ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΗΜΑ ΙΑΤΡΙΚΗΣ

ΜΗΧΑΝΙΣΜΟΙ ΚΥΤΤΑΡΙΚΟΥ ΘΑΝΑΤΟΥ ΤΩΝ ΝΕΥΡΩΝΩΝ ΤΟΥ ΕΓΚΕΦΑΛΟΥ ΣΤΗ ΝΟΣΟ ΤΟΥ ALZHEIMER

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΓΕΩΡΓΙΟΣ ΒΟΛΟΥΔΑΚΗΣ

Επιβλέπων καθηγητής: Α. Γραβάνης

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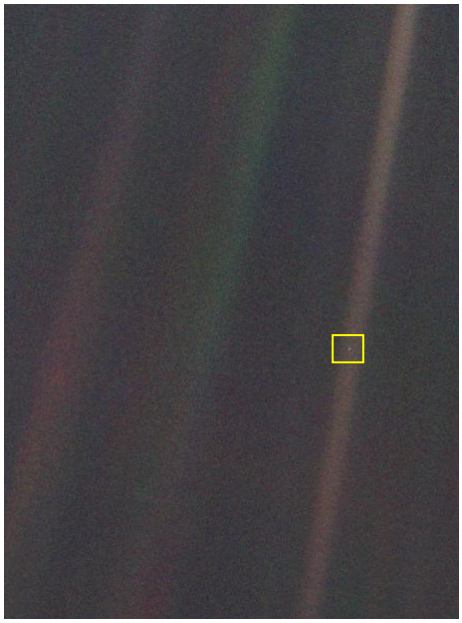

Figure 1. This narrow-angle color image of the Earth, dubbed 'Pale Blue Dot', is a part of the first ever 'portrait' of the solar system taken by Voyager 1. The spacecraft acquired a total of 60 frames for a mosaic of the solar system from a distance of more than 4 billion miles from Earth and about 32 degrees above the ecliptic. From Voyager's great distance Earth is a mere point of light, less than the size of a picture element even in the narrow-angle camera. Earth was a crescent only 0.12 pixel in size. Coincidentally, Earth lies right in the center of one of the scattered light rays resulting from taking the image so close to the sun. This blown-up image of the Earth was taken through three color filters – violet, blue and green – and recombined to produce the color image. The background features in the image are artifacts resulting from the magnification.

Look again at that dot. That's here. That's home. That's us. On it everyone you love, everyone you know, everyone you ever heard of, every human being who ever was, lived out their lives. The aggregate of our joy and suffering, thousands of confident religions, ideologies, and economic doctrines, every hunter and forager, every hero and coward, every creator and destroyer of civilization, every king and peasant, every young couple in love, every mother and father, hopeful child, inventor and explorer, every teacher of morals, every corrupt politician, every "superstar", every "supreme leader", every saint and sinner in the history of our species lived there; on a mote of dust suspended in a sunbeam. The Earth is a very small stage in a vast cosmic arena. Think of the endless cruelties visited by the inhabitants of one corner of this pixel on the scarcely distinguishable inhabitants of some other corner, how frequent their misunderstandings, how eager they are to kill one another, how fervent their hatreds. Think of the rivers of blood spilled by all those generals and emperors so that, in glory and triumph, they could become the momentary masters of a fraction of a dot.

Our posturings, our imagined self-importance, the delusion that we have some privileged position in the Universe, are challenged by this point of pale light. Our planet is a lonely speck in the great enveloping cosmic dark. In our obscurity, in all this vastness, there is no hint that help will come from elsewhere to save us from ourselves.

The Earth is the only world known so far to harbor life. There is nowhere else, at least in the near future, to which our species could migrate. Visit, yes. Settle, not yet. Like it or not, for the moment the Earth is where we make our stand.

It has been said that astronomy is a humbling and character-building experience. There is perhaps no better demonstration of the folly of human conceits than this distant image of our tiny world. To me, it underscores our responsibility to deal more kindly with one another, and to preserve and cherish the pale blue dot, the only home we've ever known. — Carl Sagan, *Pale Blue Dot: A Vision of the Human Future in Space*

ΠΕΡΙΛΗΨΗ

ΣΚΟΠΟΣ

Η διδακτορική διατριβή είχε σαν στόχο να αναγνωρίσει και να μελετήσει τους μηχανισμούς κυτταρικού θανάτου των νευρώνων του εγκεφάλου στη νόσο του Alzheimer (AD). Η πλειοψηφία των μεταλλαγών που προκαλούν Οικογενή νόσο του Alzheimer (Familial Alzheimer's Disease, FAD) έχουν αναγνωριστεί στο γονίδιο της Πρεσενιλίνης 1 (Presenilin 1, PS1). Καθώς η PS1 είναι ένα κεντρικό μόριο στην παθοφυσιολογία της AD, και η AD είναι μία νευροεκφυλιστική ασθένεια, δημιουργείται το εύλογο ερώτημα: «Επηρεάζει η PS1 τους μηχανισμούς κυτταρικού θανάτου;». Είναι γνωστό από τη βιβλιογραφία ότι αναπτυξιακοί παράγοντες μπορούν να προστατέψουν τους νευρώνες από επιβλαβή ερεθίσματα όπως η νευροτοξικότητα γλουταμικού οξέους, το οξειδωτικό στρες και τη στέρηση θρεπτικών συστατικών. Μερικοί από αυτούς τους αναπτυξιακούς παράγοντες δρούνε μέσω της πρόσδεσης τους στον υποδοχέα του επιδερμικού αναπτυξιακού παράγοντα (epidermal growth factor receptor, EGFR) που έχει σαν αποτέλεσμα την ενεργοποίηση σηματοδοτικών μονοπατιών επιβίωσης. Εκτός από το ρόλο του EGFR στη νευροπροστασία, ο υποδοχέας αυτός μπορεί εν δυνάμει να μεταβιβάσει σηματοδότηση κυτταρικού πολλαπλασιασμού, κυτταρικής και ιστικής διαφοροποίησης. Επίσης, πρόσφατες μελέτες τοποθετούν τον EGFR σε κομβικό σημείο των μονοπατιών των νευρομεταβολικών ασθενειών όπως η AD και το γήρας. Στην παρούσα μελέτη, έθεσα το ερώτημα, αν η PS1 είναι απαραίτητη για τις νευροπροστατευτικές ιδιότητες των προσδετών του EGFR έναντι της νευροτοξικότητας γλουταμικού οξέως και πως.

ΜΕΘΟΔΟΣ

Για να απαντήσω στα κύρια ερωτήματα της μελέτης μου, χρησιμοποίησα μια σειρά ποντικών στους οποίους έχει διαταραχτεί η έκφραση της PS1 (PS1 knockout, PS1KO). Καθώς οι νεογέννητοι ποντικοί που δεν εκφράζουν PS1 (PS1-/-) αποβιώνουν αμέσως μετά τη γέννα, δεν καθίσταται δυνατό να μελετήσω τους μηχανισμούς κυτταρικού θανάτου των νευρώνων του εγκεφάλου σε ενήλικους ποντικούς. Για το λόγο αυτό μελέτησα τα επίπεδα έκφρασης των πρωτεϊνών που με ενδιέφεραν σε αυτή τη μελέτη με ανοσοϊστοτύπωμα κατά Western (WB) και ανοσοϊστοχημεία (IHC) σε εγκεφάλους από έμβρυα 15.5 ημερών (E15.5). Μπόρεσα επίσης να μελετήσω ιστοειδικά μοριακά μονοπάτια, παρασκευάζοντας πρωτογενείς καλλιέργειες νευρώνων (PCNC), ινοβλαστών (pMEF) και γλοιϊκών κυττάρων (pGlia; κυρίως αστροκυττάρων). Πραγματοποίησα πειράματα νευρωνικής επιβίωσης έναντι της νευροτοξικότητας γλουταμικού οξέως σε WT (αγρίου τύπου) και PS1-/- PCNCs για να αξιολογήσω την δυνατότητα των κυττάρων να αξιοποιούν τους προσδέτες του EGFR (EGF και HB-EGF) προς την ελάττωση του κυτταρικού θανάτου από τη νευροτοξικότητα γλουταμικού οξέος. Η νευρωνική επιβίωση εκτιμήθηκε και ποσοτικοποιήθηκε με τη χρήση της δοκιμασίας MTT η οποία μετράει την αναγωγική ισχύ του κυττάρου καθώς και με μελέτη της πυρηνικής μορφολογίας των κυττάρων χρησιμοποιώντας τη χρώση Hoechst. Η δυνατότητα των προσδετών του EGFR να ενεργοποιούν σημαντικά μονοπάτια επιβίωσης αξιολογήθηκε με τη μέτρηση των επιπέδων φωσφορυλίωσης των πρωτεϊνών AKT και ERK με WB. Η ποσοτικοποίηση των επιπέδων mRNA του EGFR έναντι του γονιδίου ελέγχου (GAPDH) πραγματοποιήθηκε με αλυσιδωτή αντίδραση πολυμεράσης πραγματικού χρόνου (Real-time PCR). Για τις

ανάγκες αυτής της μελέτης χρειάστηκε οξεία μείωση των επιπέδων έκφρασης του EGFR η οποία πραγματοποιήθηκε χρησιμοποιώντας την τεχνολογία siRNA. Αντιθέτως στις περιπτώσεις που χρειάστηκε να αυξηθούν τα επίπεδα έκφρασης του EGFR ή να επαναφερθούν τα επίπεδα έκφρασης της PS1 χρησιμοποιήθηκαν φορείς έκφρασης βασιζόμενοι σε λεντιϊό.

Επιπροσθέτως, εκτός από το PS1KO μοντέλο, χρησιμοποιήθηκε και το PS2KO μοντέλο για να εκτιμηθεί η ειδικότητα των αποτελεσμάτων που βρέθηκαν στους νευρώνες για την PS1 και τη δράση γ-σεκρετάσης.

ΑΠΟΤΕΛΕΣΜΑΤΑ ΚΑΙ ΣΥΜΠΕΡΑΣΜΑΤΑ

Στην παρούσα μελέτη είδαμε για πρώτη φορά ότι η απώλεια της PS1 οδηγεί σε δραματική μείωση των επιπέδων έκφρασης του EGFR (>95%) σε νευρώνες (PCNCs) και σε σημαντική μείωση (περίπου 40%) στους εγκεφάλους E15.5 ποντικών. Οι PS1-/- PCNCs περιέχουν ελάχιστο EGFR και δεν μπορούν να αξιοποιήσουν προσδέτες του EGFR για να ενεργοποιήσουν τα κυτταρικά μονοπάτια επιβίωσης ώστε να προστατευτούν από τη νευροτοξικότητα του γλουταμικού οξέος, ωστόσο η αύξηση των επιπέδων έκφρασης του EGFR στους PS1-/- νευρώνες αρκεί για να επαναφέρει τις δύο αυτές λειτουργίες. Επί απουσίας της PS1, η μείωση των επιπέδων του EGFR φαίνεται ότι οφείλεται στη μείωση των επιπέδων σύνθεσης του EGFR, καθώς τα επίπεδα του *Egfr* mRNA μειώνονται >95%, ενώ ο ρυθμός αποδόμησης της πρωτεΐνης και του mRNA του EGFR παραμένει αμετάβλητος. Η επανεισαγωγή της PS1 σε PS1-/- νευρώνες με φορέα έκφρασης αυξάνει τα επίπεδα του *Egfr* mRNA και της πρωτεΐνης EGFR, ενώ η μείωση των επιπέδων έκφρασης της PS1 σε PS1+/- νευρώνες μειώνει τα επίπεδα του *Egfr* mRNA και της πρωτεΐνης EGFR. Η επίδραση που έχει η PS1 στα επίπεδα έκφρασης του EGFR είναι ειδική για τους νευρώνες καθώς δεν παρατηρήθηκε αλλαγή των επιπέδων έκφρασης σε PS1-/- πρωτογενείς καλλιέργειες ινοβλαστών ή γλοιϊκών κυττάρων. Επιπροσθέτως, η PS1 επηρεάζει τα επίπεδα έκφρασης του EGFR με τρόπο που δεν εξαρτάται από την ενζυμική δραστηριότητα γ-σεκρετάσης, ούτε από την PS2. Τα δεδομένα της μελέτης αποκάλυψαν ότι η PS1 δρα ως ένας θετικός μεταγραφικός ρυθμιστής του νευρωνικού EGFR. Η σημαντική μείωση των επιπέδων του EGFR επί απουσίας της PS1 μπορεί να συνεισφέρει στις αναπτυξιακές ανωμαλίες και στο θανατηφόρο φαινότυπο που χαρακτηρίζει τους PS1-/-, αλλά όχι τους PS2-/- ποντικούς. Επιπροσθέτως, η PS1 μπορεί να επηρεάζει τους νευροπροστατευτικούς μηχανισμούς στη νόσο του Alzheimer ελέγχοντας τη σηματοδότηση των μονοπατιών επιβίωσης του νευρωνικού EGFR. Εν συντομία, η PS1 είναι απαραίτητη για τη νευρωνική αλλά όχι γλοιϊκή έκφραση του EGFR και της επαγόμενης από αυτού νευροπροστασία με ένα τρόπο που είναι ανεξάρτητος από την ενζυμική δράση γ-σεκρετάσης της PS1.

ABSTRACT

AIM OF THE STUDY

The study was aimed at elucidating the mechanisms of cortical neuronal cell death in the Alzheimer's disease. Presenilin 1 (PS1) mutations are responsible for the majority of the identified cases of patients with Familial Alzheimer's Disease (FAD). That begs the question of the role of PS1 in neuronal cell death and neuroprotection. It has been known in the literature that several growth factors are able to protect neurons from harmful stimuli such as excitotoxicity from excitatory amino acid neurotransmitters, oxidative stress, and nutrient deprivation. Likewise, epidermal growth factors (EGFs) protect neurons from toxic insults by binding epidermal growth factor receptor (EGFR) and stimulating survival signaling. Apart from its role in neuroprotection, EGFR plays pivotal roles in cell proliferation, differentiation, and tissue development, and recent evidence implicates this receptor in neurometabolic disorders like AD and ageing. In this study, I asked the question of whether PS1 is necessary for the neuroprotective capacity of ligands of the EGFR against L-glutamate excitotoxicity, and what is the mode of regulation of this neuroprotective ability.

METHODS

For this study, we predominantly used the PS1 knockout (PS1-/-) mouse model. Since the PS1-/- pups die shortly after birth, I could not study cortical neuronal cell death in adult mice. For that reason I studied the expression levels of the proteins of interest by Western Blot (WB) and immunohistochemistry (IHC) in embryonic day 15.5 (E15.5) mouse brains. Fortunately, a variety of primary cell cultures can be obtained from the embryos to dissect the molecular pathway of interest. In this study, I prepared primary cortical neuronal cultures (PCNC), primary glial cultures (pGlia; mostly astrocytes) and primary mouse embryonic fibroblasts (pMEF). Survival experiments were performed against glutamate excitotoxicity in PCNCs to evaluate the ability of EGFR ligands (EGF and HB-EGF) to reduce neuronal cell death under excitotoxicity. Neuronal viability was evaluated by MTT assay which measures the reduction potential of the cell and also by the gold-standard nuclear morphology assay by employing a Hoechst dye. The ability of the EGFR ligands to activate key survival pathways was assessed by visualizing the level of phosphorylation of AKT and ERK with WB. Quantification of mRNA levels of EGFR against a housekeeping gene (GAPDH) was performed by Real-time PCR. Finally, I was able to manipulate the levels of expression of PS1 and EGFR in our cell cultures by employing siRNA technology to reduce the levels of PS1 mRNA, and a mammalian expression vector (based on a lentiviral backbone) for expressing either PS1 or EGFR.

In addition to the PS1KO mouse model, the PS2KO mouse model was also employed to evaluate the specificity of the findings relative to PS1 and γ-secretase function in PCNCs.

RESULTS & CONCLUSIONS

We show that absence of PS1 results in a dramatic decrease (>95%) of neuronal EGFR and that PS1-/- brains have reduced amounts (around 60% of WT) of this receptor. PS1−/− cortical neurons contain little EGFR and show no epidermal growth factor–induced survival signaling or protection against excitotoxicity, but exogenous EGFR rescues both functions even in absence of PS1. *Egfr* mRNA is greatly reduced (>95%) in PS1−/− neurons, and PS1−/− brains contain decreased amounts of this mRNA, although PS1 affects the stability of neither EGFR nor its mRNA. Exogenous PS1 increases neuronal *Egfr* mRNA, while down-regulation of PS1 decreases it. These effects are neuron-specific, as PS1 affects the EGFR of neither glial nor fibroblast cells. In addition, PS1 controls EGFR through novel mechanisms shared with neither γ-secretase nor the paralog PS2. Our data reveal that PS1 functions as a positive transcriptional regulator of neuronal EGFR controlling its expression in a cell-specific manner. Severe downregulation of EGFR may contribute to developmental abnormalities and lethal phenotype found in PS1, but not PS2, null mice. Furthermore, PS1 may affect neuroprotection and Alzheimer disease by controlling survival signaling of neuronal EGFR. In summary, Presenilin 1 is necessary for neuronal, but not glial, EGFR expression and neuroprotection via γ-secretase-independent transcriptional mechanisms.

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CHAPTER 1 INTRODUCTION

1.1 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a neurodegenerative disorder which has a lot of medical, social and economical consequences. AD is the most common type of age-related dementia affecting up to 80% of the people in specific age groups (Abbott, 2011). The cloud that shrouds so many people when they grow old depriving them of their memories and identities was largely considered a natural part of ageing like arthritic pain and the need to use glasses for presbyopia. Nonetheless for a person that was unfortunate enough to experience their loved ones fading away due to AD, the cognitive decline never looks like a natural phenomenon (Brody *et al*, 2011).

More specifically, AD is characterized by loss of recent memory, diminished critical thinking, personality changes and progressive cognitive decline. The diagnosis cannot be made on the basis of the clinical presentation alone, and a definite diagnosis requires pathological examination of brain tissue. Autopsy studies show that the brains of most people over 65, even without clinical dementia, contain a few neuritic plaques (NPs), and neurofibrillary tangles (NFTs) in the hippocampus and entorhinal cortex, which suggests that their formation is part of the ageing process. Characteristic neuropathology that is more common in demented patients is characterized from the presence of a significant number of NPs, and NFTs not only in the limbic cortex but also in the neocortex and other regions (Newell *et al*, 1999). Neuritic plaques are made up of a peptide called Aβ, which derives from the amyloid precursor protein expressed in human brains (Robakis *et al*, 1987). Neurofibrillary tangles are composed of phosphorylated tau. It is of great importance to note though, that none of the above histopathological depositions are pathognomonic for AD, as NPs as well as NFTs can be found in young healthy individuals in smaller quantities. The etiologies and mechanisms of increased neuronal cell death remain unknown, but most of the studies point towards genetic and environmental factors.

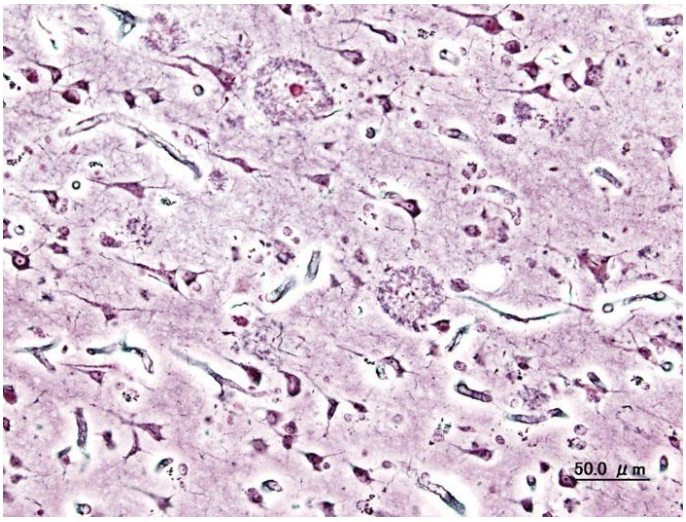


Figure 2. Histopathologic image of senile plaques seen in the cerebral cortex in a patient with Alzheimer disease of presenile onset. Silver impregnation. Wikimedia, CC BY-SA 3.0

Age remains the most important risk factor for developing AD. Based on the age of onset, AD is divided in early onset AD (EOAD; less than 60-65 years old) and late onset AD (LOAD; more than 60-65 years old).

The potential benefits from elucidating the pathophysiological mechanism are staggering. AD is a disease that puts a tremendous pressure on modern societies, at the level of individuals, families and countries as a whole; the care cost and the productivity loss are estimated to exceed 210 billion Euros per year and they are on the rise following the increase of the incidence of the disease. There are more people now than ever reaching old age but what awaits 6 out of 100 people that pass the 60th year of their life is dementia (Abbott, 2011).

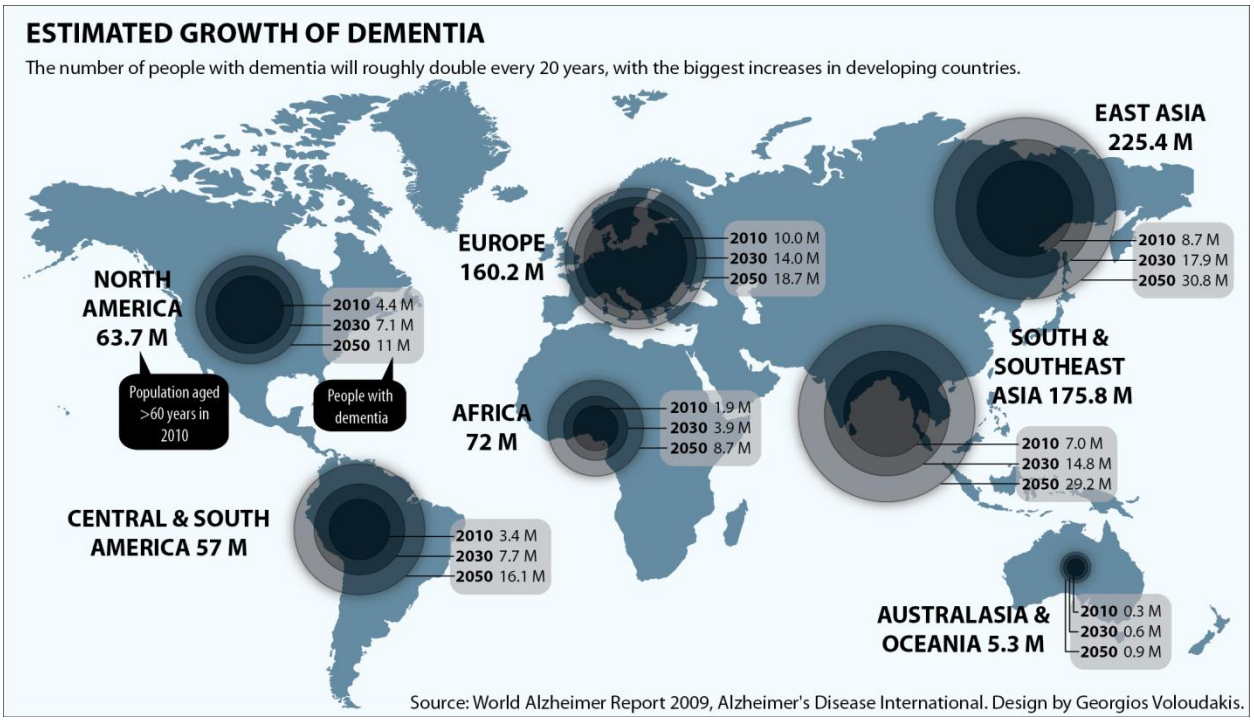


Figure 3. Estimated population growth of people with dementia. I have designed the figure such as the area of the circles to be proportional to the population size denoted by the arrows.

1.1.1 Sporadic Alzheimer's disease (sAD)

Most cases of AD occur after the ages of 65 to 70 years old (LOAD) and are called sporadic because there seems to be no clear correlation with the genetic background. Nevertheless, the genetic predisposition of the non-Mendelian form of AD is considerable, even for LOAD, with a heritability estimate of 60-80%, and several low penetrance polymorphisms have already been identified which increase the risk for developing the disease (Van Cauwenberghe *et al*, 2015). These patients constitute more than 90% of the incidence of AD (Bertram & Tanzi, 2004). The only gene that has been systematically associated with sAD in numerous genetic studies is the one encoding for Apolipoprotein E (*APOE*) and its allele ε^4 that is associated with an increased risk. But even though ε^4 carriers have increased risk of developing AD, some carriers never present with disease symptoms even at very late age, as reviewed in (Bekris *et al*, 2010).

1.1.2 Familial Alzheimer's disease (FAD)

There is a small proportion of AD patients (around 5%) that develop AD due to the presence high-penetrant mutations in three genes (deterministic) and the patients who carry them are classified as Familial Alzheimer's Disease (FAD). The FAD cases usually present as more aggressive forms of the disease and in their majority follow an autosomal dominant pattern of inheritance (Van Cauwenberghe *et al*, 2015). The brain neuropathology is similar in most cases of FAD and sAD, which allows us to conclude that there is a common cellular mechanism underlying both forms of the disease. On the basis of this, we could gain insights in the pathophysiology of the sporadic disease by studying the familial forms. Three genes have been identified that are associated with autosomal dominant AD: PS1, PS2 and APP, out of which mutations in PS1 account for 18-50% of all FAD cases (Theuns *et al*, 2000).

1.2 PATHOPHYSIOLOGY OF AD

1.2.1 Amyloid cascade hypothesis in AD

Identification of Aβ as the main constituent of neuritic plaques, as well as mutations of *APP*, *PSEN1* and *PSEN2* in FAD has resulted in the formulation of the amyloid cascade hypothesis (Glenner & Wong, 1984; Haass & Selkoe, 1998; Hardy & Selkoe, 2002). Aβ is a cleavage fragment of APP and PS1 and PS2 are part of the γ-/ε-secretase complexes. According to this hypothesis, deposition of Aβ is the initial pathological trigger of the disease, leading to formation of neurofibrillary tangles, neuronal death and dementia. Over the course of the years, the theory has evolved to accommodate new findings. For example, initially it was thought that Aβ monomers were the toxic species but it is now not only known that they are neuroprotective under certain conditions, but also that their aggregation may deprive neurons from their protective activity (Giuffrida *et al*, 2009). It is now believed that the form of Aβ that carries the most destructive capacity is the soluble oligomers of $A\beta_{42}$ species, which are reported to cause acute synaptotoxicity. There are 2 pathways by which APP can be cleaved as described below, and only in one of the two is Aβ produced:

1. As depicted in Figure 4, Aβ (a type I transmembrane protein) is produced via the amyloidogenic cleavage pathway. In the amyloidogenic pathway, APP is cleaved by β-secretase and then γ-secretase (Presenilins) to produce Aβ peptides, which are a family of small proteins with heterogeneous ends containing 35-43 amino acids.
2. In the non-amyloidogenic pathway (Figure 4), APP is processed by combined action of α-secretase (ADAM family) (Allinson *et al*, 2003) and γ-secretase. The former cleaves the APP within the Aβ sequence producing a membrane-bound carboxyl terminal fragment (APP-CTF1; α-stub in Figure 4), as a result, no Aβ is formed in the non-amyloidogenic pathway. In addition, γ-secretase (which also serves as ε-secretase) cleaves APP-CTF1 at the ε-site, which is seven to ten amino acids downstream of the γ-cleavage site, and produces the APP intracellular domain (AICD).

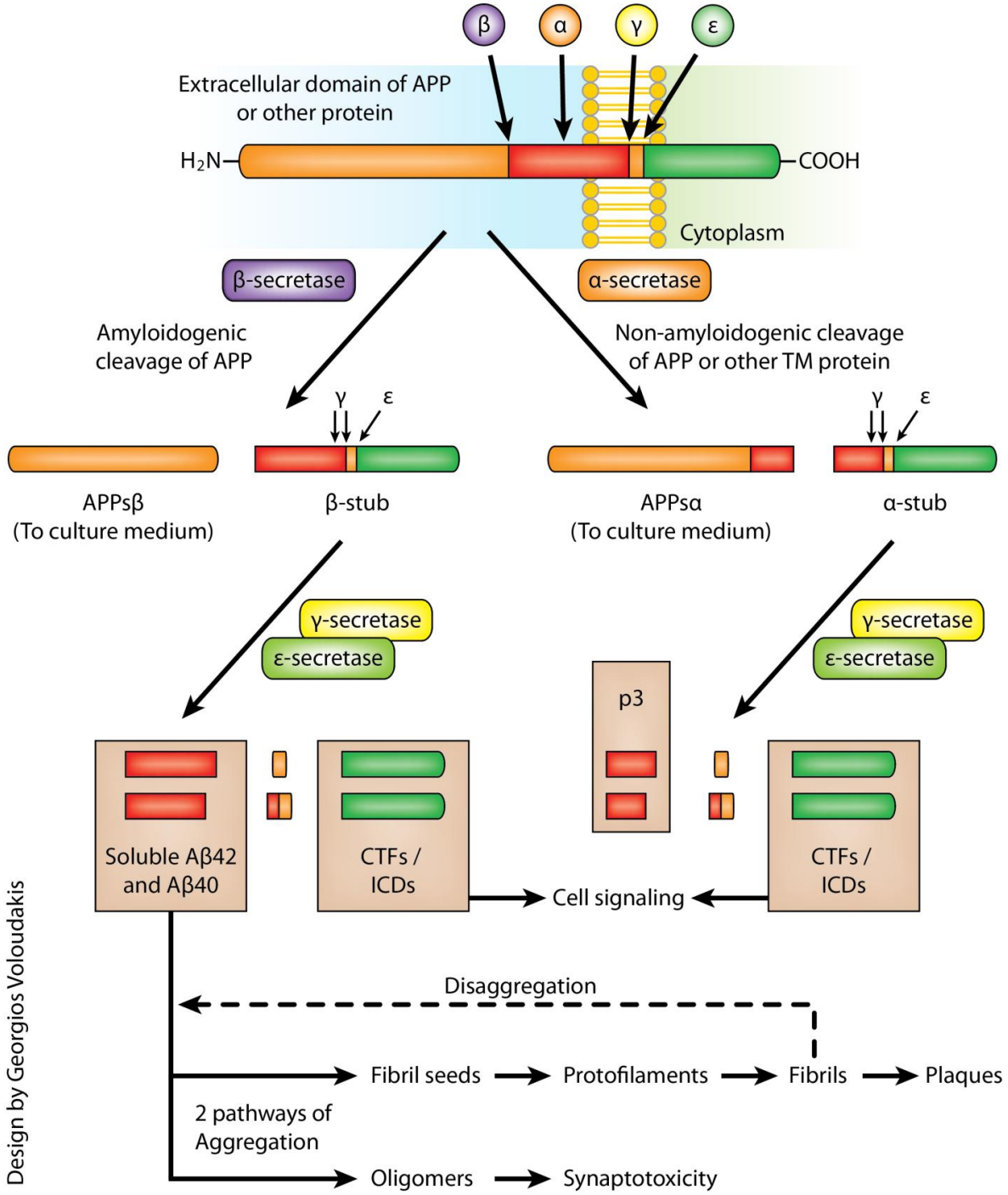


Figure 4. Proteolytic processing of APP, which the amyloid cascade hypothesis is based upon.

Despite being the dominant theory of the AD field, many researchers doubt that the amyloid cascade hypothesis explains the etiology of AD (Neve & Robakis, 1998).

1. Many groups challenge the principle that there is significant correlation between neuritic plaques in the brain with the degree of dementia or neuronal loss (Crystal *et al*, 1988; Arriagada *et al*, 1992; Davis *et al*, 1999; Bouras *et al*, 2006; Robakis, 2011).

2. Amyloid depositions at levels found in AD patients are often detected in normal aged people (Crystal *et al*, 1988; Davis *et al*, 1999)
3. Although FAD mutations initially supported the amyloid hypothesis, recent work showed that many PS1 FAD mutations do not increase Aβ or Aβ42/40 ratio (Shioi *et al*, 2007; Batelli *et al*, 2008).
4. Amyloid depositions are neither sufficient nor necessary to induce neurodegeneration or abnormal electrophysiological functions in transgenic animal models (Hsia *et al*, 1999; Mucke *et al*, 2000)
5. Most drugs in clinical trials based on this hypothesis, have failed to show beneficial effects on cognitive behaviors despite an efficient reduction in amyloid plaque pathology (Holmes *et al*, 2008; Mullard, 2012). In defense of this hypothesis stands only the fact that these therapies do not selectively target the oligomeric-Aβ species.

1.2.2 Glutamate excitotoxicity and AD

L-glutamate (L-Glu) is the principal excitatory amino acid (EAA) neurotransmitter in cortical and hippocampal neurons (Orrego & Villanueva, 1993). As reviewed in Hynd *et al*, 2004, the ability of L-Glu and a number of related EAAs to excite CNS neurons was first demonstrated in 1959. Free glutamate concentration is very finely regulated and is around 0.6μM in the synaptic cleft at resting conditions, then spikes at 1.1 mM for around 100-200μs and can be present at concentrations >100μM for more than 1ms. Importantly the concentration of glutamate cannot be greater than 50μM (the concentration we use for our excitotoxicity assays) for more than 5ms (Clements *et al*, 1992). One of the receptors activated by glutamate is the NMDA receptor which is involved in learning and memory (Danysz & Parsons, 1998). Excessive NMDA stimulation can be induced by ischemia and lead to excitotoxicity, suggesting that agents that block pathologic stimulation of NMDA receptors may protect against further damage in patients with vascular dementia (VaD) (Lancelot & Beal, 1998). In addition, the physiologic function of the remaining neurons could be restored, resulting in symptomatic improvement (Kornhuber *et al*, 1994). The apparent implication of glutamate excitotoxicity in AD is further supported by the relative efficiency of memantine (see 1.3.2 Memantine) in moderate to severe AD.

1.3 TREATMENT OF AD

AD, even today, is mostly symptomatically managed: treatment of behavioral disturbances, environmental manipulations to support function, and counseling with respect to safety issues. Drugs do exist that ameliorate symptoms such as memory loss and confusion, but their effect is at least modest and short-lived.

1.3.1 Cholinesterase inhibitors

Patients with AD have reduced cerebral content of choline acetyl transferase, which leads to a decrease in acetylcholine synthesis (ACh) and impaired cortical cholinergic function. For mild to

moderate AD, cholinesterase inhibitors have been shown to have a modest effect which in most cases results in a small improvement in cognition and activities of daily living. A meta-analysis concluded that 12 patients would need to be treated for one to benefit by achieving minimal improvement or better (Lanctôt *et al*, 2003), another systematic review concluded that the benefit of cholinesterase inhibitors are clinically marginal but statistically significant (Raina *et al*, 2008). One of the possible explanations for the limited efficacy of cholinesterase inhibitors may be that they benefit only a subpopulation of the patients since 30-50% of patients show no benefit at all (Clark & Karlawish, 2003).

1.3.2 Memantine

Memantine is an NMDA receptor antagonist that is supposed to be neuroprotective *in vivo* (please look at the section 1.2.2 Glutamate excitotoxicity and AD for more information). Memantine appears to have modest although statistical significant benefits for the majority of patients with moderate to severe AD (Raina *et al*, 2008). There is possibility that memantine is a disease-modifying drug, even though it has no effect at early disease stages.

1.3.3 Experimental drugs based on the amyloid cascade hypothesis

Most of the drugs in clinical trials are developed based on the amyloid cascade hypothesis.

1.3.3.1 Gamma secretase inhibitors

Gamma secretase inhibitors aim to reduce the levels of Aβ produced. They have failed in all the clinical trials so far (Doody *et al*, 2013; De Strooper, 2014). Although there is some criticism on the studies' design (De Strooper, 2014), there are solid arguments on why there is a fundamental error of principle for this class of drugs such as a) they are not selective enough for APP or b) as mentioned above, cleavage of APP may even be beneficial.

1.3.3.2 Immunization against Aβ

This strategy employs passive and active immunization directed against Aβ. Aβ-targeting antibodies have failed to show any cognitive improvement even when amyloid plaque deposition burden was decreased (Mullard, 2012; Doody *et al*, 2014).

1.4 Functions of Presenilins (PS): PS1 και PS2

Mutations in the genes that code for the Presenilins (PS), PS1 and PS2 cause aggressive forms of early-onset familial Alzheimer's disease (FAD). More than 150 mutations have been identified in the gene *PSEN1* (PS1 gene; Figure 5) that cause AD (Cruts *et al*, 2012; Robakis, 2014) and new mutations are still being discovered (Deng *et al*, 2014), compared to the 9 mutations that have been identified for *PSEN2* (PS2 gene).

1.4.1 The Presenilins as γ-/ε- secretases

Presenilins as mentioned above are believed to constitute the catalytic subunit of γ-/ε-secretase, which is a complex comprised of PS1 or 2, APH-1, PEN-2 and Nicastrin (De Strooper, 2003). PS1 is a

44 kDa multi-transmembrane protein where both N-terminal and C-terminal ends are located in the cytoplasm (Figure 5, reviewed in (Marambaud & Robakis, 2005)). It is ubiquitously expressed and enriched in neural cells in the brain (Elder *et al*, 1996; Marambaud & Robakis, 2005). The cellular localization of PS1 is in the ER, Golgi apparatus as well as plasma membrane (Marambaud & Robakis, 2005). PS1 is cleaved between the predicted transmembrane domains VI and VII (Thinakaran *et al*, 1996) and activated (Wolfe, 2006).

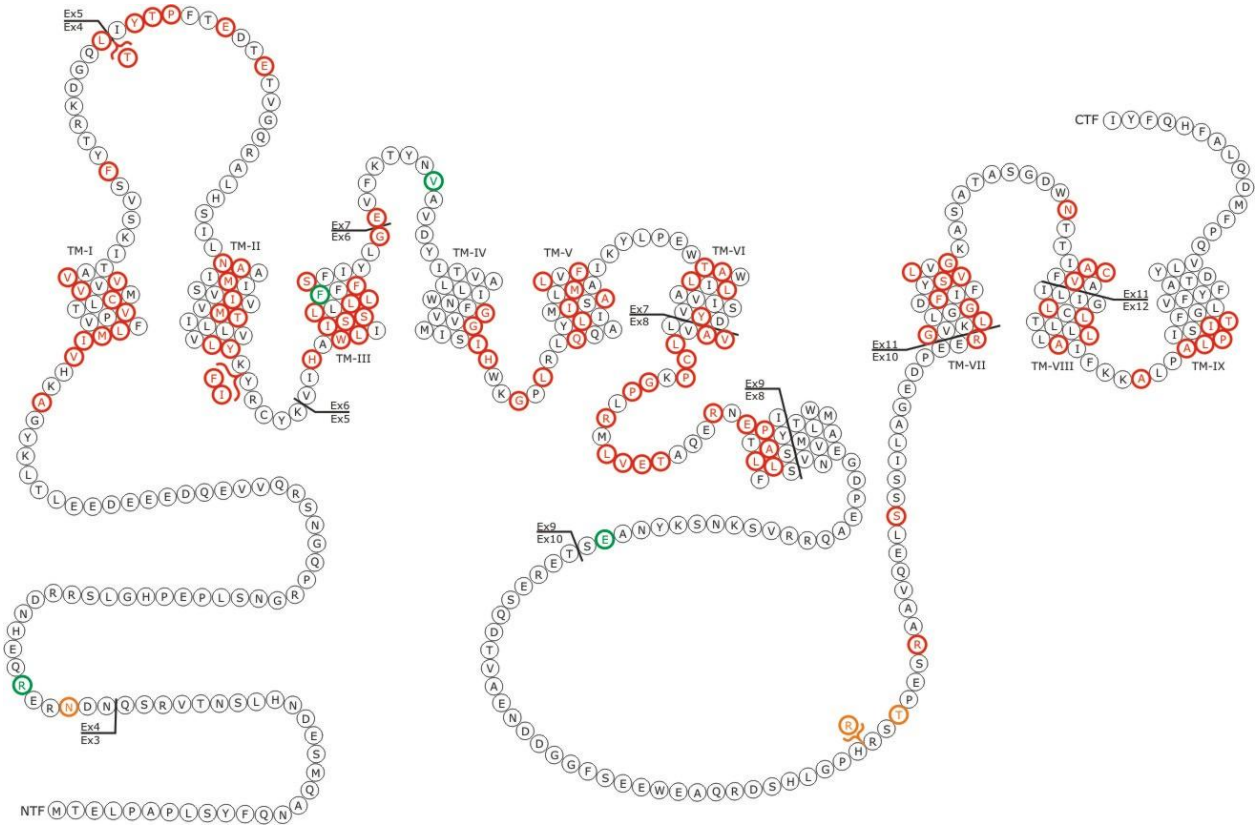


Figure 5. Human Presenilin 1 showing all known mutated amino acids. Mutations that lead to AD are red, not pathogenic in green and when the pathogenic nature is unknown, they are orange. Source: Alzheimer Disease & Frontotemporal Dementia Mutation Database (AD&FTDMDB).

In addition to APP processing which is documented in greater detail in the section above, there are more than 30 type I membrane proteins (single pass proteins with their N-terminal domain facing the extracellular space) identified so far that also undergo sequential cleavage by α- and γ-secretase, producing soluble ICDs. Along those transmembrane proteins are Notch-1 (De Strooper *et al*, 1999), cadherins (Marambaud *et al*, 2002), Erbb4 (Lee *et al*, 2002) and EphB receptors. Some of the ICDs like NICD (Notch ICD) enter the nuclei and regulate gene expression, while others remain in the cytoplasm and partake in signaling pathways (Marambaud & Robakis, 2005). Probably due to the many substrates (verified and unknown) of γ-secretase, non-selective γ-secretase inhibitors used in the past in clinical trials have caused side effects such as hematological and gastrointestinal toxicity, skin reactions and hair color changes. It has also been reported that many PS1 FAD mutations inhibit γ-secretase processing of proteins such as APP, Notch-1, and N-cadherin (Song *et al*, 1999; Marambaud *et al*, 2003).

1.4.2 Gamma-secretase independent functions of PS1

In addition to its γ-secretase-dependent function, PS1 has been implicated in important cellular functions such as cell signaling, intracellular transport and neuronal survival (Pimplikar *et al*, 2010; Barthet *et al*, 2013). PS1 has been directly or indirectly implicated in the regulation of trafficking and metabolism of many proteins including APP (Naruse *et al*, 1998; Cai *et al*, 2003), APLP1 (APP homolog) (Naruse *et al*, 1998), nicastrin (Leem *et al*, 2002), TrkB and EphB2 (Barthet *et al*, 2013). One possible mechanism is that PS1 regulates trafficking and post-translational modification of proteins in synthetic compartments such as ER and Golgi (Leem *et al*, 2002; Cai *et al*, 2003). Previous studies also indicate that PS1 is essential for lysosomal proteolysis and autophagy (Pimplikar *et al*, 2010). Absence of PS1 impairs acidification of lysosomes by disrupting delivery of vacuolar ATPase (vATPase) to lysosomes (Lee *et al*, 2010). Lysosomal proteolysis and autophagy are important for maintenance of normal neuronal function since they clear damaged, misfolded or aggregated proteins during ageing or disease states (Wong & Cuervo, 2010; Pimplikar *et al*, 2010). Last but not least, PS1 has also been implicated in calcium homeostasis regulation (Yang & Cook, 2004; Tu *et al*, 2006)

1.4.3 The role of PS1 in neuronal growth factor signaling and neuroprotection against excitotoxicity

Overstimulation by excitatory neurotransmitters such a glutamate can lead to neuronal cell death. There have been numerous publications in the literature demonstrating the potential of several growth factors to rescue primary cortical or hippocampal neurons from excitatory cell death.

It has been reported that PS1 is necessary for the neuroprotective functions of specific growth factors including brain-derived neurotrophic factor (BDNF) and ephrinB (Barthet *et al*, 2013). The molecular mechanism was later dissected and it was found that surface expression of their respective receptors, TrkB and EphB2 was decreased in PS1-/- neurons. Ligand-induced endocytosis and degradation of the two receptors were compromised in PS1-/- neuronal cultures (Barthet *et al*, 2013). Up until this study, no evidence for a PS1 role in the neuroprotective functions of EGFR or its ligands has been reported.

1.5 THE ROLE OF NEURONAL EPIDERMAL GROWTH FACTOR RECEPTOR IN THE BRAIN

EGFR is a protein tyrosine kinase receptor with key roles in cell growth, differentiation, tissue function and transformation acting as an integrator where extracellular growth and survival signals converge and transform into intracellular outputs (Berasain *et al*, 2011; Avraham & Yarden, 2011). Ligands to this receptor, known as epidermal growth factors (EGFs), are found in brain where they regulate neuronal development, function and survival (Opanashuk *et al*, 1999; Farkas & Krieglstein, 2002; Hanke *et al*, 2004; Enwere *et al*, 2004). In addition, binding of EGFs to their cognate receptor stimulates EGFR-dependent phosphorylation of survival kinases such as Akt and Erk1/2 thus promoting neuronal survival against toxic insults such as excitotoxicity, oxidative stress and

ischemia (Casper *et al*, 1991; Peng *et al*, 1998; Opanashuk *et al*, 1999; Farkas & Krieglstein, 2002; Hanke *et al*, 2004; Sun *et al*, 2010). On the other hand overstimulation of EGFR signaling by exposure of cortical cultures in vitro to prolonged EGF exposure gradually leads to oxidative neuronal injury and neuronal cell death within 48-72 hours (Cha *et al*, 2000).

Functions of EGFR have been implicated in a spectrum of neurometabolic disorders including diabetes, AD and aging (Avraham & Yarden, 2011; Siddiqui *et al*, 2012). The crucial roles the EGF ligand-receptor system plays in development was shown by data that mouse embryos lacking either EGFR or its brain-enriched ligand heparin binding EGF-like growth factor (Hb-EGF), die at birth (Miettinen *et al*, 1995; Sibilia & Wagner, 1995; Threadgill *et al*, 1995; Kornblum *et al*, 1998; Iwamoto *et al*, 2003) while surviving animals suffer from cortical neurodegeneration (Sibilia *et al*, 1998).

Importantly, emerging genetic evidence suggests a role of this receptor in the development of AD. Thus, recent analysis of genome-wide association studies and protein-protein interaction studies identify EGFR as a significant risk factor for AD (Talwar *et al*, 2014) while transcriptional profiling studies indicate that the AD-associated ApoE4 allele changes the brain expression of EGFR (Conejero-Goldberg *et al*, 2011). Furthermore, it has been reported that EGFR functions mediate Aβ42-induced memory loss in experimental animal models (Wang *et al*, 2012). Additional work indicates that EGFR interacts with Presenilin1 (PS1), a protein with pivotal roles in familial AD (FAD). Thus, PS1 has been reported to regulate neural progenitor cell differentiation through EGFR (Gadadhar *et al*, 2011). Interestingly, as is described above, similar to phenotypes observed in EGFR-null Tg mice, PS1 null mice also die at birth displaying severe neuronal abnormalities (Shen *et al*, 1997; Donoviel *et al*, 1999) highlighting the importance of both PS1 and EGFR in development and brain function.

1.5.1 Regulation of EGFR by PS1

Recently, several groups using artificially immortalized or cancer cells reported variable conclusions for the effects of PS1 and γ-secretase on the expression and function of Epidermal Growth Factor Receptor (EGFR) (Zhang *et al*, 2007; Repetto *et al*, 2007; Li *et al*, 2007; Rocher-Ros *et al*, 2010).

Several groups reported that PS1 suppresses expression of EGFR in immortalized mouse embryonic fibroblast cells (iMEFs) but proposed mechanisms are unclear as both pre- and post-translational mechanisms of EGFR inhibition by PS1 were proposed (Zhang *et al*, 2007; Repetto *et al*, 2007; Rocher-Ros *et al*, 2010). Furthermore, evidence for and against involvement of γ-secretase in the regulation of cellular levels of EGFR has been described in immortalized cells (Zhang *et al*, 2007; Repetto *et al*, 2007; Li *et al*, 2007; Rocher-Ros *et al*, 2010). Here we present data that in contrast to results in iMEF cell lines, PS1 functions as a positive and specific regulator of neuronal EGFR. Thus, compared to wild type (WT), PS1 null neurons contain very little EGFR and show no EGF-dependent signaling and neuroprotection against glutamate excitotoxicity. Absence of PS1 however, has no effect on the

EGFR of primary glia or fibroblast cells while PS1 null brain tissue shows a significant reduction of this receptor. Importantly, our data reveals that PS1, but not PS2, increases the levels of neuronal EGFR by transcriptional mechanisms independent of protein turnover and γ-secretase activity.

1.6 Evaluation of the PS1KO PCNC model for the study of neuronal cell death as a model for neuronal cell death in AD

Unfortunately there is no undisputable theory that can explain the pathogenesis of AD in all cases and maybe AD is really a syndrome and not a disease, meaning that several different causes lead to the same symptoms. Although there are several theories in the literature that have enough supporting evidence, no theory (not even the amyloid cascade theory) holds its ground in every AD case. One old theory that is regaining ground lately is the theory of the "Presenilin loss of function" (PS1 & PS2), which can explain many of the paradoxes that are in conflict with the amyloid theory. Based on the loss of function theory, loss of an integral function of the Presenilins leads to the disease. As it has previously been shown by studies conducted in Dr. Robakis laboratory, mutations of PS1 that have been associated with FAD, decrease the enzymatic activity of the γ-secretase complex against specific substrates (Marambaud *et al*, 2003; Georgakopoulos *et al*, 2006; Litterst *et al*, 2007).

To examine the physiological *in vivo* role of PS1, groups have generated mice that do not express Presenilin 1 (Wong *et al*, 1997; Shen *et al*, 1997). Homozygous mutant mice (PS1KO) die within the first ten minutes after birth. The most striking phenotype observed in PS1KO embryos was a severe deficit in the development of the axial skeleton and ribs (left panel of Figure 6), which was traced to defective somitogenesis (Wong *et al*, 1997). In addition, all PS1KO embryos exhibited intraparenchymal hemorrhages as it can be seen in the brains of E15.5 embryos in the right panel of Figure 6. Anatomical analysis by hematoxylin and eosin staining of day E17.5 embryos revealed gross abnormalities in the lung and brain. The posterior portion of the PS1-/- brain is characterized by symmetric cavitations as a result of severe loss of neural progenitor cells. It is of note though, that most PS1 FAD mutations that have been introduced in mice retain normal function during mammalian embryonic development.

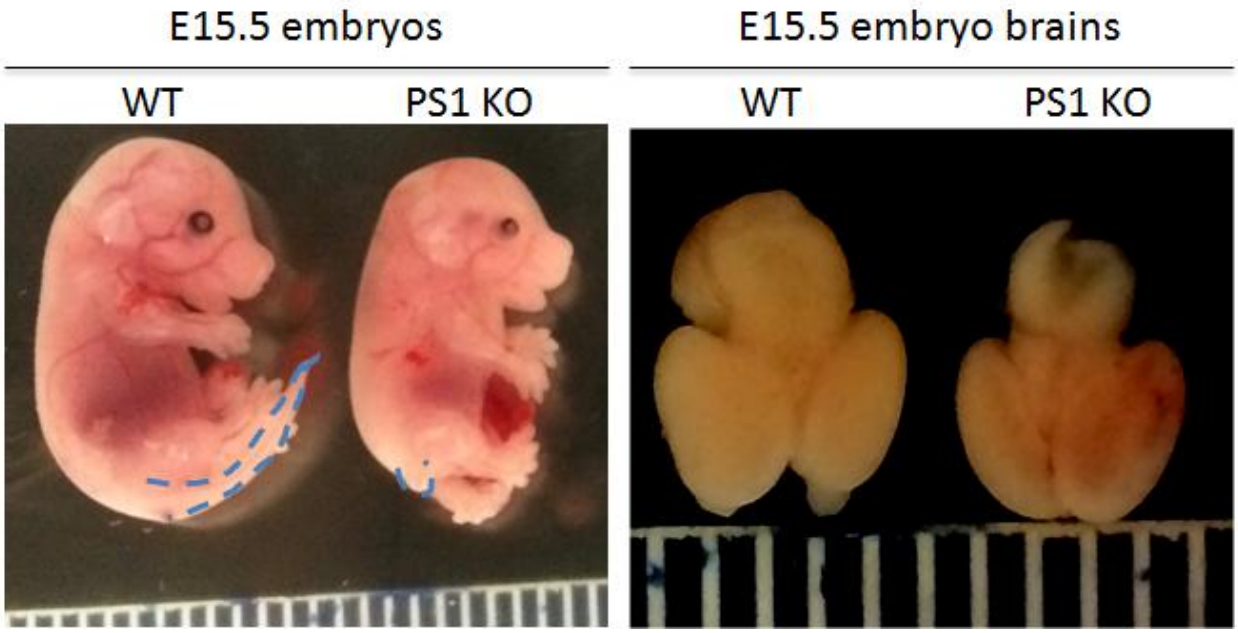


Figure 6. PS1KO embryos at E15.5 (left panel) and of their brains (right panel). On the left panel, please note the shorter body of the PS1KO embryos and the shorter tails marked with blue dotted lines. On the right panel please note the intraparenchymal cortical hemorrhage and the malformed hindbrain.

In our lab we use the PS1KO mouse generated by (Wong *et al*, 1997). Due to the fact that PS1KO mice die shortly after birth (within the first 10 minutes), it is impossible to study the adult mice, therefore many researchers resort to histological studies of embryo brains and produce primary cultures to study the physiologic function of PS1 in different cell types and during brain development. It has been reported that in the brains of PS1KO mice, the ventricular zone is thinner by E14.5 and the massive neuronal loss in specific subregions is apparent after E16.5. These observations have been interpreted to indicate that PS1 is required for normal neurogenesis and neuronal survival (Shen *et al*, 1997). In the presence of the cofounding cerebral hemorrhage, drawing conclusions about neuronal survival is risky. That is why the conditional disruption of PS1 in the forebrain (Yu *et al*, 2001) with the αCaMKII promoter, shed some light by revealing no gross abnormalities in the aforementioned areas. The latter study demonstrated that the likelihood that the neuronal absence of PS1 leads to *in vivo* neurodegeneration is small, but the fact that the mice do exhibit deficits in long-term spatial memory suggests that there may be functional differences. To make things more complicated, a study that used lentiviral vectors-expressing siRNA against PS1 can induce the differentiation of neural progenitor cells (NPC) into neurons, astrocytes and oligodendrocytes, suggesting that a) the multipotentiality of NPC is not affected by PS1 b) there is a different mechanism by which the depletion of the ventricular zone in the PS1KO mice can be explained.

In order to study the mechanisms of survival of cortical neurons, we used Primary Cortical Neuronal Cultures (PCNC) produced from embryonic day 15.5 (E15.5) mouse embryos. Knocking out PS1 leads in the accumulation of protein fragments that result from metalloproteinase cleavage but not γ-secretase cleavage, for example Figure 7Ashows increased accumulation of APP/CTF1 and N-cad/CTF1 fragments known to occur in the absence of PS1 (Barthet *et al*, 2011, 2012). The PCNCs were produced as described in the section 2.5.1 Primary Cortical Neuronal Culture Preparation. Neurons from PS1-/- mice are indiscernible under the light microscope, and exhibit similar staining patterns for MAP2 and GFAP as seen Figure 7B.

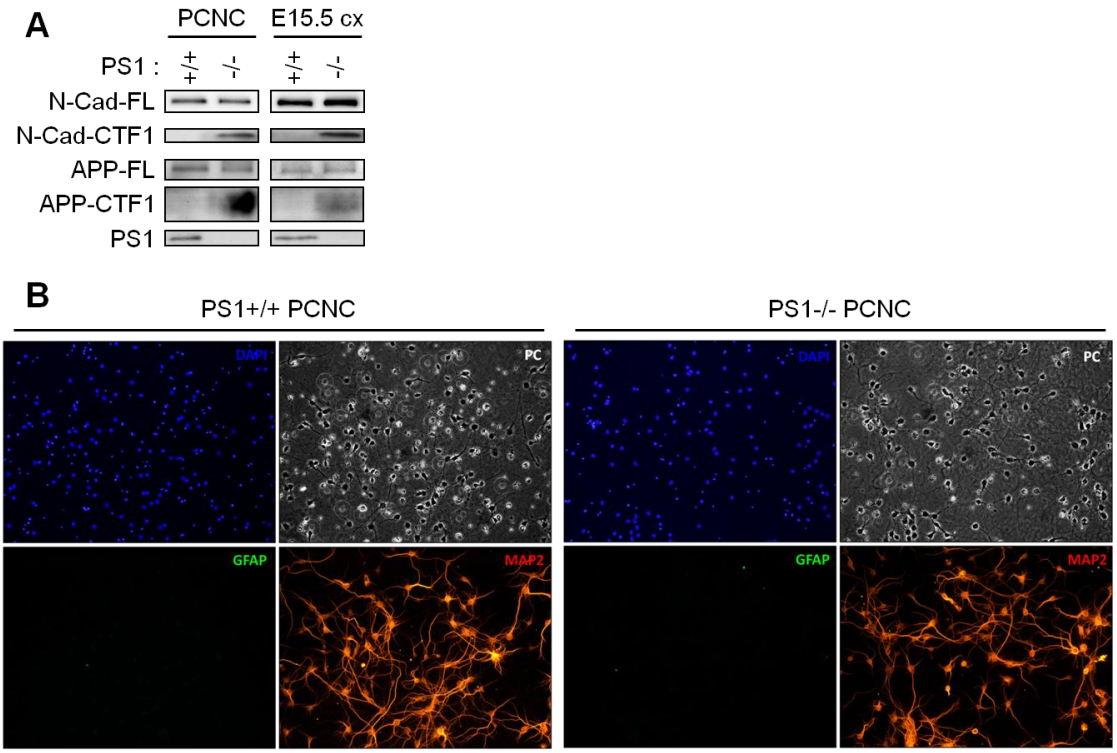


Figure 7. A) Absence of PS1 results in accumulation of γ-secretase substrates APP/CTF1 and N-cad/CTF1 due to decreased γ-secretase activity. Lysates from WT and PS1-/- PCNCs of 9DIV or from E15.5 brain cortex from WT and PS1-/- mice were probed on WBs for indicated proteins B) Representative field of our PCNC at 9 DIV. MAP2 immunostaining shows that the PCNCs from PS1+/+ and PS1-/- are composed predominantly by MAP2+ cells (>95%). GFAP staining reveals a small number of astrocytes (green).

A recent study (Xia *et al*, 2015) showed that there are two PS1 FAD mutations (PS1 L435F and C410Y), that when introduced to mice (by mutating the relevant amino acid in the endogenous mouse PS1 generating a knock-in mouse) cause complete loss of PS1 function in the homozygous mice (i.e. *Psen1*$^{L435F/L435F}$) *in vitro*, including full loss of γ-secretase enzymatic activity. The homozygous mice described above have the same phenotype as do the PS1KO (*Psen1*$^{-/-}$) mice and die postnatally (during or shortly after birth), they also exhibit similar developmental abnormalities characterized by a short body and short tail. The fact that there are human PS1 FAD mutations that phenotypically, histologically and biochemically resemble what would be expected from a loss of PS1 function increase the value of the PS1KO model for the study of Alzheimer's disease.

Based on the above we believe that the PS1KO PCNC will be a valid model to study the physiological role of PS1 on neuronal cell death and draw some conclusions that may apply to Alzheimer's Disease.

CHAPTER 2 MATERIALS AND METHODS

2.1 ANTIBODIES

Table 1 Antibodies used

Antibody	Application	Catalog number/ preparation name	Company/ reference for non-commercial
Anti-EGFR	WB	06-847	Millipore, Billerica, MA, USA
Anti-EGFR	IHC	ab15669	Abcam, Cambridge, MA, USA
Anti-MAP2	IHC/ICC	ab5392	
Anti-GFAP	IHC	Ab4674	
Anti-GFAP	ICC	ab7260	
Anti-Actin	WB	ab3280	
Anti-Tubulin (β)	WB	sc-9104	Santa Cruz, Dallas, TX, USA
Anti-MAP2	WB	M-1406	Sigma-Aldrich, St. Louis, MO, USA
Anti-Actin	WB	3700	Cell Signaling Technology, Danvers, MA, USA
Anti-phospho-EGFR(Y1068)	WB	3777	
Anti-Doublecortin	WB	4604	
Anti-AKT	WB	9272	
Anti-phospho-AKT (S473)	WB	4051	
Anti-phospho-AKT (T308)	WB	9275	
Anti-ERK	WB	9102	
Anti-phospho-ERK (T202/Y204)	WB	9101	
Anti-PS1 (CTF)	WB	33B10	(Marambaud *et al*, 2002)
Anti-APP antiserum (C-term)	WB	R1	
Alexa Fluor 633 Goat Anti-Rabbit IgG	IHC/ICC	A21070	Life Technologies, Grand Island, NY, USA
Alexa Fluor 555 Goat Anti-Chicken IgG	IHC/ICC	A21437	

2.2 IMPORTANT CHEMICALS AND INHIBITORS

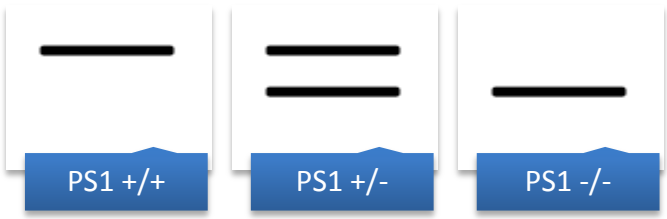
Table 2 Chemicals and inhibitors used

Chemical	Class	Application	Catalog number/ preparation name	Company/ reference for non-commercial
Erlotinib	inhibitor		1023	Selleck Chemicals LLC, Houston, TX, USA
AG-1478			2728	
EGF	Growth factor			Sigma-Aldrich, St. Louis, MO, USA
HB-EGF				
Cycloheximide	Inhibitor			
U0126				
Wortmannin				
L—654,458				
Accell negative control SMARTPOOL SiRNA	siRNA	PCNC/pMEF	D0019101005	Thermo Fisher Scientific, Lafayette, CO, USA
Accell mouse PSEN1 SMARTPOOL SiRNA		PCNC/pMEF	NC0494888	
ON-TARGETplus Non-targeting Pool		iMEF	D-001810-10-05	
ON-TARGETplus Mouse Psen1 (19164) siRNA - SMART pool		iMEF	L-048761-01-0005	
DharmaFECT 1 Transfection Reagent		iMEF	T-2001-01	

2.3 PS1KO (PS1-/-) MOUSE MODEL

Mice with a targeted disruption of the PS1 gene (*Psen1*) were generated as characterized in (Shen *et al*, 1997) and described extensively in the section 1.6 Evaluation of the PS1KO PCNC model for the study of neuronal cell death as a model for neuronal cell death in AD. The disruption of the PS1 was achieved by replacing exon 3 with a pgk-neo cassette. Genotype analysis of the embryos can be done by PCR using a PS1 intron 1-specific primer (P1, ACCTCAGCTGTTTGTCCCGG), a cassette-specific primer (P2, GCACGAGACTAGTGAGACGTG) and a PS1 exon 3-specific primer (P3, TCTGGAAGTAGGACAAAGGTG). The wild-type PS1 generates a PCR product of 345bp and the mutated allele a band of 300bp. DNA is extracted from a tail piece of adult mice or the head without the brain from E15.5 mouse embryos by using the Stratagene DNA Extraction Kit (Cat #200600, Agilent Technologies, Santa Clara, CA). The PCR protocol is as follows:

Temp		Time
94°C		4’
X 32 times	94°C	30”
	56°C	30”
	72°C	45”
72°C		10’
4°C		∞



Running the PCR product on a 1.5% agarose gel, provides good separation.

2.4 PS2KO (PS2-/-) MOUSE MODEL

Mice with a targeted disruption of the PS2 gene (*Psen2*) were generated as characterized in (Steiner *et al*, 1999). Mice that are homozygous for the targeted mutation are viable, fertile, normal in size and don’t display any gross physical or behavioral abnormalities. Alveolar wall thickening, fibrotic deposits, hemorrhages in alveoli and airways are observed by histological analysis. Mice carrying an ablated *Psen2* gene were created by targeting exon 5 of the mouse gene in ES cells. A small deletion in exon 5 and insertion of the neomycin resistance gene was sufficient to disrupt translation.

2.5 CELL CULTURES

All animal experiments were carried out according to regulations of Mount Sinai Medical Center

2.5.1 Primary Cortical Neuronal Culture Preparation

Primary cortical neuronal cultures (PCNC) were prepared from E15.5 mouse embryo brains, plated on poly-d-lysine-coated plates in Neurobasal® Medium (Cat#211039-49, ThermoFisher Scientific, Waltham, MA) supplemented with B-27® supplement (Cat#17504-044, ThermoFisher Scientific, Waltham, MA) as reported (Baki *et al*, 2008; Xuan *et al*, 2013; Barthet *et al*, 2013). Cortices of mouse embryonic brains of embryonic day 15.5 (E15.5) were dissected out, treated with trypsin, and mechanically dissociated in Krebs-Ringer Bicarbonate Buffer. Neurons were suspended in the growth medium described above (Neurobasal/B27) and plated on poly-D-lysine-coated dishes. Neurons were kept 3, 5, 7 or 9 days *in vitro* (DIV) as indicated in figure legends or text. Under these conditions, postmitotic neurons represent more than 95% of cultured cells ((Baki *et al*, 2008) and Figure 7).

2.5.2 Primary mouse embryonic fibroblast (pMEFs) preparation

Primary mouse embryonic fibroblasts were obtained as previously described (Greber *et al*, 2007) from E12.5 mouse embryos. As outlined in greater detail (Xu, 2005), by the use of forceps, the liver, intestines, the heart and the brain were removed, the remaining fetus was washed in PBS to remove as much blood as possible. The fetuses were teased into fine pieces in a dish with 0.25% trypsin-EDTA. Using a pipette, the materials were transferred in to a falcon tube, supplemented with ice cold 0.25% trypsin-EDTA and left at 4°C overnight to allow trypsin to diffuse inside the tissues.

Trypsin was activated the following day by incubation at 37°C. The tissues were mechanically dissociated in MEF medium and the cell suspension was collected and cultured.

2.5.3 Immortalized mouse embryonic fibroblast cell lines (iMEFs)

MEF cells were isolated from trypsinized mouse tissue at embryonic day 15 (E15), immortalized using SV40 T antigen (pSV3neo) and clones were maintained in the presence of G418 (400-100 μg/ml). Single clones were selected at low density using cloning cylinders and individually propagated. Immortalized MEF cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS), p/s in 5% CO_2 at 37°C. Extra care was taken so that the fibroblasts do not become confluent since their growth is affected after that.

2.5.4 Primary glial cultures (pGlia)

Primary glial cultures containing mostly astrocytes were prepared from mouse embryo brains as described (Kim & Magrané, 2011). 1 to 2 day old neonatal mouse pups (P1-P2) were anesthetized, decapitated and the brain cortices were extracted. The tissues were mechanically dissociated, and passed through a 100um strainer, the supernatant was collected and then passed through a 70um strainer, centrifuged and plated. Glial cells were plated at 3×10^5 cells/cm^2 in DMEM supplemented with 10% FBS, p/s in 5% CO_2 at 37°C. Medium was replaced 24 hours after plating and cells were left growing for 8 days. Cells were then trypsinized and replated at a density of 3×10^4 cells/cm^2 until desired confluence.

2.6 Western Blots (WBs)

Cell lysates were prepared in either: a) RIPA (50mM Tris-HCl, pH7.4, 150mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100 and 0.1% w/v SDS) supplemented with protease (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (20mM NaF, 5mM Na_3VO_4, 1mM sodium pyrophosphate, and 100nM microcystin-LR) or b) 1% SDS solubilization buffer (50mM Tris-HCl, pH7.4, 150mM NaCl, 1% w/v SDS). Mouse brain lysates were prepared from E15.5 embryos by mechanical dissociation and sonication in RIPA or 1% SDS solubilization buffer. Samples were centrifuged at 14,000g and protein in supernatants was measured using BCA assays (Pierce, Waltham, MA, USA). Aliquots were diluted with 4X Laemmli buffer, denatured in a boiling water bath and equal amounts of protein were resolved on SDS-PAGE (using the Criterion™ midi-format protein electrophoresis system, Bio-Rad, Hercules, CA), transferred to polyvinylidene fluoride (PVDF), followed by Western blotting and protein detection with antibodies as described (Barthet *et al*, 2013).

2.7 Immunocytochemistry (ICC)

Immunocytochemistry was performed as previously described in (Baki *et al*, 2008). Neurons on poly-D-lysine coated coverslips were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100, washed with TBS and blocked in Superbock blocking Buffer (Pierce, Rockford, IL) for 1 hour.

Samples were then incubated with anti-MAP2 or anti-GFAP antibodies (Abcam, Cambridge, UK) overnight and then with Alexa Fluor antibody conjugates (Molecular Probes, Eugene, OR) for 1 h. Coverslips were rinsed, mounted on PermaFluor Mountant (ThermoFisher Scientific, Waltham, MA) and images obtained with an inverted Olympus microscope (Olympus Corporation)

2.8 IMMUNOHISTOCHEMISTRY (IHC)

Embryonic brain immunohistochemistry was performed as described (Ge *et al*, 2010) except the antigen retrieval step which was performed as in (Muñoz-Elias *et al*, 2004). Briefly, brains were fixed in 4% (w/v) paraformaldehyde and 0.2% (v/v) picric acid in Dulbecco's Phosphate Buffered Saline w/o Calcium/Magnesium (DPBS; Lonza, Basel, Switzerland) at 4°C, washed 3 times with DPBS, and placed in 30% (w/v) sucrose overnight at 4 °C. Tissue was then embedded in OCT Compound (Tissue-Tek, Fisher Healthcare, Houston TX), frozen in ethanol/dry ice and coronal cryostat sections of 20μm mounted on microscope slides and stored at -80 °C. For immunostaining, slides at room temperature (RT) were washed in DPBS, microwaved in 10mM sodium citrate and returned to RT, a process repeated 3 times. Sections were then washed with DPBS, blocked for 1 hour at RT with 10% goat serum and 0.2% Triton X-100 in DPBS and treated with anti-MAP2 and anti-EGFR antibodies overnight at 4 °C. Sections were washed with DPBS, incubated with secondary antibodies conjugated with Alexa Fluor (Molecular Probes, Eugene, OR) for 1 hour at RT, and washed as above. For nuclear staining sections were placed in 0.8 μg/ml Hoechst dye (Sigma-Aldrich, St. Louis, MO) in DPBS for 5 minutes and washed as above. Slides were rinsed with water and images taken on a Zeiss Axio Imager (Carl Zeiss Microscopy, Göttingen, Germany) with a Hamamatsu image acquisition device (Hamamatsu Photonics, Hamamatsu, Japan).

2.9 CELL VIABILITY/SURVIVAL ASSAYS

2.9.1 Excitotoxicity assay

All excitotoxicity assays were performed in Hank's Balanced Salt Solution supplemented with 1.3mM $CaCl_2$ (HBSS; Cat#14175095; ThermoFisher Scientific, Waltham, MA), from now on noted simply as HBSS. Glutamate in final concentration of 50uM was used to induce excitotoxicity for 3 hours.

2.9.2 MTT assay

Neuronal cell survival was evaluated using the MTT assay as described (Barthet *et al*, 2013). Following incubation with growth factors and glutamate, 0.05% 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (w/v) in HBSS was added to neurons and incubated for 3 h at 37°C under a 5% CO_2. Cultures were then washed once with DPBS. Then 200uL of 0.04M HCl in isopropanol was added and incubated under shaking for 5 minutes. Finally, 100μl of the solute were transferred into a fresh 96-well plate and absorbance measured at OD560 with background subtraction at 620nm.

2.9.3 Neuronal nuclear staining assay

Independent assays of indicated number were used to assess neuronal cell death from glutamate excitotoxicity as described by using neuronal nuclear staining (Hoechst staining kit, Sigma-Aldrich, St. Louis, MO, USA) as described (Xu *et al*, 2011; Barthet *et al*, 2013). The details of the each experimental treatment can be found in the figure legends or main text. When the treatments were over, the liquid was aspirated from the wells and the cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature and stained with 0.8μg/ml Hoechst 33342 solution in DPBS for 8 minutes. Stained nuclei images were acquired with fluorescence inverted microscope, and counted according to manufacturer's instructions (Sigma). Numbers of viable neurons were determined in five fields per well, and in each experiment, four identical wells were evaluated per condition per plate. The average number of four wells was used as one experiment per condition in the statistical analysis.

2.10 REAL-TIME PCR, DNA CONSTRUCTS AND TRANSFECTIONS

Total RNA was isolated using the mirVana extraction kit and quantified according to manufacturer's protocol (Ambion, Austin, TX, USA). cDNA was synthesized using 320ng RNA, oligo(dT) primers, and Superscript III Reverse transcriptase according to instructions (Invitrogen, Waltham, MA, USA). PCR primers were as follows: EGFR, 5'-gccatctgggccaaagatacc-3' and 5' -gtcttcgcatgaataggccaat -3'; GAPDH, 5'–aggtcggttgtgaacggatttg-3' and 5'-tgtagaccatgtagttgaggtca-3'. PCR amplification mixtures were prepared using QuantiFast SYBR Green PCR kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA), and real-time PCR assay was performed on an *ABI PRISM 7900HT sequence detection system* (Applied Biosystems, Foster City, CA, USA). Mouse PS1- (FUGW-mmPS1) or EGFR- (FCbAIGW-mmEGFR) expressing plasmids were constructed by cloning mouse PS1 or EGFR cDNA with upstream Kozak sequences into the FUGW or FCbAIGW lentiviral backbone vectors respectively and transduction of neurons was performed using Amaxa technology and Nucleofector Kit (Lonza, Basel, Switzerland) Primary Neurons (program O-005) according to manufacturer's instructions.

CHAPTER 3 RESULTS

3.1 EGFR-MEDIATED SIGNALING AND NEUROPROTECTION ARE ABOLISHED IN PS1 NULL NEURONS.

3.1.1 EGFR-mediated signaling is abolished in PS1-/- PCNCs

Recent literature reports that PS1 negatively regulates EGFR (Zhang *et al*, 2007; Repetto *et al*, 2007; Rocher-Ros *et al*, 2010) suggesting signaling by this receptor may increase in the absence of PS1. To investigate PS1 effects on survival signaling of neuronal EGFR, brain primary cortical neuronal cultures (PCNC) from WT, PS1 heterozygous (PS1+/-) and homozygous (PS1-/-) KO mice were treated with EGFR ligands EGF and Hb-EGF and AKT and ERK phosphorylation signaling mediated by EGFR stimulation was analyzed (Jin *et al*, 2005). As expected, these treatments caused rapid increases in the phosphorylation of both kinases in WT and PS1+/- neurons. Surprisingly however, the EGFs failed to stimulate phosphorylation of either kinase in PS1 null (PS1-/-) neurons, indicating that EGFR signaling is attenuated in the absence of PS1 (Figure 8A). In contrast, BDNF, a factor that signals to AKT and ERK kinases through TrkB receptor, stimulated phosphorylation of these kinases in both WT and PS1-/- neurons indicating that signaling of neuronal EGFR specifically decreases in the absence of PS1 (Figure 8B). To ensure that activation of ERK and AKT was not delayed in PS1-/- neurons, we examined phosphorylation kinetics in response to Hb-EGF, an EGFR ligand abundantly expressed in the brain (Opanashuk *et al*, 1999). Hb-EGF caused a sustained phosphorylation of both kinases in WT and PS1 +/- but not in PS1-/- neurons (Figure 8C). Thus, contrary to expectations, our data suggested that absence of PS1 severely attenuates EGF-induced neuronal signaling and that presence of even one PS1 allele is sufficient for full signaling (Figure 8A and C).

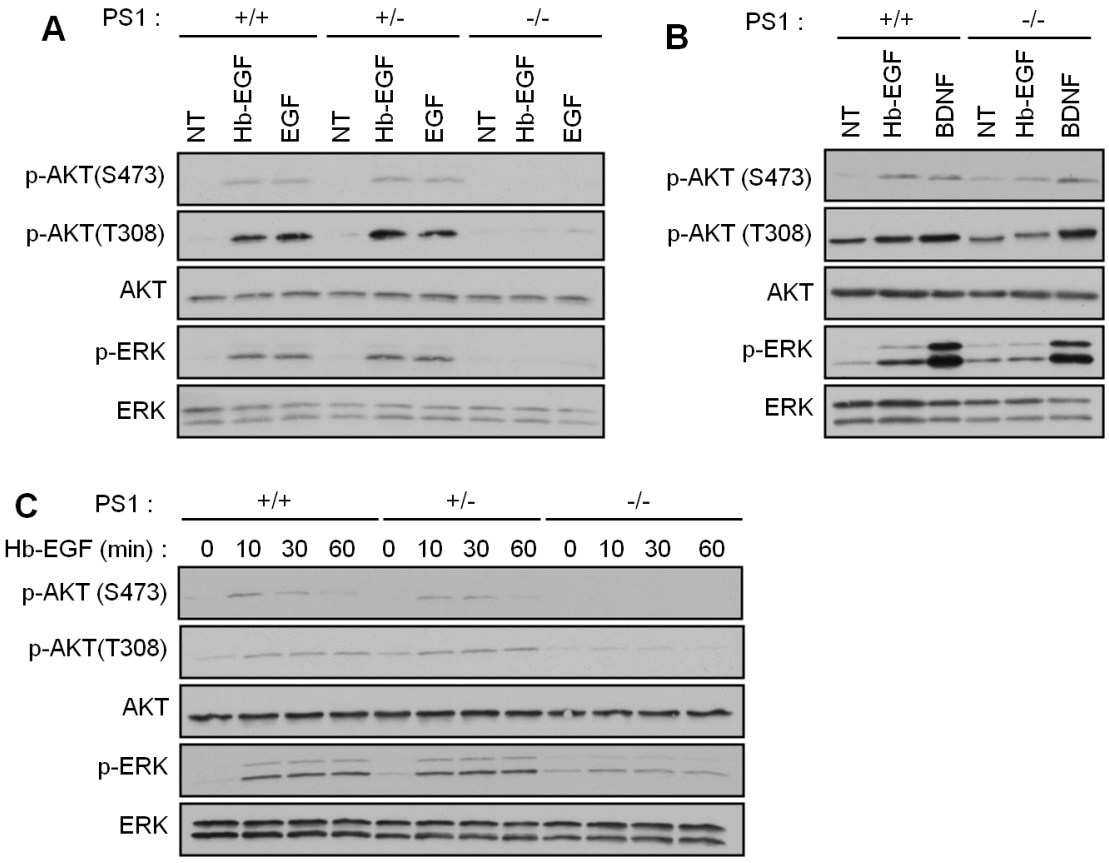


Figure 8. EGF-dependent activation of ERK and AKT survival signaling is abolished in PS1-deficient neurons. *A*) Primary cortical neuronal cultures (PCNC) from WT (+/+), PS1 heterozygous (+/-) and PS1 homozygous (-/-) KO embryonic mouse brains cultured in 6-well plates were treated at 9DIV with 20ng/ml EGF or Hb-EGF for 15 minutes. Following incubation, lysates were collected and probed on Western blots (WBs) for the indicated proteins. *B*) WT or PS1-/- PCNC as above were treated at 9DIV with 20ng/ml Hb-EGF or 100ng/ml BDNF for 15 minutes. Non-treated cultures (NT) were used as controls. Following incubation, lysates were collected and assayed on WBs for the indicated proteins. *C*) WT, PS1+/-, or PS1-/- PCNC prepared as above were treated at 9DIV with 20ng/ml Hb-EGF. Following incubation, lysates were collected at different time points and assayed on WBs for the indicated proteins. Data were obtained from at least three separate experiments.

3.1.2 EGFR-mediated neuroprotection against glutamate excitotoxicity is abolished in PS1-/- PCNCs

Since ERK and AKT kinases mediate survival signaling of the neuroprotective system EGF/EGFR (Farkas & Krieglstein, 2002; Jin *et al*, 2005), we asked whether EGF-dependent neuroprotection changes in the absence of PS1. To this end, neuronal cultures were treated with glutamate in the presence or absence of either EGF or Hb-EGF. Figure 9A shows that although these ligands decreased the glutamate-induced neuronal death in both PS1 +/+ and +/- cultures, they were unable to decrease neuronal death in PS1-/- cultures indicating that PS1 is required for EGF-dependent neuroprotection. In contrast, progranulin rescues PS1 null neurons from excitotoxicity regardless of PS1 gene dosage (Figure 9A and (Xu *et al*, 2011)) confirming that EGF-dependent neuroprotection is specifically regulated by PS1. To examine which kinase is required for EGF neuroprotection, neuronal cultures were treated with either MEK/ERK1/2 inhibitor U0126 or PI3K/AKT inhibitor wortmannin followed by glutamate. Although these treatments decreased the Hb-EGF-induced

activating phosphorylation of ERK and AKT respectively (Figure 9B), only inhibition of PI3K/AKT had a significant effect on the Hb-EGF-induced neuroprotection (Figure 9C). Together, our data indicate that PS1 is necessary for EGF-induced phosphorylation of both AKT and ERK but AKT activation is mainly responsible for EGF-dependent neuroprotection against excitotoxicity *in vitro*.

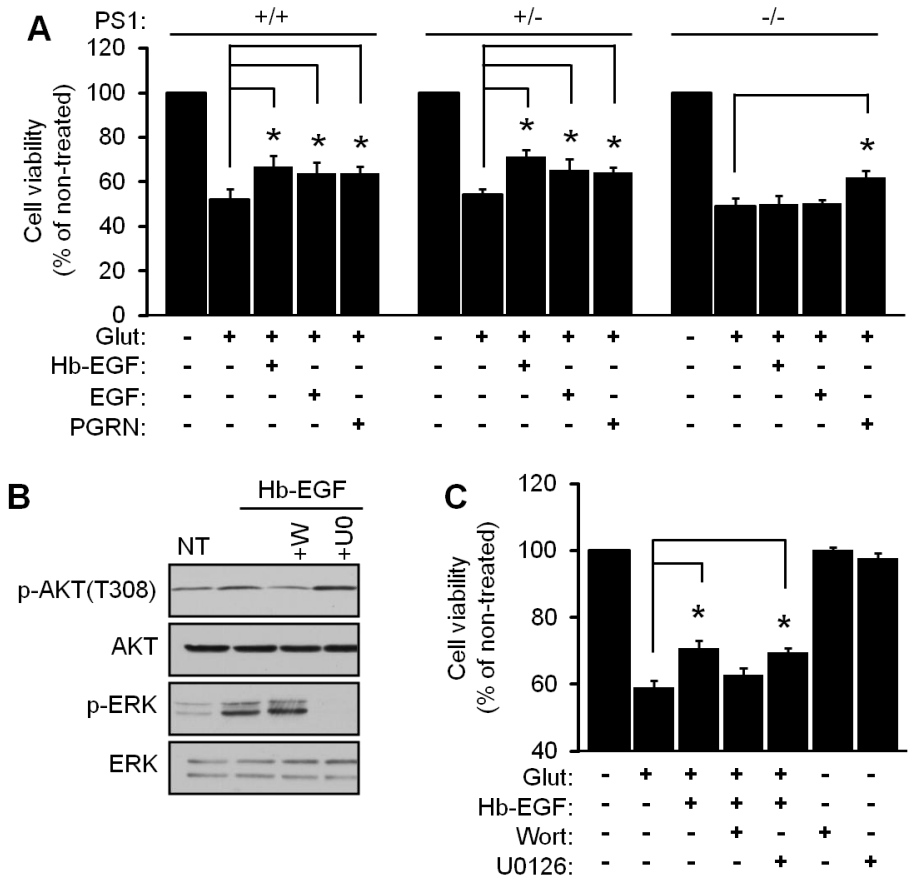


Figure 9. EGF neuroprotection against excitotoxicity is abolished in PS1-deficient neurons. *A*) WT, PS1+/-, and PS1-/- mouse PCNC were grown in 24-well plates in Neurobasal Media plus B27 supplement. At 9 DIV, neurons were treated with 20ng/ml Hb-EGF or EGF or 35nM PGRN overnight. The next day, the medium was switched to HBSS containing Hb-EGF, EGF or PGRN for 30 minutes followed by 50μM glutamate incubation for 3 hours and cell viability was evaluated by MTT assay and normalized to non-treated cells as described (3, 29). No significant effect compared to non-treated was observed when growth factors alone were added to cultures. *B*) WT PCNC in 6-well plates were treated at 9DIV either with ERK inhibitor U0126 (U0, 5μM) or PI3K/AKT inhibitor wortmannin (W, 50nM) for 30 minutes prior to addition of 20ng/ml Hb-EGF for 15 minutes in Neurobasal Media plus B27 supplement. Following incubation, lysates were collected and assayed on WBs for the indicated proteins. *C*) Mouse PCNC grown as above (see A) were treated at 9DIV either with ERK inhibitor U0126 (5μM) or PI3K/AKT inhibitor wortmannin (50nM) for 30 minutes prior to addition of 20ng/ml Hb-EGF. Three hours later medium was switched to HBSS plus Hb-EGF and inhibitors for 30 minutes followed by 50μM glutamate incubation for 3 hours. Cell viability was evaluated by MTT assay and normalized to non-treated cells. Results (mean ± SEM) were summarized from at least four independent experiments. In each experiment each condition is the average of four identical wells. *, p<0.05 comparing between cultures treated with glutamate in the presence or absence of Hb-EGF and/or inhibitors (paired *t-test*).

3.1.3 EGFR-mediated neuroprotection against glutamate excitotoxicity is restored after reintroduction of PS1 in PS1-/- PCNCs

PS1 is known to play an essential role in neuronal differentiation during neural development (Gadadhar *et al*, 2011), which raises the possibility that PS1-/- PCNCs are at a different developmental stage when we start culturing them. To see whether expression of PS1 is sufficient to restore the EGFR-mediated neuroprotection against glutamate excitotoxicity we reintroduced PS1 in PS1-/- PCNCs. Indeed, following reintroduction of PS1 the neurons respond to EGF and Hb-EGF neuroprotective effects (Figure 10)

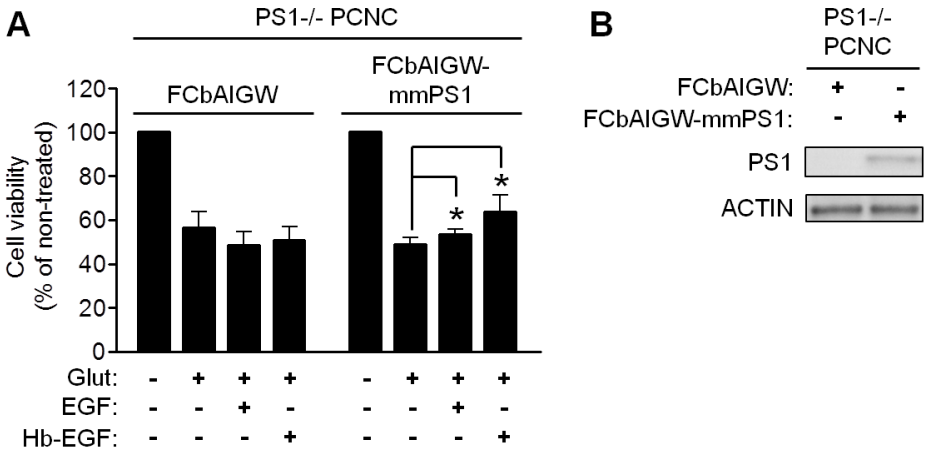


Figure 10. Reintroduction of PS1 in PS1-/- restores the EGFR-mediated neuroprotection against glutamate excitotoxicity. PS1-/- neurons were transfected with either empty vector (FCbAIGW) or a PS1-expressing construct (FCbAIGW-mmPS1) and at 8 DIV, neurons were treated with 20ng/ml EGF or Hb-EGF overnight as indicated in Figure. Next day, the medium was switched to HBSS containing EGF or Hb-EGF for 30 minutes followed by 50μM glutamate incubation for 3 hours. Cell viability was then measured counting healthy nuclei with Hoechst kit 33342 (Methods) and normalized to non-treated cells. Results (mean ± SEM) are from three independent experiments (n=4). *, p<0.05 (paired t-test) *B*) PS1-/- neurons were transfected with PS1-expressing plasmid FCbAIGW-mmPS1 or vector alone (FCbAIGW) and at 8 DIV lysates were then collected and probed on WBs for indicated proteins.

3.2 PS1 POSITIVELY REGULATES NEURONAL EGFR.

To determine the mechanism by which PS1 facilitates survival signaling of EGF ligands, we examined the PS1 effects on the expression of neuronal EGFR. Surprisingly, in contrast to reports that PS1 negatively regulates this receptor in iMEF (Zhang *et al*, 2007; Repetto *et al*, 2007; Rocher-Ros *et al*, 2010), levels of neuronal EGFR were dramatically decreased (> 95%) in PS1-/- neurons compared to WT (Figure 11A). This outcome was replicated in several independent neuronal cultures prepared from different embryonic mouse brains derived from distinct pregnancies. Notably, PS1+/- neurons contain similar amounts of EGFR as WT neurons (Figure 11A) showing absence of one PS1 allele has little effect on neuronal EGFR an outcome consistent with similar signaling and neuroprotective activities of EGFs in WT and PS1+/- neurons (Figure 9). Additional experiments showed that absence of PS1 causes a dramatic EGFR reduction in all neuronal cultures

regardless of age (Figure 11B) and that tissue from PS1-/- brains probed by either WBs (Figure 11C) or immunohistochemistry (Figure 11D) also contains reduced amounts of this receptor.

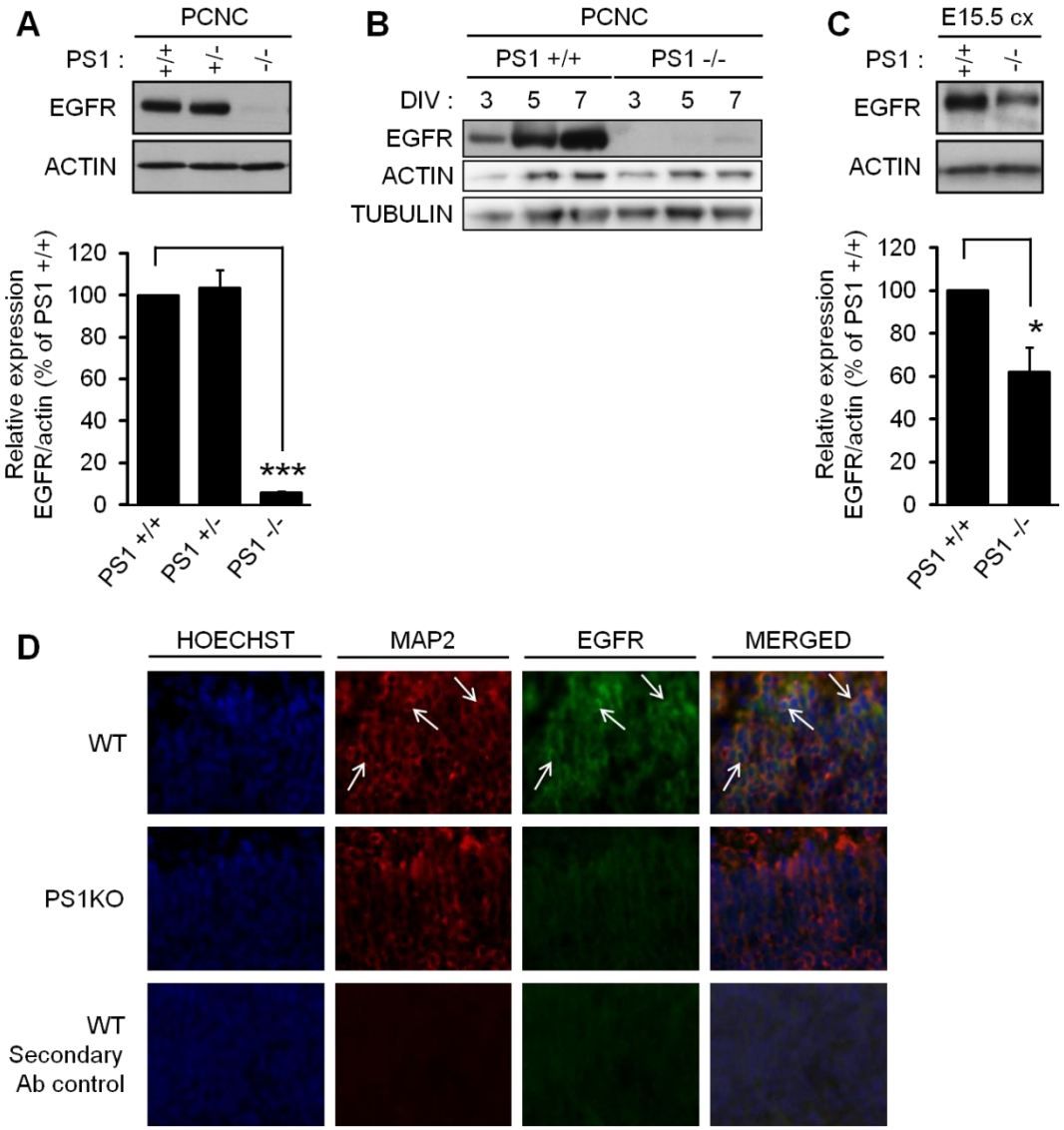


Figure 11. PS1 positively correlates with cellular levels of neuronal EGFR in vitro and in vivo. *A*) (Upper): Lysates from WT, PS1+/-, or PS1-/- PCNC grown in 6-well plates as above were prepared at 9DIV and probed on WBs for the indicated proteins. (Lower): densitometric analysis of the relative amounts of EGFR in PCNC is expressed as ratio of EGFR to actin. *B*) Lysates from WT (+/+) or PS1-/- PCNC grown as above were prepared at 3, 5 or 7 DIV and probed on WBs for indicated proteins. *C*) (Upper): lysates from embryonic brain cortex (E15.5 cx) from wild-type or PS1 homozygous KO were prepared as described in the methods section. (Lower): densitometric analysis of the relative amounts of EGFR in embryonic cortices is expressed ratio of EGFR to actin. D) MAP2 and EGFR immunoreactivity of superficial cortical layers of E15.5 mouse brain embryos. Arrows show neurons from WT mouse brain express both MAP2 (red) and EGFR (green). In contrast, no EGFR-specific staining is observed in PS1KO neurons of the same brain area. Data were respectively obtained from at least four separate experiments. *, p<0.05; ***, p<0.001 (paired *t-test*).

Furthermore, examination of neuronal markers DCX and MAP2 showed that the amounts of these proteins in our cultures and brain tissue are independent of PS1 (Figure 12A) further supporting specific PS1 effects on EGFR. Since absence of PS1 causes a smaller EGFR reduction in brain tissue

than in PS1-/- neurons (40% *vs.* 95% respectively, Figure 11A and C), we asked whether PS1 deficiency exerts a less dramatic effect on EGFR of non-neuronal brain cells. Figure 12B shows that the levels of this receptor in primary glia from PS1-/- brains do not differ significantly from those in glia from WT brains suggesting that absence of PS1 results in specific reduction of neuronal EGFR. This outcome is consistent with data that absence of PS1 causes a more dramatic decrease in neuronal than brain EGFR as total amount of brain EGFR is determined by the sum of its expression in all brain cells.

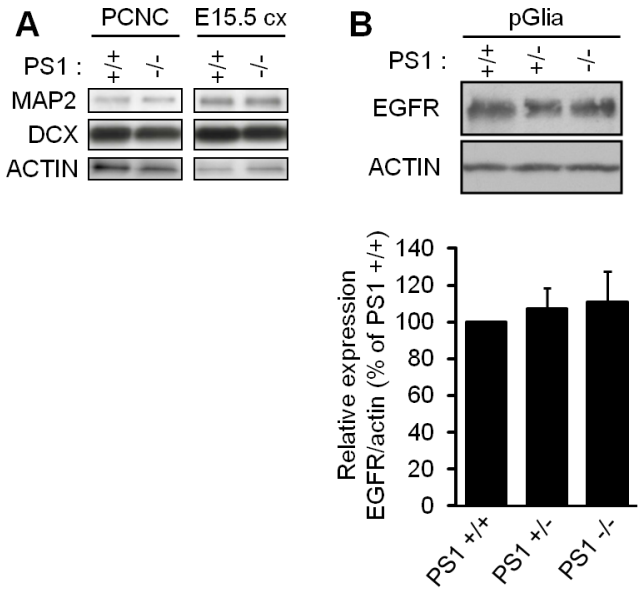


Figure 12. A) (Left) Lysates from WT or PS1-/- PCNC grown as above were prepared at 9 DIV and probed on WBs for the indicated proteins. (Right) Lysates from WT and PS1-/- embryonic brain cortex (E15.5 cx) were prepared and probed on WBs for indicated proteins. B) (Upper): primary glial (pGlia) cultures from wild-type, PS1 heterozygous or homozygous KO were obtained. Cells were cultured in 6-well plates. When cells reached about 80% confluence, lysates were collected and assayed on Western blotting for the proteins indicated. (Lower): densitometric analysis of the relative amounts of EGFR in primary glial cultures is expressed as ratio of EGFR to actin. Data were respectively obtained from at least four separate experiments. *, p<0.05; ***, p<0.001 (paired *t-test*).

To examine whether acute downregulation of PS1 has similar effects on expression and signaling of neuronal EGFR as chronic absence of PS1, we used anti-PS1 siRNAs. Since PS1+/- neurons have similar levels of EGFR and exhibit similar EGF signaling as WT neurons and to ensure efficient downregulation of EGFR by siRNAs, we used these neurons in our experiments. As shown in Figure 13, acute knockdown of neuronal PS1 decreased both, the amounts of EGFR (Figure 13A) and the EGF-dependent neuroprotection against glutamate excitotoxicity (Figure 13B). These data show that acute downregulation of PS1 has similar effects on the expression of EGFR and EGF-dependent neuroprotection as those observed under conditions of chronic absence of PS1. Finally, re-introduction of mouse PS1 in PS1 null neurons caused a robust increase of EGFR (Figure 13C) further supporting the conclusion that PS1 strongly stimulates the neuronal expression of EGFR.

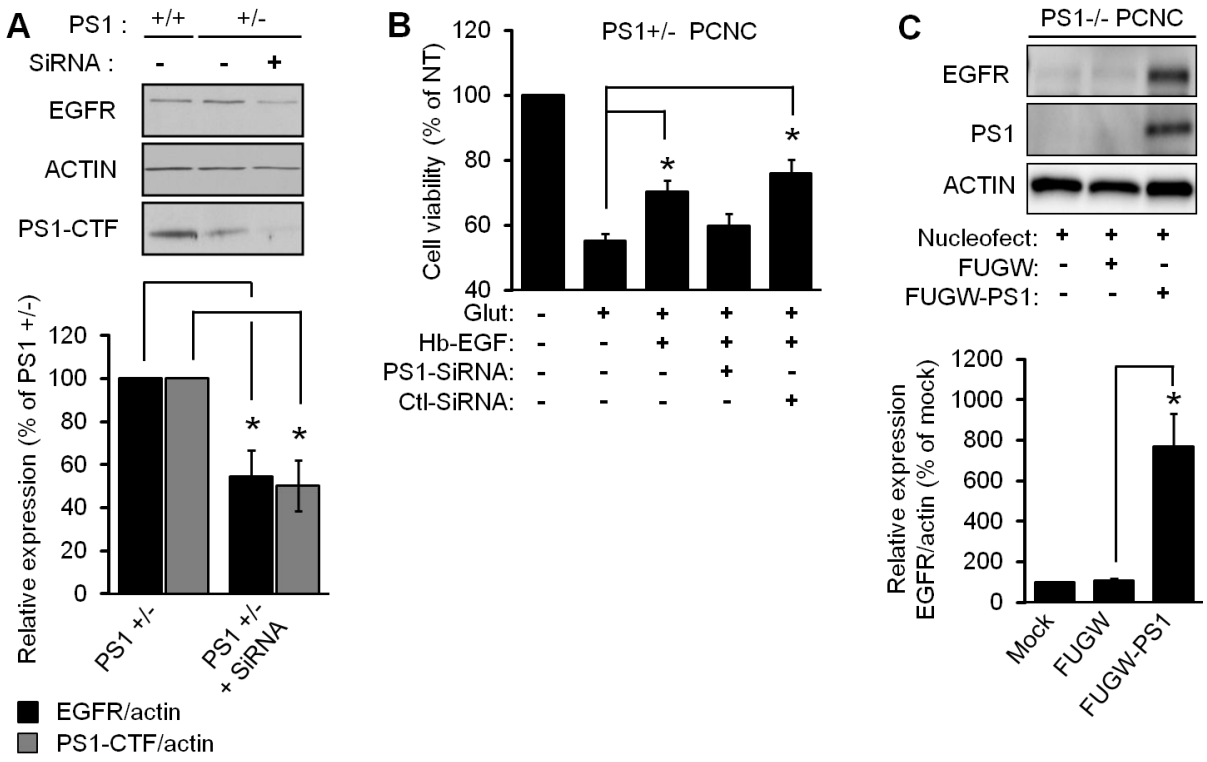


Figure 13. Acute knockdown of PS1 decreases EGFR and abolishes EGF neuroprotection while reintroduction of PS1 in PS1 -/- neurons increases EGFR. *A*) (Upper): PCNC from PS1 heterozygous KO (+/-) were cultured in 12-well plates and at 5 DIV were treated with 1μM Accell SMARTpool SiRNA against PS1 for 72 hours. Non-treated cultures were used as controls. Following incubation, lysates were collected and assayed by Western blotting for the indicated proteins. WT neurons (+/+) were also included as control. (Lower): densitometric analysis of the relative amounts of EGFR and PS1-CTF in PCNC described above is expressed as ratios to actin and then normalized as a percentage of EGFR or PS1-CTF amount in the non-treated WT neurons. Data were respectively obtained from four separate experiments. *, p<0.05; (paired *t-test*). No effect was observed on PS1 when neurons were treated with 1μM Accell SMARTpool scrambled control (non-targeting) SiRNA for 72 hours (data not shown). *B*) PS1+/- PCNCs were grown in 24-well plates with Neurobasal Media plus B27 supplement. At 5 DIV neurons were treated with 1μM Accell SMARTpool SiRNA for 72 hours and then incubated with 20ng/ml Hb-EGF overnight. The next day, the medium was switched to HBSS containing Hb-EGF for 30 minutes followed by 50μM glutamate incubation for 3 hours. Cell viability was evaluated by MTT assay and normalized to non-treated cells. Results (mean ± SEM) were summarized from six independent experiments and in each experiment each condition is the average of four identical wells. *, p<0.05 comparing between cultures treated with glutamate in the presence or absence of Hb-EGF, PS1-SiRNA or non-targeting SiRNA (paired *t-test*). *C*) (Upper): PS1-/- PCNC were mock transfected (left lane) or transfected with either mouse PS1 in FUGW vector or vector alone. Following incubation, lysates were collected at 8 DIV and assayed on WBs for the indicated proteins. (Lower): densitometric analysis of the amounts of EGFR in transfected PS1-/- neuronal cultures above is expressed as ratio of EGFR to actin and normalized to mock nucleofected neurons. Bars represent means and error bars SEM. Data were obtained from four independent experiments. *, p<0.05 (paired *t-test*).

To establish a direct link between reduced neuronal survival in the absence of PS1 and expression of EGFR, we expressed exogenous EGFR in PS1-/- neurons. Figure 14A shows that exogenous EGFR rescues the ability of EGF to increase neuronal survival even in the absence of PS1. Furthermore, expression of EGFR restores the ability of EGF to stimulate phospho-EGFR and activate its downstream targets AKT and ERK kinases even in the absence of PS1 (compare Figure 14B to Figure 8A). Importantly, Figure 14C shows that in our neuronal cultures, EGFR dominates the survival

signaling of both EGF and Hb-EGF as treatment with EGFR inhibitor Erlotinib blocks induced phosphorylation of AKT and ERK. These data reveal a direct link between EGFR and PS1-dependent phenotypes on neuronal survival and activation of AKT kinase.

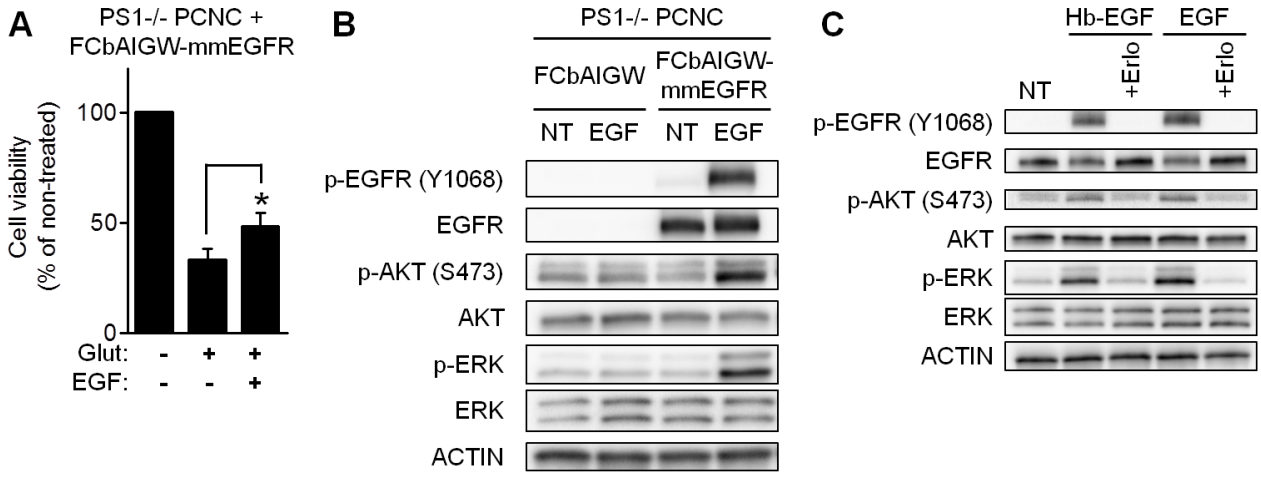


Figure 14. Expression of exogenous EGFR restores the PS1-dependent ability of EGF to rescue neurons from excitotoxicity and stimulate survival signaling. *A*) PS1-/- neurons were transfected with either empty vector (FCbAIGW) or an EGFR-expressing construct (FCbAIGW-EGFR) and at 8 DIV, neurons were treated with 20ng/ml EGF overnight as indicated in Figure. Next day, the medium was switched to HBSS containing EGF for 30 minutes followed by 50μM glutamate incubation for 3 hours. Cell viability was then measured counting healthy nuclei with Hoechst kit 33342 and normalized to non-treated cells as described. Results (mean ± SEM) are from three independent experiments (n=3). *, p<0.05 (paired *t-test*) *B*) PS1-/- neurons were transfected with EGFR-expressing plasmid FCbAIGW-EGFR or vector alone (FCbAIGW) and at 8 DIV cultures were treated with 20ng/mL EGF for 15 minutes. Lysates were then collected and probed on WBs for indicated proteins. C) WT PCNC of 8 DIV were incubated with 10uM Erlotinib (Erlo) for 30 minutes and then treated with 20ng/mL of either Hb-EGF or EGF. Cultures were incubated for an additional 15 minutes and lysates were assayed for indicated proteins.

3.3 NEITHER GAMMA-SECRETASE NOR PS2 REGULATE EXPRESSION OF NEURONAL EGFR.

PS1 has been shown to have both γ-secretase-dependent and independent functions (see 1.4.2 Gamma-secretase independent functions of PS1 and (Pimplikar *et al*, 2010; Barthet *et al*, 2013)). Use of immortalized cell systems however to examine the effects of γ-secretase on the cellular levels of EGFR yielded inconsistent answers as evidence were reported against (Repetto *et al*, 2007; Rocher-Ros *et al*, 2010) and for (Zhang *et al*, 2007; Li *et al*, 2007) involvement of γ-secretase activity in the regulation of this receptor. To examine effects of γ-secretase on neuronal EGFR we treated primary neuronal cultures with L-685,458, a potent γ-secretase inhibitor widely used in our and other laboratories (Marambaud *et al*, 2002; Zhang *et al*, 2007; Barthet *et al*, 2011). Figure 15A shows that although L-685,458 causes a robust accumulation of APP-derived γ-secretase substrates APP-CTFs, this treatment has no effect on neuronal EGFR indicating γ-secretase is not involved in the regulation of the expression of this receptor. To further explore this issue, we probed iMEFs which have been widely used to explore the role of γ-secretase in the regulation of EGFR (Zhang *et al*, 2007; Repetto *et al*, 2007; Li *et al*, 2007; Rocher-Ros *et al*, 2010). Figure 15B shows that in agreement with data in primary neurons, inhibition of γ-secretase has no effect on the EGFR of iMEF cells. Since γ-

secretase activity cleaves a large number of type I transmembrane proteins (Barthet *et al*, 2012) we asked whether EGFR, a type I protein, may be processed by this activity. Our efforts however to detect EGFR-derived peptides expected from the γ-secretase processing of proteins (Marambaud *et al*, 2002; Georgakopoulos *et al*, 2006; Litterst *et al*, 2007) or complexes between PS1 and EGFR using co-immunoprecipitation protocols were unsuccessful (Georgakopoulos et al., unpublished).

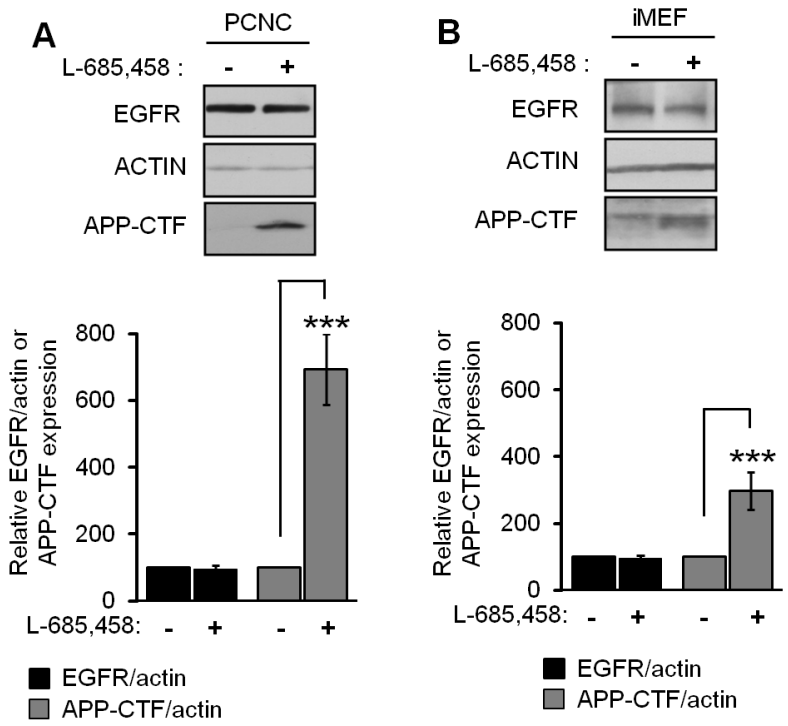


Figure 15. Inhibition of γ-secretase has no effect on the expression of EGFR. *A*) WT PCNC were grown in 6-well plates for 9 days and then treated with γ-secretase inhibitor L-685,458 (500nM) overnight (+). WT control (-) cultures were treated with the same solution without the inhibitor. Following incubation, lysates were collected and assayed on WBs for the indicated proteins. Lower panel: densitometric analysis of the relative amounts of EGFR and APP-CTFs in PCNC normalized to actin and expressed as percent of the amounts in non-treated controls. Data were from four independent experiments. ***, p<0.001 (paired *t-test*). *B*) Cultures of immortalized mouse embryonic fibroblasts (iMEF) were treated overnight with γ-secretase inhibitor L-685,458 (+) as above or with medium without inhibitor as controls (-). Following incubation, lysates were collected and assayed on WBs for indicated proteins. Lower panel: densitometric analysis of the relative amounts of EGFR and APP-CTFs in iMEFs as above. Data are from four separate experiments as above.

Similar to PS1, its paralog PS2 also functions as a catalytic component of γ-secretase complexes that processes substrates including APP and Notch1 (Xia *et al*, 1997; Steiner *et al*, 1999). We thus used PS2 null (PS2-/-) neurons to ask whether PS2 regulates neuronal EGFR. Figure 16 shows that absence of PS2 affects neither the levels of this receptor nor its neuroprotective activity, supporting the conclusion that PS1 controls EGFR through specific mechanisms independent of both γ-secretase and PS2.

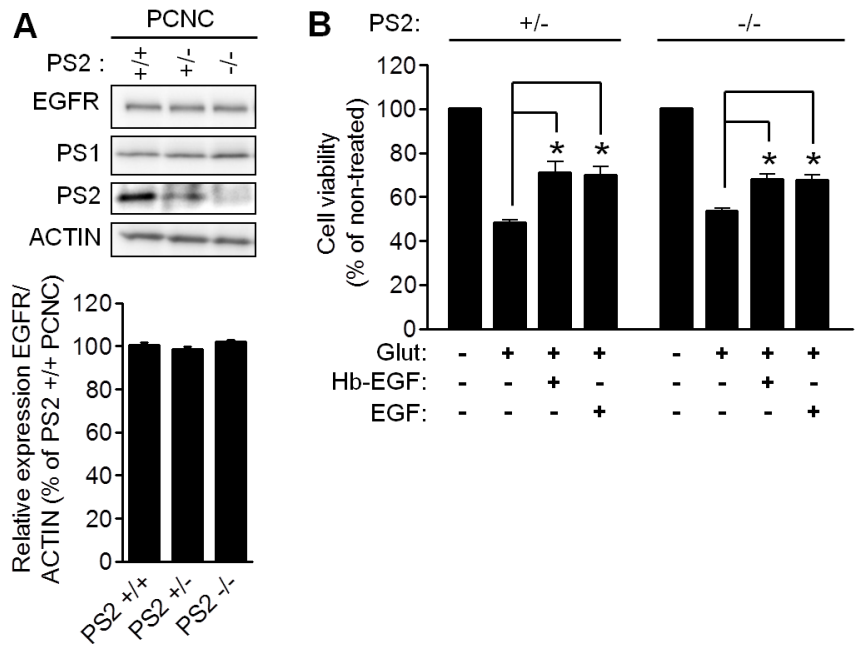


Figure 16. PS2 affects neither EGFR expression nor EGF-dependent neuroprotection. *A*) (Upper): PCNC from WT (+/+) and PS2 heterozygous (+/-) and homozygous (-/-) KO embryonic mouse brains were cultured in 6-well plates. At 9DIV, lysates were collected and probed on WBs for the indicated proteins. (Lower): densitometric analysis of the relative amounts of EGFR shown above expressed as EGFR to actin ratio. *B*) WT, PS2+/- and PS2-/- mouse PCNC were grown in 24-well plates. At 9 DIV cultures were treated overnight with 20ng/ml of either Hb-EGF or EGF. Next day, the medium was switched to HBSS containing Hb-EGF or EGF for 30 minutes followed by 50μM glutamate incubation for 3 hours (see Figure 9A). Cell viability was evaluated by MTT assay and normalized to non-treated cells. Treatment with growth factors alone had no effect on neuronal viability compared to untreated cultures. Bars represent means and error bars SEM. Data were respectively obtained from at least four separate experiments. *, p<0.05; (unpaired *t-test* for A, paired *t-test* for B).

3.4 MECHANISMS OF REGULATION OF NEURONAL EGFR.

Recent data obtained in iMEF cell lines indicate that PS1 regulates EGFR post-translationally by promoting its degradation through the proteasomal and lysosomal systems (Repetto *et al*, 2007; Rocher-Ros *et al*, 2010). In contrast, others reported that PS1 negatively regulates transcription of EGFR in iMEF cell lines (Zhang *et al*, 2007). We measured the turnover rate of EGFR in WT and PS1 KO neurons using the protein synthesis inhibitor cycloheximide (CHX). Our data show that EGFR turnover in PS1-/- neurons is undistinguishable from that in WT neurons (Figure 17A). To examine involvement of transcriptional controls we measured the *Egfr* mRNA by quantitative real-time PCR using independent neuronal preparations. We found that the levels of this mRNA in PS1-/- neurons were reduced by more than 95% compared to WT neurons (Figure 17B). Thus, protein and mRNA data indicate that PS1 positively regulates EGFR at the mRNA level. Measurements of brain mRNA also support a positive regulation of *Egfr* mRNA by PS1 as this mRNA is significantly decreased in PS1-/- embryonic brains compared to WT (Figure 17C). Furthermore, similar to the relative decrease of EGFR protein in PS1-/- neurons and brain tissue (Figure 11), reduction of *Egfr* mRNA in PS1 null brains is smaller than its reduction in PS1-/- neurons in agreement with a specific decrease

of neuronal mRNA in brain. Furthermore, similar to results in PS1-/- neurons, acute downregulation of PS1 reduced the neuronal *Egfr* mRNA (Figure 17D) supporting the conclusion that PS1 positively regulates this mRNA. To examine whether PS1 controls the stability of this mRNA, we used actinomycin to arrest transcription. Figure 17E shows that *Egfr* mRNA declines at similar rates in the presence or absence of PS1 indicating PS1 regulates the *Egfr* mRNA at a step before synthesis of mature transcripts.

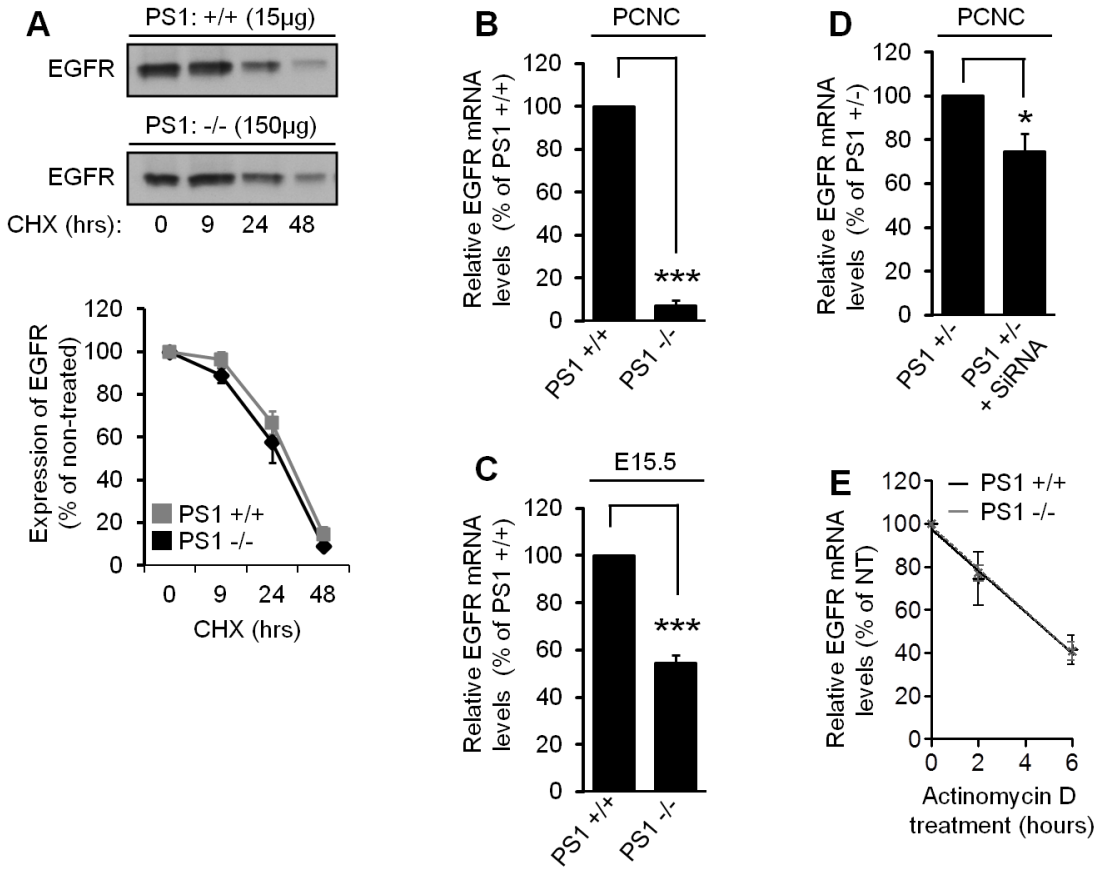


Figure 17. PS1 positively regulates expression of neuronal *Egfr* mRNA. *A*) (Upper): WT (PS1+/+) or PS1 homozygous KO (PS1-/-) PCNC in Neurobasal Media plus B27 supplement were treated at 7DIV with 50µg/ml cycloheximide (CHX) for the indicated times. Non-treated (0 time) cultures were used as controls. Following incubation, lysates (15µg from WT or 150µg from PS1-/-) were collected and probed on WBs for EGFR. (Lower): kinetic analysis of the relative amounts of EGFR in PCNC following CHX treatment expressed as a percentage of EGFR amount in 0-time samples. Data were obtained from four independent experiments. *B-C*) Total mRNA was isolated from WT (PS1+/+) or PS1 null (PS1-/-) mouse PCNC of 8DIV (*B*) or embryonic cortex (E15.5) (*C*). Following cDNA amplification using E15.5 cortical mRNA, quantitative real-time PCR assay was performed as described in the experimental procedures. Data were respectively obtained from at least four separate experiments, ***, p<0.001 (paired *t-test*). *D*) PS1 heterozygous KO (PS1+/-) PCNC grown on 12-well plates were treated at 5DIV with 1µM Accell SMARTpool SiRNA against PS1 for 72 hours. Non-treated cultures were used as controls. Following incubation, total mRNA was isolated, followed by cDNA amplification and quantitative real-time PCR performed as described in Methods. Data were respectively obtained from at least four separate experiments, *, p<0.05 (paired *t-test*). *E*) PCNC of WT (PS1+/+) or PS1 null (PS1-/-) mice were treated with actinomycin D (2µg/mL) to arrest transcription and total RNA was isolated at 0, 2, or 6 hours later. RNA Samples were then subjected to cDNA amplification and quantitative real-time PCR and relative amounts of *Egfr* mRNA were calculated using GAPDH as an internal control (ΔCt). Amounts of *Egfr* mRNA are

expressed as % of its amounts at time zero and plotted against time using the $2^{-\Delta\Delta Ct}$ method. The data were fitted to the linear regression model and the slope of the curve was similar between genotypes: -0.159 in WT vs. -0.162 in PS1KO (n=3, 95% ci).

3.5 PS1 DOES NOT REGULATE THE EXPRESSION OF EGFR IN FIBROBLASTS

In contrast to our data that PS1 positively regulates expression of neuronal EGFR, several groups reported that PS1 inhibits expression of this receptor in non-neuronal systems. Since however, evidence of a negative correlation between PS1 and EGFR was obtained using immortalized double KO (DKO) fibroblast cells line lacking both PS2 and PS1 (Zhang *et al*, 2007; Repetto *et al*, 2007; Rocher-Ros *et al*, 2010) we examined the expression of EGFR in primary fibroblasts lacking only PS1. Similar to data obtained in primary glia cells, absence of either one or both alleles of PS1 has no effect on EGFR, a result consistently obtained in several independent preparations of primary fibroblasts (Figure 18A). Importantly, measurements in distinct iMEF cell lines revealed clonal variability of the EGFR levels independent of PS1 expression or genotype (Figure 18B) suggesting that clonal selection of iMEFs used to examine PS1 effects on FGFR may contributed to literature disagreements on the role of PS1 on EFGR (Zhang *et al*, 2007; Repetto *et al*, 2007; Li *et al*, 2007; Rocher-Ros *et al*, 2010). We thus examined the effects of acute downregulation of PS1 on the EFGR of iMEFs using anti-PS1 siRNA. Figure 9C shows that downregulation of PS1 in two independent PS1 heterozygous KO (PS1+/-) iMEF clones had no significant effect on the levels of EGFR. Furthermore, in contrast to data in neurons (Figure 18C), re-introduction of PS1 in PS1 KO iMEF clones showed no effects on EGFR (Figure 18D). These experiments indicate that PS1 may not regulate the EGFR of fibroblast cells, a conclusion strongly supported by absence of PS1 effects on the EGFR of primary fibroblasts (Figure 18A). Together, our data show that PS1 positively regulates neuronal EGFR but has no effects on the expression of this receptor in glia or fibroblast cells. Our results however, do not exclude the possibility that PS1 may regulate this receptor in other cell systems not examined here (Gadadhar *et al*, 2011).

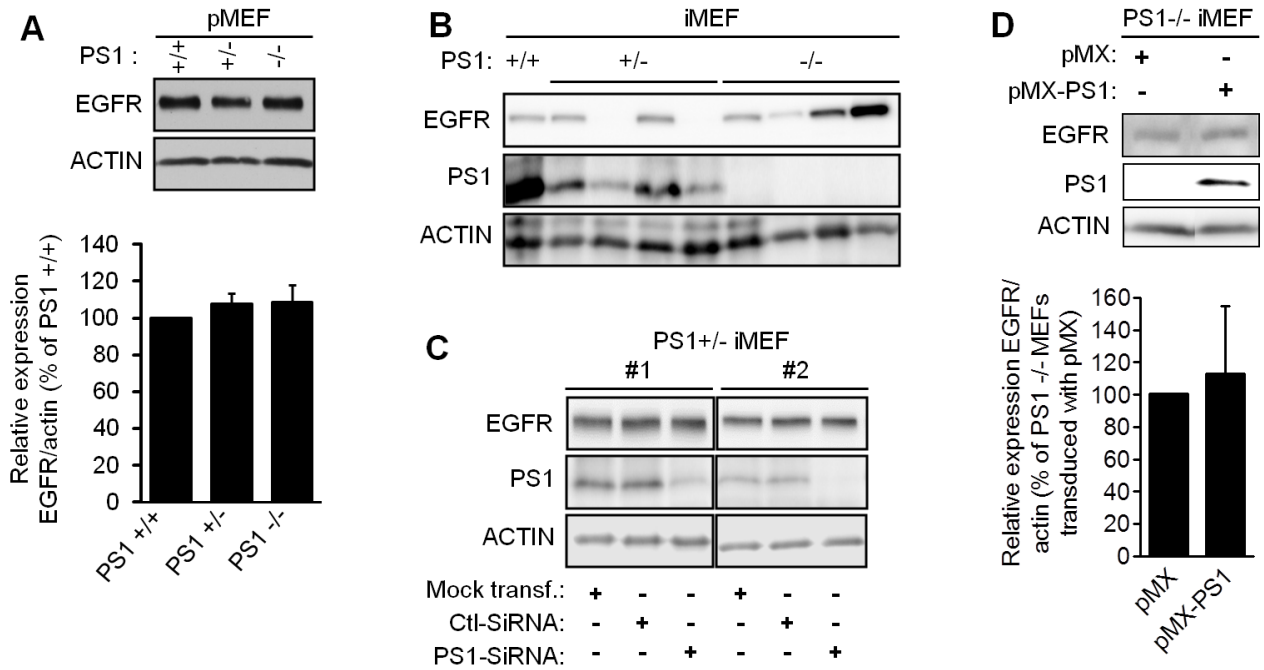


Figure 18. Absence of PS1 has no effect on EGFR expression of primary fibroblasts or iMEFs. *A*) (Upper): primary mouse fibroblasts (pMEF) from WT (+/+), PS1 +/- and -/- mouse embryos were obtained. Cells were cultured in 6-well plates at 80% confluence and lysates were prepared and assayed on WBs for the indicated proteins. (Lower): Relative amounts of EGFR in pMEFs of the genotypes shown in upper figure are expressed as EGFR to actin ratio (n=6). *B*) Lysates from WT (+/+), PS1+/-, and PS1-/- MEF cell lines (iMEF) were assayed on WBs for the indicated proteins. Each cell line is derived from an independent iMEF clone. *C*) Immortalized PS1 heterozygous KO MEFs (PS1+/- iMEF) were transfected with 25nM of anti-PS1 siRNA for 72 hours. Mock-transfected and scrambled siRNA-transfected (Ctl-siRNA) cultures were used as controls. Following incubation, lysates were collected and assayed by WBs for the indicated proteins. *D*) (Upper): PS1 null iMEFs (PS1-/- iMEF) were stably transduced with either human PS1 in pMX vector or vector alone. Lysates were prepared and assayed on WBs for indicated proteins. (Lower): densitometric analysis of relative amounts of EGFR in 4 different PS1 KO iMEF clones transfected either with pMX vector or pMX-PS1. Amount of EGFR in each PS1-transfected clone is normalized to the EGFR of the vector-transfected clone. Bars represent means, error bars SEM.

CHAPTER 4 DISCUSSION AND FUTURE DIRECTIONS

4.1 DISCUSSION

EGFR is a key tyrosine kinase growth factor receptor expressed in many cell types where it regulates growth, migration, and differentiation (Peus *et al*, 1997; Hudson & McCawley, 1998; Avraham & Yarden, 2011). In addition, this receptor plays pivotal roles in tumor development and proliferation (Normanno *et al*, 2006; Avraham & Yarden, 2011) while other studies reveal that EGFR signaling controls neuronal function and survival (Opanashuk *et al*, 1999; Farkas & Krieglstein, 2002; Hanke *et al*, 2004; Enwere *et al*, 2004). Thus, ligands to this receptor such as Hb-EGF and EGF protect brain neurons from excitotoxicity, oxygen/glucose deprivation, and traumatic injuries, insults implicated in the pathogenesis of neurodegenerative disorders and stroke (Casper *et al*, 1991; Peng *et al*, 1998; Opanashuk *et al*, 1999; Hanke *et al*, 2004; Sun *et al*, 2010). Recently, it was reported that PS1 is necessary for neuroprotective activities of brain-derived neurotrophic factor (BDNF) and ephrinB, a function independent of γ-secretase activity (Barthet *et al*, 2013). Here we show that in the absence of PS1, cortical neurons are unable to activate the EGFR neuroprotective signaling in response to EGFs, an outcome consistently obtained under both chronic absence of PS1 in PS1-/- neurons and acute downregulation of neuronal PS1 using siRNAs. Thus, in contrast to WT neurons, neither EGF nor Hb-EGF showed any neuroprotective activity against excitotoxicity in PS1-/- neuronal cultures, suggesting that PS1 is indispensable for EGF-dependent neuroprotection. Furthermore, in absence of PS1, EGFs are unable to stimulate phosphorylation of survival kinase AKT, an event critical to the neuroprotective function of EGF factors against excitotoxicity. Interestingly, in contrast to BDNF- and ephrinB-dependent neuroprotection in which expression of both PS1 alleles is required (Barthet *et al*, 2013), one PS1 allele is sufficient for full EGF-induced neuroprotective activity against excitotoxicity. This difference may reflect variations in the mechanisms by which PS1 mediates neuroprotective activities of specific ligand-receptor systems.

We found that lack of EGF-dependent neuroprotection correlates with decreased levels of neuronal EGFR. Furthermore, re-introduction of exogenous PS1 in PS1-/- neurons increased cellular EGFR and neuroprotective potential while acute downregulation of neuronal PS1 decreased both, the levels of EGFR and neuroprotective activity of EGF ligands. Together, these data show that PS1 is a critical positive regulator of the expression of neuronal EGFR and that due to a dramatic decrease of EGFR, PS1 null neurons are unable to use EGF factors to activate survival signaling. Importantly, expression of exogenous EGFR restores the ability of EGFs to phosphorylate EGFR, activate its targets AKT and ERK and rescue neurons from excitotoxicity even in the absence of PS1 supporting the conclusion that the main role of PS1 in EGF neuroprotection is to regulate expression of EGFR. In contrast to the crucial role PS1 plays in the regulation of neuronal EGFR, PS1 has no effect on the EGFR of primary glia and fibroblast cells indicating that PS1 specifically regulates the neuronal receptor. This conclusion is also supported by our finding that absence of PS1 causes a more

dramatic drop in neuronal than brain EGFR as total amount of this receptor in brain is determined by its expression in all cells.

PS1 functions as catalytic component of γ-secretase complexes that process many type I transmembrane proteins including receptors. Previous work on the role of γ-secretase on EGFR however, yielded contradictory evidence for (Zhang *et al*, 2007; Li *et al*, 2007) and against (Repetto *et al*, 2007; Rocher-Ros *et al*, 2010) a role of this activity in the regulation of the receptor. Our data show that γ-secretase activity has no significant effect on neuronal EGFR. Furthermore, we found that PS1 has no effects on the stability of neuronal EGFR. We thus asked whether PS1 might stimulate expression of *Egfr* mRNA. Indeed, we found that this mRNA is greatly decreased in PS1-/- neurons but this decrease is smaller in PS1 null brains, an observation consistent with our data that PS1 does not affect the EGFR levels of astrocytes. Acute downregulation of neuronal PS1 resulted in a robust decrease of *Egfr* mRNA providing strong evidence that PS1 directly regulates this mRNA. Importantly, our experiments showed that PS1 has no effect on the stability of *Egfr* mRNA indicating that PS1 controls a step prior to synthesis of mature receptor mRNA. Together, our data show that PS1 functions as a critical positive regulator of neuronal *Egfr* mRNA. Interestingly, absence of PS2 affects neither the cellular levels of EGFR nor its neuroprotective functions suggesting that a novel PS1-dependent transcriptional mechanism controls expression of neuronal EGFR independent of both PS2 and γ-secretase. Although the detailed mechanisms by which PS1 stimulates transcription of neuronal EGFR remain to be elucidated, it is tempting to speculate that recently described γ-secretase-independent functions of PS1 in cell signaling may be involved (Pimplikar *et al*, 2010).

Our conclusions that PS1 stimulates neuronal, but not glial or fibroblast, EGFR mRNA was unexpected as evidence obtained in iMEFs indicate that PS1 negatively regulates cellular levels of EGFR. Although the source of this inconsistency is unclear, use of PS1 and PS2 DKO iMEFs and tumor cells resulted in inconsistent conclusions for both, the role of γ-secretase in the regulation of EGFR (Zhang *et al*, 2007; Repetto *et al*, 2007; Li *et al*, 2007; Rocher-Ros *et al*, 2010; Song *et al*, 2012) and the mechanisms by which PS1 suppresses this receptor (Zhang *et al*, 2007; Repetto *et al*, 2007; Rocher-Ros *et al*, 2010). In addition, it has been found that DKO iMEFs lacking both PS1 and PS2 have defects independent of Presenilins (Watanabe *et al*, 2009) whilst conditional PS1 KO in PS2 KO background (PS1cKO/PS2KO) mice may still express PS1 (Yu *et al*, 2001; Feng *et al*, 2001; Saura *et al*, 2004). Thus, transformed cells where gene expression may be distorted by immortalization, genomic rearrangements and clonal selection, may not be reliable indicators for *in vivo* effects of PS1 on EGFR. In support of this possibility, we observed large variations in the levels of EGFR among distinct iMEF cell lines regardless of PS1 genotype. More importantly, in our experiments neither downregulation nor overexpression of PS1 had significant effects on the EGFR of iMEFs, an outcome consistent with absence of PS1 effects on the EGFR of primary fibroblasts. Thus, our data brings into

new focus previous inconsistent conclusions on the role of PS1 and γ-secretase in the expression of EGFR and reveal novel pathways by which PS1 regulates neuronal gene expression *via* γ-secretase independent mechanisms.

In contrast to severe developmental abnormalities and lethal phenotypes caused by absence of PS1 in transgenic mouse models (Wong *et al*, 1997; Shen *et al*, 1997), PS2 null mice survive free of serious phenotypes (Steiner *et al*, 1999; Herreman *et al*, 1999). It is currently believed that reduction of γ-secretase cleavage of Notch1 receptor is responsible for the lethal phenotype of PS1 null mice (De Strooper *et al*, 1999). This suggestion however does not explain the absence of phenotypes in PS2 null mice, even though similar to PS1, PS2 also catalyzes the γ-secretase processing of Notch1 (Steiner *et al*, 1999). Our finding that PS1 is indispensable for the expression of neuronal EGFR, a function displayed neither by PS2 nor γ-secretase, raises the intriguing possibility that developmental abnormalities in PS1 null mice are, at least in part, due to reduced neuronal EGFR. Importantly, deficiencies in either EGFR or Hb-EGF cause early postnatal mortality with abnormalities similar to those observed in PS1 null mice (Sibilia *et al*, 1998) while recent evidence reveals that PS1 regulates proliferation of brain progenitor cells through EGFR (Gadadhar *et al*, 2011). Recently it was reported that the tyrosine kinase activity of EGFR regulates biogenesis of miRNAs involved in cellular response to hypoxia (Shen *et al*, 2013). Thus, it is reasonable to infer that by controlling the levels of neuronal EGFR, PS1 tightly regulates and fine-tunes the functions of this receptor including its roles in development, production of miRNAs, and neuronal survival and function. Importantly, emerging evidence indicates that the role of this receptor in the development of AD has been unappreciated. Recent analysis of genome-wide studies and protein-protein interaction modeling identify EGFR as a significant risk factor for sporadic AD (Talwar *et al*, 2014) while transcriptional profiling studies indicate that the AD-associated apoE4 allele changes the brain expression of EGFR compared to apoE3 (Conejero-Goldberg *et al*, 2011). Furthermore, additional work indicates that EGFR functions mediate Aβ42-induced memory loss in experimental animal models (Wang *et al*, 2012). Thus, by controlling EGFR, PS1 may regulate the course of many diseases known to be modulated by this receptor including cancer and sporadic AD.

4.2 FUTURE DIRECTIONS

4.2.1 Studying the mechanisms of transcriptional regulation of neuronal EGFR

This study has determined that PS1 is a positive transcriptional regulator of neuronal EGFR and that this is achieved by expressing higher levels of *Egfr* mRNA. There is very little information about how PS1 regulates the levels of neuronal *Egfr* mRNA, and this would be a very interesting topic for further studies.

4.2.1.1 Identify transcriptional factors that differentially bind the EGFR promoter in the presence and absence of PS1

To examine whether decreased production of EGFR mRNA in PS1-/- neurons correlates with changes in protein binding to the EGFR promoter, I could use DNA pull-down assays to search for proteins that differentially bind this promoter in WT and PS1-/- neurons. Briefly, nuclear extract from WT and PS1-/- neurons would be incubated with artificially constructed biotinylated EGFR promoter and bound proteins would be precipitated by streptavidin. As bait I would use a specific DNA fragment containing the mouse EGFR promoter sequence which will include transcription factor binding sites (Brandt *et al*, 2006) and is part of the active mouse *Egfr* promoter region (based on cortical H3K4me3 ChIP seq data and brain DNaseI hypersensitivity assays of the Mouse ENCODE data (Figure 19 and (Stamatoyannopoulos *et al*, 2012)). The resultant precipitate would contain the proteins that bind on the active promoter region of EGFR.

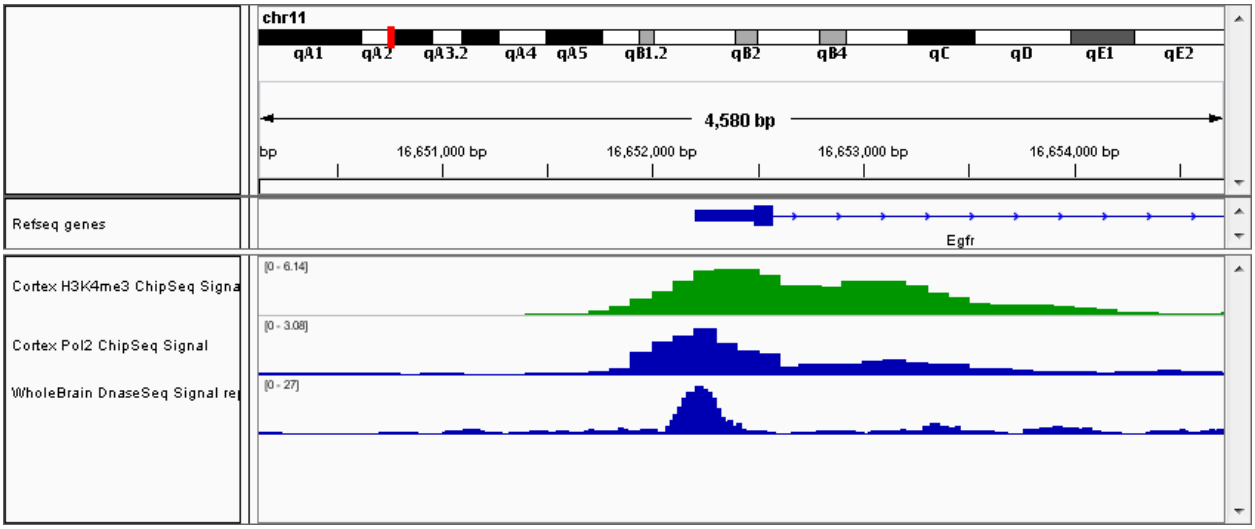


Figure 19. Mouse EGFR promoter region as visualized in IGV. The mouse genome sequence is loaded in IGV and three additional tracks are displayed: a) Cortex H3K4me3 ChipSeq: to identify active promoter regions, b) Pol2 ChipSeq: to identify Pol2 binding site, and c) Whole Brain DNAse seq to identify areas where protein complexes are bound,.

I would then dissociate the proteins from the precipitate and separate them by SDS-PAGE. I would stain the gel to look for protein signals (with colloidal Coomassie staining) that are only detected in the samples derived from WT or PS1-/- neuronal nuclear extracts. I would then cut these bands and analyze them with mass spectrometry to find the proteins that they are corresponding to. Verification of the presence of these proteins on the promoter would be performed by WB of the precipitates. Once the proteins had been identified, a series of experiments can be performed to verify the role of these proteins in regulating neuronal EGFR. Briefly some of the experiments that can be done are outlined below:

1. Downregulate or overexpress the protein in neurons to establish direct link of its expression with EGFR expression levels.

2. Perform chromatin immunoprecipitation (ChIP) assay to verify *in vivo* binding in PCNC and brains.
3. Use a luciferase reporter assay with the *Egfr* promoter to demonstrate that this protein is sufficient to increase transcriptional activity

Finally, we will determine how PS1 affects this protein's function. For example does it regulate its amounts, subcellular localization, phosphorylation state or activity? If PS1 affects neither of the above properties of this protein then PS1 regulates protein binding to the *Egfr* promoter via other pathways such as DNA methylation.

4.2.1.2 Examine whether the difference in Egfr mRNA expression is due to differential methylation of the EGFR promoter in the absence of PS1

Epigenetic mechanisms, such as DNA methylation are dynamically regulated in post-mitotic neurons in the adult nervous system (Feng *et al*, 2010), where they function in activity-dependent neuronal plasticity and memory formation (Levenson & Sweatt, 2005; Lubin *et al*, 2008; Roth & Sweatt, 2009). Furthermore, evidence shows that DNA methylation is involved in neurodegenerative and neuropsychiatric disorders (Mattson, 2003; Weaver *et al*, 2004). EGFR is a 170 kDa protein encoded by 28 exons and a member of the HER/ERB-B family of tyrosine receptor kinases. As reported above (3.4 Mechanisms of regulation of neuronal EGFR.), levels of neuronal EGFR mRNA decrease significantly but its stability is unaffected suggesting a reduction in the synthesis of mature mRNA. In most mammalian genomes, the cytosines are methylated at CpG dinucleotide sites. Interestingly, adjacent to the promoter regions of actively transcribed genes, short DNA stretches of this dinucleotide (called CpG islands, CGIs) remain unmethylated and the chromatin structure as a result is more relaxed. Methylation of these CGIs however, may block initiation of transcription and lower rates of gene expression often correlate with *de novo* methylation of promoter CGIs (Gal-Yam *et al*, 2006). Thus, cytosine methylation of promoter-associated CGIs is an important mechanism of gene silencing and decreased rates of mRNA synthesis. The promoter of EGFR has one CpG island of about 1Kb that extends into exon 1. It has been found that transcriptional downregulation of EGFR in human colorectal and breast cancers correlates with increased methylation of this CGI close to the transcription start site (TSS) (Scartozzi *et al*, 2011). Dynamic changes in the methylation of this CGI as a means to control expression of EGFR mRNA has already been reported in humans (Scartozzi *et al*, 2011)

Since, as described above, EGFR expression levels decrease in PS1-/- neurons, and methylation of the EGFR promoter is one of the main ways by which production of *Egfr* mRNA is regulated, it is worth investigating whether PS1 affects the methylation levels of the EGFR promoter. Analysis of EGFR promoter methylation would be performed using the bisulfite-based DNA modification procedure (Tost & Gut, 2007) that converts cytosine to uracil and then thymine (after PCR amplification), but leaves 5-methylcytosine (5mC) intact. The 5mC pattern is mapped by subsequent

pyrosequencing and analysis of the percent methylation at the CpG sites of the promoter. Genomic DNA from WT and PS1KO PCNC would be treated by bisulfate using. PCR would then be performed with biotinylated primers to produce single-stranded DNA templates followed by pyrosequencing.

4.2.2 Determining whether other Receptor Tyrosine Kinase pathways are also affected

It has been previously shown that in PS1-/- neurons, ligand-induced endocytosis and degradation of the receptors EphB2 and TrkB were compromised in neuronal cultures and thus the ability to utilize ephrinB1 and BDNF against glutamate excitotoxicity was also affected (Barthet *et al*, 2013). Here we report that PS1 affects the EGF-mediated neuroprotection against glutamate excitotoxicity via regulation of the expression levels of EGFR, another receptor tyrosine kinase (RTK). Since the discovery of the first RTK more than 25 years ago, many members of this family of cell-surface receptors have emerged as key regulators of critical cellular processes, such as proliferation and differentiation, cell survival and metabolism, cell migration, and cell-cycle control (reviewed in (Lemmon & Schlessinger, 2010)). Given the importance of RTKs in cellular functions and the molecular crosstalk between them, a large scale unbiased approach like RNA sequencing could shed some light on these pathways to identify more abnormalities of neuronal RTK expression in the absence of PS1 or in the presence of PS1 FAD mutations.

RNA would be extracted from PS1+/+ and PS1-/- PCNCs, subsequently cDNA libraries would be produced to capture RNA with poly(A) tails (mature mRNA). Extra care would be needed to assign distinct oligonucleotide tags to each sample. This last step would give us the ability to multiplex our samples (load all samples together in the same lanes to reduce lane-specific variations). We would then run our samples in a next generation sequencing machine like Illumina HiSeq 2500 with 100 nucleotide/single read setting, ensuring enough number of reads for each sample. After the sequencing is completed we would receive the raw reads, and the data would have to be analyzed as described below:

1. The raw reads would be mapped on the mouse genome (using tophat or equivalent)
2. The raw reads would be assembled into complete transcripts (using cufflinks or equivalent)
3. A reference transcripts dataset would be produced by merging the transcripts datasets produced above (using cuffmerge or equivalent)
4. I would use cuffdiff (or equivalent) to evaluate differential expression of the genes but also identify novel transcripts/isoforms.

Once the differential expression of the genes (DEG) is obtained, the relative expression of RTKs can be evaluated, and their respective KEGG pathways (Kanehisa, 2000) evaluated. The pathway analysis will be very important to assess downstream effectors of the pathways. Once the targets have been identified, mRNA expression levels can be validated by Real-time qPCR, and the protein levels by WB. Apart from the usefulness of identifying other specific deregulated pathways in the PS1-/- or PS1FAD neurons, the neuron specific DEG data could be compared to Alzheimer's Disease' Weighted

Gene Co-Expression Networks signatures (after performing expression deconvolution for neuronal subtype) to further assess the relevance of the PS1KO and PS1FAD mouse model to the disease.

4.2.3 Studying the role of EGFR on PS1KO embryo lethality

As mentioned above, the similarity of severe developmental abnormalities and lethal phenotypes caused by absence of PS1 in transgenic mouse models (Wong *et al*, 1997; Shen *et al*, 1997) and deficiencies in either EGFR or Hb-EGF (Sibilia *et al*, 1998), raise the question of whether neuron-specific reintroduction of EGFR in PS1KO embryos could rescue the PS1KO phenotype. If viable offspring could emerge, we would then be able to study the non-neuronal effects of the absence of PS1 in adult mice.

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Presenilin1 is necessary for neuronal, but not glial, EGFR expression and neuroprotection *via* γ-secretase-independent transcriptional mechanisms

Abbreviated title: PS1 controls expression of neuronal receptors.

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Abbreviations: AD; Alzheimer's Disease, AKT; Protein Kinase B, APP; Amyloid Precursor Protein, APP-CTF; Amyloid Precursor Protein - C-terminal fragment, BDNF; Brain-Derived Neurotrophic Factor, CHX; cycloheximide, DCX; doublecortin, DMEM; Dulbecco's Modified Eagle's medium, E15; Embryonic day 15, EGFR; Epidermal Growth Factor Receptor, EGFs; Epidermal Growth Factors, EphB; Ephrin B Receptors, ERK; Extracellular-signal Regulated Kinases, FAD; Familial Alzheimer's Disease, FBS; Fetal Bovine Serum, Hb-EGF; Heparin-binding EGF, iMEF; immortalized Mouse Embryonic Fibroblasts, MAP2; Microtubule-Associated Protein 2, MEF; Mouse Embryonic Fibroblasts, mmEGFR; *Mus musculus* EGFR, mmPS1; *Mus musculus* PS1, MTT assay; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay, PCNC; Primary Cortical Neuronal Cultures, pGLIA; primary glial cultures, PI3K; Phosphoinositide 3-kinase, pMEF; primary Mouse Embryonic Fibroblasts, PS1; Presenilin 1, PS2; Presenilin 2, RIPA buffer; Radioimmunoprecipitation assay buffer, SDS-PAGE; Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis, siRNA; small interfering RNA, WB; Western Blot, WT; wild type, KO; Knockout.

Abstract

Epidermal growth factor receptor (EGFR) plays pivotal roles in cell proliferation, differentiation and tissue development while EGFs protect neurons from toxic insults by binding EGFR and stimulating survival signaling. Furthermore, recent evidence implicates this receptor in neurometabolic disorders like AD and aging. Here we show that absence of presenilin1 (PS1) results in dramatic decrease (>95%) of neuronal EGFR and that PS1 null (PS1-/-) brains have reduced amounts of this receptor. PS1-/- cortical neurons contain little EGFR and show no EGF-induced survival signaling or protection against excitotoxicity but exogenous EGFR rescues both functions even in absence of PS1. EGFR mRNA is severely reduced (>95%) in PS1-/- neurons and PS1-/- brains contain decreased amounts of this mRNA although PS1 affects the stability of neither EGFR nor its mRNA. Exogenous PS1 increases neuronal EGFR mRNA while downregulation of PS1 decreases this mRNA. These effects are neuron-specific as PS1 affects the EGFR of neither glial nor fibroblast cells. In addition, PS1 controls EGFR through novel mechanisms shared with neither γ-secretase nor PS2. Our data reveal that PS1 functions as a positive transcriptional regulator of neuronal EGFR controlling its expression and function in a cell-specific manner. Deregulation of EGFR may contribute to developmental abnormalities and lethal phenotypes found in PS1, but not PS2, null mice. Furthermore, PS1 may affect neuroprotection and AD by controlling survival signaling of neuronal EGFR.

Key words: Alzheimer disease · transcription of neuronal receptors · neurodegeneration · EGFR mRNA · survival signaling

Introduction

Mutations in genes encoding presenilin (PS) proteins PS1 and PS2 cause aggressive forms of early-onset familial Alzheimer's disease (FAD). Both PS proteins are found as important functional components of the proteolytic γ-secretase complexes that cleave many typeI transmembrane proteins including the amyloid precursor protein (APP), cadherins, Notch1, ErbB4, and EphB receptors (1). In addition, PS1 has γ-secretase independent functions in cell signaling, intracellular trafficking and neuronal survival (2, 3). Recently, several groups using artificially immortalized or cancer cells reported variable conclusions for the effects of PS1 and γ-secretase on the expression and function of Epidermal Growth Factor Receptor (EGFR) (4–7).

EGFR is a protein tyrosine kinase receptor with key roles in cell growth, differentiation and tissue development and function acting as an integrator where extracellular growth and survival signals converge and transform into intracellular outputs (8, 9). Furthermore, EGFR is known to play pivotal roles on human cell transformation and cancer (8, 9) and ligands to this receptor, known as epidermal growth factors (EGFs), are found in brain where they regulate neuronal development, function and survival (10–13). Binding of EGFs to their cognate receptor stimulates EGFR-dependent phosphorylation of survival kinases thus increasing neuronal survival against toxic insults such as excitotoxicity, oxidative stress and ischemia (11–16). Functions of EGFR have been implicated in a spectrum of neurometabolic disorders including diabetes, AD and aging (8, 17). The crucial roles the EGF ligand-receptor system plays in development was shown by data that mouse embryos lacking either EGFR or its brain-enriched ligand heparin binding EGF-like growth factor (Hb-EGF), die at birth (18–22) while surviving animals suffer from cortical neurodegeneration (23). PS1 null mice also die at birth displaying severe neuronal abnormalities highlighting the importance of both PS1 and EGFR in development and brain function. Importantly, although both PS1 and PS2 have γ-secretase activity only absence of PS1 results in severe developmental abnormalities and lethal phenotypes (24, 25).

Recently, we reported that PS1 is necessary for the neuroprotective functions of specific growth factors including brain-derived neurotrophic factor (BDNF) and ephrinB (3), but no evidence for a PS1 role in the neuroprotective functions of EGFR or its ligands has been reported. In contrast, several groups reported that PS1 suppresses expression of EGFR in immortalized mouse embryonic fibroblast cells (iMEFs) but proposed mechanisms are unclear as both pre- and post-translational mechanisms of EGFR inhibition by PS1 were proposed (4–6). Furthermore, evidence for and against involvement of γ-secretase in the regulation of cellular levels of EGFR has been described in immortalized cells (4–7).

Here we present data that in contrast to results in iMEF cell lines, PS1 functions as a positive and specific regulator of neuronal EGFR. Thus, compared to wild type (WT), PS1 null neurons contain very little EGFR and show no EGF-dependent signaling and neuroprotection against glutamate excitotoxicity. Absence of PS1 however, has no effect on the EGFR of primary glia or fibroblast cells while PS1 null brain tissue shows a significant reduction of this receptor. Importantly, our data reveals that PS1, but not PS2, increases the levels of neuronal EGFR by transcriptional mechanisms independent of protein turnover and γ-secretase activity.

MATERIALS AND METHODS

Materials

Antibodies against EGFR were from Millipore, Billerica, MA, USA, Tubulin from Santa Cruz, Dallas, TX, USA and MAP2 from Sigma-Aldrich, St. Louis, MO, USA. Antibodies against Actin, doublecortin (DCX), phospho-EGFR (Y1068), AKT, phospho-AKT (S473 and T308), ERK, and phospho-ERK (T202/Y204) were from Cell Signaling Technology, Danvers, MA, USA. Anti-PS1 monoclonal antibody 33B10 and R1 antiserum against C-terminal APP were described previously (26). Erlotinib and AG-1478 were from Selleck Chemicals, Houston, TX, USA. EGF, Hb-EGF, Cycloheximide (CHX), U0126, wortmannin and γ-secretase inhibitor L-645,458 were from Sigma-Aldrich, St. Louis, MO, USA. A SMARTpool of Accell anti-PS1 siRNAs (Dharmacon, Lafayette, CO, USA) containing a mixture of four (4) siRNAs against PS1 and non-targeting SMARTpool SiRNAs (ON-TARGETplus for immortalized fibroblasts and Accell for neurons and primary fibroblasts) were from Dharmacon, Lafayette, CO, USA.

Cell cultures

Wild type (WT) and PS1 heterozygous (PS1+/-) knockout (KO) mouse colonies as well as production and genotyping of WT, PS1+/- and PS1 homozygous KO (PS1-/-) mouse embryos were as described (3). In addition, Supplementary Fig. 1 shows increased accumulation of APP/CTF1 and N-cad/CTF1 fragments known to occur in the absence of PS1 (1, 27) All animal experiments were carried out according to regulations of Mount Sinai Medical Center. Primary cortical neuronal cultures (PCNC) were prepared from E 15.5 mouse embryo brains, plated on poly-D-lysine-coated plates in Neurobasal medium as reported (3, 28, 29) and kept *in vitro* before use as indicated in figure legends. At 9 days *in vitro* (DIV) postmitotic neurons represent more than 95% of cultured cells (3 and Supplementary Fig. 2).

Immortalized MEF cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS), p/s in 5% CO_2 at 37°C. Primary fibroblast (30) and primary glial cultures containing mostly astrocytes were prepared from mouse embryo brains as described (31). Glial cells were plated at 3×10^5 cells/cm^2 in DMEM supplemented with 10% FBS, p/s in 5% CO_2 at 37°C. Medium was replaced 24 hours after plating and cells were left growing for 8 days. Cells were then trypsinized and re-plated at a density of 3×10^4 cells/cm^2 until desired confluency. MEF cells were isolated from trypsinized mouse tissue at embryonic day 15 (E15), immortalized using SV40 T antigen (pSV3neo) and clones were maintained in the presence of G418 (400-100 μg/ml). Single clones were selected at low density using cloning cylinders and individually propagated.

Western blots (WBs)

Cell lysates were prepared in RIPA (50mM Tris/HCl, pH7.4, 150mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100 and 0.1% w/v SDS) supplemented with protease (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (20mM NaF, 5mM Na_3VO_4, 1mM sodium pyrophosphate, and 100nM microcystin-LR). Mouse brain lysates were prepared from E15.5 embryos by mechanical dissociation and sonication in RIPA buffer. Samples were centrifuged at 14,000xg and protein in supernatants was measured using BCA assays (Pierce, Waltham, MA, USA). Aliquots were diluted with 3X Laemmli buffer, denatured in a boiling water bath and equal amounts of protein were resolved on SDS-PAGE followed by Western blotting and protein detection with antibodies as described (3).

Cell viability

Neuronal cell survival was evaluated using the MTT assay as described (3). Briefly, following incubation with growth factors and glutamate, MTT was added to neurons and incubated for 3 h at 37^{o}C under a 5% CO_2. Cultures were then washed with PBS and Isopropanol/HCl 1N, incubated under shaking for 5 minutes and then 100μl of supernatant was transferred into a fresh 96-well plate and absorbance measured at OD560-620nm. Use of neuronal nuclear staining (Hoechst staining kit, Sigma-Aldrich, St. Louis, MO, USA) as described (3, 32) gave similar results. For convenience, MTT assays were used in this work.

Real-time PCR, DNA constructs and transfections

Total RNA was isolated using the mirVana extraction kit and quantified according to manufacturer's protocol (Ambion, Austin, TX, USA). cDNA was synthesized using 320ng RNA, oligo(dT) primers, and Superscript III Reverse transcriptase according to instructions (Invitrogen, Waltham, MA, USA). PCR primers were as follows: EGFR, 5'-gccatctgggccaaagatacc-3' and 5' -gtcttcgcatgaataggccaat -3'; GAPDH, 5'–aggtcggttgtgaacggatttg-3' and 5'-tgtagaccatgtagttgaggtca-3'. PCR amplification mixtures were prepared using QuantiFast SYBR Green PCR kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA), and real-time PCR assay was performed on an *ABI PRISM 7900HT sequence detection system* (Applied Biosystems, Foster City, CA, USA). Mouse PS1- (FUGW-mmPS1) or EGFR- (FCbAIGW-mmEGFR) expressing plasmids were constructed by cloning mouse PS1 or EGFR cDNA with upstream Kozak sequences into the FUGW or FCbAIGW lentiviral backbone vectors respectively and transduction of neurons was performed using Amaxa technology and Nucleofector Kit (Lonza, Basel, Switzerland) Primary Neurons (program O-005) according to manufacturer's instructions.

RESULTS

EGFR-mediated signaling and neuroprotection are abolished in PS1 null neurons.

Recent literature reports that PS1 negatively regulates EGFR (4–6) suggesting signaling by this receptor may increase in the absence of PS1. To investigate PS1 effects on survival signaling of neuronal EGFR, brain primary cortical neuronal cultures (PCNC) from WT, PS1 heterozygous (PS1+/-) and homozygous (PS1-/-) KO mice prepared as described (3) were treated with EGFR ligands EGF and Hb-EGF and AKT and ERK phosphorylation signaling mediated by EGFR stimulation was analyzed (33). As expected, these treatments caused rapid increases in the phosphorylation of both kinases in WT and PS1+/- neurons. Surprisingly however, the EGFs failed to stimulate phosphorylation of either kinase in PS1 null (PS1-/-) neurons, indicating that EGFR signaling is attenuated in the absence of PS1 (Fig. 1A). To ensure that activation of ERK and AKT was not delayed in PS1-/- neurons, we examined phosphorylation kinetics in response to Hb-EGF, an EGFR ligand abundantly expressed in the brain (13). Hb-EGF caused a sustained phosphorylation of both kinases in WT and PS1 +/- but not in PS1-/- neurons (Fig. 1B). Thus, contrary to expectations, our data suggested that absence of PS1 severely attenuates EGF-induced neuronal signaling and that presence of even one PS1 allele is sufficient for full signaling (Figs 1A and B). In contrast, BDNF, a factor that signals to AKT and ERK kinases through TrkB receptor, stimulated phosphorylation of these kinases in both WT and PS1-/- neurons indicating that signaling of neuronal EGFR specifically decreases in the absence of PS1 (Fig. 1C). Since ERK and

AKT kinases mediate survival signaling of the neuroprotective system EGF/EGFR (11, 33), we asked whether EGF-dependent neuroprotection changes in the absence of PS1. To this end, neuronal cultures were treated with glutamate in the presence or absence of either EGF or Hb-EGF. Fig 2A shows that although these ligands decreased the glutamate-induced neuronal death in both PS1 +/+ and +/- cultures, they were unable to decrease neuronal death in PS1-/- cultures indicating that PS1 is required for EGF-dependent neuroprotection. In contrast, progranulin rescues PS1 null neurons from excitotoxicity regardless of PS1 gene dosage (Fig 2A and (32)) confirming that EGF-dependent neuroprotection is specifically regulated by PS1. To examine which kinase is required for EGF neuroprotection, neuronal cultures were treated with either MEK/ERK1/2 inhibitor U0126 or PI3K/AKT inhibitor wortmannin followed by glutamate. Although these treatments decreased the Hb-EGF-induced activating phosphorylation of ERK and AKT respectively (Fig 2B), only inhibition of PI3K/AKT had a significant effect on the Hb-EGF-induced neuroprotection (Fig 2C). Together, our data indicate that PS1 is necessary for EGF-induced phosphorylation of both AKT and ERK but AKT activation is mainly responsible for EGF-dependent neuroprotection against excitotoxicity *in vitro*.

PS1 positively regulates neuronal EGFR.

To determine the mechanism by which PS1 facilitates survival signaling of EGF ligands, we examined the PS1 effects on the expression of neuronal EGFR. Surprisingly, in contrast to reports that PS1 negatively regulates this receptor in iMEF (4–6), levels of neuronal EGFR were dramatically decreased (> 95%) in PS1-/- neurons compared to WT (Fig 3A). This outcome was replicated in several independent neuronal cultures prepared from different embryonic mouse brains derived from distinct pregnancies. Notably, PS1+/- neurons contain similar amounts of EGFR as WT neurons (Fig. 3A) showing absence of one PS1 allele has little effect on neuronal EGFR an outcome consistent with similar signaling and neuroprotective activities of EGFs in WT and PS1+/- neurons (Fig. 2). Additional experiments showed that absence of PS1 causes a dramatic EGFR reduction in all neuronal cultures regardless of age (Fig. 3B) and that tissue from PS1-/- brains probed by either WBs (Fig. 3C) or immunohistochemistry (Supplementary Fig. 3) also contains reduced amounts of this receptor. Furthermore, examination of neuronal markers DCX and MAP2 showed that the amounts of these proteins in our cultures and brain tissue are independent of PS1 (Fig. 3D) further supporting specific PS1 affects on EGFR. Since absence of PS1 causes a smaller EGFR reduction in brain tissue than in PS1-/- neurons (40% *vs* 95% respectively, Figs. 3A and C), we asked whether PS1 deficiency exerts a less dramatic effect on EGFR of non-neuronal brain cells. Fig 3E shows that the levels of this receptor in

primary glia from PS1-/- brains do not differ significantly from those in glia from WT brains suggesting that absence of PS1 results in specific reduction of neuronal EGFR. This outcome is consistent with data that absence of PS1 causes a more dramatic decrease in neuronal than brain EGFR as total amount of brain EGFR is determined by the sum of its expression in all brain cells.

To examine whether acute downregulation of PS1 has similar effects on expression and signaling of neuronal EGFR as chronic absence of PS1, we used anti-PS1 siRNAs. Since PS1+/- neurons have similar levels of EGFR and exhibit similar EGF signaling as WT neurons and to ensure efficient downregulation of EGFR by siRNAs, we used these neurons in our experiments. As shown in Fig 4, acute knockdown of neuronal PS1 decreased both, the amounts of EGFR (Fig. 4A) and the EGF-dependent neuroprotection against glutamate excitotoxicity (Fig. 4B). These data show that acute downregulation of PS1 has similar effects on the expression of EGFR and EGF-dependent neuroprotection as those observed under conditions of chronic absence of PS1. Finally, re-introduction of mouse PS1 in PS1 null neurons caused a robust increase of EGFR (Fig. 4C) further supporting the conclusion that PS1 strongly stimulates the neuronal expression of EGFR.

To establish a direct link between reduced neuronal survival in the absence of PS1 and expression of EGFR, we expressed exogenous EGFR in PS1-/- neurons. Fig 5A shows that exogenous EGFR rescues the ability of EGF to increase neuronal survival even in the absence of PS1. Furthermore, expression of EGFR restores the ability of EGF to stimulate phospho-EGFR and activate its downstream targets AKT and ERK kinases even in the absence of PS1 (compare Fig. 5B to 1A). Importantly, Fig. 5C shows that in our neuronal cultures, EGFR dominates the survival signaling of both EGF and Hb-EGF as treatment with EGFR inhibitor Erlotinib blocks induced phosphorylation of AKT and ERK. These data reveal a direct link between EGFR and PS1-dependent phenotypes on neuronal survival and activation of AKT kinase.

Neither γ-secretase nor PS2 regulate expression of neuronal EGFR.

PS1 has been shown to have both γ-secretase-dependent and independent functions (2, 3). Use of immortalized cell systems however to examine the effects of γ-secretase on the cellular levels of EGFR yielded inconsistent answers as evidence were reported against (4, 6) and for (5, 7) involvement of γ-secretase activity in the regulation of this receptor. To examine effects of γ-secretase on neuronal EGFR we treated primary neuronal cultures with L-685,458, a potent γ-secretase inhibitor widely used in our and other laboratories (5, 26, 27). Fig. 6A shows that although L-685,458 causes a robust accumulation

of APP-derived γ-secretase substrates APP-CTFs, this treatment has no effect on neuronal EGFR indicating γ-secretase is not involved in the regulation of the expression of this receptor. To further explore this issue, we probed iMEFs which have been widely used to explore the role of γ-secretase in the regulation of EGFR (4–7). Figure 6B shows that in agreement with data in primary neurons, inhibition of γ-secretase has no effect on the EGFR of iMEF cells. Since γ-secretase activity cleaves a large number of typeI transmembrane proteins (1) we asked whether EGFR, a typeI protein, may be processed by this activity. Our efforts however to detect EGFR-derived peptides expected from the γ-secretase processing of proteins (26, 34, 35) or complexes between PS1 and EGFR using co-immunoprecipitation protocols were unsuccessful (Georgakopoulos et al., unpublished). Similar to PS1, its homolog PS2 also functions as a catalytic component of γ-secretase complexes that process substrates including APP and Notch1 (36, 37). We thus used PS2 null (PS2-/-) neurons to ask whether PS2 regulates neuronal EGFR. Fig. 7 shows that absence of PS2 affects neither the levels of this receptor nor its neuroprotective activity, supporting the conclusion that PS1 controls EGFR through specific mechanisms independent of both γ-secretase and PS2.

Mechanisms of regulation of neuronal EGFR.

Recent data obtained in iMEF cell lines indicate that PS1 regulates EGFR post-translationally by promoting its degradation through the proteasomal and lysosomal systems (4, 6). In contrast, others reported that PS1 negatively regulates transcription of EGFR in iMEF cell lines (5). We measured the turnover rate of EGFR in WT and PS1 KO neurons using the protein synthesis inhibitor cycloheximide (CHX). Our data show that EGFR turnover in PS1-/- neurons is undistinguishable from that in WT neurons (Fig. 8A). To examine involvement of transcriptional controls we measured the *Egfr* mRNA by quantitative real-time PCR using independent neuronal preparations. We found that the levels of this mRNA in PS1-/- neurons were reduced by more than 95% compared to WT neurons (Fig. 8B). Thus, protein and mRNA data indicate that PS1 positively regulates EGFR at the mRNA level. Measurements of brain mRNA also support a positive regulation of *Egfr* mRNA by PS1 as this mRNA is significantly decreased in PS1-/- embryonic brains compared to WT (Fig. 8C). Furthermore, similar to the relative decrease of EGFR protein in PS1-/- neurons and brain tissue (Fig. 3), reduction of *Egfr* mRNA in PS1 null brains is smaller than its reduction in PS1-/- neurons in agreement with a specific decrease of neuronal mRNA in brain. Furthermore, similar to results in PS1-/- neurons, acute downregulation of PS1 reduced the neuronal *Egfr* mRNA (Fig. 8D) supporting the conclusion that PS1 positively regulates this mRNA. To examine whether PS1 controls the stability of this mRNA, we used actinomycin to arrest

transcription. Fig. 8E shows that *Egfr* mRNA declines at similar rates in the presence or absence of PS1 indicating PS1 regulates the *Egfr* mRNA at a step before synthesis of mature transcripts.

In contrast to our data that PS1 positively regulates expression of neuronal EGFR, several groups reported that PS1 inhibits expression of this receptor in non-neuronal systems. Since however, evidence of a negative correlation between PS1 and EGFR was obtained using immortalized double KO (DKO) fibroblast cells line lacking both PS2 and PS1 (4–6) we examined the expression of EGFR in primary fibroblasts lacking only PS1. Similar to data obtained in primary glia cells, absence of either one or both alleles of PS1 has no effect on EGFR, a result consistently obtained in several independent preparations of primary fibroblasts (Fig. 9A). Importantly, measurements in distinct iMEF cell lines revealed clonal variability of the EGFR levels independent of PS1 expression or genotype (Fig. 9B) suggesting that clonal selection of iMEFs used to examine PS1 effects on FGFR may contributed to literature disagreements on the role of PS1 on EFGR (4–7). We thus examined the effects of acute downregulation of PS1 on the EFGR of iMEFs using anti-PS1 siRNA. Figure 9C shows that downregulation of PS1 in two independent PS1 heterozygous KO (PS1+/-) iMEF clones had no significant effect on the levels of EGFR. Furthermore, in contrast to data in neurons (Fig. 4C), re-introduction of PS1 in PS1 KO iMEF clones showed no effects on EGFR (Fig. 9D). These experiments indicate that PS1 may not regulate the EGFR of fibroblast cells, a conclusion strongly supported by absence of PS1 effects on the EGFR of primary fibroblasts (Fig. 9A). Together, our data show that PS1 positively regulates neuronal EGFR but has no effects on the expression of this receptor in glia or fibroblast cells. Our results however, do not exclude the possibility that PS1 may regulate this receptor in other cell systems not examined here (38).

DISCUSSION

EGFR is a key tyrosine kinase growth factor receptor expressed in many cell types where it regulates growth, migration, and differentiation (8, 39, 40). In addition, this receptor plays pivotal roles in tumor development and proliferation (8, 41) while other studies reveal that EGFR signaling controls neuronal function and survival (10–13). Thus, ligands to this receptor such as Hb-EGF and EGF protect brain neurons from excitotoxicity, oxygen/glucose deprivation, and traumatic injuries, insults implicated in the pathogenesis of neurodegenerative disorders and stroke (12–16). Recently, we reported that PS1 is necessary for neuroprotective activities of brain-derived neurotrophic factor (BDNF) and ephrinB, a function independent of γ-secretase activity (3). Here we show that in the absence of PS1, cortical neurons are unable to activate the EGFR neuroprotective signaling in response to EGFs, an outcome consistently obtained under both, chronic absence of PS1 in PS1-/- neurons and acute downregulation of

neuronal PS1 using siRNAs. Thus, in contrast to WT neurons, neither EGF nor Hb-EGF showed any neuroprotective activity against excitotoxicity in PS1-/- neuronal cultures, suggesting that PS1 is indispensable for EGF-dependent neuroprotection. Furthermore, in absence of PS1, EGFs are unable to stimulate activating phosphorylation of survival kinase AKT an event critical to the neuroprotective function of EGF factors against excitotoxicity. Interestingly, in contrast to BDNF- and ephrinB-dependent neuroprotection in which expression of both PS1 alleles is required (3), one PS1 allele is sufficient for full EGF-induced neuroprotective activity against excitotoxicity. This difference may reflect variations in the mechanisms by which PS1 mediates neuroprotective activities of specific ligand-receptor systems.

We found that lack of EGF-dependent neuroprotection correlates with decreased levels of neuronal EGFR. Furthermore, re-introduction of exogenous PS1 in PS1-/- neurons increased cellular EGFR while acute downregulation of neuronal PS1 decreased both, the levels of EGFR and neuroprotective activity of EGF ligands. Together, these data show that PS1 is a critical positive regulator of the expression of neuronal EGFR and that due to a dramatic decrease of EGFR, PS1 null neurons are unable to use EGF factors to activate survival signaling. Importantly, expression of exogenous EGFR restores the ability of EGFs to phosphorylate EGFR, activates its targets AKT and ERK and rescues neurons from excitotoxicity even in absence of PS1 supporting the conclusion that the main role of PS1 in EGF neuroprotection is to regulate expression of EGFR. In contrast to the crucial role PS1 plays in the regulation of neuronal EGFR, PS1 has no effect on the EGFR of primary glia and fibroblast cells indicating that PS1 specifically regulates the neuronal receptor. This conclusion is also supported by our finding that absence of PS1 causes a more dramatic drop in neuronal than brain EGFR as total amount of this receptor in brain is determined by its expression in all cells.

PS1 functions as catalytic component of γ-secretase complexes that process many typeI transmembrane proteins including receptors. Previous work on the role of γ-secretase on EGFR however, yielded contradictory evidence for (5, 7) and against (4, 6) a role of this activity in the regulation of the receptor. Our data show that γ-secretase activity has no significant effect on neuronal EGFR. Furthermore, we found that PS1 has no effects on the stability of neuronal EGFR. We thus asked whether PS1 might stimulate expression of *Egfr* mRNA. Indeed, we found that this mRNA is greatly decreased in PS1-/- neurons but this decrease is smaller in PS1 null brains, an observation consistent with our data that PS1 does not affect the EGFR levels of astrocytes. Acute downregulation of neuronal PS1 resulted in a robust decrease of *Egfr* mRNA providing strong evidence that PS1 directly regulates this mRNA.

Importantly, our experiments showed that PS1 has no effect of the stability of *Egfr* mRNA indicating that PS1 controls a step prior to synthesis of mature receptor mRNA. Together, our data show that PS1 functions as a critical positive regulator of neuronal *Egfr* mRNA. Interestingly, absence of PS2 affects neither the cellular levels of EGFR nor its neuroprotective functions suggesting that a novel PS1-dependent transcriptional mechanism controls expression of neuronal EGFR independent of both PS2 and γ-secretase. Although the detailed mechanisms by which PS1 stimulates transcription of neuronal EGFR remain to be elucidated, it is tempting to speculate that recently described γ-secretase-independent functions of PS1 in cell signaling may be involved (2).

Our conclusions that PS1 stimulates neuronal, but not glial or fibroblast, EGFR mRNA was unexpected as evidence obtained in iMEFs indicate that PS1 negatively regulates cellular levels of EGFR. Although the source of this inconsistency is unclear, use of PS1 and PS2 DKO iMEFs and tumor cells resulted in inconsistent conclusions for both, the role of γ-secretase in the regulation of EGFR (4–7, 42) and the mechanisms by which PS1 suppresses this receptor (4–6). In addition, it has been found that DKO iMEFs lacking both PS1 and PS2 have defects independent of presenilins (43) whilst conditional PS2/PS1 DKO mice may still express PS1 (44–46). Thus, transformed cells where gene expression may be distorted by immortalization, genomic rearrangements and clonal selection, may not be reliable indicators for *in vivo* effects of PS1 on EGFR. In support of this possibility, we observed large variations in the levels of EGFR among distinct iMEF cell lines regardless of PS1 genotype. More importantly, in our experiments neither downregulation nor overexpression of PS1 had significant effects on the EGFR of iMEFs an outcome consistent with absence of PS1 effects on the EGFR of primary fibroblasts. Thus, our data brings into new focus previous inconsistent conclusions on the role of PS1 and γ-secretase in the expression of EGFR and reveal novel pathways by which PS1 regulates neuronal gene expression *via* γ-secretase independent mechanisms.

In contrast to severe developmental abnormalities and lethal phenotypes caused by absence of PS1 in transgenic mouse models (24, 47), PS2 null mice survive free of serious phenotypes (37, 48). It is currently believed that reduction of γ-secretase cleavage of Notch1 receptor is responsible for the lethal phenotype of PS1 null mice (49). This suggestion however does not explain the absence of phenotypes in PS2 null mice, even though similar to PS1, PS2 also catalyzes the γ-secretase processing of Notch1 (37). Our finding that PS1 is indispensible for the expression of neuronal EGFR, a function displayed neither by PS2 nor γ-secretase, raises the intriguing possibility that developmental abnormalities in PS1 null mice are, at least in part, due to reduced neuronal EGFR. Importantly, deficiencies in either EGFR

or Hb-EGF cause early postnatal mortality with abnormalities similar to those observed in PS1 null mice (23) while recent evidence reveals that PS1 regulates proliferation of brain progenitor cells through EGFR (38). Recently it was reported that the tyrosine kinase activity of EGFR regulates biogenesis of miRNAs involved in cellular response to hypoxia (50). Thus, it is reasonable to infer that by controlling the levels of neuronal EGFR, PS1 tightly regulates and fine-tunes the functions of this receptor including its roles in development, production of miRNAs, and neuronal survival and function. Importantly, emerging evidence indicates that the role of this receptor in the development of AD has been unappreciated. Recent analysis of genome-wide studies and protein-protein interaction modeling identify EGFR as a significant risk factor for sporadic AD (51) while transcriptional profiling studies indicate that the AD-associated apoE4 allele changes the brain expression of EGFR compared to apoE3 (52). Furthermore, additional work indicates that EGFR functions mediate Aβ42-induced memory loss in experimental animal models (53). Thus, by controlling EGFR, PS1 may regulate the course of many diseases known to be modulated by this receptor including cancer and sporadic AD.

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Figure 1. EGF-dependent activation of ERK and AKT survival signaling is abolished in PS1-deficient neurons. *A*) Primary cortical neuronal cultures (PCNC) from WT (+/+), PS1 heterozygous (+/-) and PS1 homozygous (-/-) KO embryonic mouse brains cultured in 6-well plates were treated at 9DIV with 20ng/ml EGF or Hb-EGF for 15 minutes. Following incubation, lysates were collected and probed on Western blots (WBs) for the indicated proteins. *B*) WT, PS1+/-, or PS1-/- PCNC prepared as above were treated at 9DIV with 20ng/ml Hb-EGF. Following incubation, lysates were collected at different time points and assayed on WBs for the indicated proteins. Data were obtained from at least three separate experiments. *C*) WT or PS1-/- PCNC as above were treated at 9DIV with 20ng/ml Hb-EGF or 100ng/ml BDNF for 15 minutes. Non-treated cultures (NT) were used as controls. Following incubation, lysates were collected and assayed on WBs for the indicated proteins.

Figure 2. EGF neuroprotection against excitotoxicity is abolished in PS1-deficient neurons. *A*) WT, PS1+/-, and PS1-/- mouse PCNC were grown in 24-well plates in Neurobasal Media plus B27 supplement. At 9 DIV, neurons were treated with 20ng/ml Hb-EGF or EGF or 35nM PGRN overnight. The next day, the medium was switched to HBSS containing Hb-EGF, EGF or PGRN for 30 minutes followed by 50μM glutamate incubation for 3 hours and cell viability was evaluated by MTT assay and normalized to non-treated cells as described (3, 29). No significant effect compared to non-treated was observed when growth factors alone were added to cultures. *B*) WT PCNC in 6-well plates were treated at 9DIV either with ERK inhibitor U0126 (U0, 5μM) or PI3K/AKT inhibitor wortmannin (W, 50nM) for 30 minutes prior to addition of 20ng/ml Hb-EGF for 15 minutes in Neurobasal Media plus B27 supplement. Following incubation, lysates were collected and assayed on WBs for the indicated proteins. *C*) Mouse PCNC grown as above (see A) were treated at 9DIV either with ERK inhibitor U0126 (5μM) or PI3K/AKT inhibitor wortmannin (50nM) for 30 minutes prior to addition of 20ng/ml Hb-EGF. Three hours later medium was switched to HBSS plus Hb-EGF and inhibitors for 30 minutes followed by 50μM glutamate incubation for 3 hours. Cell viability was evaluated by MTT assay and normalized to non-treated cells. Results (mean ± SEM) were summarized from at least four independent experiments. In each experiment each condition is the average of four identical wells. *, $p<0.05$ comparing between cultures treated with glutamate in the presence or absence of Hb-EGF and/or inhibitors (paired *t-test*).

Figure 3. PS1 positively correlates with cellular levels of neuronal EGFR in vitro and in vivo. *A*) (Upper): Lysates from WT, PS1+/-, or PS1-/- PCNC grown in 6-well plates as above were prepared at 9DIV and probed on WBs for the indicated proteins. (Lower): densitometric analysis of the relative amounts of EGFR in PCNC is

expressed as ratio of EGFR to actin. *B*) Lysates from WT (+/+) or PS1-/- PCNC grown as above were prepared at 3, 5 or 7 DIV and probed on WBs for indicated proteins. *C*) (Upper): lysates from embryonic brain cortex (E15.5 cx) from wild-type or PS1 homozygous KO were prepared as described in the methods section. (Lower): densitometric analysis of the relative amounts of EGFR in embryonic cortices is expressed ratio of EGFR to actin. *D*) (Left) Lysates from WT or PS1-/- PCNC grown as above were prepared at 9 DIV and probed on WBs for the indicated proteins. (Right) Lysates from WT and PS1-/- embryonic brain cortex (E15.5 cx) were prepared and probed on WBs for indicated proteins. *E*) (Upper): primary glial (pGlia) cultures from wild-type, PS1 heterozygous or homozygous KO were obtained as described in Methods. Cells were cultured in 6-well plates. When cells reached about 80% confluence, lysates were collected and assayed on Western blotting for the proteins indicated. (Lower): densitometric analysis of the relative amounts of EGFR in primary glial cultures is expressed as ratio of EGFR to actin. Data were respectively obtained from at least four separate experiments. *, $p<0.05$; ***, $p<0.001$ (paired *t-test*).

Figure 4. Acute knockdown of PS1 decreases EGFR and abolishes EGF neuroprotection while reintroduction of PS1 in PS1 -/- neurons increases EGFR. *A*) (Upper): PCNC from PS1 heterozygous KO (+/-) were cultured in 12-well plates and at 5 DIV were treated with 1μM Accell SMARTpool SiRNA against PS1 for 72 hours. Non-treated cultures were used as controls. Following incubation, lysates were collected and assayed by Western blotting for the indicated proteins. WT neurons (+/+) were also included as control. (Lower): densitometric analysis of the relative amounts of EGFR and PS1-CTF in PCNC described above is expressed as ratios to actin and then normalized as a percentage of EGFR or PS1-CTF amount in the non-treated WT neurons. Data were respectively obtained from four separate experiments. *, $p<0.05$; (paired *t-test*). No effect was observed on PS1 when neurons were treated with 1μM Accell SMARTpool scramble SiRNA for 72 hours (data not shown). *B*) PS1+/- PCNC were grown in 24-well plates with Neurobasal Media plus B27 supplement. At 5 DIV neurons were treated with 1μM Accell SMARTpool SiRNA for 72 hours and then incubated with 20ng/ml Hb-EGF overnight. The next day, the medium was switched to HBSS containing Hb-EGF for 30 minutes followed by 50μM glutamate incubation for 3 hours. Cell viability was evaluated by MTT assay and normalized to non-treated cells. Results (mean ± SEM) were summarized from six independent experiments and in each experiment each condition is the average of four identical wells. *, $p<0.05$ comparing between cultures treated with glutamate in the presence or absence of Hb-EGF, PS1-SiRNA or non-targeting SiRNA (paired *t-test*). *C*) (Upper): PS1-/- PCNC were mock transfected (left lane) or transfected with either mouse PS1 in FUGW vector or vector alone. Following incubation, lysates were collected at 8 DIV and assayed on WBs for the indicated proteins. (Lower): densitometric analysis of the amounts of EGFR in transfected PS1-/- neuronal

cultures above is expressed as ratio of EGFR to actin and normalized to mock nucleofected neurons. Bars represent means and error bars SEM. Data were obtained from four independent experiments. *, $p<0.05$ (paired *t-test*).

Figure 5. Expression of exogenous EGFR restores the PS1-dependent ability of EGF to rescue neurons from excitotoxicity and stimulate survival signaling. *A*) PS1-/- neurons were transfected with either empty vector (FCbAIGW) or an EGFR-expressing construct (FCbAIGW-EGFR) and at 8 DIV, neurons were treated with 20ng/ml EGF overnight as indicated in Figure. Next day, the medium was switched to HBSS containing EGF for 30 minutes followed by 50μM glutamate incubation for 3 hours. Cell viability was then measured counting healthy nuclei with Hoechst kit 33342 (Methods) and normalized to non-treated cells as described (3). Results (mean ± SEM) are from three independent experiments (n=3). *, $p<0.05$ (paired *t-test*) *B*) PS1-/- neurons were transfected with EGFR-expressing plasmid FCbAIGW-EGFR or vector alone (FCbAIGW) and at 8 DIV cultures were treated with 20nf/mL EGF for 15 minutes. Lysates were then collected and probed on WBs for indicated proteins. *C*) WT PCNC of 8 DIV were incubated with 10uM Erlotinib (Erlo) for 30 minutes and then treated with 20ng/mL of either Hb-EGF or EGF. Cultures were incubated for an additional 15 minutes and lysates were assayed for indicated proteins.

Figure 6. Inhibition of γ-secretase has no effect on the expression of EGFR. *A*) WT PCNC were grown in 6-well plates for 9 days and then treated with γ-secretase inhibitor L-685,458 (500nM) overnight (+). WT control (-) cultures were treated with the same solution without the inhibitor. Following incubation, lysates were collected and assayed on WBs for the indicated proteins. Lower panel: densitometric analysis of the relative amounts of EGFR and APP-CTFs in PCNC normalized to actin and expressed as percent of the amounts in non-treated controls. Data were from four independent experiments. ***, $p<0.001$ (paired *t-test*). *B*) Cultures of immortalized mouse embryonic fibroblasts (iMEF) were treated overnight with γ-secretase inhibitor L-685,458 (+) as above or with medium without inhibitor as controls (-). Following incubation, lysates were collected and assayed on WBs for indicated proteins. Lower panel: densitometric analysis of the relative amounts of EGFR and APP-CTFs in iMEFs as above. Data are from four separate experiments as above.

Figure 7. PS2 affects neither EGFR expression nor EGF-dependent neuroprotection. *A*) (Upper): PCNC from WT (+/+) and PS2 heterozygous (+/-) and homozygous (-/-) KO embryonic mouse brains were cultured in 6-well plates. At 9DIV, lysates were collected and probed on WBs for the indicated proteins. (Lower): densitometric analysis of the relative amounts of EGFR shown above expressed as EGFR to actin ratio. *B*) WT,

PS2+/- and PS2-/- mouse PCNC were grown in 24-well plates. At 9 DIV cultures were treated overnight with 20ng/ml of either Hb-EGF or EGF. Next day, the medium was switched to HBSS containing Hb-EGF or EGF for 30 minutes followed by 50μM glutamate incubation for 3 hours (see Fig. 2A). Cell viability was evaluated by MTT assay and normalized to non-treated cells. Treatment with growth factors alone had no effect on neuronal viability compared to untreated cultures. Bars represent means and error bars SEM. Data were respectively obtained from at least four separate experiments. *, $p<0.05$; (unpaired *t-test* for A, paired *t-test* for B).

Figure 8. PS1 positively regulates expression of neuronal *Egfr* mRNA. *A*) (Upper): WT (PS1+/+) or PS1 homozygous KO (PS1-/-) PCNC in Neurobasal Media plus B27 supplement were treated at 7DIV with 50μg/ml cycloheximide (CHX) for the indicated times. Non-treated (0 time) cultures were used as controls. Following incubation, lysates (15μg from WT or 150μg from PS1-/-) were collected and probed on WBs for EGFR. (Lower): kinetic analysis of the relative amounts of EGFR in PCNC following CHX treatment expressed as a percentage of EGFR amount in 0-time samples. Data were obtained from four independent experiments. *B-C*) Total mRNA was isolated from WT (PS1+/+) or PS1 null (PS1-/-) mouse PCNC of 8DIV (*B*) or embryonic cortex (E15.5) (*C*). Following cDNA amplification using E15.5 cortical mRNA, quantitative real-time PCR assay was performed as described in the experimental procedures. Data were respectively obtained from at least four separate experiments, ***, $p<0.001$ (paired *t-test*). *D*) PS1 heterozygous KO (PS1+/-) PCNC grown on 12-well plates were treated at 5DIV with 1μM Accell SMARTpool SiRNA against PS1 for 72 hours. Non-treated cultures were used as controls. Following incubation, total mRNA was isolated, followed by cDNA amplification and quantitative real-time PCR performed as described in Methods. Data were respectively obtained from at least four separate experiments, *, $p<0.05$ (paired *t-test*). *E*) PCNC of WT (PS1+/+) or PS1 null (PS1-/-) mice were treated with actinomycin D (2μg/mL) to arrest transcription and total RNA was isolated at 0, 2, or 6 hours later. RNA Samples were then subjected to cDNA amplification and quantitative real-time PCR and relative amounts of *Egfr* mRNA were calculated using GAPDH as an internal control (ΔCt). Amounts of *Egfr* mRNA are expressed as % of its amounts at time zero and plotted against time using the $2^{-\Delta\Delta Ct}$ method. The data were fitted to the linear regression model and the slope of the curve was similar between genotypes: -0.159 in WT vs -0.162 in PS1KO (n=3, 95% ci).

Figure 9. Absence of PS1 has no effect on EGFR expression of primary fibroblasts or iMEFs. *A*) (Upper): primary mouse fibroblasts (pMEF) from WT (+/+), PS1 +/- and -/- mouse embryos were obtained as described in Methods. Cells were cultured in 6-well plates at 80% confluence and lysates were prepared and assayed on

WBs for the indicated proteins. (Lower): Relative amounts of EGFR in pMEFs of the genotypes shown in upper figure are expressed as EGFR to actin ratio (n=6). *B*) Lysates from WT (+/+), PS1+/-, and PS1-/- MEF cell lines (iMEF) were assayed on WBs for the indicated proteins. Each cell line is derived from an independent iMEF clone. *C*) Immortalized PS1 heterozygous KO MEFs (PS1+/- iMEF) were transfected with 25nM of anti-PS1 siRNA for 72 hours. Mock-transfected and scrambled siRNA-transfected (Ctl-siRNA) cultures were used as controls. Following incubation, lysates were collected and assayed by WBs for the indicated proteins. *D*) (Upper): PS1 null iMEFs (PS1-/- iMEF) were stably transduced with either human PS1 in pMX vector or vector alone. Lysates were prepared and assayed on WBs for indicated proteins. (Lower): densitometric analysis of relative amounts of EGFR in 4 different PS1 KO iMEF clones transfected either with pMX vector or pMX-PS1. Amount of EGFR in each PS1-transfected clone is normalized to the EGFR of the vector-transfected clone. Bars represent means, error bars SEM.

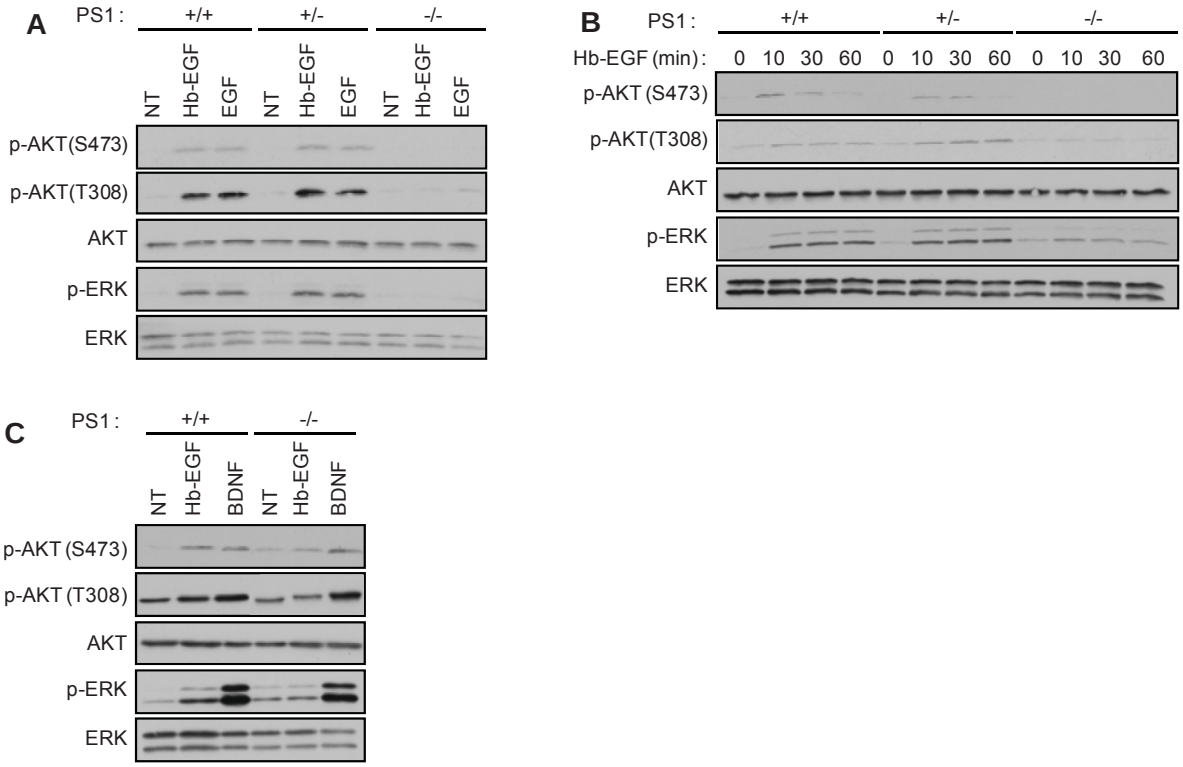
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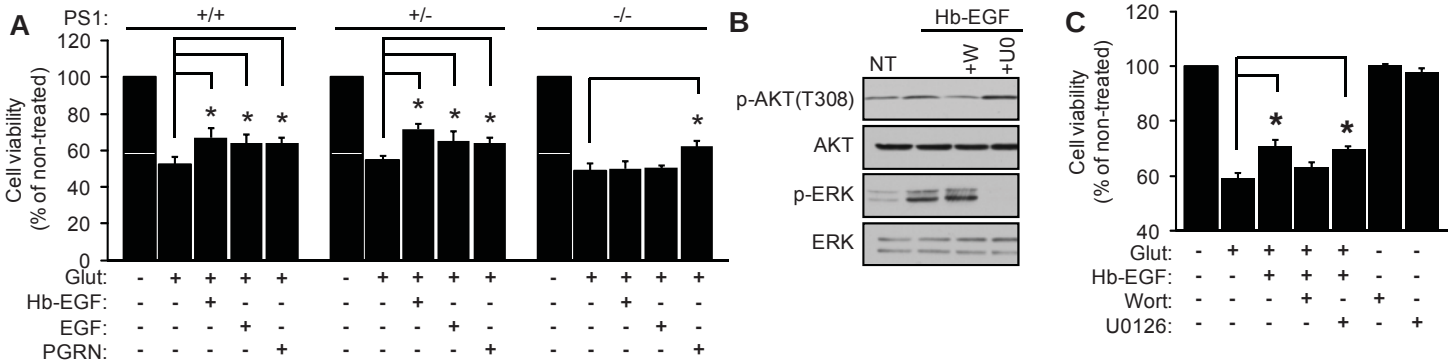
Figure 1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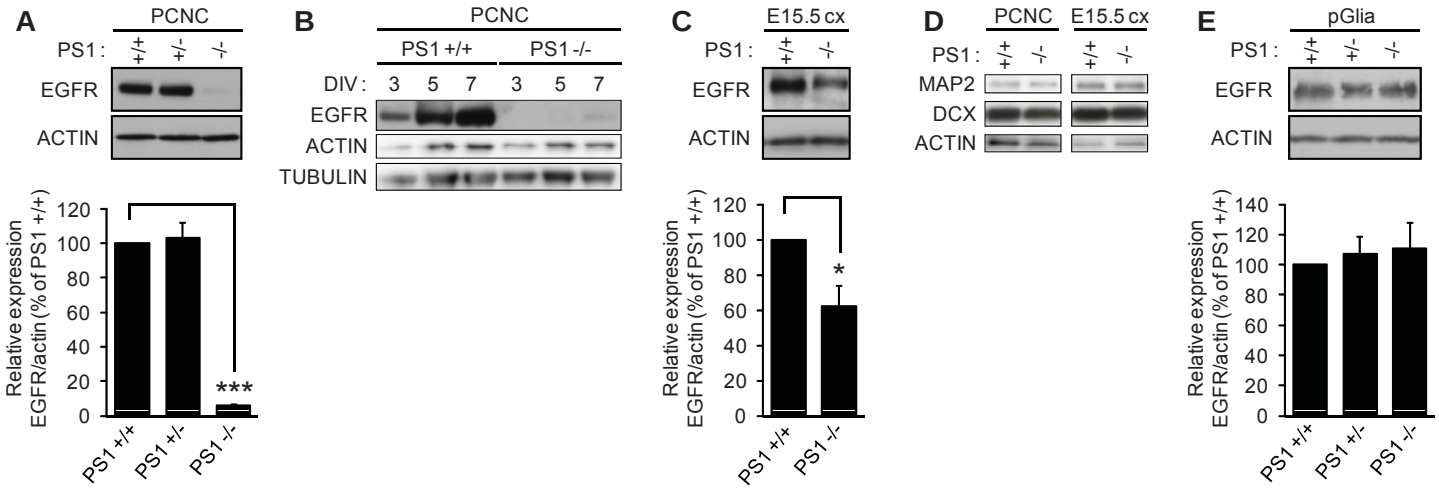
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PS1 -/-

Figure 3

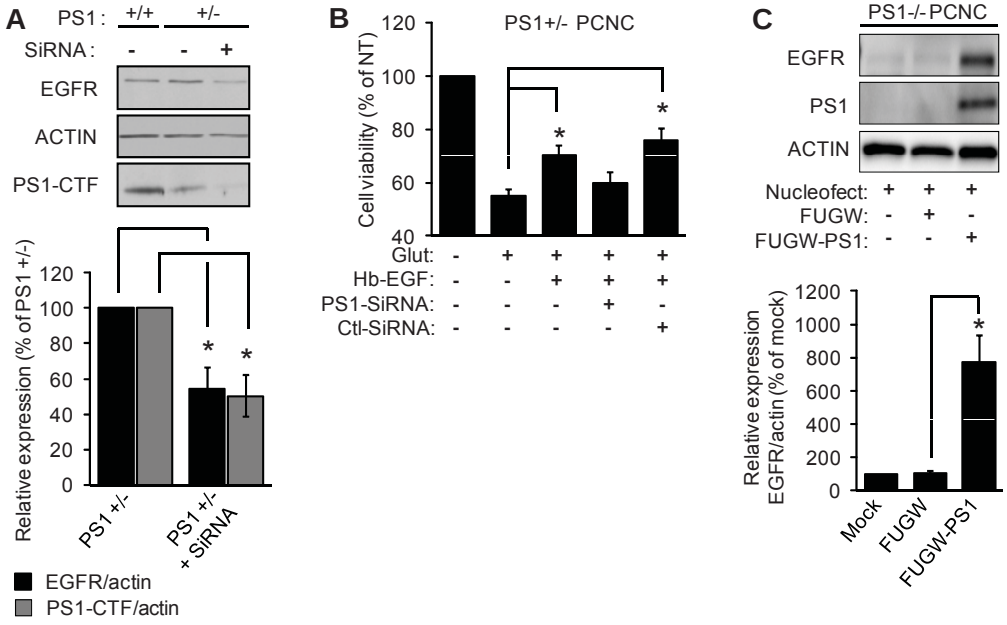

A
PS1 : +/+ +/-
SiRNA : - - +
EGFR
ACTIN
PS1-CTF
Relative expression (% of PS1 +/-)
120
100
80
60
40
20
0
*
*
PS1 +/-
PS1 +/- + SiRNA
EGFR/actin
PS1-CTF/actin
B
PS1+/- PCNC
Cell viability (% of NT)
120
100
80
60
40
*
*
Glut: - + + + +
Hb-EGF: - - + + +
PS1-SiRNA: - - - + -
Ctl-SiRNA: - - - - +
C
PS1-/- PCNC
EGFR
PS1
ACTIN
Nucleofect: + + +
FUGW: - + -
FUGW-PS1: - - +
Relative expression EGFR/actin (% of mock)
1200
1000
800
600
400
200
0
*
Mock
FUGW
FUGW-PS1

Figure 4.

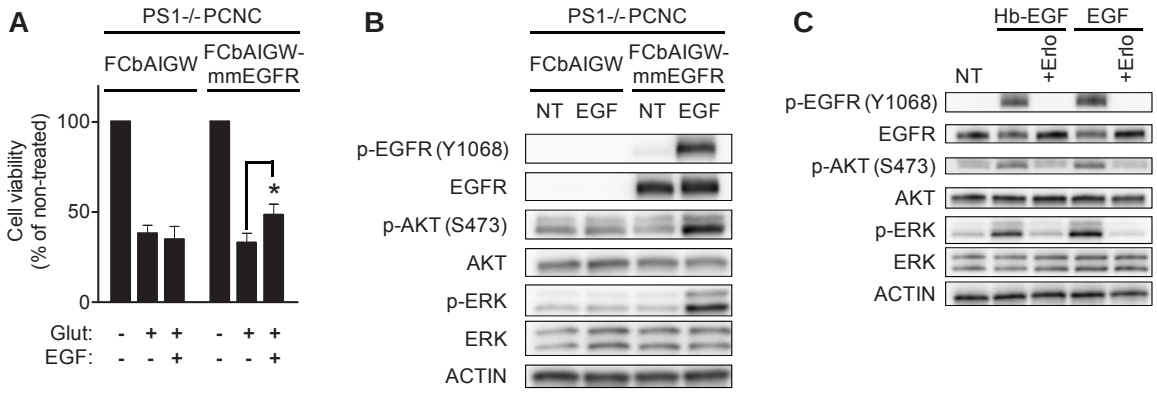

A
PS1-/-PCNC
FCbAIGW
FCbAIGW-mmEGFR
Cell viability (% of non-treated)
100
50
0
*
Glut: - + + - + +
EGF: - - + - - +
B
PS1-/-PCNC
FCbAIGW
FCbAIGW-mmEGFR
NT EGF NT EGF
p-EGFR (Y1068)
EGFR
p-AKT (S473)
AKT
p-ERK
ERK
ACTIN
C
Hb-EGF
EGF
NT
+Erlo
+Erlo
p-EGFR (Y1068)
EGFR
p-AKT (S473)
AKT
p-ERK
ERK
ACTIN

Figure 5

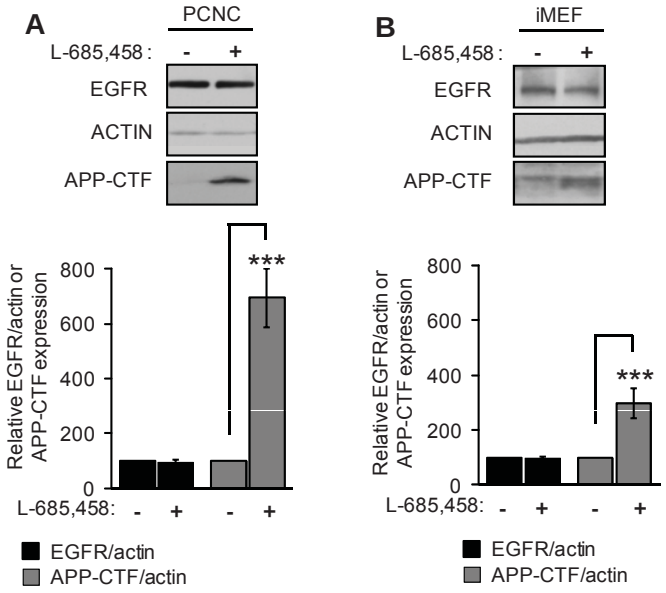

A
PCNC
L-685,458 :
-
+
EGFR
ACTIN
APP-CTF
Relative EGFR/actin or APP-CTF expression
800
600
400
200
0
***
L-685,458:
-
+
-
+
EGFR/actin
APP-CTF/actin
B
iMEF
L-685,458 :
-
+
EGFR
ACTIN
APP-CTF
Relative EGFR/actin or APP-CTF expression
800
600
400
200
0
***
L-685,458:
-
+
-
+
EGFR/actin
APP-CTF/actin

Figure 6

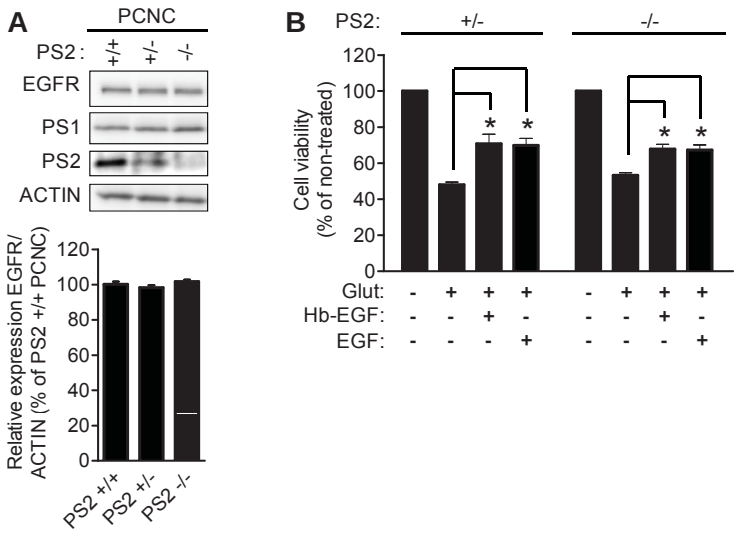

A
PCNC
PS2 :
+/+
+/-
-/-
EGFR
PS1
PS2
ACTIN
Relative expression EGFR/ ACTIN (% of PS2 +/+ PCNC)
120
100
80
60
40
20
0
PS2 +/+
PS2 +/-
PS2 -/-
B
PS2:
+/-
-/-
Cell viability (% of non-treated)
120
100
80
60
40
20
0
*
*
*
*
Glut: - + + + - + + +
Hb-EGF: - - + - - - + -
EGF: - - - + - - - +

Figure 7

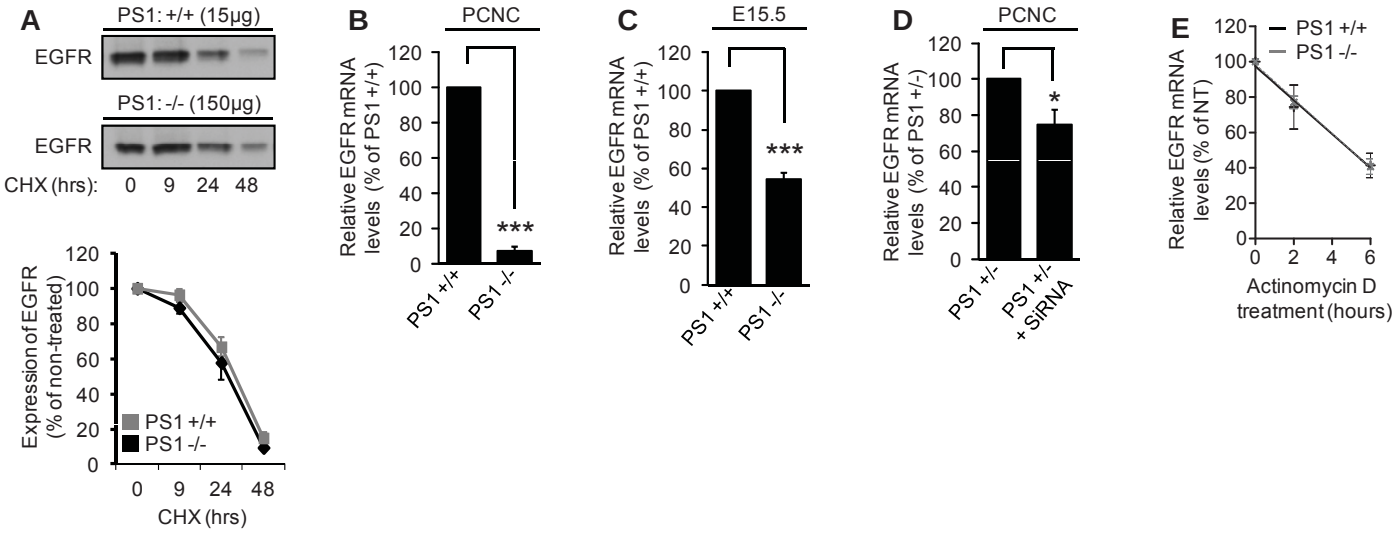

A
PS1: +/+ (15µg)
EGFR
PS1: -/- (150µg)
EGFR
CHX (hrs): 0 9 24 48
Expression of EGFR (% of non-treated)
PS1 +/+
PS1 -/-
CHX (hrs)
B
PCNC
Relative EGFR mRNA levels (% of PS1 +/+)
***
PS1 +/+
PS1 -/-
C
E15.5
Relative EGFR mRNA levels (% of PS1 +/+)
***
PS1 +/+
PS1 -/-
D
PCNC
Relative EGFR mRNA levels (% of PS1 +/-)
*
PS1 +/-
PS1 +/- + SiRNA
E
PS1 +/+
PS1 -/-
Relative EGFR mRNA levels (% of NT)
Actinomycin D treatment (hours)

Figure 8

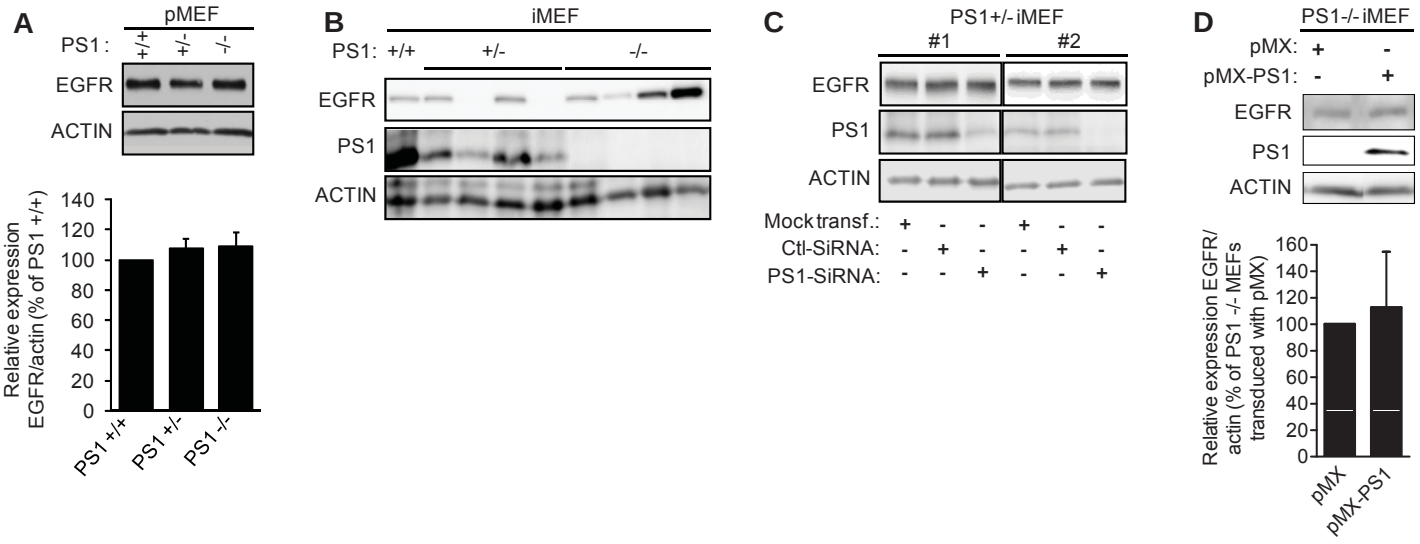
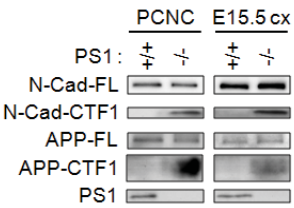
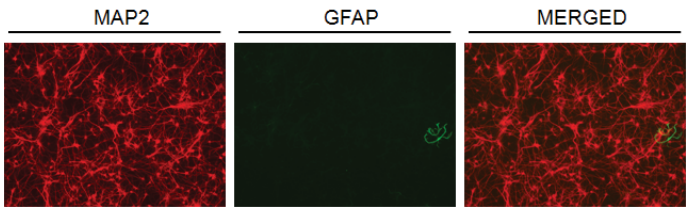

A
pMEF
PS1 :
+/+
+/-
-/-
EGFR
ACTIN
Relative expression EGFR/actin (% of PS1 +/+)
140
120
100
80
60
40
20
0
PS1 +/+
PS1 +/-
PS1 -/-
B
iMEF
PS1: +/+
+/-
-/-
EGFR
PS1
ACTIN
C
PS1+/- iMEF
#1
#2
EGFR
PS1
ACTIN
Mock transf.: + - - + - -
Ctl-SiRNA: - + - - + -
PS1-SiRNA: - - + - - +
D
PS1-/- iMEF
pMX: + -
pMX-PS1: - +
EGFR
PS1
ACTIN
Relative expression EGFR/ actin (% of PS1 -/- MEFs transduced with pMX)
160
140
120
100
80
60
40
20
0
pMX
pMX-PS1

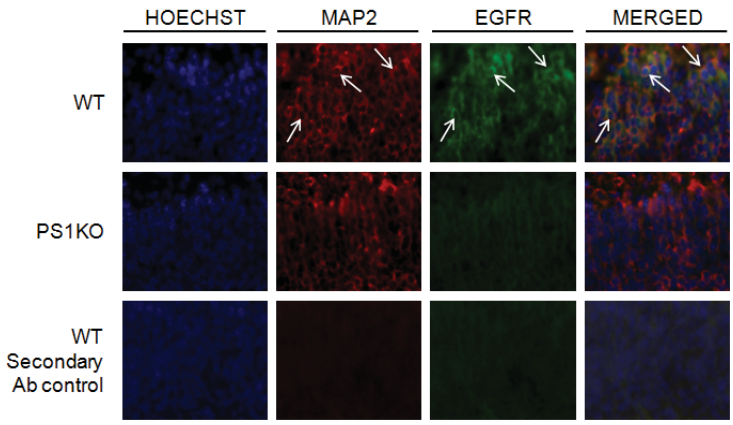
Figure 9



Supplementary Figure 1. Absence of PS1 results in accumulation of γ-secretase substrates APP/CTF1 and N-cad/CTF1 due to decreased γ-secretase activity. Lysates from WT and PS1-/- PCNCs of 9DIV or from E15.5 brain cortex from WT and PS1-/- mice were probed on WBs for indicated proteins.



Supplementary Figure 2. Representative field of our PCNC at 9 DIV. MAP2 immunostaining shows neurons are the predominant cell type in our cultures (>95%, see also Methods). GFAP staining reveals a small number of astrocytes (green). Immunocytochemistry was performed as we described (28). Briefly, neurons on poly-D-lysine coated coverslips were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100, washed with TBS and blocked in Superbock blocking Buffer (Pierce, Rockford, IL) for 1 hour. Samples were then incubated with anti-MAP2 or anti-GFAP antibodies (Abcam, Cambridge, UK) overnight and then with Alexa Fluor antibody conjugates (Molecular Probes, Eugene, OR) for 1 h. Coverslips were rinsed, mounted on PermaFluor Mountant (ThermoFisher Scientific, Waltham, MA) and images obtained with an inverted Olympus microscope (Olympus Corporation).



Supplementary Figure 3. MAP2 and EGFR immunoreactivity of superficial cortical layers of E15.5 mouse brain embryos. Arrows show neurons from WT mouse brain express both MAP2 (red) and EGFR (green). In contrast, no EGFR-specific staining is observed in PS1KO neurons of the same brain area. Brain immunohistochemistry was as described (1) except antigen retrieval was as in (2). Briefly, brains were fixed in 4% (w/v) paraformaldehyde and 0.2% (v/v) picric acid in Dulbecco's Phosphate Buffered Saline w/o Calcium/Magnesium (DPBS; Lonza, Basel, Switzerland) at 4°C, washed 3 times with DPBS, and placed in 30% (w/v) sucrose overnight at 4 °C. Tissue was then embedded in OCT Compound (Tissue-Tek, Fisher Healthcare, Houston TX), frozen in ethanol/dry ice and coronal cryostat sections of 20µm mounted on microscope slides and stored at -80 °C. For immunostaining, slides at room temperature (RT) were washed in DPBS, microwaved in 10mM sodium citrate and returned to RT, a process repeated 3 times. Sections were then washed with DPBS, blocked for 1 hour at RT with 10% goat serum and 0.2% Triton X-100 in DPBS and treated with anti-MAP2 and anti-EGFR antibodies overnight at 4 °C. Sections were washed with DPBS, incubated with secondary antibodies conjugated with Alexa Fluor (Molecular Probes, Eugene, OR) for 1 hour at RT, and washed as above. For nuclear staining sections were placed in 0.8 µg/ml Hoechst dye (Sigma-Aldrich, St. Louis, MO) in DPBS for 5 minutes and washed as above. Slides were rinsed with water and images taken on a Zeiss Axio Imager (Carl Zeiss Microscopy, Göttingen, Germany) with a Hamamatsu image acquisition device (Hamamatsu Photonics, Hamamatsu, Japan).

References to Supplemental Data.

1. Ge, X., Frank, C. L., Calderon de Anda, F., and Tsai, L. H. (2010) Hook3 Interacts with PCM1 to Regulate Pericentriolar Material Assembly and the Timing of Neurogenesis. *Neuron.* **65**, 191–203
2. Muñoz-Elias, G., Marcus, A. J., Coyne, T. M., Woodbury, D., and Black, I. B. (2004) Adult bone marrow stromal cells in the embryonic brain: engraftment, migration, differentiation, and long-term survival. *J. Neurosci.* **24**, 4585–4595