An aging pathway controls the TrkA to p75^{NTR} receptor switch and amyloid β -peptide generation

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Aging of the brain is characterized by marked changes in the expression levels of the neurotrophin receptors, TrkA and p75^{NTR}. An expression pattern in which TrkA predominates in younger animals switches to one in which p75^{NTR} predominates in older animals. This TrkA-to-p75^{NTR} switch is accompanied by activation of the second messenger ceramide, stabilization of β -site amyloid precursor protein-cleaving enzyme-1 (BACE1), and increased production of amyloid β -peptide (A β). Here, we show that the insulin-like growth factor-1 receptor (IGF1-R), the common regulator of lifespan and age-related events in many different organisms, is responsible for the TrkA-to-p75^{NTR} switch in both human neuroblastoma cell lines and primary neurons from mouse brain. The signaling pathway that controls the level of TrkA and p75^{NTR} downstream of the IGF1-R requires IRS2, PIP3/Akt, and is under the control of PTEN and p44, the short isoform of p53. We also show that hyperactivation of IGF1-R signaling in p44 transgenic animals, which show an accelerated form of aging, is characterized by early TrkA-to-p75^{NTR} switch and increased production of A β in the brain.

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Introduction

Compelling evidence indicates that the insulin/insulin-like growth factor 1 (IGF1) signaling pathway plays a major role in controlling maximum lifespan and, to some extent, the incidence or prevalence of age-associated diseases such as diabetes. Hypomorphic alleles of the *Igf1-r* gene extend lifespan, whereas increased activation of insulin/IGF1 signaling accelerates the progression of aging (Longo and Finch, 2003; Kenyon, 2005). A role for the IGF1 receptor (IGF1-R) in aging

has been demonstrated for all species analyzed so far, from yeast to *Caenorhabditis elegans*, *Drosophila melanogaster*, and, most recently, mammals (Longo and Finch, 2003; Kenyon, 2005). Caloric restriction, which is the only intervention shown to extend maximum lifespan and delay many of the biological changes that are associated with aging in mammals (Weindruch and Sohal, 1997), works at least in part by blocking IGF1-R signaling. Conversely, increased activation of the IGF1-R signaling pathway accelerates the progression of aging and shortens maximum lifespan (Longo and Finch, 2003; Kenyon, 2005).

Late-onset Alzheimer's disease (AD) is the World's most prevalent form of dementia and one of the most common age-related diseases. Because of the ongoing increase in life expectancy, AD is projected to affect as many as 45 million individuals worldwide by the year 2050. The single most important risk-factor for late-onset AD is age.

The abnormal accumulation of amyloid β -peptide (A β) in the form of senile (or amyloid) plaques and amyloid angiopathy is one of the main neuropathological hallmarks of AD. A β is a 39–43 amino acid long peptide generated by sequential proteolysis of amyloid precursor protein (APP) at β and γ sites (Puglielli *et al.*, 2003b; Selkoe, 2004). The β -site cleavage represents the rate-limiting step in A β generation and is catalyzed by β -site APP-cleaving enzyme-1 (BACE1). Studies from both transgenic and 'knockout' mice indicate that the abnormal accumulation of A β in the brain represents an important step for the development of the Alzheimer form of neurodegeneration (Gotz *et al.*, 2004).

We have recently shown that A β generation in the brain is activated by a switch from the TrkA to p75^{NTR} neurotrophin receptor (Costantini *et al.*, 2005). During normal aging, there is a progressive increase in the level of p75^{NTR} and a parallel decrease in the level of TrkA expression, suggesting that this switch may be physiologically relevant in the pathogenesis of AD (Costantini *et al.*, 2005). Coordinate regulation of neurotrophin signaling during aging leads to activation of neutral sphingomyelinase (nSMase) and liberation of the lipid second messenger ceramide, which in turn is responsible for the molecular stabilization of BACE1 and the increased production of A β (Puglielli *et al.*, 2003a; Costantini *et al.*, 2005). These events can be blocked by caloric restriction, inhibition of nSMase, and genetic disruption of *Ngfr*, the gene that codifies for p75^{NTR} (Costantini *et al.*, 2005). In contrast to nSMase inhibitors, however, caloric restriction acts earlier in the pathway and blocks or reverts the switch from TrkA to p75^{NTR}. The ability of caloric restriction to block events upstream of the neurotrophin receptors suggested that the TrkA to p75^{NTR} molecular switch might be under the general aging program mediated by the insulin/IGF1 signaling pathway. To test this hypothesis, we analyzed the effect of the IGF1-R, the main regulator of life span and age-dependent events, on the TrkA/p75^{NTR} switch and A β generation during mammalian aging.

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Results

IGF1 induces the TrkA to p75^{NTR} switch in SH-SY5Y cells

We initially used a human neuroblastoma cell line (SH-SY5Y) that expresses both the IGF1-R and neurotrophin receptors TrkA and p75^{NTR}, and that is responsive to IGF1 (Kim *et al*, 2004). When treated with IGF1, SH-SY5Y cells responded with an increase in the expression of p75^{NTR} and a decrease in TrkA (Figure 1A). This response reached a plateau after ~4 days and was more evident with p75^{NTR} than TrkA. This is consistent with our previous results showing that aging induces a stronger effect on p75^{NTR} than on TrkA (Costantini *et al*, 2005). The TrkA-to-p75^{NTR} switch was accompanied by a marked increase in the endogenous levels of ceramide (Figure 1B) and β -secretase activity (Figure 1C), and on the steady-state levels of C99 (Figure 1E). C99 (also called β -APP-CTF) is the intermediate product of β cleavage of APP and a direct indication of BACE1 activity. A parallel increase in C83 (also called α -APP-CTF), produced by α cleavage of APP, was also evident (Figure 1E); however, this cleavage does not lead

to the generation of A β . The increased secretion of the N-terminal sAPP α and sAPP β fragments of APP into the conditioned media (Supplementary Figure 1A) confirmed that the increased levels of C99 and C83 were a direct consequence of an increased processing of APP, rather than an indirect product of reduced γ cleavage. In fact, sAPP α and sAPP β result from α and β cleavage of APP, respectively, and closely follow the steady-state levels of C99 and C83.

The activation of β cleavage of APP reached a plateau after ~6–8 days, consistent with the fact that molecular stabilization of BACE1 requires ~4 days of ceramide treatment (Puglielli *et al*, 2003a). Concomitant analysis of β - and γ -secretase activities *in vitro* showed a progressive increase in β -secretase activity with the highest levels of activation at days 6–8 (Figure 1C), which paralleled the steady-state levels of C99 (Figure 1E). No effect was observed on γ -secretase activity *in vitro* (Figure 1D) or on the steady-state levels of the different members of the γ -secretase complex (PS1, Nicastrin, Aph1, and Pen2; Supplementary Figure 1A), which is consistent with our previous results showing no overall change in γ cleavage during aging (Costantini *et al*, 2005).

Figure 1 IGF1 regulates APP processing by acting upstream of p75^{NTR}/TrkA in SH-SY5Y human neuroblastoma cell lines. (A–F) SH-SY5Y cells were treated with IGF1 in the absence of serum. (A) p75^{NTR} and TrkA steady-state levels were assessed by immunoblotting of total cell lysates. (B) Endogenous ceramide was analyzed by TLC. Error bars represent the s.d. of four different assays. Asterisk indicates statistical significance from control (no treatment). (C, D) β - (C) and γ - (D) secretase activities from cell homogenates were analyzed *in vitro* by using the QTL lightspeed assay. Error bars represent the s.d. of four different assays. Asterisk indicates statistical significance from control (no treatment). (E) Western blot analysis of APP processing following IGF1 treatment. Mature and immature APP, together with C83 and C99, are indicated. (F) Treatment of SH-SY5Y cells with IGF1 was repeated in the presence or absence of either the nSMase inhibitor GW4869 (5 μ M) or the ceramide-specific glucosyltransferase inhibitor NB-DGJ (50 μ M).

or following activation of the second messenger ceramide (Puglielli *et al*, 2003a; Costantini *et al*, 2005).

The effect induced by IGF1 on both APP processing and BACE1 steady-state levels was blocked by the nSMase inhibitor GW4869 (Figure 1F), confirming that the cell-surface activation of the second messenger ceramide is a required step for the regulation of APP/A β metabolism downstream of IGF1-R and p75^{NTR}. No effect was observed when IGF1 was administered in the presence of *N*-butyldeoxygalactonojirimycin (NB-DGJ), which blocks the biosynthesis of glycosphingolipids (Puglielli *et al*, 2003a) without affecting the hydrolysis of cell surface sphingomyelin (Figure 1F). Finally, no apparent effect was observed on the steady-state levels of BACE2, a close homolog of BACE1, or on TACE and ADAM 10, two regulated forms of α secretase (Supplementary Figure 1A).

Analysis of cholesterol distribution on the cell surface revealed no difference in clustering between cholesterol-poor (CP) and cholesterol-rich (CRD; also called lipid rafts) domains (Supplementary Figure 1B). Similarly, no apparent effect was observed on the cellular levels of GM1, a glycosphingolipid highly enriched in lipid rafts (Supplementary Figure 1C). Taken together, the above results confirm our previous conclusion (Puglielli *et al*, 2003a) that the ceramide-dependent regulation of APP processing does not require changes in the dynamics of lipid rafts. Finally, IGF1 treatment did not affect the subcellular distribution of APP (Supplementary Figure 1D), the translocation of newly synthesized APP to the plasma membrane (Supplementary Figure 1E), or the steady-state levels of cell surface APP (Supplementary Figure 1F).

The major pathway responsible for the transduction of IGF1-R signaling involves phosphorylation of phosphatidylinositol biphosphate (PIP2) by phosphatidylinositol 3-kinase (PI3K), with consequent activation of PKB/Akt. In order to identify the specific signaling cascade acting downstream of the IGF1-R in the regulation of the TrkA to p75^{NTR} molecular switch, we treated SH-SY5Y cells with either LY294002, a general PI3K inhibitor (Nauc *et al*, 2004), or PD98059, which blocks the Raf/MEK/ERK pathway (Schulze *et al*, 2004). As shown in Figure 2A, LY294002 completely blocked the molecular switch induced by IGF1 treatment. This effect was observed on both p75^{NTR} and TrkA levels, confirming that the two receptors are under the same signaling pathway. PD98059 had a slight effect, but there was no additive effect following incubation with both LY294002 and PD98059 (Figure 2A), suggesting a possible cross-talk between these two main branches of IGF1-R signaling.

Activation of PI3K and Akt, down-stream of IGF1-R, requires the recruitment of insulin/IGF1 receptor substrates (IRS) (Waters and Pessin, 1996; Bondy and Cheng, 2004). Of the four IRS proteins identified so far, both IRS1 and IRS2 are highly expressed in the brain, where they seem able to provide some degree of signal specificity (Waters and Pessin, 1996). In order to differentiate between IRS1 and IRS2, we analyzed SHEP neuroblastoma cells, which express only IRS1, and compared them to SH-SY5Y cells, which express only IRS2 (Figure 2B; see also Meyer *et al*, 2001). Both neuroblastoma cell lines were responsive to IGF-1 and were able to induce phosphorylation/activation of IGF1-R and Akt/PKB (Figure 2B). However, SHEP cells were not able to activate the expression of p75^{NTR} in response to IGF-1

(Figure 2B), suggesting that IRS1 cannot transduce IGF1-R signaling down to p75^{NTR}. Consistent with this conclusion, SHEP cells became able to activate p75^{NTR} expression following transfection with human IRS2 (Figure 2C), whereas SH-SY5Y lost the same ability following treatment with siRNA targeted against *Irs2* (Figure 2D). The silencing of IRS2 in SH-SY5Y cells also blocked the ability of IGF1 to stimulate the *in vitro* activity of β secretase (Figure 2E) and the β cleavage of APP (Figure 2D). Finally, expression of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) blocked the ability of IGF1 to activate the expression of p75^{NTR} in the above SHEP/IRS2 background (Figure 2C). PTEN acts by suppressing PI3K signaling downstream of IGF1-R through its PtdIns(3,4,5)P(3) lipid phosphatase activity that converts the signaling active second messenger phosphatidylinositol triphosphate (PIP3) into the inactive PIP2 (Solari *et al*, 2005). Therefore, when taken together, the above results indicate that IRS2, PI3K, and PIP3 are required elements for the IGF1-mediated activation of p75^{NTR} expression downstream of the IGF1-R, and that PTEN acts as a negative control of IGF1 signaling.

IGF1-R controls the TrkA to p75^{NTR} switch and A β generation in primary neurons

Analysis of primary neurons maintained *in vitro* revealed marked changes in the expression of the TrkA and p75^{NTR} receptors as the cultures aged. As shown in Figure 3A, the expression of TrkA decreased, while the expression of p75^{NTR} increased. Furthermore, the TrkA to p75^{NTR} switch followed the onset of IGF1-R expression (Figure 3A) and was accompanied by progressive increases in endogenous ceramide (Figure 3B), BACE1 steady-state levels and β cleavage of APP (Supplementary Figure 2; panels labeled 'control'), as well as A β secretion into the culture medium (Figure 3C). These events required a functionally active p75^{NTR} and activation of nSMase; indeed, they were completely blocked by both genetic disruption of the ligand-binding domain of p75^{NTR} in neurons from p75^{NTR}^{-/-} mice and by pharmacologic inhibition of nSMase by manumycin A in wild-type neurons (Figure 3B and C, and Supplementary Figure 2). Reduction of BACE1 steady-state levels, β cleavage of APP, and A β secretion in wild-type but not in p75^{NTR}^{-/-} neurons by treatment with manumycin A confirms that nSMase and ceramide act downstream of p75^{NTR} (Costantini *et al*, 2005). Finally, treatment of cultured neurons with the nerve growth factor (NGF) increased the steady-state levels of BACE1 and C99 in 24, but not 3, days old cultures (Supplementary Figure 3A), confirming our previous results in neuroblastoma cell lines where we showed that transfection with p75^{NTR} was sufficient in activating β cleavage of APP (when compared to TrkA expressing cells), and that NGF treatment caused a further increase under the above p75^{NTR} background (Costantini *et al*, 2005).

Analysis of the cultured media recovered from 3 and 24 days cultures revealed the presence of IGF1, which was not observed in the fresh media before incubation, indicating that IGF1 is normally produced and secreted by cultured neurons (Supplementary Figure 3B). Together with the fact that both IGF1-R and Akt/PKB were found phosphorylated in 24-days, but not in 3-days, neurons (Supplementary Figure 3C), these results indicate that the activation of IGF1-R observed in our cultures is also accompanied by activation of IGF1 signaling.

Figure 2 The TrkA-to-p75^{NTR} switch downstream of IGF1-R requires IRS2, PI3K, and the second messenger PIP3. **(A)** Western blot analysis of SH-SY5Y cells treated with IGF1 in the presence of LY294002 (LY; 10 μ M) and/or PD98059 (PD; 20 μ M). p75^{NTR} and TrkA are indicated. **(B)** Western blot analysis of IRS1 and IRS2 expression levels in SH-SY5Y and SHEP neuroblastoma cells in the absence of IGF1 treatment. Akt phosphorylation and p75^{NTR} steady-state levels were analyzed both before and after IGF1 treatment. **(C)** Western blot analysis of PTEN, IRS2, and p75^{NTR} in SHEP neuroblastoma cells prior and after transfection with human IRS2 and PTEN. **(D, E)** SH-SY5Y cells were treated with IGF1 before and after silencing of IRS2. siRNA against *Irs2* contained a pool of several siRNA duplexes targeted against *Irs2*. Treatment was started the day before incubation with IGF1 and was repeated 3 days later. **(D)** Western blot analysis of IRS2, p75^{NTR}, and APP. **(E)** β -Secretase activity from cell homogenates was analyzed *in vitro* by using the QTL lightspeed assay. Asterisk indicates statistical significance.

Even if unlikely, the *in vitro* culture of neurons might create conditions that select neurons expressing IGF1-R and p75^{NTR} skewing results in favor of p75^{NTR} signaling. In order to address this issue and demonstrate that these events required the IGF1-R, we used siRNA targeted to *Igfr-1* to reduce expression in neurons grown for 18 and 24 days *in vitro*. As shown in Figure 3D, reduction in the level of the IGF1-R was accompanied by a marked decrease in the level of p75^{NTR} with consequent reduction in the steady-state levels of BACE1 and C99. At the same time, there was a parallel increase in the level of TrkA (Figure 3D), further confirming that IGF1-R acts upstream of the TrkA/p75^{NTR} switch. Very similar results were obtained when we used antisense oligo-

nucleotides overlapping the translation start site of mouse *Igfr-1* to reduce expression (Figure 3E).

Next, we used a different genetic approach to determine if increased expression of the IGF1-R had a reciprocal effect on p75^{NTR} signaling and β -amyloid peptide formation. Overexpression of the short isoform of p53 (p44) in mice causes hyperactivation of IGF1 signaling and increased expression of the IGF1-R (Maier *et al*, 2004). Compared to neurons from wild-type (nontransgenic) mice, primary neurons from p44 transgenic mice show an early (days 3 and 6 *in vitro*) and dramatic activation of IGF1-R expression (Figure 3F). This is accompanied by parallel changes in the cascade of events leading to A β production: upregulation of

Figure 3 IGF1-R acts upstream of p75^{NTR}/TrkA during neuronal life in culture. (A–E) Primary neurons from wild-type (WT) mice were maintained in serum-free media up to 24 days. (A) Western blot analysis of IGF1-R, p75^{NTR}, and TrkA expression levels at different time points. (B) Endogenous ceramide was analyzed by TLC following pre-labeling with [³H]palmitic acid during days 0–3 of culture. Error bars represent the s.d. of four different assays. Asterisks indicate statistical significance from day 3 (*) or age-matched (#) controls. Manumycin A (Man A) was added in the media 3 days before the time-point indicated in the figure, and used at the final concentration of 100 μ M. (C) A β levels in the conditioned media were determined by standard sandwich ELISA using an antirodent A β antibody. Error bars represent the s.d. of six different determinations. (D, E) Western blot analysis of neurons treated with either siRNA duplexes or antisense oligonucleotides designed against *Igf1-r*. siRNA and antisense strategies were used with day 18 and 24 neurons because they showed the highest levels of IGF1-R activation (A). (D) siRNA against *Igf1-r* contained a pool of several siRNA duplexes targeted against *Igf1-r*. Treatment was carried out once at day 15 (for experiments performed at day 18) or day 21 (for experiments performed at day 24). (E) The effects produced by the antisense were compared to an *Igf1-r* sense oligonucleotide. Both oligos were used at 10 μ M final concentration. Treatment was started 6 days before the experiment and the oligos were added every three days together with fresh media. (F) Neurons from WT and p44^{+/+} mice were prepared as in (A) and then analyzed by immunoblotting. (G) Cerebral cortex from 3 and 30-month-old WT mice fed either a control or a restricted diet was analyzed for IGF1-R expression. Western blot of three different animals for each group is shown.

p75^{NTR} with concomitant downregulation of TrkA, followed by a marked increase in both BACE1 and C99 steady-state levels (Figure 3F).

An identical pathway controls A β production in the aging animal

The results presented above demonstrate that signaling through the IGF1-R controls the level of the neurotrophin receptors TrkA and p75^{NTR} in both neuroblastoma cell lines and primary neurons, and that the IGF1-R acts upstream of p75^{NTR} and ceramide in the regulation of A β generation. To determine the physiological relevance of IGF1-R-mediated A β production in the aging animal, we compared the level of IGF1-R in the brains of wild-type mice at 3 and 30 months of age. As shown in Figure 3G, there is a naturally occurring increase in receptor level in animals fed a normal diet, which was completely blocked by caloric restriction. Caloric restriction is known to reduce insulin/IGF1 signaling in a number of different organisms, including mice. As we demonstrated in a previous paper (Costantini *et al*, 2005), caloric restriction was also able to block the age-associated TrkA to p75^{NTR} switch and consequent activation of β cleavage.

These results suggested that increased activation of IGF1-R signaling associated with premature and accelerated aging in p44^{+/+} mice (Campisi, 2004; Maier *et al*, 2004) should have the opposite effect. As shown in Figure 4A, analysis of cerebral cortex from p44 transgenic mice did, in fact, reveal a dramatic increase in the level of p75^{NTR}, which was paralleled by decreased levels of TrkA. It is worth stressing that the levels of both p75^{NTR} and TrkA observed in 1-month-old p44 transgenic mice were similar to those observed in 30-month-old control animals, and that no further change in p75^{NTR} and TrkA levels occurred over their remaining lifespan (Figure 4A). The marked increase in p75^{NTR} expression in p44 transgenic mice was paralleled by similar changes in the levels of ceramide, BACE1, and C99, and by increased production of A β (Figure 4A), further confirming our previous conclusion that the p75^{NTR}-ceramide signaling pathway regulates β cleavage of APP (Costantini *et al*, 2005). Treatment of p44 transgenic mice with the nSMase inhibitor manumycin A reduced the levels of ceramide in the cortex, and produced a significant reduction in both the level of BACE1 and β cleavage of APP (Figure 4B). Very similar results were obtained when manumycin A was administered to cultured primary neurons from p44 mice (Figure 4C and D). Together, these results demonstrate that nSMase acts downstream p75^{NTR} in this model of accelerated aging, as well as in normally aging wild-type mice.

Discussion

A model of the upstream events that control A β generation in neurons is presented in Figure 5. Our results indicate that the TrkA to p75^{NTR} molecular switch, which regulates the rate of A β generation during aging, is mediated by the IGF1 receptor. The signaling cascade downstream of IGF1-R requires IRS2, the second messenger PIP3, and activation of Akt. The induction of p75^{NTR} is followed by activation of nSMase and liberation of the second messenger ceramide, which in turn is responsible for the molecular stabilization of BACE1 (Puglielli *et al*, 2003a), the rate-limiting enzyme in A β generation. This sequence of events can be blocked by both

genetic and biochemical manipulations (indicated in red at the appropriate steps in Figure 5) that target either the level of IGF1-R or its downstream signaling cascade. Similar results can also be observed after genetic disruption of *Ngfr* or biochemical inhibition of nSMase, which block the age-associated activation of A β biogenesis (Costantini *et al*, 2005). Conversely, dysregulation of the p53:p44 ratio (indicated in blue at the appropriate step in Figure 5) can result in hyperactivation of IGF1-R signaling and premature TrkA to p75^{NTR} switch.

The entire sequence of events represented in the model shown in Figure 5 could be observed with neuronal cells grown *in vitro*. Although cellular systems like these are more feasible for the kind of biochemical or genetic manipulations used to obtain the data presented in Figure 3 and in the Supplementary data, they are also subject to artifact that can arise when cells are grown in the artificial environment of the tissue culture dish. For example, it was formally possible that the *in vitro* culture of neurons might have been accompanied by a progressive, selective death of TrkA expressing neurons, skewing results in favor of p75^{NTR} expressing neurons. However, the fact that we also observed the TrkA to p75^{NTR} transition in neuroblastoma cell lines following IGF1 treatment argues in favor of the validity of these results. In addition, both antisense oligonucleotides and siRNA against IGF1-R reversed this effect in neurons, decreasing the steady-state levels of p75^{NTR} while increasing TrkA. Finally, the primary neurons used for these experiments had been cultured for 18 or 24 days and should have already undergone any possible selection of p75^{NTR} expressing neurons over TrkA expressing neurons.

IGF1 is secreted into the bloodstream by the liver, where its synthesis is regulated by pituitary growth hormone (GH). However, many other tissues, including the brain, are able to synthesize IGF1 locally, where it is not under the control of circulating GH (Bondy and Cheng, 2004). Indeed, we were able to detect IGF1 in the conditioned media of neuronal cultures (Supplementary Figure 3B), indicating that this factor is normally released by neurons. Consistent with our results, Ames dwarf mice (Prop 1^{df}), which have a defect in the production and secretion of GH by the anterior pituitary, show undetectable levels of both GH and IGF1 in the serum but completely normal levels of IGF1 in the brain (Sun *et al*, 2005). In addition, the same animals present normal levels of IGF1 mRNA and normal activation of IGF1 signaling downstream of IGF1-R in the hippocampus (Sun *et al*, 2005).

In contrast to serum IGF1, which follows the decrease in GH that occurs after puberty (Bondy and Cheng, 2004), IGF1 levels in the central nervous system (CNS) show either no absolute change or a slight increase during aging (Carro and Torres-Aleman, 2004). At the same time, serum insulin levels tend to increase during aging, most likely caused by progressive peripheral insulin resistance. Insulin resistance is known to be a risk-factor for the development of AD and has been implicated in the etiology of several aspects of AD neuropathology, including the production or secretion of A β (Galasko, 2003; Carro and Torres-Aleman, 2004).

Both the IGF1-R and the insulin receptor (IR) are widely expressed in the brain (Bondy and Cheng, 2004). They show co-expression in many areas, including cerebral cortex, hippocampus, and in the fenestrated capillary beds where they mediate high efficiency translocation of insulin and IGF1

Figure 4 p44^{+/+} mice show an early and dramatic activation of p75^{NTR} expression and APP/A β metabolism. **(A)** Analysis of cerebral cortex from wild type (WT) and p44^{+/+} mice. The steady-state levels of p75^{NTR}, TrkA, BACE1, and C99 were analyzed by Western blot with the appropriate antibodies. Ceramide was quantified by both ESI-MS and TLC, whereas A β levels were determined by standard sandwich ELISA using an antirodent A β antibody. The average values of endogenous A β_{total} obtained for 1-month-old animals were 6.4 ± 0.9 pmol/mg and are in the same range of previously found values for endogenous A β_{total} using murine-specific reporter antibodies (Bowen *et al.*, 2004; Costantini *et al.*, 2005). Results are expressed as percent of 1-month-old WT mice. Error bars represent the s.d. of 12 different determinations. Asterisk indicates a significant difference from 1-month-old animals (*) or age-matched controls (#). **(B)** 9-month-old p44^{+/+} mice were treated with manumycin A for 1 month (3.5 mg per animal over a period of 1 month) and then analyzed at the end of treatment as described in (A). Results are expressed as in (A). Error bars represent the s.d. of 12 different determinations. Asterisk indicates a significant difference from untreated animals (*). **(C–D)** Primary neurons (day 6 in culture) from WT and p44^{+/+} mice were prepared as described in Figure 3. **(C)** Western blot of cell lysates was performed with the indicated antibodies. **(D)** β -Secretase activity from cell homogenates was analyzed *in vitro* by using the QTL lightspeed assay. Error bars represent the s.d. of four different assays. Asterisks indicate statistical significance from WT (nontransgenic) neurons (*) and from p44^{+/+} neurons in the absence of treatment (#).

across the BBB (Bondy and Cheng, 2004). The IGF1-R and IR share a high degree of identity, can form hybrid receptors (Federici *et al.*, 1997), and partially overlap in their function (Bondy and Cheng, 2004). Although IGF1 levels in the CNS remain constant or increase slightly during aging (Carro and Torres-Aleman, 2004), the IGF1-R shows a marked increase in

both cortex and hippocampus. The increase in IGF1-R levels in the hippocampus, the region of the brain important for learning and memory, correlates with learning deficits in aging rats (Stenvers *et al*, 1996; Chung *et al*, 2002a,b). In humans, a recent study of the brains of normally aged individuals has found an age-associated increase in the

Figure 5 A model of the molecular pathways controlling A β generation during aging. Genetic and pharmacological manipulations described in the text are indicated in red or blue next to the steps in the pathway that they affect. Manipulations indicated in red inhibit signaling, while the blue manipulation amplifies signaling.

expression of the IR (Lu *et al*, 2004). It is also worth mentioning that both IGF1 and insulin can promote phosphorylation of τ , the principal component of neurofibrillary tangles, in primary cortical neurons (Lesort and Johnson, 2000), and that hyperphosphorylated τ accumulates in hippocampal neurons expressing p75^{NTR} in patients with AD (Hu *et al*, 2002). Finally, both IR and IGF1-R might also be involved in the regulation of A β clearance either in the brain or through the brain–blood interface of the choroids plexus (Carro *et al*, 2005a). In fact, the main enzyme responsible for insulin degradation, insulin-degrading enzyme (IDE), is also one of the major enzymes responsible for the degradation of A β (Leissring *et al*, 2003). Changes in the levels of one of the substrates (i.e., insulin) are likely to affect the affinity of IDE for the other substrate (i.e., A β). On this regard, it is also important to consider that brain insulin delays/impairs A β clearance across the brain–blood barrier (Shiiki *et al*, 2004), and that impaired IGF1-R expression in the choroids plexus also affects A β clearance from the brain (Carro *et al*, 2005b).

IGF1-R and its homologues are the common regulators of lifespan and age-associated events in all organisms studied to date, including yeast, *C. elegans*, *D. melanogaster* and mammals. Hypomorphic alleles of the *Igf1-r* gene extend lifespan, whereas increased activation of the insulin/IGF signaling accelerates the progression of aging and shortens the maximum-lifespan (Longo and Finch, 2003; Kenyon, 2005). Our results have now connected the IGF1 receptor to neurotrophin signaling and to AD, the most common age-associated dementia.

One question that remains concerns the functional significance of the TrkA to p75^{NTR} switch. Both receptors bind NGF with similar affinities, and appear to interact in order to fine

tune their signaling properties (Kalb, 2005). The old paradigm that TrkA transduces only ‘life’ signals whereas p75^{NTR} transduces only ‘death’ signals has been strongly challenged by new data showing not only that the opposite is possible, but also that it happens quite frequently. Indeed, TrkA can cause cells to die and p75^{NTR} can promote cell survival (Kalb, 2005). In addition, TrkA can block p75^{NTR} signaling (Plo *et al*, 2004), promote proteolysis of p75^{NTR} (Kanning *et al*, 2003), and potentially form heterogeneous signaling complexes with p75^{NTR} (Zampieri and Chao, 2004; Kalb, 2005). How this complex set of signals regulates brain functioning during aging remains unknown and will need further evaluation by using appropriate aging paradigms.

Materials and methods

Animals and dietary manipulations

Animals were maintained under specific pathogen-free conditions until killing, in accordance to guidelines for the ethical care and treatment of animals from the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison. Both nSMase inhibition and the normal husbandry of the mice were described previously (Maier *et al*, 2004; Costantini *et al*, 2005). At the completion of treatment, mice were killed and brains were rapidly removed for isolation of cortices and hippocampi. Tissue was immediately processed for further analysis.

Cell culture

Human neuroblastoma cells SH-SY5Y were obtained from American Type Culture Collection (clone #CRL-2266) and grown in a 1:1 mixture of F12 and MEM media (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Mediatech, Inc.). SHEP neuroblastoma cells were maintained in DMEM supplemented as above. Cells were maintained in a humidified atmosphere with 6% CO₂.

For neuronal cultures, hippocampi and frontal cortices were dissected from embryonic-day 16–18 (E16–18) mice and placed in DMEM (Gibco BRL) (Puglielli *et al*, 2003a; Costantini *et al*, 2005). The tissue was mechanically dissociated by pipetting and neurons were plated on poly-(L-lysine)-coated six-well plates (Becton Dickinson Labware) for 2 h. Neurons were then changed to Neurobasal medium containing 2% B27 supplement (Gibco BRL) in the absence of serum or antibiotics. Cultures grown in serum-free media yielded ~99.5% neurons and 0.5% glia. Microscopically, glial cells were not apparent in cultures at the time they were used for experimental analyses. However, some of the experiments aimed at the analysis of APP processing and IGF1-R, p75^{NTR}, and TrkA expression levels were also performed in the presence of 10 μ M cytosine β -D-arabinofuranoside hydrochloride (Sigma) in order to exclude any effect produced by possible proliferation of glial cells. Medium was changed every 3 days.

Lipid labeling and extraction

Labeling of sphingolipids was performed using [9,10-³H(N)]palmitic acid (60 Ci/mmol) (NEN Life Science) as described (Puglielli *et al*, 2003a; Costantini *et al*, 2005). For lipid extraction, cells were washed twice in PBS, scraped and extracted in chloroform:methanol (2:1, v/v). The lipid phase was dried, resuspended in chloroform, and applied, together with standards, to a Silica Gel-G (EM Science) thin layer chromatography (TLC) plate. Plates were developed as described (Puglielli *et al*, 2003a; Costantini *et al*, 2005); spots were then scraped and counted in a liquid-scintillation counter.

For ceramide quantification in the brain, brain membrane extracts were analyzed by both electrospray ionization mass spectrometry (ESI-MS) and TLC as described previously (Costantini *et al*, 2005). Identity and quantification of TLC spots was further confirmed by ESI-MS (performed at the Mass Spectrometry Facility of the University of Wisconsin Biotechnology Center). Pixel densities of TLC spots were calculated from scanned images with Adobe Photoshop; densitometry was confirmed with the EpiChem³ DarkroomTM (UVP Bioimaging Systems) using Labworks Image Acquisition and Analysis Software 4.5.

Antibodies and Western blot analysis

Protein extracts were prepared in GTIP buffer (100 mM Tris pH 7.6, 20 mM EDTA, 1.5 M NaCl) with 1% Triton X-100 (Roche), 0.25% NP40 (Roche), plus a complete protein inhibitors cocktail (Roche) and a mixture of protein phosphatases inhibitors (cocktail set I and set II; Calbiochem).

Western blot analysis was performed as described (Puglielli *et al*, 2003a; Costantini *et al*, 2005). The following antibodies were used throughout this study: APP (polyclonal; Chemicon International); BACE1 (polyclonal; Abcam); p75^{NTR} (polyclonal; Promega); TrkA and IGF1-R (polyclonal; Santa Cruz Biotechnologies and Cell Signaling); phospho-Akt, phospho-IGF1-R, IRS1, and IRS2 (monoclonal; Cell Signaling); actin (polyclonal; Cell Signaling). Secondary antibodies (Amersham) were used at a 1:6000 dilution. Binding was detected by chemiluminescence (LumiGLO kit; KPL, Gaithersburg, MD).

Pixel densities (for signal-area) of scanned images were calculated with Adobe Photoshop; densitometry (for signal-density) was analyzed with the EpiChem³ DarkroomTM (UVP Bioimaging Systems) using Labworks Image Acquisition and Analysis Software 4.5.

Antisense/siRNA treatment

Phosphodiester oligonucleotides, including *Igf1-r* antisense (5'-CAGACTTCATTCCTTT-3') and sense (5'-AAAGGAATGAAGTCTG-3'), were synthesized at the University of Wisconsin Biotechnology Center and purified on reverse-phase high-performance liquid chromatography. Both oligos were used at 10 μ M final concentration. Treatment was started 6 days before the experiment and the oligos were added every 3 days together with fresh media.

The pools of small interfering RNA (siRNA) duplexes designed against human *Igf1-r* and human *Irs2* were obtained from Upstate (cat. #M-003012) and Dharmacon (cat. #MQ-003554-01), respectively. Scrambled siRNA was used as the control siRNA. siRNAs were transfected into cells by using the siIMPORTER Transfection Reagent (Upstate; cat. #64-101) as suggested by the manufacturer. Treatment of primary neurons with siRNA against *Igf1-r* was performed once at day 15 of their life in culture (for experiments performed at day 18) or day 21 (for experiments performed at day 24). Treatment of SH-SY5Y cells with siRNA against *Irs2* was started the day before incubation with IGF1 and was repeated 3 days later.

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A β determination

For A β determinations in the brain, cortices and hippocampi were homogenized separately as described before and analyzed by standard sandwich ELISA (Costantini *et al*, 2005). We used antibodies 9131 (for A β 1–40) and 9134 (for A β 1–42) as capture antibodies, and 9154 (specific for rodent A β) and 4G8 as biotinylated reporter antibodies. The above antibodies were all from Signet Laboratories. For each sample, the levels of A β ₄₀, A β ₄₂, and A β _{total} were quantified as triplicate based upon standard curves run (on every ELISA plate) and then expressed as pmol A β /mg of protein. A β ₄₂ was constantly found to be ~25–30% of total A β values.

β - and γ -secretase activity in vitro

Tissue homogenates from hippocampi and cortices were assayed *in vitro* using the QTL Lightspeed Assay (QTL Biosystems) as described (Costantini *et al*, 2005). Each sample was analyzed as duplicate of two different concentrations. Values were calculated over background (blank; no enzyme) and expressed as arbitrary units per mg of protein.

Statistical analysis

The data were analyzed by one-way ANOVA and Student's *t*-test comparison, using GraphPad InStat3 software. Statistical significance was reached at *P* < 0.05.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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