. These data imply that there is an inherent plasticity in embryonic NSCs located along the anteroposterior and dorsoventral axes of the mammalian brain; furthermore, NSCs are competent to respond to local inductive cues. Generally, we assume that the local environment can regulate differentiation. Whether or not it directly underscores the common stem cell characteristic of self-renewal is unknown.

Components of the Notch signaling pathway have been implicated in mediating a key NSC behavior: self-renewal [5–9]. In fact, an intact Notch pathway is required for both embryonic and adult forebrain NSCs to retain this elusive characteristic. Utilizing *presenilin-1 (PS1)* mutants as a model for Notch signaling, Hitoshi et al. [7] demonstrated that a reduction in *PS1* – and hence, Notch signaling – reduced the capacity of ganglionic eminence-derived NSCs to undergo symmetrical self-renewing divisions. Within the adult, though, Notch signaling

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was shown to modify the cell cycle time of the adult forebrain NSC. Not only does this underlie the importance of Notch signaling in NSC self-renewal, but it also indicates a conspicuous difference between embryonic and adult NSCs. This distinction can be explained in one of two ways: modifications to the cell cycle may alter the mode of division, which alleviates the inconsistency, or the function of Notch signaling may be dependent on the niche/environment in which it is acting. In fact, others have reported that the influence of Notch signaling on gliogenesis [10], proliferation [11–13], and self-renewal [14] differs depending on the environmental context. This begs the question: is the function of Notch signaling in NSCs context-dependent?

Employing mice that have a deficiency in *PS1,* we asked whether Notch signaling is functionally conserved in NSCs that are located in different niches – that is, does the function of Notch signaling vary spatially and temporally? At embryonic day (E) 14.5, Notch signaling is critical for all NSCs to self-renew and to expand their populations by undergoing symmetric self-renewing divisions; this function is independent of the spatial context. Additionally, Notch signaling appears to mediate the self-renewal of adult NSCs by modifying the length of the cell cycle. These data demonstrate a functional conservation of Notch signaling in NSCs, and imply a conservation of the mechanism by which stem cells self-renew.

Materials and Methods

Animals and Genotyping

The generation and genotyping of *PS1* mutant mice have previously been described [15, 16]. *PS1–/–* and their heterozygous and wild-type littermates were on a C57Bl6/129 hybrid background. In addition, a specified gestational age was utilized: the afternoon of the day the vaginal plug was observed was considered to be E0.5.

Dissections

Generating neurospheres from the embryonic brain has also been previously described [4, 17]. At E14.5, tissue was obtained from multiple regions of the embryonic brain: dorsal cortex (Ctx), ganglionic eminences (GE; both lateral and medial), and rostral hindbrain region (HB). Concurrent with the embryonic brain dissections, the eyes were excised from the head primordium and transferred to D-PBS. Subsequently, the eyes were placed into dispase (Collaborative Research) for 45 s at 37°C, facilitating removal of the extraocular tissue and separation of the retinal pigmented epithelium (RPE) from the neural retina (NR). RPE and NR were separately placed into serum-free media (SFM). Each tissue sample, whether brain-derived or retina-derived, was dissociated into a single cell suspension with the use of a small-bore, fire-polished Pasteur pipette. Cell suspensions were plated according to the cell culture conditions listed below.

Adult dissections have also been described for both, the brain and eye [18, 19]. For the brain, the medial and lateral portions of the lateral ventricle were dissected from both hemispheres; tissue samples were then subjected to an enzymatic digestion (1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid) for 50 min at 37°C. Concurrent with the brain dissections, the eyes were aseptically excised. Next, extracted ciliary marginal zones (the region where the RPE is thrown into a series of folds at the periphery of the retina) were placed into dispase (Collaborative Research) for 10 min at 37°C, followed by an additional 15-min enzymatic treatment (contents as per the adult brain) at 37°C. After the enzyme exposure, all tissue samples were transferred to SFM containing 1 mg/ml trypsin inhibitor (Roche Diagnostics) and triturated with a small-bore, fire-polished Pasteur pipette to acquire a single cell suspension. Plating conditions are listed below.

Cell Culture

Cells were cultured in the NSC colony-forming assay called the neurosphere assay [18–20]. Briefly, cells from either the embryonic brain, adult brain, or adult ciliary marginal zone were plated at a low cell density of 10 cells/μl – ensuring clonality of the spheres – in 24-well (0.5 ml/well, 1.9 cm^2 per well) uncoated plates (Nunclon) in SFM containing either 10 ng/ml FGF2 (human recombinant; Sigma) and 2 μg/ml heparin (Sigma) (F + H), or 10 ng/ml FGF2, 2 μg/ml heparin and 20 ng/ml EGF (mouse submaxillary; Sigma) (E + F + H). Embryonic RPE, however, were plated at half an eye per well in a 24-well plate in the presence of F + H. After a week in vitro, the presence of a stem cell was demarcated by the presence of a free-floating, clonally derived neurosphere; thus, the number of neurospheres served as an in vitro index of the in vivo stem cell population.

In order to ascertain the capacity of the NSCs and retinal stem cells (RSCs) to self-renew and expand in vitro, the free-floating primary neurospheres were subcloned or passaged: dissociating the sphere into a single cell suspension and reculturing for 1 week. For single sphere passaging, individual neurospheres of an equivalent size were mechanically dissociated and plated in the presence of SFM containing E + F + H in an uncoated, 48-well (0.3 ml/well; 1.1 cm^2 per well) Nunclon plate. Additionally, adult-derived neurospheres were passaged in bulk and plated in the presence of E + F + H at a low cell density of 10 cells/μl. At each passage, trypan blue exclusion was used to determine the percentage of viable cells. In contrast, the RSC-derived spheres (i.e. from either the ciliary marginal zone of the adult or the RPE of the embryo) required a previously described enzymatic treatment for dissociation [19]. The enzymatic treatment contains the same enzymes as per the dissection procedures, with the addition of collagenase (1 mg/ml; Sigma). Retinal spheres remained in this enzymatic solution for approximately 1 h. Following the treatment, enzymes were inhibited with SFM containing trypsin inhibitor (1 mg/ml; Roche Diagnostics). Subsequently, the cell suspension was mechanically dissociated using a small-bore, fire-polished Pasteur pipette, and plated in media containing F + H at a density of 10 cells/μl.

Spheres can also be obtained from a non-stem-cell-containing region: the embryonic NR. These spheres (derived by plating E14.5 NR at a density of 20 cells/μl in SFM containing F + H) are not able to self-renew – and hence, passage – in vitro. Passing is, therefore, required to determine whether a sphere is, indeed, stem-cell-derived, rather than progenitor-derived.

Real-Time PCR

Total RNA was isolated using the RNeasy extraction kit (Qiagen). One microgram of the total RNA was used with random hexamer primers (Fermentas) and MoMLV reverse transcriptase (Superscriptase III, Invitrogen) in a 20-μl first-strand cDNA synthesis reaction carried out at 42°C for 60 min. The 20-μl PCR mixture consisted of 0.25 μl cDNA, 0.1 μ*M* of each of 5′ and 3′ primers, and SBYR green PCR master mix (Applied Biosystems). Real-time PCR was run on an ABI Prism SDS 7000 with the following program: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The sense and antisense primers used were as follows: *Hes1,* sense 5′-CCAGCCAGTGTCAACACGA-3′; antisense 5′-AATGCCGGGAGCTATCTTTCT-3′; *Hes5,* sense 5′-AGTCCCAAGGAGAAAAACCGA-3′; antisense 5′-GCTGTGTTTCAGGTAGCTGAC-3′, and HPRT, sense-5′-AAACAATGCAAACTTTGCTTTCC-3′; antisense 5′-GGTCCTTTTCACCAGCAAGCT-3′. For comparative quantification, the $\Delta\Delta C_T$ value was calculated as follows. First, the ΔC_T for each animal was assessed. In a second step, Notch signaling pathway genes were normalized to HRPT (calculated as $\Delta\Delta C_T$) per animal. Next, the $\Delta\Delta C_T$ was calculated per genotype, which was examined by Student's t test. Finally, the data were calibrated to *PS1+/–*.

Table 1. Notch signaling is reduced in adult-derived *PS1+/–* neurospheres relative to *PS1+/+*

Gene of interest	PS1+/–	PS1+/+	p value
Hes1	1 (0.84–1.19)	1.34 (1.10–1.63)	>0.05
	1 (0.64–1.57)	1.19 (1.00–1.41)	>0.05
Hes5	1 (0.90–1.11)	1.83 (1.59–2.11)	<0.01
	1 (0.90–1.11)	1.26 (1.14–1.39)	<0.05

Summary of the real-time PCR analyses for two Notch signaling pathway targets, *Hes1* and *Hes5.* The levels of expression were compared between adult-derived neurospheres from six animals (n = 3 *PS1+/+,* n = 3 *PS1+/–*). HRPT was used to normalize the samples (see methods for more details). Note that only *Hes5* consistently displayed a significantly higher level of expression in *PS1+/+* relative to *PS1+/–*. Figures in parentheses indicate ranges.

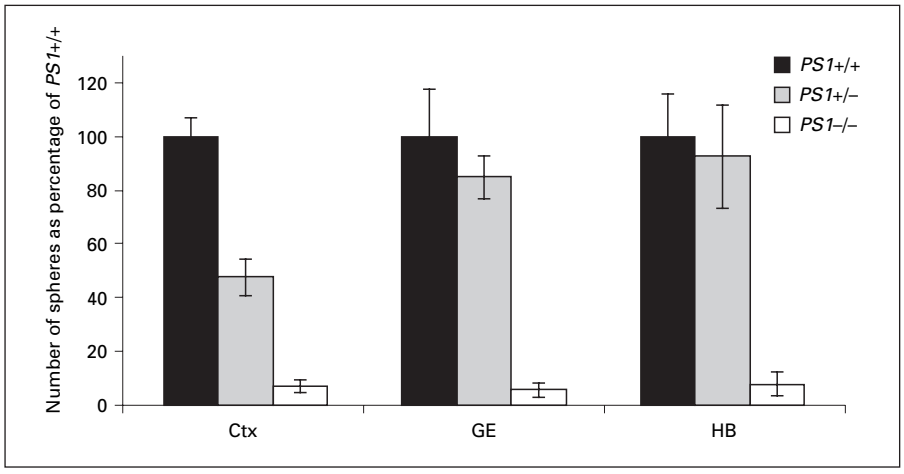
Results

At E14.5, NSCs from the GE, HB and Ctx of PS1 Mutants All Display a Diminished Capacity to Self-Renew, Which Is Sensitive to the Gene Dosage

Operationally, all NSCs possess the ability to proliferate, self-renew and give rise to multiple lineages; this behavior can be empirically tested in the colony-forming neurosphere assay. In order to ascertain whether or not Notch signaling is functionally conserved in NSC populations residing in distinct locations, we utilized mice that have a deficiency in the *PS1* gene [15, 16]. These *PS1* mutants serve as an ideal model to elucidate the role of endogenous Notch signaling because, unlike many other Notch pathway mutants [21, 22], the *PS1–/–* survive until birth. Moreover, both *PS1–/–* [7, 23] and *PS1+/–* (table 1) express a reduced level of *Hes5* (a target gene of Notch signaling) relative to *PS1+/+*, without an apparent change in the expression of *Hes1.* This profile – maintenance of *Hes1* with a reduction in *Hes5* – is consistent with two other Notch signaling pathway mutants: *Notch1–/–* and *RBP-Jκ–/–* [7, 24]. From a functional perspective, we have shown that these *PS1* mutants display a diminution in the ability of their FGF2-responsive NSCs to self-renew when generated from the E14.5 GE primordium [7]. Not only was this phenotype sensitive to the gene dosage, but it could also be (mostly) rescued by the overexpression of the active form of the Notch1 receptor. Together, these data are inconsistent with the notion that other known functions of the *presenilin* genes, such as the promotion of β-catenin degradation, account for the self-renewal deficit [25]. In fact, these findings demonstrate that the deficit was, indeed, brought about by reduced Notch signaling, and further support the use of *PS1* mutants as a model for Notch signaling.

A question remains: can the self-renewal deficit be recapitulated in other regions of the brain? To determine this, we generated FGF2-responsive neurospheres from germinal zones of three distinct neural compartments – Ctx, GE, and HB – in the *PS1* mutant brain, with each region differing in its position along the dorsoventral or anteroposterior axes. A significant interaction effect of region and genotype was revealed [$F(4, 68) = 7.30$, $p < 0.01$]. Post hoc analyses exposed a dramatic depletion in the in vivo NSC population: irrespective of the region of origin, approximately 95% fewer neurospheres were observed in the *PS1–/–* neural primordia relative to wild type (for each region $p < 0.01$, fig. 1). Conversely, the only *PS1+/–* region displaying a significant reduction in primary sphere formation relative to *PS1+/+* was the Ctx ($p < 0.01$). Follow-up analyses, moreover, established a significant depletion in the NSC population derived from the Ctx, relative to that derived from either the GE or HB of the *PS1+/–* mutant brain ($p < 0.01$ for each region). In contrast, there was not a significant difference in primary sphere formation between the GE and HB of the *PS1+/–* mutant ($p > 0.05$).

A reduction in primary sphere formation can be explained by a number of scenarios. First, loss of *PS1* renders the NSC incapable of proliferation. Inconsistent



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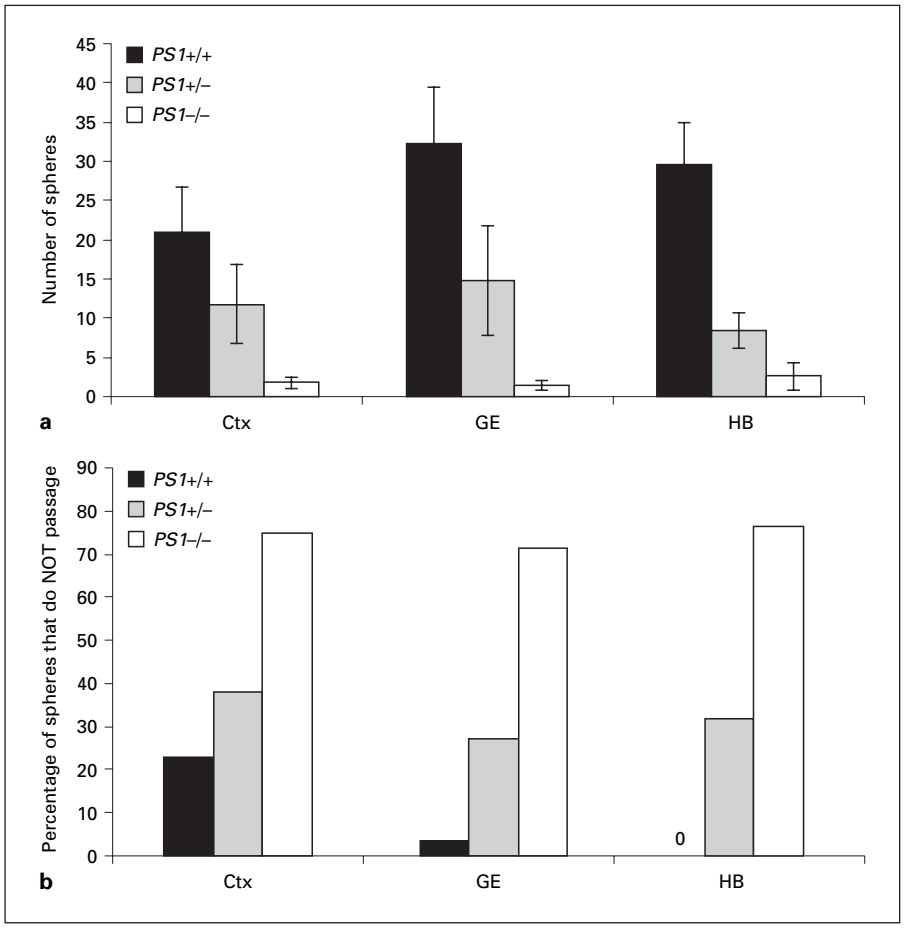

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Fig. 1. Despite spatial context or niche, the NSC population is reduced in the *PS1–/–* brains. Shown here are the FGF2-responsive NSCs isolated from the Ctx, GE, and HB germinal zones of E14.5 *PS1+/+*, *PS1+/–*, and *PS1–/–* embryos (n = 6–18 per genotype per region). Comparatively, the 95% depletion exhibited in the Ctx of *PS1–/–* relative to *PS1+/+* is also displayed by the GE and HB (p values <0.01). In contrast, the *PS1+/–* Ctx shows an additional decrement in the number of neurospheres and hence, NSCs (52%, $p < 0.01$) relative to *PS1+/+*. This depletion in the *PS1+/–* Ctx was significantly different relative to either the *PS1+/–* GE or HB (p values <0.05). Conversely, no decrement in neurosphere formation was evident in the GE or HB germinal zones from *PS1+/–* relative to *PS1+/+* controls. These results are depicted as means ± SEM.

Fig. 2. Loss of *PS1* results in a reduced capacity of FGF2-responsive NSCs to self-renew and expand in vitro: this reduction is sensitive to the gene dosage, and independent of the spatial context. **a** Self-renewal was assessed by manually dissociating single FGF2-responsive primary neurospheres of approximately similar sizes, and reculturing in the presence of E + F + H. Secondary sphere formation is depicted for Ctx-derived, GE-derived, and HB-derived neurospheres (n = 17–65 per genotype per region). Both, *PS1–/–* and *PS1+/–* spheres (p values <0.01), demonstrate a decrement in the ability of the NSC to undergo expansionary symmetric divisions relative to *PS1+/+* controls, irrespective of the spatial context. **b** The aforementioned data are re-expressed as the percentage of single primary neurospheres that do not form secondary spheres upon reculturing. These data demonstrate an increase in the number of terminal symmetric divisions, resulting in the loss of self-renewal (see results for statistics); this loss occurs regardless of the spatial context or niche in which the NSC resides. Results are expressed as means ± SEM.

with this interpretation, the neurospheres derived from *PS1–/–* mutants were equivalent in size relative to wild type. Furthermore, spheres (or clumps) below our criteria of 100 μm were not present (data not shown). These data, thus, invalidate arguments that suggest a decrease in proliferation or survival. Second, a diminution in sphere formation may be achieved in a non-cell-autonomous manner; that is, a deficiency in Notch signaling may compromise another cell population, subsequently altering the NSC behavior in vivo. Third, a homozygous mutation in

the *PS1* gene may directly alter the capacity of NSCs to self-renew, which would be evident by a reduction in secondary sphere formation. To distinguish between the latter two possibilities, we subcloned single primary neurospheres of similar size and determined the number of new secondary spheres that grew 7 days later in the presence of E + F + H (secondary neurospheres, like adult-derived neurospheres, are responsive to both FGF and EGF). Figure 2a shows a gene-dosage effect on the ability of NSCs to self-renew in vitro that is independent of the spatial context. Neither a significant interaction effect of region and genotype, nor a main effect of region [F values <1.0, p values >0.05] were evident. There was, however, a significant main effect on genotype [$F(2, 310) = 13.95$, $p < 0.01$]. Post hoc examinations displayed a significant reduction in the number of secondary spheres that formed not only from the *PS1–/–* ($p < 0.01$), but also from the *PS1+/–* relative to wild-type littermate controls ($p < 0.01$). Moreover, there was a significant depletion in sphere formation from the *PS1–/–* relative to *PS1+/–* ($p < 0.05$). These data demonstrate that in all cases, the ability of NSCs to self-renew and expand is susceptible to the loss of *PS1* in a cell-autonomous manner, which occurs in a gene-dosage-sensitive manner.

In addition to assessing the capacity of the NSC to self-renew and proliferate, the process of subcloning establishes the mode of division by which the NSC fulfills this critical behavior. The presence of at least two secondary neurospheres proves that the original stem cell, upon generating the primary sphere, undergoes at least one symmetrical division [20]. We call this type of division an expansionary symmetric division. Alternatively, a reduction in secondary sphere formation could be generated by either an increase in asymmetric divisions (a steady-state mode of division) or an increase in terminal symmetric divisions. This latter mode of division results in the loss of the stem cell fate (as assessed by the lack of sphere formation) with the concomitant production of two progenitor cells. A reduction in sphere formation is, therefore, not necessarily synonymous with a decline in self-renewal capacity: an increase in asymmetric divisions will decrease the number of secondary spheres that form, but fulfills the self-renewal criteria.

In order to determine whether reduced *PS1,* and presumably Notch signaling, resulted in a loss of self-renewal, we reexpressed the passaging data as the percentage of spheres that do not passage (fig. 2b). A χ^2 test revealed an overall significant effect between conditions ($\chi^2 = 181.80$, $n = 320$, $p < 0.01$). First, post hoc analyses comparing genotypes within a particular region revealed a significant increase in the percentage of spheres that did not passage from the *PS1–/–* NSCs relative to wild-type controls in all regions investigated: Ctx ($\chi^2 = 27.33$, $n = 93$, $p < 0.01$), GE ($\chi^2 = 22.23$, $n = 42$, $p < 0.01$), and HB ($\chi^2 = 27.69$, $n = 42$, $p < 0.01$). There was also a significant loss of self-renewal in the *PS1+/–* NSCs residing in the Ctx ($\chi^2 = 5.81$, $n = 123$, $p < 0.05$), GE ($\chi^2 = 6.5$, $n = 72$, $p < 0.05$) and HB ($\chi^2 = 9.87$, $n = 66$, $p < 0.05$) relative to the wild type. Additionally, a significant effect was exposed between the *PS1–/–* NSC relative to *PS1+/–* in the Ctx ($\chi^2 = 10.38$, $n = 86$, $p < 0.01$), GE ($\chi^2 = 8.80$, $n = 58$, $p < 0.01$), and HB ($\chi^2 = 9.74$, $n = 58$, $p < 0.01$). These data show that, with a decrease in *PS1,* there is an increase in the loss of the stem cell fate.

Digressing from the *PS1* mutation, there is an additional effect that is evident in the wild-type-derived spheres and is, therefore, independent of *PS1.* A significant effect is unveiled when comparing the *PS1+/+* Ctx-derived NSCs relative to either the *PS1+/+* GE-derived ($\chi^2 = 3.98$, $n = 95$, $p < 0.05$) or the *PS1+/+* HB-derived NSCs ($\chi^2 = 5.33$, $n = 90$, $p < 0.05$). This difference in wild-type self-renewal, however, was not evident between the GE and HB ($\chi^2 = 0.91$, $n = 53$, $p > 0.05$). In accordance with this, the Ctx has been shown to contain a sphere-producing progenitor population that possesses a modest level of self-renewal capacity [26]. Thus, the Ctx has a reduced propensity for self-renewal that is independent of the *PS1* mutation.

All together, our results show that there is a correlation between the loss of self-renewal capacity and the loss of *PS1*: a reduction in *PS1* occurs concomitantly with a reduction in self-renewal capacity. We suggest that this loss is achieved by an increase in the number of terminal symmetric divisions. Consistently, *PS1–/–* (and a positive trend in *PS1+/–*) GE-derived neurospheres were previously shown to contain more differentiated cells (neurons and astrocytes) [7]. This is of particular importance as it implies that the NSCs, rather than dying, underwent a terminal symmetric division (which yielded two progenitor cells at the expense of the NSC fate). Arguably, loss of the stem cell fate can be accredited to, rather than a change in the self-renewing division mode, a decrease in NSC survival. However, a decrease in NSC survival is less likely given the comparable numbers of primary neurospheres (excluding the Ctx) generated in the *PS1+/–* relative to *PS1+/+*. In this scenario, the hypothetical survival challenge would have to have been acquired during secondary sphere formation as it was clearly not present during primary sphere formation. These data are, thus, in agreement with an increase in terminal symmetric di-

visions underpinning the loss of the NSC fate. The remaining deficit (both *PS1–/–* and *PS1+/–* relative to *PS1+/+*, p values <0.05, data not shown) – not accounted for by *PS1* mutant-derived spheres failing to generate secondary spheres – can be accredited to either more terminal symmetric divisions or an increase in asymmetric divisions. Regardless, Notch signaling clearly plays a role in maintaining the NSC fate, and this function, moreover, appears to be conserved between all regions investigated.

E14.5 RSCs Are Reduced in PS1*–/– Mice, and Those Present Have a Reduced Capacity to Self-Renew*

A population of stem cells can be isolated from the embryonic and adult mammalian retina [19]. Specifically, these RSCs reside in the RPE (most peripheral portion) during embryogenesis and, later on in adulthood, the ciliary marginal zone. Like the NSCs from the brain, RSCs are also characterized by their ability to self-renew and their multipotency. However, these RSCs are distinct from those residing in the brain [19, 27, 28]. This provides an alternative population of stem cells to determine whether the extent of Notch signaling is functionally conserved within stem cells at E14.5.

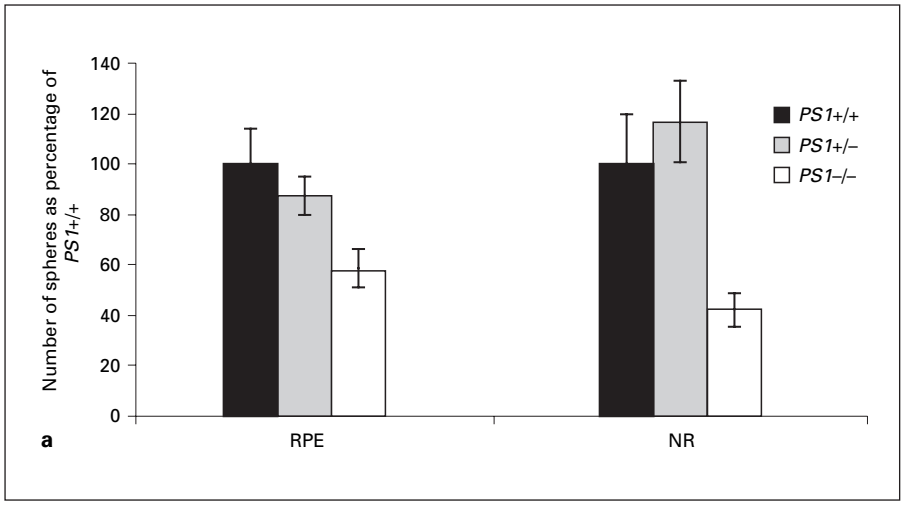
We isolated retinal spheres in the presence of FGF2 from the *PS1* mutants to determine whether this loss elicited a deficit similar to the loss found in the brain. Interestingly, the RSC result was marginal in comparison to the brain-derived NSC reductions in the *PS1–/–* (fig. 3a). A significant interaction effect of region – retina and GE – and genotype was unveiled [$F(2, 38) = 20.85$, $p < 0.01$]. Post hoc analyses established that the *PS1–/–* retinal anlage was reduced by 57% ($p < 0.05$) in sphere-forming cells relative to wild-type littermate controls, which is statistically disparate from the 95% depletion in the *PS1–/–* GE ($p < 0.01$). As in the brain, the *PS1–/–* retinal spheres that did form were equivalent in size to both the wild-type and heterozygote spheres. Once again, this suggests that neither a proliferation deficit nor a survival deficit underscores the phenotype.

Unlike the brain, however, the deficit in the *PS1–/–* RSC population may be the result of a non-cell-autonomous effect. The fact that the RSCs are dividing is a secondary effect; the stem cells are simply trying to replenish a progenitor population, which is potentially the primary effect of *PS1.* Subsequently, we isolated NR-derived spheres from the *PS1* mutants. The result: sphere-forming *PS1–/–* NR progenitors are diminished at E14.5 (fig. 3a), resulting in a significant main effect of genotype [$F(2, 30) = 6.43$, $p < 0.01$]. Follow-up analyses established a significant decline (58%) in the number of *PS1–/–* NR progenitor spheres relative to *PS1+/–* ($p < 0.01$). Importantly, these spheres were not able to passage. This phenotype is consistent with the notion that they are, in fact, progenitor cells, and excludes the possibility that the RSCs are residing in an ectopic location (data not shown). Although these NR spheres represent a small portion of the NR population (approximately 0.1%), there were no obvious differences between the total number of NR cells initially isolated at E14.5 from the *PS1+/–* and *PS1–/–* relative to *PS1+/+.* Together, these data make it difficult to decipher the primary effect of *PS1* in the retina: RSCs or NR progenitor cells.

In order to elucidate which deficit – RSC or NR – is the primary effect, we bulk passaged the RSC-derived spheres from the RPE to determine whether or not there was a cell-autonomous deficit in self-renewal capacity. This resulted in a reduction in retinal secondary sphere formation that was sensitive to the gene dosage (fig. 3c). A one-way ANOVA revealed that there was a significant main effect on genotype [$F(2, 20) = 326.56$, $p < 0.05$]. Post hoc analyses showed that, relative to the wild type, there was a 50% ($p < 0.01$) and 100% ($p < 0.01$) depletion in secondary sphere formation from the *PS1+/–* and *PS1–/–* retinal anlage, respectively. Arguably, there were depletions in both NR-derived and RPE-derived spheres from the *PS1–/–,* which shrouds the conclusion as to whether the primary effect is cell-autonomous or non-cell-autonomous. It is of interest, however, that while the *PS1+/–* RSC population had a diminished capacity for self-renewal, there was not a reduction in the number of progenitor cells in vivo. These data imply that the decline in retinal sphere formation may be due to a cell-autonomous effect: the RSC self-renewal deficit initially depletes the in vivo *PS1–/–* RSC population and subsequently, the number of stem cell progeny – NR progenitor cells – born in vivo. Thus, Notch signaling appears to be critical for the maintenance of the RSCs, and is sensitive to the gene dosage.

Both, Retinal-Derived and Brain-Derived NSC Populations of the PS1 *+/– Adult Are Depleted at 24 Weeks of Age, and the Brain-Derived NSC Shows a Progressive Loss of Self-Renewal in vitro*

As development proceeds, the behavior of NSCs is modified: there is an inflation in the cell cycle time from 26.5 h at E14.5 to a relatively quiescent cycle in which the cells divide only once every 15 days in adulthood [29, 30]. In conjunction with this inflation, the mode of division switches from predominately symmetric divisions, which



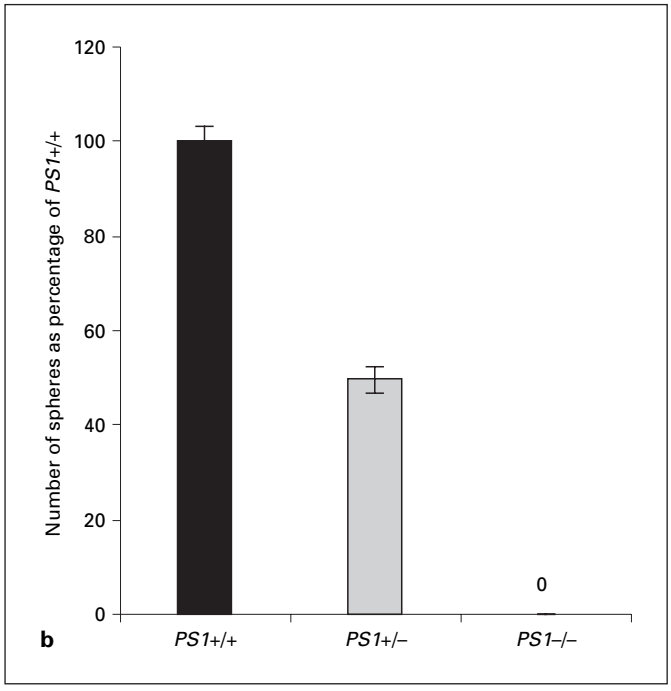


Fig. 3. Loss of *PS1* results in a diminished capacity of RSCs to self-renew at E14.5. **a** The *PS1*–/– RPE contains fewer RSCs, as evidenced by the 43% reduction ($p < 0.01$) in retinal spheres, relative to both *PS1*+/– and *PS1*+/+ (n = 13–20). In addition, the progenitor population, which is derived from the NR, is reduced in the eye of *PS1*–/– ($p < 0.01$) relative to *PS1*+/– and *PS1*+/+ (n = 5–17 per genotype). **b** Primary retinal spheres derived from both the RPE of *PS1*–/– and *PS1*+/– displayed a reduced capacity to form secondary spheres, relative to *PS1*+/+ (n = 6–9 per genotype; p values <0.05) in the presence of F + H and at a low cell density (10 cells/μl). These data demonstrate a diminution in the ability of RSCs to self-renew in a gene-dosage-sensitive manner. Data are expressed as means ± SEM.

expand the NSC population, to a steady-state asymmetric mode of division. Previously, we had shown that during this time there was a diminution in the NSC population of the perinatal/adult *PS1*+/– brain relative to wild-type controls [7]. At 24 weeks of age, we demonstrated that the reduced NSC population was proliferating at a faster rate without an apparent effect on the progenitor population. Questions remain regarding these data. How does this reduction in cell cycle time modulate the ability of the NSC to self-renew? Moreover, does a modification to the cell cycle time translate into a change in the division mode?

We generated neurospheres – forebrain NSC-derived and RSC-derived – from *PS1*+/– mice at 24 weeks of age in the presence of E + F + H. Both *PS1*+/– regions generated consistently fewer neurospheres relative to wild-type littermate controls, indicating depletions in both in vivo stem cell populations (fig. 4a). A two-way ANOVA did not establish a significant interaction effect of region and genotype, nor did it suggest a main effect of region [F values <1.0, p values >0.05]. There was, however, a significant main effect on genotype [$F(1, 5) = 75.96$, $p < 0.01$]. Subsequent analyses revealed that the number of sphere-

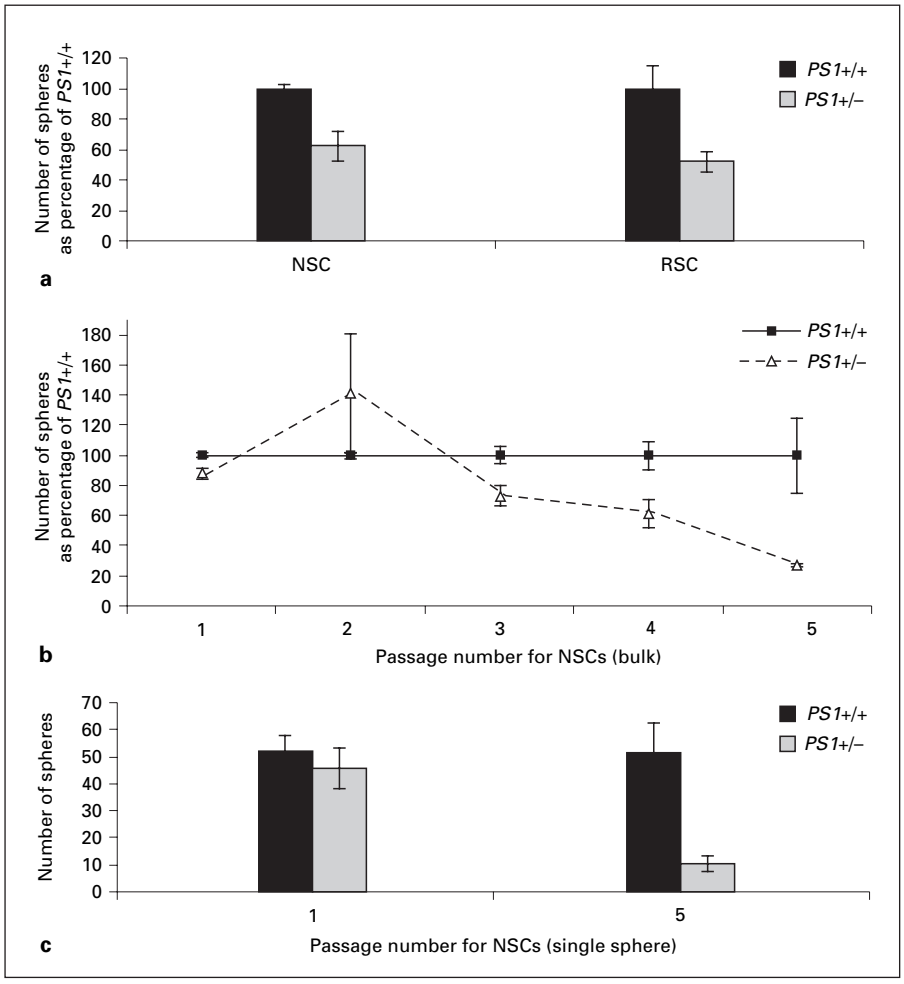


Fig. 4. Both the forebrain NSC and RSC populations are reduced in the adult *PS1*+/– and display a progressive loss of self-renewal. **a** The number of neurospheres and retinal spheres derived from the *PS1*+/– ($p < 0.05$) 24-week-old adult subependyma (NSC) and ciliary margin zone (RSC) are decreased by approx. 40% with respect to *PS1*+/+ (n = 5–6 per region per genotype. **b** The relative numbers of neurospheres were plotted across subsequent passages. The neurospheres derived from the *PS1*+/– (n = 4) forebrain displayed a progressive loss in the number of spheres upon serial passages when plated in the presence of E + F + H and at low cell densities (10 cells/μl) relative to *PS1*+/+ spheres (n = 3). There was a significant decline at passages 3, 4 and 5 (p values <0.05). **c** The loss exhibited in **b** was recapitulated by single sphere passaging. Similarly sized spheres derived from both the *PS1*+/– (n = 29) and *PS1*+/+ (n = 27) forebrains gave rise to a comparable number of secondary spheres (passage 1). In contrast, during their 5th passage, single *PS1*+/– (n = 17, $p < 0.01$) neurospheres produced 78% fewer spheres upon reculturing relative to *PS1*+/+ (n = 20). (The spheres used in passage 5 were of similar size to those used in passage 1.) These data demonstrate a loss of self-renewal in both the NSC and RSC population; the NSC loss is progressive both in vivo and in vitro. The graphed data are shown as means ± SEM.

forming cells had diminished by approximately 40% ($p < 0.05$) in the *PS1*+/– relative to *PS1*+/+.

In order to ascertain how self-renewal was impacted, we bulk passaged the forebrain-derived neurospheres. Figure 4b depicts the results. A significant interaction effect of genotype and passage number was confirmed by a two-way ANOVA [$F(4, 15) = 4.94$, $p < 0.01$]. In fact, post hoc analyses showed that from passage 3 to 5 there was a significant reduction in sphere formation (27, 39 and 73%, respectively; $p < 0.01$ at each passage). There are a few key points to mention here regarding these data. First, there were no significant differences in the viability of *PS1*+/– cells relative to *PS1*+/+ at each passage, as assessed by trypan blue exclusion (data not shown). Second, bulk passaging results are delineated from frequency data. This is pertinent as it implies that the *PS1*+/– NSC frequency increased early on relative to *PS1*+/+ [from approx. 60% (fig. 4a) to a level that was comparable to (trending towards greater than) wild type at passages 2 and 3 (fig. 4b)]. Without a change in viability, an increase in NSC frequency can occur by either an increase in NSC expansionary symmetric divisions or a change in the overall growth rate. To decipher the cause, we passaged single primary neurospheres from both the *PS1*+/– and *PS1*+/+ (fig. 4c). This function did not expose an inherent propensity by individual *PS1*+/– NSCs to divide, either symmetrically or asymmetrically, relative to wild-type controls. Together, these data indicate a change in the overall growth rate of the *PS1*+/– NSC population. In fact, a crude estimation of sphere size (number of cells divided by the number of spheres collected to generate cells) indicated that there were roughly 1.32 times more cells in a *PS1*+/– secondary neurosphere relative to *PS1*+/+. These data are consistent with the in vivo increase in *PS1*+/– NSC proliferation rate [7]. However, further subcloning showed that the growth rate of *PS1*+/–

dwindles relative to *PS1+/+*. In contrast to passage 1, we revealed at passage 5 that *PS1+/–* spheres of the same size produced dramatically fewer neurospheres relative to wild-type controls (fig. 4c). A two-way ANOVA established a significant interaction of genotype and passage number [$F(1, 28) = 9.85$, $p < 0.01$]. A 78% reduction ($p < 0.01$) in sphere formation from *PS1+/–* NSC relative to *PS1+/+* NSC at the fifth passage was shown to be significant by follow-up analyses. These data indicate that, as passaging proceeded, the ability of the NSC to undergo expansionary symmetric divisions diminished, even in spheres of equivalent size. Importantly, this reduction did not occur with a concomitant increase in the percentage of spheres that failed to self-renew (that is, generate at least one new sphere): 100% of the adult-derived neurospheres passaged, irrespective of passage number or genotype. Together, these data suggest that the ability to generate spheres (and hence, progenitor cells) is still present and precludes the notion that the stem cells are simply dying. Finally, the late emergence of the change in NSC division mode implies that this phenomenon is not a direct result of diminished Notch signaling.

Discussion

Notch Signaling Is Functionally Conserved in Embryonic NSCs That Vary Spatially and Phenotypically

Although previous studies [5, 7, 9] established that Notch signaling was required by GE-derived NSCs to self-renew, it was unclear whether this function was conserved in other NSC populations. To determine if Notch signaling was functionally conserved, we used the colony-forming neurosphere assay to assess whether the E14.5 phenotype of diminished self-renewal within the GE of the *PS1* mutants was exhibited within similar NSCs that differ in their regional specification (Ctx and HB) and in another NSC population, the RSC, which is distinct from those residing in the brain. Primary sphere formation in all regions elicited varying deficits in both, *PS1–/–* and *PS1+/–* stem cell populations. Of the four regions studied, the Ctx exhibited the gravest deficit: while the other *PS1+/–* regions possessed an intact NSC population relative to *PS1+/+,* the *PS1+/–* Ctx was reduced by 50%. In contrast, the *PS1–/–* retina generated a relatively marginal depletion of 50% relative to the 95% depletion in all other NSC populations studied. These incongruent data can be explained in two ways: first, the requirement for Notch signaling is spatially differential; second, the self-renewal phenotype could be masked by a differential number of self-renewing divisions between regions. The latter interpretation is consistent with the notion that RSCs emerge (and expand) later in development than brain-derived stem cells [19, 31], and that cortical NSCs possess an inherent reduction in self-renewal that is independent of the genetic mutation (fig. 2b) [26]. In accordance with this, passaging revealed a depletion in the self-renewal capacity, independent of the spatial context: both *PS1–/–* and *PS1+/–* NSCs displayed 95–100% and 50% reduction, respectively, in secondary sphere formation. Passaging data are thus in agreement with the supposition that the disparity is attributed to a difference in the number of self-renewing divisions in vivo. Furthermore, the decline in secondary sphere formation could be accredited – in a gene-dosage-sensitive manner – to an increase in NSCs that failed to self-renew (fig. 2b). We attribute the loss of self-renewal to an increase in terminal symmetric divisions, whereby the stem cell fate is lost along with the production of two progenitor cells. Collectively, these data posit Notch signaling as a critical mediator of self-renewal: Notch signaling retains cells in an *undifferentiated* state and promotes expansionary symmetric divisions in all regions investigated. Functional conservation of Notch signaling is, moreover, consistent with another distinct stem cell system: hematopoietic. Notch signaling has been shown to retain hematopoietic stem cells in an *undifferentiated* state [32–34], though the exact manner by which it sustains the hematopoietic stem cell fate remains to be determined.

Finally, our data are in alignment with the notion that self-renewal mechanisms are conserved between divergent stem cells. In addition to Notch signaling, both neural and hematopoietic stem cells have been shown to exploit other molecular mechanisms in a similar manner: loss-of-function studies for p21 and *Bmi1* revealed comparable self-renewal deficits [35–38]. Collectively, these data point to universal mechanisms which underscore a common stem cell behavior: self-renewal.

Both Adult Forebrain NSCs and RSCs Require Notch Signaling to Maintain Themselves

During development, we had previously demonstrated that the *PS1+/–* NSC population undergoes a progressive loss, such that by 24 weeks of age there is a 40% reduction as assessed by the neurosphere assay [7]. Additionally, 24-week-old *PS1+/–* mice had a 57% increase in the number of label-retaining cells within the subependyma. Although not further characterized in this report or our prior report [7], these label-retaining cells in the

subependyma have been demonstrated to continue to proliferate in vivo [39]. This is in agreement with these cells representing NSCs, a relatively quiescent population. Subsequently, we argued that the in vivo increase in label-retaining cells and a concomitant reduction in the in vivo NSC population demarcated a decrease in the cell cycle time of the *PS1*+/– NSC (approximated to be 38% of the wild type). This alteration to the cell cycle time can be explained in two ways. First, Notch signaling augments the cell cycle time of adult forebrain NSCs, and the loss of *PS1* – and presumably Notch signaling – results in a reduction of the NSC cell cycle time. Alternatively, the modification to the *PS1*+/– NSC cell cycle time could be an in vivo artifact of a diminishing stem cell pool attempting to compensate for the loss, and not a direct result of the *PS1* mutation. A critical question remains: does Notch signaling directly control the cell cycle time of adult NSCs, and what impact does that have on self-renewal?

In order to elucidate whether the altered cell cycle time of the *PS1*+/– adult NSC was maintained in vitro while also impacting self-renewal capacity, we performed both, single sphere passaging and serial subcloning. Initially, the ability of the *PS1*+/– NSC to self-renew was no different than its wild-type counterpart as assessed by single sphere passaging. However, the overall *PS1*+/– growth rate was increased (without an apparent change in survival) relative to *PS1*+/+. The increase in growth rate is consistent with the in vivo increase in NSC proliferation rate [7]. As passaging proceeded, the capacity for self-renewal and expansion diminished, such that by passage 5 neurosphere formation was reduced by 78% relative to wild-type controls. This finding could be recapitulated by passaging single spheres of similar size. Importantly, there were no changes in the frequency of spheres that did not passage. Together, these results suggest that the reduction is neither due to apoptosis (the progenitor population clearly formed), nor stem cell dilution. Moreover, the rather late emergence of a change in NSC division mode suggests that this phenomenon is not a direst result of the *PS1* mutation and hence, diminished Notch signaling.

A similar phenotype – progressive decline in neurosphere formation – was exhibited by a cyclin-dependent kinase inhibitor knockout, $p21^{CIP1/WAF1}$ (p21 hereafter) [36]. *p21*–/– NSCs proliferate at a faster rate. The result: NSCs exhaust their extensive, but finite, self-renewal capacity at an accelerated rate relative to wild-type controls both in vivo and in vitro. This enhanced rate of proliferation did not, however, occur with a switch in the mode of division. Thus, it is argued that p21 is required by the NSC to maintain the relatively quiescent state, ensuring preservation of the population by preventing replicative senescence [36]. These data are particularly intriguing as Notch signaling has been shown to directly modulate the levels of p21 which subsequently lengthens the cell cycle time [40, 41]. Moreover, the effects of diminished *PS1,* like those in *p21*–/– mutants, cannot be explained by a proliferation deficit, a survival deficit, a change in division mode, or a non-cell-autonomous (progenitor) effect. By exclusion, a hypothesis remains: Notch signaling directly alters (increases) the cell cycle time of the adult forebrain NSCs to prevent NSCs from exhausting their self-renewal capacity and thereby, preserving the population. Arguably, differences do exist between the two mutants: there is an increase in the perinatal *p21*–/– NSC population, which is never exhibited by any of the Notch mutants; additionally, neurospheres derived from *p21*–/– mutants do not generate more neurons and glia, as evidenced by neurospheres derived from *PS1*–/– mutants [7, 34, this paper]. These differences, however, can potentially be explained by other known cell cycle factors that are modified by Notch signaling, such as cyclin D1, cdk2, and (indirectly) cdc25 [42, 43]. It follows then that both the in vivo and in vitro diminution of the *PS1*+/– NSC population may be attributed to the augmented proliferation rate of the NSCs, which consequently causes the NSCs to reach their Hayflick number (number of divisions a cell can undergo before reaching replicative senescence) at a faster rate. Furthermore, our data suggest that, as the adult *PS1*+/– NSC approaches its hypothesized Hayflick number, the mode of division becomes biased towards an asymmetric steady-state mode of division. A similar phenomenon is evident in old mice. The NSC population is diminished in senescent mice [44]. Although the 'old NSCs' can still self-renew, their ability to expand starts to approach a plateau in vitro; this indicates a predominance of asymmetric divisions and stands in stark contrast to the exponentially expanding young NSC population [Alexson et al., unpubl. data]. Thus, we suggest that as the population reaches replicative senescence, the cell cycle time becomes elongated. (This increase in cell cycle length may be attributed to elevated levels of molecules controlling the G1 to S transition, including $p16^{INK4A}$ and p53 [45], and occurs independently of Notch signaling.) Elongation, we suggest, promotes asymmetric divisions (cell cycle and division mode are discussed in further detail below).

All together, our adult data imply that Notch signaling – at least preliminarily speaking – functions to maintain the relatively quiescent state of adult NSCs by modifying the cell cycle time. This function prevents the stem

cell population from exhausting their finite self-renewal capacity and thereby preserves the NSC population.

Are the Embryonic and Adult Data Out of Sync, or Is There Unity in the Role of Notch Signaling in NSCs across the Life Span?

We have argued thus far that, spatially, Notch signaling is functionally conserved within NSCs. When we consider the data in terms of temporal control, there appears to be a conspicuous discrepancy: in the embryo, Notch signaling appears to regulate the mode of division, while in the adult we argued that it modifies the cell cycle time without an apparent change in division mode. Arguably, both roles can impact the self-renewal status of a stem cell. The crux of the matter is whether or not the manner in which Notch signaling mediates self-renewal is subject to variable temporal control throughout the life span (i.e. two independent functions of Notch signaling). Admittedly, the answer could be quite simply yes. There are, however, alternative interpretations that could rectify this inconsistency, including progenitor contamination, stem cell heterogeneity, and the alteration of division mode by changes in cell cycle time.

First, the differential results could be accredited to progenitor contamination within the embryonic data. Two populations of cells exist within the perinatal brain that possess both, the capacity for multipotentiality and self-renewal. Sphere-forming cells residing in the anterior lateral ventricle are definitive NSCs exhibiting extensive longevity (maintenance of self-renewal concomitant with multipotency); sphere-forming cells derived from the striatum, on the other hand, are a transient progenitor population and thus, endowed with limited longevity [26]. Within the embryonic brain, it is plausible that the progenitor population dominates and subsequently, skews the embryonic passaging data. It follows then that, if the function of Notch signaling is dependent upon the cell type in which it is utilized, we should be able to spatially separate the effects at postnatal day 1: the mode of division effect will localize to the striatal-derived spheres (the transient progenitor population), while the adult phenotype of cell cycle time alteration will be present in the anterior lateral ventricle (the definitive NSC).

Second, Notch signaling may modify the cell cycle time of both, the embryonic and adult NSCs. Although the capacity for self-renewal of the stem cell population is extensive, the propensity for self-renewal amongst individual stem cells could be heterogeneous. This interpretation supposes a predetermined and random distribution of self-renewal capacity ranging from marginal to extensive. During development, stem cells located at the lower end of the distribution curve – NSCs possessing marginal self-renewal – will exhaust this capacity. Consequently, we predict that the 'marginal population' will be lost and the more 'extensive population' will be preserved by adulthood. When comparing the distribution of expansionary symmetric divisions of E14.5 GE-derived and adult subependyma-derived neurospheres, there appears to be a difference (fig. 5a). Most noticeable is the presence of embryonic NSCs with a more limited capacity for expansionary symmetric divisions. This variability translates into a significant reduction in in vitro neurosphere expansion of E14.5 GE-derived relative to adult-derived NSCs (compare GE in figure 2a and passage 1 of figure 4c; $p < 0.05$). With respect to the *PS1* mutant, it would be expected that the faster cell cycle time during development would enhance the loss of the stem cells possessing a marginal capacity for self-renewal (fig. 5b). This results in the preservation and concentration of NSCs possessing a tremendous capacity for self-renewal in the *PS1*+/– adult subependyma (fig. 5c). Thus, according to this hypothesis, E14.5 *PS1*+/– NSCs displaying a high degree of expansion will portray a more adult-like phenotype upon subsequent passaging – that is, a progressive loss without an apparent increase in the proportion of spheres that do not passage. In contrast, the spheres at the lower end of the distribution curve should show a dramatic increase in the number of spheres that do not passage. If this theory is valid, it would imply that stem cells with a limited capacity for self-renewal could pervert the passaging data; the distortion occurs due to an acceleration of marginal stem cell exhaustion as opposed to a switch in division mode.

Finally, the third hypothesis also assumes that Notch signaling modifies the cell cycle time of NSCs; however, the regulation of the cell cycle is under different constraints across the life span. These divergent constraints ultimately result in varying degrees by which Notch signaling can impact the duration of the cell cycle. This notion will be discussed in further detail below. Regardless, this supposition implies that, in addition to preventing exhaustion, manipulations to the cell cycle can alter the mode of division.

Is cell cycle the ultimate regulator of self-renewal? Although there is no direct evidence, correlations can be made between the in vivo cell cycle time of NSCs and their division mode: lengthening of the NSC cell cycle time is associated with a conversion from an expansionary symmetric to an asymmetric division mode [29, 46]. Furthermore, there is evidence from *Drosophila* that links

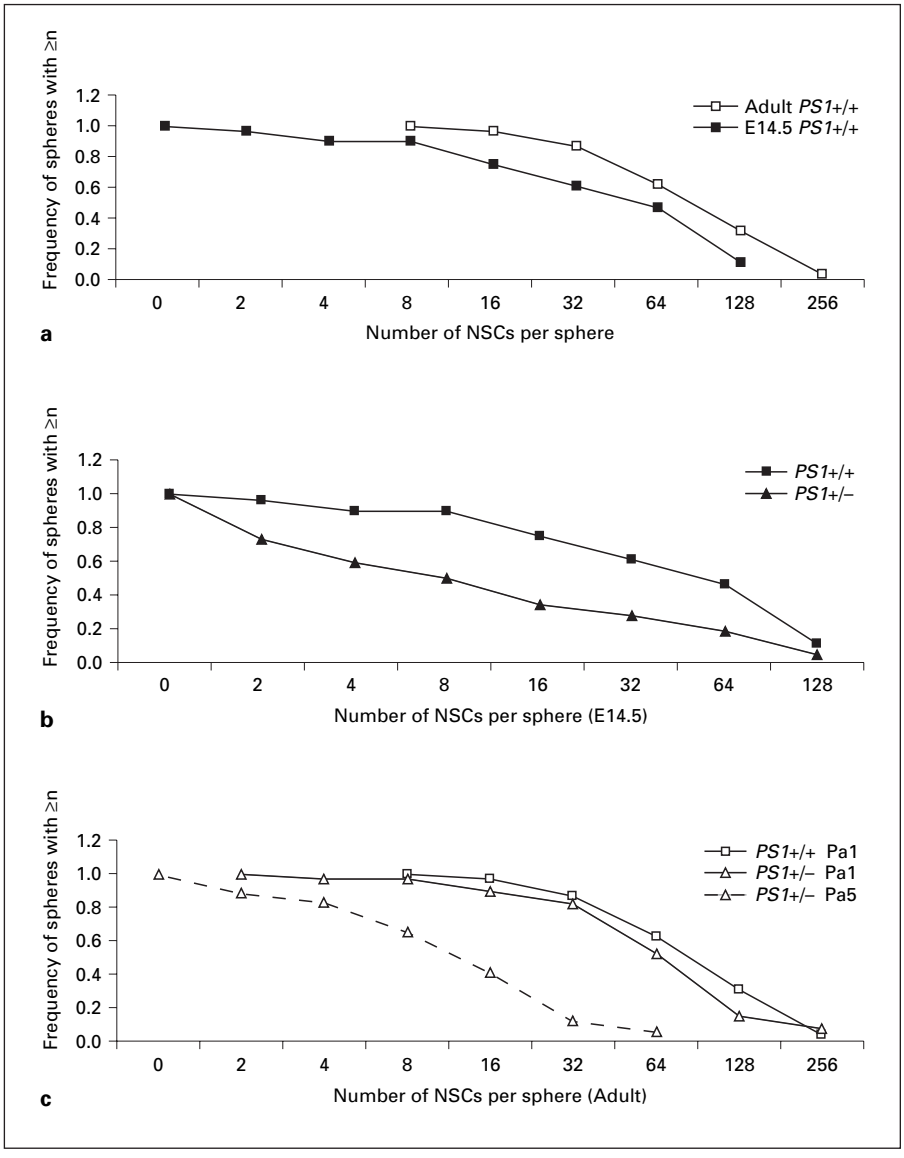


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Fig. 5. The single sphere passaging data are reformatted to determine the distribution of self-renewal capacity. The assumption that we make is that the ability to undergo expansionary symmetric divisions is correlated to the capacity for self-renewal. Therefore, the most conservative approach in determining self-renewal capacity is to consider only those spheres with high degrees of expansion capacity in vitro to have extensive self-renewal capacity, and those displaying minimal degrees of expansion in vitro to have marginal capacity for self-renewal. Subsequently, each neurosphere is classified according to its self-renewal capacity, n, which is also equivalent to the number of NSCs present in each neurosphere. As a population, the distribution is determined for each point. To highlight the NSCs that possess extensive self-renewal capacity, the distribution is then expressed as the frequency of obtaining a neurosphere with a value greater than or equal to n for each point. Finally, it should be mentioned that these are preliminary comparisons which will require additional data points for statistical comparison. **a** This graph compares the self-renewal profile of wild-type spheres from a temporal perspective. E14.5 GE-derived NSCs start to decline earlier than their adult counterparts. This implies a more varied distribution with a greater number of spheres possessing marginal self-renewal capacity. **b** Relative to wild-type littermate controls, the *PS1+/–* NSC population at E14.5 is redistributed: stem cells with a limited potential are lost and stem cells that possess a high degree of self-renewal are retained. **c** The self-renewal profile for adult *PS1+/–* is essentially equivalent to *PS1+/+* during the first passage (Pa1). By passage 5 (Pa5), the *PS1+/–* distribution shifts to the left, indicating a reduced capacity to self-renew.

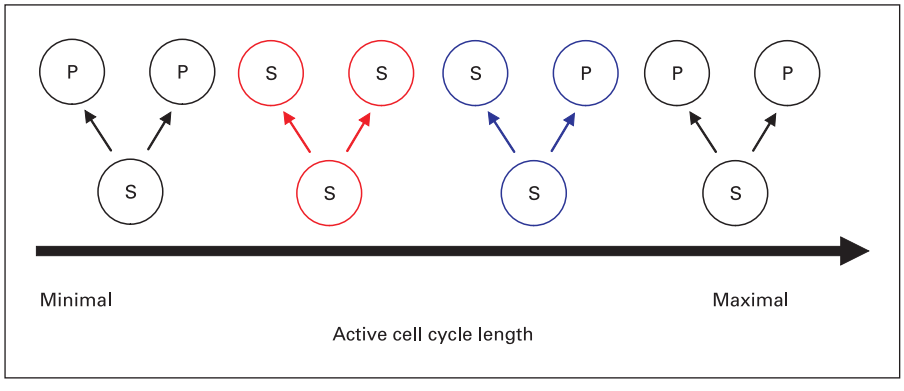

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Fig. 6. A cell cycle model: the ultimate regulator of self-renewal. In its simplest form, the cell cycle model for self-renewal is the following: with increasing cell cycle time (note that this is pertaining to an active cell cycle time and does not include G0), a terminal symmetric division can be converted into an expansionary symmetric, then to an asymmetric division, and finally to a terminal symmetric division. S represents a stem cell, and P denotes a progenitor cell.

components of the cell cycle to the mode of division, such as cdc25 and cdc2 [47–49]. At this point, we are mechanistically incognizant of how these division modes are achieved. It is intriguing, however, to assume that these correlations are not merely coincidental, and that the cell cycle may serve as the gatekeeper to self-renewal. Consequently, we propose a cell cycle model: with increasing cell cycle time, a terminal symmetric division (TSD) can be converted to an expansionary symmetric, then to an asymmetric division and finally back to a TSD (fig. 6). This model proposes that tight regulation of a stem cell's active cell cycle (excluding G0, which acts as a buffering phase) is required in order to ensure competency. Generally speaking, a short cell cycle time will promote an expansionary symmetric division in stem cells. This is attributed to the cell's inability to receive a differentiation signal. Conversely, augmenting the length of the cell cycle allows for the reception of a differentiation signal and favors division modes that promote cellular diversity. Initially, an asymmetric division is favored: the time allotted is sufficient for both, the reception of differentiation signals, and for the coordination of the asymmetric localization of intrinsic determinants and mitotic spindle orientation. If the cell cycle time becomes either too long or too short, a TSD ensues. Excessive lengthening of the cell cycle skews the ratio of differentiation signal versus competence factor towards differentiation: the result is a TSD. Alternatively, shortening the cell cycle time too much can wreak havoc on the entire process. Potential errors (including erroneous DNA synthesis and a back-lag due to inadequate time for the upregulation of factors required for the subsequent phase) are incompatible with competency and hence, the stem cell fate. Once again, a TSD ensues. Altogether, this is the basis for the cell cycle model.

Revisiting the Notch data, we can use the cell cycle model to explain the conspicuous difference between the *PS1*+/– effects within the embryo versus the adult. At E14.5, the NSCs proliferate at a considerably faster rate relative to the adult: 26.5 h versus 15 days [29, 30]. The extent by which Notch signaling modifies the overall cell cycle time varies over the life span. Within the embryo, the change in the active cell cycle length is significant enough to alter the mode of division (switches from expansionary symmetric division to TSD). In contrast, the modification to the 15-day adult NSC cell cycle time causes the NSC to proliferate faster, without an apparent change in the division mode (change occurs mostly in G0). Alternatively, the cell cycle model can explain differences due to the dosage of Notch signaling. NSC heterogeneity – with respect to self-renewal capacity – can explain the *PS1*+/– effect (see above); the change in cell cycle time does not cause a change in division mode at either age. This hypothesis, however, does not explain the severity of the *PS1*–/– effect. Thus, we argue that the homozygous mutation causes a drastic alteration to the active cell cycle and subsequently, a change in division mode. In either scenario, Notch signaling functions to modify the length of the NSC cell cycle. The cell cycle itself not only controls the rate of NSC proliferation, but also serves as a biological switch regulating self-renewal and cell fate determination.

In conclusion, we have shown that Notch signaling is a critical mediator of NSC self-renewal. We suggest that the function of Notch signaling is conserved between similar and disparate stem cell populations at E14.5, promoting expansionary symmetric divisions. In the adult, Notch signaling appears to alter the cell cycle time of two divergent NSCs as a means of preventing stem cell exhaustion. It remains to be determined, however, whether the function of Notch is subject to temporal control in NSCs, or whether modifications to the cell cycle time can alter the mode of division.

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