

On the Molecular Basis Linking Nerve Growth Factor (NGF) to Alzheimer's Disease

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SUMMARY

1. Alzheimer's disease (AD) is pathologically defined by the deposition of amyloid peptide and neurofibrillary tangles and is characterized by a progressive loss of cognition and memory function, due to marked cortical cholinergic depletion.

2. Cholinergic cortical innervation is provided by basal forebrain cholinergic neurons. The neurotrophin Nerve Growth Factor (NGF) promotes survival and differentiation of basal forebrain cholinergic neurons.

3. This assertion has been at the basis of the hypothesis developed in the last 20 years, whereby NGF deprivation would be one of the factor involved in the etiology of sporadic forms of AD.

4. In this review, we shall summarize data that lead to the production and characterization of a mouse model for AD (AD11 anti-NGF mice), based on the expression of transgenic antibodies neutralizing NGF. The AD-like phenotype of AD11 mice will be discussed on the basis of recent studies that have posed NGF and its precursor pro-NGF back to the stage of AD-like neurodegeneration, showing the involvement of the precursor pro-NGF in one of the cascades leading to AD neurodegeneration.

KEY WORDS: Alzheimer's disease; nerve growth factor; TrkA; P75NTR; sortilin; transgenic mice; neurodegeneration.

Alzheimer's disease (AD) is a progressive, incurable disease representing the major cause of dementia in the elderly. Two forms of AD exist, a familial one (multiple family members are affected) and a sporadic one, in which one or a few members of a family have the disease (Selkoe, 2001). From the neuropathological point of view, the presence of two characteristic hallmarks defines the disease: plaques of β -amyloid protein and neurofibrillary tangles, mainly constituted of paired helical filaments of abnormally phosphorylated tau proteins (Selkoe, 2001). As the disease progresses, neuronal death appears. In particular, cholinergic neurons of the basal forebrain are lost (Bartus *et al.*, 1982; Whitehouse *et al.*, 1982), accounting for the development of cognitive impairments (Perry *et al.*, 1978; Collerton, 1986; DeKosky *et al.*, 1992).

For over a decade, the predominant viewpoint among researchers and clinicians has been that the deposition of β -amyloid is the primary etiological event

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in AD, a theory known as the “amyloid cascade hypothesis” (Golde, 2005). According to this hypothesis, β -amyloid would originate from the proteolytic cleavage of the amyloid precursor protein (APP), and it would deposit and aggregate in extracellular insoluble forms to constitute plaques. These processes would cause neurotoxic damage and the consequent neurodegeneration characterizing AD. However, doubts can be raised about the reliability of the amyloid cascade hypothesis. One of the major issues rests in the experiments performed in transgenic mice, where the over-expression of mutated forms of APP linked to the familial forms of AD did not trigger the amyloid cascade (Selkoe, 2002). In the brain of these mice β -amyloid plaques can be found, but not tangles, suggesting that tau pathology, which is an essential part of the dementing process, does not depend on the altered processing of APP. Such observations led to argue that some common factors should induce both plaque and tangle formation. Nerve growth factor (NGF) might be one of these.

MATURE NGF AND ITS RECEPTORS

NGF is a highly conserved protein that was first identified in two sarcoma tissues (Levi-Montalcini and Hamburger, 1951) and in certain snake venoms (Cohen, 1959). Murine and human NGF genes code for two transcripts to produce 34 and 27 kDa precursors (Scott *et al.*, 1983; Ullrich *et al.*, 1983). These NGF precursors are cleaved by convertases such as furin and convertases 1 and 2 to give rise to mature processed NGF of 13.2 kDa (Seidah *et al.*, 1996). When fully processed, NGF exists as a non-covalently bound homodimer (Stach and Shooter, 1974) and it is released through constitutive secretory pathways (Mowla *et al.*, 1999). NGF acts through binding to two classes of cell surface receptors, the tyrosine kinase receptor A (TrkA) and p75NTR.

TrkA is a type I transmembrane protein member of the receptor tyrosine kinase superfamily. It selectively binds to NGF, with a dissociation constant (kd) equal to 10^{-11} M (Cordon-Cardo *et al.*, 1991; Kaplan *et al.*, 1991; Klein *et al.*, 1991). Binding of the NGF homodimer causes receptor dimerization (Jing *et al.*, 1992) followed by autophosphorylation on tyrosine residues within the activation loop and by phosphorylation of the seven intracellular tyrosine residues (Cunningham *et al.*, 1997). These phosphorylated residues represent docking sites for signaling molecules which regulate cell growth and survival through different signaling pathways (Kaplan and Miller, 2000; Huang and Reichardt, 2003).

The second NGF receptor is represented by p75NTR (Chao *et al.*, 1986; Johnson *et al.*, 1986). It non-specifically binds to all neurotrophins with similar affinity (kd = 10^{-9} M) (Rodriguez-Tebar *et al.*, 1990; Squinto *et al.*, 1991). P75NTR is a type I transmembrane protein with an extracellular domain (ECD) constituted by four cysteine-rich repeated domains (CRDs) (Yan and Chao, 1991; Baldwin *et al.*, 1992). CRD3 is the domain responsible for the interaction with neurotrophins (Yan and Chao, 1991; Baldwin *et al.*, 1992; Chapman and Kuntz, 1995; Shamovsky *et al.*, 1999). The intracellular domain (ICD) contains several regions that mediate the interaction with signaling elements (Roux and Barker, 2002).

NGF AND ALZHEIMER'S DISEASE: THE CLASSICAL VIEW

During the last 20 years, several scientific supports have been given to the hypothesis that NGF might be one of the factors involved in the cascade of events leading to AD. NGF maintains and regulates the cholinergic phenotype of basal forebrain neurons (Mobley *et al.*, 1986; Li *et al.*, 1995) through the retrograde transport of the NGF/TrkA signaling complex from the cortex and hippocampus, where NGF is produced, to the basal forebrain (Mufson *et al.*, 1999). NGF protects cholinergic neurons, following age-related atrophy (Holtzman *et al.*, 1993; Smith *et al.*, 1999) and experimental surgical lesions (Koliatsos *et al.*, 1990, 1991a,b; Hefti *et al.*, 1993) and contribute to enhance memory in aged rodents (Fischer *et al.*, 1987; Markowska *et al.*, 1994, 1996).

In AD brains, the levels of NGF protein may be increased in the cortex and hippocampus (Crutcher *et al.*, 1993; Scott *et al.*, 1995; Fahnstock *et al.*, 1996; Hellweg *et al.*, 1998; Hock *et al.*, 2000). By contrast, NGF protein levels in the basal forebrain are reduced (Mufson *et al.*, 1994, 1995; Scott *et al.*, 1995). The increased levels of NGF in the cortex and hippocampus have been correlated to a decreased expression of TrkA receptors (Mufson *et al.*, 1996, 1997, 2000; Boissiere *et al.*, 1997; Hock *et al.*, 1998; Counts *et al.*, 2004) which are necessary to transport NGF to the basal forebrain.

Thus, according to this classical view, the atrophy of cholinergic neurons in AD patients would be the consequence of a lack of trophic support due to the impairment of NGF retrograde transport system and the consequent accumulation of NGF protein in target areas (Mufson *et al.*, 1999; Salehi *et al.*, 2003).

A confirmation to this hypothesis could have been obtained from animal models. However, in no animal model a direct link to AD was found. A difficulty was also due to the fact that mice, in which NGF synthesis was deleted by gene homologous recombination (*ngf* $-/-$ mice), die during the early postnatal period and do not allow to study effects of long term NGF deprivation (Crowley *et al.*, 1994). Heterozygous NGF knockout mice can survive until adulthood but no signs of AD-like neurodegeneration can be observed, with the exception of a 20% loss of basal forebrain cholinergic neurons (Chen *et al.*, 1997).

To overcome the problems encountered with *ngf* $-/-$ mice, a new approach was used to neutralize NGF activity. The activity of proteins can be blocked using antibodies that can not only recognize a given protein but neutralize its activity (neuroantibody approach) (Cattaneo, 1998).

In the initial study, the efficiency of antibody secretion by different type of cells was analyzed and it was found that the secretion by neuronal and glial cells was very high, with efficiency comparable to that of lymphoid cells transfected with the same antibody genes (Cattaneo and Neuberger, 1987). Thus, after achieving the proof of principle by producing transgenic mice expressing recombinant antibodies neutralizing substance P (Piccoli *et al.*, 1995), anti-NGF transgenic mice were generated (Ruberti *et al.*, 2000).

AD11 ANTI-NGF MICE: THE α D11 ANTIBODY

AD11 anti-NGF mice express the recombinant version of the monoclonal antibody (mAb) α D11. This antibody was selected after immunization of rats with mouse NGF and neutralizes NGF activity by a direct competition between NGF itself and the TrkA receptor (Cattaneo *et al.*, 1988). The epitope of mAb α D11 on NGF includes the loop region from residues 41–49, which contributes to the interaction surface between NGF and its high affinity receptor TrkA and distinguishes NGF from other members of the neurotrophin family (Ibanez *et al.*, 1991). The neutralizing properties of mAb α D11 and the specificity of this inhibition were extensively proven both *in vitro* and *in vivo*. In the first set of analysis, α D11 prevented the NGF-induced elongation of processes in PC12 cells and inhibited the survival of neurons from dorsal root ganglia (Molnar *et al.*, 1998). In the same experiment, mAb α D11 was not able to inhibit the pro-survival action of BDNF, NT-3 or NT-4 (Molnar *et al.*, 1998). *In vivo*, the implantation of hybridoma cells secreting mAb α D11 induces the atrophy of NGF-dependent BFCNs in young rats (Molnar *et al.*, 1998).

AD11 ANTI-NGF MICE: A COMPREHENSIVE MODEL FOR AD-LIKE NEURODEGENERATION

The starting point to obtain AD11 anti-NGF mice was to make it detectable against the mouse IgGs. For this purpose, the variable regions of the light and heavy chains of mAb α D11 were cloned and reassembled with the constant regions of the K and γ 1 chains of human immunoglobulins (Ruberti *et al.*, 1993). The chimeric α D11 antibody was placed under the transcriptional control of the early promoter region of human cytomegalovirus in two separate plasmids. The linearized DNA was then individually injected in mouse eggs (Ruberti *et al.*, 2000).

Mice expressing the neutralizing antibody α D11 were obtained by crossing the line of mice expressing the light chain of the recombinant version of the α D11 antibody (AD11-VK mice) with mice expressing the heavy chain of the same recombinant antibody (AD11-VH mice) (Ruberti *et al.*, 2000).

The two-tier approach used to obtain AD11 transgenic mice, allowed us to circumvent the effect of an early exposure to functional antibodies during the embryonic stage of development. Indeed, at embryonic day 13, transgenic antibodies are detectable but then their levels become undetectable in the prenatal and postnatal period during which NGF influences mouse development (Capsoni *et al.*, 2000a). Only after postnatal day 45 the antibody levels reach values above the detection threshold (Capsoni *et al.*, 2000a). The expression of the recombinant α D11 antibody allows neutralizing up to 50% of unbound NGF (Ruberti *et al.*, 2000).

AD11 mice are characterized by a progressive neurodegeneration which resembles many features of AD. The characterization of the AD-like neurodegeneration in the brain of AD11 mice was performed using immunohistochemical and biochemical techniques. A detailed time course was performed to assess the presence of behavioral deficits, using different paradigms such as the object recognition test, the

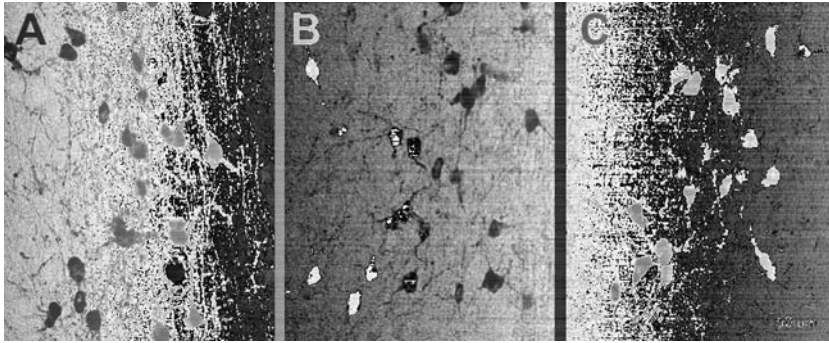


Fig. 1. Progressive atrophy and loss of choline acetyltransferase-positive neurons in the basal forebrain of AD11 mice. (A) WT mice (B) 2-month-old and (C) 15-month-old AD11 mice. Scale bar = 50 μ m.

radial and the Morris water maze tests. As expected from previous studies on the effects of NGF deprivation in the basal forebrain (Molnar *et al.*, 1997, 1998), AD11 mice are characterized by an atrophy and loss of cholinergic neurons in this brain region, that starts from 2 months of age remaining stable thereafter (Capsoni *et al.*, 2000, 2002b) (Fig. 1(A) and (B)) and progresses until 6 months of age, remaining stable thereafter (Capsoni *et al.*, 2002b) (Fig. 1(C)).

In the cortex and hippocampus, NGF deprivation provokes a re-distribution of the phosphorylated form of the microtubule associated protein tau. In AD, phosphorylated tau is the main component of intracellular neurofibrillary tangles found in cell bodies, neuropil threads and dystrophic neurites (Binder *et al.*, 2005; Iqbal *et al.*, 2005). The phosphorylated state of tau influences the solubility of this protein which becomes insoluble and accumulates in form of fibrils (the so-called paired helical filaments (PHFs) (Iqbal *et al.*, 2005). The regional progression of neurofibrillary accumulation is a characteristic of AD. Phosphorylated tau can be found first in transentorhinal and entorhinal regions of human brain (Braak and Braak, 1991). Interestingly, in 2-month-old AD11 mice the first accumulation of phosphorylated tau can also be found in the entorhinal region, spreading with age to other cortical and hippocampal areas (Capsoni *et al.*, 2002b). At subcellular level, tau accumulates in neuronal perikarion and then in dystrophic neurites (Capsoni *et al.*, 2000b, 2002b). From the biochemical point of view, aged AD11 mice show an accumulation of insoluble tau (Capsoni *et al.*, 2000b, 2002b). Electron microscopy studies performed on insoluble brain extracts revealed that insoluble tau assembles in aggregates that morphologically resemble those found in human AD PHFs (Capsoni *et al.*, 2002b) (Fig. 2).

In AD, abnormally phosphorylated tau provokes the disassembly of neuronal microtubules (Mandelkow *et al.*, 1996; Alonso *et al.*, 1997). Interestingly, in concomitance with appearance the progressive accumulation of phosphorylated tau, also in AD11 mice a re-distribution of neuronal microtubules can be observed in cortical areas (Capsoni *et al.*, 2002b).

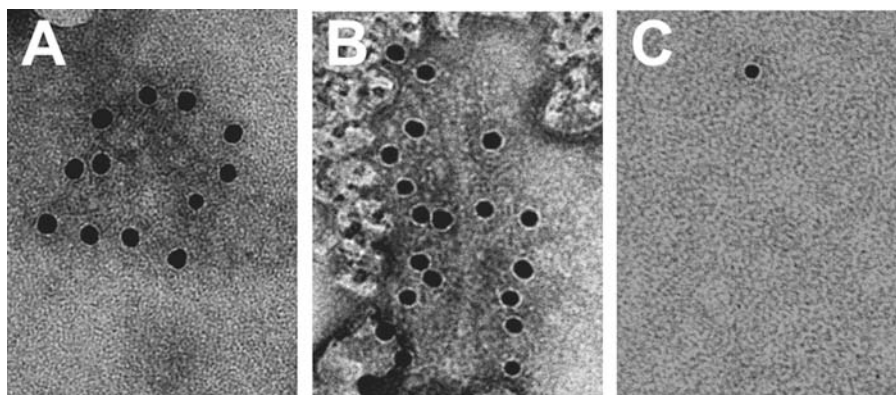


Fig. 2. Immunoelectron microscopy demonstrating the presence of PHF-like insoluble aggregates of the microtubule associated protein tau in brains from (A) AD11 mice and (B) human AD. Black dots correspond gold particles.

In AD11 mice, β -amyloid, which represents another principal endpoint of AD, is found in intracellular compartments, localized in MAP-2 positive dystrophic neurites of the hippocampus (Fig. 3), starting from 6 months of age (Capsoni *et al.*, 2002b). In aged AD11 mice, β -amyloid is also found in extracellular, plaque-like deposits (Capsoni *et al.*, 2002a) (Fig. 4). Interestingly, in AD11 mice, the deposition of β -amyloid occurs as a consequence of an altered processing of the endogenous β -amyloid precursor protein APP. This observation allows defining AD11 mice as a model for the sporadic form of AD, in opposition to other transgenic mice over-expressing mutated forms of human APP.

In AD11 mice, the neurodegeneration is accompanied by functional alterations that match with the appearance of the endpoints characterizing the neurodegeneration. Object recognition deficits appear early (at 4 months of age) soon after the onset of cholinergic deficits and tau accumulation (De Rosa *et al.*, 2005). At 6 months of age, when β -amyloid intracellular deposits start to appear, AD11 mice show impairment of synaptic plasticity in the cortex (Pesavento *et al.*, 2002) and hippocampus (E. Sola, S. Capsoni, A. Cattaneo, and E. Cherubini, unpublished data) and a progression of object recognition deficits (De Rosa *et al.*, 2005). In aged AD11 mice, synaptic plasticity deficits are severe and can no more be reverted by cholinesterase inhibitors (N. Origlia, S. Capsoni, L. Domenici, and A. Cattaneo, unpublished data). Behavioral deficits are severe, with impairment in working and spatial memory (Capsoni *et al.*, 2000b; Ruberti *et al.*, 2000; De Rosa *et al.*, 2005).

The complexity of the phenotype raised the doubt that only the effects of the local, decreased NGF signaling to basal forebrain neurons might not explain it.

Administering this neurotrophin to AD11 mice proved the fact that this neurodegeneration was specifically due to NGF deprivation. Since NGF, when injected peripherally, cannot cross the blood brain barrier, the classical approach used to deliver NGF to the brain is to directly inject NGF in brain parenchyma (Blesch and Tuszynski, 2004). In the case of AD11 mice, it was decided to apply an

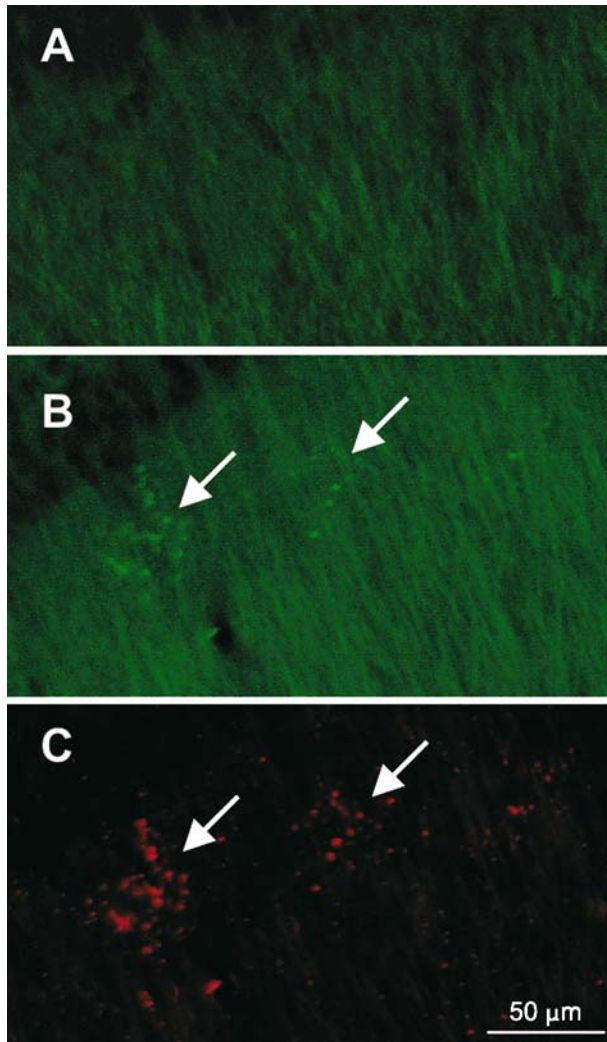


Fig. 3. Microtubule associated protein 2 (MAP2) immunohistochemistry in (A) WT and (B) AD11 mouse demonstrating the presence of dystrophic neurites (green) in the radial layer of the AD11 mouse hippocampus. (C) These dystrophic neurites are positive for β -amyloid (red). Arrows point to clusters of dystrophic neurites. Scale bar = 50 μ m.

approach first discovered by Frey *et al.* whereby NGF can be delivered to the brain in pharmacologically relevant concentrations through non-invasive intranasal injections (Frey *et al.*, 1997; Chen *et al.*, 1998). In AD11 mice, the intranasal injections were performed at early and at moderate stages of neurodegeneration, characterized by a loss of cholinergic neurons, intracellular accumulation of

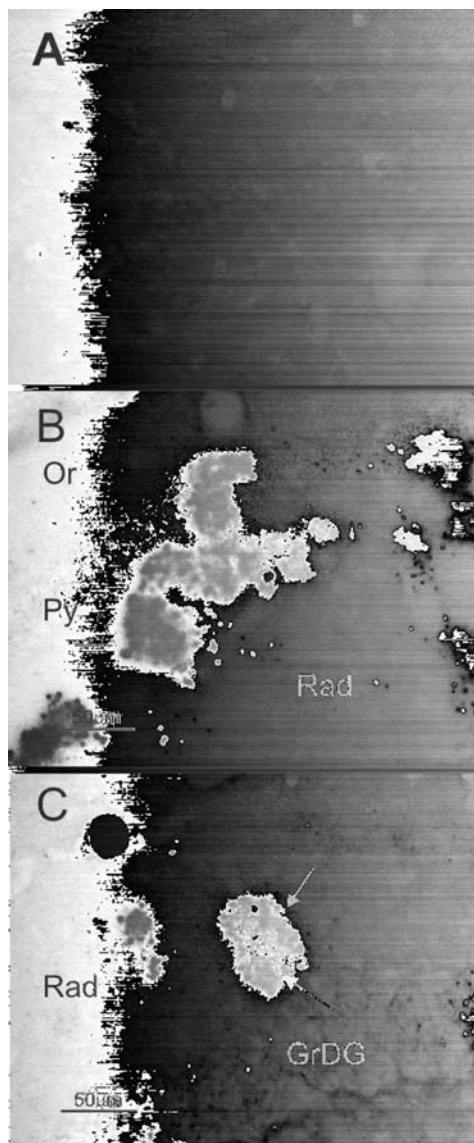


Fig. 4. β -amyloid immunohistochemistry in 15-month-old (A) WT and (B, C) AD11 mouse brain demonstrating the absence of plaques in WT mice and the accumulation of extracellular deposits in AD11 mice. In panel (B), extracellular deposition is intermingled to dystrophic neurites while in (C) plaques are constituted by a core of extracellular material (red arrow) surrounded by dystrophic neurites (black arrow). GrDG = granule layer of dentate gyrus; Py = pyramidal layer; Or = oriens layer; rad = stratum radiatum of the hippocampus. Scale bar = 50 μ m.

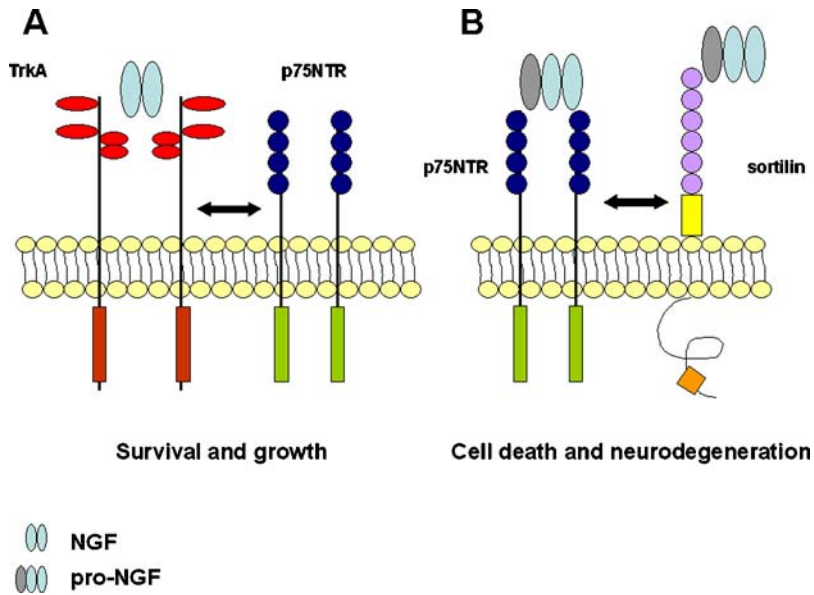


Fig. 5. Schematic representation of TrkA and p75NTR receptors. (A) According to the classical concept, p75NTR interacts with the TrkA receptor and increases its affinity for NGF, determining cell growth and survival. (B) The p75NTR/sortilin complex binds pro-NGF, determining cell death and neurodegeneration.

hyperphosphorylated tau and β -amyloid and behavioral deficits (Capsoni *et al.*, 2002c). In all cases, NGF was able to rescue the cholinergic deficit, the over-expression of hyperphosphorylated tau and beta amyloid (Capsoni *et al.*, 2002c). In these experiments, testing the ability of AD11 mice to recognize new objects from the familiar ones assessed behavioral deficits. AD11 mice do not recognize the two types of objects, while wild-type mice explore more the new object. The intranasal administration of NGF rescued the object recognition deficits in AD11 mice (De Rosa *et al.*, 2005). Thus, these experiments demonstrate that the AD-like neurodegeneration is due to alterations in NGF signaling. However, these experiments were inconclusive to clarify whether the rescue of the cholinergic phenotype was sufficient and essential to determine the rescue of the other relevant AD endpoints, such as phosphorylated tau and β -amyloid phenotype. Indeed, intranasal NGF might act not only on cholinergic neurons but also on other neuronal populations. The hint came from experiments during which the acetylcholinesterase inhibitors galantamine and donepezil were administered to AD11 mice (Capsoni *et al.*, 2002c, 2004). Both drugs were able to rescue basal forebrain cholinergic neurons but neither of them determined the amelioration of the tau-related phenotype (Capsoni *et al.*, 2000c, 2004). Thus, it might be concluded that the complex AD-like phenotype in AD11 mice was not a mere consequence of atrophy of basal forebrain cholinergic neurons, and a new hypothesis, linked to the new complexity of NGF signaling, had to be formulated.

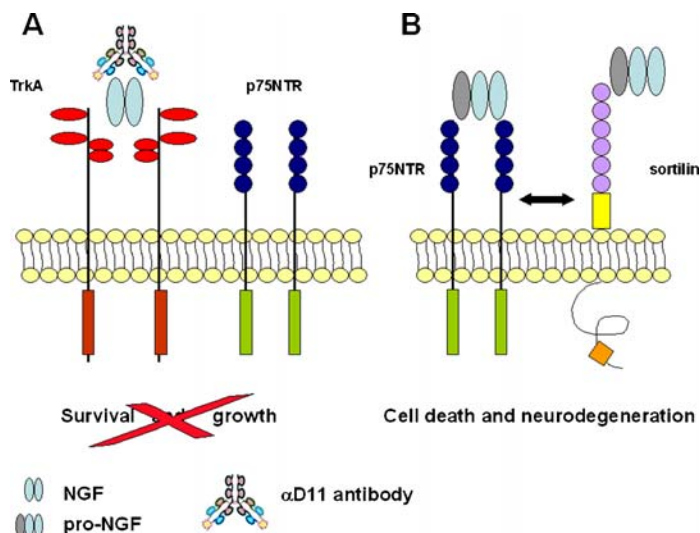


Fig. 6. Schematic diagram showing the effects of the α D11 antibody on pro-NGF/NGF signaling. (A) The α D11 antibody prevents binding of mature NGF to the TrkA/p75NTR complex determining a decrease in cell survival. (B) MAb α D11 does not bind pro-NGF that is free to interact with the p75NTR/sortilin complex, triggering neurodegeneration.

NGF AND ALZHEIMER'S DISEASE: THE NEW PERSPECTIVE

Two recent findings changed the perspective of the involvement of NGF and p75NTR in AD and helped in formulate a new theory.

Fahnestock *et al.* performed the first observation. They found that the major form of NGF accumulating in cerebral cortex and hippocampus of AD brains is indeed the precursor pro-NGF (Fahnestock *et al.*, 2001). The consequences of this accumulation might be explained by the second finding. In the past, it was a dogma thinking that the precursor pro-NGF was just a molecule devoid of signaling properties, with the mere function of being the precursor of mature NGF (Shooter, 2001). The only signaling properties taken into consideration for NGF-induced cell survival/death were those mediated by the TrkA receptors and p75NTR. While the first receptor is by definition the cell survival mediator (Fig. 5(A)), p75NTR has a twofold role. P75NTR increases the affinity and specificity of TrkA to NGF (Hempstead *et al.*, 1991; He and Garcia, 2004) but it also mediates cell apoptosis, in absence of TrkA signaling (Fig. 5(A)) (Kaplan and Miller, 2000). Recently, it was reported that pro-NGF preferentially binds, in presence of a co-receptor belonging to the Vps10p-domain receptors, sortilin, to p75NTR receptor (Nykjaer *et al.*, 2004). The binding of pro-NGF to the p75NTR/sortilin complex determines cell death and apoptosis in neuronal cells (Fig. 5(B)) (Lee *et al.*, 2001; Harrington *et al.*, 2004).

These findings prompted us to explore the possibility that the recombinant antibody α D11 preferentially binds to one of the two forms of NGF. Indeed, using plasmon resonance assays, it was found that mAb α D11 binds with picomolar

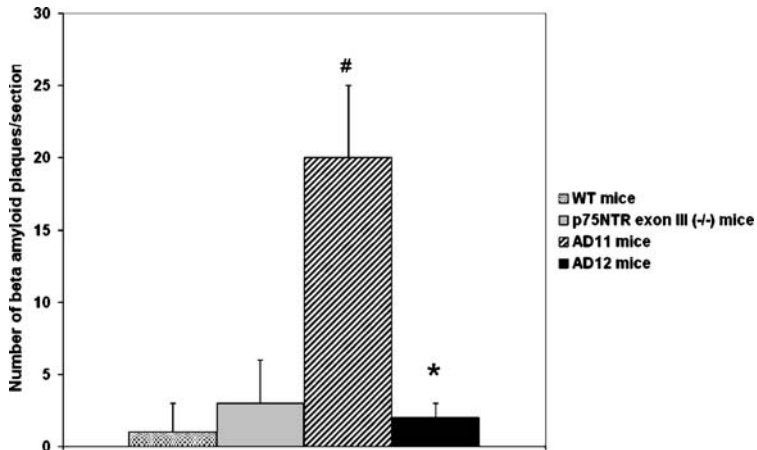


Fig. 7. The graph shows that β -amyloid plaques are not detectable in AD12 mice in which the neutralizing antibody anti-NGF is still expressed, but the p75NTR receptor was knocked out (# $P < 0.05$ AD11 mice vs. WT and p75NTR knockout mice; * $P < 0.05$ AD12 mice vs. AD11 mice).

affinity to mature NGF, while it has a very fast dissociation constant (and thus a very low affinity) for pro-NGF (F. Paoletti and A. Cattaneo, unpublished data). As a consequence, we hypothesized that the AD-like phenotype in AD11 mice could be due to the fact that, while mature NGF activity is neutralized by the recombinant anti-NGF antibody (Fig. 6(A)), pro-NGF is left free to interact with the p75NTR/sortilin complex (Fig. 6(B)). A first demonstration of the goodness of this hypothesis comes from the results obtained by crossing a line of mice expressing the recombinant anti-NGF antibody to p75 knockout mice, in which the ectodomain of the receptors has been ablated (Lee *et al.*, 1992). Thus, we obtained a line of mice (AD12 mice) in which mature NGF is neutralized and pro-NGF cannot signal through p75NTR. The result obtained so far showed that, in AD12 mice, no β -amyloid intracellular deposits or extracellular plaques could be found (Fig. 7). Thus, the study of the interactions between pro-NGF/NGF and their receptors holds great promise as one of the keys to clarifying how they are involved in the signaling cascade leading to AD. As a consequence, new diagnostic and therapeutic perspective will be designed and developed in the attempt to cure AD.

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