Nerve Growth Factor, Aging and Alzheimer's disease

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September, 2007

A thesis submitted to McGill University in partial fulfillment
of the requirements of the degree of

Doctor of Philosophy

This thesis is dedicated to my family, specially to my wife Carolina

Abstract

Since the cholinergic hypothesis of geriatric memory dysfunction was proposed by Bartus and colleagues in 1982, studies conducted in animals and humans so far have failed to obtain evidence for the involvement of NGF in normal Aging and/or in the pathophysiology of Alzheimer's disease (AD). It has been hypothesized that age-related degeneration of basal forebrain cholinergic neurons (BFCN) may be caused by the altered *endogenous* NGF maturation either by reduced responsiveness to NGF, by reduced NGF transport or by the failure in coupling to second messengers. NGF administered in the CNS of AD patients led to undesirable side effects, most likely mediated by p75 neurotropin receptor ($p75^{NTR}$) rather than through its specific TrkA receptor. Thus, we decided to treat behaviorally characterize age-impaired (AI) rats with small proteolytic- resistant peptide mimetic of the TrkA receptor, named D3. This selective partial agonist of the TrkA receptor reversed the atrophy of the BFCN, ameliorating the cognitive decline observed in AI rats.

The realization that the precursor of NGF (proNGF) might play a biological role in the CNS, raised questions regarding the regulatory mechanisms leading to its release, as well as the control of the proNGF to NGF ratio and, ultimately, the degradation of the NGF molecule. To answer these questions, we performed *in vitro* and *in vivo* studies aimed at elucidating the preferential NGF form released from the cerebral cortex, and the biochemical pathway leading to NGF maturation and degradation. These studies have revealed that proNGF is the main releasable form of the neurotrophin and that the maturation and degradation of NGF largely occurs in the extracellular space with the involvement of a complex protease cascade.

The newly described mechanism for NGF conversion and degradation was found compromised in Alzheimer's disease. In brief, we found a failure in the conversion of proNGF to NGF, which was exacerbated by an increased NGF degradation, leading to proNGF accumulation in the Alzheimer's brain. In addition, and due to the widespread oxidative damage present in Aging and AD brains, we

investigated whether proNGF was targeted by reactive oxygen species. Furthermore, we have found that the accumulated proNGF in AD brains occurred in a nitrated form. Thus, elevated levels of nitrated proNGF were found to be the predominant molecular form of NGF in the AD brain. In *in vitro* maturation studies, we found that nitrated proNGF is converted to nitrated NGF. This nitrated NGF displayed reduced trophic support actions in cultured DRG neurons and displayed reduced capacity to activate TrkA when injected *in vivo.* Finally, we have found that cognitively aged-impaired rats also contain nitrated proNGF when compared to cognitively unimpaired age-matched controls.

In conclusion, we suggest that the well established vulnerability of NGF-dependent forebrain cholinergic neurons in Aging and AD is caused by a profound dismetabolism of the complex protease cascade, which is responsible for the maturation and degradation of NGF, along with the deleterious brain environment characterized by chronic neuroinflammation and oxidative stress. This results in the nitration of pro and of mature NGF, provoking a “trophic factor disconnection” for the maintenance of the cholinergic phenotype of basal forebrain neurons.

Résumé

Depuis que l'hypothèse cholinergique sur la dysfonction de la mémoire chez la personne âgée a été proposée par Bartus et collègues en 1982, toutes les études cherchant à mettre en évidence l'implication du NGF chez les sujets normaux âgés et ceux atteints de la Maladie D'Alzheimer (MDA) ont échoué à la fois chez l'homme et chez l'animal. L'hypothèse émise fût que la dégénérescence des Neurones Cholinergiques du Cerveau Antérieur Basal (NCCAB) liée à l'âge pourrait être due soit à une maturation altérée du NGF *endogène* soit à une réponse réduite au NGF, par un transport déficient, ou bien encore à un défaut de couplage aux seconds messagers. Le NGF administré dans le SNC de patients atteints de MDA conduit à des effets secondaires indésirables principalement causés par p75 ou le récepteur neurotropique ($p75^{NTR}$) plutôt qu'à travers son récepteur spécifique, TrkA. Par conséquent, nous avons décidé de traiter des rats caractérisés comme âgé-déficients (AD) avec de petits peptides résistant à la protéolyse appelés D3 et mimant l'action du NGF sur les récepteurs TrkA. Cet agoniste partiel du récepteur TrkA remédie à l'atrophie des neurones cholinergiques du CAB, réduisant le déclin cognitif observé chez les rats AD.

Le fait que le précurseur du NGF (proNGF) pourrait jouer un rôle biologique, soulève des questions quant aux mécanismes régulant sa libération, ainsi que ceux contrôlant le ratio proNGF/NGF et enfin ceux contrôlant la dégradation finale du NGF. Pour répondre à ces questions, nous avons réalisé des expériences *in vitro* et *in vivo* afin de savoir sous quelle forme préférentielle le NGF était libéré dans le cortex cérébral, et afin de connaitre les chemins biochimiques menant à la maturation et la dégradation du NGF. Ces études ont révélé que le proNGF représentait la forme principale de libération de cette neurotrophine et que la maturation et la dégradation du NGF avaient lieu dans le milieu extracellulaire avec l'implication d'une cascade de complexe de protéases.

Nous avons démontré que ce mécanisme nouvellement décrit pour la transformation et la dégradation du NGF est compromis durant la MDA. En bref, nous avons trouvé un dysfonctionnement de la transformation de proNGF en NGF

qui est exacerbé par un manque de dégradation du NGF ce qui conduit à une accumulation de proNGF dans le cerveau des patients atteints de MDA. De plus, du fait des dommages oxydatifs se produisant dans le cerveau âgés et atteints de MDA, nous avons cherché à savoir si le proNGF était ciblé par les réactions oxydatives. Nous avons trouvé que le proNGF accumulé chez les patients MDA l'était sous une forme nitrée. Par conséquent des niveaux élevés de proNGF nitrique s'accumulent et représentent la forme prédominante de NGF dans les cerveaux MDA. Dans des études de maturation *in vitro*, nous avons montré que la forme proNGF nitrique était transformée ne NGF nitrique. Le NGF sous sa forme nitrique produit une action de support trophique réduite dans des cultures de neurones des GRD (Ganglions Rachidiens Dorsaux) et montre une capacité réduite à activer TrkA quand il est injecté *in vivo*. Finalement, nous avons trouvé que les rats âgés cognitivement déficients présentent des taux plus élevés en proNGF nitrique que les rats contrôles âgés cognitivement normaux.

En conclusion, nous suggérons que la déjà bien établie vulnérabilité dépendante du NGF des neurones cholinergiques du cerveau antérieur chez les rats âgés et MDA est causée par un trouble profond du complexe de cascades des protéases qui est responsable de la maturation et de la dégradation du NGF accompagné d'un environnement cérébral délétère caractérisé par une neuro-inflammation chronique et un stress oxydatif. Ces résultats montrent que la nitration du pro-NGF et NGF mature provoque «une déconnection en facteur trophique» indispensable au maintien du phénotype des neurones cholinergiques du cerveau antérieur basal.

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Acknowledgments

Since many people have helped me throughout this thesis, I will try to thank everybody…
I would like to thank first **Dr. Claudio A. Cuello**, for giving me the chance to succeed in this project, trusting me with experimental freedom. Thanks for your unconditional support during all these years.
I would like also to thanks **Adriana Ducatenzeiler** and **Vanessa Partridge**, two wonderful friends, always there to talk, laugh or help! You have made my stay at McGill more pleasant. Thanks **Wanda Leon** for your help and valuable contribution to this thesis. I would like to thanks **Karen Bell**, for being an amazing friend.
Thank **Catalina D'Alessio**, thank for your help during all these years, you helped me with everything! Thanks for being there always!
Thanks to my friends **Marcos Ceretti** and **Federica Pritz**, thanks for your unconditional support; thanks to you both, my friends. Gustavo Buffanti, I appreciate your help with flash animation and for being a great friend.
Thanks to **Helene Duplessis** for being always with a smile ready to help!
Thanks to **Alfredo Ribeiro-Da-Silva** and the people working with you, especially **Louis-Etienne Lorenzo** for his help with the french-version of the abstracts.
Thanks **Vannessa P**. for your editing help and to **Adriana D** for the labels…
I would like to thank **Lisa Bolduc** for her sense of humor and for being so friendly.

Thank to my family, for being so supportive and understanding, I owe it all to you; Caro, Lola, Agus, Lu y Vicky.

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Contributions of Authors

This doctoral thesis is the product of data generated in 3 separate manuscripts, found in Chapters 2 through 4.

Dr A. Claudio Cuello: as my supervisor, he is co-author on all manuscripts and he has been the main intellectual influence for the work described. ACC revised and edited all manuscripts.

Martin A. Bruno: was the main investigator for all described projects. MAB designed, in consultants with ACC, all of the experimental work and ran the vast majority of the experiments. For all publications, MAB drafted the figures and generated the complete final drafts of all manuscripts.

Chapter 2

"Long-Lasting Rescue of Age-Associated Deficits in Cognition and the CNS Cholinergic
Phenotype by a Partial Agonist Peptidomimetic Ligand of TrkA *The Journal of Neuroscience* 24(37):8009–8018, 2004.
By **Martin A. Bruno,** Paul B. S. Clarke, Alicia Seltzer, Remi Quirion, Kevin Burgess, A. Claudio Cuello and H. Uri Saragovi.

- **Paul B. S. Clarke**: helped with the statistical analysis.
- **Alicia Seltzer**: performed a pilot experiment with D3 in aged animals in collaboration with Dr H. Uri Saragovi and Dr Remi Quirion (data not included in the publication).
- **Remi Quirion**: participated in the pilot experiments and revised the manuscript prior to its publication.
- **Kevin Burgess**: provided us with D3 and D3-biotin compounds.
- **H Uri Saragovi**: participated in the design of experiments and writing/editing the manuscript. (HUS and ACC were senior authors with equal contribution).

Chapter 3

"Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade" *PNAS*, 103, 17:6735–6740, 2006. By **Martin A. Bruno** and A. Claudio Cuello

Chapter 4

"Abeta-induced NGF trophic disconnection in Alzheimer's disease" *PNAS*, submitted
By **Bruno MA**, Leon WC, Fragoso, G, Mushynski WE, Almazan, G and Cuello, AC, submitted

- **Wanda C Leon**: made the injection of Abeta oligomers and nitrated NGF into the hippocampus of the rats and performed some of the Western blots.

- **Gabriela Fragoso**: performed the *in vitro* studies in DRG neuron cultures with nitrated NGF.

- **G. Mushynsky**: the tissue culture was performed by GF at his lab. GM assisted with manuscript editing.

- **Guillermina Almazan**: provided assistance with the tissue culture experiments and participated in editing the manuscript.

CHAPTER 1

General Introduction

1A- Preface

The correlation of clinical dementia ratings with the reduction in a number of cortical cholinergic markers suggests a tight association between cholinergic hypofunction and cognitive deficits. Cholinergic neurons of the basal forebrain have been described to undergo degenerative changes during aging, resulting in cholinergic hypofunction directly related to the progressing memory deficits with aging. In addition, basal forebrain cholinergic neuron atrophy is also a feature of Alzheimer's disease, which has been suggested to cause, at least partly, the cognitive decline observed, and has led to the formulation of the cholinergic hypothesis of geriatric memory dysfunction.

Nerve growth factor (NGF) plays a role in protecting and maintaining the phenotype of basal forebrain cholinergic neurons, which are highly sensitive to failures in NGF content and trophic support that may induce cholinergic cellular atrophy and cognitive decline. Interestingly, the age-related cholinergic atrophy and cell loss in normal brain, as well as in Alzheimer's disease, is not complemented by reductions in the levels of NGF as could be expected. In contrast, elevated levels of the precursor form of the NGF (proNGF) has been found in cortical brain samples from early and late stages of Alzheimer's patients, which hints to either a deficiency in NGF biological activity and/or alteration in its maturation, leading to a progressive cholinergic atrophy. In consequence, altered cognitive abilities diminish with age, but the causes of NGF support failure in aging and in Alzheimer's disease have yet to be elucidated.

Chronic oxidative stress has been hypothesized to be a major contributor to the aging process, resulting from the cytotoxic consequences of an inescapable side-product of oxidative metabolism, the production of reactive oxygen species (ROS). The continued exposure to ROS damages critical cellular structures and may be responsible for some age-related pathologies, including Alzheimer's disease. NGF has been shown to positively modulate the levels and activity of the enzymes involved in the metabolic degradation of ROS, which represent the main endogenous antioxidant defense. Interestingly, age-related memory impairment is correlated with a decrease in brain antioxidant mechanisms and, despite the NGF regulation of antioxidant enzymes, it has been shown that ROS can

negatively influence NGF signaling. However, the relationship between NGF/ROS interaction and the possible negative impacts on the cholinergic phenotype in aging and Alzheimer's disease remain elusive.

The following Introduction reviews the literature concerning Nerve Growth Factor and its tight relationship with basal forebrain cholinergic neurons (BFCN), and focuses on their functional interactions in Aging and Alzheimer's disease.

1B- Nerve Growth Factor

1B1 Nerve Growth Factor Discovery

The NGF story began in 1949, when Rita Levi-Montalcini discovered that the tumor tissues induced hyperinnervation of internal organs. She hypothesized that the transplanted tissues released a diffusible agent that stimulated the growth and differentiation of the developing nerve cells. She observed that the tumor could release a diffusible factor that promoted neurite outgrowth directly, in addition to nerve cell differentiation (Levi-Montalcini, 1952;Levi-Montalcini and Hamburger, 1951). Furthermore, this effect was seen as dose-dependent and, in consequence, Levi-Montalcini devised a semi-quantitative method for determining the biological activity of the substance released from the tumor tissue. She named the active substance nerve growth–promoting factor. In the early 1950's, the finding that the adult male mouse submaxillary glands were found to be a rich source of nerve growth factor (NGF) (Cohen et al., 1954;Levi-Montalcini and Booker, 1960). Following this discovery, Levi-Montalcini and Stanley Cohen isolated and purified the molecule and demonstrated that it was a protein (Cohen, 1960;Levi-Montalcini, 1964;Levi-Montalcini and Booker, 1960).

With the availability of large amounts of purified mouse salivary NGF, Levi-Montalcini and Cohen could produce large quantities of antibodies against NGF (Cohen, 1960;Levi-Montalcini and Angeletti, 1966) and, through the use of these antibodies, they demonstrated the functional significance of NGF in the *in vivo* development of sympathetic and sensory ganglia. In the early 1970's, Levi-Montalcini include the effect of NGF on cell types other than sympathetic and sensory neurons, the influence of NGF on the central cholinergic system (CNS), and the ability of NGF to act as a chemotropic agent. In October

1986, the Nobel Prize was awarded jointly to Rita Levi-Montalcini and Stanley Cohen for their discoveries of NGF and epidermal growth factor (EGF). However, further studies demonstrated that only a limited number of neurons were found to be NGF responsive in the CNS, and the isolation of brain-derived neurotrophin factor (BDNF) from the brain helped establish the concept that the fate and the shape of most vertebrate neurons can be regulated by diffusible growth factors (Hofer and Barde, 1988).

Currently, the NGF-superfamily includes a group of structurally and functionally related molecules: NGF, BDNF, neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin 6 (NT-6) and these proteins share between 50-60% amino acid homology (Ebendal, 1992). In term of the receptors, neurotrophins bind to tyrosine kinase receptors gene family, which includes TrkB, the receptor for BDNF and NT-4, TrkC, the receptor for NT-3 and TrkA, the receptor for NGF. In addition, all neurotrophins bind to p75 neurotrophin receptors ($p75^{NTR}$) (Bothwell, 1995;Kaplan and Miller, 1997). Discussion of specific aspects of the NGF interactions with TrkA and $p75^{NTR}$ will follow (**Section 1B3 and Figure 1-1**).

1B2 Nerve Growth Factor Structure, Synthesis and Release

In 1971, Angeletti and Bradshaw demonstrated that the NGF protein is part of a precursor molecule, and that proteolytic activity remains associated with NGF. This protein assembly form a large, multimolecular complex defined as 7S NGF. The 7S complex consists of three different molecular species; the α-subunit, the function of which is largely unknown; the γ-subunit, which possesses protease activity; and the β-subunit, which is the biologically active form of NGF (Angeletti and Bradshaw, 1971). Further studies demonstrated that it is unlikely that 7S NGF or a similar complex exists anywhere other than in mouse or rat submaxillary gland, and the properties and functions of the α - and γ - subunits are probably irrelevant to the functions of NGF (Shooter, 2001). After sequencing NGF, two studies in the early 1970's led to the conclusion that NGF was a dimer of (almost) identical peptide chains (Angeletti and Bradshaw, 1971). One, the A-chain, contained 118 amino acid residues; the second, the B-chain, was identical to the A-chain but lacked the first eight residues at the N terminus. The molecular weight of an intact NGF dimer was

calculated to be approximately 26-KDa, containing by the two ~13-KDa polypeptide chains, each of which has three intrachain disulfide bridges.

The first successful crystallization of NGF was achieved in 1975 (Wlodawer et al., 1975). The preliminary X-ray analysis of the crystals showed that the unit cell consisted of 6 molecules of the NGF dimmer, in 12 assymetrical units. It took another 16 years before the three-dimensional structure was solved (McDonald et al., 1991). This novel structure defined the first member of the neurotrophin superfamily with predominantly -strand secondary structure and a unique cystine-knot domain in the protein monomer. In the NGF dimer, two of these monomers (chains) assembled about their flat faces and with their long axes roughly parallel. The hydrophobic nature of the dimer interface readily accounted for the extremely tight association of the two NGF chains observed by Bothwell & Shooter (Bothwell and Shooter, 1977) and again emphasized that the NGF dimer and not the NGF monomer chain, was the active NGF entity under physiological conditions.

In the early 1980's, cDNAs for mouse and human NGF mRNAs were first characterized (Scott et al., 1983;Ullrich et al., 1983) and in 1986, Edwards and coworkers reported that the NGF gene was located on human chromosome 1 (Edwards et al., 1986). Sequence data predicts that NGF is generated from a ~35-KDa precursor containing hydrophobic signal peptides at its N-termini, followed by pro-regions containing sequences of contiguous basic amino acids. The mature, fully processed form of the biologically active NGF dimer appears to be similar in all tissues. Following the first demonstration that proNGF could be processed intracellularly in both constitutive (e.g. glial and fibroblast) and regulated (neurons) secreting cells (Edwards et al., 1988), Bresnahan and coworkers demonstrated that the serine proteinase furin is capable of processing mouse proNGF to NGF (Bresnahan et al., 1984). In support of this, Seidah et al. reported that the conversion of proNGF to NGF at its N terminus used the convertase furin (Seidah et al., 1996). This enzyme was able to cleave proNGF in cells containing regulatory and/or constitutive secretion pathways in order to release mature NGF. N-linked glycosylation of the pro-sequence and trimming of the oligosaccharide chains were required for transport of proNGF from the endoplasmic reticulum to the trans Golgi network, where furin is located and the conversion to mature NGF occurs. The observation that furin and NGF were co-localized in the tubule cells of the submaxillary gland from early development supported this view

(Farhadi et al., 1997). Furin is ubiquitously expressed in cells that generate NGF (Day et al., 1993;Schafer et al., 1993) and it is produced early in embryonic development, before the appearance of NGF (Zheng et al., 1994).

Interestingly, Lee and coworkers showed that the proforms of nerve growth factor (proNGF) were secreted by transfected cells and cleaved extracellularly by the serine protease plasmin, suggesting that the biological action of nerve growth factor may be regulated by proteolytic extracellular cleavage(Lee et al., 2001). In this regard, the role of neurotrophin prodomains have been thought to be limited to promoting the folding of the mature domain (Rattenholl et al., 2001;Suter et al., 1991), and the sorting of neurotrophins to either constitutive or regulated secretory pathways (Farhadi et al., 2000). However, sequence comparison of proneurotrophins revealed regions of the prodomain that are highly conserved across species, suggesting that they may mediate additional biological actions (Heinrich and Lum, 2000). This particular subject is presented in the **Section 1B4**.

1B3 Nerve Growth Factor Receptors and NGF Signaling Mechanisms

The biological actions of NGF are mediated by two structurally distinct transmembrane receptors, TrkA (Barbacid et al., 1991;Kaplan et al., 1991a;Klein et al., 1991) and p75 neurotrophin receptor ($p75^{NTR}$) (Bothwell, 1995;Kaplan and Miller, 1997). There are two classes of binding sites for NGF; low-affinity (i.e. $K_d \sim 10^{-9}$ M) and high-affinity binding sites (i.e. $K_d \sim 10^{-11}$M). When $p75^{NTR}$ was cloned (Chao et al., 1986;Klein et al., 1991) transfection studies revealed that $p75^{NTR}$ bound NGF at lower of these affinities, and the receptor was therefore termed the low-affinity NGF receptor. However, several studies demonstrated that NGF binds the two receptors with similar affinities, and both $p75^{NTR}$ and TrkA receptors have been shown to contribute to the high affinity binding site (for reviews see (Huang and Reichardt, 2003;Reichardt, 2006;Roux and Barker, 2002).

The TrkA receptor is a single-pass transmembrane polypeptide chain that serves as a receptor tyrosine kinase of NGF and belongs to the tropomyosin receptor kinase family (for review see Huang and Reichardt, 2003). Genetic analysis revealed that in normal cells the proto-oncogene encoded a 140-KDa glycosylated protein, whereas a little over half of the receptor forms the extracellular region (a short, single transmembrane domain) and an intracellular domain encoding a tyrosine kinase (Martin-Zanca et al., 1989). Following its

initial discovery in 1986 from human colon carcinoma (Martin-Zanca et al., 1986), the receptor remained an "orphan receptor" for several years, when in 1991 it was discovered that NGF evoked a rapid tyrosine phosphorylation of endogenous TrkA in PC12 cells, and of exogenous TrkA in transfected fibroblast cells (Kaplan et al., 1991a;Kaplan et al., 1991b;Klein et al., 1991). Sequence and mutagenesis analysis (Wiesmann et al., 1999) has indicated that the extracellular region of the receptor can be divided into five domains flanked by two cysteine cluster regions: domains 1 and 3 (C1 and C3 depicted in **Figure 1-1**) a leucine-rich motif (domain 2, LR in **Figure 1-1**) followed by two immunoglobulin superfamily domains (4 and 5, Ig1 and Ig2 in **Figure 1-1**). Additional studies showed that all of the NGF-binding activity resides in the extracellular domain 5 of TrkA (Holden et al., 1997;Robertson et al., 2001;Wiesmann et al., 1999).

Upon binding of NGF, the TrkA receptor is subjected to a series of events including receptor dimerization and transphosphorylation of activation loop tyrosines leading to activation of kinase activity, followed by autophosphorylation of tyrosines outside of the activation loop (Cunningham et al., 1997), and activating signal transduction components including 1) Shc 2) extracellular signal-regulated kinases (ERKs), 3) phosphatidylinositol 3'-kinase (PI3'K) and 4) phospholipase C γ-1 (PLC γ-1) (Kaplan & Stephens, *J Neurobiol*. 1994) (see **Figure 1-1**). In vitro studies indicates that TrkA autophosphorylates at tyrosines 490, 670, 674, 675 and 785 (Loeb et al., 1994;Stephens et al., 1994). These autophosphorylation sites serve as binding sites for specific signaling proteins and adaptors, including the Ras signaling cascade after tyrosines 490 and 785 transphosphorylation (Basu et al., 1994;Loeb et al., 1994) using the Shc adaptor, thereby activating the MAP kinase signaling cascade and transcriptional events leading to the transcription of the *c-fos* gene (Gille et al., 1995;Hill et al., 1993). *c-fos* is an immediate early gene that is rapidly transcribed in response to many extracellular stimuli, including NGF, and is an early component of a series of transcriptional events necessary for initiation and maintenance of differentiation (Ginty et al., 1994;Greenberg et al., 1986). The cAMP regulatory element binding protein (CREB) is a transcription factor that binds the DNA within the *c-fos* promoter at the site called the CRE (cAMP responsive element) (Berkowitz et al., 1989). NGF signaling leads to CREB phosphorylation at serine 133 for several hours via Ras-dependent mechanisms (Ginty et al., 1994). This may permit accumulated immediate early

gene proteins, leading to either proliferation or differentiation (Marshall, 1994). In this regard, the Ras-MAP kinase cascade activation which persists for several hours downstream from TrkA, may be very important for NGF-induced differentiation via regulation of *c-fos* (Xing et al., 1996;York et al., 1998). On the other hand, convergence of control over the MAP kinase pathway may also occur between Ras, protein kinase C (PKC) and Src kinases, which regulate a wide range of events, ranging from cell proliferation, cytoskeletal alterations, and differentiation, to survival, adhesion, and migration (Alema et al., 1985;Erpel and Courtneidge, 1995;Hagag et al., 1986;Kremer et al., 1991;Wooten et al., 2000). On the other hand, upon NGF binding, phosphorylation of Tyrosine 785 near the C terminus of TrkA induces phosphorylation and activation of PLC γ-1, mediating the hydrolysis of inositol 1-4-5-P_3 (IP_3). This originates the release of intracellular calcium from the endoplasmic reticulum and diacylglycerol (DAG), which is a potent activator of PKC. This downstream mechanisms from the TrkA activation leads to phosphorylation of several proteins critical to survival and differentiation (Coleman and Wooten, 1994;Wooten et al., 1997;Wooten et al., 2000).

Internalization of the NGF-TrkA complex plays an important role in intracellular signaling, particularly in neurons, where retrograde transport of the "signal" from distant axon terminals is required to trigger a trophic response in the cell body (Heerssen and Segal, 2002). Considerable evidence suggests that this internalization involves endocytosis and the formation of "signaling endosomes", organelles in which NGF continues to be bound to its activated receptors (Grimes et al., 1996;Grimes et al., 1997;Riccio et al., 1997;Tsui-Pierchala and Ginty, 1999;Ure and Campenot, 1997). These findings are consistent with the concept that NGF signaling initiates the endocytosis of specialized membrane regions that are enriched with TrkA, allowing the downstream signaling second-messenger (Bilderback et al., 1999;Delcroix et al., 2003;Howe et al., 2001;Huang et al., 1999). However, studies in compartmentalized cultures of sympathetic neurons have demonstrated that only a minor fraction of Trk receptors are retrogradely transported (Tsui-Pierchala and Ginty, 1999;Ure and Campenot, 1997), and a recent study identified a specific sequence in the TrkA juxtamembrane region that is both necessary and sufficient for rapid recycling of the internalized receptor, which is predominantly recycled in a signal-dependent manner (Chen et al., 2005)

The $p75^{NTR}$ is a transmembrane glycoprotein that binds all members of the neurotrophin family with equal affinity. It was the first identified NGF receptor, and for many years was believed to be the only such receptor (Johnson et al., 1986). $P75^{NTR}$ is the first identified member of the roughly 25 members of the tumor necrosis factor receptor (TNF-R) superfamily (Bazan, 1990;Cosman et al., 1990;Gruss and Dower, 1995). These receptors share several common signaling features, including the ability to control cell viability via regulation of apoptosis. The extracellular domain of $p75^{NTR}$ consists of four cysteine-repeat domains (CR, depicted in **Figure 1-1**), and experimental and structural modeling studies have suggested that CR2 and CR3 may be primarily responsible for neurotrophin-binding interactions (Baldwin et al., 1992;Shamovsky et al., 1999;Yan and Chao, 1991). One of the $p75^{NTR}$ signal transduction pathways identified is related to apoptotic signaling. It involves generation of a lipid second messenger: ceramide (Dobrowsky et al., 1994). This apoptotic signaling pathaway is related to a death region in the cytoplasmatic domain of $p75^{NTR}$ (Liepinsh et al., 1997). Another death signaling domain present within the $p75^{NTR}$ juxtamembrane region is named chopper. This domain is necessary and sufficient to induce death in neurons (Coulson et al., 2000).

Furthermore, another $p75^{NTR}$ apoptotic mediated pathway is related to the JNK pathway. Once phosphorylated, c-Jun induces apoptosis in neurons subjected to NGF withdrawal by inducing pro-apoptotic genes (Harris and Johnson, Jr., 2001). Moreover, $p75^{NTR}$ JNK dependent activation in cortical neurons also induces pro-apoptotic signaling through caspase activation (Bhakar et al., 2003). Recently, in vivo studies have reported that $p75^{NTR}$/proNGF interactions through JNK activation induced caspase 6 and 3-dependent apoptotic signaling (Volosin et al., 2006).

NGF binding to $p75^{NTR}$ also activates the transcription factor NF-*k*B, which in some systems it exhibits anti-apoptotic signaling (Maggirwar et al., 1998;Mattson et al., 1997), but in others it is associated with pro-apoptotic signaling (Schneider et al., 1999;Schwaninger et al., 1999). The balance of these two pathways, as modulated by TrkA in some cells (Yoon et al., 1998), may control the ultimate fate of the cell. Binding NGF to either $p75^{NTR}$ or TrkA receptors can also lead to NF-*k*B activation (Carter et al., 1996;Mattson and Meffert, 2006), representing a possible crosstalk between the $p75^{NTR}$ and TrkA pathways (see **Figure 1-1**). In this regard, NF-*k*B has long been known to function as a critical regulator of apoptosis and

often induces genes favoring cell survival, including the enzyme superoxide dismutase, an antioxidant enzyme which protects neurons from oxidative stress damage (Mattson et al., 1997).

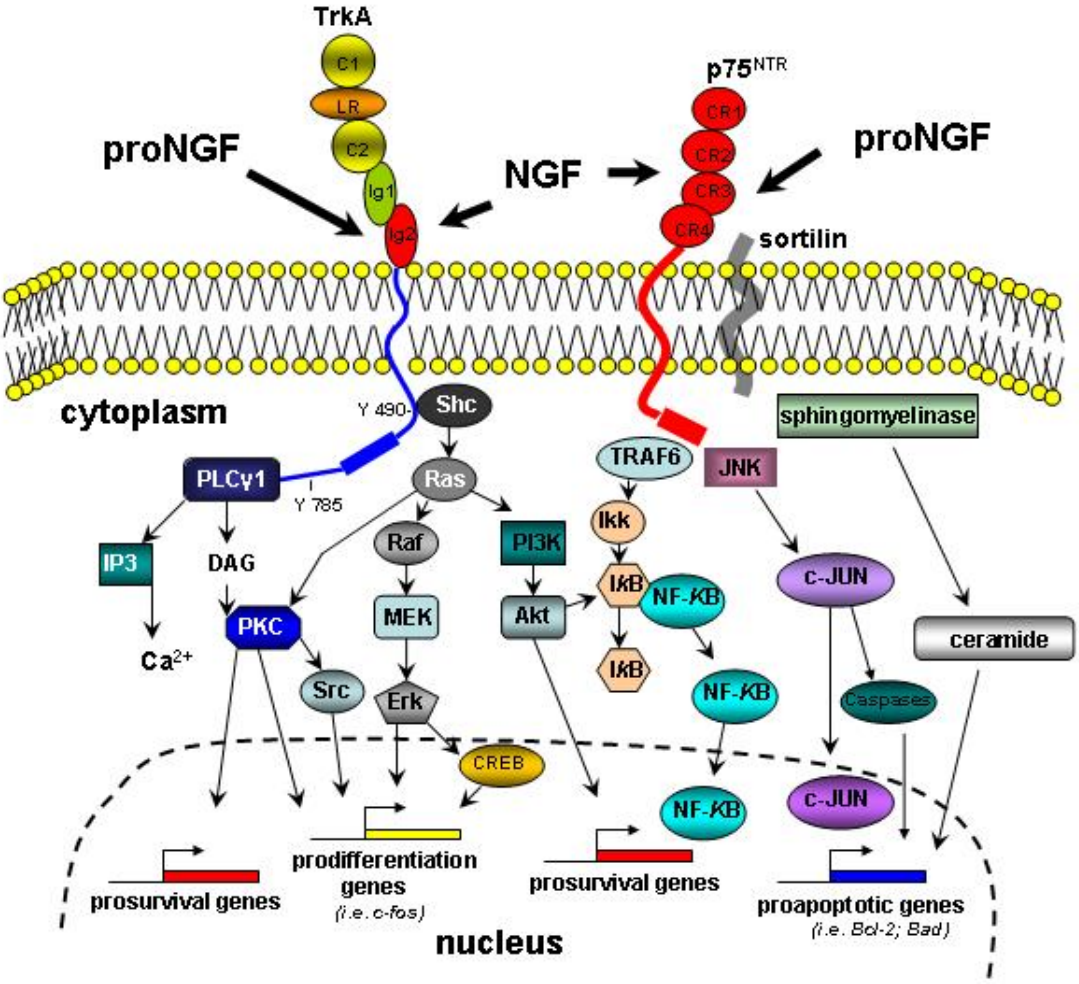

Figure 1-1 NGF-mediated TrkA and p75^{NTR} intracellular signaling cascades

1B4 Controversial proNGF activity: pro-apoptotic or pro-neurotrophic molecule?

Despite the fact that NGF is found as a precursor in different tissues with almost no detectable mature NGF [including the CNS (Fahnestock et al., 2001); dorsal root ganglia (Reinshagen et al., 2000); prostate (Delsite and Djakiew, 1999); retina (Chakrabarti et al., 1990); thyroid gland (Dicou et al., 1986); skin (Yiangou et al., 2002) and hair follicle (Yardley et al., 2000)] it has been widely assumed that proNGF has little or no biological activity. However, in a recent study in 2001, Lee and coworkers suggested that the cleavage-resistant precursor of NGF is a high-affinity, functional ligand for $p75^{NTR}$, binding the receptor with five times greater affinity than mature NGF to mediate apoptosis in treated smooth muscle cells. Moreover, proNGF displayed reduced binding affinity to TrkA, did not induce TrkA phosphorylation, and proNGF-treated neurons and PC12 cells displayed reduced neurite outgrowth, as compared with mature NGF. On the other hand, they showed that the proteolytically cleaved mature NGF is the preferred ligand for TrkA, for the survival and differentiation of cultured cells (Lee et al., 2001).

Other studies have demonstrated that proNGF creates a signaling complex by simultaneously binding to $p75^{NTR}$ and the sortilin receptor, suggesting that sortilin may act as a co-receptor and molecular switch governing the $p75^{NTR}$-mediated pro-apoptotic signal induced by proNGF (Nykjaer et al., 2004). On the other hand, Fahnestock and colleagues reported that proNGF exhibits neurotrophic activity in survival and neurite outgrowth assays in cultured neurons and PC12 cells similar to mature NGF, but is approximately fivefold less active. Moreover, they demonstrated that proNGF binds TrkA and it is slightly less active than mature NGF in promoting phosphorylation of TrkA and its downstream signaling effectors, suggesting that proNGF could be responsible for much of the biological activity normally attributed to mature NGF in vivo (Fahnestock et al., 2004b). In sum, the biological significance of proNGF is not fully resolved and remains a fascinating and very controversial issue in the field.

1C Basal Forebrain Cholinergic Neurons and Nerve Growth Factor Interactions

Nerve Growth Factor is considered to be the most prominent regulator of the basal forebrain cholinergic neurons (BFCN) phenotype thus far reported. This view is largely supported by the following experimental findings:

1) the NGF expression mirrors the location of BFCN and its innervated target sites;
2) the NGF receptors expression on BFCN
3) the capacity of NGF to enhance phenotype-specific markers of BFCN
4) BFCN are highly sensitive to NGF signaling
5) atrophy in BFCN can be reversed by the application of exogenous NGF

Thus, this section will review the literature regarding the anatomical and functional relationships between NGF and BFCN, in normal and injured CNS.

1C1 Anatomy of Basal Forebrain Cholinergic Neurons

Cortical cholinergic mechanisms have been extensively investigated because of the postulated role of this transmitter system in arousal, learning, and memory (Bartus et al., 1982;Hasselmo, 1999). Although acetylcholine was the first neurochemical to be proposed as a central nervous system transmitter (Feldberg, 1945;Feldberg, 1976;Vogt, 1969), it was only in the early 1980's that it was possible to microscopically identify CNS cholinergic neurons and fiber pathways. The earliest attempts to define the localization of CNS cholinergic neurons were made by the application of histochemical method to reveal the presence of acetyl cholinesterase (AChE) activity by Shute and Lewis (Shute and Lewis, 1975).

During the early 1980's when antibodies against choline acetyltransferase (ChAT) were developed (Eckenstein and Thoenen, 1982;Levey et al., 1983;Rossier et al., 1973) a number of studies arose applying immunohistochemistry enabling for the first time a depiction of the localization of CNS ChAT-immunoreactive (-IR) cell bodies and fiber tracts (Mesulam, 1990;Mesulam et al., 1983b;Mesulam et al., 1983a;Mesulam et al., 1984;Wainer et al., 1985) describing the organization of cholinergic neurons in the brain. Some of the most comprehensive studies about CNS cholinergic neurons and major cholinergic pathways were made by Mesulam and collaborators, introducing the Ch classification in which cholinergic forebrain projections are classified into six main central pathways (Ch1-Ch6) and linked to origin of the nuclei where the cholinergic fibers arise (Mesulam et al., 1983b).

The basal forebrain cholinergic neurons are comprised of the nucleus basalis, also called the nucleus basalis of Meynert in human beings and subhuman primates, the horizontal and vertical diagonal bands of Broca (HDBB and VDBB respectively) and the medial septal nucleus

(MS). Neurons located at HDVV and VDBB predominantly innervate the anterior cingulated cortex and the olfactory bulb respectively. On the other hand, neurons situated at the MS project largely to the hippocampus and are referred to as the septohippocampal cholinergic pathway, and the NBM provides cholinergic innervations to the amygdala and innervates all areas of the cortical mantle in a topographic-specific manner, constituting the basalocortical cholinergic pathway (Mesulam, 1996;Page and Sofroniew, 1996). These pathways are represented, in a simplified fashion (without the Ch classification), in **Figure 1-2**, where the basalocortical and the septohippocampal pathways are highlighted in gray.

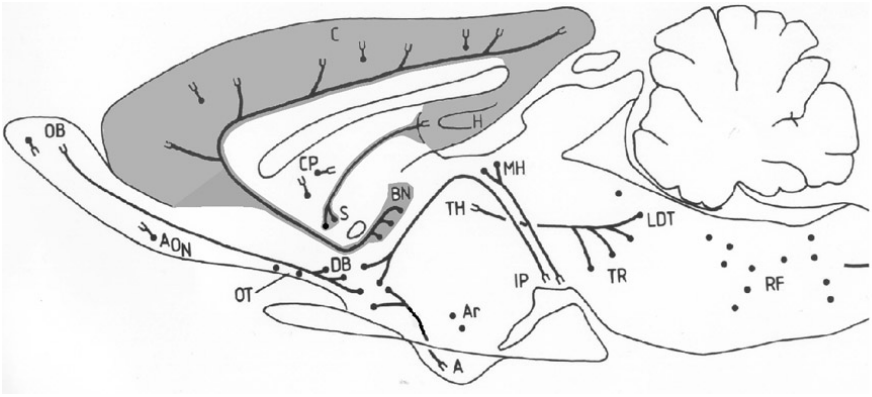

Figure 1-2 Schematic representation of the cholinergic pathways in the CNS. Highlighted in grey, the basalocortical and septohippocampal cholinergic pathways

Fisher and coworkers have shown that among the population of neurons in the nucleus basalis projecting to the cortex, 30% to 35% are GABAergic neurons (Fisher et al., 1988), whose axons preferentially connect cortical GABAergic interneurons (Freund and Meskenaite, 1992) to desinhibit them (Jimenez-Capdeville et al., 1997). Thus, it is now being increasingly realized that the physiology of the basal forebrain cholinergic neurons may also be tightly linked to the basal forebrain GABAergic population, which intermingle with the cholinergic neurons (Fisher et al., 1988;Kohler et al., 1984;Toth et al., 1993;Walker et al., 1989).

Several studies have confirmed that TrkA and $p75^{NTR}$ are expressed in basal forebrain cholinergic neurons, in both basal forebrain nuclei (e.g., medial septum, diagonal band of Broca and nucleus basalis magnocellularis) and also at their target regions (e.g., hippocampus, amygdala, cerebral cortex and olfactory bulb) (Altar et al., 1991;Buck et al., 1987;Kawaja and Gage, 1991a;Pioro et al., 1990;Pioro and Cuello, 1990;Richardson et al., 1986;Riopelle et al.,

1987;Steininger et al., 1993) and co-localize in close to all basal forebrain cholinergic neurons (Gibbs and Pfaff, 1994;Lee et al., 1998;Merlio et al., 1992;Sobreviela et al., 1994). Similarly, NGF mRNA and protein levels are selectively expressed in basal forebrain cholinergic targets in the adult CNS, with the highest expression being found in areas of intense basal forebrain cholinergic innervation, including hippocampus, amygdala, cerebral cortex and olfactory bulb (Ayer-LeLievre et al., 1988;Goedert et al., 1986;Korsching et al., 1985;Large et al., 1986;Maisonpierre et al., 1990;Shelton and Reicherdt, 1986;Whittemore et al., 1986;Whittemore et al., 1988). Despite the fact that the expression of NGF mRNA is at least a five-fold lower than in target regions, the basal forebrain contains high levels of NGF protein (Conner et al., 1992;Korsching et al., 1985;Large et al., 1986). This could be explained by the active retrograde transport of NGF from the cerebral cortex and hippocampus to the nucleus basalis and medial septum, respectively (Ferguson and Johnson, Jr., 1991;Hefti et al., 1984;Schweitzer, 1987;Seiler and Schwab, 1984).

1C2 Role of Basal Forebrain Cholinergic Neurons in Cognitive Functions

The involvement of central cholinergic function in learning and memory was first postulated by Deutsch in 1971 (Deutsch, 1971), corroborated later in 1974 by pharmacological studies in young healthy patients, demonstrating that anticholinergic drugs impaired cognitive performance to levels detectable in dementing disorders (Drachman and Leavitt, 1974b), while enhancement of central cholinergic function improved the performance of aged patients (Drachman, 1977). Further to this, a vast number of experimental studies administering anticholinergic drugs in rodents and humans have provided unequivocal evidence regarding the important role of the basal forebrain cholinergic neurons in cognitive processes (Christensen et al., 1992;Fibiger, 1991;Molchan et al., 1992).

Activated BFCN release acetylcholine from their nerve terminals in the cerebral cortex and hippocampus, and it is thought that this transmitter modulates synaptic efficacy in cortical tissues (Conner et al., 2003;Kilgard and Merzenich, 1998;Mesulam and Geula, 1988). Cholinergic neurotransmission is mediated by CNS nicotinic and muscarinic receptors. Acetylcholine exerts its effects on the CNS by interacting with G-protein-coupled muscarinic and ligand-gated ion channel nicotinic receptors. Most studies have focused on muscarinic receptors because this family has more established roles in central cholinergic transmission and they are known to play central roles in facilitating cognitive functions, such as learning and memory (Bartus et al., 1982;Coyle et al.,

1983;Iversen, 1997). Five metabotrophic muscarinic acetylcholine receptors have been cloned and identified by differential affinities for antagonist, m_1-m_5 muscarinic receptor subtypes (Bonner et al., 1987;Hulme et al., 1990). All of the subtypes appear to be present in the brain, albeit in different distributions and relative abundance. In the forebrain region, m_1, m_2 and m_4 are the most abundant subtypes. For example, in the hippocampus and several regions of the neocortex in the human brain, m_1 ranges from 35-60% of all muscarinic receptor binding sites, whereas m_2 and m_4 account for about 15-25% of receptors in the same areas (Flynn et al., 1995). The m_3 and m_5 subtype receptors are expressed only at very low levels in brain. In neocortical and hippocampal regions, the m_1 receptor has a postsynaptic distribution, suggesting that this subtype may play a general role in cholinergic modulation of glutamatergic transmission (Markram and Segal, 1992;Mrzljak et al., 1993). In this regard it is of interest that cholinergic synapses terminate preferentially on glutamatergic pyramidal neurons (Casu et al., 1999). The m_2 subtype is a presynaptic autoreceptor expressed at high levels in the cholinergic neurons, as well as at non-cholinergic neurons that project to the cortex and hippocampus (Levey et al., 1995a), and it is generally assumed that it presynaptically inhibits acetylcholine release (Mash et al., 1985). The m_4 subtype is fairly abundant in cortex and hippocampus, and it may be presynaptically located in the cerebral cortex and noncholinergic axons that comprise the septohippocampal pathway (Levey et al., 1995b;Rouse and Levey, 1996;Volpicelli and Levey, 2004).

For the nicotinic receptor family, 11 genes that encode 8 alpha (α_2-α_9) and 3 beta ($ß_2$- $ß_4$) receptors subunits have been identified (Colquhoun and Patrick, 1997;McGehee and Role, 1995). A wide variety of subtypes of these receptors arise from combinations of subunits that comprise the channel-receptor complex, and their dendritic, somal, axonal, presynaptic, and postsynaptic locations contribute to the varied roles these receptors play in the CNS. Presynaptic nicotinic receptors enhance neurotransmitter release, while postsynaptic receptors contribute for small minority of fast excitatory transmission. By modulating activity-dependent events, nicotinic receptors participate in fundamental aspects of synaptic plasticity that are involved in attention, learning and memory (Aramakis et al., 2000;Dani and Bertrand, 2007;Ge and Dani, 2005;Ji et al., 2001).

1C3 Endogenous Nerve Growth Factor Trophic Support to Basal Forebrain Cholinergic Neurons

During development, acting in a target-derived manner, NGF promotes the survival and maturation of several populations of neurons expressing TrkA and $p75^{NTR}$, including basal forebrain cholinergic neurons (Kew et al., 1996;Lee et al., 1998;Li et al., 1995;Svendsen et al., 1994). Chen and colleagues have shown that NGF (+/-) mice displayed a marked atrophy and reduction in the number of basal forebrain cholinergic neurons, as well as a reduction in the cholinergic innervation of the hippocampus (Chen et al., 1997). Moreover, disruption of the TrkA gene reduced the number and size of basal forebrain cholinergic neurons (Fagan et al., 1997). On the other hand, $p75^{NTR}$ gene disruption appears to increase the number and size of basal forebrain cholinergic neurons (Greferath et al., 2000;Peterson et al., 1997;Yeo et al., 1997). The role of TrkA signaling during development is well established as the main signaling mechanism for survival and differentiation of BFCN. Thus, the embryonic development of the forebrain cholinergic system is highly dependent on the expression of NGF and TrkA receptors and, to lesser extent, on the expression of $p75^{NTR}$ (Fagan et al., 1997;Klein, 1994;Tucker et al., 2001).

In early postnatal stages, relatively high levels of NGF are expressed, which decrease substantially shortly after birth (Lu et al., 1989). Forebrain cholinergic neurons of early postnatal naïve rats remain highly sensitive to exogenously administered NGF. When NGF is applied exogenously in neonatal stages, it provokes a pronounced up-regulation of cholinergic markers in the hippocampus (Fusco et al., 1989). Conversely, a down-regulation of cholinergic markers in naïve animals occurs when FAB fragments of anti-NGF antibodies are administered (Vantini et al., 1989). In adults, both NGF receptors and NGF expression continue to be dynamically regulated to very low basal levels by many cells types throughout adult life and aging. Once mature, most neurons lose absolute dependence on target-derived growth factor for survival. In adults, the focus of NGF signaling shifts away from the regulation of neuronal survival to the regulation of neuronal phenotype and function (Cuello et al., 1992;Cuello, 1996;Sofroniew et al., 2001;Svendsen et al., 1991). Evidence of this is the observation that cholinergic neurons of the nucleus basalis shrink but do not die after extensive cortical infarct types of lesions in the cerebral cortex, which provide 'target-derived' NGF to these neurons (Sofroniew et al., 1983). Furthermore, more recently it has been shown that endogenous NGF is responsible for the "steady-state" number of cholinergic synapses in the adult cerebral cortex (Debeir et al., 1999).

Taken together, it is clear that neurotrophins appear to be necessary for the maintenance of CNS neuronal phenotypes and their continuous cell remodeling during adulthood (Cuello et al.,

1990;Thoenen, 1995). In contrast to neonatal animals, forebrain cholinergic neurons of mature animals appear to be less sensitive to exogenously applied NGF. Thus, exogenously applied NGF in adult naïve rats does not significantly affect the morphologic or biochemical phenotype of CNS cholinergic neurons (Garofalo and Cuello, 1994). However, the presence of CNS insults makes the forebrain cholinergic neurons more responsive to trophic factor stimulation. Neuronal NGF expression in vivo is markedly upregulated by seizures, forebrain ischemia and tissue injury (Gall and Isackson, 1989;Herrera et al., 1993;Lindvall et al., 1994;Zafra et al., 1991). Moreover, it has been shown that NGF produced by astrocytes and microglia is markedly upregulated following local tissue injury and inflammation (Arendt et al., 1995;Yoshida and Gage, 1992), and also in response to reactive oxygen species, ischemia and traumatic brain injury (Friedman et al., 1990;Gottlieb and Matute, 1999;Yoshida and Gage, 1991).

1C4 Exogenous Nerve Growth Factor protective effects on Basal Forebrain Cholinergic Neurons

In addition to the mentioned function of endogenous NGF on BFCN that express TrkA and $p75^{NTR}$, several studies demonstrated that these neurons exhibit neuritic outgrowth in response to exogenous NGF administration in adults, particularly after injury (Hefti, 1986;Kawaja and Gage, 1991b;Kordower et al., 1994;Kromer, 1987;Williams et al., 1986). Thus, this observation suggests that NGF may stimulate regrowth and reorganization of connectivity of receptor-bearing neurons after CNS insults. In this regard, several works reported that exogenous application of NGF prevented the down-regulation of cholinergic markers in septal cholinergic neurons after injury (Hefti, 1986;Kromer, 1987). Interestingly, in the adult septum, axotomy-induced cell death is not due to loss of NGF signaling, but rather from a drastic injury too close to the cell somata (Kordower et al., 1993;Sofroniew et al., 1990;Sofroniew et al., 1993). However, in this model, cell death can be largely prevented in both rodent and primates by exogenous NGF infusions at the time of the axotomy (Hefti, 1986;Tuszynski and Gage, 1995). Furthermore, it was shown that NGF is also able to recover atrophic BFCN as consequence of a stroke-type lesion in the cerebral cortex (Cuello et al., 1994). Likewise, Rossner and colleagues reported that immunolesion-induced basal forebrain cholinergic neuron degeneration was ameliorated after NGF treatment (Rossner et al., 1996).

In 1993, Rylett and colleagues have shown that the continuous intracerebral administration of exogenous NGF to adults rats increases choline acetyl transferase (ChAT) and choline uptake activities and enhances acetylcholine synthesis and release (Rylett et al., 1993). In addition, Dekker and coworkers reported that exogenous NGF ameliorated cholinergic and behavioral deficits after nucleus basalis lesions (Dekker et al., 1991;Dekker and Thal, 1992). Previous studies in our lab, using the stroke-type lesions of the neocortex, have reported a complete reversal of the nucleus basalis cholinergic cell shrinkage along with a recovery of the lost ChAT activity in the microdissected basalis area after exogenous NGF delivered into the CNS either incorporated in biodegradable microspheres (Maysinger et al., 1992) or grafting NGF secretor fibroblasts (Piccardo et al., 1992). Furthermore, NGF mRNA expression has been reported within basal forebrain cholinergic neurons and can also be produced by GABAergic neurons located at the nucleus basalis, suggesting an autocrine/paracrine role of NGF in the CNS (Lauterborn et al., 1991). In this regard, our lab produced direct evidence for such an autocrine/paracrine role of NGF, by the local application of exogenous NGF at the somatodendritic region of the nucleus basalis, which modulates the cholinergic phenotype in rats (Hu et al., 1997). Similar approaches have been applied to primates (Tuszynski et al., 1996), and more recently applied to humans AD patients in clinical trials (Tuszynski et al., 2005).

1D Nerve Growth Factor and Basal Forebrain Cholinergic Neurons in Aging

1D1 Age-Related Atrophy of Basal Forebrain Cholinergic Neurons

Age-dependent cognitive impairments in mammalians, including primates, are accompanied by substantial structural changes in BFCN at the cell soma and its ascending nerve terminals. Neurochemical studies indicate a significant involvement of cholinergic mechanisms in the aging process (Armstrong et al., 1988;Meyer et al., 1984;Michalek et al., 1989;Rylett and Williams, 1994;Williams et al., 1993). Furthermore, there is considerable evidence showing that age-related cognitive impairments correlate with cellular atrophy of TrkA and $p75^{NTR}$ expressing BFCN (Gage et al., 1988b;Koh et al., 1989). In fact, similar atrophic changes can also be induced in young animals by disrupting the connection of these neurons with their targets cells, which produce NGF (Kordower et al., 1993;Sofroniew et al., 1990).

BFCN are apparently highly sensitive to interruptions in the NGF supply, such that an initially small failure in NGF signaling may be rapidly exacerbated by the down-regulation of NGF receptors (Sofroniew and Mobley, 1993). As discussed above, developmentally NGF-dependent neurons become independent of a supply of target-derived NGF for acute survival once they have established their connections. As mentioned in **Section 1C2,** *in vivo* studies have shown that in mature rats, provoking either the immunoneutralization of endogenous NGF or the blockade of their TrkA receptors results in the loss of pre-existing cortical cholinergic pre-synaptic sites. This is a strong argument favoring a role of endogenous NGF in the maintenance of the steady-state number of cholinergic connections in mature animals (Debeir et al., 1999).

Despite the overwhelming evidence for atrophy of the NGF-dependent BFCN in aging, there is no persuasive data for a decline in NGF content or NGF mRNA in the cerebral cortex of aged animals (Alberch et al., 1991;Crutcher and Weingartner, 1991). These observations suggest that a failure in endogenous NGF trophic support may contribute to the atrophy of BFCN in aging. Available evidence suggests that intrinsic neuronal changes might compromise the ability of aged BFCN to derive neurotrophic support. Specifically, it has been demonstrated in aged rats that BFCN exhibit a reduced capacity for generalized retrograde transport of NGF, thus cholinergic neurons that do not transport NGF are severally shrunken and downregulate TrkA expression (Cooper et al., 1994;De Lacalle et al., 1996). Other possibilities might include a compromise in the TrkA signaling system or diminished NGF release.

Interestingly, Williams and colleagues recently reported altered NGF response but not release in the aged septo-hippocampal cholinergic pathway, suggesting that besides the known diminished retrograde transport, the acute loss of response to NGF in the basal forebrain cholinergic neurons during aging may also play a role in the degenerative process (Williams et al., 2005). Nevertheless, the progressive decline in spatial memory and integrity of BFCN in rats during aging (Armstrong et al., 1993) could be reversed by chronic administration of exogenous NGF (Fisher et al., 1987), by NGF-secreting neural progenitors grafted to the basal forebrain (Martinez-Serrano et al., 1995), or through *ex vivo* nerve growth factor gene transfer to the basal forebrain (Martinez-Serrano and Bjorklund, 1998).

Based on the lessons gathered so far on the responses of the cholinergic neurons in mature and aged animals to *endogenous* and *exogenous* NGF, as well as on the status of this transmitter system in aged animals and humans, I speculated that a link might exist between the availability of

biologically active NGF and the phenotypic characteristics of BFCN in aging, ultimately leading to a progressive cognitive deterioration. This is one of the main issues covered in this thesis.

1D2 Basal Forebrain Cholinergic Dysfunction in Alzheimer's disease

Age is the greatest risk factor for Alzheimer's disease (AD) and is characterized by a progressive loss of memory and deterioration of higher cognitive functions. The cholinergic hypothesis of geriatric memory dysfunction (Bartus et al., 1982) posits the degeneration of the cholinergic neurons in the basal forebrain and the loss of cholinergic transmission in the cerebral cortex and other areas as the principal cause of cognitive dysfunction in patient with AD. This hypothesis was reinforced by several further studies reporting a profound reduction of cholinergic markers in the hippocampus and cerebral cortex of patients with Alzheimer's disease. The depletion of CNS cholinergic markers was originally reported by Bowen (Bowen et al., 1976) and Davies and Maloney ((Davies and Maloney, 1976) and consequently confirmed and extended in several studies (Etienne et al., 1986;Francis et al., 1999;Perry et al., 1977;Quirion et al., 1986).

The brain of an individual with Alzheimer's disease exhibits extracellular plaques of aggregated ß-amyloid proteins (Aß) surrounded by dystrophic neurites, activated microglia and reactive astrocytes, intracellular neurofibrilary tangles that contain hyperphosphorylated tau protein and a profound loss of basal forebrain cholinergic neurons (for reviews see (Hardy and Selkoe, 2002;Schliebs and Arendt, 2006;Walsh and Selkoe, 2004). A growing body of evidence suggests that altered processing of amyloid precursor protein (APP) is one of the early events in the pathogenesis of AD. The APP gene maps to chromosome 21 in humans (George-Hyslop et al., 1987;Kang et al., 1987;Tanzi et al., 1987) and is ubiquitously expressed, but most abundant, in the brain (Selkoe, 1994). Although Aß accumulation may trigger or contribute to the process of neurodegeneration, the mechanism whereby Aß induces basal forebrain cholinergic cell loss and cognitive impairment remains to be elucidated. Aß peptides are a group of hydrophobic peptides of 39-43 amino acids residues, derived by proteolytic cleavage of amyloid precursor protein (APP), a single transmembrane glycoprotein with a long N-terminal extracellular region and a short C-terminal cytoplasmatic tail (Kang et al., 1987;Selkoe, 2001;Wisniewski et al., 1997) (**see Figure 1-3**). Mature APP is processed by distinct α-secretase or ß-secretase pathways. The α-secretase activity cleaves the Aß domain within Lys^{16} and Leu^{17} yielding a soluble N-terminal APPα and a 10-KDa C-terminal APP fragment that can be further processed by γ-secretase to generate $Aß_{17-40}$

or $A\beta_{17-42}$, also known as the P3 peptides. The ß-secretase pathway is mediated by the sequential actions of ß-secretase (ß-APP cleaving enzyme [BACE]) (Cai et al., 2001) and γ-secretase enzymes, resulting in the formation of the full length $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides, depending on the exact point of cleavage (Esch et al., 1990;Vassar et al., 1999). The γ-secretase is a large molecular complex, within which the presenilins contain the catalytic activity, in cooperation with other protein such as Nicastrin, Aph-1, and Pen-2 (De Strooper, 2003;Wolfe, 2002)

The Aß peptide had been early identified from CNS amyloid plaques by Glenner and Wong (Glenner and Wong, 1984) and its sequence was confirmed by Masters and coworkers (Masters et al., 1985). The Aß is present in the brain and cerebrospinal fluids (CSF) of normal humans throughout life (Haass et al., 1992;Ida et al., 1996;Seubert et al., 1992;Vigo-Pelfrey et al., 1993;Walsh et al., 2000). The physiological role of Aß, if any, are not fully defined but it has been recently proposed to be a modulator of the CRE regulated gene expression (Echeverria et al., 2005). The presence of Aß *per se* does not cause neurodegeneration. However, increased Aß production/accumulation appears sufficient to cause early onset AD (Cai et al., 1993;Citron et al., 1992;Kumar-Singh et al., 2006;Rovelet-Lecrux et al., 2006). Under normal conditions, about 90% of secreted Aß peptides are $A\beta_{1-40}$, which is a soluble form of the peptide and is eliminated from the brain. In contrast, about 10% of secreted Aß peptides are $A\beta_{1-42}$ species that are highly fibrilogenic and deposited early in individuals with AD (Price and Sisodia, 1998;Selkoe, 2001;Seubert et al., 1992;Suzuki et al., 1994). Thus, the major amyloid peptides found in plaques are $A\beta_{1-42}$ and $A\beta_{1-40}$ peptides. $A\beta_{1-42}$ is deposited first in the brain with AD and is the predominant form in the senile plaque, whereas $A\beta_{1-40}$ is deposited later in the diseases process (Jarrett et al., 1993;Jarrett and Lansbury, Jr., 1993).

The apparent lack of correlation between the amount of fibrilar Aß deposition and the severity of dementia (Dickson et al., 1995;Katzman, 1986;Terry et al., 1991) strengthened the proposition that soluble, and not fibrillar, forms of Aß may be responsible for the alteration of neuronal function and viability in AD (Hardy and Selkoe, 2002;Klein, 2002;Tanzi, 2005). Thus, emerged data over the past decade demonstrated that diffusible soluble oligomers of Aß are neurotoxic at low concentrations, can induce inhibition of long-term potentiation (Walsh et al., 2002), disrupt synaptic plasticity (Klyubin et al., 2005;Rowan et al., 2004) and provoke cognitive dysfunction in rodents (Cleary et al., 2005b). The Aß amount in the brain correlate with the

severity of AD and, most relevant, brain's levels of soluble Aß species appear to better correlate with the extent of synaptic loss and the severity of cognitive impairment than density of plaque deposition (Lue et al., 1999;McLean et al., 1999;Naslund et al., 2000;Wang et al., 1999).

Figure

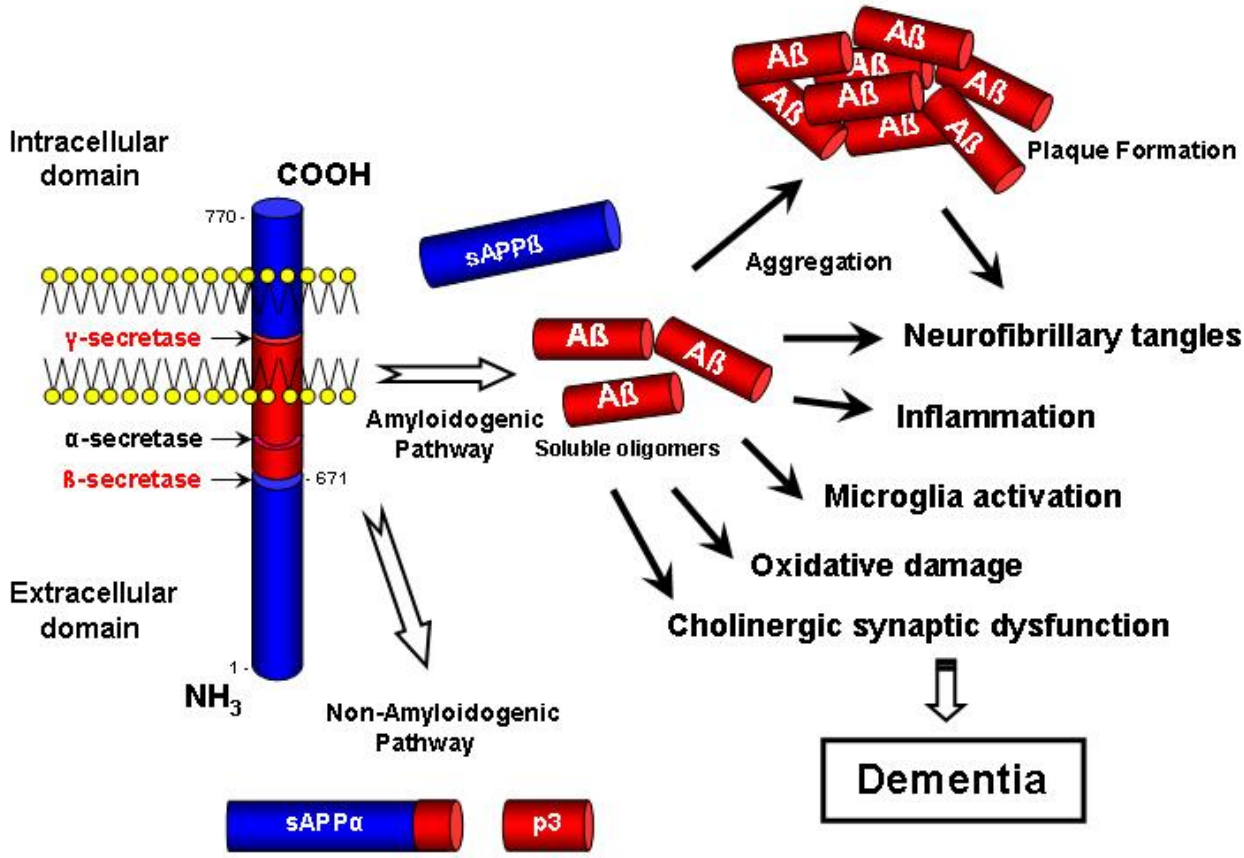

Figure 1-3 Schematic representation of amyloidogenic *vs.* non-amyloidogenic APP pathways

A clear connection has been established between the cholinergic system and APP metabolism. APP processing pathways can be influenced by a variety of factors, including the stimulation of acetylcholine receptors and NGF (Mills and Reiner, 1999;Roberson and Harrell, 1997). Nitsch and colleagues were the first to demonstrate the cholinergic regulation of APP processing (Nitsch et al., 1992). They reported that a nonselective muscarinic receptor agonist, carbachol, significantly increased the release of soluble APPα in cells expressing m_1 and m_3, but not in cells expressing m_2 or m_4 receptor subtypes. Activation of muscarinic m_1-receptor-transfected cells not only enhanced soluble APPα secretion but also reduced the secretion of Aß.

Conversely, Quirion and coworkers have shown that Aß inhibits the evoked acetylcholine release from cortical and hippocampal slices (Kar et al., 1996).

Similarly, muscarinic m_1 and m_3 receptor agonists have shown to stimulate the release of soluble APPα from rat cortical slices (Pittel et al., 1996). Aß-related peptides are produced constitutively by brain cells and picomolar-nanomolar concentrations can have a neuromodulatory role in the regulation of normal cholinergic functions, through their negative effects on acetylcholine biosynthesis and release. Acetylcholine may in turn reciprocally regulate APP synthesis and processing. Lesion of the basal forebrain cholinergic neurons, or transient inhibition of cortical acetylcholine release, can elevate local APP synthesis (Lin et al., 1999;Roberson and Harrell, 1997;Wallace et al., 1993) whereas agonist-induced activation of muscarinic m_1 and m_3 receptor subtypes increases the secretion of soluble APP derivatives and reduces the production of amyloidogenic Aß peptides (Buxbaum et al., 1992;Hellstrom-Lindahl, 2000;Nitsch et al., 1992). Similar effects have been found in the human species with the boosting of the cholinergic system, by the application of anti-cholinesterases (Giacobini, 1987) or muscarinic-receptor agonists in AD (Hock et al., 2000b;Nitsch et al., 2000). These results suggest a mechanism whereby normal cholinergic innervation facilitates the non-amyloidogenic maturation of APP via the α-secretase pathway, whereas the amyloidogenic-related Aß peptides depress the activity of cholinergic neurons.

Nicotinic receptors have also been described as modulators of APP processing, both in cell culture (Efthimiopoulos et al., 1996;Seo et al., 2001) and *in vivo* (Utsuki et al., 2002), favoring the non-amyloidogenic pathway when treated with low doses of nicotine (Lahiri et al., 2002). A recent study in tobacco smoking elderly people further supported the idea of a beneficial effect of nicotine for Alzheimer's disease patients, who demonstrated less ß-amyloid deposition in the enthorinal cortex as compared to non-smokers (Court JA et al., 2005).

Furthermore, NGF has also been observed, in cells and *in vivo*, to modulate the cholinergic control of APP processing. In PC12 cells transfected with the m_1 acetylcholine receptor subtype, NGF promoted soluble APPα secretion following m_1 agonist stimulation (Haring et al., 1995). Chronic treatment of transgenic Tg2576 Alzheimer mice with the synthetic xanthine propentofylline, known to stimulate NGF synthesis and release, resulted in increased NGF mRNA levels and a major shift in the balance of APP processing from the amyloidogenic to the non-

amyloidogenic pathway, compared to untreated controls (Chauhan et al., 2005;Chauhan and Siegel, 2003).

Although the beneficial effects of NGF on neuronal maintenance and rescue from damage appeared promising for the therapeutic potential of NGF (Martínez-Serrano and Björklund, 1998;Rahimi and Juliano, 2001;Rossner et al., 1996), complications of NGF delivery in the CNS and its side effects have been shown to limit its clinical efficacy (Jonhagen, 2000;Williams et al., 2006b). The intraventricular administration of NGF factor to Alzheimer patients (Eriksdotter et al., 1998) and rodents (Winkler et al., 2000), besides its modest beneficial effects on cognitive performance, produced a number of unwanted side-effects, including severe back pain, risk of infection and weight loss (Jonhagen, 2000;Nabeshima and Yamada, 2000;Rattray, 2001). This may partly be due to the diversity of cells responding to NGF signaling (Sofroniew et al., 2001), thus minimizing the exposure of NGF to non-targeted brain areas, though intraparenchimal administrations have been proven to be more efficacious in reducing undesirable side-effects (Mahoney and Saltzman, 1999;Tuszynski, 2000). However, this approach has inherent difficulties and risks for a prolonged CNS administration (Tuszynski et al., 2005).

The most widely applied strategy for boosting CNS cholinergic neurotransmission has been the use of inhibitors of acetylcholinesterase (AChE); blocking the major enzyme involved in the degradation of acetylcholine and thus elevating and/or maintaining its level in the synaptic cleft, which prolongs its action on postsynaptic muscarinic and nicotinic receptors (Giacobini, 2004;Nordberg, 2006). A second cholinergic approach has been aimed to develop specific M_1 postsynaptic agonists, which have been shown to have cognition-enhancing effects in animals (Caccamo et al., 2006). Moreover, another cholinergic approach to Alzheimer's disease has been the development of antagonists of the M_2 autoreceptors, thus increasing the levels of acetylcholine (Lachowicz et al., 2001;Quirion et al., 1995). Furthermore, an alternative approach to enhance cholinergic neurotransmission includes the increase of acetylcholine release, by activation of presynaptic nicotinic cholinergic receptors through appropriate nicotinic agonists (Oddo and LaFerla, 2006), an approach recently shown to improve cognitive deficits in Alzheimer patients (Levin and Rezvani, 2002).

1D3 Oxidative Stress and microglial activation in Aging and Alzheimer's disease

In 1956, Harman predicted that the lifespan of an organism could be increased by augmenting antioxidant defenses, the "free-radical theory of aging" (HARMAN, 1956). An inescapable side-product of oxidative metabolism is the production of reactive oxygen species (ROS), which induces lipid peroxidation, protein oxidation and oxidative modifications in nuclear and mitochondrial DNA (Hamilton et al., 2001;Lin and Beal, 2006;O'Donnell and Lynch, 1998). The continued exposure to ROS generated by oxidative metabolism damages critical cellular structures and may be responsible for some age-related pathologies, including Alzheimer's disease. Neurons have an age-related decrease in the capacity to compensate for a progressive imbalance between antioxidant defenses and intracellular concentrations of reactive oxygen leading to irreversible injury within cells, thus contributing to the pathogenesis of neurodegenerative diseases (Perry et al., 2002;Perry and Smith, 1998;Smith et al., 1995). Chronic oxidative stress has been hypothesized to be a major contributor to the aging process (Meister, 1984), resulting from the cytotoxic consequences of reactive oxygen species on imbalances in oxidative homeostasis in the brain, whose end point is neuronal death (Basaga, 1990).

The increasingly recognized principal reactive oxygen species generated in neurons, as a mayor contributor to neurodegenerative processes, involves the interaction of the superoxide anion radical (O_2-$^{\bullet}$). This arises from the electron transport process in mitochondria, with nitric oxide (NO) resulting in the formation of peroxynitrite. In turn, peroxynitrite promotes membrane lipid peroxidation at the plasma membrane, and at the mitochondrial and endoplasmic reticulum (ER) membranes, as well as nitration of proteins on tyrosine residues (Beckman and Crow, 1993;Keller et al., 1998). A prominent mechanism of generating superoxide in neurons is the elevation of cytoplasmatic calcium levels which results in increased mitochondrial superoxide production as a consequence of mitochondrial calcium uptake. Since calcium also stimulates nitric oxide sinthase, calcium is also a powerful inducer of peroxynitrite formation (Bringold et al., 2000;Ghafourifar and Richter, 1997;Tatoyan and Giulivi, 1998). In addition to the ROS produced within neurons, ROS arising from other cells may be important contributors to oxidative stress in neurons. For example, activated microglia produce large amounts of nitric oxide, superoxide and peroxynitrite, and adversely affect adjacent cells (McGeer and McGeer, 1995b;Minghetti and Levi, 1998). Activated microglia may also produce excitotoxins, inducing calcium influx and ROS production in neurons (Gremo et al., 1997).

To protect against ROS-induced damage, neurons have endogenous antioxidant defense mechanism to quench ROS, including an enzymatic antioxidant system and cellular molecules. Cells possess two different superoxide dismutases (SOD): (a) Mn-SOD which is localized in mitochondria and (b) Cu/Zn-SOD which is localized primary in the cytoplasm. These two enzymes, along with catalase and glutathione peroxidase, constitute the most effective mechanism involved in the direct elimination of active oxygen species. The enzyme superoxide dismutase scavenges superoxide radical by converting them to molecular oxygen and hydrogen peroxide (H_2O_2), which is in turn inactivated by glutathione peroxidase and catalase (Fridovich, 1974;Rotilio et al., 1985;Zelko et al., 2002). In addition, many cellular molecules are active antioxidants in the body, including glutathione, ascorbate (vitamin C), α-tocopherol (vitamin E), ß-carotene, uric acid, NADPH, bilirubin and reduced CoQ10, all involved in protecting the cells during normal metabolism (Liu et al., 2007).

Age-related memory impairment is correlated with a decrease in brain antioxidants (Berr, 2000;Perkins et al., 1999;Perrig et al., 1997;Rinaldi et al., 2003). A decrease in the activities of Mn-SOD, Cu/Zn-SOD, catalase, glutathione peroxidase, and intracellular glutathione concentration has been found in the brain of different aged animals (Chen and Lowry, 1989;Gupta et al., 1991;Navarro et al., 2004;Siqueira et al., 2005;Wang et al., 2003b). Thus, age-related decreases in antioxidant activity may lead to an age-related accumulation of oxidatively modified proteins, as described in senescent animals (Berlett and Stadtman, 1997). Interestingly, administration of free radical scavengers to animals reverses protein oxidation in aged brains and restores the performance of animals on behavioral tests, strongly suggesting an age-related decline in mental performance associated with a reversible process involving the oxidation of protein components in the brain (Carney et al., 1991;Floyd and Hensley, 2000). The above evidence suggests that oxidative modifications to cellular proteins increases with age, and is consistent with a large body of evidence demonstrating that oxidation/nitration-dependent post-translational modifications are responsible for the altered stability and decreased function observed for a range of different proteins isolated from aged animals (Gafni, 1997).

The definitive causes of sporadic (non-genetic) forms of Alzheimer disease are still unresolved. However, an increasing amount of evidence shows that oxidative damage is one of the earliest neuronal and pathological changes observed, when compared to other pathological manifestations (Nunomura et al., 2001;Pratico et al., 2002). Thus, oxidative stress is one of the

postulated causative factors of Alzheimer's disease (Christen, 2000;Markesbery, 1997;Perry and Smith, 1998;Smith et al., 1995). Biochemical analysis of tissue homogenates from Alzheimer's disease and control brain samples have provided evidence for increased oxidative damage. This is more prominent in vulnerable regions of Alzheimer's pathology (Lovell et al., 1995;Smith et al., 1991). Moreover, additional evidence for increased oxidative stress in Alzheimer's disease comes from studies showing alterations of antioxidant enzymes in vulnerable regions of Alzheimer's brain. For example, protein and activity levels of catalase were observedly decreased relative to control brains, while levels of Cu/Zn-SOD and Mn-SOD were found to increase in vulnerable regions of AD brains (Delacourte et al., 1988).

Recently, there has been an increasing amount of evidence showing that microglial activation may be involved in the pathogenesis of Alzheimer's disease, and Aß may be the molecular link between oxidative stress and Alzheimer's disease-associated neuronal cell degeneration. In addition to assisting with the removal of injured cells and cellular debris, microglia could aggravate cellular insults. Microglia may lead to cellular damage in Alzheimer's disease, whereby markers that are indicative of activated microglia have been found significantly increased in vulnerable regions from mild and early stages of the disease, suggesting that microglial activation is an early event in the pathogenesis of Alzheimer's disease (Cagnin et al., 2001;Rogers and Lue, 2001). Interestingly, in patients with Alzheimer's disease, Aß induces microglia activation and expression of inducible nitric oxide, NO and peroxynitrite overproduction, therefore increasing the overall radical burden in Aß-loaded brain regions (Combs et al., 2001;McGeer and McGeer, 1995b).Furthermore, microglia co-localize with the perivascular deposits of Aß and microglia activation have been observed to occur in concert with the evolution of ß-amyloid deposition (Sheng et al., 1997). The generation of oxidative stress by microglia during Aß deposition suggests that microglia may play an important role in the pathogenesis of Alzheimer's disease, whereas the spread of peroxynitrite can lead to damage of neuronal cells (Smith et al., 1997) and nitration of key proteins (Di Stasi et al., 1999) thus affecting signaling transduction pathways.

1D4 Peroxynitrite, Nerve Growth Factor and Cholinergic Signaling

In addition to the adverse effects of Aß on acetylcholine production and release, *in vivo* studies have also provided evidence that oxidative stress plays an important role in aging and also

in the Alzheimer's disease related cholinergic deficits. Administration of anti-oxidants to aged rats results in improved performance in learning and memory tasks dependent on cholinergic function (Carney et al., 1991). Moreover, maintenance of rats on a reduced caloric diet, a well established method for increasing lifespan in rodents, has been shown to retard the age-related deficits in muscarinic signaling (Joseph et al., 1995) by a mechanism likely involving reduced oxidative stress (Sohal and Weindruch, 1996). Furthermore, Nistico and colleagues have reported that long-term intraventricular administration of NGF in aged rats restores the levels and activity of the main enzymes involved in the metabolic degradation of ROS, including Cu/Zn-SOD, catalase and glutathione peroxidase (Nistico et al., 1991;Nistico et al., 1992). In fact, regulation of antioxidant enzyme expression by NGF was reported by Sampath & Perez-Polo (Sampath and Perez-Polo, 1997) and further *in vitro* studies supported and provided additional information about the intracellular mechanism underlying this former observation. Primary basal forebrain cultures were protected against oxidative stress via activation of NF-*k*ß (Gu et al., 2000) and NGF have shown to prevent peroxynitrite production and protein tyrosine nitration, regulating the expression of Cu/Zn-SOD via the PI3K/Akt/NF-*k*ß pathway (Rojo et al., 2004). Moreover, induction of Mn-SOD through NF-*k*ß activation protected neurons from Aß-induced oxidative stress (Mattson et al., 1997).

In addition to the NGF regulation of antioxidant enzymes, it has been shown that peroxynitrite inhibited NGF signaling (Jonnala and Buccafusco, 2001), transforming NGF into an apoptotic factor (Pehar et al., 2006) and stimulating activated astrocytes to produce and release proNGF (Domeniconi et al., 2007). Taken together, these results strongly suggest that chronic peroxynitrite production in the brain could impair normal cholinergic function in aging and in Alzheimer's disease through alteration in NGF production, maturation and /or activity, leading to the progressive cognitive deterioration. This idea is reinforced by several *in vivo* studies in which administration of either antioxidant agents or exogenous NGF prevented the age-related basal forebrain cholinergic degeneration, ameliorating or reversing the memory-associated deficits. Our observations, discussed in **Chapter 4**, bring new light to this problem and offer possible explanations, linking the Aß-induced oxidative stress with the metabolism and post-translational changes of NGF.

1E- Thesis Objectives

In view of the above scenarios and particularly due to the marked vulnerability of the basal forebrain cholinergic neurons during aging and Alzheimer's disease, this thesis is aimed at improving our general understanding of the mechanisms underlying the interrelationship between (a) disruption in NGF trophic support (b) cholinergic dysfunction (c) cognitive decline and (d) oxidative stress.

It includes the following issues:

1) A therapeutic approach strategy aimed at reversing the atrophy of the basal forebrain cholinergic neurons and at ameliorating the cognitive impairments displayed by behaviorally characterized aged rats.

2) Studies defining the molecular form of NGF released from the cerebral cortex in an activity-dependent manner and the maturation and degradation of NGF in the extracellular space.

3) Studies in Alzheimer's disease human brain tissue revealing the states of the protease cascade responsible for NGF maturation and degradation. Also, studies aimed at clarifying whether Aß oligomers can replicate observations made in AD brain, and *in vitro* and *in vivo* studies on the biological effects of oxidized NGF.

These topics are addressed as individual Chapters resulting from published or submitted papers, followed by a General Discussion which offers an integrated perspective of the data presented here and focuses on potential further therapeutical applications.

CHAPTER 2

Long-Lasting Rescue of Age-Associated Deficits in Cognition and the CNS Cholinergic Phenotype by a Partial Agonist Peptidomimetic Ligand of TrkA

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The Journal of Neuroscience, September 15, 2004 24(37):8009–8018

2A Abstract

Previously, we developed a proteolytically stable small molecule peptidomimetic termed D3 as a selective ligand of the extracellular domain of the TrkA receptor for the NGF. *Ex vivo* D3 was defined as a selective, partial TrkA agonist. Here, the *in vivo* efficacy of D3 as a potential therapeutic for cholinergic neurons was tested in cognitively impaired aged rats, and we compared the consequence of partial TrkA activation (D3) versus full TrkA/p75 activation (NGF).We show that *in vivo* D3 binds to TrkA receptors and affords a significant and long-lived phenotypic rescue of the cholinergic phenotype both in the cortex and in the nucleus basalis. The cholinergic rescue was selective and correlates with a significant improvement of memory/learning in cognitively impaired aged rats. The effects of the synthetic ligand D3 and the natural ligand NGF were comparable. Small, proteolytically stable ligands with selective agonistic activity at a growth factor receptor may have therapeutic potential for neurodegenerative disorders.

2B Introduction

Neurotrophins are growth factors that regulate the development and maintenance of the peripheral and the central nervous systems (Lewin and Barde, 1996). Neurotrophins and their receptors are targets for therapeutic intervention in neurodegeneration (Eide et al., 1993;Hefti, 1994;Saragovi and Gehring, 2000;Thoenen, 1995).

The neurotrophin nerve growth factor (NGF) acts by binding to receptors TrkA and p75. NGF–TrkA binding (approximate *K*d, 10^{-11}M) activates the intrinsic tyrosine kinase of the receptor, causing tyrosine phosphorylation of TrkA and associated signaling partners, and activates "traditional" neurotrophic biological signals. These signals promote cell survival or differentiation (Kaplan and Miller, 2000). The $p75^{NTR}$ receptor is a member of the tumor necrosis factor receptor superfamily. Depending on the cellular environment and the type of ligand, $p75^{NTR}$ can transducer pro-survival, pro-apoptotic, or pro-differentiation signals (Barker, 1998;Rabizadeh et al., 1999;Saragovi and Zaccaro, 2002;Zaccaro et al., 2001). These features make $p75^{NTR}$ biology complex and the behavior of $p75^{NTR}$ ligands unpredictable.

Mature cholinergic basal forebrain (CBF) neurons, which innervate the cerebral cortex and hippocampus, play a key role in cognition and memory (Lad et al., 2003). These neurons express NGF receptors (Backman et al., 1997) and are dependent for their health and cholinergic

phenotype on NGF receptor activation (Debeir et al., 1999;Sofroniew et al., 2001). In aging and cognitive disorders such as Alzheimer's disease (AD), CBF neurons undergo atrophy and eventually degenerate (Casu et al., 1999;Turrini et al., 2001). This process correlates with a progressive reduction of TrkA density before cellular death (Mufson et al., 1997;Mufson et al., 2000;Sendera et al., 2000). Indeed, TrkA (but not of $p75^{NTR}$) depletion is a reliable marker of progressively deteriorating cognitive ability (Counts et al., 2004). Thus, exogenous NGF has been used as an experimental therapeutic agent in aging and stroke animal models (Backman et al., 1997;Frick et al., 1997;Garofalo et al., 1992) and in human AD patients (Gage et al., 1988a). Exogenous NGF reversed the age-dependent changes in CBF neurons and improved performance in spatial memory tasks. The effect of NGF was long-lived after discontinuation of delivery. However, NGF therapy for AD patients failed because of CNS delivery problems and pleiotropic and adverse side effects (Barinaga, 1994;Jonhagen, 2000;Verrall, 1994). Moreover, it is hard to interpret the outcome of NGF– $p75^{NTR}$ interactions in vivo in the aged brain.

We reasoned that it would be advantageous to apply ligands that bind TrkA and exclude $p75^{NTR}$. To this end, we developed a TrkA-selective NGF peptidomimetic termed D3. D3 induces TrkA tyrosine phosphorylation, stabilizes TrkA–TrkA homodimers, enhances ChAT production/activity, and induces neurotrophic activities in PC12 cells and primary neuronal cultures (Maliartchouk et al., 2000). D3 has partial agonistic activity but could be best described as a potentiator of NGF action. In addition, D3 is a small (mass, 580 Da), water-soluble and proteolytically stable molecule. In view of these drug-like features, we tested whether D3 has superior in vivo pharmacological properties compared with the NGF polypeptide.

2C Materials and Methods

Animals Aged (24 months old) and young (6 months old) male Fisher-344 rats were used. Efforts were made to minimize the number of animals used and their suffering. All procedures were approved beforehand by the Animal Care Committee of McGill University and followed the guidelines of the Canadian Institutes of Health Research.

D3 distribution in brain D3 and its analog D3-biotin (D3 conjugated with biotin) bind TrkA with similar properties (Maliartchouk et al., 2000), and D3-biotin can be followed via the biotin label (Maliartchouk et al., 2000). To assess whether D3 can diffuse when given

intracerebroventricularly, male Fischer-344 aged rats (n=2) were anesthetized, placed in a stereotaxic apparatus, and given a single injection of D3-biotin. A relatively high dose of D3-biotin (10 μg in 10 μl) was used because our pilot studies (data not shown) demonstrated it yields a good signal in these diffusion studies. D3-biotin was injected into the lateral ventricle (anteroposterior, -0.8 mm; lateral, 1.3 mm; vertical, 3.5 mm) (Paxinos and Watson, 1986). Without perfusion (to avoid washout during perfusion–fixation), rats were killed after an intracerebroventricular injection at 0.25, 2, 4, 8, 12, and 24 hr. Fixed (4% paraformaldehyde; 15 min), free-floating sections (50μm) were cut using a freezing sledge microtome, followed by 30 min of blocking with 3% normal mouse serum (Chemicon, Temecula, CA). After rinsing, sections were incubated at room temperature for 2 hr with a mouse IgG anti-biotin–Alexa-488 (1:300; Molecular Probes, Eugene, OR). Sections were rinsed three times with PBS and then mounted onto gelatin-coated slides using Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA).

Cellular distribution and D3–TrkA localization studies Aged rats (n = 4) received a single intracerebroventricular injection of D3-biotin (1 μg in 10 μl), and 24 hr later, their brains were perfused– fixed (24 hr because the bio-distribution studies above indicated good penetration). Then, 35 μm-thick sections containing the nucleus basalis and cerebral cortex regions were prepared, and double immunostaining was done to identify neurons, glia, or TrkA receptors. Neurons were identified with mouse anti-neural nuclei (NeuN) antibody (1:100; Chemicon), followed by goat anti-mouse coupled with rhodamine (1: 100; red; Jackson ImmunoResearch, West Grove, PA), and D3-biotin on neurons was identified with goat anti-biotin–FITC (green; Sigma, St. Louis, MO). Glia were identified using mouse anti-glial fibrillary acidic protein (GFAP; 1:100; Chemicon), followed by goat anti-mouse coupled with rhodamine (1:100; red; Jackson ImmunoResearch), and D3-biotin on glia was identified with goat anti-biotin–FITC (green; Sigma). TrkA receptors were identified using rabbit anti-TrkA antibody (1:500; Chemicon), followed by donkey anti-rabbit coupled with rhodamine red X (1:100; red; Jackson ImmunoResearch), and D3-biotin co-localizing with TrkA was identified with mouse IgG anti-biotin–Alexa-488 (1:300; green; Molecular Probes). In all cases, sections were pre-incubated and blocked with the corresponding normal serum secondary antibody. The co-localization of D3-biotin and neuronal, glial, or TrkA markers was examined with an LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

Behavioral task We resorted to segregating animals in spatially cognitively impaired and unimpaired groups applying well accepted protocols using the Morris water maze test (Chen et al., 1995;Fischer et al., 1987;Gage et al., 1988a), to avoid the potential confounding effects of studying drug effects on a global and heterogeneous population of aged rats.

Acquisition phase Aged (n = 120) and young (n = 10) rats were screened for spatial memory deficits using the Morris water maze as described previously (Aubert et al., 1995;Issa et al., 1990). The animals were required to find a submerged platform in a 1.4 m diameter pool of white, nontoxic colored water using only distal and spatial clues available in the testing room. Throughout, all tests were always carried in the same room and set up. The center of the escape platform (15 cm diameter) was located 45 cm from the pool wall, in the northeast quadrant. Animals were tested in 15 trials over 5 consecutive days (three trials per day with an intertrial time of 20 min) with the platform 2 cm below the water. At the end of the testing periods, all animals were given three trials in which the platform was raised 2cm above the water to exclude visual deficits as the cause of poor performance, as described (Rowe et al., 2003). The swim speeds and distances were recorded using a video tracking system (HVS Image, Buckingham, UK) to exclude motor deficits as the cause of poor performance, as described (Rowe et al., 2003). For excluding thigmotaxis and a motivational status as the cause of poor performance (Graziano et al., 2003;Rowe et al., 2003), concentric zones of the pool defined by the software program (HVS Image) were used to average swim patterns during days 2–5 of the acquisition phase. The latency to locate the escape platform was used to segregate rats into aged cognitively unimpaired and aged cognitively impaired groups. Aged rats whose individual mean latencies to locate the platform were within 0.5 SDs of the latency of young rats were considered cognitively unimpaired (n= 19). Aged rats whose individual average latencies to locate the platform were >2 SDs from those of young rats on days 2–5 of the acquisition phase were considered cognitively impaired (n= 31), as described (Rowe et al., 2003;Tombaugh et al., 2002). Rats displaying an intermediate performance between unimpaired and impaired (i.e., rats with <2 SDs but >0.5 SDs) were eliminated from this study (n= 70) because this “cognitively mildly impaired population” was expected to yield highly variable behavioral data.

Testing phase Throughout, all tests were always carried in the same room and the same Morris water maze was set up. Aged cognitively impaired rats were treated with saline, NGF, or D3 (see below) during 2 weeks, allowed to recover from surgery for 1 week (week 3), and then they were retested in the Morris water maze (week 4) with the platform submerged in the same position as in the acquisition phase. Animals were tested in nine trials over 3 consecutive days (three trials per day with an inter-trial time of 20 min) with the platform 2 cm below the water. The latency of each aged cognitively impaired group (vehicle, NGF, or D3) was also analyzed as the average time spent in each quadrant of the pool using the same software program (HVS Image).

Memory test At the 5th and 12th weeks after drug delivery, rats were placed in the water maze with no platform, for three trials of 60 sec with an inter-trial interval of 20 min in a single day. The data are expressed as percentage of time spent in each quadrant ±SEM, the "target quadrant" being where the platform had been hidden before. This experiment is reflective of memory because there is no new learning.

Surgical procedures and CNS administration Cognitively impaired rats were anesthetized and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). They were implanted with cannulas (Alzet) into the lateral ventricle, according to the following coordinates from bregma: anteroposterior, -0.8 mm; lateral, 1.3 mm; vertical, 3.5 mm (Paxinos and Watson, 1986). The cannulas were connected to sterile coiled polyethylene tubing filled with an air–oil spacer (Vahlsing et al., 1989;Varon et al., 1989) at the pump end. Rats receiving control vehicle (saline group; n= 8) had pumps filled with phosphate-buffered artificial CSF (150 mM NaCl, 1.8 mM $CaCl_2$, 1.2 mM MgSO4, 2 mM K_2HPO4, 10 mM glucose, and 0.001% rat serum, pH 7.4). Rats receiving positive control (NGF group; n= 7) had 1.4μg/d of 2.5 S NGF (Cedarlane Laboratories, Ontario, Canada) diluted in saline vehicle (a total of ~20μg NGF). This concentration of NGF was established previously to be neuroprotective in dose–response studies *in vivo* (Garofalo and Cuello, 1995). Rats receiving test peptidomimetic (D3 group; n= 7) had 2.8μg/d D3 diluted in saline vehicle (a total of ~40 μg of D3). This concentration of D3 was chosen based on its potency and affinity relative to NGF. The tubing was connected from its oil spacer end to Alzet 2002 (14 d life span) osmotic minipumps filled with dye (0.1% methylene blue), which were pre-tested to confirm their delivery rates. After the end of the drug delivery, the pump placed subcutaneously in the

neck/shoulder area and the tubing were removed from anesthetized rats, and the total infusion volume was determined for each animal to confirm the dosage administered. Animals were allowed to rest for 1 week, and the testing phase of the memory task was performed as described above.

Phenotypic analyses After completion of the behavioral studies, rats were anesthetized and perfused transcardially with a mixture of 4% paraformaldehyde, 0.5% glutaraldehyde, and 15% saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4, for 30 min, followed by the same fixative without glutaraldehyde for an additional 30 min. The tissue was subsequently postfixed for 4 hr in the latter fixative mixture and left at 4°Cwith a solution of 30% sucrose in 0.1 M PBS. Then, 35μm-thick sections between coordinates from bregma (0.5mm and -3.0 mm, which comprise the parietal region of the cerebral cortex and the nucleus basalis area) (Paxinos and Watson, 1986) were cut using a sledge microtome (Sliding Microtome, SM2000 R; Leica Microsystems, Quebec, Canada) equipped with a freezing stage. After rinsing in PBS, the free-floating sections were incubated for 1 hr in a solution containing 1% bovine serum and 10% normal goat serum in PB. Sections were then incubated overnight at 4°C with an anti- vesicular acetylcholine transporter (VAChT) antibody (1:10,000; a gift from Dr. R. Edwards, University of California–San Francisco, San Francisco, CA) or anti-synaptophysin antibody (1:200) (Gilmor et al., 1996) as described. After washing, the tissue was incubated in biotinylated goat anti-rabbit antibody (1:800; 2 hr at room temperature; Vector Laboratories). After several washes, the tissue was incubated in 0.6% diaminobenzidine (Sigma) in Tween 20 in PBS (15 min at room temperature). Subsequently, H2O2 was added to the diaminobenzidine solution. After washing, the sections were mounted on gelatin-coated glass slides and were dehydrated and coverslipped with Entellan (Merck, Darmstadt, Germany). Omission of the primary antibody served as a negative control.

Image analysis The density of VAChT-immunoreactive (IR) or synaptophysin-IR pre-synaptic boutons in lamina V of the parietal cortex (bregma coordinates: lateral, 4.7–5.7 mm; ventral, 3.0–4.2 mm) and the size and density of the VAChT-IR neurons at the nucleus basalis (lateral, 2.3–3.1 mm; antero-posterior, -1.2 to -1.8 mm; ventral, 6.3– 7.3 mm) per 1000 μm^2 were measured using a BH-2 Olympus microscope connected to an image analysis system (MCID Elite; Imaging

Research, St. Catharines, Ontario, Canada) as described (Debeir et al., 1999). Briefly, five images per area, from both hemispheres, were taken from five different slices per rat (50 images per rats for each region basalis and cortex).For lamina V of the parietal cortex and the nucleus basalis, five animals were used in each group: young adult, aged unimpaired, aged impaired, aged impaired plus saline, and aged impaired plus D3. For the NGF group, 4 animals were analyzed. Because our previous studies have shown that sham surgeries (no infusion of saline control) on mature or in aged rats have no impact on behavior or basal forebrain cholinergic markers (Garofalo and Cuello, 1994;Garofalo and Cuello, 1995), we opted to forgo these additional surgical controls. For quantification of lamina V of the parietal cortex, a total area of 80,000 μm^2 was analyzed per rat. For the nucleus basalis, a total of 50,000 μm^2 were analyzed per rat. Results are expressed per 1000 μm^2. For analyses of cell size, a total of 10,000 VAChT-IR cells per rat were studied.

Preparation of D3 and NGF D3 and D3-biotin were prepared as described (Maliartchouk et al., 2000). The material was analyzed as homogeneous single peak by analytical HPLC, by matrix-assisted desorption ionization mass spectrometry, and by ^1H NMR (300 MHz; DMSO-d_6) (Pattarawarapan et al., 2002), and the expected structure was confirmed. D3 was prepared in saline buffer at ~3.5 mg/ml. 2.5 S NGF (Cedarlane Laboratories, Ontario, Canada) was prepared in saline buffer.

Data analysis Statistical analyses were performed using commercial software (Systat 10.0; SPSS Inc.,Chicago, IL). Data were subjected to univariate and multivariate ANOVA; for repeated measures, the Huyhn-Feldt *p* value is reported. Between-group comparisons were made using Tukey's test. All probability values were two-tailed; a level of 5% was considered significant. Data are reported as the mean ±SEM.

2D Results

The D3 peptidomimetic TrkA ligand distributes throughout the CNS parenchyma and co-localizes with the TrkA receptor

D3-biotin was given intracerebroventricularly to assess whether small peptidomimetics penetrate the CNS parenchyma efficiently. After an intracerebroventricular injection of D3-biotin (10 μg), rats were killed without perfusion at times 0.25, 2, 4, 8, 12, and 24 hr and stained with mouse anti-biotin–Alexa-488 (**Figure 2-1**). At 2 hr (**Figure 2-1B**) and 4 hr (**Figure 2-1C**) after injection, D3-biotin started to penetrate the CNS parenchyma. A maximal signal is seen at the 8 hr (**Figure 2-1D**) and 12 hr (**Figure 2-1E**) time points. At 24 hr (**Figure 2-1F**), no signal is apparent in the choroid plexus or ventricular surface. Hence, D3-biotin is efficient at penetrating brain parenchyma.

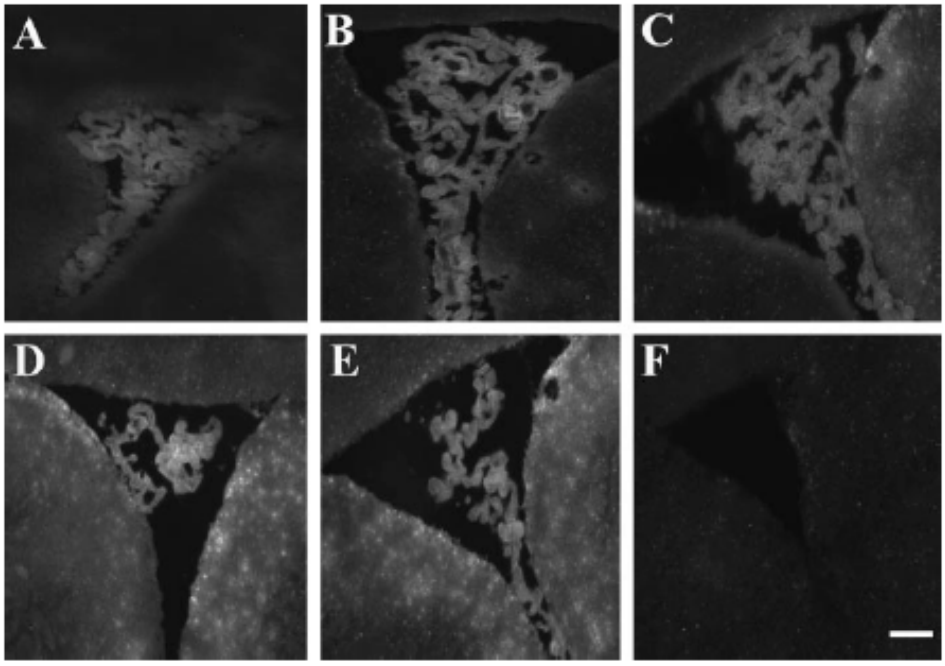

Figure 2-1 Peptidomimetic D3 efficiently penetrates the CNS parenchyma.
Images of the lateral ventricle of fresh, unfixed tissue after D3-biotin injection at time points: 15 min (A); 2 hr (B); 4 hr (C); 8 hr (D); 12 hr (E); 24 hr (F). At 2 hr after injection, D3-biotin started to penetrate the CNS parenchyma and gave a maximal signal at the 8 and 12 hr. At 24 hr, no D3-biotin is apparent in the choroid plexus or ventricular surface. Scale bar, 200μm.

More detailed co-localization studies were performed using D3-biotin and subjecting the rats to a perfusion–fixation step to remove unbound D3-biotin 24 hr after injection (**Figure 2-2**). Most of the D3-biotin was washed off by perfusion, and it is presumed that those D3-biotin molecules were not bound to a receptor. Specific analyses of target areas where TrkA is expressed (e.g., cerebral

cortex and nucleus basalis) revealed bound D3-biotin remaining after perfusion and could not be washed off, suggesting that it is tightly bound to a receptor.

These D3-biotin molecules accumulated at the cell surface of lamina V parietal cortical neurons, the nuclei of which were identified with the NeuN marker (**Figure 2-2A**). Additional analysis using glial markers demonstrated no co-localization of D3-biotin signal with GFAP in parietal cortex lamina V (**Figure 2-2B**). Finally, studies in the nucleus basalis demonstrated co-localization of the D3-biotin signal with TrkA immunoreactivity (**Figure 2-2C**). Together, these data indicate that D3 binds to cell surface TrkA expressed in neurons.

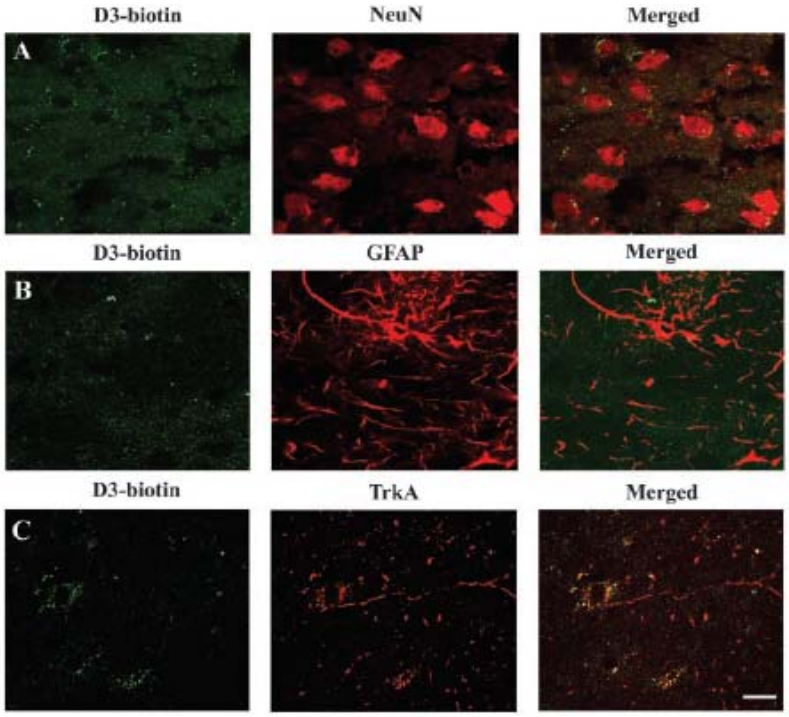

Figure 2-2 Neuronal binding by D3 and colocalization with TrkA receptors
Confocal microscopy of parietal cortex lamina V (A, B) or nucleus basalis (**C**) 24 hr after injection of D3-biotin. Brains were perfused, and sections were immunostained. (**A**) Colocalization of D3-biotin and NeuN. (**B**) Lack of colocalization of D3-biotin and GFAP. (**C**) Colocalization of D3-biotin and TrkA. Scale bar, 20μm.

Pre-selection of aged, cognitively impaired rats

An aged rat model of cognitive impairment was used to test *in vivo* the TrkA partial agonist D3 (**Figure 2-3A**). From of a pool of 120 aged rats (24-month-old males), the Morris water maze allowed pre-selection of 31 rats that were severely cognitively impaired (**Figure 2-3B**). In the

Morris water maze rats, use visual clues to learn and recall the position of a platform hidden underwater. Visual impairment was ruled out as the cause of poor performance by testing with the platform exposed (**Figure 2-3B**) (day 6). All data were analyzed for swim speed to rule out motor deficits. Thirty-one cognitively impaired aged rats were thus selected. In the visual platform task (**Figure 2-3B**) (day 6), latencies to reach the platform were low in all three groups. However, Tukey's test revealed significant between-group differences ($p< 0.05$ to $p<0.0001$) in which young animals tended to reach the platform soonest and the aged impaired last. This difference may be attributable to swim speed because this measure differed between the three groups of animals ($F = 37.1$; df 2, 57; $p <0.0001$). This group difference did not differ across days (day X group interaction: $F = 1.1$; df 8, 228; $p >0.3$), and the young animals swam fastest (22.3 ± 0.5 cm/sec).

Importantly, the swim speed of the two aged groups (unimpaired and impaired) was indistinguishable (18.7 ± 0.1 and 18.7 ±0.2, respectively). Therefore, swim speed did not account for poor performance in the aged rats. Additionally, poor performance by cognitively impaired aged rats was not attributable to thigmotaxis, which is a tendency of the animal to swim in contact with the pool wall (Graziano et al., 2003). All rats (young, aged unimpaired, aged impaired) swam in each zone (**Figure 2-3C**) for a similar relative amount of time (**Figure 2-3D**). Therefore, thigmotaxis did not account for poor performance in the aged impaired rats. Thus, 31 aged cognitively impaired rats were defined and selected. Of these 31, two died of natural causes. Of the remaining 29 aged cognitively impaired rats, 24 were used for drug testing, followed by behavioral analyses (see **Figures 2-4, 2-5**) and studies of the cholinergic phenotype (see **Figures 2-3, 2-4**). The remaining five aged cognitively impaired rats (together with five aged unimpaired rats and five young adult rats) were used as controls for analyses of the density of pre-synaptic cholinergic boutons in the parietal cortex, and the density and size of cholinergic neurons in the nucleus basalis in rats not exposed to drugs (see **Figures 2-3, 2-4**).

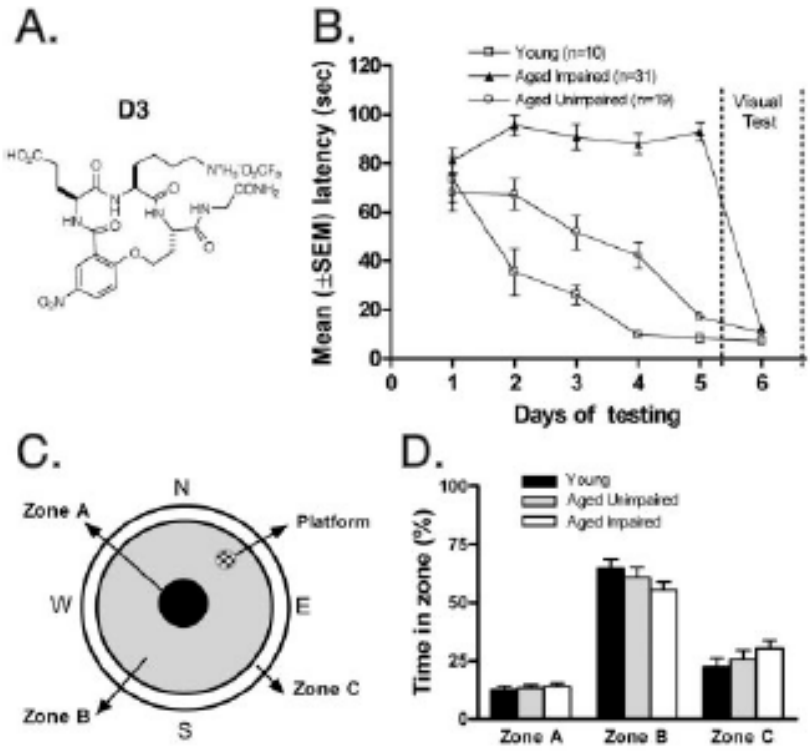

Figure 2-3 Preselection of cognitively impaired aged rats
(**A)** TrkA partial agonist D3 peptidomimetic. (**B**) Segregation of cohorts of aged impaired rats in the Morris water maze(see Materials and Methods for details). Latency scores were subjected to a two-way ANOVA with two factors: days (within-subject) and group (between-subject). This confirmed that the three groups did indeed differ. The main effects of day and group, and their interaction, were all highly significant ($F=99.0$; df 2, 228; $p<0.0001$; $F=18.1$; df 4, 228; $p<0.0001$; $F=10.3$; df 8, 228; $p< 0.0001$, respectively). In the visual platform task (day 6), all three groups reached the platform rapidly. (**C**) Graphic representation of the pool divided into three concentric zones (Zone A, 20 cm diameter; Zone B, 45 cm width; Zone C, 15 cm from the outer wall). The location of the escape platform is indicated in the northeast, with its center 45 cm from the outer wall. (**D**) The young, aged unimpaired, and aged impaired groups spend similar relative amounts of time in the three zones averaged for days 2–5 of the acquisition phase. In particular, there was no group difference in the proportion of time spent in the outer Zone C, which is a measure of thigmotaxis ($F= 0.99$; df 2, 57; $p> 0.3$).

The peptidomimetic TrkA ligand, D3, affords rescue of age-associated cognitive deficits in vivo

Twenty-four cognitively impaired aged rats were randomly allocated to three treatments. One group received vehicle (saline; n= 8), another received NGF-positive control (n= 8), and another received D3 (n= 8). Over 14 d, a total of 20μg of NGF was administered, a dose previously

shown to be neuroprotective (Garofalo and Cuello, 1995). Over 14 d, a total of ~40μg of D3 was administered, a dose chosen from previous pilot studies with aged rats (data not shown) reflecting the lower *ex vivo* TrkA binding affinity and efficacy of D3 compared with NGF. On week 4 after initiation of drug delivery, rats were retested for learning and memory in the Morris water maze, as shown in the experimental flowchart of **Figure 2-4**. All rats in the saline and the D3 groups survived surgery and testing with no signs of side effects. In the NGF group, there were two rats with tremors and weight loss (a known side effect of applying NGF therapeutically). All the Morris water maze testing data from these two rats in the NGF group were completely discarded. Also, there were two deaths each in the NGF and the D3 groups attributable to natural causes. Thus, the n values were n= 8 (vehicle), n= 6 (D3), and n = 4 (NGF).

Rats that received D3 or NGF had significantly improved performance compared with the saline group from trial 2 onward of the testing phase (**Figure 2-4A**). However, NGF and D3 were not significantly different from each other. Swim speeds of aged impaired animals receiving treatments were not a factor because they were closely similar between groups and across trials. The mean swim speeds (centimeters per second) for the vehicle, D3, and NGF groups were, respectively, 18.2 ± 0.2, 18.2 ± 0.2, and 18.1 ± 0.2 (ANOVA main effects and interaction; $p > 0.7$).

The data in **Figure 2-4A** were further evaluated by quadrant analyses, specifically comparing trial 1 and trial 7 on week 4 post-drug delivery. The data are presented as the percentage of time the animals spend in the trained quadrant (where the platform is located) versus the other three quadrants (**Figure 2-4B**). The analyses for the vehicle, NGF, and D3 groups are shown for trial 1 (**Figure 2-4C**) and trial 7 (**Figure 2-4D**). As expected, in trial 1, there were no significant differences among the vehicle, NGF, or D3 groups (**Figure 2-4C**), but in trial 7, the NGF and the D3 groups had a significant improvement at targeting the trained quadrant compared with vehicle (**Figure 2-4D**). Tukey's test showed that this measure differed significantly between groups on trial 7 but not on trial 1 (ANOVA; $p < 0.0001$ and $p > 0.09$, respectively). In trial 7, comparisons for all three groups were significant ($p < 0.05$ to $p < 0.0001$). These results are suggestive of improved learning and/or memory by the NGF and D3 groups.

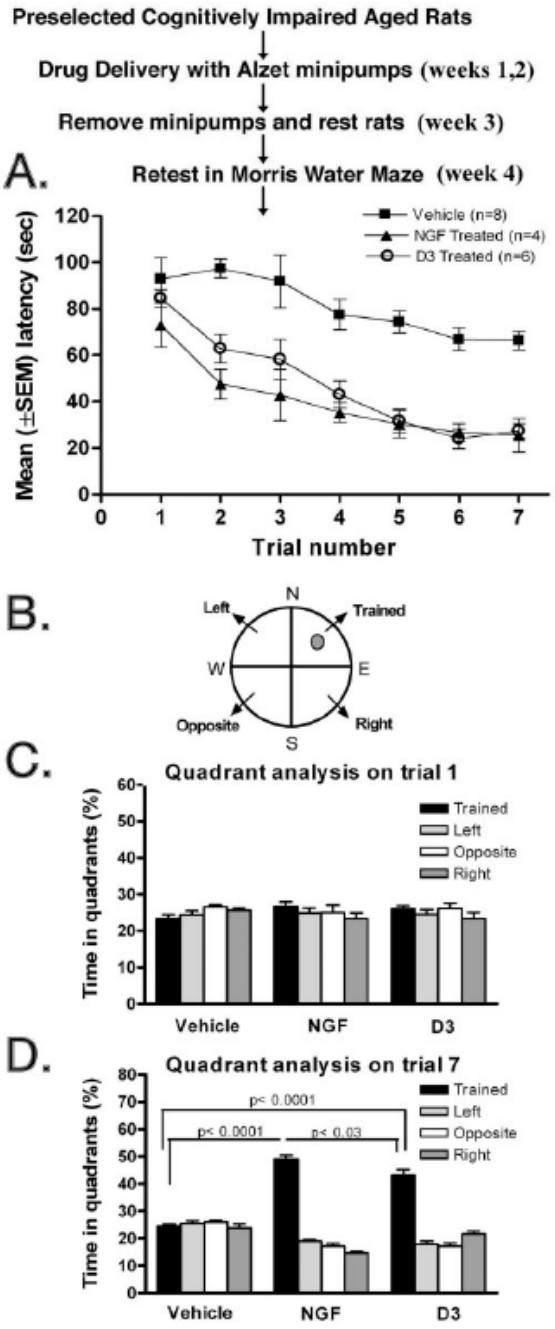

Figure 2-4 D3 and NGF improve performance in cognitively impaired aged rats

(**A**) Cognitively impaired rats were treated as indicated and then were retested, as described in Materials and Methods, in the Morris water maze on week 4 after initiation of drug delivery. ANOVA of latencies to find the platform revealed significant main effects of group ($F=44.8$; df 2, 15; $p< 0.0001$) and trial ($F=18.6$; df 6, 90; $p<0.0001$). The latencies of D3- and NGF-treated rats were similar in all trials, whereas both groups diverged significantly from the vehicle-treated control group (group x linear trend of trial: $F=4.62$; df 2, 15; $p< 0.05$). Drug effects were evident, for example, for data pooled across the last four trials (Tukey's test: D3 or NGF *vs.* vehicle, $p<0.0001$). (**B**) Schematic graph of the pool showing the quadrants and the position of the submerged escape platform. Data for Figure 4 A were evaluated by quadrant analyses and presented as the percentage of time spent in the trained quadrant (where the platform is located) versus the other three quadrants. (**C**) Analyses for the vehicle, NGF, and D3 groups for trial 1. D, Analyses for the vehicle, NGF, and D3 groups for trial 7. Statistical comparisons refer to
Tukey's test.

D3 affords a long-lasting rescue of age-associated memory deficits in vivo

To discriminate between learning and memory, the rats were tested in the same pool for a fixed time of 1 min, but without the hidden platform, thus offering no escape and no new learning. Because there is no successful resolution of the task, because there is not platform to find, the time spent in the target quadrant (where the platform had been placed) does not reflect new learning but only memory. Representative swim paths from individual rats at 12 weeks after drug delivery are shown in **Figure 2-5A**. The NGF and the D3 groups spent a significantly higher proportion of time in the target quadrant at week 5 (**Figure 2-5B**) and week 12 (**Figure 2-5C**) after drug delivery. In contrast, saline-treated rats spent very similar amounts of time in each quadrant (**Figure 2-5B, C**). These data indicate that the behavioral improvement afforded by D3 and NGF is very long-lived and attributable, at least in part, to improved memory.

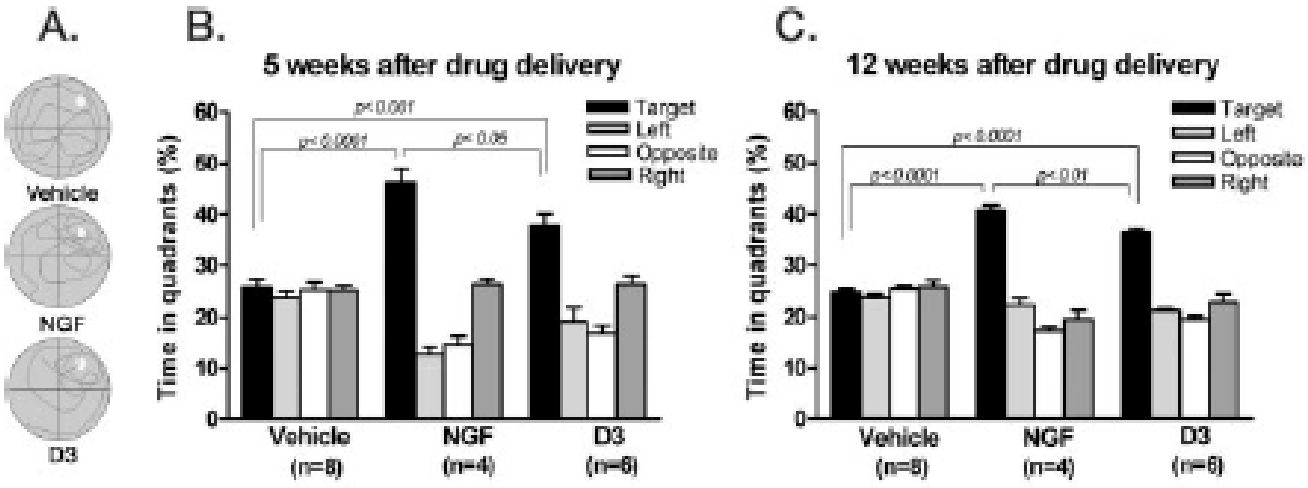

Figure 2-5 D3 and NGF improve memory in cognitively impaired aged rats
(**A**) Typical swim path for individual rats recorded over a 1 min period in week 12. Target quadrant is where the escape platform had been located formerly (northeast quadrant). The summary of quadrant analysis for the vehicle and D3 groups and NGF group at week 5 (**B**) and week 12 (**C**) after drug treatment. Statistical comparisons refer to Tukey's test.

D3 rescues the cholinergic neuronal phenotype in vivo

All the aged rats used for behavioral testing were killed 12 weeks after drug delivery, their brains were perfused and fixed, and multiple serial cryosections were prepared. As controls, an

additional cohort not subjected to drug treatment or surgery (five young rats, five aged unimpaired rats, and five aged impaired rats) were studied as well. Sections were immunostained with antibodies to VAChT. This marker was chosen because cortical changes in VAChT accompany age-dependent cognitive impairments in mammals (Bartus et al., 1982;Casu et al., 1999;Hasselmo, 1999;Turrini et al., 2001). The density of VAChT-IR presynaptic boutons in the lamina V of the parietal cortex and the size of soma in the nucleus basalis were studied quantitatively. Concomitant analyses of synaptophysin immunostaining and density were also done to test for cholinergic specificity. In untreated rats, computer-assisted image analysis of the number of VAChT-IR boutons in lamina V of the parietal cortex demonstrated a reduction in cognitively impaired aged rats compared with young rats ($p < 0.001$) and between cognitively impaired aged rats and unimpaired aged rats ($p< 0.01$). There were no statistical differences between the young rats and unimpaired aged rats (**Figure 2-6A**).

In cognitively impaired aged rats, treatment with D3 or NGF significantly ($p< 0.001$) increased the density of cortical cholinergic (i.e., VAChT-IR) presynaptic boutons to a similar extent, ~ 40% (**Figure 2-6A**). It is noteworthy that the phenotypic changes in forebrain VAChT expression brought about by D3 and NGF treatments were long-lived and lasted up to 12 weeks after drug treatment.

Control immunostaining of all cortical presynaptic sites with synaptophysin antibodies in adjacent serial sections demonstrated no variation in that marker (**Figure 2-6B**), except a slight difference between the young and aged impaired groups ($p< 0.05$). This control indicates that D3 is a fairly selective agent for NGF sensitive cholinergic neurons. The microscopic appearance of images of VAChT cortical presynaptic sites is shown to illustrate the VAChT changes in each of the test groups (**Figure 2-6C**).

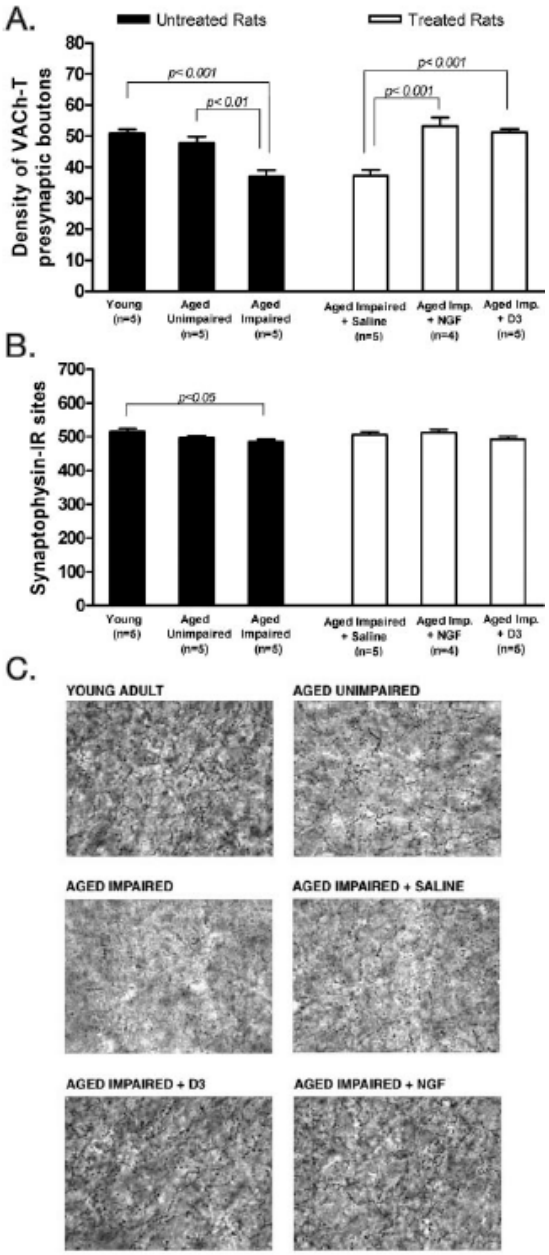

Figure 2-6 Selective effect of D3 on the cholinergic phenotype of cortical neurons
(**A**) Number (boutons/1000 μm2) ± SEM of cholinergic presynaptic boutons (VAChT immunoreactive). (**B**) Total presynaptic boutons (synaptophysin immunoreactive) in lamina V of the parietal cortex of aged Fischer-344 rats. In A and B, a total area of 80,000 μm2 was analyzed per rat (n= 4or 5). Aged impaired versus aged impaired plus vehicle were not different from each other but presented a significantly decreased number of cholinergic presynaptic boutons versus young. Restoration of the size in the cholinergic presynaptic boutons in aged impaired plus NGF and aged impaired plus D3 when compared with aged impaired and aged impaired plus vehicle is shown. For synaptophysin staining, there were no significant differences between any groups. (**C**) Representative pictures of lamina V of the parietal cortex immunostained with anti-VAChT antibodies.Statistical comparisons refer to Tukey's test.

The size of VAChT-immunostained cell somata in the nucleus basalis was determined by computer-assisted image analysis. In untreated rats, mean somal size was significantly ($p< 0.01$) reduced in cognitively impaired aged rats compared with young rats (**Figure 2-7A**). There was a small but significant difference between unimpaired aged rats compared with impaired aged rats ($p< 0.05$), but not between young rats and unimpaired aged rats.

In cognitively impaired aged rats, treatment with either D3 or NGF increased significantly ($p< 0.001$) the somal size of nucleus basalis neurons by ~30% (**Figure 2-7A**). Because the size of D3- or NGF-treated rats was not significantly different from each other or from young rats, the data would suggest that drug treatment reversed shrinkage. As was the case with cholinergic terminals, the phenotypic changes in cell somata brought about by D3 and NGF were long-lived because they lasted up to 12 weeks after drug treatment. There were no significant changes observed in the density of nucleus basalis cholinergic cell bodies (**Figure 2-7B**), thus reinforcing the notion that atrophy (rather than cholinergic forebrain cell loss) is a feature in age-related cholinergic decline.

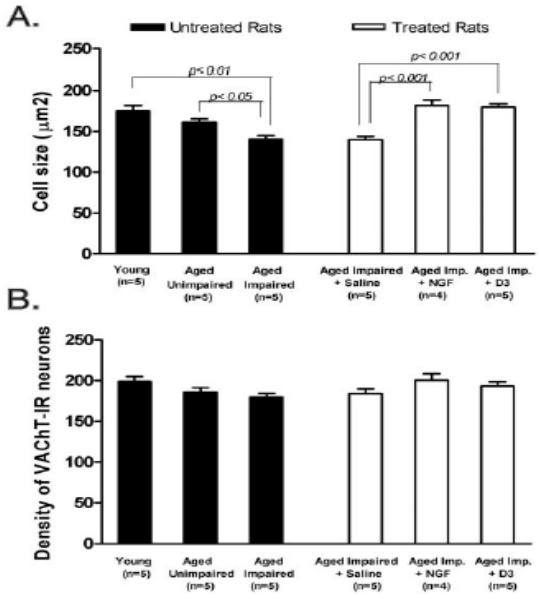

Figure 2-7 Selective effect of D3 on the size and cholinergic phenotype of nucleus basalis neurons

(**A**) Cell size of the soma (μm2) ± SEM of VAChT-IR cholinergic neurons in the nucleus basalis of Fischer-344 rats. For cell size analyses, a total of 10,000 VachT-positive cells per rat (n= 4 or 5) were studied. Aged impaired and aged impaired plus vehicle were not different from each other but displayed significantly lower cell size than young. Restoration of the cell size of cholinergic neurons after receiving either D3 or NGF when compared with aged impaired and aged impaired plus vehicle. (**B**) Density of VAChT-IR neurons, representative of neuronal numbers in the nucleus basalis. Statistical comparisons refer to Tukey's test. For VAChT density, a total of 50,000 VachT-positive cells per rat (n= 4 or 5) were studied, and data are expressed per 1000 μm2.

2E Discussion

We demonstrated the biological actions and usefulness of a peptidomimetic small molecule TrkA agonist named D3 (Maliartchouk et al., 2000) in an *in vivo* model of cognitive impairment. D3 affords a significant, long-lasting, and selective enhancement of the CNS cholinergic phenotype in cognitively impaired aged rats, accompanied with a significant, long-lasting improvement in the cognitive ability of aged rats thus treated. In these investigations, D3 and NGF afforded comparable results at the doses tested.

Targeting TrkA in cognitive disorders

In the normal adult human CNS, the cortical and nucleus basalis neurons that comprise the cholinergic system participate in higher brain functions such as learning and memory. These neurons express the TrkA receptor (Mufson et al., 1997) and are responsive to NGF. In neurodegenerative processes such as mild cognitive impairment (MCI), loss of TrkA density correlates with neuronal atrophy and precedes neuronal death and severe cognitive impairment in AD (Counts et al., 2004). Indeed, in MCI–AD progression, and in Down's syndrome, loss of TrkA correlates with cognitive decline (Mufson et al., 2000;Mufson et al., 2002). Likewise, in basal forebrain neurons of the aged rat, there is reduced expression of NGF receptors, which is reversed by delivery of NGF (Backman et al., 1997). Although published evidence linking a compromised TrkA– NGF system and progressive cognitive impairment is substantial, consistent for many models (MCI, AD, Aging, and Down's syndrome), and NGF reverts cognitive impairment, the relationship seems to be casual rather than causal. Additional studies will be needed to address causality.

Selective improvement to cognition and the cholinergic system

We demonstrate that the age-dependent cognitive impairment is accompanied by marked cholinergic synaptic loss, reduction in the size of cholinergic cell soma (but not in cell numbers), and by reduced expression of VAChT. These behavioral and phenotypic deficits are reverted by NGF and D3. The pharmacological effects are long lasting and were observed for 3 months after the initiation of drug treatment. It is gratifying that our data using exogenous NGF as control is consistent with previous reports (Chen et al., 1995;Fischer et al., 1987). The in vivo cholinergic

selectivity of D3 was evidenced by a specific enhancement of VAChT-IR boutons, but an absence of an effect on the overall pre-synaptic population (e.g., synaptophysin-IR boutons), and was anticipated because D3 is a TrkA ligand. One interesting finding corollary to our study of D3 provides additional confirmation that structural changes (reduced density of VAChT-IR pre-synaptic boutons in the neocortex and smaller size of somata in the nucleus basalis) may be directly implicated in aged-associated cognitive decline because such changes occur only in the cognitively impaired aged rats but not in the unimpaired aged rats.

Our results suggest that it may be possible to delay the chronic and progressive cholinergic neurodegeneration in aging or in pathological states with the use of neurotrophin receptor agonists. If selective agonists of TrkA were to be applied before extensive CNS damage (e.g., in MCI), it is conceivable that selective protection of cholinergic forebrain neurons and cognitive improvements could be achieved. Additionally, single or concomitant use of pharmacological modulators of $p75^{NTR}$ action or potentiators of NGF activity may also prove to be useful (Maliartchouk et al., 2000;Maliartchouk and Saragovi, 1997).To our knowledge, D3 is the first reported small molecule ligand capable of inducing selective survival of the cholinergic neurons. Other small molecules with drug-like properties, such as GM1 or the immunophilins, have been nonselective (Saragovi and Gehring, 2000).

Efficacy and selectivity

Selectivity, rather than potency or affinity, is the most relevant feature that impacts on drug efficacy *in vivo* (Mattie et al., 1989;Troke et al., 1990;Vella and Floridia, 1998;Waldman, 2002). For example, despite its high TrkA binding affinity (K_d, 10–100 pM), NGF has a narrow therapeutic window partly because of poor selectivity (e.g., it binds $p75^{NTR}$), partly because of pleiotropic activation, and partly because of a short half-life. In contrast, the TrkA binding affinity of D3 is ~5 μM. However, D3 does not bind another target, and it does not activate other Trk receptors (Maliartchouk et al., 2000). Therefore, D3 may afford a large therapeutic window. D3 can be detected associated with TrkA receptors for a relatively long time (as shown in **Figure 5C**). Improved penetration and stability for D3 (compared with NGF) may result in better access to target tissue from the site of administration.

Targeting TrkA in vivo

Anti-TrkA antiserum revealed TrkA protein in four spatial locations: at the surface of the cell soma and the neuritic terminals and intracellularly at the cytoplasma and the neurites. In contrast, we noted that D3 seems to localize preferentially at TrkA receptors present on the cell surface of the somata. There is very little D3 on the cell surface of neuritic terminals or inside cells either at the cytoplasma or inside neurite extensions. There are five possible explanations for these differences. First, there may be lower relative signal intensity for D3 versus anti-TrkA serum because the latter has multiple reactivities. Second, the TrkA protein present in neurite terminals (seen with anti-TrkA serum after fixation/permeabilization) may be mostly intracellular and not cell surfaced, and hence it is not accessible to the D3- biotin injected in vivo. Third, relative receptor density in the neurites may be lower than in the cell somata. Fourth, intracellular retrogradely transported D3 may be processed (i.e., the biotin label is cleaved off) and becomes undetectable. Fifth, perhaps the D3–TrkA complex does not internalize efficiently. *In vitro* studies of neuronal cultures documented that ligand-activated TrkA neurotrophic signals do not require internalization of the NGF–TrkA complex (Neet and Campenot, 2001); thus, our in vivo studies would be compatible with those results.

Pharmacological advantages

Two properties of D3 are desirable in a drug candidate: proteolytic stability and no incidence of side effects compared with NGF. The *ex vivo* proteolytic stability of D3 has been shown previously (Maliartchouk et al., 2000). D3 is extremely stable to pepsin, chymotrypsin, papain, trypsin, and other peptidases. Preliminary pharmacokinetic studies on D3 (data not shown) showed a serum half-life of ~4.5 hr and the appearance of two metabolites. In contrast, the serum half-life of NGF is in the order of minutes (Verrall, 1994).

The effective dose of NGF (nearly the lowest effective dose identified unpublished reports) (Garofalo and Cuello, 1995) caused noteworthy weight loss and tremors as side effects in two of eight (25%) of the tested rats. In contrast, the effective dose of D3 did not appear to cause these side effects or any obvious signs of toxicity, but we have not performed objective pathological studies. Note that all the data with NGF toxemic rats were discarded. Had that data been considered, the neuroprotective and behavioral effect of D3 would have been significantly better than NGF.

Although D3 is a partial agonist of TrkA, its intrinsic activity is relatively low and it is best revealed when acting as a synergistic potentiator of NGF (Maliartchouk et al., 2000). This property may be advantageous when testing in vivo because, in some neurogenerative disorders, NGF protein is increased (Fahnestock et al., 1996;Hock et al., 2000a;Hock et al., 2000c); but this NGF protein is either less active or has different activity (Fahnestock et al., 2004a;Fahnestock et al., 2004b). For that reason, a selective small molecule NGF potentiator such as D3 may be extremely effective.

Also, D3 binds at the IgC2 ectodomain of TrkA, which is defined as a functional "receptor hot spot." In contrast, NGF seems to bind at least two hot spots on TrkA (Saragovi and Zaccaro, 2002;Zaccaro et al., 2001). A potentially attractive feature is that small molecules could target receptors at single, defined, activation, or regulatory hot spots (e.g., only the Trk IgC2 ectodomain) while excluding co-receptors (e.g., $p75^{NTR}$). Thus, binding to a single receptor site could afford partial receptor activation and higher selectivity.

Summary

Disregulation of the neurotrophins or their receptors is relevant to human neuropathologies, including chronic or acute neurodegeneration, pain, and cancer (Eide et al., 1993;Hefti, 1994;Saragovi and Gehring, 2000). Neurotrophin clinical trials have been disappointing because of *in vivo* instability, side effects produced by the activation of signals that were not intentionally targeted, and drawbacks inherent to proteins when used as drugs. For these reasons, small molecule peptidomimetics that have better pharmacokinetic properties may be useful therapeutics. From a drug development perspective, it may be desirable to have small molecules that uncouple trophic and neuritogenic signals (Saragovi et al., 1998;Zhang et al., 2000) because they can be selective therapeutics in conditions in which a single activity is required.

2-E Acknowledgments

This work was supported by Canadian Institutes of Health Research Grants MT-13265 (H.U.S.) and MOP 62735 (A.C.C.) and National Institutes of Health Grant CA82642 (K.B.). M.A.B. received a fellowship from Consejo Nacional de Investigaciones Cientıficas y Tecnicas, Argentina. We thank Dr. H. Nedev and M. C. Zaccaro (McGill University, Montreal, Quebec, Canada) for aid

with compound synthesis and characterization and K. Bell and E. Tsang for aid with image analysis and histology.

Connecting Text: Chapter 2 to 3

As discussed in the **Introduction**, the cholinergic system is compromised in aging, and even more in AD. Interestingly, the age-related cholinergic atrophy and cell loss in normal brain, as well as in Alzheimer's disease, is not complemented by reductions in the levels of NGF as could be expected. In contrast, elevated levels of the precursor form of the NGF (proNGF) have been found in cortical brain samples from early and late stages of Alzheimer's patients. This situation could be attributed either to a failure of the NGF retrograde axonal transport system from cerebral cortex to the cell soma, or to the inability of cholinergic terminals to bind and internalize TrkA/NGF complexes. However, we considered that the cholinergic atrophy, among with diminished TrkA levels and reduced NGF retrograde transport could be due to the failure of the maturation of proNGF into a biologically active NGF. Favoring such interpretation, we reasoned:

1) As reported in **Chapter 2**, atrophic cholinergic neurons respond positively to the administration of D3 mimetic peptide, similar to exogenous NGF, reversing the age-dependent cortical cholinergic synaptic losses as well as ameliorating memory traits in cognitively impaired aged rats.

2) Lesion-induced trophic disconnection of the nucleus basalis (Figueiredo, *Neuroscience* 1995) or the septal nucleus (Venero et al, Neuroscience 1994) leads to down regulation of the TrkA mRNA message. The down regulated TrkA message in atrophic neurons in both models (septum and nucleus basalis) still responds positively to the *exogenous* administration of NGF by up-regulating the TrkA mRNA message (Figueiredo et al, Neuroscience 1995, Venero et al, Neuroscience 1994).

3) ProNGF is the predominant form of NGF in the brain, whereas the mature form is undetectable (Fahnestock et al, *Moll Cell Neurosci* 2001).

In view of the above, we deem it necessary to investigate *"de novo"* mechanisms for the stimulus-coupled release of NGF in the CNS. To achieve this, we established an *"ex-vivo"* superfusion system of cerebral cortex tissue, stimulated with depolarizing agents and classical

transmitter receptor agonists. To our surprise, we found that proNGF was the molecular form released in an activity-dependent manner (and not mature NGF), and that the NGF conversion and degradation occurs in the extracellular space, mediated by a complex cascade of convertases and endogenous inhibitors. These findings, elucidating an issue unresolved for over three decades, are described in **Chapter 3.**

CHAPTER 3

Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade

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PNAS, April 25, 2006 vol. 103; no.17: 6735–6740

3A Abstract

In this report, we provide direct demonstration that the neurotrophin nerve growth factor (NGF) is released in the extracellular space in an activity-dependent manner in its precursor form (proNGF) and that it is in this compartment that its maturation and degradation takes place because of the coordinated release and the action of proenzymes and enzyme regulators. This converting protease cascade and its endogenous regulators (including tissue plasminogen activator, plasminogen, neuroserpin, precursor matrix metalloproteinase 9, and tissue inhibitor metalloproteinase 1) are colocalized in neurons of the cerebral cortex and released upon neuronal stimulation. We also provide evidence that this mechanism operates in *in vivo* conditions, as the CNS application of inhibitors of converting and degrading enzymes lead to dramatic alterations in the tissue levels of either precursor NGF or mature NGF. Pathological alterations of this cascade in the CNS might cause or contribute to a lack of proper neuronal trophic support in conditions such as cerebral ischemia, seizure and Alzheimer's disease or, conversely, to excessive local production of neurotrophins as reported in inflammatory arthritis pain.

3B Introduction

The neurotrophin family of growth factors plays a critical role for neuronal survival and differentiation (Bibel and Barde, 2000;Levi-Montalcini, 1987). They are produced and liberated in an activity-dependent manner (Thoenen, 1995) and are responsible for maintaining neuronal phenotype in the adult CNS (Sofroniew et al., 1990), including the regulation of the steady state number of synapses (Debeir et al., 1999). These actions are normally attributed to mature neurotrophins, although recently a biological role for precursor neurotrophin molecules has also been proposed (Fahnestock et al., 2004b;Lee et al., 2001). Despite this wealth of knowledge, it is not clear whether these neurotrophins convert to their mature and biologically active form intracellularly or extracellularly, nor is it clear whether, upon their activity-dependent release into the CNS, they are in their mature or precursor form. For example, NGF has been proposed to be released in its mature form (Blochl and Thoenen, 1995;Blochl and Thoenen, 1996;Canossa et al., 1997), while in the case of BDNF recent experimental data suggests that the precursor form (proBDNF) was released and was then processed extracellularly to elicit long term potentiation (LTP) (Pang et al., 2004).

These issues are of functional significance as recent *in vitro* studies with cells transfected with furin-resistant mutated forms of immature NGF (proNGF) have shown that unprocessed proNGF interacts preferentially with the ubiquitous $p75^{NTR}$ instead of the high affinity NGF receptor, TrkA, facilitating an apoptotic mechanism in embryonic cells of the PNS (Lee et al., 2001). Moreover, it has been proposed that, in the adult CNS, proNGF expression is up-regulated following CNS lesions, probably contributing to cell death through $p75^{NTR}$ and sortilin (Harrington et al., 2004;Nykjaer et al., 2004). However, other authors have provided evidence suggesting a neurotrophic role for proNGF, albeit to a lesser degree than that of the mature NGF (Fahnestock et al., 2004a).

The realization that proNGF might play a biological role in the CNS raised questions regarding the regulatory mechanisms leading to its release, as well as the control of the proNGF to mNGF ratio and ultimately the degradation of the mature NGF molecule. To answer these questions we embarked on a series of *in vitro* and *in vivo* studies aimed at elucidating the preferential NGF form released from the cerebral cortex and the pathway leading to NGF maturation and degradation. These studies have revealed that proNGF is the main releasable form of the neurotrophin and that the maturation and degradation of mature NGF largely occurs in the extracellular space with the involvement of a complex protease cascade.

3C Materials and Methods

Animals Six months old Fischer-344 rats were used in this study. Efforts were made to minimize the number of animal used and their suffering. All procedures were approved beforehand by the Animal Care Committee of McGill University and followed the guidelines of the Canadian Council on Animal Care.

Perfusion System and Release Experiments Animals were decapitated, the cerebral cortex was rapidly dissected out at 4ºC, cut in small blocks using a TC-2 Tissue Sectioner (Sorvall Instruments) and placed into a perfusion system (chamber containing a 8.0 µM pore size membrane, Falcon). The tissue was constantly superfused with modified Hanks buffer equilibrated with 95% O_2 and 5% CO_2 with a flow rate of 0.25 ml/min at 37ºC as described (Canossa et al., 1997). The tissue was equilibrated for 30 min before stimulation. Tissue stimulation was induced either by carbachol (100nM), glutamate (60µM) or KCl (50mM) applied over a 5-min period.

Samples were collected at 5-min intervals. Specific calcium chelators (10 μM BAPTA, 10μM BAPTA/AM, Calbiochem) were added in the perfusion buffer 45-min after the beginning of the perfusion and maintained during the second collection period until the end of the experiment (n = 6, for every condition).

Western blots ProNGF, tPA, plasminogen, neuroserpin, proMMP-9 and TIMP-1 were identified by SDS-PAGE and Western blotting from fractional release samples. For NGF and proNGF Western blot analysis, a well characterized rabbit polyclonal anti-NGF H-20 (1:750; Santa Cruz Biotechnology, CA) was applied. Mature mouse NGF (5ng; Cedarlane, Canada) was used as a standard protein. For tPA Western blots, monoclonal anti-tPA (1:1000) and single-chain tPA (3ng) as a control were used (American Diagnostica). For neuroserpin Western blotting, rabbit polyclonal anti-neuroserpin (1:1000; kindly provided by Dr. Daniel A. Lawrence, Maryland) and neuroserpin (6ng; kindly provided by Dr. David A. Lomas, Cambridge UK) were used. For plasminogen Western blots, rabbit anti-mouse plasminogen 1:500 (Molecular Innovations) was used. ProMMP-9 was detected with rabbit anti-MMP-9 (1:1000; H-129, Santa Cruz Biotechnology) and TIMP- 1 with goat anti-TIMP-1 (1:500; R-18, Santa Cruz Biotechnology). Rat cerebral cortex homogenates containing 30μg protein/well were loaded as controls.

Enzymatic assays For proNGF maturation assays, superfusate samples of carbachol-stimulated tissue were rapidly centrifuged at 4ºC. Supernatants were treated for 1h at 37ºC for maturation assays with the following enzymes: mouse MMP-2 (2 μg/μl R&D System), MMP-7 (2.1μg/μl Calbiochem), mouse MMP-9 (2 μg/μl R&D System), proMMP-9 (2μg/μl Oncogene, San Diego, CA), mouse plasmin (Molecular Innovations, Inc., Southfield, MI, USA), and α2- antiplasmin (5μg/μl Sigma). The cleaved products were proved by Western blotting with an antibody to NGF (H-20; Santa Cruz Biotechnology, Inc.). Every condition was repeated 3 times. NGF degradation assays utilized purified mature mouse NGF (Cedarlane, Canada). ProMMP-9 or MMP-9 were pre-incubated for 1h at 37ºC with either 2 μg/μl plasmin or 1.6 μg/μl of mouse plasminogen (Molecular Innovations Inc) and 2 μg/μl of mouse tPA (American Diagnostica) in order to induce their activation. NGF degradation reactions were performed for 1h at 37ºC. GM6001 and the GM6001 negative control were both applied at 1 μg/μl (Calbiochem). The results of the enzymatic reaction were proved by Western blotting with anti-NGF H-20.

Immunostaining and Confocal Microscopy Brain slices were processed as described by us previously (Bruno et al., 2004). Briefly, rats were anesthetized and perfused trancardially, and the brain tissue removed and post-fixed. Thirty five µm-thick free-floating sections were incubated for 1h with the corresponding blocking sera at 10% in PBS-Triton X-100 (0.1%) and incubated overnight at 4ºC with a monoclonal antibody against tPA (1:50, American Diagnostica, Inc.), rabbit anti-proNGF (1:100, Alomone Lab), rabbit anti-mouse-plasminogen (1:100, Molecular Innovations, Inc.), rabbit anti-neuroserpin (1:100, Dr. Daniel A. Lawrence, American Red Cross, Maryland), rabbit anti-MMP-9 (1:100 Santa Cruz Biotechnology) and goat anti-TIMP-1(Santa Cruz Biotechnology). After washing, the sections were incubated for 2 hr at room temperature with the corresponding secondary antibodies: goat anti-mouse biotinylated IgG (1:200, Vector Laboratories Inc) followed by strepto-avidin Alexa 488 conjugate (1:200, Molecular Probes) and donkey anti-rabbit coupled with rhodamine red (1:100, Jackson ImmunoResearch). The immunoreactive sites were examined with an LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

Surgical procedures and intracortical administration Rats were anesthetized and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Cannulae (Alzet 2001, 24µl/day) were implanted into the parietal cerebral cortex, bilaterally, one side receiving the experimental compound while the other received the control solution for 72 hours, using the following coordinates from Bregma: anteroposterior, -1.3 mm; lateral, ± 4.0 mm; vertical, 1.6 mm. The cannulae were connected to sterile coiled polyethylene tubing filled with an air-oil spacer at the pump end. Rats receiving vehicle (n=4) had pumps filled with phosphate-buffered artificial CSF (150 mM NaCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM K_2HPO_4, 10 mM glucose, and 0.001% rat serum, pH 7.4). Some rats (n=4) received a total dose of 36 µg of neuroserpin, 0.5µg/µl diluted in saline vehicle. The same protocol was followed for GM6001 treated rats. Both, GM6001 and the GM6001 negative control (100mg/kg) (Wang and Tsirka, 2005) were diluted in artificial CSF.

3D Results

Previous studies have demonstrated two different neuronal NGF release mechanism, the constitutive and the activity-mediated secretion (Blochl and Thoenen, 1996). To determine whether proNGF or mature NGF, or a combination of the two, is released from mature CNS neurons, we superfused small slices of the frontal and parietal cerebral cortex of young Fisher 344 rats. To test the constitutive secretion mechanism, the tissue was superfused at 37°C for 60 min without stimulation, and collecting samples were analyzed by Western blot. Neither proNGF nor NGF was detectable under these conditions in our experiments. To test the activity-mediated neuronal secretion, after 30 min of tissue stabilization, the cortical tissue was stimulated with carbachol (100nM), glutamate (60μM) or KCl (50mM), and superfusates were analyzed by Western blot. Contrary to the results obtained under nonstimulation conditions, our results consistently demonstrate the presence of a single immunoreactive 40-kDa band, after KCl or transmitter receptor stimulation, corresponding to proNGF. Contrary to our expectations, NGF (14-kDa) was undetectable in these conditions (**Figure 3-1A**). These results indicate that the precursor proNGF, instead of mature NGF, is the molecular form preferentially released by neurons in an activity-dependent manner.

To determine whether this activity-dependent mechanism of proNGF release was reliant on intracellular or extracellular calcium, we applied at the second carbachol (100nM) stimulation, plasma membrane permeable (BAPTA/AM), or plasma membrane impermeable (BAPTA) calcium chelators. The neutralization of intracellular calcium before the second carbachol stimulation with BAPTA/AM resulted in the total blockage of the second proNGF peak (**Figure 3-1B**). On the other hand, when the non permeable calcium chelator, BAPTA, was included in the perfusion buffer, the second stimulus-coupled proNGF secretion was unimpeded (**Figure 3-1C**). These results strongly indicate that the stimulus coupled release of proNGF is independent of extracellular calcium but tightly dependent on intracellular calcium stores.

We next investigated how proNGF is converted to nature NGF in the extracellular milieu upon its stimulus-coupled neuronal release. A mechanism involving plasminogen and the tissue plasminogen activator (tPA) was suspected because it has been shown that tPA plays a key role in synaptic plasticity (Qian et al., 1993)and, more recently, that tPA activation of plasmin for the cleavage of precursor BDNF into mature BDNF is essential for the formation of hippocampal long-term potentiation (Pang et al., 2004). To establish whether tPA and plasminogen also are

released from the cerebral cortex in an activity dependent manner, we stimulated cortical tissue samples with carbachol, glutamate, or KCl, as above. In these experiments, we consistently found tPA immunoreactive material in the superfusate after neuronal stimulation with a secretion pattern similar to that of proNGF (**Figure 3-1 D–F**). We also found that the stimulus-coupled tPA release also depended on intracellular calcium (**Figure 3-1E**) and independent of the presence of extracellular calcium (**Figure 3-1F**). To localize the cell sites containing proNGF and tPA, we used confocal microscopy to investigate both proNGF and tPA immunoreactive sites by using highly specific antibodies. A close colocalization of the proneurotrophin with tPA in numerous pyramidal neurons of the rat cerebral cortex was revealed (**Figure 3-1G**), indicating that both the substrate proNGF and the enzymatic cleavage complex would be liberated on demand from the same or similar neuronal stores and by analogous release mechanisms. Such a system would require the extracellular availability of the tPA substrate and an inhibitory loop to terminate the convertase reaction. The obvious tPA substrate is the inactive zymogen plasminogen. tPA converts plasminogen into the active protease plasmin, which ultimately cleaves proNGF into mature NGF.

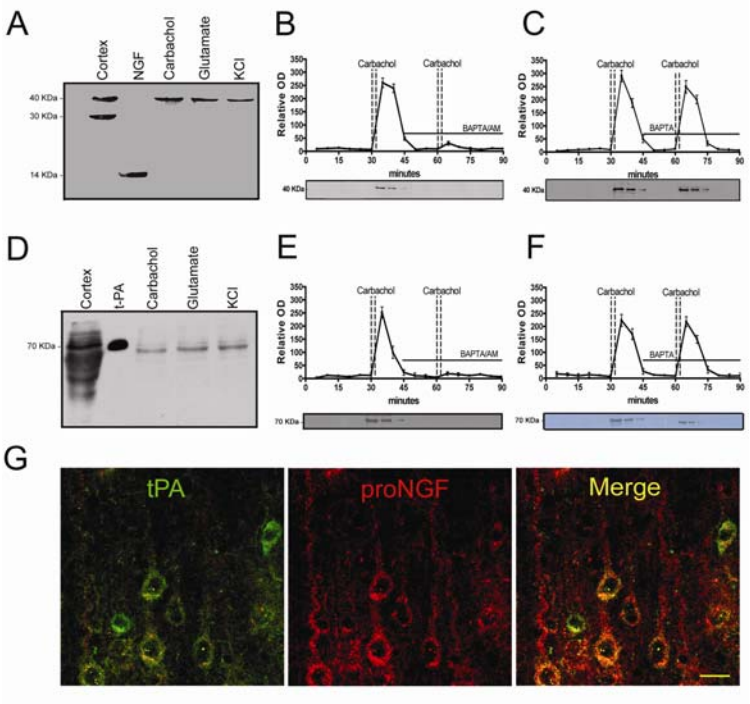


Figure 3-1 Neuronal colocalization and stimulus-coupled release of proNGF and tPA

Western blots demonstrating proNGF (**A**) and tPA (**D**) released from cerebral cortex after stimulation (see Materials and Methods). First lane illustrates immunoreactive bands from cortical homogenates. In the second lane, 5ng NGF (**A**) or 3ng tPA (**D**) are loaded as control. Time course

of proNGF (**B**) and tPA (**E**) released from cerebral cortex tissue. Activity-dependent release of neuroactive proteins was induced by two consecutive carbachol stimulations. The presence of the intracellular calcium chelator, BAPTA/AM (10 μM), in the superfusion buffer inhibited the release of proNGF (**B**) and of tPA (**E**) but the presence of the extracellular calcium chelator, BAPTA (10 μM), did not affect proNGF (**C**) or tPA (**F**) release (mean ±SEM). (**G**) Localization of tPA (green) and proNGF (red) in cortical pyramidal neurons; colocalization illustrated with merged images (yellow). (Scale bar: 20μm.)

A likely endogenous negative regulator for this system would be neuroserpin, a member of the serine proteinase inhibitor family, which is known to be secreted from axonal growth cones of the CNS, where it is thought to control tPA activity (Barker-Carlson et al., 2002;Hastings et al., 1997;Osterwalder et al., 1998). We further investigated whether plasminogen and neuroserpin also could be released from the cerebral cortex upon neuronal activation. We found that plasminogen and neuroserpin (the endogenous brain inhibitor of tPA) both were released upon neuronal stimulation with carbachol (**Figure 3-2A** and **B**) in an intracellular calcium dependent-manner (**Figure 3-8 A** and **B**, which is published as supporting information on the PNAS web site).

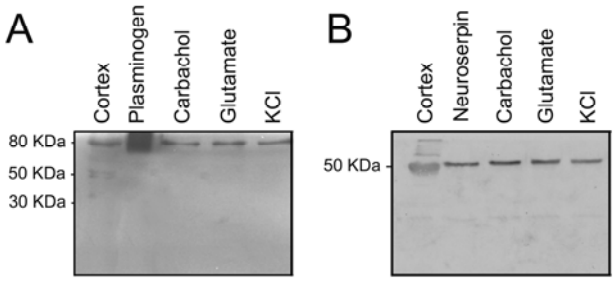


Figure 3-2 Plasminogen and neuroserpin release conditions

Western blots of plasminogen **(A)** and neuroserpin **(B)** released after carbachol, glutamate and KCl stimulation. The first lane illustrates immunoreactive bands from cortical homogenates; the second lane, positive controls for mouse plasminogen (10ng) and neuroserpin (6ng), respectively.

In agreement with previous reports (Hastings et al., 1997;Osterwalder et al., 1998;Tsirka et al., 1997), immunocytochemical investigations by using confocal microscopy revealed that plasminogen and neuroserpin also were colocalized in pyramidal neurons (**Figure 3-3 A** and **B**). Taken together, these results indicate the presence of a storage pool of proNGF, plasminogen, tPA, and neuroserpin in CNS cortical neurons, which is ready to be liberated to the extracellular space in response to neuronal activity. Thus, our investigations provide direct evidence for the activity-dependent release of the neurotrophin precursor protein (proNGF) and that of the protease cascade complex required for its maturation in the extracellular space. The rate of proNGF to mature NGF conversion appears to be regulated by the endogenous tPA inhibitor neuroserpin.

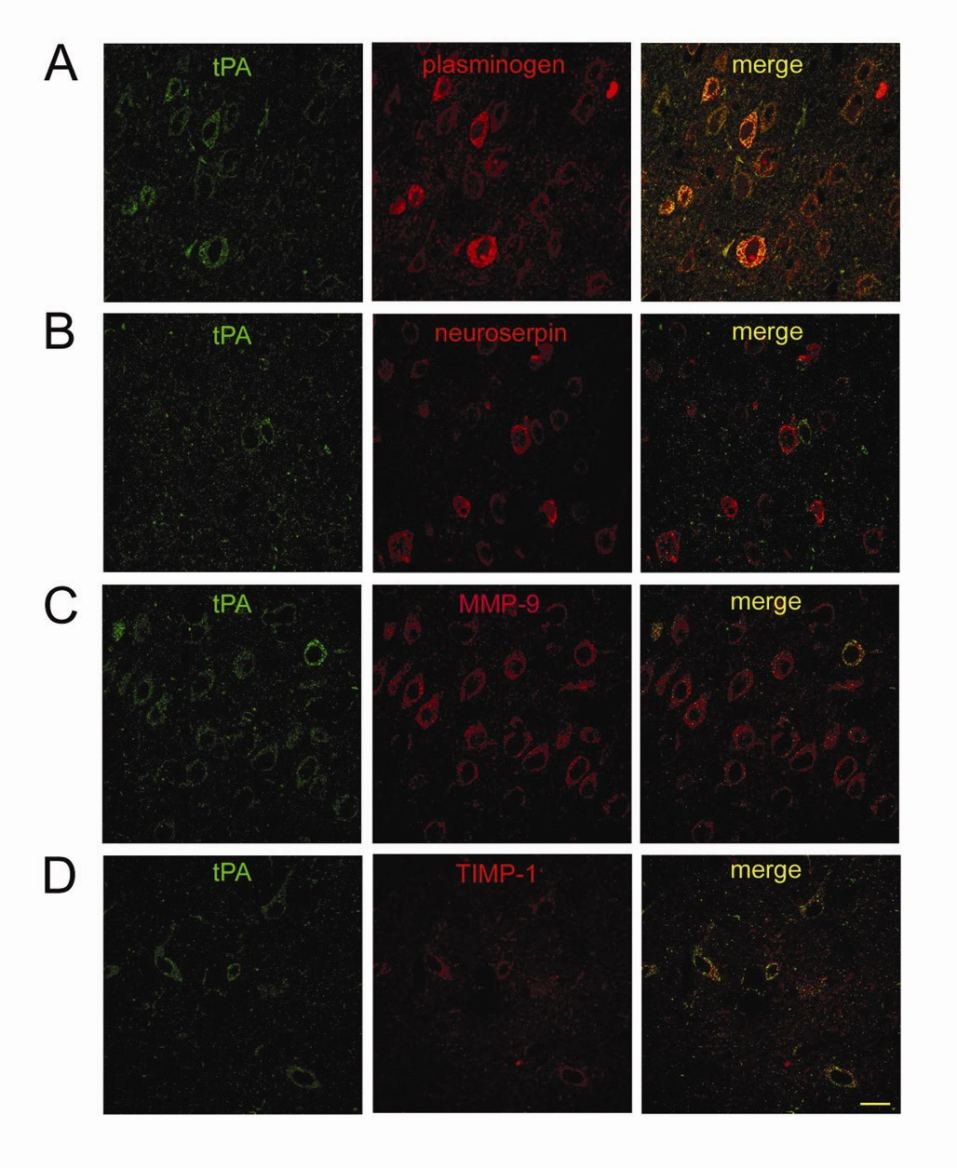


Figure 3-3 Colocalization of tPA with plasminogen, neuroserpin, proMMP-9 and TIMP-1 at pyramidal neurons

(Left) tPA immunolocalization is shown in green. (Center) Immunolocalization of plasminogen, neuroserpin, proMMP9, and TIP-1 are shown in red. (Right) Merged images with tPA on left column; yellow indicates co-localization in the same neuron. (Scale bar: 20μm.)

Both plasmin and some members of the matrix metalloproteinase (MMP) family have been suspected of being responsible for the conversion of proNGF into mature NGF (Lee et al., 2001). In neurons, several components of the MMP family are expressed. Their proteolytic activity is controlled by endogenous tissue inhibitors (TIMP). The major targets of the TIMP/MMP system are proteins of the extracellular matrix. The MMPs are produced and released by cells in an inactive MMP (proMMP) form, and its activation is controlled by a cascade of steps involving other MMPs and the plasmin system (Dzwonek et al., 2004). To confirm the presence of these proteins in the cerebral cortex and to ascertain whether MMP-9 and TIMP-1 follow the same release pattern as proNGF and that of the tPA/plasminogen protease cascade members, the same immunocytochemical and neurochemical protocols described above were applied, showing that MMP-9 and TIMP-1 colocalized in neocortical pyramidal neurons (**Figure 3-3C** and **D**). As we found for the tPA/plasminogen protease cascade, proMMP–9 and TIMP-1 were identified in the stimulated superfusate samples, indicating that both are released by neurons (**Figure 3-4A** and **B**) in a manner dependent on intracellular, but independent of extracellular, calcium upon carbachol stimulation (see **Figure 3-8E–H** below)

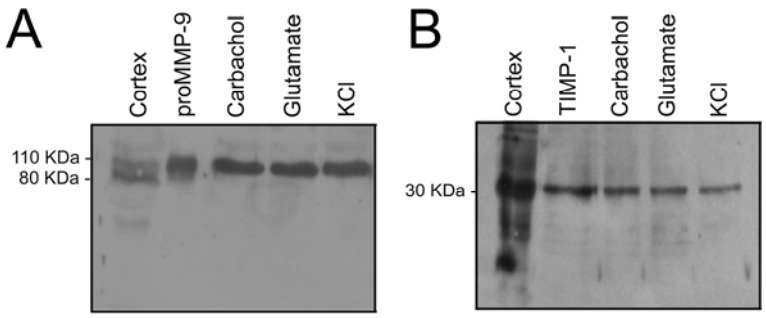


Figure 3-4 Activity-dependant release of proMMP-9 and TIMP-1

Representative Western blots of proMMP-9 **(A)** and TIMP-1 **(B)** released by cerebral cortical tissue upon stimulation with carbachol (100nM), glutamate (60μM) or KCl (50mM).

ProNGF, tPA, plasminogen, neuroserpin, proMMP-9, and TIMP-1 were released in an intracellular calcium-dependent manner after stimulation with either glutamate (60μM) or KCl (50mM) (data not shown). It is interesting that all these proteins shared these unusual stimulus-

coupled release properties in contrast to the extracellular calcium regulated release of conventional transmitters and neuroactive peptides, such as substance P. In our tissue, superfusion model cortical substance P release followed the ‘‘canonical’’ extracellular dependency reported in previous studies (Iversen et al., 1976). Thus, evoked substance P release was completely abolished in the absence of calcium in the superfusion buffer or by including the extracellular calcium chelator BAPTA (see **Figure 3-9** below, which is published as supporting information on the PNAS web site).

To clarify which of these proteases is capable of converting the endogenously produced and released proNGF into mature NGF, we incubated superfusate samples from stimulated cortical tissues with diverse convertase candidates. Under these conditions, only plasmin was capable of converting endogenous proNGF to mature NGF (see **Figure 3-5A**). The application of the inhibitor α2-antiplasmin to plasmin containing samples blocked the conversion of proNGF to mNGF confirming the specificity of such reaction (see **Figure 3-5A**, lane 7). A further demonstration of the specificity of this metabolic network (the convertase tPA, the tPA substrate plasminogen, the inhibitor neuroserpin) is provided by our finding that none of them, on their own, were capable of activating the conversion of proNGF to its mature form (see **Figure 3-5B**). This conversion only took place in the presence of tPA plus plasminogen (**Figure 3-5B**, lane 5), and it was completely blocked when the tPA/plasminogen complex was incubated in the presence of neuroserpin (**Figure 3-5B**, lane 6).

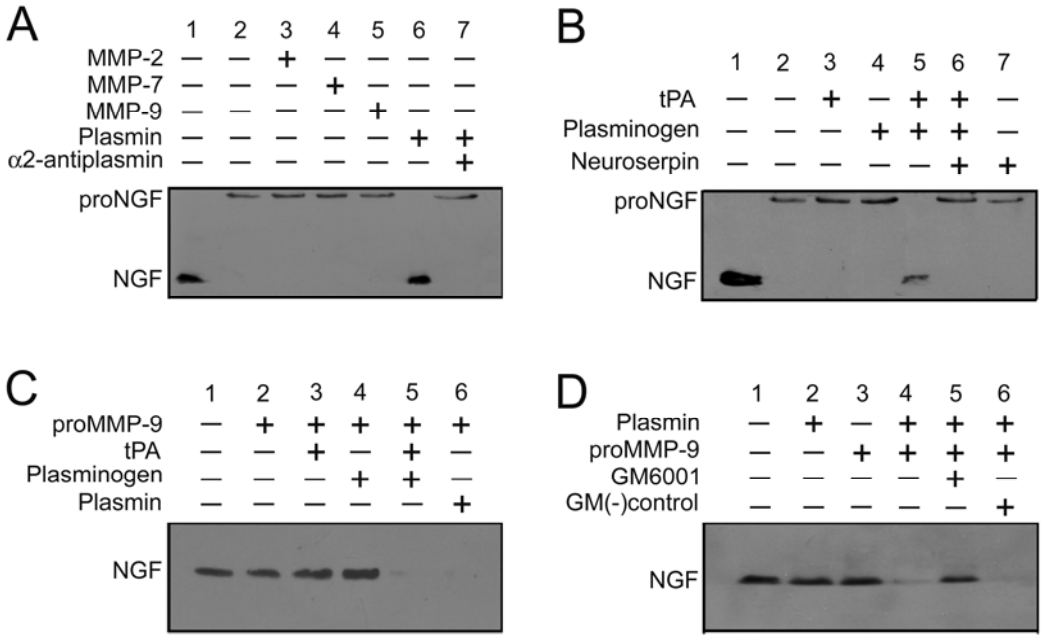


Figure 3-5 Maturation of released proNGF and degradation of mature NGF

(**A**) Plasmin-induced conversion of endogenously released proNGF into NGF. The plasmin protease activity on proNGF was inhibited by α2-antiplasmin. MMP-2, MMP-7, and MMP-9 failed to convert proNGF into NGF. (**B)** tPA, plasminogen, or neuroserpin alone (lanes 3, 4 and 7, respectively) were not able to convert proNGF on their own; it was only when both plasminogen and tPA were present that the conversion took place (lane 5). The convertase activity was inhibited by neuroserpin (lane 6). NGF (A, B, and D, lane 1) and proNGF (A and B, lane 2) are reference controls (**C**). MMP-9 alone was not able to degrade NGF (B, lane 2) whereas NGF degradation occurred in the presence of MMP-9, tPA, and plasminogen (lane 5) or with MMP-9 plus plasmin (lane 6). (**D**) Neither plasmin nor proMMP-9 alone degraded NGF (lane 2 and 3, respectively). The plasmin activated MMP-9 degradation of NGF (lane 4) was blocked by the metalloproteinase inhibitor GM6001 (lane 5) but not by the negative control GM6001 (lane 6).

During the course of our investigations, we found that the newly generated mature NGF disappears promptly from the extracellular space. To determine whether the enzymatic components actively released upon neuronal stimulation could degrade NGF, we examined their effects on mature NGF alone and in combination with putative proteases (**Figure 3-5C**). In these experiments, neither latent inactive MMP-9, plasmin, plasminogen, nor tPA alone were capable of provoking the degradation of mature NGF. However, our results showed that when MMP-9 is activated by plasmin, NGF degradation takes place (**Figure 3-5C**, lanes 5 and 6). To determine whether the NGF degradation was mediated by the activated MMP-9, we incubated NGF with plasmin-activated MMP-9 in the presence of the broad-spectrum metalloproteinase inhibitor, GM6001, or in the presence of the GM6001 negative control. The results obtained (**Figure 3-5D**) indicate that the degradation of NGF is blocked by the MMP inhibitor, GM6001, but not by the inactive congener. These observations support the notion that plasmin-activated MMP-9 is the most likely protease responsible for the rapid enzymatic inactivation of remnant, unused mature NGF.

An obvious question emerged from these results: Do these proposed mechanisms have an in vivo relevance? To test the in vivo impact on the proNGF/NGF extracellular conversion to mature NGF and its eventual degradation by the tPA/plasmin/MMP-9 proteolytic cascade, we decided to disrupt this cascade locally in the cerebral cortex. Thus, we intervened at two levels of the proposed cascade. First, we blocked plasmin formation by inhibiting tPA action by infusing the tPA endogenous inhibitor, neuroserpin, and, second, by inhibiting the activated MMP-9 by infusing the broad spectrum MMP inhibitor, GM6001. The first approach should define, in an in

vivo situation, the relevance of tPA/plasminogen activation in the conversion of endogenous proNGF into its mature form, whereas the second should define whether endogenous MMP-9 is able to provoke the degradation of mature NGF. The continuous, unilateral infusion of neuroserpin into the cerebral cortex of young rats for 72 h provoked a several fold increment in proNGF tissue levels when compared with the contralateral, vehicle injected, side (see **Figure 3-6A**). In these experiments, no change was observed in cytoskeletal ß-tubulin or neuropsin, a well characterized secretory serine protease present in pyramidal neurons and released in an activity-dependent manner for the cleavage of extracellular matrix proteins (Shimizu et al., 1998). The levels of proNGF returned to basal level 48 h after the neuroserpin infusion was stopped (data not shown). On the other hand, the unilateral infusion of the MMP-9 inhibitor, GM6001 in the cerebral cortex of young rats for 72 h caused a dramatic rise of mature NGF content with a significant decrease in proNGF levels when compared with values from the contralateral side, which received either the GM6001 negative control or saline (see **Figure 3-6B**). To investigate whether GM6001 could have a direct effect on proNGF maturation, released proNGF was incubated at 37°C. The MMP-9 inhibitor did not show any effect on proNGF levels or in the rate of conversion of proNGF into NGF (data not shown). Therefore, the marked change in the proNGF/NGF ratio in vivo caused by the MMP-9 inhibition of the NGF degradation suggests that the NGF accumulation might unleash a negative feedback mechanism on the proNGF production. The GM6001 effect on NGF degradation disappeared 48 h after discontinuation of treatment, whereas the proNGF/NGF levels returned to basal levels (data not shown).

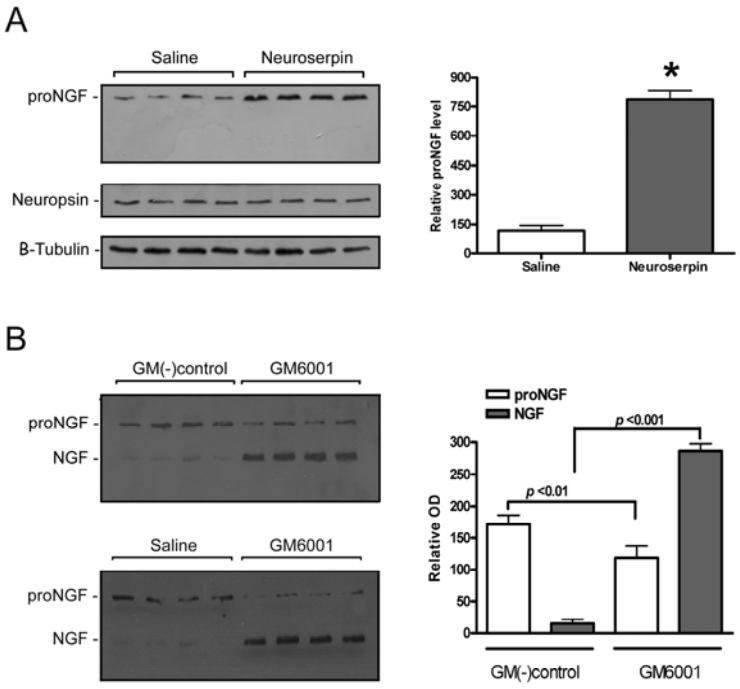


Figure 3-6 The cortical proNGF/NGF ratio is changed by the application of neuroserpin or MMP-9 inhibitors

(**A**) Increased amount of cortical proNGF in neuroserpin-treated animals (mean ±SEM; $P < 0.001$; t test). (**B**) The inhibitor of matrix metalloproteinase GM6001 significantly increased the cortical NGF ($P < 0.001$) and decreased proNGF ($P < 0.01$) when compared with the GM6001 negative or saline control-treated (mean ± SEM). The levels of neuropsin, a serine protease secretory protein present in pyramidal neurons and ß-tubulin were not altered in these experiments.

3E Discussion

In human, mouse and rat brain tissue, little or no mature NGF is detected (Fahnestock et al., 2001). The lack of mature NGF signals can be explained, in part, by the rapid internalization and retrograde transport in the form of NGF–TrkA complexes within signaling endosomes, as elegantly demonstrated by Grimes and coworkers (Grimes et al., 1996). However, our results strongly indicate that newly generated NGF, which is not membrane- bound to TrkA receptors nor rapidly internalized, is promptly removed from the extracellular space by activated MMP-9 via the enzymatic degradation of mature NGF.

NGF is generated from a precursor molecule, proNGF, which undergoes processing (conversion) to generate mature NGF. For more than two decades, it has been assumed that the

mature NGF form accounts for the neurotrophin's biological activity, including cell survival, neurite outgrowth, and neuronal differentiation. Likewise, it has been assumed that proNGF had little or no biological action. However, Fahnestock et al. (Fahnestock et al., 2001) have recently shown that proNGF is abundant in CNS tissue, whereas mature NGF is undetectable, suggesting that proNGF either may have a function distinct from its role as a precursor or that the precursor is processed to mature NGF intracellularly before secretion to the extracellular milieu. Hempstead et al. (Lee et al., 2001) generated a cDNA construct of a mutated, furin cleavage-resistant form of proNGF to ''impair intracellular proteolysis''. This artificial, recombinant, transgene form of proNGF was shown to bind preferably p75 neurotrophin receptor and promote apoptosis in primary superior cervical ganglion neurons and smooth muscle cells, in vitro. In contrast, Fahnestock et al. (Fahnestock et al., 2004b), by using a different recombinant cleavage-resistant form of proNGF, showed that this precursor exhibits neurotrophic activity similar to mature NGF, but is ~5-fold less active. This artificial proNGF binds to TrkA but is less active in promoting phosphorylation of TrkA and its downstream signaling effectors, Erk1/2, in PC12 and NIH 3T3 cells.

Our study provides direct evidence for the differential activity dependent release of endogenous precursors of mature neurotrophin in the fully differentiated and adult CNS. A similar activity mediated secretion of NGF was found by Thoenen et al. (Blochl and Thoenen, 1995;Blochl and Thoenen, 1996), where the analysis was performed under similar release conditions in native hippocampal slices and from NGF-cDNA transfected hippocampal neurons. However, in those experiments, NGF was quantified by highly sensitive ELISA, which does not discriminate mature NGF from the proNGF forms. Although the less-sensitive Western blot analysis does not allow to accurately investigating the so-called constitutive (spontaneous) release, it allows us to define with precision the molecular NGF forms involved in the activity-dependent release of this neuroptrophic peptide. This work reports direct evidence of an activity-dependent release of the components of the proteolytic cascade responsible for the extracellular conversion of proNGF to mature NGF. It also demonstrates the mechanism ultimately leading to in vivo enzymatic inactivation (degradation) of mature NGF. These findings may be of great importance if pathological alterations of this cascade in the CNS cause or contribute to a lack of proper neuronal trophic support in conditions such as cerebral ischemia, seizure, and Alzheimer's disease.

All of the members of the cascade reported here are known to be highly expressed in the CNS (Hastings et al., 1997;Osterwalder et al., 1998;Tsirka et al., 1997). Thus, the gelatinase MMP-9 protein is highly expressed, predominantly in neurons of the hippocampus and cerebral cortex, where in line with our observation, it is suspected of playing a role in synaptic plasticity (Szklarczyk et al., 2002) and spatial learning (Wright et al., 2003). Its inhibitor, TIMP-1, also is present in cortical (Newton et al., 2003) and hippocampal neurons (Rivera et al., 1997). These observations are consistent with the present report of a coordinated and simultaneous release of neurotrophin precursors and corresponding proteases.

Our studies reveal that, once converted, mature NGF is enzymatically degraded by activated MMP-9. We demonstrate that neuronal stimulation releases the required inactive proMMP-9, which is activated extracellularly by plasmin. The activated MMP-9, in consequence, causes the proteolytic degradation of mature NGF. Furthermore, we were able to demonstrate that such a NGF degradative mechanism is operative in the adult CNS in vivo. The combined in vivo experiments illustrates that it is possible, at least experimentally, to manipulate the ratio of proNGF to mature NGF in the adult CNS. This observation suggests previously undescribed therapeutic opportunities for conditions in which the up-regulation of endogenous mature NGF would be desirable.

In summary, the present results indicates the existence of a CNS protease cascade responsible for the conversion of proNGF into mature NGF and, ultimately, for its degradation within extracellular space. These events are schematically illustrated in **Figure 3-7 A** and **B**). Our findings support the view that the protease cascade, as well as the neurotrophin precursor, is stored in neuronal comportments from which it is simultaneously liberated with the NGF precursor upon functional demand after neuronal stimulation. These observations are consistent with the idea that metabolic networks do not interact at random but instead in a tightly coordinated manner (Jeong et al., 2000).

The deregulation of the protease cascade controlling proNGF conversion and NGF degradation should open new vistas on trophic responses in the adult CNS and in pathological circumstances, such as seizure, cerebral ischemia, andAlzheimer's disease, where an up-regulation of proNGF is well documented (Fahnestock et al., 2001). This pathway might be also of relevance in chronic arthritic pain, where NGF production is a prominent proinflammatory factor (Iannone and Lapadula, 2003).

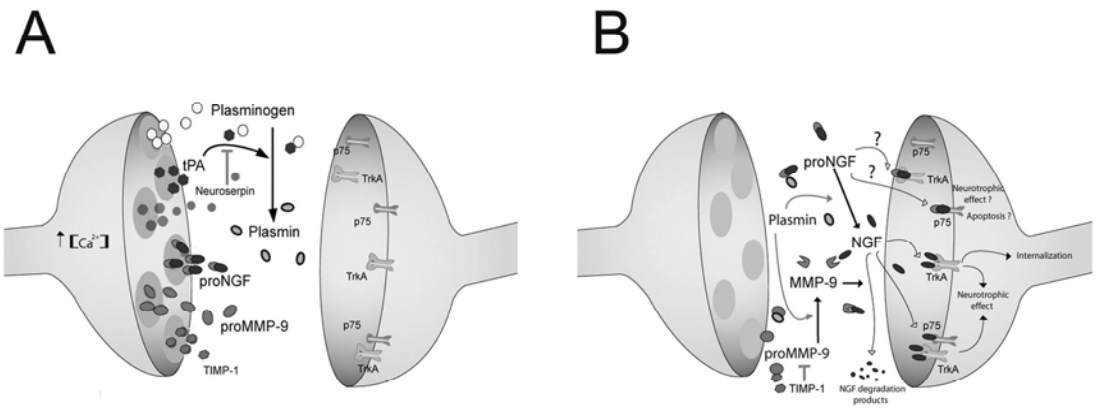


Figure 3-7 Schematic representations of events leading to proNGF conversion into mature NGF and its degradation

Neuronally stored proNGF, plasminogen, tPA, neuroserpin, proMMP-9, and TIMP1would be released into the extracellular space upon neuronal stimulation. Released tPA would induce the conversion of plasminogen to plasmin, where its activity is tightly regulated by secreted neuroserpin. The generated plasmin would convert proNGF into mature NGF and activate proMMP-9 into active MMP-9. Mature NGF would interact with its cognate receptors (TrkA and $p75^{NTR}$) or suffer degradation by activated MMP-9.

3F Acknowledgments

This work was supported by Canadian Institute of Health Research Grant MOP 62735. We thank Dr Alfredo Ribeiro-da-Silva for advice on confocal microscopy, Adriana Ducatenzailer and Vanessa Partridge for technical support, and Sid Parkinson for editorial assistance. The authors are most grateful to Dr Elena Miranda and Dr David Lomas, Cambridge Institute for Medical Research, University of Cambridge, UK, for the generous provision of neuroserpin and to Dr. Daniel A. Lawrence, American Red Cross, Maryland, USA for providing the anti-neuroserpin antibody. MAB received a fellowship from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina and ACC is the holder of the Charles E. Frosst Merck Chair of Pharmacology, McGill University.

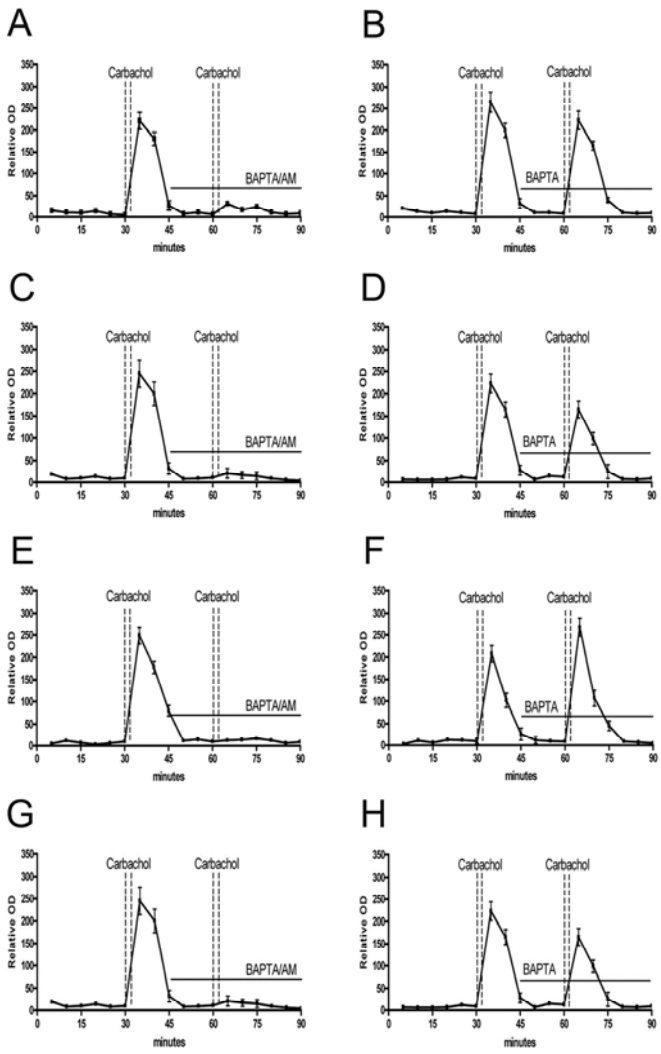


Figure 3-8 The intracellular calcium chelator BAPTA/AM inhibited the activity-dependent release of endogenous plasminogen (A), neuroserpin (C), proMMP-9 (E) and TIMP-1 (G) after the second carbachol stimulation, whereas the release of these neuroactive proteins was not affected by the presence of the extracellular calcium chelator BAPTA (B, D, F and H respectively). Every condition was repeated six times, and data were presented as the mean ±SEM.

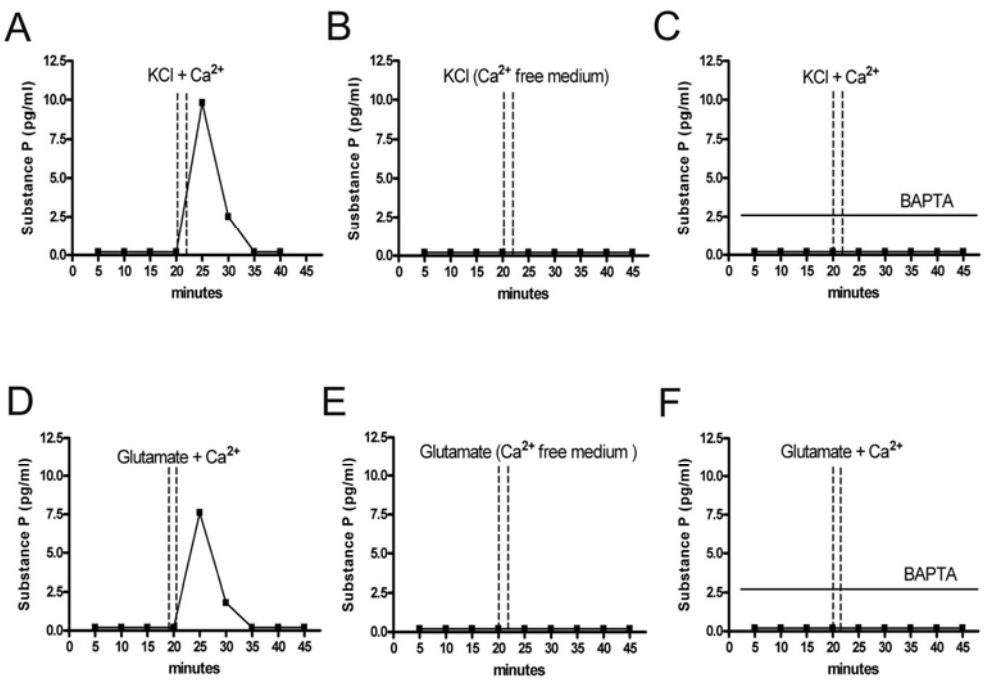


Figure 3-9 Substance P released by cerebral cortical tissue upon stimulation with KCl (50mM) (**A**) and Glutamate (60μM) (**D**). The absence of the extracellular calcium (calcium-free superfusion medium) **(B** and **E)** or the presence of the extracellular calcium chelator, BAPTA (10 μM) **(C** and **F)** abolished the stimulus-coupled substance P release. Data represents the Mean ± SEM.

Connecting Text: Chapter 3 to 4

In **Chapter 3**, we have described a novel metabolic pathway responsible for the NGF maturation and ultimately its degradation. In that study, we have also provided the first direct evidence that proNGF, and not mature NGF, is the molecular form released in an activity-dependent manner in the adult CNS. This molecule is significantly elevated in the AD brain. Our results suggest that mature NGF is difficult to detect in the CNS, probably because upon proNGF release, mature NGF binds TrkA receptors and is rapidly internalized. The remaining, unbound, NGF it is most likely degraded by MMP-9.

In view of the above, our next step was to investigate to what extent decreased proNGF maturation, and exacerbation of the proteolytic cascade responsible for NGF degradation, could explain the observed proNGF accumulation in AD brain. These investigations revealed that there is indeed an altered metabolism of NGF in the AD brain, as well as important post-translational changes in proNGF protein.

CHAPTER 4

Aβ-induced NGF trophic disconnection in Alzheimer's disease

Bruno MA, Leon WC, Fragoso, G, Mushynski WE, Almazan, G and Cuello, AC

Submitted

4A Abstract

The causative mechanisms for the atrophy of forebrain cholinergic neurons in Alzheimer's disease remain undefined. Consequently, we investigated the status of the NGF maturation and degradation protease cascades in Alzheimer's brains. We found evidence for a diminished conversion of the NGF precursor molecule to its mature form and for increased NGF-degradation in Alzheimer's brain samples, resulting in an up-regulation of proNGF levels. We further observed that Aß-oligomers injections provoked a similar up-regulation of proNGF levels in the rat hippocampus. The Aβ-induced activation of inflammatory microglia was accompanied by hyperactivity of the NGF degrading enzyme MMP-9, and increased inducible nitric oxide synthase which facilitated CNS accumulation of peroxynitrite-modified proNGF. Finally, we observed both *in vitro* and *in vivo* that oxidative modifications of NGF notably diminished its biological activity. These combined Aβ effects on NGF metabolism would explain the well known cholinergic atrophy found in Alzheimer's disease

4B Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by a progressive loss of memory and deterioration of higher cognitive functions. The remarkable vulnerability of the cholinergic system to the amyloid ß (Aß) pathology is not clearly understood. A profound loss/atrophy of basal forebrain cholinergic neurons (BFCN) innervating the hippocampus and the cerebral cortex contributes to the dementia-related cognitive decline and remains a main target for therapeutic intervention in AD. However, why this transmitter system is particularly vulnerable in the Alzheimer's neuropathology remains unclear.

Experimental work in transgenic models of the AD-like amyloid pathology would indicate that this cholinergic depletion is driven by the progressive CNS Aß accumulation of Aβ in the CNS (Bell et al., 2006;Wong et al., 1999). Furthermore, multiple lines of evidence indicate that accumulation of neurotoxic oligomeric aggregates of Aß may be a central event in the pathogenesis of AD (Walsh and Selkoe, 2007). It has been shown that soluble Aß species are neurotoxic at low concentrations, induce inhibition of long-term potentiation (Walsh et al., 2002), disrupt synaptic plasticity (Klyubin et al., 2005;Rowan et al., 2004) and provoke cognitive

dysfunction in rodents (Cleary et al., 2005b). However, there is no direct experimental evidence explaining why the Aß burden is particularly harmful to BFCN.

The maintenance of the biochemical-morphological phenotype of BFCN is highly dependent on the supply of endogenous NGF (for review see Cuello, 1996;Thoenen, 1995). Furthermore, the steady state number of cholinergic synapses in the cerebral cortex is highly dependent on minute amounts of endogenous NGF (Debeir et al., 1999). Nevertheless, in AD there is no evidence for diminished translational activity or for a lack of the neurotrophins but, paradoxically, an up-regulation in the levels of proNGF (NGF precursor protein) has been documented (Fahnestock et al., 2001).

Recently, we have gathered experimental evidence in rodents for the involvement of a protease cascade responsible for both the maturation of NGF from proNGF to mature NGF and for the degradation/inactivation of NGF in the extracellular space, due to the coordinated release and activation of the matrix metalloproteinase 9 (MMP-9).We further provided evidence that this mechanism is also operative in *in vivo* conditions, as CNS application of inhibitors of converting and degrading enzymes leads to dramatic alterations in the tissue levels of either proNGF or mature NGF (Bruno and Cuello, 2006).

In this present study, we hypothesize that amyloid ß-induced alterations in this cascade causes or contributes to the disruption of trophic support of BFCN in the Alzheimer's brain. Specifically, we propose that the cholinergic system is compromised by amyloid ß burden which interferes with the rate of NGF maturation and degradation and further increases NGF oxidation by nitrogen reactive species. Therefore, we investigated the state of the components of the NGF maturation/degradation cascade in cortical human AD and in non-demented age-matched control brain samples. We further investigated if the changes observed were directly due to the Aß burden. For this, we infused soluble amyloid-ß oligomers into the rat hippocampus, triggering an inflammatory response as expressed by up-regulation of inducible nitric oxide synthase (iNOS), enhanced MMP-9 activity and proNGF/NGF oxidation. We finally investigated *in vitro* whether the observed Aß-induced peroxynitration of NGF alters the trophic support of dorsal root ganglia neurons. These investigations point toward a direct involvement of Aß in the disruption of trophic support of NGF-dependent neurons by NGF. Our *in vivo* experiments, comparing native NGF with peroxynitrite-modified NGF revealed a decreased capacity of the latter to induce TrkA phosphorylation and, consequently, diminished biological activity.

4C Material and Methods

Study Subjects. Frozen post-mortem tissue samples obtained from the Netherlands Brain Bank, Netherlands Institute for Brain Research were used in these investigations. The clinical history of patients is archived at the Netherlands Brain Bank. This project has been approved by the independent Ethical Committee of the Netherlands Brain Bank and by the McGill University Review Board, following the Tri-Council Policy on Ethical Conduct for Research Involving Humans. Efforts were made to minimize the number of animals used and their suffering. All procedures were approved beforehand by the Animal Care Committee of McGill University and followed the guidelines of the Canadian Council on Animal Care.

Tissue Processing Frozen tissues (from middle frontal gyrus) of non-demented (n=7) and Alzheimer's disease (n=7) patients were homogenized without thawing in 1 ml of homogenizing buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidep P-40, 0.1% SDS, 0.1% deoxycholic acid, 2µg/ml of aprotinin, 2µg/ml of leupeptin, 100µg/ml PMSF, pH 7.4). The homogenates were incubated for 15 min on ice and then centrifuged at 14,000g for 30 min at 4ºC, and the supernatants protein contents were determined using the DC protein assay as described by the manufacturer (Bio-Rad Laboratories, Hercules, California, USA). The same protocol was followed for hippocampal rat tissue.

Antibodies and Reagents Antibodies from Santa Cruz Biotechnology Inc.(California, USA) were: TrkA (H-190), NGF (H-20), Plasminogen (H-90), TIMP-1 (H-150), MMP-9 (H-129), CD40 (C-20), Neuroserpin (H-90), tPA (H-90) and iNOS (N-20). Polyclonal anti-proNGF (from Alomone Labs, Israel); mouse monoclonal anti-Nitro Tyrosine (ab24496, Abcam Inc., MA, USA); anti-phospho TrkA (Cell Signaling Technology), A11 (Gift from Dr Glabe, Irvine, CA, USA) and 6E10 monoclonal antibody from Chemicon (USA) Monoclonal anti-Neurofilament antibody (N52, Sigma-Aldrich, Oakville, ON, Canada). Secondary antibodies were from Jackson Immunoresearch Laboratories (Cedarlane, ON, Canada). Peroxynitrite and degraded-perxynitrite (Upstate,USA), recombinant proNGF (Alomone Labs, Israel) and NGF (Cedarlane Laboratories, Ontario, Canada).

Plasmin (Molecular Innovations Inc., Southfield, MI, USA), Aß (1-42) peptide and the reverse control peptide (42-1) from American Peptide Company and minocycline from Sigma-Aldrich (Oakville, ON, Canada).

Immunoprecipitation In order to obtain elution of the protein with little antibody contamination, cross-linking of the antibodies (TrkA and NGF) to Sepharose A beads (Sigma-Aldrich, Canada) was performed following Abcam online protocol (see Abcam protocols, USA). Then**,** the beads were resuspended in PBS containing 0.1% Tween-20 and incubated with 300µg of brain homogenates overnight at 4ºC under agitation. After washing the beads, loading buffer was added, loaded and run on a SDS-PAGE.

Western blot 40-100µg of each sample was separated on 8-12% polyacrylamide resolving gels. Proteins were then transferred onto nitrocellulose membranes and blocked 1-2 hours at room temperature in TBS-T (Tris buffer, 10% Tween-20) with 5% (w/v) nonfat milk powder (Carnation). Incubations with primary antibodies were performed overnight at 4ºC. After washing, membranes were incubated in HRP-conjugated secondary antibodies for 1h at RT. For detection, an ECL chemiluminescense system (Amersham-Pharmacia) was used according to manufacturer's instructions. The immunoreactive bands were determined by densitometry of the films using MCID image analysis system. Values were normalized to ß-III tubulin.

Gelatin Zymography 60µg of each sample was separated on 10% polyacrylamide gels containing 0.1% gelatin. After running, the gel was incubated in Zymogram Renaturing Buffer (Triton X-100, 25% (v/v) in water) with gentle agitation during 30 min at room temperature. After decanting the Zymogram Renaturing Buffer, gels were incubated overnight at 37ºC with gentle agitation in Developing Buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM CaCl2, 0.02% Brij 35, pH 7.6). The gels were then stained with Coomassie Blue R-250 (0.5% in ethanol 40%) for 30 min. Areas of protease activity appeared as clear bands against a dark blue background after incubation of the gel with an appropriate destining solution, Methanol: Acetic acid: Water (40: 10: 50).

Aß-oligomers preparation Aß-oligomers were prepared as described by Demuro et al., 2005. Briefly, soluble Aß-oligomers were prepared by dissolving 1.0 mg of peptide in 400 µl of

hexafluoroisopropanol at room temperature. Then, 100 µl of the resulting seedless solution was added to 900 µl double-distilled water and centrifuged for 15 min at 14,000 x *g*, and the supernatant fraction was transferred to a new tube and subjected to a gentle stream of N_2 for 5–10 min to evaporate the hexafluoroisopropanol. Finally, the samples were stirred at 500 rpm using a Teflon-coated microstir bar for 24–48 h at 22 °C. The reverse control peptide (42-1) followed the same processing.

Dot blot Using a narrow-mouth pipette, 2 µl of fresh monomeric Aß peptide and 2 µl of soluble Aß-oligomeric solution were spotted onto the nitrocellulose membrane. After drying, the membrane was soaked in 5% BSA in TBS-T (1hr, RT) in order to block non-specific sites. Then, the membrane was incubated with two different primary antibodies (A11 and 6E10 diluted 1:1000 in BSA/TBS-T) for 1h at RT. The membrane was washed (3x5min) with TBS-T and incubated with the corresponding secondary antibody conjugated with HRP (1:10000) for 30min at RT. After washing (3x5min) the membrane was incubate with ECL reagent for 1 min and expose X-ray film in the dark room.

Peroxynitrite-modified NGF and proNGF preparations Mature NGF (1µg) or recombinant proNGF (1µg) were treated with peroxynitrite in 50mM sodium phosphate buffer and 20mM sodium bicarbonate, pH 7.4, following the protocol described by Pehar et al., 2006. At the time of the experiment, peroxynitrite concentration (by absorbance at 302nm) was $\varepsilon = 1690\ M^{-1\ cm-1}$. Fresh stock solutions were prepared in 0.01M NaOH and the reaction was performed at 37ºC and peroxynitrite was added to the top of the tube (containing NGF or proNGF) 5 times and mixed by vortexing for 3s to reach the final peroxynitrite concentration of 1mM. The same procedures were applied for degraded peroxynitrite diluted in NaOH 0.01M.

Dorsal root ganglion neuron culture Purified DRG neuron cultures were prepared using methods described previously (Giasson and Mushynski, 1996). DRG neurons were obtained from Sprague-Dawley rat embryos at 15-16 days gestation, dissociated with trypsin and plated onto rat-tail collagen-coated dishes. The cultures were maintained with 25ng/ml NGF in serum-free N1 medium and treated with anti-mitotic agents to get rid of proliferating Schwann cells and fibroblasts. After 3

weeks DRG neurons are morphologically mature, displaying a profuse axonal network. The medium was replenished every 2 days.

Immunofluorescence DRG neurons plated on 12-mm glass coverslips were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature and then with methanol for 5 min at –20ºC. Afterward the cells were washed three times with PBS and blocked for 15 min in PBS containing 10% goat serum and 0.2% Triton X-100. Monoclonal anti-Neurofilament (N52, IgG1) antibody was diluted in the same solution and applied for 1hr at RT. The secondary goat anti-mouse IgG1-FITC was applied for 1hr at RT. The coverslips were mounted on glass slides and examined under a Leitz Diaplan epifluorescent microscope.

DRG number and cross-sectional measurement The number of cells determined by counting cell nuclei stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:1000) and the mean cross-sectional areas measured in the plane of the nucleus (N52-FITC-IR positive neurons) were performed on the digitized images (25x) with a computer-assisted imaging analysis system (MCID Elite; Imaging Research, Ontario, Canada). Cell counting was performed in six fields of 0.39 mm^2 and repeated with two independent cultures for each treatment (native NGF and peroxynitrite-modified NGF) and each culture period (4 DIV and 28 DIV). For neuronal mean cross-sectional areas, 30 cells were randomly chosen for every condition and expressed as mean cross-sectional area μm^2 (mean ± SEM).

Surgical procedures and Hippocampal injections Four months old males Fischer-344 rats were divided in three experimental groups: the first group of rats (n=5) were injected bilaterally into the hippocampus (following coordinates from bregma: anteroposterior -0.45mm; lateral ± 0.35mm; vertical 0.35mm) with 1µg Aß-oligomers, the second group (n=5) received bilaterally 1µg of the reverse control peptide 42-1 and the third group (n=5) was treated 24hs prior to the Aß-oligomers injections (1µg) with minocycline (IP, 50mg/kg, (Lee et al., 2006), treated daily with the same dose of minocycline (IP) during 72hs and then sacrificed. Each animal was anesthetized and placed in the stereotaxic apparatus (Davis Kopf Instruments, Tujunga, CA) and received a single injection of 3µl solution containing Aß-oligomers or the 42-1 reverse control peptide diluted in phosphate-buffered artificial CSF (150 mM NaCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM K_2HPO_4, 10 mM

glucose, and 0.001% rat serum, pH 7.4), using a Hamilton syringe pump (flow rate 0.25µl/minute). The needle was left in place for 5 min before the injection was started, and then the solutions were injected slowly over a period of 12min. The needle was left for ~2-5 min before retracting slowly to prevent backwash up the needle tract. When all injections were made, the skull was swabbed, and the skin overlying the skull was closed with surgical stitches. Animals were kept alive for 72hs, then sacrified and the hippocampus removed and homogenized. For native NGF *vs*. peroxynitrite-modified NGF intraparenchymal administration, the procedures followed were exactly as described above but animals (n=5) were sacrificed 2h after the injection. The doses administered (150ng) diluted in artificial CSF (final volume 3µl/12min) has been previously shown to produce a pronounced tyrosine phosphorylation of Trk-type proteins 2 after the injection (Venero et al., 1996).

Statistical Analysis Statistical significance between pixel values for control and AD groups was calculated using t test and bars represents mean intensities ± SEM for each group.

4D Results

Altered NGF maturation and cortical proNGF accumulation in AD brain

NGF is the most important trophic factor for the phenotypic maintenance of BFCN. Alterations in NGF and its high-affinity receptor (TrkA) have been observed in early and late-stages of AD. More specifically, increased levels of proNGF (Fahnestock et al., 2001;Peng et al., 2004) and loss of cortical TrkA (Counts and Mufson, 2005) have been found in subjects with Mild Cognitive Impairment (MCI) and AD. However, the mechanism in AD by which the Aβ pathology causes atrophy of BFCN, despite the abundance of NGF precursor proteins, remains obscure. We speculated that the pathway responsible for the NGF maturation/degradation could be involved in the Alzheimer's pathology (Bruno and Cuello, 2006). In other words, that the Aß pathology would provoke alterations of the NGF-maturation/degradation mechanism, thereby causing or contributing to proNGF accumulation, leading to the remarkable vulnerability of forebrain cholinergic neurons in AD. We therefore investigated the presence of the components of this protease cascade in human cortical tissue samples from AD and non-demented age-matched controls. We found increased levels of proNGF ($P< 0.01$; **Figure 4-1A**) and decreased levels of

plasminogen ($P< 0.05$; **Figure 4-1B**) and tissue plasminogen activator (tPA) ($P< 0.05$) in the Alzheimer brain and a correspondingly significant decrease in plasmin formation ($P< 0.001$; **Figure 4-1C**), the agent which ultimately converts proNGF into mature NGF (Bruno and Cuello, 2006;Lee et al., 2001). We also found a significant decrease in levels of neuroserpin, the brain inhibitor of tPA, in the AD samples, ($P < 0.05$; **Figure 4-1D**). These results suggest that in AD there is a marked compromise of the cascade responsible for NGF maturation causing progressive accumulation of the NGF precursor. Our next step was to study whether the NGF degradation component of this pathway was also altered in AD.

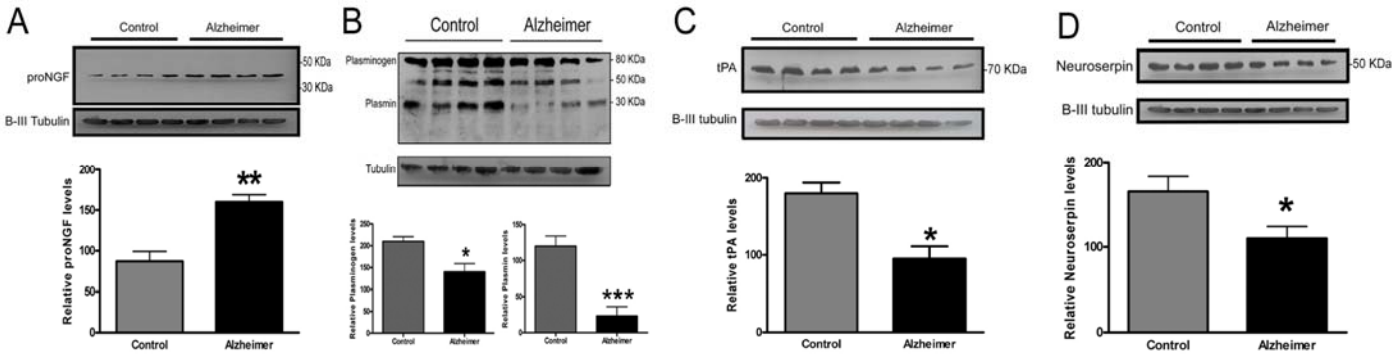


Figure 4-1 Altered cortical levels of the NGF maturation cascade components in Alzheimer disease

Western blot analysis from middle frontal gyrus of AD brain homogenates compared with age-matched controls revealed **(A)** increased amount of proNGF ($P< 0.01$) **(B)** decreased levels of plasminogen ($P< 0.05$) and plasmin ($P< 0.001$) **(C)** decreased level of tPA ($P< 0.05$) and **(D)** decreased levels of neuroserpin, the brain tPA inhibitor ($P< 0.05$). This reduction in plasmin formation lead to a reduction in NGF maturation and would explain the observed proNGF accumulation in cortical AD brain. Quantification of the immunoreactive bands was determined by densitometry of the films using MCID program and pixel values were normalized to ß-III tubulin values for each sample. (n=7 for each group).

Increased NGF degradation pathway in AD

Elevated levels of MMP-9 were identified in Alzheimer hippocampal tissue (Backstrom et al., 1992) and in plasma when compared with age-matched controls (Lorenzl et al., 2003). Furthermore, it has been shown that MMP-9 degrades Aß peptide (Backstrom et al., 1996) and amyloid-ß fibrils suggesting a role in amyloid clearance (Yan et al., 2006). We have observed increased levels and activity of MMP-9 in cortical human brain tissue, as measured by Western

blot analysis ($P< 0.001$; **Figure 4-2A**) and gelatin zymography ($P< 0.001$; **Figure 4-2A**). Moreover, no significant changes in the tissue inhibitor of MMP-9, TIMP-1 were observed in AD cortical tissue compared with age-matched controls ($P> 0.05$; **Figure 4-2B**).

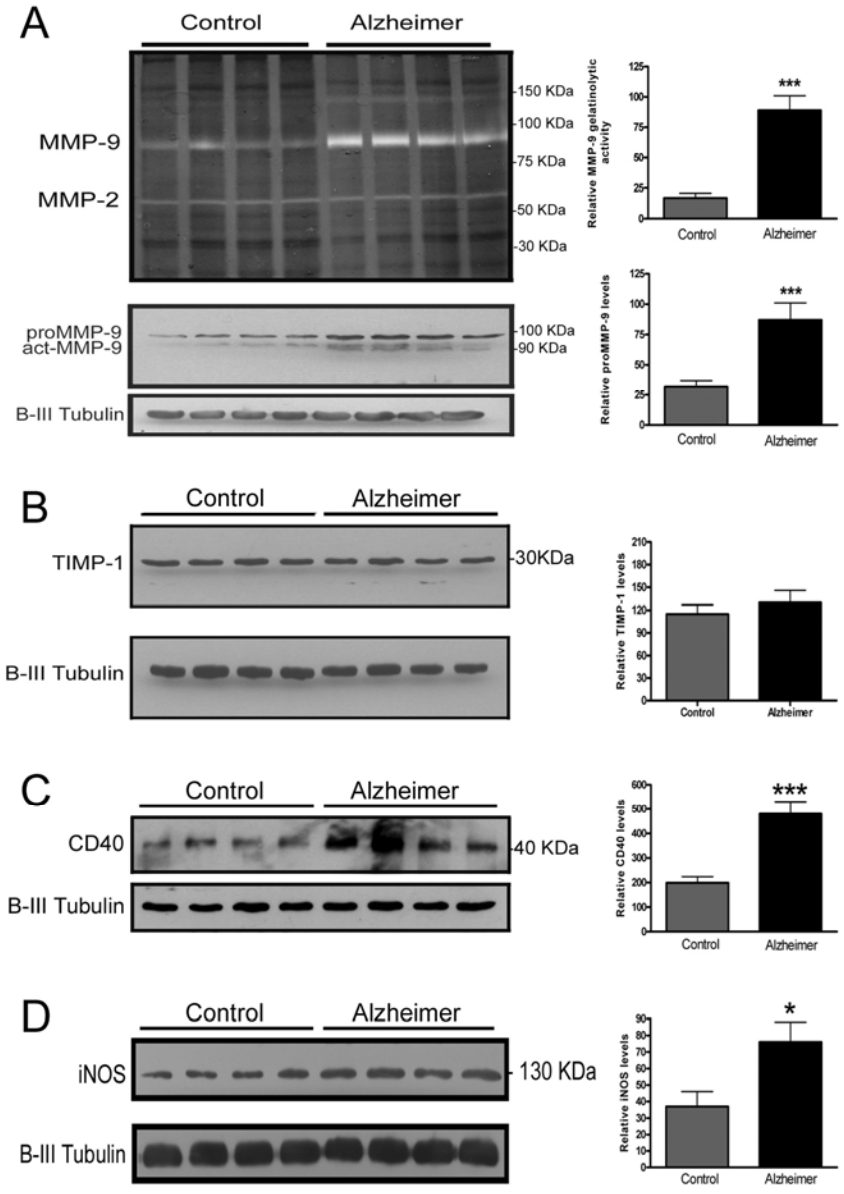


Figure 4-2 Increased NGF degradation, microglia activation and NO production in cortical Alzheimer disease tissue

(A) The upper panel illustrates the increased MMP-9 gelatinolytic activity in the middle frontal gyrus of AD *vs.* control, by gelatin zymography ($P< 0.001$). In the lower panel, increased pro- and active- MMP-9 levels were found in AD by western blot ($P<0.001$) **(B)** No significant differences ($P> 0.05$) were found, by western

blotting analysis, of the MMP-9 inhibitor TIMP-1, in the middle frontal gyrus of AD samples as compared with age-matched controls ($P > 0.05$). **(C)** Statistically significant increment in CD40 protein ($P < 0.001$) in AD samples, indicating microglia activation. **(D)** The expression of inducible nitric oxide synthase (iNOS) was found also elevated in AD brain tissue ($P < 0.05$). Increased MMP-9 activity and NO production may lead to increased NGF degradation in AD brain. Quantification of the immunoreactive bands was determined by densitometry of the films using MCID program and pixel values were normalized to ß-III tubulin values for each sample (n=7 for each group)

MMP-9 is released in an activity-dependent manner as a propeptide (proMMP-9) and can be then activated by plasmin (Bruno and Cuello, 2006). However, we have found increased proMMP-9 and MMP-9 levels in cortical AD samples, strongly suggesting an alternative mechanism for MMP-9 activation since the tPA/plasminogen/plasmin system is down regulated in AD. S-nitrosylation of proMMP-9 by nitric oxide (NO) has been described as an alternative mechanism for activation of MMP-9 in response to oxidative stresses (Gu et al., 2002) and Aß directly induces the expression of MMP-9 (Walker et al., 2006). We therefore investigated whether NO production could be involved in the MMP-9 activation observed in AD brains.

Microglia activation and Nitric Oxide production in AD

Aggregated-Aß activation of microglia is a central feature in the chronic inflammatory pathology in AD (for review see (McGeer and McGeer, 1995a) and a direct indicator of an ongoing chronic inflammatory process. NO is synthesized by a family of nitric oxide synthase enzymes (NOS) including inducible NOS (iNOS); endothelial NOS (eNOS) and neuronal NOS (nNOS). iNOS is found in activated microglia whereas NO production (Smith et al., 1996) and aberrant expression of iNOS is found in AD brains (Luth et al., 2001;Luth et al., 2002). We investigated the levels of activated microglia and iNOS expression in cortical AD tissue and compared these with age-matched controls. CD40 expression is a well-established marker of microglial activity, which is up-regulated in the presence of Aß peptides (Nguyen et al., 1998;Tan et al., 1999). CD40 is also up-regulated by activated microglia in AD brains (Calingasan et al., 2002;Togo et al., 2000).Consistent with these previous reports, we have found increased levels of both, CD40 ($P < 0.001$, **Figure 4-2C**) and iNOS ($P < 0.05$; **Figure 4-2D**) indicating increased NO production in the AD brain. Augmenting the content of NO followed by hyperactivity of MMP-9, increases the degradation of mature NGF, further altering the ratio proNGF/NGF and leading to

accumulation of the pro-apoptotic proNGF (Bruno and Cuello, 2006). Based on this, we performed further studies to determine whether this microglia activation and increased NO production is triggered by the amyloid-ß burden.

In vivo effects of injected soluble Aß-oligomers on proNGF levels and MMP-9 activity

Brain levels of soluble Aß in AD show a stronger correlation with cognitive impairments than plaque density (Lue et al., 1999;McLean et al., 1999;Naslund et al., 2000). Consequently, our next step was to investigate whether the altered expression of proNGF, MMP-9, CD40 and iNOS observed in AD brains were triggered by Aß. We administered a single injection of soluble Aß-oligomers (ipsilateral, 1µg/3µl) *vs*. the reverse peptide 42-1 as control (contralateral, 1µg/3µl) in the rat hippocampus. 72hs later the tissue was dissected and processed. Soluble Aß-oligomers were prepared as described previously (Demuro et al., 2005) and the Aß-oligomerization confirmed by dot blot analysis using A11 and 6E10 antibodies and the monomeric peptide form as control (**Figure 4-3A**). In the hippocampal Aß-oligomers-injected side we found increased levels of proNGF ($P< 0.01$) and MMP-9 activity ($P< 0.001$) when compared to the reverse peptide injected side (**Figure 4-3B** and **C**).

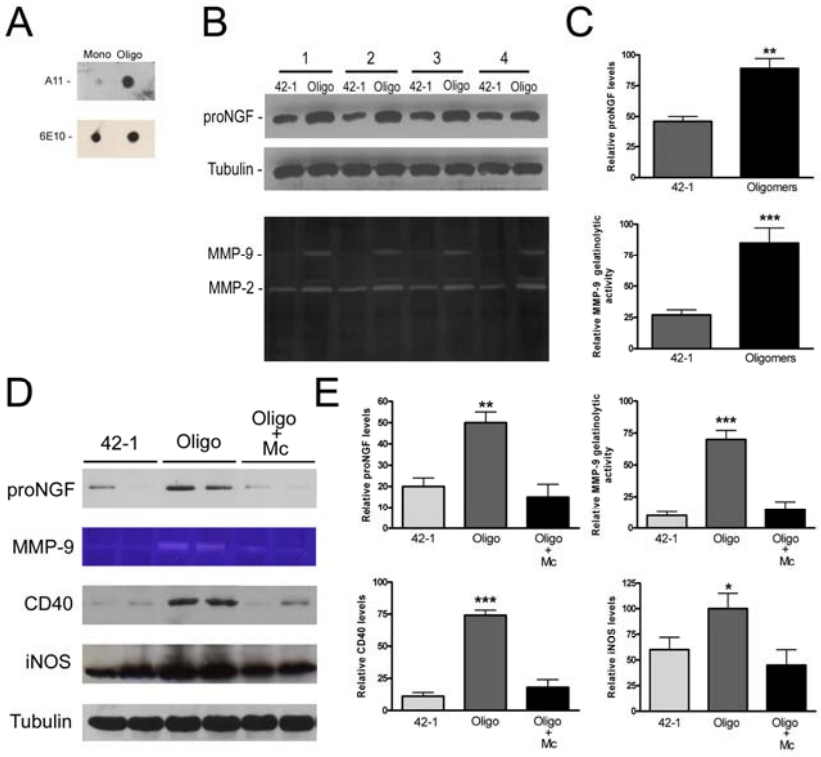


Figure 4-3 In vivo effects of soluble Aß-oligomers in the NGF maturation/degradation cascade

(A) Before hippocampal injections, the formation of soluble Aß-oligomers were confirmed by dot blot analysis**,** using A11 antibody which recognizes the presence of oligomers, and 6E10 antibody, which recognizes monomers and oligomeric forms of Aß. **(B)** 72hs after Aß-oligomers *vs.* 42-1 reverse peptide were injected into the hippocampus and the levels of proNGF were determined by Western blot and MMP-9 activity analyzed by gelatin zymography. Note the up-regulation of proNGF levels and of MMP-9 activity in hippocampi receiving Aβ-oligomers **(C)** Histogram representation of the relative values of proNGF and MMP-9 activity. Quantification of bands was performed by densitometry using MCID program and values normalized to ß-III tubulin for western blots (proNGF) and to MMP-2 for zymograms (MPP-9 activity). Statistical differences ** ($P< 0.01$) and *** ($P< 0.001$) were found in Aß-oligomers injected side samples compared with the control 42-1 reverse peptide injected side samples (n=5). **(D)** and **(E)** Minocycline (Mc) treatment (n=5) prevented the increase in proNGF ($P<0.01$), MMP-9 ($P<0.001$), microglia activation (CD40; $P<0.001$) and iNOS ($P< 0.05$) up-regulation induced by Aß-oligomers.

Minocycline prevented Aß oligomer-induced alterations on the NGF maturation/degradation cascade and microglial activation

These observations led us to speculate that targeting microglia activation to prevent iNOS expression and NO production in AD could have therapeutic potential in protecting and preventing further deterioration of the basal forebrain cholinergic neurons. In order to achieve this, rats infused with soluble Aß-oligomers were treated with minocycline, a tetracycline derivative that has been shown to effectively attenuate microglia activation by down regulating the production of pro-inflammatory molecules (Familian et al., 2007), lowering iNOS expression (Amin et al., 1996;Ryu et al., 2004) and also inhibiting MMP-9 (Lee et al., 2006). Minocycline was administered (IP, 50mg/kg, (Lee et al., 2006) 24hs before soluble Aß-oligomers injections and daily afterwards for 72hs when animals were sacrificed and hippocampal tissue processed for neurochemical analysis. Minocycline-treated animals infused with Aß-oligomers were significantly protected from the Aß-induced up-regulation of proNGF ($P< 0.01$), MMP-9 ($P< 0.001$) and, of the microglial CD40 ($P< 0.001$) and iNOS ($P< 0.05$) expression when compared with Aß-oligomers infused rats without minocycline treatment (**Figure 4-3D** and **E**). No statistical significant differences were found between minocycline-treated rats injected with soluble Aß-oligomers and 42-1 reverse control peptide injected rats. Our next step was to investigate whether the increased NO production could

modify the trophic activity of NGF since recent reports indicate altered trophic function of peroxynitrite-modified NGF (Domeniconi et al., 2007;Pehar et al., 2006).

Peroxynitrite-mediated nitrotyrosine formation in the proNGF and NGF molecule

Nitrotyrosine immnunoreactivity is five to eight folds higher in areas of neurodegeneration in the AD brain than in cognitively normal subjects (Hensley et al., 1998;Luth et al., 2002;Smith et al., 1997). In agreement with this, using antibodies directed against nitrotyrosine, we observed a clear increment in the content and number of nitrotyrosine immunoreactive bands in AD brain samples compared with age-matched control (**Figure 4-4A**).

We further investigated whether the increased cortical levels of proNGF seen in AD also contains nitrotyrosine residues. To achieve this, cortical brain homogenates from AD patients and age-matched controls were immunoprecipitated using an anti-NGF antibody and resolved in two different experiments by Western blot analysis. In order to identify whether the immunoprecipitated material corresponded to the precursor, the mature protein or to both, we used two different antibodies, one against mature NGF and another against its precursor form, proNGF. In both cases, the results were the same, with a high-molecular band corresponding to proNGF (**Figure 4-4B**). Equally loaded immunoprecipitated material was resolved by Western blot analysis with an antibody against nitrotyrosine where we found nitrotyrosine-positive bands in AD brain samples, with almost undetectable nitrated proNGF in age-matched controls (**Figure 4-4C**). These results indicated that there is a clear accumulation of peroxynitrite-modified proNGF in cortical tissue of AD brains.

As shown in **Figure 4-3D**, a single injection of soluble Aß-oligomers into the rat hippocampus increased levels of iNOS at 72hs. To test the possibility that under such conditions peroxynitration of protein does occur, the brain homogenates were further resolved by Western blot analysis using the nitrotyrosine antibody. As expected, additional nitrotyrosine-positive bands appeared in the samples injected with Aß-oligomers compared with the control samples injected with 42-1 reverse peptide. Moreover, this peroxynitrite-mediated oxidative damage could be prevented by the treatment with minocycline (**Figure 4-4D**). To investigate if soluble Aß-oligomers are specifically able to induce the nitration of tyrosine residues in proNGF/NGF molecules, these proteins were immunoprecipitated with NGF antibodies. This revealed an

accumulation of nitrated proNGF in the samples injected with soluble Aß-oligomers which was also prevented by minocycline treatment (**Figure 4-4E**).

To better understand whether the proNGF accumulation observed in AD cortical tissue could be induced by the peroxynitrite-mediated oxidation of proNGF, recombinant purified proNGF and mature NGF were treated with peroxynitrite ($ONOO^-$, 1mM) as described previously (Pehar et al., 2006). The reaction products were resolved by Western blot analysis using anti-NGF (**Figure 4-4F**) and anti-nitrotyrosine antibodies (**Figure 4-4G**). Native NGF (line 1) and proNGF (line 4) were also treated with decomposed peroxynitrite (ROA, 1mM, lines 2 and 5, respectively) and used as control. No changes were observed after ROA treatment compared with native NGF and recombinant proNGF without any treatment. However, after peroxynitrite treatment, nitrotyrosine-positive bands were observed in both, NGF and proNGF (lines 3 and 6, respectively). In addition, we tested whether plasmin was capable of maturing peroxynitrite-modified proNGF. In **Figure 4-4F** line 7, two NGF positive bands corresponding to proNGF and mature NGF can be observed, indicating that plasmin was able to convert peroxynitrite-modified proNGF into also nitrotyrosine-containing mature NGF. After plasmin treatment this reactive product was resolved using a nitrotyrosine antibody, and a 14-KDa band corresponding to mature NGF was observed (**Figure 4-4G**, line 7). This result indicates that peroxynitrite-modified proNGF is converted by plasmin into nitrated mature NGF. We performed further studies testing the ability of nitrated NGF to exert trophic neuronal support when compared with native NGF.

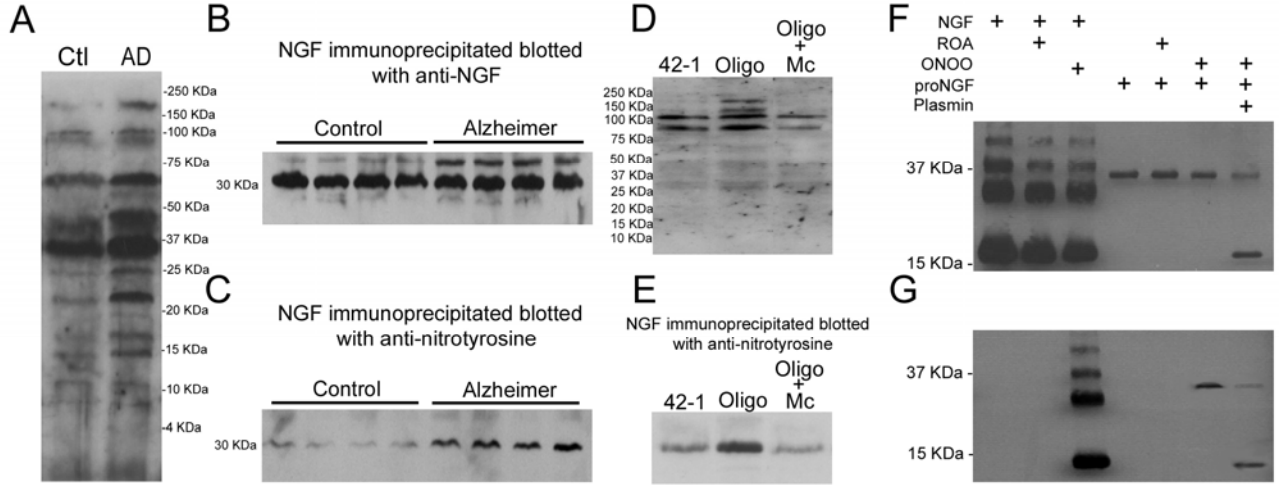


Figure 4-4 Peroxynitration of proNGF and NGF in Alzheimer disease

(A) Representative Western blots showing the high proportion of nitro-tyrosine protein contents in middle frontal gyrus of AD samples *vs.* age-matched controls (Ctl), using a monoclonal anti-Nitro-tyrosine antibody **(B)** 300µg of middle frontal gyrus tissue from AD and controls were immunoprecipitated with NGF antibody and resolved by Western blot and further **(C)** blotted with the monoclonal anti Nitro-tyrosine antibody revealing a marked tyrosine nitration of proNGF molecules in AD brain samples. **(D)** 60µg of hippocampal homogenates from rats injected with 42-1 reverse control peptide, soluble Aß-oligomers and minocycline-treated rats also injected with soluble Aß-oligomers (5 animals injected for each condition) were separated and blotted with anti Nitro-tyrosine antibody. Several nitrotyrosine-positive bands can be observed in Aß injected rats. The minocycline treatment markedly diminished the nitrotyrosine modifications of proteins in Aβ-oligomers treated samples. **(E)** Hippocampal homogenates from injected rats were immunoprecipitated with NGF antibody, separated and blotted with Nitro-tyrosine antibody showing that Aß-oligomers caused nitration of tyrosine residues in proNGF which was prevented by minocycline's treatment. **(F)** Representative Western blot showing the effects of peroxynitrite ($ONOO^-$, 1mM) on native NGF (10ng loaded in each well) and recombinant proNGF (50ng loaded in each well): NGF (line 1), NGF treated with degraded peroxynitrite (ROA, 1mM) (line 2), NGF treated with peroxynitrite (line 3), proNGF (line 4); proNGF treated with ROA (line 5); proNGF treated with peroxynitrite (line 6) and proNGF treated with peroxynitrite and plasmin (2µg/µl) (line 7). The final reaction products were separated by SDS-PGE and blotted with anti-NGF antibody. **(G)** The same reaction products were immunoblotted with anti Nitro-tyrosine antibody revealing the presence on peroxynitrite-modified NGF (line 3), peroxynitrite-modified proNGF (line 6) and peroxynitrite-modified mature NGF derived from peroxynitrite-modified proNGF and converted (matured) by the action of plasmin (line 7). These results would indicate that peroxynitrite-induced nitration of proNGF and NGF is mediated by activated microglia in response to soluble Aß-oligomers burden and that the nitrated-proNGF can be converted to nitrated-NGF by the action of endogenous plasmin.

Altered trophic support of peroxynitrite-modified NGF to DRG neurons

The question which remained to be investigated was whether peroxynitrite-modified mature NGF could exert its biological activity by activating TrkA receptors. Previous studies have shown that NGF plays a critical role in the survival, differentiation, and maintenance of dorsal root ganglion (DRG) sensory neurons (Levi-Montalcini and Angeletti, 1963;Shadiack et al., 2001;Sofroniew et al., 2001) and that its application to the distal axons of sympathetic neurons in compartmentalized cultures causes the extremely rapid appearance within cell bodies of a number of phosphorylated proteins, including TrkA (Senger and Campenot, 1997). Thus, we tested the

ability of native *vs*. oxidized NGF to offer trophic support to DRG neurons. In initial experiments, neurons were treated immediately after plating but did not survive in the presence of peroxynitrite-modified NGF (data not shown). Next, DRG neurons were maintained with native NGF for the first 4 days in vitro (DIV) and then switched to peroxynitrite-modified NGF for 4 more days (**Figure 4-5A** upper panel). A marked shrinkage of DRG neuronal soma was observed, expressed as mean cross-sectional area ($P < 0.001$, **Figure 4-5B**), along with decreased neuronal processes in remaining neurons, and a statistically significant decrease in the number of neurons ($P < 0.001$, Figure **4-5D**). Thus, oxidized NGF had a clear impact on both cell survival and phenotypic maintenance.

In a second set of experiments, after 28 DIV, mature and stable DRG cultured neurons were treated with peroxynitrite-modified NGF for 4 consecutive days (**Figure 4-5A**, lower panel). This resulted in moderate atrophy of the cells expressed as statistically significant changes in the mean cross-sectional area of DRG neurons ($P < 0.05$, **Figure 4-5C**) but no change in cell survival ($P > 0.05$; **Figure 4-5D**) when compared with native NGF treatment; indicating that after neuronal differentiation this negative effect of peroxynitrated NGF is restricted to the maintenance of the phenotypic characteristics without altering cell survival.

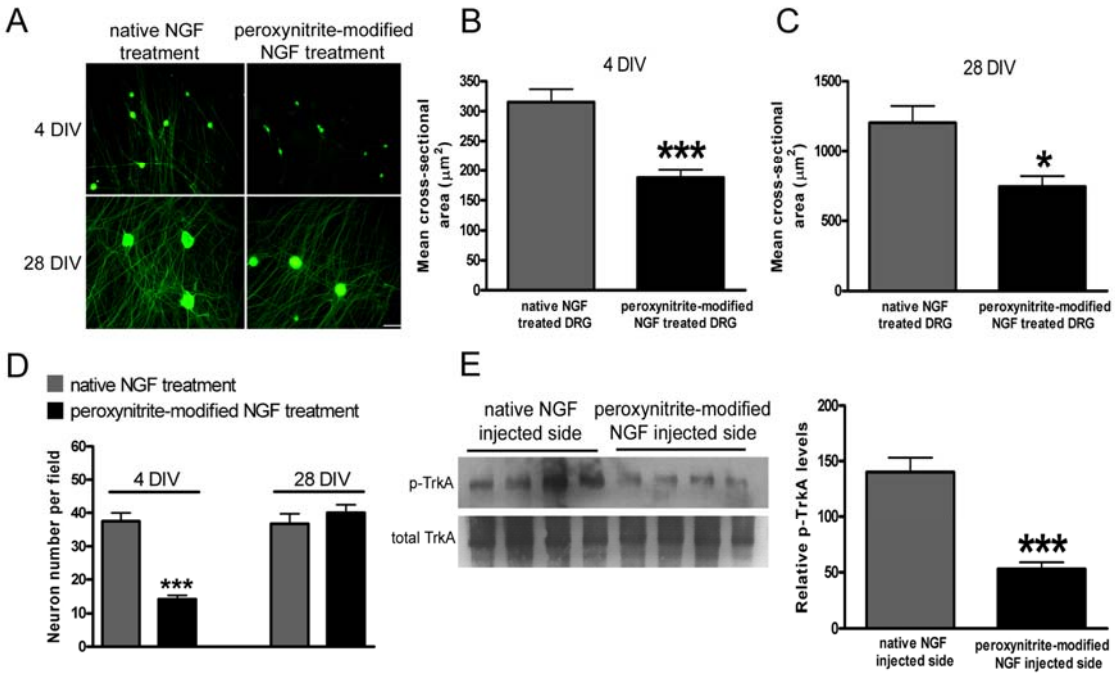


Figure 4- 5 Altered biological activity of peroxynitrite-modified NGF compared with native NGF, *in vitro* and *in vivo* observations.

(A) DRG (neurofilament-immunoreactive, revealed by immunofluorescence) neurons at 4 DIV (upper panel) and 28 DIV (lower panel) treated with native NGF (left side) or peroxynitrite-modified NGF (right side) 25x, Scale bar 50μm. **(**B and C**)** The peroxynitrite-modified NGF treatment caused a statistically significant reduction in the cell soma of DRG neurons expressed as mean cross-sectional area (μm^2) **(B)** 4 DIV (n=30, $P< 0.001$) and **(C)** 28 DIV (n=30, $P< 0.05$); as well as a marked reduction in neural processes treated at both time-points. **(D)** Statistically significant reduction in DRG cell survival (six fields of 0.39 mm^2 per condition) was observed in peroxynitrite-modified NGF at 4 DIV ($P< 0.001$). However, at 28 DIV, there was not significant reduction in cell number between native NGF *vs*. peroxynitrite-modified NGF, indicating that nitrated NGF lose the capacity of maintaining the survival of newly cultured DRG neurons or maintaining the phenotype of mature and fully developed DRG cultured neurons. **(E)** Reduced capacity of peroxynitrite-modified NGF to induce TrkA phosphorylation in *in vivo* conditions. 150ng of native NGF or peroxynitrite-modified NGF were injected into the rat hippocampus and 2hs later the animals where sacrificed and the tissue conducted to immunoprecipitation with TrkA antibody and separated by Western blot. When compared with native NGF injected side, peroxynitrite-modified NGF displayed a statistically significant reduction (n=5; $P< 0.001$) on its capacity to induce TrkA phosphorylation, explaining its failure in eliciting trophic support for either cell survival of differentiating (4 DIV) or maintenance of phenotype in differentiated DRG neurons (28 DIV).

Altered capacity of peroxynitrite-modified NGF to phosphorylate TrkA in vivo

Finally, we investigated whether the lack of trophic support of oxidized NGF displayed *in vitro* is also validated under *in vivo* conditions. Native NGF and peroxynitrite-modified NGF were injected unilaterally into the rat hippocampus and 2 hours latter the levels of TrkA autophosphorylation were investigated, as described previously (Venero et al., 1996). Each rat received a single injection of 150 ng of native NGF in one hemisphere and 150ng of peroxynitrite-modified NGF in the other. Two hours later the hippocampi were dissected, homogenized and subjected to immunoprecipitation using an anti-TrkA antibody. Western blots of phosphorylated TrkA and total TrkA (**Figure 4-5E**) revealed a significant reduction in the level of TrkA phosphorylation ($P< 0.001$) in the peroxynitrite-modified NGF injected side compared with the native NGF injected side. These results suggest that peroxynitrite modifies NGF activity by reducing its competence to induce TrkA autophosphorylation, thus disrupting the orthodox NGF signaling cascade.

4E Discussion

Basal forebrain cholinergic neurons (BFCN) are relevant to higher CNS functions, including learning and memory. The vulnerability of these NGF-dependent neurons is a consistently recognized feature of AD neuropathology. The cholinergic atrophy is reproduced in transgenic models of the Alzheimer's-like amyloid pathology (Bell et al., 2006;Wong et al., 1999). However, the mechanisms linking the Aß amyloid burden and the atrophy of BFCN are still unclear. In the present study, we found a marked alteration in the levels of the protease cascade responsible for NGF maturation in human AD cortical tissue. Diminished plasmin formation in the AD brain would reduce the NGF maturation process, facilitating the accumulation of proNGF. In addition, in AD cortical tissue we found increased levels and proteolytic activity of MMP-9, a protease with capacity to degrade mature NGF (Bruno and Cuello, 2006). Based on this, in the Alzheimer's brain we detected a double jeopardy for the trophic support of BFCN. This is due to the combined effects of decreased NGF maturation and increased degradation of mature NGF. Thus, it is plausible that alterations in the NGF maturation/degradation balance in the CNS might cause or importantly contribute to the remarkable vulnerability of forebrain cholinergic neurons in AD.

We investigated whether the observed impaired processing of proNGF and the increased NGF degradation was related to the ß-amyloid accumulation in the brain. Current evidence supports the view that soluble ß-amyloid peptides induce impairments of memory formation (Cleary et al., 2005b;Lesne et al., 2006;Stepanichev et al., 2003), a mechanism probably involving the basal forebrain cholinergic system (Olariu et al., 2001;Tran et al., 2001). In these investigations, by applying soluble Aß-oligomers in the rat hippocampus we demonstrated that a significant accumulation of proNGF also occurs. Furthermore, this treatment led to increased levels and activity of MMP-9 signifying an Alzheimer's-like increment of the NGF degradation in rats treated with soluble Aß-oligomers.

There is increasing evidence indicating that oxidative damage to proteins and other macromolecules is a salient feature of the pathology of AD (Luth et al., 2002;Smith et al., 1997;Torreilles et al., 1999;Xie et al., 2002) Microglial cells are normally in a resting and immunodepressed state, but in AD they become gradually activated with simultaneous production of superoxide and NO (Smith et al., 1996;Smith et al., 1997). In vitro studies have shown that

activated microglia can become neurotoxic by generating peroxynitrite, a powerful oxidant molecule resulting from the reaction of superoxide with NO (Dringen, 2005;Li et al., 2005;Wang et al., 2004). This is a potent oxidant and nitrating agent involved in oxidation and nitration of tyrosine residues in proteins (for review see Alvarez and Radi, 2003;Torreilles et al., 1999). In our study, after the delivery of soluble Aß-oligomers into the rat hippocampus we found a marked microglia activation leading to iNOS production analogous to that found in AD brains. We further provide evidence that this up-regulation of iNOS results in peroxynitrite-mediated nitration of proteins in response to the injection of soluble Aß-oligomers.

Recently, it has been shown that peroxynitrite transforms NGF into an apoptotic factor for motor neurons, triggering the formation of stable high-molecular weight oligomers of NGF inducing tyrosine nitration in a dose-dependent manner (Pehar et al., 2006). Contrarily, another group of investigators (Domeniconi et al., 2007) proposed that in response to peroxynitrite treatment, reactive astrocytes secreted proNGF, and this precursor form of NGF and not the mature form, induces motor neuron cell death. We have investigated in particular the possible oxidative effects of peroxynitrite on proNGF and NGF molecules since activated microglia generates peroxynitrite in response to soluble Aß-oligomers.

Previous studies have shown that peroxynitrite transform NGF into an apoptotic agent for motor neuron, acting through p75 neurotrophin receptor ($p75^{NTR}$) (Pehar et al., 2006). Interestingly, in AD cortical tissue there is an accumulation of proNGF, another proposed pro-apoptotic proposed molecule, which binds with high affinity to $p75^{NTR}$ and lacks the capacity to bind TrkA receptor (Volosin et al., 2006). Since we found high levels of peroxynitrite-modified proNGF in the AD brain, and this can be converted to peroxynitrite-mediated NGF nitration, we investigated whether these peroxynitrite-mediated alterations in the NGF molecule changes biological responses on TrkA expressing neurons. We observed that nitrated NGF loses the property of maintaining the survival of newly cultured DRG neurons or maintaining the phenotype of mature and fully developed DRG cultured neurons. These observations strongly suggest that peroxynitrite radically changes the biological properties of mature NGF as a trophic molecule.

Our observations that peroxynitrite-modified NGF injected to the rat hippocampus was unable to induce TrkA autophosphorylation as compared with native NGF would further reinforce this concept. These findings add a new perspective to the poorly understood vulnerability of the BFCN in AD. The well-noted reduction of TrkA proteins in AD (Counts and Mufson, 2005)

would be in line with the present findings of perturbed NGF maturation, increased degradation and peroxynitration of NGF, as the TrkA gene expression is induced by NGF (Figueiredo et al., 1995;Holtzman et al., 1992;Li et al., 1995;Wong et al., 1999).

In summary, our results would suggest that in the Aβ-induced Alzheimer's pathology, there is a profound alteration in the conversion, degradation, and degree of peroxynitration of NGF. This scenario will drastically reduce the biological actions of NGF for the phenotypic maintenance of the NGF-dependent basal forebrain cholinergic neurons as a consequence of the Aß burden Alzheimer's pathology. Our findings also indicate that some of these alterations to NGF metabolism are provoked by the Aβ-induced microglia activation, and that early Alzheimer's therapies with minocycline or related compounds could prevent the atrophy of BCFN, and also the deleterious peroxynitration of key CNS proteins.

4F Acknowledgments

We would like to thank Adriana Ducatenzeiler and Vanessa Partridge for excellent technical support and Mona-Lisa Bolduc for editorial assistance. The authors would also like to thank Charles Glabe (Irvine, CA, USA) for the generous provision of A11 antibodies. This work was supported by the Canadian Institute of Health Research Grant MOP 62735 and the US Alzheimer's Association IIRG-06-25861. Martin A. Bruno received a fellowship from Consejo Nacional de Investigaciones Cientıficas y Tecnicas (CONICET), Argentina, and A. Claudio Cuello holds the Charles E. Frosst Merck Chair in Pharmacology, McGill University, Montreal, QC, Canada.

Chapter 5

General Discussion

In the preceding Chapters I have shown:

1) That aged-impaired rat responds to the exogenous administration of NGF and D3, a small peptide mimetic of TrkA receptor, improving their behavioral performance and reversing BFCN atrophy (Chapter 2).
2) That cerebral cortex tissue releases proNGF in an activity and intracellular calcium-dependent manner. We have found that proNGF is matured and degraded in the extracellular space by the coordinated action of convertases and proteases (Chapter 3).
3) That proNGF is accumulated and nitrated in cortical AD brain and that there is a dysregulation of the enzymatic cascade leading to proNGF maturation and NGF degradation. Further to this, *in vitro* and *in vivo* experiments revealed that nitrated NGF is not biologically active (Chapter 4).

In this section I will discuss how these findings relate to the corresponding field of science, as well as ongoing experiments aimed at increasing our understanding in the field. In addition, I will highlight the new questions this research has provoked and the future research opportunities regarding Alzheimer's disease therapeutics.
This section is divided in subsections, as follows:

5A Alterations in endogenous NGF trophic support in Aging.

5A1- ProNGF maturation/degradation cascade in Aging
5A2- Erk-MAPK cascade signaling in Aging
5A3- ChAT activity in Aging
5A4- Oxidative stress damage and proNGF in Aging
5A5- Increased oxidative stress in Aging
5A6- Mitochondrial dysfunction and calcium homeostasis, two proposed Hypotheses of Aging
5A7- Microglial activation, nitric oxide production and the "Inflammation Hypothesis" of Aging
5A8- Impaired protein turnover in Aging

5B Aß, Oxidative stress, Neuroinflammation and Cholinergic dysfunction in Alzheimer's disease

5B1- The “Amyloid Hypothesis” and the “Oxidative stress Hypothesis” of Alzheimer’s disease

5B2- The “Calcium Hypothesis” and the “Mitochondrial Hypothesis” of Alzheimer’s disease

5B3- Microglial activation, nitric oxide production and Neuroinflammation in Alzheimer’s disease

5B4- The “Cholinergic Hypothesis” and the “Neurotrophic Hypothesis” of Alzheimer’s disease

5C Summary

5A Alterations in endogenous NGF trophic support in Aging

5A1 Pro-NGF maturation/degradation cascade in Aging

Evidence accumulated so far led to the assumption that BFCN are highly dependant on a continuous supply of biologically active NGF and that an interruption in this support might be a direct cause of cholinergic neuronal atrophy in aging. However, as highlighted previously, the evidence for a decreased production of NGF in aging is not compelling. Reports regarding age-dependent changes in the levels of NGF protein or its mRNA in the cerebral cortex and hippocampus vary; from a decrease, to no change, or to an elevation and, in many occasions, a dissociation of these two markers (Alberch et al., 1991;Henriksson et al., 1992;Katoh-Semba et al., 1998;Larkfors et al., 1987;Larkfors et al., 1988). Nevertheless, a possible explanation for such discrepancy may lay in the fact that these studies were performed without considering the cognitive status of the aged animals. For instance, in **Chapter 3** we have demonstrated that the preferential cortical cholinergic synaptic loss and the basal forebrain cholinergic somata shrinkage is not a widespread predicament in aging, but is rather specific to cognitively age-impaired rats. In consequence, we would expect a correlation between NGF levels and the cognitive status of aged rats. Interestingly, the reported data is fairly consistent, indicating that brain NGF levels are maintained at normal or, in some areas, at supranormal levels in rats with severe learning and memory impairment (Hasenohrl et al., 1997;Hellweg et al., 1990;Lindner et al., 1994;Sugaya et al., 1998). Based on these results, we formulated a new hypothesis, assuming that BFCN trophic uncoupling was a consequence of increased proNGF levels. Thus, we quantified, by Western blot, the cortical levels of both proNGF and NGF in behaviorally characterized unimpaired (AU) and impaired (AI) aged rats **(Appendix A)**, using Morris water maze, as described in detail in **Chapter 2, Section 2C** .

Surprisingly, we did not find any statistically significant differences in the levels of cortical proNGF between young, AU and AI rats **(Appendix B)**, whereas mature NGF was undetectable. Thus, we switched the direction of our investigation towards the state of the NGF maturation/degradation proteolytic cascade members, hypothesizing that any alteration in this complex cascade could lead to an alteration in content affecting NGF biological activity. Again, and in agreement with the data obtained for proNGF levels, we did not find any statistically significant differences (by Western blot) in either in the levels of plasminogen (**Appendix C)**, tissue plasminogen activator , tPA **(Appendix D)**, neuroserpin **(Appendix E)** nor in activity (by gelatin zymography) of MMP-9 **(Appendix F)**

among the three investigated groups (Young, AU and AI). Based on these results, I assumed that the NGF processing in aging was normal, despite its cognitive abilities. In consequence, I focused my attention on investigating whether the NGF/TrkA intracellular mechanism becomes activated by endogenous NGF.

5A2 Erk-MAPK cascade signaling in Aging

So far, no evidence of increased proNGF levels or alteration in its maturation/degradation cascade was observed in age-impaired rats. Our next step was to further investigate whether BFCN received proper trophic support from endogenous NGF in AI rats. We tested and compared the cortical levels (by Western blot) of the MAP kinase activation pathway, an intracellular NGF/TrkA signaling mechanism (described in **Section 1B3** and depicted in **Figure 1-1)** in Young, AU and AI rats. It is important to mention that we specifically selected this intracellular pathway because the Erk-MAP kinase cascade has been repeatedly shown to play a critical role in learning and memory processes, including spatial memory tasks (for reviews see Adams and Sweatt, 2002;Silva, 2003;Sweatt, 2001;Weeber and Sweatt, 2002). In this case, as shown in **Appendix G**, we found in our blots a significant decrease in cortical phosphorylated Erk in AI animals compared to AU ($p< 0.01$) and AI compared to young rats ($p< 0.01$). Interestingly, no statistical differences were found in phosphorylated ErK between young and AU rats ($p> 0.5$). Moreover, no changes in total ErK were observed among groups ($p> 0.5$). These findings are consistent with recent observation revealing an age-dependent loss of NGF signaling with disrupted Erk-MAPK activation in the rat BFCN (Williams et al., 2007;Williams et al., 2006a).

5A3 ChAT activity in Aging

At this stage of the research, we were narrowing the possibilities, and based on the collected information, we reasoned that the disconnection was likely due to NGF/TrkA interaction, leading to a failure in the coupling of the second messenger cascade. In this regard, it has been shown that NGF, through TrkA receptors, enhances a robust acetylcholine release, acetylcholine content, and the activity of the enzyme in charge of synthesizing acetylcholine, ChAT, in BFCN cultures (Auld et al., 2001b;Auld et al., 2001a;Berse et al., 1999;Cuello et al., 1989;Nonner et al., 2000). In addition, administration of exogenous NGF into the brain leads to a significant increase in the level and activity of ChAT (Garofalo and Cuello, 1994;Hefti, 1986;Kromer, 1987;Mobley et al., 1985;Williams and

Rylett, 1990) ameliorating learning deficits in age rats (Backman et al., 1996;Gage et al., 1988b;Hellweg et al., 1990;Martínez-Serrano et al., 1995). The reported data and our studies presented in **Chapter 2** strongly indicate that despite the occurrence of decreased TrkA levels in AI rats (Hasenohrl et al., 1997), BFCN positively respond to the exogenous administration of either NGF or the TrkA mimetic peptide D3.

Based on the information gathered so far regarding the tight NGF/ChAT interactions, we decided to seek for new clues as to whether diminished cortical ChAT activity is differentialy expressed in the CNS of aged animals according to their cognitive status. Thus, we measured and compared the cortical ChAT activity in Young, AU and AI rats (**Appendix H**) Interestingly, cognitively impaired animals (AI) displayed a significant decrease in cortical ChAT activity compared with young ($p< 0.001$) and AU rats ($p< 0.01$), whereas no differences where found between young and AU rats. This data reinforced and validated the essential role of acetylcholine neurotransmission and NGF trophic support in learning and memory functions. We reasoned then that in AI rats, endogenous NGF was failing to maintain the biochemical phenotype of BFCN, displaying lower ChAT activity and thus compromising the cognitive function of these rats.

5A4 Oxidative stress damage and proNGF in Aging

Finally, in our last attempt to elucidate the possible cause of such NGF trophic disconnection observed in AI rats, we tested whether proNGF was targeted and nitrated by peroxynitrite. In this regard, it has been shown that a progressive accumulation of nitrated and oxidatively-modified proteins increases with age, with a progressive decrease in the endogenous antioxidant activity (Oliver et al., 1987). Reversed protein oxidation in aged brains was observed after the administration of free radical scavengers, restoring also the performance of animals on behavioral tests. This suggests that age-related decline in mental performance is tightly associated with a reversible process involving the oxidation of protein components in the brain (Carney et al., 1991;Floyd and Hensley, 2000).

As shown in **Chapter 4 (Figure 4-4)**, we have seen that peroxynitrite can cause nitration of tyrosine residues on the proNGF molecule, that nitrated proNGF is the predominant molecular form of NGF, and that its concentration increased in AD brain cortical tissue when compared with age-matched controls. We demonstrated that nitrated proNGF can be matured into nitrated NGF, which displayed reduced capacity for inducing TrkA phosphorylation and activation (**Figure 4-5, Chapter 4**). In consequence, we decided to investigate whether the lack of endogenous NGF trophic support to

BFCN in cognitively segregated aged animals was due to the widespread oxidative damage reported in aging. We followed the same protocol described in **Chapter 4 (Section 4C)**, thereby cortical proNGF/NGF were immunoprecipitated from behaviorally characterized rat brain homogenates and then resolved with an antibody against nytrotyrosine, since a hallmark of peroxinitrite action is the nitration of tyrosine residues in proteins. As expected, cortical proNGF in AI rats was also a target of peroxynitrite actions (**Appendix I)**, directly pointing toward an increased peroxynitrite-mediated oxidative damage in these age-impaired rats.

The above information is consistent with several studies reporting a tight correlation between age-related memory impairment and a decrease in brain antioxidants mechanisms (Berr, 2000;Perkins et al., 1999;Perrig et al., 1997;Rinaldi et al., 2003). A decrease in the activities of Mn-SOD, Cu/Zn-SOD, catalase, glutathione peroxidase, and intracellular glutathione concentration has been found in the brain of differently aged animals (Chen and Lowry, 1989;Gupta et al., 1991;Navarro et al., 2004;Siqueira et al., 2005;Wang et al., 2003b). Administration of anti-oxidant agents to aged rats results in improved performance in learning and memory tasks dependent on cholinergic function (Carney et al., 1991). Long-term intraventricular administration of NGF in aged rats restores the levels and activity of the main enzymes involved in the metabolic degradation of ROS, including Cu/Zn-SOD, catalase and glutathione peroxidase (Nistico et al., 1991;Nistico et al., 1992).

Since the cholinergic hypothesis of geriatric memory dysfunction was proposed (Bartus et al., 1982), studies conducted in animals and humans have so far failed to obtain evidence for the involvement of NGF in normal aging and/or in the pathophysiology of Alzheimer's disease. It has been hypothesized that age-related degeneration of BFCN may be caused by the altered *endogenous* NGF maturation, by reduced responsiveness to NGF, by reduced NGF transport, or by the failure in coupling to "second messengers". However, we have found that endogenous NGF is being targeted by peroxynitrite, an important mediator of oxidative damage. This is not surprising, since peroxynitrite has been shown to alter acetylcholine synthesis and ChAT activity (Guermonprez et al., 2001;Ohkuma et al., 1995) and acetylcholine release (Hara et al., 1998;Tran et al., 2003). Moreover, peroxynitrite can directly inactivate the main antioxidant enzymes in the brain by tyrosine nitration mechanisms, Cu/Zn-SOD and Mn-SOD (Aoyama et al., 2000;Bayir et al., 2007;Demicheli et al., 2007;Filipovic et al., 2007;Yamakura et al., 1998).

In sum, the information and data provided in **Chapter 2**, and discussed here in the above sections should explain the vulnerability of the basal cholinergic system in aging. It seems that during

the aging process proNGF and mature NGF are confronted in the CNS with a scenario of chronically increased ROS concentrations and decreased antioxidant defense mechanisms.

5A5 Increased oxidative stress in Aging

Approximately one-half of intracellular proteins are oxidized in aged animals, suggesting that during aging, there is a widespread nonselective oxidation of many cellular proteins (Gafni, 1997). A logical question arises: Why do reactive oxygen species increase with age, causing oxidative damage? The answer to this question is likely related to mitochondria, calcium homeostasis and microglial activation.

In 1956, Professor Denham Harman, the founder of the free radical theory of aging, proposed that oxygen free radicals are formed endogenously from normal oxygen-utilizing metabolic processes and play an essential role in the aging process (HARMAN, 1956). In the past 50 years, Harman's hypothesis has been refined to encompass not only free radicals, but also other forms of activated oxygen, leading to the modification of the free radical theory. As mentioned in the Introduction **(Section 1D3)** Meister hypothesized in 1984 that one of the major contributors to the aging process is chronic oxidative stress (Meister, 1984). The major source of free radicals and oxidants is through the respiratory generation of ATP-using oxygen (Ames et al., 1993;Beckman and Ames, 1998;Pierrefiche and Laborit, 1995). A molecule carrying an unpaired electron, which makes it extremely reactive and ready to acquire an electron in any way possible, is termed a free radical. In the process of acquiring an electron, the free radical will attach itself to another molecule, thereby modifying it biochemically (Imlay et al., 2003). Radicals like nitric oxide and superoxide have little propensity to react with non-radical biomolecules. However, the combination between them generates peroxynitrite, a potent oxidant and nitrating agent capable of attacking and modifying proteins, lipids and DNA (Demiryurek et al., 1998;Kelm et al., 1997;Szabo, 2003). In **Chapter 4**, we have shown a clear example of peroxinitrite actions, showing the loss of nitrated NGF biological activity as consequence of peroxynitration.

Because the concentrations of SOD and superoxide are relatively constant in a given tissue, the primary driven force for peroxynitrite formation is the nitric oxide concentration. Nitric oxide reacts with superoxide three-fold faster than the SOD enzymatic activity, thus nitric oxide is the only known biomolecule capable of out competing SOD for available superoxide (Torreilles et al., 1999). Superoxide is formed mainly as a by-product of the mitochondrial respiratory chain. In the case of

mitochondrial dysfunction, the excessive intracellular calcium accumulation leads to the abnormal production of NO within neurons, forming peroxynitrite (for reviews see Heales et al., 1999;Moncada and Bolanos, 2006;Radi et al., 2002;Stewart and Heales, 2003). This is likely the first step, whereby the initial insult within neurons may activate microglial cells, which produce large amounts of nitric oxide through activation of inducible NOS (iNOS), leading to the generation and release of peroxynitrite. With its strong oxidizing and nitrating properties, peroxynitrite may thus be playing a pivotal role in exacerbating neurodegenerative processes during aging. One clear example of these deleterious effects of peroxynitrite was described in **Chapter 4** and confirmed in **Section 5A4** and in **Appendix I**. In consequence, we have concluded that increased nitrated proNGF in Aging and Alzheimer's disease may contribute importantly to the chronic trophic factor disconnection of BFCN. We based our conclusion on previous *in vivo* studies that have shown that in mature rats, provoking either the immunoneutralization of endogenous NGF or the blockade of their TrkA receptors results in the loss of pre-existing cortical cholinergic pre-synaptic sites. This is a strong argument favoring a role of endogenous NGF in the maintenance of the steady-state number of cholinergic connections in mature animals (Debeir et al., 1999) and a mechanism which is being disrupted by peroxynitrite-mediated nitration of NGF.

5A6 Mitochondrial dysfunction and calcium homeostasis, two proposed Hypotheses of Aging

As expected, many mitochondrial parameters are significantly affected by age, including changes in mitochondrial membrane fluidity and composition, overall reduction in the activity of the mitochondrial electron transport chain (or respiratory chain), accumulation of mutations in the mitochondrial DNA (mtDNA), accumulation of products of oxidative stress (peroxidation and oxidative protein damage) and the development of megamitochondria (Droge, 2002;Lee and Wei, 2001;Lenaz, 1998;Toescu et al., 2000;Wakabayashi, 2002). Overall, the above strongly support the well established "mitochondrial theory of aging" (Economos, 1980).

This theory implies that mitochondria in the aged neurons are less effective in responding to the increase the cell energy demands. Mitochondrial ATP production depends on the role of calcium, by the action of key enzymes of the Krebs cycle (for review see McCormack et al., 1993). Thus, neuronal stimulation that induces calcium signals will also activate mitochondrial calcium uptake pathways, resulting in an overall increase in mitochondrial calcium (Brookes and Darley-Usmar, 2002;Peng et al., 1998) and a consequent mitochondrial depolarization (Duchen, 2000;Nicholls and

Budd, 2000;Toescu and Verkhratsky, 2003). The activation of the respiratory chain causes production of ATP with the recovery of resting mitochondrial membrane potential. In the aged neurons, the mitochondrial depolarization proceeds normally, similar to the response in young neurons, but the repolarization process proceeds at a significantly reduced rate (Brown et al., 2004;Mattson, 2007;Xiong et al., 2002). This reduced rate of mitochondrial repolarization in aging is correlated with the degree of impairment of intracellular calcium recovery observed in basal cholinergic neurons in aging (Murchison et al., 2004;Murchison and Griffith, 2007).

The "calcium hypothesis of neuronal aging" (Khachaturian, 1994) implied that aging induces a small perturbation in one or another of the calcium homeostatic mechanisms, such that small changes in intracellular calcium and accumulation over larger periods of time will result in neuronal dysfunction and, most importantly, in neuronal damage and cell death. This agrees with studies of aging rodent neurons (Landfield and Pitler, 1984;Parihar and Brewer, 2007). A prominent mechanism of generating superoxide in neurons is the elevation of cytoplasmic calcium levels. This would result in increased mitochondrial superoxide production, due to mitochondrial calcium uptake. Since calcium also stimulates nitric oxide synthase (NOS), this ion is responsible for peroxynitrite production. Thus, overproduction of peroxynitrite produces substantial oxidative damage to mitochondrial DNA, impairing mitochondrial functions (Hamilton et al., 2001;Mecocci et al., 1993). In addition, the calcium regulatory protein calmodulin (CaM), pumps calcium in both endoplasmic reticulums (ER) and the plasma membrane, and calcium channels (e.g. ryanodine receptor) are preferentially damaged by ROS (Eu et al., 2000;Squier and Bigelow, 2000).

Alterations in mitochondrial function and in calcium homeostasis during aging are in agreement with genetic studies performed in the human cerebral cortex. Genes involved in calcium homeostasis and calcium-binding signaling were found to be down-regulated with aging, and several mitochondrial-related genes were also reduced in aged human cortex (Lu et al., 2004). However, the same study revealed that the human cortex is also associated with an increased expression of genes that mediate stress responses and genes involved in inflammatory responses during aging (Lu et al., 2004). The time in life when brain aging starts is undefined (Blalock et al., 2003;Jiang et al., 2001;Lee et al., 2000), however, reduced expression of genes playing important roles in calcium homeostasis and mitochondrial function start early in adult life; after 40 years of age. The young adult (< 40 years old) and the aged human population (>70 years old) are relatively homogeneous in their gene expression patterns. However, the above observations suggest that the middle-aged individuals

(between 40 and 70 years of age) exhibit much greater heterogenecity in the intracellular calcium homeostasis and mitochondrial function. Thus, individuals may diverge in their rates of "brain-aging" as they transit from middle to old age (Lu et al., 2004).

5A7 Microglial activation, nitric oxide production and the "Inflammation Hypothesis" of Aging

As mentioned previously (**Section 5A5**), neuronal alterations in calcium homeostasis and mitochondrial dysfunction in aging leads to increased production of reactive oxygen species (ROS), which can rapidly activate microglia. The microglial cell, originally described by Del Rio-Hortega in 1932 (*In:Cytology and cellular pathology of the nervous system, New York, PB Hoeber p481-534, Penfield W; Editor*), is a member of the monocyte/macrophage family and is the brain–resident tissue macrophage. Although the function of resting microglia remains elusive, it is well known that microglia rapidly transform to an activated state in response to a wide array of stimuli, including ROS (for review see Chung et al., 2006;Glezer et al., 2007). Once activated, microglia release proinflamatory molecules through the redox-sensitive transcription factor NF-*k*B, including interleukins (IL-1 ß, IL-6), tumor necrosis factor α (TNF- α) and enzymes like iNOS (Akama et al., 1998;John et al., 2003;Lue et al., 2001). NF-*k*B is among the most important transcription factors shown to respond directly to oxidative stress conditions (Haddad, 2003). ROS enhance the signal transduction pathways for NFκB activation in the cytoplasm and translocation into the nucleus (Kabe et al., 2005). Because of its sensitivity to the oxidative status, the regulation of NF-*k*B is greatly influenced by the intracellular redox status and plays a major role in the regulation of inflammation processes during aging (Helenius et al., 1996;Korhonen et al., 1997).

Among proinflammatory genes that encode proteins for orchestrating inflammatory responses, many signaling proteins such as cytokines and enzymes (e.g. iNOS) are regulated by the oxidative-responsive transcription factor NF-*k*B (for reviews see (Baeuerle and Baltimore, 1996;Schreck et al., 1992). Under normal physiological conditions, NF-*k*B activation in response to proinflammatory signals is short-lived, and the reaction stops quickly once the signal is terminated. However, if the activation signal persists, as in the aging process, a chronic inflammatory condition would have far reaching effects. Indeed, some of the NF-*k*B-induced proteins are known to act as potent NF-*k*B activators, creating an auto-activating loop (Handel et al., 1995), and consequently more synthesis of inflammatory mediators.

Inflammation is a primary defense against threats to homeostasis. With aging, inflammatory responses may be overreactive or even cause damage. For example, consensual findings show the enhanced levels of NF-*k*B activity in the brain during aging (Korhonen et al., 1997) including basal forebrain cholinergic neurons (Toliver-Kinsky et al., 1997), along with increased iNOS expression (Law et al., 2000) and cytokines levels (Ye and Johnson, 1999;Ye and Johnson, 2001). Based on this accumulating evidence, Hae Young Chang and colleagues proposed the "molecular inflammation hypothesis of Aging" (Chung et al., 2006). This hypothesis highlights the important role of molecular inflammation as a possible prime factor underlying age-related disorders.

There is a considerable debate as to whether activated microglia are beneficial or harmful. This may, however, depend on the degree of activation. Microglial activation and proinflammatory signaling is believed to play a beneficial role in host defense and to have neuroprotective elements (for review see (Bruce-Keller, 1999;Conde and Streit, 2006). Indeed, some studies have shown that inhibition of microglia is, in contrast, associated with more damages or less repair (for review see (Glezer et al., 2007). On the other hand, the information described above suggests that microglial activation and inflammation both seems to be hallmarks of aging and neurodegenerative processes. In these phenomena, it is now clear that the redox- responsive transcription factor NF-*k*B in microglia is playing a key role during the aging process, as a key transcription factor involved in the production of proinflammatory cytokines, ROS and nitric oxide (Akama et al., 1998;John et al., 2003). On the other hand, the requirement of NF-*k*B transcription factor activation in cognitive function such as learning and memory has been revealed in a number of behavioral assays. For example, pharmacological and/or genetic inhibition of NF-*k*B results in impairment in several learning and memory tasks in mice (for review see Kaltschmidt et al., 2005;Meffert and Baltimore, 2005).

A potential unifying hypothesis concerning the role of NF-*k*B activation could be that it promotes survival in neurons, whereas the chronic activation of NF-*k*B in activated microglia may induce the increased production of cytokines, ROS and nitric oxide, and might indirectly promote neuronal degeneration in aging (Mattson and Meffert, 2006). Because neurons and glial cells express NF-*k*B, it is likely that ideal agents would be cell-type-selective in their actions. For example, inhibitors of NF-*k*B that selectively target microglial cells might suppress damaging neuronal inflammation without affecting the function of NF-*k*B in neurons. Ideally, unraveling the cell-type-specific functions of NF-*k*B in more detail is likely to aid therapeutics efforts enormously.

5A8 Impairment protein turnover in Aging

As described above, damage to macromolecules, and in particular proteins, implicated in the cellular degeneration that occurs during the aging process is corroborated by the accumulation of oxidative end-products over time. Oxidized protein accumulation is commonly seen as a hallmark of cellular aging. Besides increased alteration of cellular proteins, damaged protein accumulation has been proposed to originate from the dysfunction of the synthesis/degradation balance: protein turnover. During aging, the accumulation of oxidized proteins raises problems regarding their efficiency and their degradation by the proteasome; a system implicated not only in the the removal of altered proteins, but also in the continuous renewal of intracellular proteins (for reviews see (Coux et al., 1996;Grune et al., 1997;Voges et al., 1999). Thus, the rapid clearance of oxidized proteins by the proteasome is therefore critical in minimizing the accumulation of oxidatively modified proteins.

Oxidized proteins are degraded by the proteasome, resulting in peptides of 3-20 residues long (Coux et al., 1996;Voges et al., 1999) which are further degraded by cellular peptidases (Tomkinson, 1999). Almost all amino acids within proteins can be oxidized by ROS; sulfur-containing (cystein and methionine) and aromatic (tyrosine and triptophan) amino acids being the most susceptible to the attack (Berlett and Stadtman, 1997;Davies and Delsignore, 1987). Thus, oxidized proteins are less active and present decreased stability than their native forms (Fisher and Stadtman, 1992). Moreover, alteration of secondary and/or tertiary structure has also been reported for oxidized proteins (Ferrington et al., 2001). These findings agree with our observations of decreased trophic activity and reduced capacity of oxidized NGF to activate TrkA receptors (**Chapter 4, Figure 4-5**).

The age-related accumulation of oxidized proteins and the slowing down of protein turnover raised the possibility that the proteasome degradation pathway is impaired with age. In this regard, proteasome activity has been reported to decline with age in a variety of tissues, including the hippocampus and cerebral cortex (Keller et al., 2000;Zeng et al., 2005). These findings suggest that oxidized proteins accumulate with age because they are not efficiently eliminated by the proteasomal machinery. In addition, particular attention has been paid to the fate of proteasome when subjected to oxidative stress. Interestingly, it has been shown that when exposed to ROS, two of the enzymes that cleave peptides in the proteasome catalytic subunit are inactivated (Conconi et al., 1996;Conconi et al., 1998). Then, the age-related proteasome dysfunction is likely induced upon oxidative stress. In conclusion, we can assume that the accumulation of oxidized proteins during aging can be explained

by increased protein oxidation and nitration, decreased protein degradation, or the combination of both.

5B Aß, Oxidative Stress, Neuroinflammation and Cholinergic Dysfunction in Alzheimer's disease

5B1 The "Amyloid Hypothesis" and the "Oxidative Stress Hypothesis" of Alzheimer's disease

More than a century has elapsed since the description of Alois Alzheimer's patient August D. As mentioned in the Introduction (**Section 1D2**), age is the greatest risk factor for Alzheimer's disease. Current knowledge of early cellular changes in AD progression is limited, but a growing body of evidence suggests that altered processing of amyloid precursor protein (APP) is one of the early events in the pathogenesis of AD but the initial trigger(s) is still not known (**see Figure 1-3**). According to the "amyloid hypothesis", accumulation of Aß in the brain is the primary influence driving AD pathogenesis (Hardy and Selkoe, 2002). In this regard, data emerging over the past decade demonstrated that diffusible and soluble oligomers, and not fibrilar or monomer forms of Aß, may be responsible for the alteration of neuronal function and viability in AD (Hardy and Selkoe, 2002;Klein, 2002;Tanzi, 2005). This assumption lies in the fact that at low concentrations, soluble oligomers of Aß can induce inhibition of long-term potentiation (Walsh et al., 2002), disrupt synaptic plasticity (Klyubin et al., 2005;Rowan et al., 2004) and provoke cognitive dysfunction in rodent (Cleary et al., 2005a;Lesne et al., 2006). Moreover, several studies confirmed that the degree of cognitive impairment in AD correlates much better with soluble Aß species (which are invisible to immunohistochemistry) than with histologically-determined plaque counts (Lesne et al., 2006;Lue et al., 1999;McLean et al., 1999;Naslund et al., 2000). Over the last decade, evidence has been generated to support the premise that Aß can directly lead to the generation of ROS. Interestingly, the ROS generation by Aß appears to be strongly influenced by the aggregational states of the peptides, such that inhibition of further Aß aggregation from soluble Aß oligomers to fibril formation can reduce neuronal toxicity and ROS generation (Guilloreau et al., 2007;Tabner et al., 2005;Tomiyama et al., 1996).

Based on the "chronic oxidative stress" condition observed in aging, the following question arises: is Aß exacerbating the deleterious network increasing the oxidative damage? There is overwhelming evidence that brain tissue in patients with AD is exposed chronically to oxidative stress

during the course of the disease. Thus, oxidized proteins localized to neuronal cell bodies and nuclei of both neurons and glial cells are restricted to brain regions of AD pathology and, absent from control cases (Castegna et al., 2003;Hensley et al., 1998;Smith et al., 1996;Smith et al., 1997). The increased oxidative stress parameters in Mild Cognitive Impairment (MCI) brain regions (Butterfield et al., 2007b;Butterfield et al., 2007a;Butterfield and Sultana, 2007) suggest that oxidative stress may be an early event in the progression from normal brain to AD pathology. These results support the "oxidative stress hypothesis" of AD in which oxidative damage appears to precede the clinical and pathological manifestation of the disease (Nunomura et al., 2000;Nunomura et al., 2001).

The progressive aggregation, accumulation, and further deposition of Aß is generally unknown. Recent evidence suggests that it is not a result of increased Aß synthesis, but rather a decrease in Aß degradation (Saito et al., 2003). Aß clearance occurs primarily through the action of a group of peptidases including neprilysin (NEP), insulysin (insulin degrading enzyme, IDE) and endothelin converting enzyme (ECE). Surprisingly, and further supporting the oxidative stress hypothesis of AD, NEP and IDE were recently found to be oxidized and inactivated by ROS (Caccamo et al., 2005;Shinall et al., 2005;Wang et al., 2003a). Moreover, NEP and IDE steady-state levels were also reported to uniformly decrease in AD (Caccamo et al., 2005;Russo et al., 2005;Wang et al., 2005). Thus, the increasing Aß due to its reduced clearance by IDE and NEP oxidation is thought to enhance the formation of ROS, starting a vicious cycle leading to a spiraling effect in development and progression of the AD neuropathology. In **Chapter 4 (Figure 4-5)**, we provided a clear example of this phenomenon in which a single injection of Aß leads to the oxidation of proNGF through generation of ROS, altering its maturation, degradation and biological properties.

Under these unfavorable circumstances, how would the antioxidant defense system react to such insult? In the AD CNS it has been observed that a robust increase in both the mRNA expression and the protein content of the main antioxidant enzymes, such as Cu/Zn-SOD and Mn-SOD (Ceballos et al., 1991;Delacourte et al., 1988;Furuta et al., 1995;Marcus et al., 2006) occurs. However, it is well known that peroxynitrite can directly inactivate these enzymes in the brain by tyrosine nitration mechanisms (Aoyama et al., 2000;Bayir et al., 2007;Demicheli et al., 2007;Filipovic et al., 2007;Yamakura et al., 1998). Despite its elevated levels, several studies have reported decreased antioxidant activity due to nitration of SOD enzymes in AD (Aoyama et al., 2000;Casado et al., 2007;Marcus et al., 2006). In sum, Aß-mediated generation of ROS causes the inactivation of the

enzymes responsible for its clearance, generating a neurotoxic loop contributing to the progression of AD neuropathology.

5B2 The "Calcium Hypothesis" and the "Mitochondrial Hypothesis" of Alzheimer's disease

As mentioned previously (**Section 5B1**), several studies have shown that the ROS generation by Aß appears to be strongly influenced by the aggregation state of Aß. One mechanism proposed for Aß-induced neurotoxicity involves the ability of Aß to incorporate into the plasma membranes to form cation-selective (including calcium) ion-permeable channels (Arispe et al., 1993b;Arispe et al., 1993a). The "calcium hypothesis of AD" (Arispe et al., 1994) suggests that altered intracellular calcium or disturbances in calcium homeostasis concerns cellular mechanisms underlying neuronal pathology. It is commonly believed that calcium does not modulate amyloid toxicity from the outside, but from the inside after its cellular uptake (Bhatia et al., 2000;Kawahara et al., 2000;Lin et al., 2001). *In vitro* experiments have shown that in the absence of calcium in the medium, Aß did not induce cellular degeneration (Bhatia et al., 2000;Zhu et al., 2000). In a 3D structural analysis, Lal and coworkers have revealed that the amyloid ion channels are made of small Aß oligomers (trimers to octamers) (Lal et al., 2007). Recently, specific Aß channel blockers have been shown to prevent the Aß-induced cytotoxicity in cultured cells (Diaz et al., 2006;Simakova and Arispe, 2006).

I have already described in **Section 5A6** that the mitochondrial parameters are affected by the aging process, including changes in the mitochondrial membrane fluidity, accumulation of mutations in the mtDNA and accumulation of ROS supporting the "mitochondrial theory of aging" (Economos, 1980). Since mitochondria are very sensitive to changes in intracellular calcium concentration, the interaction of Aß oligomers with the cytoplasmic membrane to form an amyloid ion channel should cause disruption in the intracellular calcium homeostasis. This redox transition leads to mitochondrial membrane potential instability, and mitochondrial integrity (Zorov et al., 2006). Several *in vitro* studies of Aß and mitochondrial function have reported that Aß affects mitochondrial DNA and proteins, leading to the impairment of the electronic transport chain and ultimately mitochondrial dysfunction (Bozner et al., 1997;Keil et al., 2004;Pappolla et al., 1998). Recently, Lustbader *et al* (Lustbader et al., 2004) reported that Aß-binding alcohol dehydrogenase directly interacts with Aß in the mitochondria of AD patients and transgenic mice and that this interaction promotes the leakage of ROS, ultimately leading to mitochondrial dysfunction. This is supported by the demonstration that Aß accumulates progressively within mitochondria in brains of AD patients (Fernandez-Vizarra et al.,

2004). Furthermore, these observations were recently confirmed in transgenic mice models of AD (Caspersen et al., 2005;Manczak et al., 2006) showing that Aß progressively accumulates in mitochondria and that Aß is associated with diminished enzymatic activity of respiratory chain complexes.

On the other hand, the "mitochondrial hypothesis" for sporadic, late onset Alzheimer's disease (Swerdlow and Khan, 2004) is based on the fact that mitochondrial dysfunction is more anatomically widespread than is Aß deposition and therefore mitochondrial dysfunction cannot be entirely accounted for by Aß (Swerdlow and Kish, 2002). Moreover, these authors have proposed that bioenergetics dysfunction and mitochondrial ROS overproduction represents a nexus between the "mitochondrial cascade hypothesis" and the 'amyloid cascade hypothesis".

5B3 Microglial Activation, nitric oxide production and Neuroinflammation in Alzheimer's disease.

As presented earlier in **Chapter 4**, a single injection of Aß oligomers into the rat hippocampus induced microglial activation (using CD40 marker, **Figure 4-3**); and upregulation of inducible nitric oxide sinthase (iNOS, **Figure 4-3**), thus generating nitric oxide and peroxynitrite. As a consequence, increased protein oxidation was observed in the injected area (**Figure 4-4**). Microglia are exquisitely sensitive to almost any disturbance of brain homeostasis (Kreutzberg, 1996) which rapidly causes them to change their morphology and up-regulate the expression of a range of protein markers which define these cells as "activated microglia". Recent imaging studies *in vivo* show that these cells, even in their resting-state, are highly active, with their fine processes continually surveying their local microenvironment (Nimmerjahn et al., 2005). The activating stimulus could be either Aß itself or the degenerating processes on neurons generated by calcium homeostasis disruption, mitochondria dysfunction and ROS production.

The extracellular deposits of Aß peptides are markedly accumulated in AD brains and are associated with microglia. Studies have revealed microglial clearance of monomers and oligomers of Aß peptides (Kakimura et al., 2002). On the other hand, microglia releases proinflammatory cytoquines (TNF-α, IL-1ß and IL-6). Moreover, Aß-induced iNOS expression results in an overproduction of nitric oxide, which reacts with superoxide to yield peroxynitrite and may, therefore, increase the overall radical burden in Aß-loaded brain regions (Beckman, 1994;Beckman and Koppenol, 1996;McGeer and McGeer, 1995a). In support of this, aberrant expression of iNOS and

nitrated and oxidized proteins were co-localized in Aß-loaded brain regions (Luth et al., 2003) and suggest that peroxynitrite is a mediator of the toxicity of activated microglia (Xie et al., 2002). The chronic neuroinflammatory cascade produced by a local neuronal insult by the presence of Aß initiate a vicious circle, maintaining and amplifying the inflammatory cascade, contributing to the progression and pathogenesis of Alzheimer's disease (for reviews see Cacquevel et al., 2004;Eikelenboom et al., 2006).

5B4 The "Cholinergic Hypothesis" and the "Neurotrophic Hypothesis" of Alzheimer's disease

In 1974, Drachman and Leavitt observed a correlation between human memory and the cholinergic system (Drachman and Leavitt, 1974a). Two years later, a significant loss of neurochemical cholinergic markers in the cerebral cortex was reported by Davis and collaborators, and Bowen and collaborators (Bowen and Smith, 1976;Davies and Maloney, 1976). Further to this, Whitehouse and co-workers (Whitehouse et al., 1982) reported the loss (no cholinergic markers were yet available) of presumptive cholinergic neurons in the nucleus magnocellularis of Meynert (i.e. nucleus basalis) in post-mortem samples of AD sufferers. This prompted the formulation of the so-called "cholinergic hypothesis of AD" (Coyle et al., 1983); in the knowledge of the central role of cholinergic neurons in memory mechanisms. Based on this hypothesis, the severe memory loss observed with AD was related to a loss of the neurotransmitter acetylcholine. The cholinergic hypothesis postulated that a loss of cholinergic neurons and/or cholinergic transmission was responsible for at least a portion of the cognitive decline observed in AD sufferers, and that replacement of the neurotransmitter would restore normal cognitive function. In this regard, animal and human studies demonstrated a powerful stimulation of learning and memory processes when acetylcholine esterase inhibitors, such as physostigmine, were administered (Davis and Mohs, 1982;Peters and Levin, 1977;Santucci et al., 1989). Moreover, further studies demonstrated a significant correlation between cholinergic loss and memory deterioration in animal models of aging and AD (Fischer et al., 1987;Hunter et al., 2004;Veng et al., 2003). Thus, anticholinesterase treatment for AD was initiated with the hope that boosting cholinergic neurotransmission would restore memory decline. Despite rigorous cholinergic therapy, the decline in memory continued in most patients and the observed benefits of the cholinergic drugs were variable. While most studies showed significant memory improvement in AD patients compared to placebo (Francis et al., 1999;Giacobini, 2000;Rogers and Friedhoff, 1996) some did not (Forette et al., 1999;Harry and

Zakzanis, 2005). However, these therapies are still approved for symptomatic treatment and are widely accepted, likely because of a lack of viable alternatives (Kaduszkiewicz et al., 2005;Takeda et al., 2006).

The question is, why do basal forebrain cholinergic neurons deteriorate in AD? Several possibilities have been suggested, among these are: (a) decreased acetylcholine synthesis, shutting down its phenotypic expression (b) cholinergic neurons are selectively vulnerable to Aß peptides and (c) a general impairment of axonal transport, leading to the lost nutrition to the neurons. However, one of the most prevailing hypotheses on why this system undergoes consistent degeneration is the loss of trophic support. The "neurotrophin hypothesis" postulated by Appel in 1981 implied a loss of NGF trophic support to BFCN in AD. Specifically, it says that "the abnormalities would lie in failure of the hippocampal and cortical cells to supply the relevant cholinergic neurotrophic hormone with resulting impairment of medial septal and nucleus basalis neurons" (Appel, 1981). In this regard, some cholinergic markers such as TrkA and $p75^{NTR}$ have been proven to decline early in the disease progress (Mufson et al., 2000;Mufson et al., 2002) similar to what has been described previously in AD patients (Mufson et al., 1989). These authors found that the degree of basal forebrain TrkA and $p75^{NTR}$ loss correlates with the cognitive impairment, as tested by the Mini-Mental State Examination (MMSE), Global Cognitive Test and Boston Naming Test (Mufson et al., 2000). In addition to receptor level changes, proNGF has been found to be increased by 40-50% in individuals with MCI (Peng et al., 2004). Taken together, these findings suggest that the trophic support is altered in early stages of the disease and may play a role as the disease progress.

In sum, we should consider that, in the AD brain, the occurrence of a deleterious network results from the interaction of a number of negative factors. This would include alterations in calcium homeostasis, mitochondrial dysfunction, exaggerated microglial activation, increased cytokines and nitric oxide production, among with chronic neuroinflammation and widespread oxidative damage. This deleterious environment is present in the brain of cognitively-impaired aged rats, in Mild Cognitive Impairment and in early and late stages of Alzheimer's disease in humans. The basal forebrain cholinergic system seems to be particularly vulnerable under these circumstances and degenerate during aging and AD. In agreement with the "neurotrophic hypothesis" and based on the results presented here, we strongly believe that the basal forebrain cholinergic system degenerates because the deleterious environment chronically affects the NGF maturation and degradation, with the

consequence of a loss in trophic actions. This assumption is based also on previous studies showing that: (1) BFCN are highly sensitive to interruptions in the NGF supply, such that an initially small failure in NGF signaling may rapidly be exacerbated by the down-regulation of NGF receptors (Sofroniew and Mobley, 1993) (2) that in mature rats provoking either immunoneutralization of endogenous NGF or blockage of their TrkA receptors results in the loss of pre-existing cortical cholinergic pre-synaptic sites (Debeir et al., 1999) and (3) that in aged rats the BFCN exhibit a reduced capacity for retrograde transport of NGF (Cooper et al., 1994;De Lacalle et al., 1996).

5C Summary

Some of the earliest clinical trials related to NGF in the AD patient were performed in Sweden. NGF administered via cannula implanted into the lateral ventricle led to undesirable side effects and to NGF-provoked pain, which was dose-dependent and disappeared after cessation of the treatment (Jonhagen, 2000;Nordberg, 1999). Thus, as described in **Chapter 2**, we decided to test the effect of a small peptide mimetic of TrkA receptor in age impaired rats to overcome unwanted NGF side-effects. These studies allowed us to confirm that solely activating TrkA receptor in the brain was enough to reverse the decline in the basal forebrain cholinergic phenotype and ameliorate the cognitive impairment of aged animals, with no detectable side-effects. Based on these results, we reasoned that there was a clear trophic disconnection between endogenous NGF and BFCN in aged rats displaying cognitive deficits.

For over two decades it has been assumed that the mature NGF form accounts for the neurotrophin's biological activity, including cell survival, neurite outgrowth and neuronal differentiation. The realization that proNGF might play a biological role in the CNS raised questions regarding the regulatory mechanisms leading to its release, as well as the control of the proNGF to NGF ratio and ultimately the degradation of the NGF molecule. To answer these questions we embarked on a series of *in vitro* and *in vivo* studies aimed at elucidating the preferential NGF form released from the cerebral cortex and the pathway leading to NGF maturation and degradation. These studies, covered in **Chapter 3** of this thesis, have revealed that proNGF is the main releasable form of the neurotrophin and that the maturation and degradation of NGF largely occurs in the extracellular space with the involvement of a complex protease cascade. In this regard, we have gathered experimental evidence that the protease cascade responsible for both the maturation of NGF from proNGF to mature NGF is also released from cortical neurons

along with proNGF, in an activity-dependent manner. In brief, we have proposed that the conversion of proNGF to NGF takes place mainly in the extracellular –as opposed to the prevalent view that it occurs intracellularly- by the action of plasminogen-derived plasmin, and by the action of tissue plasminogen activator (tPA); a mechanism regulated by neuroserpin. Likewise, we proposed that the degradation and consequent inactivation of the NGF not bound to receptors or internalized also takes place in the extracellular space. This process is mediated by the activation of the precursor of matrix metalloproteinase 9 (MMP-9) by plasmin, and the activity is regulated by the tissue inhibitor of matrix metalloproteinase 1 (TIMP-1). As mentioned above, we have shown that in this novel protease cascade, endogenous protease regulators and the NGF precursor, proNGF are simultaneously released upon neuronal stimulation. From these observations some fundamental issues are derived: (1) that most, if not all, of the radioimmunoassable NGF in cortical tissues from past investigations demonstrate proNGF and not mature NGF, (2) the precursor form of NGF is delivered "on demand" on a neuronal activity-dependent manner and (3) that the reason why the biologically active mature NGF has been so elusive is because the newly generated NGF rapidly binds the TrkA receptors at high affinity while the remnant NGF is promptly degraded by the activated MMP-9. The occurrence of such a metabolic cascade for the conversion and ultimate degradation of NGF is perhaps one of the finest examples that metabolic protein/enzymatic complexes do not act at random, but rather they are coordinated to be synthesized and delivered to their site of action in functional clusters (**see Figure 3-7**).

We have also proposed that the levels and activity of this NGF maturation/degradation cascade will ultimately play a key role in maintaining the basal forebrain cholinergic phenotype. The uninterrupted supply of NGF should maintain the neuronal phenotype of forebrain cholinergic neurons. A failure in the CNS of adult animals of this system should provoke cholinergic atrophy, while the preservation of these neurons, in the context of Alzheimer's disease, would favor a non-amyloidogenic pathway as it has been most elegantly demonstrated by Nitsch and collaborators (Nitsch et al., 1992). Our investigations have shown that the above outlined pathway is operative in *in vivo* conditions. So far, we have intervened at two levels of the proposed cascade. Firstly, we blocked plasmin formation by inhibiting tPA action by infusing the tPA endogenous inhibitor, and, secondly, by inhibiting the activated MMP-9 by infusing the broad-spectrum MMP inhibitor, GM6001. These experiments confirmed the *in vivo* validity of the biochemical pathway. Thus, we have found that the continuous, unilateral infusion of the endogenous tPA inhibitor neuroserpin

into the cerebral cortex of young rats provoked a several-fold increment in proNGF tissue levels, when compared to the contralateral, vehicle-injected side (**Figure 3-6**). In these experiments, no change was observed in unrelated, constitutive, proteins. In contrast, the unilateral infusion of the MMP-9 inhibitor, GM6001 in the cerebral cortex of young rats for 72 h caused a dramatic rise of endogenous NGF content when compared to values from the contralateral side that received the GM6001 negative control (**Figure 3-6**). These observations reinforce the notion that the newly described NGF metabolic pathway is operative in *in vivo* conditions.

Is this newly described NGF metabolic mechanism compromised in Alzheimer's disease? If so, such an event would easily explain the cholinergic vulnerability in AD. As described in **Chapter 4**, we have indeed observed a marked dysregulation of this NGF metabolic pathway in the AD cerebral cortex. In brief, we found a failure in the conversion of proNGF to NGF (lower plasminogen/plasmin activity) which is exacerbated by an increased NGF degradation resulting from a rise in MMP-9 activity. Our *in vivo* experiments indicated that a similar dysregulation can be provoked by a single injection of soluble Aβ oligomer in the hippocampus (**Figure 4-3**). In consequence, we hypothesized that pathological alterations of this metabolic cascade in the CNS might be the ultimate cause of the vulnerability of the NGF-dependent forebrain cholinergic neurons observed in AD. In support of this hypothesis is the finding of alterations in the tPA-proteolytic cascade generated by the Aß burden in AD. In addition to decrease levels (**Figure 4-1**), it has been shown that both tPA and plasminogen are oxidized and inactivated by reactive oxygen species, likely peroxynitrite (Butterfield et al., 2006;Nielsen et al., 2004). This situation results in a diminished production of NGF from the proNGF processing, favoring the CNS accumulation of proNGF. In the AD pathology, in addition, we have observed a remarkable increase in the activity of the NGF-degrading MMP-9 enzyme (**Figure 4-2**). In such a scenario the NGF supply in AD would experience a situation of double jeopardy, i.e. diminished production and increase degradation.

Interestingly, we have found that the accumulated proNGF in AD brains has been nitrated by peroxynitrite (**Figure 4-4**). Thus, nitrated proNGF is increased and it represented the predominant molecular form of NGF in the AD brain. This is not surprising because it has also been reported that oxidative inactivation of the proteasome occurs in MCI and AD (Cecarini et al., 2007), leading to the accumulation of oxidized and nitrated proteins. In order to investigate whether nitrated proNGF can be converted by plasmin into active NGF, we performed *in vitro*

studies and we found that nitrated proNGF generates nitrated NGF, which displayed a much reduced trophic support to cultured DRG neurons and a reduced capacity to activate TrkA when injected *in vivo* (**see Figure 4-5**). This is a particularly important finding because the resulting failure of the NGF trophic stimulation would lead to the progressive TrkA receptor down-regulation, following atrophy of the basal forebrain cholinergic system and the consequent diminished contribution of the CNS cholinergic system to learning mechanisms.

In conclusion, we think that the well established vulnerability of NGF-dependent forebrain cholinergic neurons in Aging and AD is caused by a profound dismetabolism of the complex protease cascade, which is responsible for the maturation and degradation of NGF in the extracellular space. This NGF maturation/degradation alteration is a consequence of the deleterious environment characterized by chronic neuroinflammation and widespread oxidative stress. Both animal and human brain data supports this hypothesis. This situation is aggravated in AD, because Aβ *per se* exacerbates calcium homeostasis alterations, mitochondrial dysfunction, microglial activation and the generation of reactive oxygen species. These conditions provoke a vicious cycle (autotoxic loop) in AD pathology in which the progressive accumulation of Aβ will disregulate the NGF metabolic cascade provoking cholinergic atrophy, which, in turn, will favor a amyloidogenic metabolism of APP thus escalating the NGF cholinergic compromise.

Contribution to Original Knowledge

1 "Long-Lasting Rescue of Age-Associated Deficits in Cognition and the CNS Cholinergic Phenotype by a Partial Agonist Peptidomimetic Ligand of TrkA *The Journal of Neuroscience* 24(37):8009–8018, 2004. **Martin A. Bruno,** Paul B. S. Clarke, Alicia Seltzer, Remi Quirion, Kevin Burgess, A. Claudio Cuello and H. Uri Saragovi.

This work provides three major contributions to original knowledge: (1) Age-related cholinergic atrophy is not a widespread predicament in brain aging, it is more marked in aged-cognitively impaired animals. The cognitive status of the animals correlates with a specific cortical cholinergic synaptic loss and profound shrinkage of cholinergic somata of nucleus basalis neurons. (2) D3, a small partial mimetic of TrkA receptors reversed the loss of cortical cholinergic synaptic contacts and the decrease in cortical cholinergic activity. Moreover, D3 reversed the shrinkage of cholinergic neurons in spatial-cognitively impaired aged rats. (3) The D3-mediated cholinergic rescue was selective, long-lived and resulted in a significant improvement of learning/memory in cognitively impaired aged rats.

2 "Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade" *PNAS,* 103, 17:6735–6740, 2006 **Martin A. Bruno** and A. Claudio Cuello**.**

This work provides several contributions to original knowledge: (1) We have found that proNGF is the molecular form of NGF released upon neuronal stimulation from rat cerebral cortex tissue. (2) The activity-dependent release of proNGF depends on intracellular calcium stores, and independent of extracellular calcium. (3) We have identified the members of the poteolytic cascade responsible for proNGF maturation upon release. (4) We have demonstrated that the members of the proposed cascade co-localize with NGF and follow the same release-pattern that proNGF. (5) We have identified MMP-9, which degrades mature NGF and with its inhibitor tissue inhibitor of matrix metalloproteinases -1 (TIMP-1), co-localize and are co-released with proNGF upon

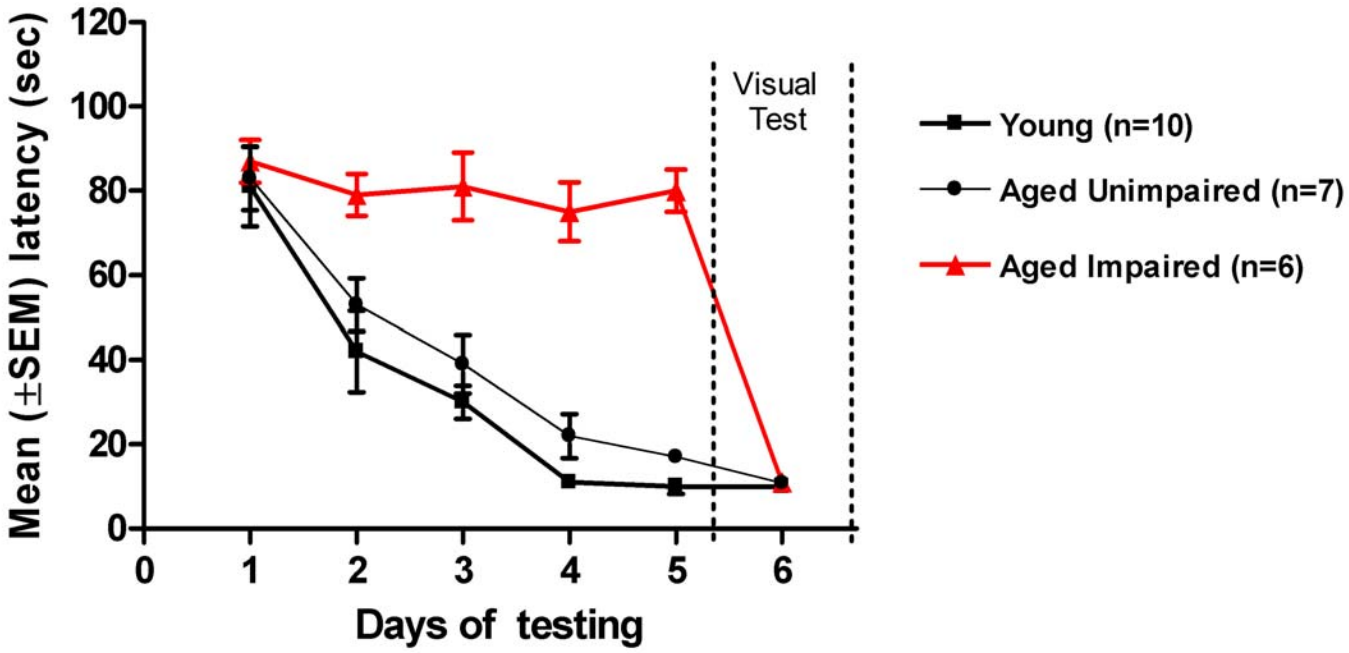
neuronal stimulation. (6) We have provided evidence that this mechanism is operative in *in vivo* situation, since the application of neuroserpin lead to accumulation of cortical proNGF, while inhibition of MMP-9 lead to decreased levels of proNGF and increased levels of mature NGF.

3 **"**Abeta-induced NGF trophic disconnection in Alzheimer's disease" *PNAS,* submitted **Bruno MA**, Leon WC, Fragoso G, Mushynski WE, Almazan G and Cuello, AC, submitted.

The present work provides several major contributions to original knowledge: (1)We have found an altered pattern of the members of the protease cascade responsible for proNGF maturation in AD cortical tissue, indicating a diminished conversion of proNGF to NGF. (2) We reported increased levels and activity of the NGF-degrading matrix metalloproteinase-9 (MMP-9) in cortical AD tissue, which degrades mature NGF (3). We have shown that increased nitrated proNGF is the predominant form of NGF in the cerebral cortex of the AD brain. (4) We observed that a single injection of soluble Aß oligomers into the rat hippocampus is sufficient to activate microglial cells and generate nitric oxide and peroxynitrite, which ultimately nitrate proNGF. (5) We demonstrated that this Aß-mediated peroxynitrite formation and proNGF nitration can be prevented by pre-treatment of Aß-injected rats with minocycline. (6) Our *in vitro* studies revealed that pre-treatment of proNGF with peroxynitrite generates peroxi-nitrated NGF once plasmin is added to the reaction medium. (7) Our studies demonstrated that peroxi-nitrated NGF loses its neurotrophic function in supporting survival of undifferentiated DRG neurons or their phenotype after differentiation. (8) Finally, our *in vivo* studies injecting peroxi-nitrated NGF into the hippocampus have revealed that once NGF is nitrated by peroxynitrite, it loses its ability to activate TrkA receptors.

Appendices

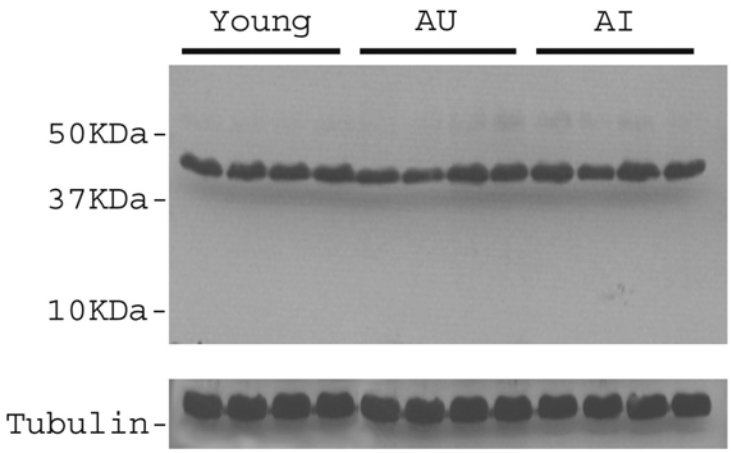
Appendix A

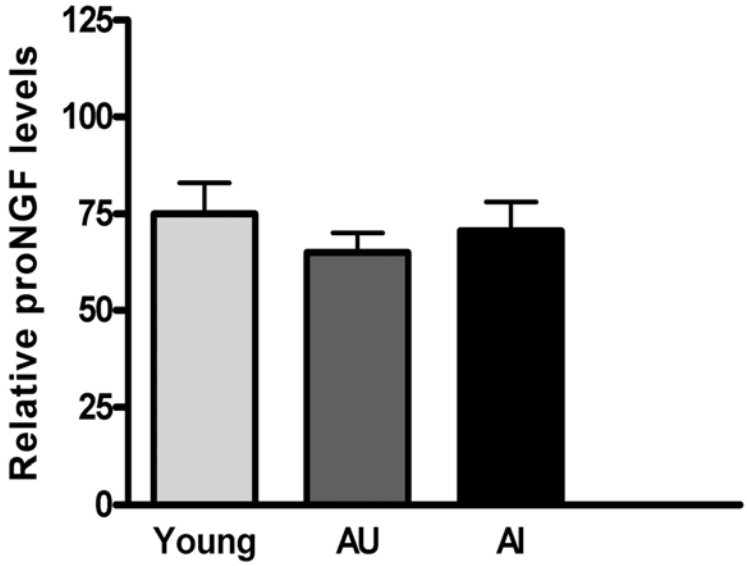


Morris water maze Aged (n = 60) and young (n = 10) rats were screened for spatial memory deficits using the Morris water maze as described in **Chapter 2, Section 2C**. The animals were required to find a submerged platform in a 1.4 m diameter pool of white, nontoxic colored water using only distal and spatial clues available in the testing room. Throughout, all tests were always carried in the same room and set up. The center of the escape platform (15 cm diameter) was located 45 cm from the pool wall, in the northeast quadrant. Animals were tested in 15 trials over 5 consecutive days (three trials per day with an intertrial time of 20 min) with the platform 2 cm below the water. At the end of the testing periods, all animals were given three trials in which the platform was raised 2cm above the water to exclude visual deficits as the cause of poor performance,

Appendix B

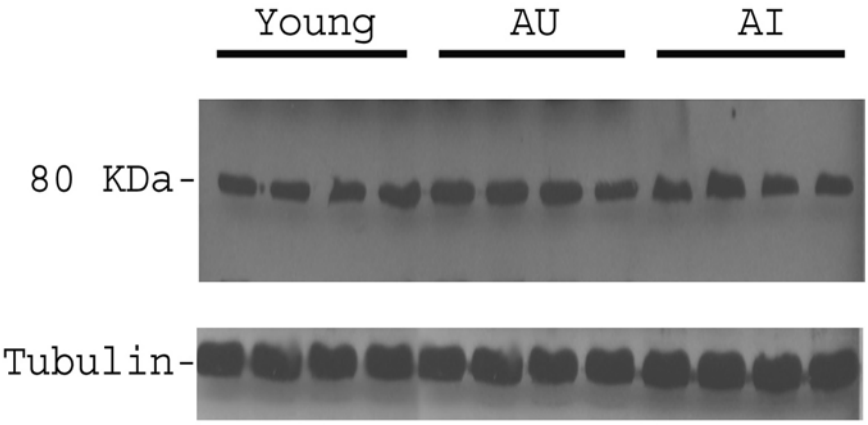
Cortical proNGF levels in young; age-unimapred (AU) and age-impaired (AI) Fischer-344 rats
(for methods see Section 3C)

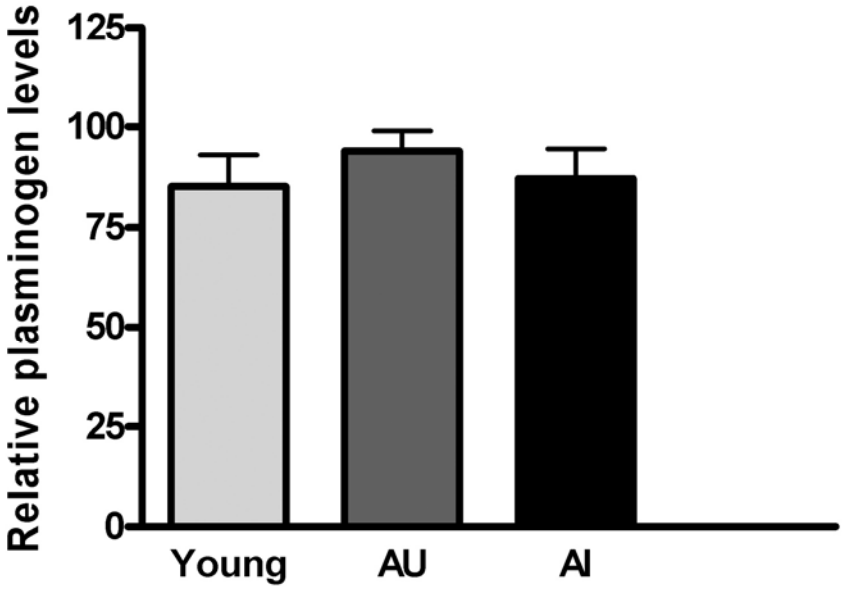




Appendix C

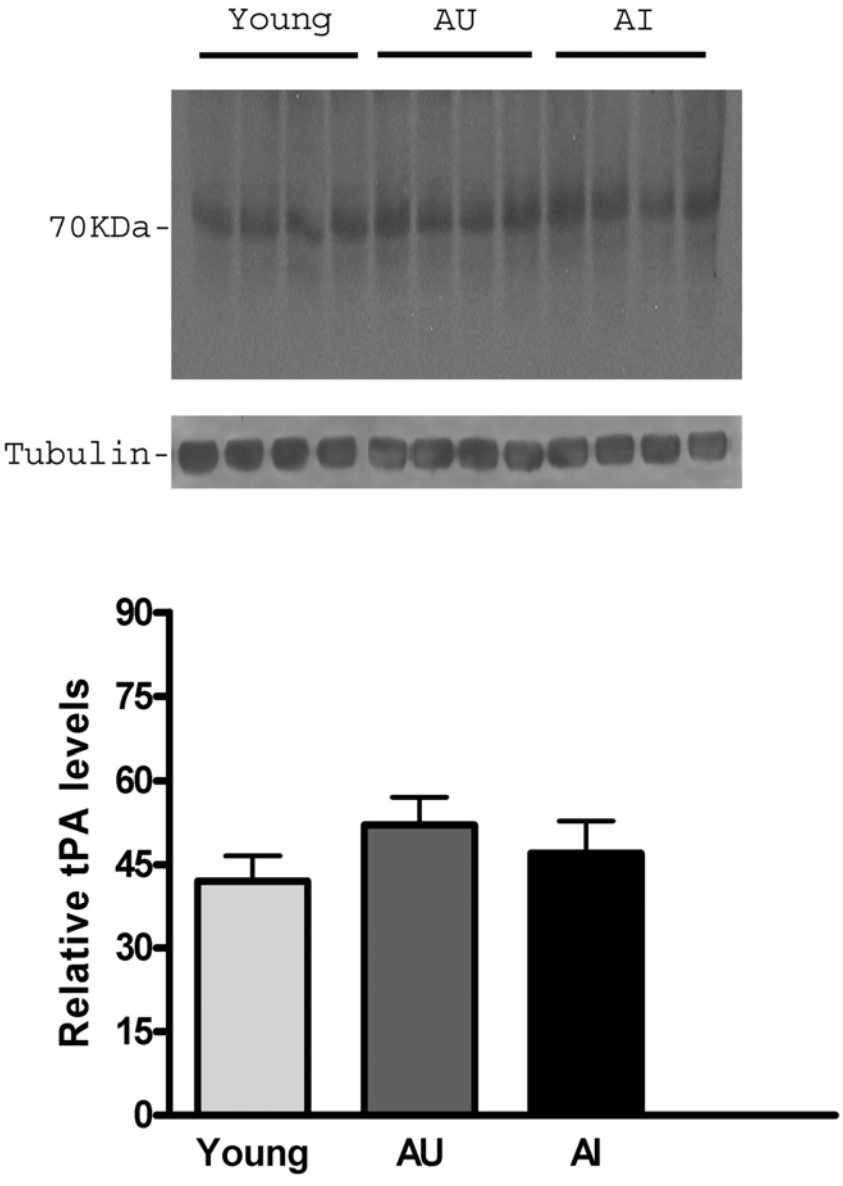
Cortical plasminogen levels in young; age-unimpaired (AU) and age-impaired Fischer-344 rats (for methods see Section 3C)





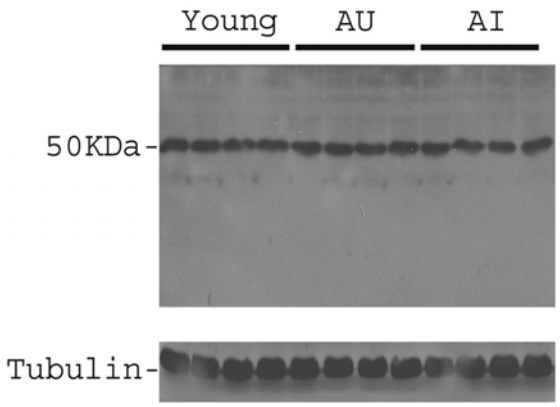
Appendix D

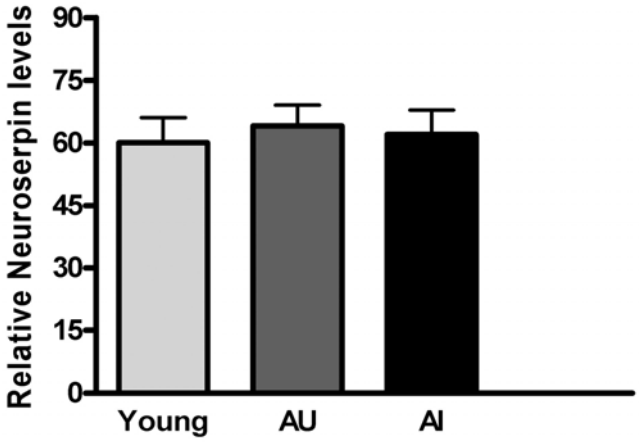
Cortical tPA levels in young, age-imapired and age-unimpaired Fischer-344 rats (for methods see Section 3C)



Appendix E

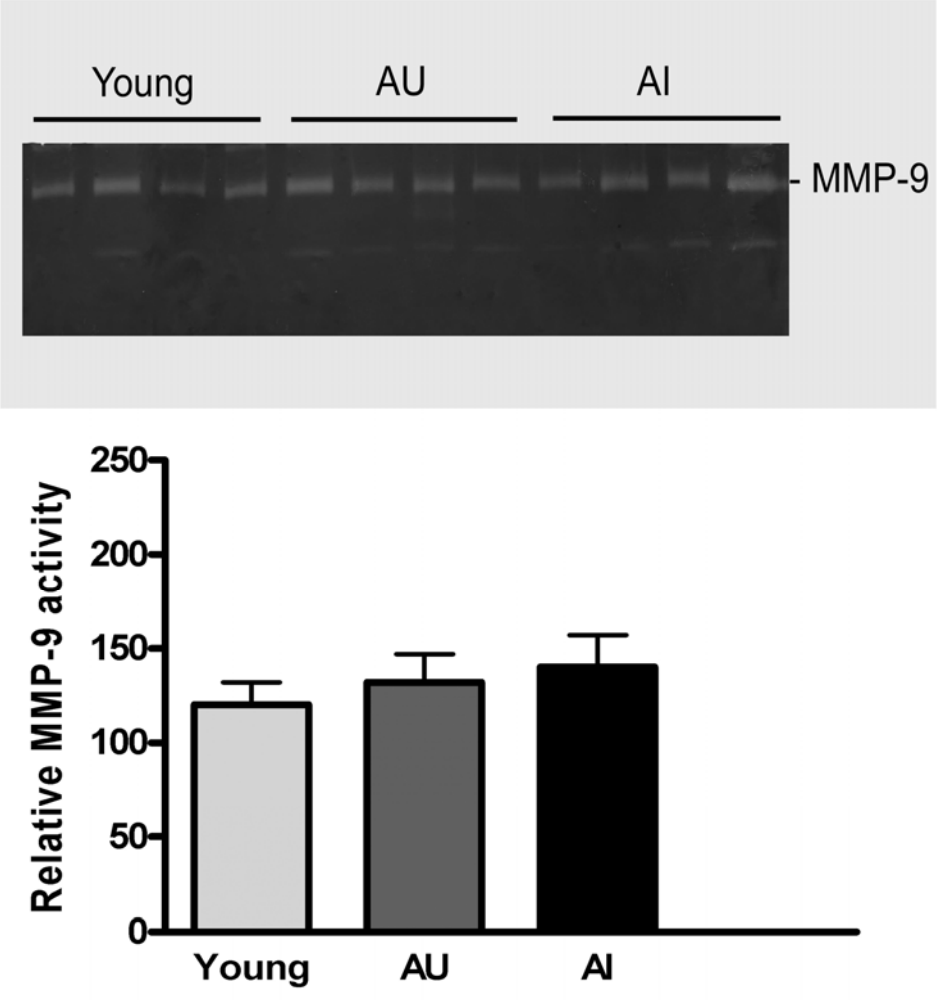
Cortical levels of neuroserpin in young, age-unimpaired (AU) and age-impaired (AI) Fischer-344 rats
(for methods see Section 3C)





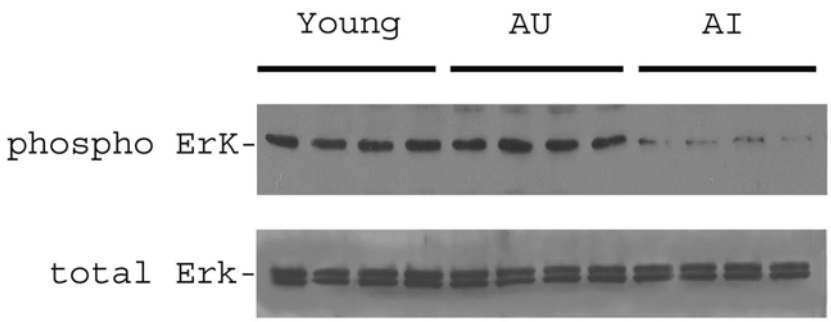
Appendix F

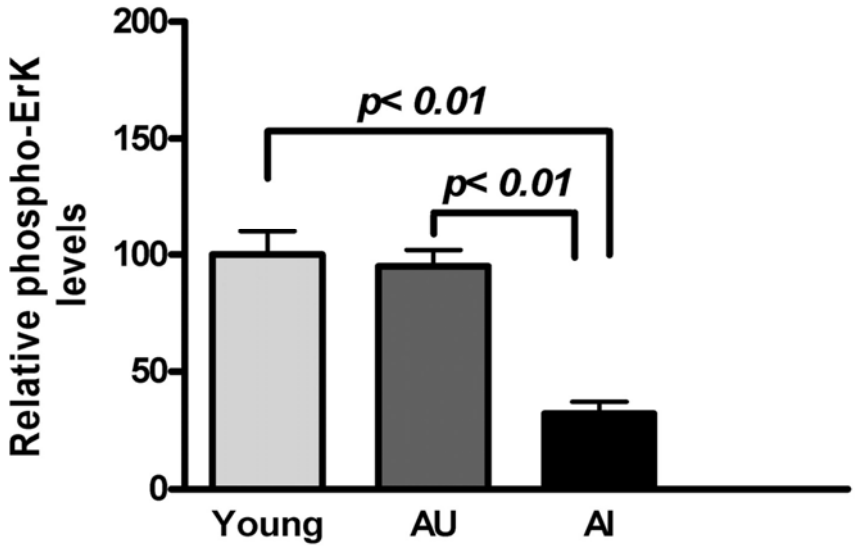
Cortical MMP-9 gelatinolytic activity in young; age-unimpaired (AU) and age-impaired (AI) Fischer-344 rats
(for detail in zymography protocol see Section 3C)



Appendix G

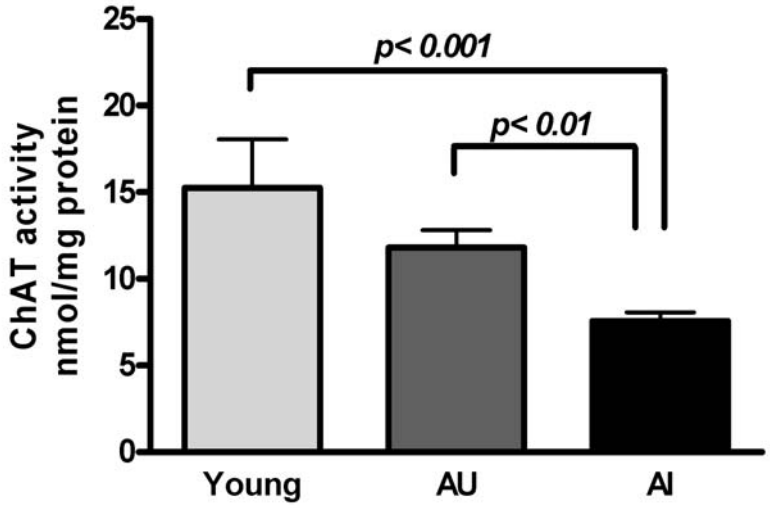
Phospho *vs.* total Erk content in cerebral cortex of young *vs.* age-unimpaired (AU) *vs.* age-impaired (AI) Fischer-344 rats





Appendix H

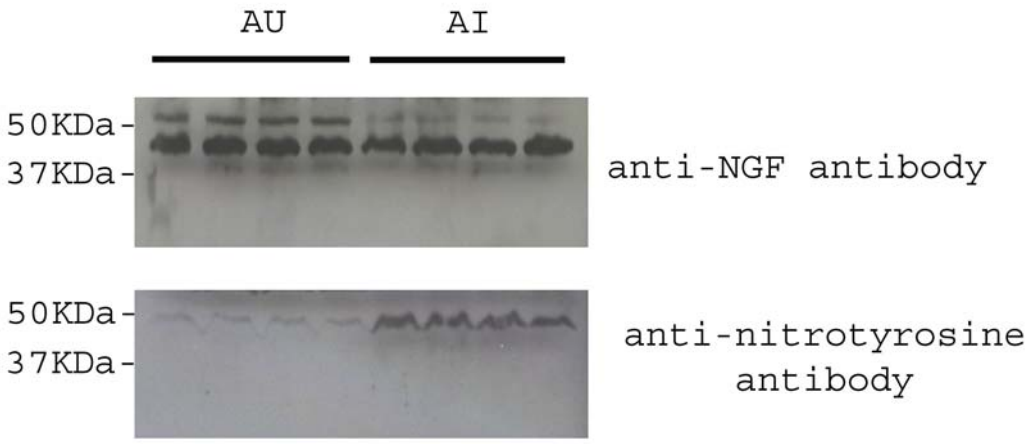
ChAT activity measurement from cerebral cortex homogenates of young; age-unimpaired (AU) and age-impaired (AI) Fischer- 344 rats



ChAT activity assays were performed directly in the wells by using Fonnum's method (described by Debeir, 1999). In brief, after incubation at 37°C for 1 h with a solution containing 25 mM EDTA, 25 mM choline chloride, 500 mM acetyl-CoA, 125 mM sodium phosphate buffer (pH 7.4), 750 mM sodium chloride, [^{3}H] acetyl- CoA, and 250 mM eserine or 0.125 units/ml acetylcholine esterase, ChAT activity was stopped by the addition of an excess of acetylcholine at 4°C. Then, [^{3}H] acetylcholine was extracted with 20 mg/ml sodium tetraphenylboron in 3-heptanone

Appendix I

Nitrated proNGF contents in the cerebral cortex of young; age-unimpaired (AU) and age-impaired Fischer-344 rats
(for Immunoprecipitation and Western blot protocols please see Section 4C)



Appendix J

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