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Egr-1 and Hipk2 are required for the TrkA to p75^{NTR} switch that occurs downstream of IGF1-R

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Abstract

The aging program mediated by IGF1-R is responsible for a naturally occurring TrkA-to-p75^{NTR} switch that leads to activation of the second messenger ceramide and increased production of the Alzheimer's disease amyloid β -peptide. Biochemical and genetic approaches that target IGF1-R signaling, p75^{NTR}, or ceramide are able to block the above events. Here, we show that the transcription factors Egr-1 and Hipk2 are required elements for the TrkA-to-p75^{NTR} switch downstream of IGF1-R signaling. Specifically, Egr-1 is required for the upregulation of p75^{NTR}, whereas Hipk2 is required for the downregulation of TrkA. In fact, gene silencing of *Egr-1* abolished the ability of IGF1 to upregulate p75^{NTR}, whereas similar approaches directed against *Hipk2* blocked the downregulation of TrkA. In addition, IGF1 treatment favored binding of Egr-1 and Hipk2 to the promoter of p75^{NTR} and TrkA, respectively. Finally, the expression levels of both Egr-1 and Hipk2 are upregulated in an age-dependent fashion. Such an event is opposed by caloric restriction, a model of delayed aging, and favored by the p44 transgene in p44^{+/+} animals, a model of accelerated aging.

Keywords

Aging; Alzheimer's disease; Egr-1; Hipk2; IGF1-R; p75^{NTR}; TrkA

1. Introduction

Studies performed in yeast, *C. elegans*, *D. melanogaster*, and mammals indicate that the signaling pathway acting downstream of the insulin-like growth factor 1 (IGF1) receptor (IGF1-R) plays an important role in the regulation of lifespan and age-related events [23,25,

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DISCLOSURE STATEMENT

The animal studies were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison. No actual or potential conflict of interest exists.

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40]. Initially discovered in lower organisms, the lifespan-regulatory function of IGF1-R has recently been proven in mammals. Indeed, a partial block of IGF1-R signaling in *Igf1-r^{+/-}* [16] and Ames dwarf [3] mice leads to a longer lifespan with no apparent manifestation of disease or cognitive impairment. In contrast, hyperactivation of IGF1-R signaling in *p44^{+/+}* [27], *p53^{+/m}* [41], and *Zmpste24^{-/-}* [43] mice accelerates the progression of aging and shortens the maximum lifespan. A partial block of IGF1-R signaling is also achieved by caloric restriction, which extends the maximum lifespan and delays many biological changes that are associated with aging [38,44].

Our group has recently shown that the aging program, mediated by IGF1-R, is responsible for a naturally occurring switch in the expression levels of neurotrophin receptors, TrkA and p75^{NTR} [7,8]. In fact, IGF1-R activates p75^{NTR} while downregulating TrkA. The TrkA to p75^{NTR} switch is responsible for an age-dependent activation of neutral sphingomyelinase (nSMase), the enzyme that converts cell-surface sphingomyelin (SM) into the second messenger ceramide. Ceramide, in turn, is responsible for the molecular stabilization of the β -site APP cleaving enzyme (BACE1), and for the increased β cleavage of the Alzheimer's disease (AD) amyloid precursor protein (APP) that accompanies aging [6,7,8,32]. These results are particularly important because aging is the single most important risk-factor for late-onset AD, which represents the most common form of dementia in the world [15,39].

The strict requirement for p75^{NTR} in the above events was proven by using animals expressing a truncated version of the receptor that lacks the ligand binding domain (*p75^{NTR}ExonIII^{-/-}*). In fact, *p75^{NTR}ExonIII^{-/-}* mice showed no apparent activation of nSMase activity and A β generation during the normal process of aging [8]. It is worth remembering that p75^{NTR} can also induce pro-death signals in a variety of cell- and animal-based models [12,34]. Therefore, the molecular mechanisms that control the TrkA to p75^{NTR} switch are crucial for the understanding of AD pathology. This conclusion is also supported by the fact that genetic disruption of p75^{NTR} resolves the high A β levels, the intracellular deposits of A β , and the plaque pathology observed in AD11 mice, a murine model of AD [4].

Here, we show that the transcription factors early growth response 1 (Egr-1; also known as nerve growth factor-induced clone A, NGFIA) and homeodomain-interacting protein kinase 2 (Hipk2) are required elements for the TrkA to p75^{NTR} switch downstream of IGF1-R signaling. We also show that the expression levels of both Egr-1 and Hipk2 are upregulated in an age-dependent fashion; such an event is opposed by caloric restriction, a model of delayed aging, and favored by the p44 transgene in *p44^{+/+}* animals, a model of accelerated aging.

2. Methods

2.1. Animals, Dietary Manipulations, and Immunostaining

Animals (on an ICR background) were maintained under specific pathogen-free conditions until sacrifice, 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. The normal husbandry was described previously [7,8,27,31]. To control caloric intake, mice were housed singly and fed less than *ad libitum* intakes [8,31]. The control group was fed 84 kcal/week of a modified formulation of AIN-76 semipurified diet (Harlan Teklad), which is ~90% of the average *ad libitum* food intake of these mice. Mice on caloric restriction were restricted in their food intake from 5 weeks of age, being fed 63 kcal/week (a 32% reduction). The restricted diet was nearly isocaloric to the control diet, but enriched in proteins, vitamins, and minerals to avoid malnutrition [8,31]. Mice (wild-type/non-transgenic, caloric restricted, and *p44^{+/+}*) were sacrificed and brains were immediately removed; cortices and hippocampi were separated and rapidly frozen by immersion in liquid nitrogen.

For immunostaining, animals were euthanized according to the NIH Guide for the Care and Use of Laboratory Animals. The brains were dissected, fixed overnight in 10% neutral buffered formalin, and paraffin-embedded using standard techniques. Coronal tissue sections (5 μ m) were prepared using a microtome. Following standard deparaffinization and rehydration, the tissue sections were processed for immunofluorescence. Antigen retrieval was performed in 100 mM citrate buffer (pH 6) heated in an autoclave. After washing with PBS, tissue sections were permeabilized with 0.1% Triton X-100 in PBS and blocked for 2 hr with 10% goat serum, 2% bovine serum albumin, and 0.1% Triton X-100 in PBS. Sections were then incubated with primary antibodies (diluted in blocking solutions) overnight at 4°C. After washing with PBS, they received the secondary antibodies (diluted in blocking solution) for 1 h at room temperature. Nuclei were counterstained with To-Pro-3 (Molecular Probes). Slides were mounted using Gel/Mount aqueous mounting medium (Biomed). The following primary antibodies were used: anti-GFAP (monoclonal; 1:500; Sigma); Egr-1 (polyclonal; 1:100; Santa Cruz Biotechnologies); Hipk2 (polyclonal; 1:100; Santa Cruz Biotechnologies). Secondary antibodies were Alexa 488- and Alexa 594-conjugated goat anti-rabbit and anti-mouse (1:500; Molecular Probes-Invitrogen). Controls were performed by omitting the primary antibody. Slides were imaged on a Nikon C1 Laser Scanning Confocal microscope. Quantification of relative optical densities (ROD) of Egr-1 and Hipk2 staining was performed on immunolabeled coronal sections imaged at X20 using Scion-Image software. Measurements were performed in three brain sections for each animal. Mean ROD values were reported as percentage of Non-Tg mice. Statistical analysis was performed using One-way ANOVA followed by Tukey-Kramer multiple comparisons test. Differences were declared statistically significant if $p < 0.05$.

2.2. 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.). Cells were maintained in a humidified atmosphere with 6% CO₂.

2.3. 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 protease inhibitor cocktail (Roche) and a mixture of protein phosphatases inhibitors (cocktail set I and set II; Calbiochem). Western blot analysis was performed as described [6,7,8,32,33]. The following antibodies were used throughout this study: Brn-3a (monoclonal; Santa Cruz Biotechnologies, cat. #sc-8429); Egr-1 (polyclonal; Santa Cruz Biotechnologies, cat. #sc-110); Hipk2 (polyclonal; Santa Cruz Biotechnologies, cat. #sc-25431); p75^{NTR} (polyclonal; Promega, cat. #G3231; and Santa Cruz Biotechnologies, cat. #sc-8317); TrkA (polyclonal; Santa Cruz Biotechnologies, cat. #sc-118); actin (polyclonal; Cell Signaling, cat. #4967); BACE1 (N-terminal, polyclonal; Abcam, cat. #ab23800). 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 EpiChemi³ DarkroomTM (UVP Bioimaging Systems) using Labworks Image Acquisition and Analysis Software 4.5.

2.4. mRNA quantitation

For mRNA quantitation of p75^{NTR} and TrkA in SH-SY5Y cells, total RNA was extracted and isolated by using the RNeasy Mini kit (Qiagen) according to manufacturer's protocol. RT-PCR was performed with Illustra Ready-To-Go RT-PCR Beads (Amersham) according to

manufacturer's protocol. The primers for p75^{NTR} (forward-5'-TGAACCAGACGCCCCACCAGAG-3'; reverse-5'-GTCCCCCGCAGAGCCGTTGAGAAG-3'), TrkA (forward-5'-GCCGTCTTTGCCTGCCTCTTCC-3'; reverse-5'-GCCCTCCCCCAGCTCCCCTTG-3'), and GAPDH (forward-5'-TTTGTCAGCTCATTTCCTGGTA-3'; reverse-5'-TTCAAGGGGTCTACATGGCAACTG-3') were designed inside the ORF and produced fragments of the expected size.

Real-time quantitative PCR was performed in a ABI PRISM 7000 Sequence Detection System using SYBR Green PCR Master Mix (Applied Biosystems). Amplifications were generated at 2 min at 52°C and 3 min at 95°C, followed by 40 cycles of denaturations at 95°C for 15 s, annealing, and synthesis (30 s at 61°C and 30 s at 72°C). Delta-delta Ct values [24] were normalized with those obtained from the amplification of GAPDH and were expressed as fold-change over control. The assay was repeated three times, with each assay containing triplicate reactions, and with each assay including an independent amplification of the probes.

2.5. Chromatin Immunoprecipitation (ChIP) analysis

SH-SY5Y cells were cultured in complete medium in 150-mm Petri dishes until 70% confluent. Cells were then fixed by the addition of 280 µl of 37% formaldehyde (Sigma) to 10 ml of culture medium for 10 min at 37 °C, harvested, and processed for chromatin immunoprecipitation (ChIP) using a commercially available kit (Active Motif). Protein-DNA immune complexes were precipitated with specific antibodies against Brn-3a, Egr-1, Hipk2, and the N-terminal domain of BACE1. PCR was carried out using different primer sets centered on the promoter region of TrkA or p75^{NTR}. The following primers were used: TrkA-region 1 (forward-5'-ACAGCAACCTTTTCCTCAACGCAGTC-3'; reverse-5'-AGCTGCCAGGCCTCCCCGACAT-3'); TrkA-region 2 (forward-5'-GGCGACCCCTTCCCTTTCT-3'; reverse-5'-GGCTCAGCGCTCCATCCT); TrkA-region 3 (forward-5'-ACAGCAACCTTTTCCTCAACGCAGTC-3'; reverse-5'-CCCCCTCTGCCCCCTCCCCTGTTA-3'); TrkA-region 4 (forward-5'-GGCGACCCCTTCCCTTTCT-3'; reverse-5'-AGCTGCCAGGCCTCCCCCGACAT-3'); p75^{NTR}-region 1 (forward-5'-CGGAGGAAGATGGGTAAGAGA-3'; reverse-5'-CCCCAGAAGCAGCAACAGC-3'); p75^{NTR}-region 2 (forward-5'-CGGAGGAAGATGGGTAAGAGA-3'; reverse-5'-TCGGGGTGGGAAGCAGAGG-3').

2.6. Preparation of cytosolic and nuclear fractions

Cytosolic extracts were prepared as described by Shetty and Idell [37] with some modifications. Cells were homogenized in homogenization buffer containing 25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, and a protease inhibitor cocktail. The homogenates were centrifuged at 14,000 × g for 15 min, and supernatants were collected as cytosolic proteins.

Nuclear extracts were prepared according to the protocol of Das *et al.* [10]. Briefly, cells were scraped into ice-cold phosphate-buffered saline and collected by centrifugation. Cell pellets were suspended in 3 volumes of lysis buffer (20 mM Hepes, pH 7.9; 10 mM KCl; 1 mM EDTA, pH 8.0; 0.2% Nonidet P-40; 10% glycerol; and protease inhibitor cocktail) followed by incubation on ice for 10 min. Cell suspensions were gently pipetted up and down; the lysates were then centrifuged at 14,000 × g for 5 min at 4 °C to obtain nuclear pellets. Nuclear pellets were washed twice with cell lysis buffer (lacking Nonidet P-40 and protease inhibitor cocktail) and then resuspended in 2 volumes of nuclear extract buffer (20 mM Hepes, pH 7.9; 10 mM KCl; 1 mM EDTA, pH 8.0; 420 mM NaCl; 20% glycerol; and 10% protease inhibitor mixture). Nuclei were extracted by incubation at 4 °C for 30 min with gentle agitation followed by centrifugation at 14,000 × g at 4 °C for 5 min; the resultant supernatant fraction was used as a nuclear extract.

2.7. Biotin-Streptavidin pull-down assay

One microgram of biotin-double-stranded DNA was used in pull-down assays performed as described previously [35]. The probes used (corresponding to primer-set n. 2 for p75^{NTR} and n. 3 for TrkA; please, see Figure 5) were incubated with 500 µg of nuclear and cytosolic extracts for 20 min at room temperature in a binding buffer containing 12% glycerol, 12 mM HEPES (pH 7.9), 4 mM Tris (pH 7.9), 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 10 µg of poly(dI-dC) competitor. Following the incubation, 30 µl of streptavidin-agarose beads (Pierce) were added to the reaction and incubated at 4 °C for 4 h. Prior to this step, 300 µl of the original streptavidin-agarose bead preparation were pre-adsorbed with 500 µl of bovine serum albumin (1 mg/ml) and 50 µg of poly (dI-dC) competitor for 30 min at 25 °C. The beads were washed three times and resuspended in 300 µl of the binding buffer. The protein-DNA-streptavidin-agarose complex was washed three times with binding buffer, and bound proteins were released by boiling in SDS loading buffer. Samples were then separated on a 4–12% Bis-Tris gel system (NuPAGE; Invitrogen) and analyzed by Western blot.

2.8. siRNA Treatment

The pools of small interfering RNA (siRNA) duplexes designed against human *egr-1*, *hipk2*, and *brn-3a* were obtained from Santa Cruz Biotechnology (*hipk2*, cat. #sc-39050; *brn-3a*, cat. #sc-29839; *egr-1*, cat. #sc-44203). 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 with siRNA was started the day before incubation with IGF1 and was repeated 3 days later. Total treatment with IGF1 (10 nM) was 4 days.

2.9. Statistical Analysis

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

3. RESULTS

3.1. Egr-1 and Hipk2 are required elements for the TrkA to p75^{NTR} switch down-stream of IGF1-R signaling

We recently reported that IGF1-R signaling upregulates p75^{NTR} while downregulating TrkA in both SH-SY5Y neuroblastoma cells and primary neurons [7]. These results were obtained by Western blot analysis of total cell lysates and in the absence of any mRNA quantitation. In order to further characterize the molecular mechanism that is responsible for the TrkA to p75^{NTR} switch, we decided to analyze the mRNA levels of both neurotrophin receptors following IGF1 treatment of SH-SY5Y cells. We have already shown that SH-SY5Y cells respond to IGF1 by phosphorylating both IGF1-R and Akt, indicating that they are able to activate IGF1-R signaling [7]. In addition, they naturally express the insulin/IGF1 receptor substrate 2 (IRS2), which is a required element for the TrkA to p75^{NTR} switch [7]. Treatment of SH-SY5Y cells with IGF1 resulted in a ~2.5-fold increase in the mRNA levels of p75^{NTR} (Fig. 1A and B); this was accompanied by a concomitant ~50% decrease in TrkA mRNA levels. These results are very similar to those obtained with signal-area and signal-density analysis of Western blot images [7]; they are also consistent with our previous conclusion that the effects induced by IGF1-R signaling and normal aging are stronger on p75^{NTR} than on TrkA [7,8]. Therefore, the above results support the conclusion that the TrkA to p75^{NTR} switch downstream of IGF1-R signaling is primarily achieved by transcriptional regulation of the respective genes.

Among the many transcription factors that have been shown to regulate the expression of p75^{NTR} or TrkA, Egr-1, Hipk2, and Brn-3a are known to be continuously expressed during

adulthood [1,5,28,29,36,42]. Our previous studies indicate that the expression levels of p75^{NTR} and TrkA are affected by normal aging; they also indicate that the effects of aging are blocked/delayed by caloric restriction and accelerated/aggravated by the p44 transgene in p44^{+/+} animals [7,8]. Therefore, we assumed that if the above transcription factors are involved in the TrkA to p75^{NTR} switch, they should follow the same trend. Figure 2 shows that Egr-1 and Hipk2, but not Brn-3a, fulfilled the above criteria. In fact, the levels of Egr-1 in the brain increased progressively during aging with a cumulative upregulation of ~1.6 fold over a period of 30 months (Fig. 2A). This age-associated effect was reduced by caloric restriction and exacerbated in p44^{+/+} mice (Fig. 2A). Very similar results were also observed with Hipk2 (Fig. 2C). In contrast, no apparent effect was observed on the expression levels of Brn-3a (Fig. 2B).

To determine if the effect of IGF-1R hyperactivation occurred in neurons, glial cells, or both, we compared the localization of Egr-1 or Hipk2 expression to that of GFAP by immunolabeling of hippocampal sections of age-matched non-transgenic and p44^{+/+} mice. As shown in Fig. 3A, the expression of Egr-1 (green) and GFAP (red) colocalized in some but not all cells in the hippocampus of both p44^{+/+} transgenic and non-transgenic mice, indicating that Egr-1 is expressed in both neurons and astrocytes (see especially the higher magnification image in (c)). In contrast, there was no overlap in the expression of Hipk2 (green) and GFAP (red), indicating that Hipk2 is found exclusively in neurons (Fig. 3B (i)). However, upregulation in p44^{+/+} animals was almost entirely restricted to neurons (compare the density of green cells in (c) to (f) for Egr-1 and in (i) to (l) for Hipk2 in Fig. 3).

Finally, to verify that the changes in expression of Egr-1, Hipk2, and Brn-3a in SH-SY5Y cells were due to activation of the IGF-1R, we treated the cells with IGF-1 and compared expression levels by Western blot analysis. As shown in Fig. 4A, both Egr-1 and Hipk2 were activated by IGF1 and their response overlapped with the effects observed on p75^{NTR} and TrkA under the same conditions [7].

Previous studies have shown that Egr-1 is required for the expression of p75^{NTR} [29], whereas Hipk2 can act as a repressor of TrkA transcription [46]. Therefore, when taken together, the above results in wild-type, caloric restricted, and p44^{+/+} mice, and in SH-SY5Y cells are consistent with the possible involvement of Egr-1 and Hipk2 in the age-associated transcriptional regulation of p75^{NTR} and TrkA. Egr-1 could be directly involved with the activation of p75^{NTR}, whereas Hipk2 could be responsible for the reduction of TrkA.

To verify this conclusion, we used siRNA targeting *Egr-1*, *Hipk2*, and *Brn-3a*. Figure 4B shows that gene silencing of *Egr-1* abolished the ability of IGF1 to upregulate p75^{NTR}, whereas similar approaches directed against *Hipk2* were able to block the downregulation of TrkA. Furthermore, there was a reciprocal effect on Egr-1 and Hipk2 such that silencing of *Egr-1* did not affect the levels of TrkA and silencing of *Hipk2* did not affect the levels of p75^{NTR}. Similar approaches directed against *Brn-3a* did not affect the expression levels of either TrkA or p75^{NTR} (Fig. 4B), confirming that Brn-3a does not play any apparent role in the TrkA to p75^{NTR} switch downstream of IGF1-R. Downregulation of Egr-1 or Hipk2 did not cause any effect in the absence of IGF1 treatment (Fig. 4C), suggesting that they do not play a significant role in maintaining the levels of neurotrophin receptors under basal conditions.

3.2. IGF1-R favors binding of Egr-1 and Hipk2 to the promoter of p75^{NTR} and TrkA, respectively

Next, we determined if Egr-1, Hipk2, and Brn-3a bind to the promoter region of TrkA and p75^{NTR} under normal conditions, in the absence of IGF1 treatment. For this purpose, we used chromatin immunoprecipitation (ChIP), which allows immunoprecipitation of protein-DNA complexes following *in vivo* cross-linking with formaldehyde, cell lysis and DNA shearing. The cross-linked DNA was immunoprecipitated with antibodies against Egr-1, Hipk2, and

Brn-3a, reversed, purified, and amplified with specific primers designed to overlap different areas upstream of the start codon (Fig. 5A and C). We found that all the above transcription factors can form *in vivo* complexes with the immediate upstream 500 bp of the promoter of TrkA and p75^{NTR} (Fig. 5). However, although each of these factors could bind to the promoter regions of the neurotrophin receptors, it was possible that IGF1-R signaling affected binding by re-directing their transcriptional activities. To address this issue, we first determined the effect of IGF1-R signaling on their nuclear localization. Figure 6A shows that the upregulation of Egr-1 and Hipk2 observed in Figure 4A is accompanied by their translocation to the nucleus. This indicates that IGF1-R signaling regulates both the expression levels and the nuclear localization of Egr-1 and Hipk2. Once again, no effect was observed on Brn-3a (Fig. 6A). Next, we performed a biotin-streptavidin pull-down assay using a PCR probe corresponding to region n. 2 of Figure 5C (for p75^{NTR}) and n. 3 of Figure 5A (for TrkA). The PCR probes were biotinylated prior to the incubation with either a nuclear or a cytosolic fraction recovered from SH-SY5Y cells cultured in the absence or presence of IGF1 in the conditioned media (as in Fig. 6A). The biotinylated DNA-protein complex was purified with streptavidin, digested with DNases, and then analyzed by SDS-PAGE and immunoblotting with specific antibodies against Egr-1, Hipk2, and Brn-3a. IGF1 appeared to favor the pull-down of Egr-1 by the p75^{NTR} probe and Hipk2 by the TrkA probe (Fig. 6B and C), confirming the specific transcriptional functions of the above elements revealed by siRNA (Fig. 4B). The results produced by DNA:protein pull-down were confirmed by coupling real-time quantitative PCR to ChIP after IGF1 treatment. In fact, Figure 6D and 6E clearly indicate that IGF1 favored binding of Egr-1 to p75^{NTR} and Hipk2 to TrkA. Once again, this effect was highly specific, such that IGF1 did not affect the binding of Hipk2 to p75^{NTR} or of Egr-1 to TrkA (Fig. 6D and E).

Therefore, when taken together, the above results indicate that the TrkA to p75^{NTR} transcriptional switch downstream of IGF1-R requires Egr-1 and Hipk2. They also indicate that IGF1-R signaling achieves the switch by favoring the interaction of Egr-1 with the promoter of p75^{NTR} and Hipk2 with the promoter of TrkA.

4. DISCUSSION

The main conclusion of this work is that Egr-1 and Hipk2 are required elements for the TrkA to p75^{NTR} transcriptional switch downstream of IGF1-R, therefore adding two new molecules to those already identified and reported in our previous work (please, see Figure 7) [7,8].

Our conclusions were validated by different observations obtained with both biochemical and genetic approaches. First, the expression levels of both Egr-1 and Hipk2 are upregulated in an age-dependent fashion in wild-type animals. The age-dependence is further emphasized by the fact that caloric restriction, which is known to delay aging and extend lifespan [38,44], was able to block/delay the activation of both Egr-1 and Hipk2. In addition, the levels of the above transcription factors appeared highly increased in p44^{+/+} mice, which display constitutive hyperactivation of IGF1-R signaling, accelerated aging, and reduced lifespan [27]. Second, both Egr-1 and Hipk2 were activated by IGF1 treatment of SH-SY5Y neuroblastoma cells, which are responsive to IGF1 and are able to activate IGF1-R signaling [7]. The results obtained with the above animal and cellular models are consistent with the behavior of TrkA and p75^{NTR} that we described before: the TrkA to p75^{NTR} switch is activated by normal aging of the brain, blocked/delayed by caloric restriction, accelerated in p44^{+/+} animals, and, finally, activated by IGF1 treatment of SH-SY5Y cells [7,8]. Third, gene-silencing approaches directed against Egr-1 and Hipk2 were able to block the TrkA to p75^{NTR} switch induced by IGF1. Downregulation of Egr-1 was able to block only p75^{NTR}, whereas downregulation of Hipk2 was able to block only TrkA, providing genetic proof to the above conclusions. Finally, ChIP and DNA:protein pull-down assays proved that Egr-1 and Hipk2 can bind to the promoter

regions of TrkA and p75^{NTR}, both *in vivo* and *in vitro*. The pull-down of Egr-1 by the p75^{NTR} probe and of Hipk2 by the TrkA probe was favored by IGF1 pre-treatment, suggesting that IGF1-R signaling may re-direct the above transcription factors to the appropriate targets. Similar conclusions were also obtained when CHIP was performed after IGF1 treatment. How this is achieved is still unknown; however, it is plausible to assume that additional “interacting-proteins” are required to provide signal specificity to the above transcriptional machinery.

Previous work has shown that Egr-1 is required for the expression of p75^{NTR} in Schwann cells [29], can act down-stream of IGF1-R [18], and can serve as a ligand-induced feedback regulator of p75^{NTR} expression down-stream of NGF [11]. In contrast, very little is known on the role of Hipk2 in the regulation of neurotrophin signaling. Indeed, the available literature is limited to the fact that Hipk2 can block the transcriptional activation of TrkA by acting as a repressor of Brn-3a [46]. In this regard, it is worth mentioning that Brn-3a knock out mice show a marked decrease in both p75^{NTR} and TrkA transcripts [14,28]. However, *Brn-3a*^{-/-} mice have severe neurological deficits and die very early during embryogenesis. Therefore, it is unclear whether Brn-3a is indeed required for the transcriptional activation of neurotrophin receptors. *Brn-3a*^{-/-} mice are able to activate (at least initially) the expression of TrkA, but are unable to sustain it in the long term, suggesting that Brn-3a is necessary only for the “long-term maintenance” of TrkA during embryogenesis, through a complex and still uncovered mechanism [14,17,26].

The present study does not seem to support any apparent involvement of Brn-3a in the transcriptional activation of TrkA or p75^{NTR} that occurs downstream of IGF1-R. It also seems to rule out that the role of Hipk2 is limited to the functional repression of Brn-3a activity. In fact, siRNA targeted against *Hipk2* was able to block the activation of TrkA, whereas siRNA directed against *Brn-3a* did not produce any effect (Fig. 4B).

Our group has shown that the TrkA to p75^{NTR} switch that occurs downstream of IGF1-R is responsible for the molecular stabilization of BACE1 and the increased generation of A β that accompanies aging [6,7,8,32]. The strict requirement for p75^{NTR} is proved by the fact that animals expressing a truncated version of the receptor (p75^{NTR}ExonIII^{-/-}; lacking the ligand binding domain) were able to activate neither ceramide nor A β generation [8]. In addition, genetic disruption of p75^{NTR} resolves the high A β levels, the intracellular deposits of A β , and the plaque pathology observed in AD11 mice, a murine model of AD [4]. In addition to its role in the generation of A β , p75^{NTR} can also induce cell-death in a variety of cell- and animal-based models [12,34], and can in part mediate the A β toxicity [9]. Therefore, the present results add additional evidence to support the involvement of the aging program in the pathogenesis of AD, one of the most common forms of age-associated diseases. It is also worth remembering that, when expressed in the same cellular system, TrkA can “silence” p75^{NTR} signaling [21, 30], in addition to promoting p75^{NTR} proteolytic clearance [13,20,22,45] through a mechanism that seems to involve both trans-activation [22] and ligand-binding [13]. Therefore, it is likely that the transcriptional downregulation of TrkA induced by Hipk2 may also result in an increased half-life and signaling of p75^{NTR} further potentiating the activation of p75^{NTR} transcription induced by Egr-1. These events may all be part of a rather complex form of regulation of neurotrophin signaling that is directly influenced by aging itself [2,7,8,19].

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Fig. 1. mRNA levels of p75^{NTR} and TrkA following IGF1 treatment of SH-SY5Y cells
 SH-SY5Y neuroblastoma cells were treated with IGF1 (10 nM) for 4 days prior to mRNA quantitation. (A) Classical RT-PCR. (B) Real-time quantitative PCR. Asterisk (*) indicates statistical significance.

Fig. 2. Effect of aging, caloric restriction, and IGF1-R hyperactivation on the expression levels of Egr-1, Brn-3a, and Hipk2

Analysis of cerebral cortex from wild-type and p44^{+/+} mice. The wild-type mice were fed either control (84 kcal/week) or caloric restricted (63 kcal/week) diet. The steady-state levels of Egr-1, Brn-3a, and Hipk2 were analyzed by Western blot with the appropriate antibodies. Results are expressed as percent of the youngest group (1–2 months of age) of wild-type mice. Error bars represent the average (N=12) + SD. Asterisk indicates a significant difference from young wild-type animals (*) or age-matched controls (#).

Fig. 3. The upregulation of Egr-1 and Hipk2 induced by the aging program is restricted to neurons (A and B) Representative microphotographs showing Egr-1 (A; green) and Hipk2 (B; green) immunoreactivity in hippocampal sections of age-matched non-transgenic (Non-Tg) and p44^{+/+} transgenic mice. Nuclei were counterstained with To-Pro-3 (blue). The To-Pro-3 staining (*b-e* and *h-k*) shows both neurons (see typical architecture of the dentate gyrus) and astrocytes (sparse outside the neuronal fields). Graphic in right panel shows relative optical density (O.D.) quantification of Egr-1 (A) and Hipk2 (B) immunoreactivity. Values are expressed as percentage of Non-Tg. Asterisk (*) indicates statistical significance from Non-Tg mice. (A) Co-labeling with anti-Egr1 (green), To-Pro-3 (blue; nuclei), and anti-GFAP (red; astrocytic marker) shows that Egr-1 is expressed in both astrocytes and neurons (*c* and *f*; they represent high-magnification of areas indicated in *a* and *d*, respectively). However, the

upregulation of Egr-1 caused by hyperactivation of IGF1-R signaling in p44^{+/+} animals appears almost entirely restricted to neurons (**a** and **d**). (B) Co-labeling with anti-Hipk2 (green), To-Pro-3 (blue; nuclei), and anti-GFAP (red; astrocytic marker) shows that Hipk2 is almost exclusively expressed in neurons (**i** and **l**; they represent high-magnification of areas indicated in **g** and **j**, respectively). Hyperactivation of IGF1-R signaling in p44^{+/+} transgenic mice resulted in increased Hipk2 immunoreactivity in hippocampal neurons (compare **j** to **g**).

Fig. 4. Egr-1 and Hipk2 are required for the downregulation of p75^{NTR} and the upregulation of TrkA, respectively

(A) SH-SY5Y cells were treated with IGF1 in the absence of serum as described [7]. Total cell lysates were separated on 4–12% Bis-Tris SDS-PAGE gels, blotted on to a PVDF membrane, and probed with the indicated antibodies. (B) SH-SY5Y cells were treated with IGF1 for 4 days, in the presence and absence of the indicated siRNAs. Cell lysates were separated on a 4–12% BisTris NuPAGE system prior to Western blot analysis. (C) SH-SY5Y cells were treated with siRNA against *Egr-1* and *Hipk2* in the absence of IGF1 treatment prior to Western blot analysis.

Fig. 5. Brn-3a, Egr-1, and Hipk2 bind to the promoter region of both TrkA and p75^{NTR}
 (A and C) Schematic view of the promoter region of TrkA (A) and p75^{NTR} (C), together with the regions covered by the primers used for ChIP analysis. The start codon is indicated. (B and D) SH-SY5Y cells were treated with formaldehyde prior to cell lysis and DNA shearing. Protein-DNA complexes were immunoprecipitated with specific antibodies (against Brn-3a, Egr-1, and Hipk2), reversed, purified, and amplified with primers designed to overlap different areas of the promoter region of TrkA and p75^{NTR}. As control, the PCR was performed on chromatin fragments isolated both before (INPUT) and after immunoprecipitation. (E) ChIP was also performed after immunoprecipitation with an anti-N-terminal-BACE1 antibody (negative control).

Fig. 6. IGF1 favors binding of Egr-1 and Hipk2 to the promoter of p75^{NTR} and TrkA, respectively (A) Western blot analysis of Egr-1, Hipk2, and Brn-3a in cytosolic- and nuclear-protein extracts. Extracts were prepared in the absence of protein phosphatase inhibitors. C, cytosol; N, nucleus. (B and C) A PCR fragment corresponding to the promoter region of p75^{NTR} (primer-set 2 of Fig. 5C) and TrkA (primer-set 3 of Fig. 5A) was biotinylated and then incubated with either a nuclear extract (indicated as N) or a cytosolic fraction (indicated as C) from SH-SY5Y cells. The DNA-protein complex was then purified with immobilized streptavidin, subjected to DNA digestion and analyzed by Western blot. (D and E) The protein-DNA complex immunoprecipitation described in Figure 5 was repeated again after IGF1 treatment and immediately followed by real-time quantitative PCR. The results obtained with the p75^{NTR} probe are shown in (D) whereas those obtained with the TrkA probe are shown in (E). Results are the average (N=3) + SD. Asterisk (*) indicates a significant difference from control (no treatment).

Fig. 7. A model of the molecular pathway controlling the TrkA to p75^{NTR} switch during aging
 The progressive activation of IGF1-R signaling in the brain that accompanies aging leads to a naturally occurring TrkA to p75^{NTR} switch. The signaling pathway downstream of IGF1-R requires IRS2, phosphatidylinositol 3-kinase (PI3K), the lipid second messenger phosphatidylinositol-triphosphate (PIP3), and is under the regulatory control of PTEN and p44/p53 [7,8]. PTEN converts PIP3 into PIP2, whereas p44 downregulates PTEN. As a result of p44 overexpression, p44^{+/+} mice display hyperactivation of IGF1-R signaling, premature aging, and an early TrkA to p75^{NTR} switch [7,27]. The TrkA to p75^{NTR} switch requires Egr-1 and Hipk2; Egr-1 activates the transcription of p75^{NTR}, whereas Hipk2 downregulates TrkA. This event most likely requires additional molecules that provide specificity to the signals mediated by Egr-1 and Hipk2 (indicated as a question mark). The downregulation of TrkA is expected to increase the half-life and signaling activities of p75^{NTR} (see text), therefore

“potentiating” the effects caused by the above transcriptional events. Finally, the activation of p75^{NTR} has been linked to neuronal death and increased production of A β (please, see text). The neuronal death may –at least in some extent- be activated by A β itself acting as a ligand for p75^{NTR} [9]. The sequence of positive events downstream of IGF1-R is shown by the green arrows. The points of inhibitory control are shown in red. Finally, the effects caused by Egr-1 and Hipk2 downstream of IGF1-R (described in this paper) are in blue.