THE EFFECTS OF ACETICOLINESTERASES
AND β-AMYLOID PATHOLOGY ON
NEUROTROPHIN SIGNALLING IN THE MOUSE BRAIN

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ACADEMIC DISSERTATION

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To my grandfathers
ACKNOWLEDGEMENTS

I first came to Neuroscience Center in fall 2007 as 24 years old master’s student. Now, almost six year later (but still clearly under 30 years of age), it is time to look back and thank those who were involved in this work. During these years I have worked, discussed, argued, laughed and travelled with so many great personalities that I regret that I am unable acknowledge everyone by name.

First, I thank my supervisor Professor Eero Castrén or, as we know him in the lab, Papa Trophin. I can only say that it has been a privilege to learn from you and work with you. Looking at the brain through neurotrophin glasses makes many things make sense, and I am grateful to you for this perspective. Furthermore, I thank you for your trust and for your positive, forward-looking attitude, which has time after time turned my doubts into optimism and made me feel good about my work.

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On a sunny July morning by the swimming pool in Kumpula, feeling calm and good about life.

Sincerely,
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ABSTRACT

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder and the leading cause of dementia. According to the prevailing amyloid cascade hypothesis of AD, degeneration results from accumulation of toxic beta-amyloid peptide (Aβ) in the brain. Therapeutic strategies based on removal of Aβ from the brain, however, have failed to stop the progression of the disease. One promising approach to tackle AD is the protection of neurons and restoration of synaptic connections with neurotrophic factors. The neurotrophin NGF (nerve growth factor) promotes survival of basal forebrain cholinergic neurons (BFCNs) through TrkA receptor and may prevent cholinergic system dysfunction in AD. Another neurotrophin, BDNF (brain-derived neurotrophic factor), and its receptor, TrkB, are critical molecular mediators of activity-dependent synaptic plasticity. BDNF is involved learning and memory, and may counteract synaptic dysfunction and cognitive impairment in AD. Neurotrophin precursors, proneurotrophins, by contrast, bind to the p75 neurotrophin receptor (p75) and promote apoptosis and synaptic depression, and possibly contribute to synaptic failure and degeneration in AD. Furthermore, p75 also mediates some of the toxic effects of Aβ. Thus, both activation of brain Trk signaling and inhibition of p75 function are potential targets for novel drugs for AD.

The first aim of this study was to examine the effects of cholinesterase inhibitors (ChEi), the first-line drugs for AD, on Trk neurotrophin receptor activity in the mouse brain. We show that donepezil and galantamine, two clinically used ChEis, rapidly phosphorylated both TrkA and TrkB receptors in the mouse hippocampus. Subsequent pharmacological studies suggest that cholinergic activation of Trk receptors required simultaneous stimulation of nicotinic and muscarinic acetylcholine receptors. Moreover, we report that drug-induced Trk receptor phosphorylation response was unaltered in Sortilin1−/− mice and Bdnf−/− mice, which show defects in anterograde transport and synaptic targeting of Trk receptors and BDNF signaling, respectively. Our results show that drugs commonly used to treat AD activate neurotrophin receptors in the mouse brain. Unveiling the mechanism of drug-induced Trk receptor activation may allow for development of better neurotrophic therapies for AD.

In the second part of this work, we wanted to elucidate the molecular mechanisms underlying impairment of BDNF-TrkB signaling in a mouse model of AD and to gain
insight to the potential of this pathway as a target for novel plasticity-enhancing drug therapies against AD. We discovered that Aβ accumulation in the APdE9 mouse model of early-onset familial AD resulted in an age-dependent increase in the levels of TrkB.T1, a dominant-negative form of TrkB in the brain. Furthermore, we detected aberrant accumulation of BDNF protein in the cortex of APdE9 mice. Moreover, we show that overexpression of TrkB.T1 and Bdnf gene deficiency aggravated the memory impairment in APdE9 mice, whereas overexpression of TrkB alleviated this memory deficit. These findings may in part explain the failure of synaptic plasticity in APdE9 mice and suggest that activation of TrkB signaling could alleviate memory impairment in patients with AD.

Finally, we developed an assay to detect signaling of p75 in vivo based on proteolytic processing of the receptor. We show that the assay can detect proteolytic processing of p75 in vitro and present our strategy to generate a p75 signaling reporter mouse based on the same methodology. Our knock-in mouse will allow accurate temporal and spatial localization of p75 activity in healthy, aging and injured nervous systems. In addition, it will serve as screening platform to discover novel drugs to inhibit the detrimental effects of the p75 receptor in AD and other diseases.

In conclusion, our results provide new evidence of the effects of ChEIs on brain neurotrophin signaling and elucidate functional and neurobiological interactions between Aβ accumulation and BDNF-TrkB signaling.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
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<tr>
<td>AChEI</td>
<td>acetylcholinesterase inhibitor</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
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<tr>
<td>Aβ</td>
<td>β-amyloid</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>BFCN</td>
<td>basal forebrain cholinergic neurons</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BuChE</td>
<td>butyrylcholinesterase</td>
</tr>
<tr>
<td>CamKII</td>
<td>calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
<tr>
<td>ChEI</td>
<td>cholinesterase inhibitor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic adenosine monophosphate response element-binding protein</td>
</tr>
<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
</tr>
<tr>
<td>DAPT</td>
<td>N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester</td>
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<tr>
<td>DON</td>
<td>donepezil</td>
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<tr>
<td>ECD</td>
<td>extracellular domain</td>
</tr>
<tr>
<td>GAL</td>
<td>galantamine</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GVP</td>
<td>Gal4/VP16</td>
</tr>
<tr>
<td>hbd</td>
<td>horizontal diagonal band</td>
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<tr>
<td>HFS</td>
<td>high-frequency stimulation</td>
</tr>
<tr>
<td>ICD</td>
<td>intracellular domain</td>
</tr>
<tr>
<td>IMI</td>
<td>imipramine</td>
</tr>
<tr>
<td>JNK3</td>
<td>c-Jun N-terminal kinase 3</td>
</tr>
<tr>
<td>Lac</td>
<td>clasto-Lactacystin β-lactone</td>
</tr>
<tr>
<td>LFS</td>
<td>low-frequency stimulation</td>
</tr>
<tr>
<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>M</td>
<td>muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>MAG</td>
<td>myelin-associated glycoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MCI</td>
<td>mild cognitive impairment</td>
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<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
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<tr>
<td>ms</td>
<td>medial septum</td>
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<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>nbM</td>
<td>nucleus basalis of Meynert</td>
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<tr>
<td>NFDM</td>
<td>non-fat dry milk</td>
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<tr>
<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>NIC</td>
<td>nicotine</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NRAGE</td>
<td>neurotrophin receptor-interacting MAGE homolog</td>
</tr>
<tr>
<td>NRIF</td>
<td>neurotrophin receptor-interacting factor</td>
</tr>
<tr>
<td>NT3, NT4</td>
<td>neurotrophins 3 and 4</td>
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<td>p75</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>OXT</td>
<td>oxotremorine</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>Rho-GDI</td>
<td>Rho protein GDP dissociation inhibitor</td>
</tr>
<tr>
<td>RIP</td>
<td>regulated intramembrane proteolysis</td>
</tr>
<tr>
<td>RIP2</td>
<td>receptor-interacting serine/threonine-protein kinase 2</td>
</tr>
<tr>
<td>TACE</td>
<td>tumor necrosis factor α converting enzyme</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
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<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor associated factor 6</td>
</tr>
<tr>
<td>Trk</td>
<td>tropomyosin-related kinase</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activation sequence</td>
</tr>
<tr>
<td>vdb</td>
<td>ventral diagonal band</td>
</tr>
<tr>
<td>VP16</td>
<td>virus protein 16</td>
</tr>
<tr>
<td>VPS10</td>
<td>vacuolar protein sorting protein 10</td>
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I  Autio H., Mätlik K., Rantamäki T., Lindemann L., Hoener M.C., Chao M., Arumäe U. & Castrén E. Acetylcholinesterase inhibitors rapidly activate Trk neurotrophin receptors in the mouse hippocampus. Neuropharmacology 61, 1291-6, 2011


IV  Autio H., Rantamäki T., Castrén E. Coordinated activation of nicotinic and muscarinic receptors mediates the effect of cholinesterase inhibitors on hippocampal Trk receptor phosphorylation. Submitted manuscript.


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1. INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative illness and the leading cause of dementia. In 2010, dementia affected 6.3 million people in Europe and 35.6 million people worldwide, and WHO has estimated that the number will reach 115 million by the year 2050. The economic burden of AD is tremendous: estimated health care costs and direct non-medical costs total over 105 billion euros in Europe alone, making AD the second most expensive disease of the brain (Olesen et al. 2012). Clearly, AD will become an enormous challenge for health care systems in ageing societies in the near future. Moreover, AD has a profound impact on the quality of living of the patients with illness, their families and their caregivers.

AD is characterized by progressive synapse loss, neurite dystrophy, neuronal degeneration, formation of neurofibrillary tangles, and accumulation of β-amyloid (Aβ) plaques in the brain (Terry et al. 1991, Querfurth and LaFerla 2010). The groundbreaking amyloid cascade hypothesis of Hardy and Higgins (1992) stated that accumulation of Aβ peptide initiates a cascade of events that leads to dysfunction and degeneration of neurons and eventually to formation of the β-amyloid plaques Dr. Alois Alzheimer described more than 100 years ago. Neuronal degeneration in AD first affects limbic structures, such as the entorhinal cortex and hippocampus, which are critically involved in consolidation of new memories (Scoville and Milner 1957, Gomez-Isla et al. 1996, Kordower et al. 2001). Degeneration eventually spreads to other brain areas and manifests as generalized cognitive impairment and behavioral disturbances (Querfurth and LaFerla 2010).

Basal forebrain cholinergic neurons (BFCNs) are among the degenerating neuron populations, which provided the rationale for development of the first successful pharmacotherapy against AD, the cholinesterase inhibitors (ChEI). These drugs inhibit the catalysis of the neurotransmitter acetylcholine (ACh) restoring cholinergic neurotransmission and improving cognition in patients (Birks 2006, Nestler et al. 2009). ChEIs remain the first-line pharmacotherapy for AD to this day, but are unable to stop progression of the disease. Efforts to develop disease-modifying drugs have been driven by the amyloid cascade hypothesis. Unfortunately, therapies based on removal of Aβ have so far failed and the need for novel disease-modifying drugs remains as great as ever (Mullard 2012).
One possible approach to confront neuronal degeneration is protection of neurons and restoration of synaptic plasticity with neurotrophic factors. In fact, the neurotrophin family of neurotrophic factors was at the forefront of AD research before the amyloid cascade hypothesis. The classic neurotrophin, nerve growth factor (NGF), discovered by Levi-Montalcini and Cohen in the 1950’s, is a specific growth factor for BFCNs capable of supporting their survival and sustaining their neurotransmitter phenotype (Gnahn et al. 1983, Hefti et al. 1985, Alderson et al. 1990). Indeed, the early neurotrophin hypothesis of AD stated that the disease would result from unavailability of target-derived NGF and suggested neurotrophins as potential treatments for AD (Appel 1981, Hefti 1983). Since then NGF has been tested in a several clinical trials and novel ways to deliver the neurotrophin to the brain are being investigated (Mandel 2010, Wahlberg et al. 2012). Appreciation of synaptic loss as a fundamental histopathological correlate to cognitive impairment in AD, on the other hand, suggests the molecular mechanisms of synaptic plasticity as potential targets for drug development. One of the candidates for such neurorestorative therapies is brain-derived neurotrophic factor (BDNF), another member of the neurotrophin family with a pivotal role in neuronal plasticity, learning and memory (Park and Poo 2013).

Furthermore, new findings in the 21st century have sparked interest in neurotrophins not only as treatments, but also as contributing factors in disease pathogenesis. NGF’s precursor proNGF, for example, is an active signaling molecule functionally very different from the mature neurotrophin and may contribute to cholinergic dysfunction and neuronal degeneration in AD (Fahnestock et al. 2001, Lee et al. 2001). At the same time, a several recent findings suggest intimate bidirectional but incompletely understood interplay between NGF and Aβ signaling in AD. Strikingly, genetic manipulation of NGF signaling in the mouse brain produces a neurodegenerative phenotype bearing more resemblance to sporadic AD than any mouse model based on mutations in APP (Capsoni et al. 2000). Moreover, p75 neurotrophin receptor (p75) mediates some of the toxic effects of Aβ (Sotthibundhu et al. 2008, Knowles et al. 2009). Thus, activation of trophic growth factor signaling and inhibition of p75 activity are potential targets for novel drugs against AD.

This review of literature will introduce the reader to the basics of neurotrophin biology and highlight the potential role of neurotrophins in the pathophysiology and treatment of AD.
2. REVIEW OF THE LITERATURE

2.1 Neurotrophins and their receptors

2.1.1 Neurotrophin synthesis, release and maturation

Neurotrophins are small, secreted proteins that regulate fundamental processes of the nervous system, such as neuronal differentiation, survival, death and plasticity (Park and Poo 2013). The mammalian neurotrophin family consists of four members: NGF, BDNF (Leibrock et al. 1989), and neurotrophins three and four (NT-3, NT-4; Hohn et al. 1990, Maisonpierre et al. 1990, Hallbook et al. 1991). Neurotrophins are widely expressed in the brain with the highest mRNA levels detected in the cerebral cortex and the hippocampus (Korsching et al. 1985, Ernfors et al. 1990, Hofer et al. 1990, Phillips et al. 1990). Cells synthesize neurotrophins as immature precursors, proneurotrophins (Fig. 1). Proneurotrophins are enzymatically cleaved to mature neurotrophins inside the cell by the proprotein convertase furin (Seidah et al. 1996a, Seidah et al. 1996b), or outside the cell by plasmin or matrix metalloproteases (MMPs; Lee et al. 2001, Pang et al. 2004, Bruno and Cuello 2006). Neuronal activity governs neurotrophin synthesis, maturation and release: seizure activity robustly increases NGF mRNA in the hippocampus (Gall and Isackson 1989), and depolarization of cultured hippocampal neurons with potassium or kainic acid promotes synthesis of BDNF and NGF (Zafra et al. 1990) and stimulates their release (Blöchl and Thoenen 1995, Goodman et al. 1996, Brigadski et al. 2005). Some studies suggest, however, that hippocampal neurons can also constitutively release NGF (Blochl and Thoenen 1995, Mowla et al. 1999, Brigadski et al. 2005). Notably, Mowla et al. (1997) reported that cultured hippocampal neurons exclusively cleave NGF intracellularly and constitutively release the neurotrophin. In contrast, others have not been able to detect constitutive NGF release in vitro at all (Griesbeck et al. 1999, Bruno and Cuello 2006). In vivo, Fahnestock et al. (2001) discovered that the majority of NGF in the human and mouse brain exists as proNGF. In agreement with this finding, overexpression of NGF in transgenic mice increased levels of the proneurotrophin in the brain (Buttigieg et al. 2007). Mature NGF, in contrast, can be detected only in very low quantities with optimized immunoblotting protocols (Allard et al. 2012). Bruno and Cuello (2006) showed that activity-evoked release of proNGF from rat cortical microslices is accompanied by the release of tissue plasminogen activator (tPA) and plasminogen. This enables conversion of
plasminogen to plasmin and permits immediate maturation of NGF (Bruno and Cuello 2006). In agreement, local inhibition of plasmin with α2-antiplasmin results in accumulation of proNGF in the rat frontal cortex (Allard et al. 2012). According to Bruno and Cuello (2006), concomitantly released MMP-9 rapidly degrades mature NGF, which could explain the difficulties in detection of this neurotrophin.

Contrary to proNGF, the amount of proBDNF is highest during synaptogenesis, whereas mature BDNF becomes the dominant form of the neurotrophin in adulthood (Matsumoto et al. 2008, Yang et al. 2009, Dieni et al. 2012). Contrary to proNGF, the mature brain contains very little proBDNF, and a carefully designed immunoprecipitation protocol is required to detect the proneurotrophin (Yang et al. 2009, Dieni et al. 2012). The question of how neurons in the central nervous system (CNS) process and secrete BDNF, however, is controversial. Based on experiments in cultured hippocampal neurons some groups have concluded that the neurons preferentially secrete proBDNF, which is cleaved by simultaneously activated plasmin (Lu et al. 2005, Nagappan et al. 2009, Yang et al. 2009). In support of this idea, Pang and coworkers (2004) detected elevated proBDNF levels in the hippocampi of plasmin and tPA knockout mice. ProBDNF did not accumulate, however, when Allard et al. (2012) inhibited plasmin locally in the rat cortex in vivo,
despite clear elevation of proNGF protein levels. Moreover, some groups have been unable to detect secretion of the proneurotrophin in vitro (Matsumoto et al. 2008). Apparently, experimental setup is critical, since detection of proBDNF may require use of plasmin inhibitors and elimination of glial cells, which potentially secrete proteases (Nagappan et al. 2009, Yang et al. 2009). Questioning the relevance of in vitro studies altogether, Dieni et al. (2012) reported that mature BDNF and the pro-domain of the neurotrophin are packaged as dissociated peptides in the same presynaptic vesicles, and suggested that intracellular proteases quickly cleave the newly synthetized proBDNF to mature BDNF in the adult brain. Disagreeing with this idea, other groups have not detected free BDNF pro-domain in the adult brain at all (Nagappan et al. 2009, Yang et al. 2009), possibly because detection requires particular Western blotting conditions (Dieni et al. 2012). Thus, mechanisms of activity-dependent BDNF release in the adult CNS remain unresolved. If activity-dependent maturation of proBDNF is indeed the main mechanism of conversion, the presence of abundant amounts of mature BDNF in the brain requires explanation. Instead, one would expect abundant presence of the proneurotrophin, and rapid and transient pulses of neurotrophin cleavage, as described for NGF (Bruno and Cuello 2006). Clarifying the mechanisms of proneurotrophin cleavage is essential since biological actions of mature and proneurotrophins are different.

2.1.2 Neurotrophin receptors

Each neurotrophin binds with high affinity to its cognate tropomyosin related kinase (Trk) receptor: NGF to TrkA, BDNF and NT4 to TrkB, and NT3 to TrkC (Fig. 1A; Hempstead et al. 1991, Kaplan et al. 1991, Klein et al. 1991, Klein et al. 1991, Soppet et al. 1991, Squinto et al. 1991, Klein et al. 1992). Trk receptors are widely expressed in the brain: TrkA-immunoreactive somas are present in the basal forebrain, olfactory tubercle, striatum and nucleus accumbens, thalamus, hypothalamus and brainstem (Sobreviela et al. 1994). In the hippocampus, the majority of TrkA is located in the cholinergic axons and terminals and glial cells, although some immunoreactivity is present in postsynaptic structures (Barker-Gibb et al. 2001). TrkB immunostaining, in contrast, is abundant in practically all brain areas, including the neocortex, all hippocampal subregions and the basal forebrain (Yan et al. 1997). In the hippocampus, TrkB is present in initial segments of axons, axon terminals and dendrites of pyramidal and granule neurons, but also in axon
terminals of inhibitory interneurons, cholinergic neurons and monoaminergic neurons (Drake et al. 1999).

In addition to Trk receptors, all neurotrophins bind to p75 neurotrophin receptor (Chao et al. 1986, Johnson et al. 1986, Radeke et al. 1987, Rodriguez-Tebar et al. 1990). P75 is widely expressed in the developing CNS (Schatteman et al. 1988, Yan and Johnson 1988, Roux and Barker 2002). During maturation, expression decreases and becomes limited to specific neuronal populations in the retina, hypothalamus, hypophysis, pineal gland, brain stem and spinal cord as well as in the brain vasculature (Schatteman et al. 1988, Roux and Barker 2002). Most prominent p75 immunoreactivity, however, is detected in BFCNs (Hefti et al. 1986, Kordower et al. 1988, Schatteman et al. 1988). In the human basal forebrain, 95% of neurons positive for choline acetyltransferase (ChAT) are also positive for p75 (Mufson et al. 1989). In contrast, p75 mRNA is absent in the adult rat hippocampus (French et al. 1999), where receptor immunoreactivity is mostly confined to cholinergic presynaptic structures (Yan and Johnson 1988, Dougherty and Milner 1999, Sanchez-Ortiz et al. 2012). Notably, some studies have also reported p75 immunoreactivity in glial cells, and in postsynaptic structures, particularly in the developing CNS (Dougherty and Milner 1999, Woo et al. 2005).

2.1.3 Neurotrophin signaling through Trk receptors

Neurotrophin binding dimerizes Trk receptors and phosphorylates tyrosine residues in the catalytic domain of the receptor (Y670, Y674, Y675 in TrkA) initiating receptor tyrosine kinase activity (Middlemas et al. 1994, Stephens et al. 1994, Segal et al. 1996). This activation leads to the phosphorylation of other tyrosine residues, which serve as docking sites for intracellular signaling molecules (Fig 1 B). The small adaptor protein Shc, for example, binds to phosphorylated tyrosine 490 of mouse TrkA (Y515 in TrkB) and activates the Ras-MAP kinase and PI3K/AKT pathways (Stephens et al. 1994, Huang and Reichardt 2003). These pathways mediate the survival and differentiation responses neurons to neurotrophins. Phosphorylated tyrosine residue 794 in TrkA (Y816 in TrkB), on the other hand, serves as a docking site for phospholipase C-γ1, which is implicated in the effects of neurotrophins on neurite outgrowth and neuronal plasticity through activation of calmodulin-dependent kinase II (CaMKII) and the transcription factor CREB (Middlemas et al. 1994, Stephens et al. 1994, Huang and Reichardt 2003). TrkB mRNA may be spliced to
produce two variants (TrkB.T1, TrkB.T2), which lack the tyrosine kinase domain (Klein et al. 1990, Middlemas et al. 1991). These variants are capable of heterodimerizing with full-length TrkB in response to BDNF, and act as dominant negative inhibitors of TrkB signaling activity (Eide et al. 1996, Haapasalo et al. 2001). Thus, the ratio between full-length and truncated TrkB isoforms may control how efficiently BDNF binding to TrkB activates downstream signaling.

2.1.4 Transactivation of Trk receptors independently of neurotrophins

In addition to neurotrophins, the G-protein coupled receptor (GPCR) ligands adenosine and pituitary adenylate cyclase-activating polypeptide (PACAP) phosphorylated TrkA and TrkB in PC12 cells and cultured hippocampal neurons, respectively (Lee and Chao 2001, Lee et al. 2002). Moreover, dopamine D1 receptor agonists activate TrkB in striatal neuron cultures (Iwakura et al. 2008). GPCR-mediated transactivation takes place in intracellular membranes and is mediated through Src family kinases (Lee and Chao 2001, Lee et al. 2002, Rajagopal et al. 2004). In addition to GPCR ligands, the small metal cation zinc activated TrkB in cultured cortical neurons (Hwang et al. 2005). The effect of zinc requires Src family kinases, but the cation also increases the release and cleavage of proBDNF (Hwang et al. 2005, Huang and McNamara 2010). Activation of TrkB by BDNF neurotrophin, on the other hand, activates Src family kinases suggesting that neurotrophin-dependent and -independent modes of Trk receptor activation are complementary (Huang and McNamara 2010). Taken together, these findings indicate that small-molecule GPCR ligands could be potentially used to activate trophic Trk receptor signaling in the brain.

2.1.5 Neurotrophin signaling through p75 receptor

P75 is a member of the tumor necrosis factor receptor (TNFR) family and thus structurally unrelated to Trk receptors (Blöchl and Blöchl 2007). The receptor is, however, the only TNFR family member known to bind neurotrophins. P75 binds multiple ligands, co-receptors and it activates various intracellular signaling pathways. Thus, the outcome of p75 activation depends on the context (Fig. 2). First, the receptor acts as a decision-making point between axonal growth or retraction through modulation of RhoA signaling (Yamashita et al. 1999, Yamashita and
Figure 2. Ligands, co-receptors and outcomes of p75 signaling in neurons. Binding of myelin-associated growth inhibitors to complex of p75 and NOGO receptor activates RhoA signaling, which leads to inhibition of neurite outgrowth (A). Neurotrophin binding to p75 enhances survival of neurons through activation of transcription factor NFkB (B). Neurotrophin binding to p75 in the absence of Trk receptor, or proneurotrophin binding to a complex of p75 and sortilin activates apoptotic signaling (C). In the presence of a cognate Trk receptor, p75 supports trophic signaling (D). MAG = myelin-associated glycoprotein, NOGO-R = NOGO receptor, RhoGDI = Rho dissociation inhibitor, RIP2 = Receptor-interacting serine/threonine-protein kinase 2, NT = neurotrophin, NFkB = nuclear factor kappa-light-chain-enhancer of activated B cells, proNT = proneurotrophin, NRAGE = neurotrophin-receptor interacting MAGE homolog, NRIF = neurotrophin receptor interacting factor, TRAF6 = tumor necrosis factor receptor associated factor 6, Trk = tropomyosin related kinase, PI3K = phosphatidylinositol 3-kinase, AKT = protein kinase B, ERK = extracellular-signal-regulated kinase, PLCy1 = phospholipase C gamma 1. The figure is based on citations in chapters 2.1.5 and 2.1.6.

Tohyama 2003). Axonal growth inhibitors derived from myelin, such as NOGO and myelin-associated glycoprotein (MAG), bind to a complex of p75 and NOGO receptor (NOGO-R; Wang et al. 2002). This ligand binding promotes interaction between p75 and protein GDP dissociation inhibitor (RhoGDI), which releases and permits activation of RhoA to prevent neurite outgrowth (Yamashita et al. 1999, Yamashita and Tohyama 2003). Neurotrophins, on the other hand, release RhoGDI from p75 allowing it to inhibit RhoA signaling and induce neurite outgrowth (Yamashita et al. 1999). Binding of NGF to p75 also promotes survival of Schwann cells in low serum conditions through interaction of p75’s death domain and receptor-interacting serine/threonine-protein kinase 2 (RIP2), and activation of the transcription factor NFkB (Carter et al. 1996, Khursigara et al. 2001, Charalampopoulos et al. 2012). Intriguingly, RhoGDI and RIP2 bind to the same
Figure 3. p75 receptor supports trophic Trk signaling when the two receptors are expressed in the same cell. Neurotrophin binding to Trk receptor activates MEK, which phosphorylates and activates metalloprotease TACE (ADAM17). TACE cleaves off p75 extracellular domain (p75ECD), which is followed by γ-secretase processing of membrane bound C-terminal domain (p75CTF) to release soluble intracellular domain (p75ICD). The p75ICD interacts with TrkA increasing its affinity to NGF and enhancing downstream trophic signaling. Adapted from Kommaddi et al. (2011).

residues of p75 making activation of the NFκB and RhoA pathways mutually exclusive (Charalampopoulos et al. 2012). Therefore, depending on the availability of neurotrophin and growth-inhibiting factors, p75 changes the balance of Ras and Rho to guide growth towards trophic support (Blöchl and Blöchl 2007).

In addition to independent trophic signaling, p75 sensitizes Trk receptors to low amounts of neurotrophins and enhances Trk signaling (Davies et al. 1993, Barker and Shooter 1994, Ceni et al. 2010, Matusica et al. 2013). The exact nature of this cooperation is incompletely understood, but recent studies suggest that the free intracellular domain of p75 (p75ICD) mediates this facilitation (Fig 3). The p75ICD is released by two-step proteolytic processing of the receptor (Skeldal et al. 2011). First, the matrix metalloprotease ADAM17, also known as tumor necrosis α converting enzyme (TACE), cleaves off the extracellular domain of p75 (p75ECD) (Weskamp et al. 2004). Second, the remaining membrane-bound C-terminal fragment is subject to regulated intramembrane proteolysis (RIP) by γ-secretase (Jung et al. 2003, Kanning et al. 2003). In PC12 cells neurotrophin binding to TrkA phosphorylates ADAM17 through the MAPK/ERK pathway, promoting ectodomain shedding and release of the p75ICD (Ceni et al. 2010, Kommaddi et al. 2011). The
p75ICD increases the affinity of NGF to TrkA, enhancing downstream signaling and promoting neurite outgrowth in PC12 cells (Ceni et al. 2010, Matusica et al. 2013).

2.1.6 p75 as a mediator of apoptosis

The role of p75 changes in the absence of trophic Trk signaling. Overexpression of p75 increases rat striatal progenitor cell death in the absence of serum (Rabizadeh et al. 1993). Moreover, neurotrophin binding to p75 in the absence of cognate Trk receptors promotes apoptosis in retinal ganglion neurons (Frade et al. 1996), cortical oligodendrocytes (Casaccia-Bonnefil et al. 1996), cultured hippocampal neurons (Friedman 2000), and sympathetic neurons (Bamji et al. 1998, Kenchappa et al. 2006). Sympathetic neurons, commonly used to study mechanisms of p75-mediated neuronal death, express p75 and TrkA, but not TrkB. Normal developmental death of sympathetic neurons is compromised both in Bdnf<sup>−/−</sup> and p75<sup>exonIII−/−</sup> mice (Bamji et al. 1998). Deppman and colleagues (2008) showed that those sympathetic neurons that are able to establish connections with their target tissues and receive NGF start secreting BDNF, which acts as a paracrine factor that binds to p75 and eliminates competing neurons that have failed to reach their targets (Fig. 4). The mechanisms of p75 mediated apoptosis of sympathetic neurons are reasonably well characterized but also disputed. Carter and colleagues showed that BDNF promotes interaction between p75ICD and neurotrophin receptor-interacting MAGE homolog (NRAGE), which is required for rapid activation of c-Jun N-terminal kinase (JNK3) and subsequent apoptosis (Bertrand et al. 2008). Moreover, activation of JNK3 promotes upregulation of TACE leading to ectodomain shedding of p75 (Kenchappa et al. 2006, Kenchappa et al. 2010). Subsequent γ-secretase processing of the receptor is required for interaction between the soluble p75ICD, neurotrophin receptor interacting factor (NRIF), and E3 ligase TRAF6 (Khursigara et al. 2001, Geetha et al. 2005, Kenchappa et al. 2006), and this complex induces a second phase of JNK3 activation (Kenchappa et al. 2010). In addition, the ubiquitination of NRIF by TRAF6 and subsequent translocation to the nucleus is involved in p75-mediated apoptosis (Geetha et al. 2005, Linggi et al. 2005, Kenchappa et al. 2006).

While most studies agree that proteolytic processing of p75 is involved in the receptor death signaling (Kenchappa et al. 2006, Coulson et al. 2008, Volosin et al. 2008), the question whether p75ICD or p75CTF is more important for apoptotic signaling is a matter of dispute. Coulson and co-workers reported that the 29 amino
Figure 4. Apoptotic p75 signaling in developing sympathetic neurons. Target-derived NGF protects sympathetic neurons from BDNF produced by competing neurons. BDNF binding to p75 activates JNK3 through NRAGE leading to ectodomain shedding of p75. The juxtamembrane chopper domain in the membrane-bound p75CTF activates inward rectifying GIRK potassium channels leading to activation of caspases. Moreover, the tertiary complex of p75ICD, NRIF and TRAF6 begins the second phase of JNK3 activation. Nuclear translocation of NRIF initiates a transcriptional program leading to apoptosis. The figure is based on articles cited in chapter 2.1.6.

acid juxtamembrane Chopper domain in the membrane-bound p75CTF activates potassium efflux through G-protein-coupled inwardly-rectifying potassium (GIRK) channels, activating caspases and promoting cell death (Coulson et al. 2000, Coulson et al. 2008). The Chopper domain only kills cells when it is membrane-bound indicating that ectodomain shedding, but not intramembrane proteolysis is required for p75-mediated death of sensory neurons. Coulson et al. (2008) argued that high-potassium culture conditions used in previous studies (Kenchappa et al. 2006) could mask GIRK-mediated cell death. Finally, studies on cultured hippocampal neurons have identified caspases 3, 6 and 9 as mediators of p75-induced apoptosis (Troy et al. 2002). Figure 4 summarizes the pathways implicated in neurotrophin-induced death of sympathetic neurons.

In addition to mature neurotrophins, p75 is a receptor for proneurotrophins. Proneurotrophins were considered as inactive precursors until Lee et al. (2001)
discovered that proNGF promotes death of sympathetic neurons and smooth muscle cells expressing p75. Later, Teng and co-workers (2005) showed that proBDNF also promotes apoptosis of sympathetic neurons derived from wildtype, but not from p75 knockout mice. In the adult CNS proneurotrophin-induced apoptosis is of particular importance after nervous system injury. P75 expression is restored, for example, in the rat entorhinal, perirhinal and piriform cortices and hippocampal formation after pilocarpine-induced seizures (Roux et al. 1999, Troy et al. 2002, Volosin et al. 2008); in the spinal cord after contusion injury (Beattie et al. 2002); in the motor cortex after axotomy of corticospinal neurons (Harrington et al. 2004); and in organotypic hippocampal slices after CA3-CA1 axotomy (Shulga et al. 2012). Upregulation of p75 is accompanied by increased production of proNGF, which, in a cytokine-like manner, signals apoptosis of injured neurons through p75. (Beattie et al. 2002, Harrington et al. 2004, Volosin et al. 2008, Shulga et al. 2012), Induction of p75 expression and proneurotrophin-induced apoptosis is not limited to these models (Ibanez and Simi 2012). Instead, upregulation of p75 may be ubiquitous response to CNS injury, permitting evaluation of viability of the damaged neuron. Drugs aimed at inhibiting upregulation of p75 or signaling may have therapeutic value in limiting the extent of post-traumatic apoptosis in the future (Ibanez and Simi 2012, Shulga et al. 2012).

Taken together, depending on the ligand and the presence of co-receptors the outcome of p75 signaling may be very different. Proteolytic processing of p75, on the other hand, takes commonly place regardless of the signaling context and therefore may serve as an indicator of receptor activity.

2.1.7 Regulation of neurotrophin signaling by sortilin

Proneurotrophin binding to p75 and consequent apoptosis requires presence of the co-receptor sortilin (Nykjaer et al. 2004, Teng et al. 2005). Sortilin (neurotensin receptor 3) belongs to a family of vacuolar protein sorting 10 (VPS10)-domain receptors (Nykjaer and Willnow 2012). In addition to its role in proneurotrophin signaling, the receptor interacts directly with Trk receptors and participates in their anterograde axonal transport to synapses (Vaegter et al. 2011). Moreover, sortilin was reported to interact with the pro-domain of proBDNF intracellularly and participate in the trafficking of the proneurotrophin to secretory granules for activity-regulated release (Chen et al. 2005).
2.1.8 Neurotrophins as regulators of synaptic plasticity

Since the body of evidence showing that BDNF-TrkB is required for synaptic plasticity is overwhelming this chapter summarizes only some of the key findings. Kang and Schuman (1995) applied neurotrophins to hippocampal slices from young rats and discovered that BDNF and NT-3 induced long-term enhancement of synaptic transmission in CA3-CA1 synapses. In agreement with this finding, LTP is impaired in Bdnf<sup>−/−</sup> mice, and can be restored by expressing BDNF locally in the pyramidal cell layer of the CA1 region (Korte et al. 1995, Korte et al. 1996). Moreover, conditional knockdown of TrkB from forebrain glutamatergic neurons impairs synaptic plasticity and hippocampus-dependent learning (Minichiello et al. 1999). Effects of BDNF on neuronal plasticity require activation of the TrkB-PLCy1 pathway (Minichiello et al. 2002).

While mature BDNF mediates synaptic strengthening, proBDNF is thought to mediate synaptic depression (Park and Poo 2013). Cleavage-resistant proBDNF enhances LTD in hippocampal slices prepared from P14 mice (Pang et al. 2004, Woo et al. 2005). Nagappan et al. (2009) showed that low-frequency stimulation (LFS), which promotes LTD, induces secretion of proBDNF from rat hippocampal slices; whereas high-frequency stimulation (HFS), which induces LTP, results in release of proBDNF, tPA and plasminogen, and thus enables neurotrophin maturation and induction of LTP. In slices from young animals, p75 was present in dendrites of pyramidal neurons of the CA1 area, and it was required for LTD induction (Woo et al. 2005). It remains unclear, however, if this model applies to the mature nervous system as well. Matsumoto et al. (2008) reported that while theta burst stimulation could not induce LTP in hippocampal slices from Bdnf knockouts, LTD induced by LFS was unaffected. The absence of proBDNF in these mice implies that the neurotrophin is dispensable for adult hippocampal LTD. Intriguingly, Martinowich and colleagues (2012) discovered that social conflict stress facilitates LTD in wildtype, but not in p75<sup>exonIII<sup>−/−</sup></sup> mice indicating that the receptor indeed plays a role in the adult LTD. This study suggested, however, that the loss of LTD in p75 knockouts may reflect abnormalities in cholinergic neurotransmission rather than absence of the receptor from pyramidal neurons.

Cholinergic system also mediates the effects of NGF on hippocampal plasticity: unlike BDNF, NGF does not elicit synaptic potentiation when applied directly to rat
hippocampal slices (Kang and Schuman 1995). Instead, the neurotrophin enhances rat hippocampal LTP in vivo when injected to the septum, but not if septal cholinergic neurons are lesioned (Conner et al. 2009). These findings suggest that efficacy of any potential plasticity-enhancing therapies based on activation of NGF-TrkA signaling is likely to depend on the state of cholinergic innervation.

2.2. Neurotrophin signaling in the basal forebrain cholinergic system

2.2.1 Cholinergic neurons of the brain

Cholinergic neurons affected by AD reside in the basal forebrain basal structure called the Meynert complex. (Fig. 5; Woolf and Butcher 2011). The Meynert complex consists of several cholinergic nuclei including the medial septum (ms); the diagonal band complex, which is divided in vertical and lateral horizontal limbs (vdb, lhdb); and the nucleus basalis of Meynert (bas, nbM). In addition, the laterodorsal tegmental (ldt) and the pedunculopontine (ppt) nuclei in the mesopontine region contain cholinergic projection neurons. Furthermore, some of the interneurons in the striatum, the olfactory bulb and the islands of Cajella complex are cholinergic. Cholinergic neurons of the ms and the vdb project to the hippocampus through white matter tract called the fimbria fornix. Cholinergic projections originating from the lhdb, on the other hand, reach out to the amygdala, olfactory bulb, and frontal cortex. Other cortical areas receive innervation mainly from the nbM. Widespread cholinergic innervation of the cortex suggests that any perturbation in the BFCN function is likely to affect information processing in almost all brain areas.

2.2.2 Cholinergic neurotransmission

Cholinergic neurons use ACh as their primary neurotransmitter. ChAT transfers an acetyl group from acetyl coenzyme A to choline to produce ACh (Nestler et al. 2009). Vesicular ACh transporter then packs the neurotransmitter into vesicles, which fuse to the cell membrane to release the neurotransmitter to the synaptic cleft upon stimulation. ACh binds to two types of receptors: ionotropic nicotinic receptors (nAChR) and G-protein-coupled muscarinic receptors (M). Nicotinic receptors are fast acting, depolarizing Na+/Ca2+ channels. The channels consist of seven subunits in various compositions, out of which the α7 homomers and α4β2 heteromers are the most common in the brain. The G-protein coupled muscarinic receptors are divided
in five subtypes (M1-M5). ACh binding to M2 and M4 receptors activates potassium channels through Gi proteins, whereas M1, M3 and M5 couple to Gq proteins and activate phospholipase C. The M1, M3, and M4 subtypes are most abundant in target areas of BFCNs, whereas M2 muscarinic receptors are autoreceptors located on cholinergic neurons. Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) catalyze the hydrolysis of ACh to acetic acid and choline, which is taken up by cholinergic terminals to be re-used in ACh synthesis.

2.2.3 The basal forebrain cholinergic system in learning and memory

The basal forebrain cholinergic system participates in cognitive processes, such as attention, perception, consciousness, and various forms of learning and memory (Woolf and Butcher 2011). The role of ACh in learning and memory, however, is complex. Although the neurotransmitter is released in the hippocampus during learning tasks, a specific lesion of cholinergic neurons with 192-IgG saporin immunotoxin produces only subtle deficits in learning and memory (Parent and Baxter 2004). Michaeu and Marighetto (2011) suggested that rather than being permissive for memory, ACh regulates memory flexibility, and coordination between different memory systems. The cholinergic system may regulate cognitive processes
by generating various brain rhythms (Woolf and Butcher 2011). For example, an
immunolesion study by Apartis and co-workers (1998) showed that the rhythmically
bursting cholinergic neurons of the septum generate a hippocampal theta rhythm. The
loss of cholinergic innervation is therefore likely to contribute to cognitive decline,
but does not alone explain memory impairment in AD.

2.2.4 Neurotrophin receptors in the basal forebrain cholinergic neurons

Unlike hippocampal and cortical neurons, the vast majority of cholinergic neurons
continue to express p75 in adulthood (Mufson et al. 1989). In addition, the majority
of BFCNs also express TrkA (Mufson et al. 1989, Holtzman et al. 1992). Sobreviela
et al. (1994) reported that 95% of TrkA-positive neurons in the septum/diagonal
band, and 80% of neurons in the nbM, were also immunopositive for p75 and ChAT.
In addition, TrkB and TrkC are present in BFCNs, although at lower levels than TrkA
(Salehi et al. 1996, Ginsberg et al. 2006). Colocalization between different Trk
receptors is common (Salehi et al. 1996). The presence of all Trk receptor subtypes
and the continuous expression of p75 distinguish BFCNs from most other neuron
populations. Striatal cholinergic interneurons, for instance, express TrkA but not p75
(Sobreviela et al. 1994). TrkA-positive cholinergic fibers are present widely in the
brain, including the hippocampus and neocortex (Sobreviela et al. 1994, Cooper et al.
2001). These areas synthesize NGF, and only very low levels of neurotrophin mRNA
are present in the basal forebrain (Goedert et al. 1986). The cholinergic terminals
take up NGF and transport it to their somas in the basal forebrain (Seiler and Schwab
1984, Kramer et al. 1999). As may be expected from the expression of TrkB and
TrkC in BFCNs, cholinergic terminals also take up and transport BDNF and NT-3
(DiStefano et al. 1992), although to a lesser extent than NGF (Kramer et al. 1999,
Cooper et al. 2001). Unlike NGF, however, these neurotrophins are also taken up by
cells in the hippocampus where they have distinct local functions and thus cannot be
considered as specific growth factors for BFCNs (DiStefano et al. 1992). From
therapeutic perspective, potential benefits from activation of TrkA signaling may be
confined to cholinergic system whereas activation of TrkB could produce effects that
are more widespread.
2.2.5 TrkA signaling in development of the basal forebrain cholinergic neurons

NGF and TrkA knockout mice are born alive but usually die by four weeks of age due to massive loss of sympathetic neurons (Crowley et al. 1994, Smeyne et al. 1994). Their BFCNs, however, develop normally and start to express cholinergic markers in the absence of the neurotrophin or its receptor (Crowley et al. 1994, Smeyne et al. 1994, Fagan et al. 1997, Sanchez-Ortiz et al. 2012). Moreover, the septohippocampal pathway in TrkA−/− mice develops normally, and the number of ChAT-positive neurons in the medial septum is unaltered at seven to eight days of age (Fagan et al. 1997, Sanchez-Ortiz et al. 2012). During the period of developmental apoptosis around P7-P13, however, more BFCNs die in TrkA−/− mice than in wildtype controls and by the age of 20-25 days the number of ChAT-positive neurons in the septum is 36% lower in the knockouts (Fagan et al. 1997). Brain-specific conditional TrkA knockouts survive to adulthood (Sanchez-Ortiz et al. 2012). By two weeks of age, however, the characteristic laminar pattern of cholinergic innervation in their hippocampi is lost and cholinergic fiber density is significantly decreased. By three months of age, p75 immunoreactivity in the hippocampus is completely absent in conditional TrkA knockouts indicating that TrkA is required for maintentance of correct target innervation and cholinergic phenotype.

2.2.6 TrkA in maintenance of cholinergic neuron phenotype and survival

NGF and BDNF augment the expression of ChAT and AChE in vitro in neuronal culture prepared from the rat embryonic septum (Hefti et al. 1985, Alderson et al. 1990). Intracerebroventricular infusion of NGF clearly increases the activity of ChAT in the neonatal hippocampus, cortex and basal forebrain, and slightly in the adult forebrain (Gnahn et al. 1983, Hefti et al. 1984, Conner et al. 2009). In contrast, infusion of an antibody against NGF into the ventricles decreases ChAT immunostaining density and enzyme activity in the vdb and the septum (Sofroniew et al. 1990). Moreover, perturbation of the balance between mature and proNGF profoundly affects BFCN phenotype: Allard et al. (2012) delivered α2-antiplasmin to the rat prefrontal cortex with an osmotic minipump and detected local accumulation of proNGF and a decrease in the number of VACHT-positive terminals and TrkA-immunoreactive structures (Allard et al. 2012). In contrast, inhibition of MMP-9,
which is responsible for degradation of mature NGF, results in accumulation of mature neurotrophin and an increase in the density of cholinergic boutons (Bruno and Cuello 2006, Allard et al. 2012).

NGF also protects cholinergic neurons in the medial septum and vdb after transection of the septohippocampal pathway (Hefti 1986). This lesion decimates 50% of AChE-positive cells on the ipsilateral side, but NGF treatment maintains the survival rate at around 92% (Hefti 1986, Morse et al. 1993). BDNF also protects ChAT- and p75-positive neurons in the basal forebrain after fimbria transection, but only when the neurotrophin is injected directly to the septum (Morse et al. 1993). Poor diffusion of BDNF to the parenchyma from the ventricles may explain this difference. Curiously, adult cholinergic neurons do not appear to depend on NGF derived from their target neurons for survival. The number of cholinergic neurons in the rat basal forebrain remained constant when Sofroniew and co-workers (1990) destroyed 90% of the hippocampal formation with N-methyl-D-aspartate (NMDA) injections. The cholinergic cell bodies shrunk, however, and expression of ChAT in the neurons was reduced. In conclusion, NGF-TrkA signaling maintains the neurotransmitter phenotype of cholinergic neurons and promotes survival of severed neurons, but may not be required for their survival in physiological conditions.

2.2.7 The role of p75 in basal forebrain cholinergic neurons

Although BFCNs express p75 at high level, relatively few studies have specifically addressed the role of the receptor in cholinergic neurons. Basic neurotrophin biology postulates at least two possible roles: on the one hand, p75 could act in synergy with Trk receptors, all of which are abundant in cholinergic neurons (2.1.2). On the other hand, p75 could act as an antagonist and mediate atrophic effects. Moreover, it is conceivable that this role depends on availability of mature and proneurotrophins and is thus subject to change during development and under pathophysiological conditions.

The majority of studies in p75 knockout mice favor an antagonistic role for p75 in BFCNs. p75exonII/- mice are viable and fertile but have deficits in sensory innervation (Lee et al. 1992). One consistent finding in these mice has been enlargement of cholinergic soma diameter in the basal forebrain (Yeo et al. 1997, Kramer et al. 1999, Greferath et al. 2000, Greferath et al. 2012). This change is accompanied by clearly
increased density of ChAT positive axons in the CA1 area (Greferath et al. 2000) and increased ChAT enzyme activity both in the basal forebrain and hippocampus (Yeo et al. 1997, Greferath et al. 2000, Krol et al. 2000). Some studies have reported reduced number of cholinergic neurons in p75 knockouts (Peterson et al. 1999, Greferath et al. 2000), but most show an increase in the number of ChAT-positive neurons (Yeo et al. 1997, Naumann et al. 2002, Greferath et al. 2012). Recently, Greferath et al. (2012) backcrossed the p75exonIII−/− mice to 129/Sv mice for 12 generations and found significantly more ChAT positive cells in the ms/vdb, hdb and in nbM of p75 knockout animals. Moreover, the group took advantage of the fact that while p75exonIII−/− mice do lack the extracellular ligand-binding domain they still express the intracellular part of the receptor (Von Schack et al. 2001). Using antibodies against p75ICD Greferath and co-workers (2012) were essentially able to stain p75-positive neurons in p75exonIII−/− mice. This approach revealed a subpopulation of p75-positive and ChAT-negative neurons in the basal forebrain. In homozygous p75 knockouts, however, all of these neurons were positive for ChAT. In other words, in the absence of p75 these neurons adopted a cholinergic phenotype. Greferath and co-workers suggested that these neurons may constitute a dormant cholinergic reservoir, which can be drafted when increased cholinergic activity is required. This finding directly indicates that p75 participates in the downregulation of cholinergic phenotype. Signaling pathways mediating these processes are, however, unknown.

On the other hand, the absence of p75 also diminishes transport of mature neurotrophins from the dorsal dentate gyrus to the basal forebrain (Kramer et al. 1999, Krol et al. 2000). Axonal transport, in general, is unaffected in p75 knockout mice. Thus, likely explanations for transport deficiency in p75 knockouts include altered neurotrophin binding to Trk receptors, reduced receptor internalization or endosome formation (Kramer et al. 1999). Indeed, antibodies against TrkA and p75 equally disrupt the internalization of mature NGF (Gatzinsky et al. 2001). Furthermore, p75 appears to be important for fine-tuning of cholinergic target innervation of the hippocampus, where cholinergic innervation in p75 knockouts is abnormally dense in the stratum radiatum and stratum oriens layers (Yeo et al. 1997). Moreover, cholinergic innervation of the supragranular layer, and the border between the former and the molecular layer of dentate gyrus appears denser (Peterson et al. 1999). Theoretically, these anomalies could limit access of the cholinergic terminals to target-derived neurotrophin.
2.3. Neurotrophin signaling in Alzheimer's disease

2.3.1 Molecular mechanisms of Alzheimer's disease

According to the prevailing amyloid cascade hypothesis, causative agent for AD is Aβ peptide (Hardy and Higgins 1992). Neurons produce Aβ from APP through two-step proteolytic processing: first, beta-site APP cleaving enzyme 1 (β-secretase, BACE) sheds APP extracellular domain and, second, γ-secretase complex releases soluble Aβ peptide in different lengths (Fig. 6, Quefurth and LaFerla 2010). Accumulation of Aβ leads to oligomerization of the peptide and Aβ oligomers disturb synaptic function, damage neurites and are toxic to neurons perturbing mitochondrial function and releasing reactive oxygen and nitrogen radicals (Shankar et al. 2008, Querfurth and LaFerla 2010). Moreover, β-amyloid pathology alters kinase and phosphatase activities resulting in hyperphosphorylation of microtubule-associated protein tau and promoting protein self-assembly to insoluble neurofibrillary tangles. Excessive Aβ further aggregates to form β-amyloid plaques that attract reactive astrocytes and activated microglia, which release cytokines and maintain harmful inflammation in the brain. Together these changes lead to widespread neuronal dysfunction and degeneration, which manifest as dementia. The strongest support for the amyloid cascade hypothesis originates from genetic studies: mutations in APP or presenilin 1 and 2 genes, which result in increased production of Aβ1-42, cause early-onset familial AD, whereas APP gene mutation (A673T), which prevents β-cleavage of APP, protects its carriers from AD and age-related cognitive impairment (Querfurth and LaFerla 2010, Jonsson et al. 2012). Animal models based on human mutations causing early-onset familial AD, such as APP695Swe mice, show impaired LTP, learning and memory (Chapman et al. 1999). Furthermore, the detrimental effects of Aβ oligomers on synaptic function are well known. Shankar et al. (2008) extracted Aβ from diseased brains and found that soluble Aβ oligomers inhibit LTP and facilitate LTD in mouse hippocampal organotypic slices. In rat hippocampal slices, the extract reduces spine density, and when injected to a ventricle, it impairs learning in the passive avoidance task. Aβ oligomers are particularly toxic for BFCNs (Sotthibundhu et al. 2008).
Figure 6. The amyloid cascade hypothesis of AD. Excessive proteolytic processing of APP by β- and γ-secretases produces soluble Aβ peptide. Particularly Aβ1-42 accumulates and forms toxic oligomers that disturb synaptic transmission and damage neurites and neurons. Aβ inhibits mitochondrial function, which causes oxidative stress and apoptosis, and alters kinase and phosphatase function, which promotes hyperphosphorylation and aggregation of tau protein to neurofibrillary tangles. Aβ further aggregates to form amyloid plaques. α = alpha secretase, β = beta-site APP cleaving enzyme (BACE), APP = amyloid precursor protein.

2.3.2 Cholinergic degeneration in Alzheimer’s disease

Loss of cholinergic cell bodies in the basal forebrain and cholinergic innervation of the hippocampus and the cortex characterize advanced AD (Davies and Maloney 1976, Whitehouse et al. 1982) and the decline in AChE and ChAT protein levels correlates with increasing numbers of β-amyloid plaques (Perry et al. 1978). These findings suggested an intimate connection between cholinergic atrophy and amyloid plaques and fuelled the formation of the cholinergic hypothesis of AD (Bartus et al. 1982). Cholinergic neuron death, however, is a rather late event in the course of the disease and is not as widespread as was previously thought. Whitehouse (1982), for example, used cell size and Nissl staining intensity to identify cholinergic neurons. When Vogels et al. (1990) neglected these markers and carefully counted the total number of neurons in all areas of the Meynert complex, they found only a 15 - 37% loss in the number of cells in the basal forebrain cholinergic nuclei. Cell loss was most prominent in the posterior nbM, but was notably almost nonexistent in the
septum (Vogels et al. 1990). Moreover, while the number of magnocellular neurons in the basal forebrain decreased, the number of small neurons was higher. Thus, most BFCNs had not died but had lost their neurotransmitter phenotype and become atrophic instead. Importantly, Vogel’s study shows that silenced cholinergic neurons remain viable even in late stages of AD. In principle, successful neurotrophic therapy could revive their function.

Furthermore, recent longitudinal clinical-histopathological studies, such as the Religious Orders Study on elderly catholic clergy, have also reported changes in BFCNs in early AD and mild cognitive impairment (MCI). Diagnostic criteria of MCI are not well established, but the term means that the patient suffers from cognitive impairment but does not meet the diagnostic criteria of dementia (Russ and Morling 2012). Amnestic MCI, in particular, may precede dementia. Surprisingly, Gilmor and colleagues (1999) discovered that the number of cholinergic neurons in the nbM of patients with MCI was unaltered, and only slightly decreased in early AD. In agreement with this, cortical ChAT and AChE enzymatic activities were reduced only in severe AD (Davis et al. 1999). ChAT activity in the hippocampus, in contrast, was actually elevated in patients with MCI (DeKosky et al. 2002). The authors suggested that the increased ChAT activity in MCI may be a compensatory response to the loss of entorhinal cortex input to the hippocampus, and could prevent the mildly impaired from succumbing to dementia.

2.3.3 Anticholinesterases

A logical consequence of the cholinergic hypothesis was the development of the first successful pharmacotherapy against AD, the ChEIs. These drugs increase cholinergic neurotransmission through nicotinic and muscarinic receptors by inhibiting the action of cholinesterase enzymes (Nestler et al. 2009). Currently three ChEIs are in clinical use: donepezil, rivastigmine and galantamine. Donepezil is a piperidine-structured, reversible and noncompetitive inhibitor of AChE with very low affinity to BuChE (Nestler et al. 2009). Galantamine, on the other hand, is a phenanthrene alkaloid from Galanthus caucasicus and a reversible and selective AChE inhibitor. In addition to inhibiting AChE, galantamine acts as an allosteric potentiating ligand (APL) of nicotinic receptors (Samochocki et al. 2003). In fact, patient plasma drug levels rarely reach concentrations required to inhibit AChE, which makes potentiation of
nicotinic receptors the possible primary mechanism of galantamine action (Nestler et al. 2009). The third ChEI, rivastigmine has a carbamate structure, and is a pseudoirreversible inhibitor of both AChE and BuChE (Nestler et al. 2009). While AChE levels decline in AD, the synthesis of BuChE increases (Perry et al. 1978). Theoretically, inhibition of BuChE could have additional benefits in the treatment of the disease. Nonetheless, all three ChEIs have similar beneficial effects on subjective and objective measures of cognition, daily activities, and behavioral disturbances in patients (Birks 2006). The drugs may stabilize the state of the patient and delay the need for hospitalization and are cost-effective treatment (Birks 2006, Bond et al. 2012).

2.3.4 NGF protein levels in Alzheimer's disease

Findings of the role of NGF as a trophic factor for cholinergic neurons formed basis for the neurotrophin hypothesis of AD, which stated that the disease develops when availability of target-derived NGF is compromised (Appel 1981, Hefti 1983). Surprisingly, post-mortem in situ hybridization studies gave no reason to believe that NGF synthesis would be compromised in patients with AD (Goedert et al. 1986, Phillips et al. 1991). In fact, NGF protein levels are elevated in cortical areas and the hippocampus in AD (Crutcher et al. 1993, Scott et al. 1995, Narisawa-Saito et al. 1996, Hock et al. 2000). Notably, Scott et al. (1995) reported that while NGF protein levels are elevated widely in the neocortex, the hippocampus, the amygdala and the putamen, the amount of NGF in the nbM is reduced by 50%. Moreover, while BFCNs in the healthy elderly are NGF-immunopositive, immunoreactivity is significantly lower or absent in patients with AD (Mufson et al. 1995). Based on these findings Mufson and colleagues (1995) hypothesized that failure of retrograde NGF transport could underlie the cortical accumulation of the protein.

2.3.5 Failure of retrograde NGF transport induced by Aβ pathology in mice

The majority of patients with Down syndrome develop early onset dementia with β-amyloid pathology similar to AD (Lott 2012). This dementia derives from an extra copy of the APP gene located in the trisomic chromosome 21. The mouse homologue of the Down syndrome critical region of this chromosome is triplicated in the Ts65Dn model (Cooper et al. 2001, Salehi et al. 2006). Cooper et al. (2001) discovered that
Ts65Dn mice almost completely fail to transport NGF from the hippocampus to the septum. Consequently, cross-sectional area and number of p75-positive neurons in the medial septal nucleus are smaller in 12-18 month old Ts65Dn mice than in diploid controls. Salehi et al. (2006) were able to partially rescue NGF transport by crossing the Ts65Dn mouse with mice hemizygous for APP in order to normalize the APP copy number. Thus, despite the 21 trisomy, these mutant mice have a normal copy number of APP gene, which indicates that overproduction of APP protein plays a role in the NGF transport defect (Salehi et al. 2006). In addition, NGF transport is deficient in many mouse models based on human familial AD mutations in APP (Salehi et al. 2006). Thus, these findings in the Ts65Dn mouse model of Down syndrome support the idea of disturbed retrograde NGF transport in AD. One possible explanation for the transport defect is post-translational modification of proNGF by β-amyloid pathology.

2.3.6 Post-translational modifications of proNGF in Alzheimer's disease

Levels of proNGF in the parietal and frontal cortex and in the hippocampus are elevated in patients with AD patients (Fahnestock et al. 2001, Bruno et al. 2009, Mufson et al. 2012). Moreover, in some brain areas this increase can be observed already in patients with MCI (Peng et al. 2004, Mufson et al. 2012). Given the contemporary findings of Lee et al. (2001) about the apoptotic actions of proNGF, Fahnestock and co-workers (2001) suggested that the accumulating proneurotrophin could contribute to the pathogenesis of Alzheimer’s disease. Moreover, Podlesniy et al. (2006) discovered that the biological activity of proNGF extracted from the frontal cortex of AD patients (AD-proNGF) differed from that of proNGF extracted from age-matched non-demented controls. Whereas control proNGF maintained the survival of serum-deprived PC12 cells, AD-proNGF promoted apoptosis instead (Podlesniy et al. 2006). This difference is explained by post-translational modifications of the proneurotrophin: AD-proNGF is nitroperoxidated, hyperglycosylated and lipoxylated, and these changes are not without effect on the biological activity of the proneurotrophin (Podlesniy et al. 2006, Bruno et al. 2009, Kichev et al. 2009). Plasmin can no longer cleave glycosylated or lipoxylated recombinant human proNGF (Kichev et al. 2009). Moreover, although plasmin does convert peroxynitrated proNGF in vitro, the resulting peroxynitrated mature NGF is less potent in phosphorylating TrkA (Bruno et al. 2009). Peroxynitrated proNGF is
absent in young rats or aged rats with normal cognitive function, but is present in aged rats with cognitive impairment (Bruno and Cuello 2012). Consistent with the idea that the modified proneurotrophin is unable to support BFCNs, the cortical ChAT levels were lower in rats with deficiencies in learning and memory than in rats with normal memory function. These post-translational modifications are associated with inflammation: infusion of soluble Aβ oligomers to the rat hippocampus activates microglia, increases expression of inducible nitric oxide synthase (iNOS), and peroxynitration proNGF (Bruno et al. 2009). These findings provide possible explanation for proNGF accumulation in target areas of cholinergic innervation in AD and in the Ts65Dn mouse model.

2.3.7 Changes in NGF receptors in Alzheimer's disease

In addition to these alterations in NGF protein levels and activity, neurotrophin receptor expression also changes in AD. The number of TrkA immunoreactive neurons in the nbM drops by almost 50% in patients with MCI or AD when compared to people with normal cognitive capabilities (Boissiere et al. 1997, Mufson et al. 1997, Mufson et al. 2000, Mufson et al. 2000). Patients with probable AD have significantly less TrkA-, TrkB-, and TrkC-positive neurons in nbM than age-matched controls (Salehi et al. 1996). Since BFCNs survive in this early stage of disease (Gilmor et al. 1999), the loss of Trk immunoreactivity may reflect decreased synthesis of the receptors in cholinergic neurons. Indeed, in situ hybridization and expression profiling of individual cholinergic neurons revealed that TrkA, TrkB and TrkC mRNAs are already downregulated in MCI (Mufson et al. 1996, Ginsberg et al. 2006). Accordingly, cortical TrkA protein levels in patients with mild to severe AD are 50 percent lower than in healthy controls (Mufson et al. 1997, Counts et al. 2004). The expression of p75 in cholinergic neurons, in contrast, remains unaltered in early AD (Mufson et al. 1996, Ginsberg et al. 2006). Since the ratio between p75 and TrkA may determine whether proNGF has trophic or atrophic effects on the cell, decreased TrkA protein levels could amplify the atrophic effects of proNGF on BFCNs (Masoudi et al. 2009). Table 1 summarizes the changes in neurotrophin and neurotrophin receptor synthesis and protein levels in MCI and AD based on post-mortem studies cited in this review of literature.
Table 1. Changes in neurotrophin synthesis and protein levels in the basal forebrain and target areas of cholinergic innervation in mild cognitive disorder (MCI) or Alzheimer’s disease (AD). ↔ = unaltered, ↓= decreased, ↑= elevated, (compared to healthy controls). BF = basal forebrain, HC = hippocampus, CX = cortex. The table is based on articles cited in chapter 2.3.

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2.3.8 The role of proNGF-p75 signaling in neuronal degeneration

Changes in proNGF biology, protein levels and receptors described in previous chapters suggest that imbalance between TrkA and p75 signaling contributes to cholinergic dysfunction in AD. It is necessary, however, to distinguish between the roles of p75 in the cholinergic system and other neurons. ProNGF becomes upregulated in the injured CNS where it promotes neuronal apoptosis through sortilin and p75 (Beattie et al. 2002, Harrington et al. 2004, Volosin et al. 2008, Shulga et al. 2012). Thus, the critical questions are: 1) is there injury-like upregulation of p75 outside the cholinergic system in AD, and 2) to what extent does proNGF-p75 signaling contribute to neuronal degeneration outside the cholinergic system. The answers to these questions clearly have major implications for the potential of p75 as a future therapeutic target in the treatment AD.

While Chakravarthy et al. (2012) detected increased p75 protein levels in hippocampal membranes in patients with AD; Counts et al. (2004) reported that p75 levels remain stable in cortical areas in all stages of the disease. Podlesniy et al. (2006), on the other hand, reported that while p75 protein levels remain stable in the entorhinal cortex and frontal cortex in advanced AD, the levels of p75CTF and p75ICD are elevated in the entorhinal cortex indicating that p75 signaling may
increase in brain areas affected by the disease. The apparent stability of cortical p75 protein levels with simultaneous loss of p75-positive cholinergic neurons suggests either increased synthesis of the receptor in the remaining cholinergic neurons, or upregulation of the receptor outside the cholinergic system. Since Ginsberg et al. (2006) showed that receptor synthesis is unaltered in BFCNs, extracholinergic upregulation could account for this discrepancy. Direct evidence supporting extracholinergic upregulation of the receptor in AD, however, is scarce. In most studies p75 protein levels were analyzed with Western blotting, which cannot discriminate the cholinergic terminals from other potential sources of the protein (Counts et al. 2004, Podlesniy et al. 2006, Chakravarthy et al. 2012). Hu et al. (2002) reported that 50% of hippocampal CA neurons in healthy aged individuals are positive for p75 and that the number increases in patients with AD. It is unclear, however, how such a widespread immunostaining would have escaped attention in previous decades of study. Finally, Mufson and Kordower (1992) described a population of small p75-positive neurons in layers II-IV of the temporal and periamygdaloid cortex and the subicular and amygdaloid complexes. These neurons were present in subjects with AD, but also in one healthy control. Notably, the authors described the neurons as healthy-looking and noted the absence of colocalization between p75 and neurofibrillary tangles. Taken together, current evidence for widespread injury-like p75 upregulation and signaling in AD is unconvincing, and the putative role of proNGF in degeneration of cortical and hippocampal neurons awaits experimental confirmation.

2.3.9 p75 as a mediator of β-amyloid toxicity

Surprisingly, p75 may directly mediate toxic effects of Aβ. Rabizadeh et al. (1994) discovered that expression of p75 in PC12 cells sensitizes the cells to Aβ toxicity. Soluble monomeric Aβ1–40 binds to p75 in transfected fibroblasts and in cultured rat embryonic cortical neurons (Yaar et al. 1997, Knowles et al. 2009). Moreover, amyloid oligomers induce apoptosis in p75-expressing NIH313 cells and p75-expressing melanocytes derived from the neural crest (Yaar et al. 1997). In contrast, cultured hippocampal neurons prepared from p75<sup>exonIII-/- </sup> mice are resistant to Aβ toxicity, and amyloid-induced neurite dystrophy (Sotthibundhu et al. 2008, Knowles et al. 2009). In addition, injection of Aβ1–42 to the hippocampus reduces the number of ChAT immunoreactive somas in the basal forebrain by 50% in wildtype mice,
whereas cholinergic neurons in p75<sup>cxonIII/-</sup> mice are unaffected by the treatment (Sothibundhu et al. 2008). Knowles et al. (2009) went on to cross mutant mice expressing two APP mutations linked to familial AD (Thy1-hAPPLond/Swe) with p75 knockouts. ChAT-positive neurites in the basal forebrain are short, and the density of cholinergic innervation of the anterior cingulate cortex is low in these APP mutants at five to seven months of age. In contrast, cholinergic innervation in double mutants lacking p75 is unaffected, indicating that the receptor mediates the dystrophic effect of Aβ on cholinergic neurons.

Recent studies have revealed some details about Aβ-induced p75 signaling. Hu et al. (2013) reported that in order to activate caspases in cultured cortical neurons Aβ requires death receptor 6 (DR6) as a novel co-receptor for p75. Intriguingly, Nikolaev and co-workers (2009) have previously connected DR6 to detrimental effects of Aβ. Moreover, elevated DR6 protein levels are present in patients with AD (Hu et al. 2013). Aβ also promotes proteolytic processing of p75 and increases accumulation of the potentially detrimental membrane-bound p75CTF (Coulson et al. 2008, Sothibundhu et al. 2008). The increased presence of p75CTF and p75ICD in the entorhinal cortex of AD patients suggests that similar signaling may take place in the AD (Podlesniy et al. 2006). Taken together, an accumulating body of evidence clearly implicates p75 in the toxic effects of Aβ. Thus, the high levels of p75 expression in BFCNs may explain the particular vulnerability of cholinergic neurons in AD.

2.3.10 Alzheimer-like neuronal degeneration in anti-NGF mice

The connection between amyloid and NGF receptors is bidirectional, and perturbation of the balance of neurotrophin signaling may result in pathological processing of APP. Ruberti et al. (2000) crossed two transgenic mice expressing either the light or heavy chain component of a recombinant antibody against NGF to produce a double transgenic mouse expressing the entire NGF antibody. Importantly, expression of the antibody was undetectable in neonates, which enabled normal pre- and postnatal development and function of the nervous system until one month of age (Capsoni et al. 2000, Origlia et al. 2006). By the age of two months, the antibody had neutralized 50% of the free NGF, and the number of ChAT positive neurons in the basal forebrain had fallen to 55% of age-matched controls. First signs of synaptic
failure appeared at two months of age (Origlia et al. 2006), and memory deficit followed at four months. By eight to nine months of age, learning and memory were significantly impaired in anti-NGF mice (De Rosa et al. 2005). This memory impairment was much more severe than what could be expected from lesions of cholinergic neurons (chapter 2.2.3). Surprisingly, Capsoni et al. (2000) described widespread apoptosis, reduced cortical thickness and ventricular dilatation in old anti-NGF mice. Moreover, immunohistochemical analysis revealed widespread presence of phospho-tau positive neurofibrillary tangles and dystrophic neurites, and significant accumulation of intra- and extracellular Aβ-positive plaques. Aβ staining appeared first in the walls of cerebral blood vessels around two months of age. By six months of age accumulation of Aβ-immunopositive clusters of cells and extracellular Aβ immunoreactivity became evident. Amyloid plaques were detected in 13 month old animals. Furthermore, the progression of Tau pathology followed the Braak staging of human AD: immunoreactivity developed first in the somas in entorhinal cortex, followed by the parietal cortex and the hippocampus (Braak and Braak 1991, Capsoni et al. 2000). Dendrites in the entorhinal cortex were affected by six months of age (Capsoni et al. 2000). Thus, progressive neuronal degeneration in the anti-NGF mouse bears marked resemblance to human sporadic AD.

Capsoni and Cattaneo (2006) suggested that the anti-NGF antibody prefers mature NGF over proNGF, and suggested that the change in the balance between pro and mature NGF could result in degeneration. Therefore, they went on to characterize the roles of NGF receptors in AD-like neurodegeneration (Capsoni et al. 2010). They used the same neuroantibody approach against TrkA and found that disturbed receptor function leads to the loss of ChAT-positive BF neurons, deficit in the object recognition task and accumulation of Aβ-immunoreactive material in both extra- and intracellular space at six months of age. In contrast, Aβ immunoreactivity fails to develop when anti-NGF mice are crossed with p75\(^\text{exonIII}^-\) mice. These findings indicate that the loss of mature NGF or TrkA promotes accumulation of amyloid through a mechanism that requires p75. In agreement with this, Constantinii et al. (2005) showed that p75 mediates age-dependent increase in β-APP-CTF protein levels and production of Aβ, while activation of TrkA with mature NGF reduces proteolytic processing of APP. In conclusion, anti-NGF mice place neurotrophin signaling upstream of Aβ pathology and suggests the intriguing possibility that Aβ accumulation could be tackled by activating NGF-TrkA signaling. Figure 7 summarizes potential interactions between NGF and Aβ signaling in AD.
Figure 7. Potential interactions between NGF and Aβ signaling in Alzheimer's disease.
Synthesis of TrkA in basal forebrain cholinergic neurons decreases in AD (1), while synthesis of p75 remains unaltered (2). ProNGF accumulates in target areas of cholinergic innervation (3). Amyloid peptide induces an inflammatory response in the CNS (4), and the oxidative stress promotes nitroperoxidation (NOX), lipoxylation and hyperglycolysis of proNGF making it resistant to maturation (5) and weakening the affinity of mature NGF to TrkA (6). These changes enhance proNGF-p75 signaling at the expense of NGF-TrkA signaling in cholinergic neurons resulting in downregulation of cholinergic markers and atrophy of cholinergic neurons. Elimination of mature NGF or TrkA (7) with antibodies expressed in transgenic mice initiates p75-dependent accumulation of Aβ, which in turn binds to p75 and activates signaling pathways leading to apoptosis (8), whereas activity of TrkA inhibits production of Aβ (9).

2.3.11 Changes in BDNF-TrkB signaling in Alzheimer's disease

Cognitive impairment in AD may ultimately reflect failure of plasticity and loss of synapses in the brain (Terry et al. 1991, Selkoe 2002). Davies et al. (1987) discovered a significant loss of synapses in the temporal and frontal cortex in AD. Later Scheff et al. (2006, 2007) reported that MCI is associated with incipient loss of synapses in the outer molecular layer of the dentate gyrus and in the stratum radiatum of hippocampal CA1 area. Synapse loss correlates with symptoms of AD better than any other histopathological hallmark of the disease (Terry et al. 1991). Given the fundamental role of TrkB-BDNF signaling in neuronal plasticity, it is unsurprising
that the loss of synapses in AD is accompanied by diminished BDNF synthesis in the brain (Phillips et al. 1991, Holsinger et al. 2000). The number of BDNF-immunopositive neurons and intensity of BDNF staining are decreased in the hippocampus and temporal cortex of patients with AD (Connor et al. 1997). AD patients also have less BDNF protein in the entorhinal cortex (Narisawa-Saito et al. 1996), frontal cortex (Ferrer et al. 1999), and hippocampus and parietal cortex (Hock et al. 2000). In the parietal cortex both pro and mature BDNF protein levels are lower in patients with MCI than in cognitively unimpaired controls, and protein levels decline further when MCI progresses to AD (Peng et al. 2005). As a notable exception, Durany et al. (2000) detected increased hippocampal BDNF levels in patients with AD. Nevertheless, cortical BDNF protein levels were still diminished. At the same time, the amount of full-length TrkB in the frontal cortex decreased, whereas TrkB.T1 expression increased (Ferrer et al. 1999). Based on in vitro findings, this change in full-length to truncated TrkB ratio is likely to perturb BDNF signaling and synaptic plasticity. Whether the observed changes in BDNF-TrkB protein levels only reflect declining numbers of synapse and neurons in the brain or play a causal role in memory impairment remains unclear.

2.4 Summary of the literature

3. AIMS OF THE STUDY

The aims of this study were:

1. To study the effects of ChEis on Trk neurotrohin receptor signaling in the mouse brain.

2. To examine the molecular mechanisms of drug-induced Trk receptor phosphorylation in the mouse brain.

3. To examine BDNF-TrkB signaling in the APdE9 mouse model of AD, and to clarify if changes in neurotrophin signaling contribute to memory impairment in this mouse model.

4. To develop a method to detect p75 receptor signaling in vivo.
4. MATERIALS AND METHODS

4.1 In vivo drug experiments and biochemical analyses

4.1.1 Animals

Table 2 summarizes mouse lines used in this work. Drug effects were tested on male mice at two to four months of age, except in the experiment on sortilin knockouts, where females were also used. C57BL6/N mice were obtained from Harlan (Netherlands) and kept in animal facilities at the University of Helsinki for at least one week before experiments. TrkB.TK mice, TrkB.T1 mice, Bdnf1−/− mice, and Sortilin knockouts (kindly provided by Dr. Anders Nykjaer, Århus, Denmark) were maintained at the University of Helsinki. The experiment on TrkB.Y816F mice was executed in the laboratory of Dr. Liliana Minichiello, EMBL Monterotondo, Italy, and frozen tissue was brought to Helsinki for analysis. APdE9 mice were from Dr. D. Borchelt and J. Jankowsky, Johns Hopkins University, Baltimore, MD, USA. These mice were crossed with TrkB.T1 mice, TrkB.TK mice, and Bdnf knockouts at University of Eastern Finland, Kuopio. Behavioral experiments were carried out in Kuopio, and frozen tissue was sent to Helsinki for biochemical analysis. Tissue from cleavage resistant proBDNF knock-in mutant mice was received from Dr. Masami Kojima in Ikeda, Japan. All animals in our hands were group-housed, and standard pellet food and water were available at all times. Light-dark cycle was 12 hours. The experiments were conducted according to the guidelines of the European Communities Council Directive (86/609/EEC) and were approved by the National Animal Experiment Board of Finland. Local authorities approved experiments done outside University of Helsinki.

4.1.2 Drug treatments

Table 3 summarizes drug experiments included in this work. Drugs were dissolved in saline or sterile-filtered tap water and the same vehicle was used as a control. In acute treatment experiments animals received a single intraperitoneal injection of donepezil-HCl (3.0 mg/kg, Toronto Research Chemicals), galantamine-HBr (3.0-9.0 mg/kg, Toronto Research Chemicals), imipramine-HCl (30.0 mg/kg Sigma-Aldrich), (-)-nicotinehydrogentartrate (1.0 mg/kg, Sigma-Aldrich) or oxotremorine-M (0.1 mg/kg, Sigma-Aldrich), or vehicle, in a volume of 5-10 ml/kg. All doses are given as
free base. We chose these doses based on pharmacokinetic and pharmacodynamic data in order to produce a robust increase in ACh levels in the mouse brain at the time points studied (Yano et al. 2009). The galantamine dose was higher since, according to previous studies, approximately threefold concentrations of galantamine compared to donepezil are needed to produce a similar level of AChE inhibition (Geerts et al. 2005). The lower galantamine dose (3 mg/kg), on the other hand, results in brain concentrations optimal for allosteric potentiation of nicotinic receptors (Geerts et al. 2005). Mice were killed 30 or 60 minutes after injection with carbon dioxide and cervical dislocation. In the repeated galantamine treatment paradigm, mice received daily intraperitoneal injection of galantamine-HBr (3.0 mg/kg) or saline. In addition to pharmacokinetic data mentioned above, we based this dosing regimen on the work of Capsoni et al. (2002), who showed that a similar dose of galantamine rescues the cholinergic deficit in the anti-NGF mouse model. Mice repeatedly treated with galantamine were sacrificed 60 minutes after final injection.

Table 2. Mouse lines used in this study.

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Mutations</th>
<th>Consequence</th>
<th>Phenotype</th>
<th>References</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrkB.TK</td>
<td>TrkB cDNA overexpressed in postnatal neurons in the cortex, hippocampus and thalamus driven by Thy1.2 expression cassette.</td>
<td>Increased phosphorylation of TrkB and PLCγ1 in the hippocampus and cortex.</td>
<td>Reduced anxiety, improved spatial and associative learning, impaired LTP.</td>
<td>Saarelainen et al. 2000</td>
<td>I, II</td>
</tr>
<tr>
<td>TrkB.T1</td>
<td>Truncated TrkB.T1 cDNA overexpressed in postnatal neurons in the cortex, hippocampus and thalamus driven by Thy1.2 expression cassette.</td>
<td>Decreased TrkB phosphorylation in the cortex.</td>
<td>Normal hippocampal LTP but impaired spatial memory.</td>
<td>Saarelainen et al. 2000</td>
<td>II</td>
</tr>
<tr>
<td>TrkB.Y816F</td>
<td>TrkB cDNA with Y816F point mutation in PLCγ binding site knocked in to juxtamembrane exon of TrkB to replace endogenous receptor.</td>
<td>Mutated TrkB is unable to activate PLCγ signaling pathway.</td>
<td>Impaired hippocampal LTP and deficient spatial and associative learning.</td>
<td>Minichiello et al. 2002</td>
<td>thesis</td>
</tr>
<tr>
<td>CR-proBDNF</td>
<td>R125M/R127L knock-in mutations in proBDNF cleavage site.</td>
<td>Accumulation of proBDNF in expense of mature BDNF in the brain.</td>
<td>unpublished</td>
<td>unpublished</td>
<td>III</td>
</tr>
<tr>
<td>Sort1Δ</td>
<td>Production of sortilin prevented by replacing part of Sort1 exon 14 with neomycin selection cassette.</td>
<td>Decreased anterograde transport and signaling of TrkA in sympathetic neurons. Decreased levels of TrkB in sympathetic fractions in the hippocampus. Impaired proneurotrophin signaling through p75.</td>
<td>Resistant to p75-mediated apoptosis of developing retinal neurons and injured corticospinal neurons.</td>
<td>Jansen et al. 2007</td>
<td>Vaeger et al. 2011</td>
</tr>
</tbody>
</table>
Table 3. Design of drug treatment experiments. H2O = sterile-filtered tap water, SAL = saline, DON = donepezil, GAL = galantamine, IMI = imipramine, NIC = nicotine, OXT = oxotremorine.

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Treatments</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>Treatment</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL6/N</td>
<td>H2O</td>
<td>3.0</td>
<td>8</td>
<td>i.p. injection, 60 min</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>9.0</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAL</td>
<td>3.0</td>
<td>12</td>
<td>i.p. injection, daily, 14 days</td>
<td>I</td>
</tr>
<tr>
<td>C57BL6/N</td>
<td>SAL</td>
<td>5.0</td>
<td>3</td>
<td>i.p. injection, 60 min</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>GAL</td>
<td>3.0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bdnf+/+</td>
<td>SAL</td>
<td>5.0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAL</td>
<td>3.0</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype</td>
<td>SAL</td>
<td>30.0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IMI</td>
<td>5.0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAL</td>
<td>30.0</td>
<td>2</td>
<td>single i.p. injection, 60 min</td>
<td>thesis</td>
</tr>
<tr>
<td>TrkB.PLCL knock-in Y816</td>
<td>SAL</td>
<td>1.0</td>
<td>8</td>
<td>i.p. injection, 30 min</td>
<td>IV</td>
</tr>
<tr>
<td>TrkB.PLCL Y816F</td>
<td>IMI</td>
<td>0.1</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TrkB.TK</td>
<td>H2O</td>
<td>3.0</td>
<td>5</td>
<td>i.p. injection, 60 min</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>5.0</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TrkB.TK</td>
<td>SAL</td>
<td>3.0</td>
<td>5</td>
<td>i.p. injection, 60 min</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>GAL</td>
<td>0.1</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL6/N</td>
<td>SAL</td>
<td>1.0</td>
<td>8</td>
<td>i.p. injection, 30 min</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>NIC</td>
<td>0.1</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL6/N</td>
<td>SAL</td>
<td>1.0 and 0.1</td>
<td>9</td>
<td>i.p. injection, 30 min</td>
<td>IV</td>
</tr>
<tr>
<td>Sort1 +/-</td>
<td>H2O</td>
<td>3.0</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sort1 +/-</td>
<td>DON</td>
<td>3.0</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H2O</td>
<td>3.0</td>
<td>4</td>
<td>i.p. injection, 30 min</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>DON</td>
<td>4.0</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.1.3 Tissue sampling, processing and the protein concentration assay

Mice were stunned with carbon dioxide and the brains were quickly dissected on an ice-cold surface. The tissue was either frozen quickly or processed immediately. The freshly dissected or frozen tissue was homogenized with a pestle in NP++ lysis buffer containing inhibitors of proteases and phosphatases (Table 4). The lysate was kept on ice for at least 30 minutes to allow lysis of cell membranes before centrifugation (16 000 g, 15 min, +4°C). The supernatant containing soluble and membrane-bound proteins was collected and stored at -80 °C. Sample protein concentrations were measured using the Lowry method (DC protein assay, Bio-Rad), which is based on the reaction between protein and alkaline copper tartrate and
Table 4. Solutions and culture mediums.

<table>
<thead>
<tr>
<th>NP lysis buffer</th>
<th>Triton X buffer</th>
<th>Laemmli buffer (2x)</th>
<th>Hanks buffer</th>
<th>Neuron culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl 137 mM</td>
<td>NaCl 137 mM</td>
<td>Tris pH 6.8 125 mM</td>
<td>NaCl 125 mM</td>
<td>B27</td>
</tr>
<tr>
<td>Tris pH 8.0 20 mM</td>
<td>Tris pH 7.4 20 mM</td>
<td>SDS 4%</td>
<td>KCl 5 mM</td>
<td>L-glutamine 1%</td>
</tr>
<tr>
<td>NaF 50 mM</td>
<td>NaF 48 mM</td>
<td>Glycerol 20%</td>
<td>NaH2PO4 1.2 mM</td>
<td>Penicillin-streptomycin 1%</td>
</tr>
<tr>
<td>Glycerol 10%</td>
<td>Triton-X 100%</td>
<td>β-mercaptoethanol 10%</td>
<td>CaCl2 1 mM</td>
<td>Neurobasal (Gibco)</td>
</tr>
<tr>
<td>NP-40 1%</td>
<td>Na3VO4 2 mM</td>
<td>Bromophenolblue 0.006%</td>
<td>MgCl2 1.2 mM</td>
<td>HEK293T culture medium</td>
</tr>
<tr>
<td>Na2VO4 2 mM</td>
<td></td>
<td></td>
<td>ZnCl2 0.001 mM</td>
<td></td>
</tr>
</tbody>
</table>

2x Complete Mini protease inhibitor (Roche)  *50X or 100X stock solutions (Gibco)

subsequent reduction of folin reagent. The reduced folin absorbs light at 750 nm. The absorbance was measured with a plate reader (Victor, Perkin Elmer), and the protein concentration was calculated based on the absorbance of protein standard samples (BSA, Bio-Rad) on the same plate.

4.1.4 Sample preparation for SDS-PAGE

The samples (20-50 g protein) were boiled (3-5 min, 100 °C) in 2x Laemmli buffer (Table 4). The buffer contains 2-mercaptoethanol, which reduces the disulfide bonds; and sodium dodecyl sulfate (SDS), which denaturates the proteins and covers them with negative charge allowing separation of proteins based on molecular weight only. Alternatively, Trk receptors were concentrated with lectin precipitation. lysate totaling 400 g of protein was incubated (2h, +4°C, rotation) with 15 Wheat Germ Agglutinin conjugated to agarose beads (EY Laboratories, San Matteo, CA, USA). This lectin has high affinity to N-acetyl-β-D-glucosaminyl residues present in the extracellular domains of Trk receptors. After precipitation the beads were centrifuged (16 000 g, 1 min, +4°C), washed once with NP++ and boiled with 20 12x Laemmli buffer to release and denaturate the proteins. In the experiment on TrkB.TK mice (I), the N-terminally FLAG-tagged TrkB receptors were pulled down by immunoprecipitation. Samples containing 400 g of protein were incubated (2h, +4°C, rotation), in precipitation buffer consisting of a 1:1 mixture of NP and Triton-X buffers (Table 4), in the presence of 5 g anti-FLAG antibody (M2, F3165, Sigma Aldrich), followed by incubation with 15 1 protein-G-sepharose (Invitrogen) to pull down the immunocomplexes. Like in lectin precipitation, the samples were then
centrifuged, washed carefully twice with precipitation buffer, and boiled in 20 12x Laemmli sample buffer.

4.1.5 SDS-PAGE and Western blotting

Samples were then loaded on polyacrylamide gels, which were either prepared from acrylamide solution (Bio-Rad), or obtained from a supplier (NuPAGE 4-12 % Mini Gels, Life Technologies). The proteins were separated under reducing conditions and were transferred (300mA for 1h at 4 °C) to a PDVF membrane (Hybond-P, Amersham). The membranes were blocked (1 h, RT) with either 3% BSA or 5% NFDM prepared in TBS with 0.01% Tween (TBS-T), depending on the choice of primary antibody (Table 5). Blocked membranes were incubated in primary antibody solution (overnight, 4°C, on rocker). On the following day the membranes were washed with TBS-T at least three times for 15 minutes, and incubated (1 h, RT) with horseradish peroxidase conjugated secondary antibodies (Table 5). After subsequent TBS-T washes, secondary antibodies were visualized using electrochemiluminescence kits (Amersham Biosciences) and a Fuji LAS-3000 Camera (Tamro Medlabs, Vantaa, Finland). Optical density of bands was quantified with ImageJ (NIH). The density of phosphoprotein bands was normalized to the density of total protein bands of the corresponding protein. Charts show averaged values of samples in treatment groups. Error bars are given as standard error of the mean (SEM). Treatment groups were statistically compared using t-test or analysis of variance, where appropriate.

4.1.6 Neurotrophin ELISAs

Neurotrophin protein concentrations were analyzed using two-site enzyme-linked immunosorbent assays (ELISA). For BDNF analyses, the tissue samples lysed in NP++ were diluted in Hanks buffer (Table 4). To improve immunodetection of neurotrophins the samples were acidified (pH 3.0) with 1 M HCl. After 15 minutes, the samples were neutralized with 1M NaOH. Samples (170 1) were then transferred to a Nunc Maxisorb ELISA plate (eBioscience), previously coated (overnight, +4°C, shaker)with anti-BDNF antibody (Roche) and blocked with 2% BSA in Hanks buffer containing 0.1% Triton-X (2h, RT, shaker). Similarly, BDNF standards (Alomone) were prepared in the Hanks buffer. Peroxidase dismutase conjugated (POD)
secondary BDNF antibody (Roche) was added to the wells and the plate was incubated overnight (+4°C, shaker). Next day, the plate was washed four times with 300 1 PBS-T, before adding 200 1 of chromogenic POD substrate (BM Blue, Roche). The peroxidase-mediated development of yellow color was stopped with 1M H2SO4 and the absorbance was measured at 490 nM. All samples were analyzed in duplicates and the BDNF concentration was calculated from the standard curve and normalized to the protein concentration in the samples.

NGF protein concentration was measured with a Chemikine NGF Sandwich ELISA kit (Millipore) according to the manufacturer’s protocol. The samples were diluted in assay diluent buffer and acid treated as described above. Strips coated with sheep anti-NGF antibody were placed in the strip well plate holder and 100 1 of the samples and NGF standards were added to the wells and incubated overnight (+4°C, shaker). On the following day the wells were washed with the assay wash buffer (4x 250 1), and the anti-mouse NGF antibody was added to the wells. After two hours of incubation the plate was washed again, and peroxidase-conjugated donkey-anti-mouse antibody was added. After another two hours washes were repeated and chromogenic TMB/E substrate was added to the wells for 10 minutes. The reaction
was stopped with the assay stop solution and absorbance at 450 nm was measured with a plate reader. All samples were analyzed in triplicates and the NGF concentration was calculated from the standard curve and normalized to the protein concentration in the samples.

4.1.7 Real-time quantitative PCR

BDNF and NGF mRNA levels were analyzed from the left hippocampus after two weeks of galantamine treatment using quantitative real-time PCR. RNA was extracted from the left hippocampus with Trizol (Invitrogen) according to the manufacturer’s instructions. The isolated RNA was treated with DNase (Roche). Complementary DNA synthesis and PCR were performed with a Phusion RT-PCR kit (Finnzymes) from 1 µg of RNA. The primers used in the cDNA synthesis were designed against the coding exons to amplify all neurotrophin isoforms. The primers were 5’- AGCTTTCTATAGGCCGCA-3’ and 5’-TCTGTGTCGCTGCTGCTGCTGCTG-3’, and 5’-GAAGGCTGCAGGCGCATAGACAAA and 5’-TACACAGGAAGTGTCTATCCTATG-3’ for NGF and BDNF, respectively. Relative levels of neurotrophin mRNA in the samples were measured based on SYBR green incorporation during the linear phase of the PCR reaction, and were calculated using the 2−ΔΔCT method (Livak and Schmittgen 2001). Neurotrophin mRNA levels were normalized to the housekeeping gene GAPDH.

4.1.8 Amyloid β toxicity in cultured cortical neurons

Primary neuronal cultures were prepared from E17 rat cortex in the Neuronal cell culture unit of Neuroscience center, University of Helsinki. Neurons were plated on 96-well culture plates (Cellstar, Greiner bio-one) coated with poly-L-lysine (Sigma). Only the inner 60 wells were used in the experiments. The cells were grown in standard Neurobasal medium (Table 4) containing B27-supplement. One third of the medium was changed twice a week. After 7-8 days in vitro, the cells were treated with galantamine (0.1 – 100.0 M) or BDNF (50 ng/ml) for 24 h or 2 h before exposure to amyloid. Aβ1-42 peptide (American Peptide Company) was diluted in acetonitrile and stored as aliquots at -80 C. One day before the experiment the peptide was diluted to 50 M in 1/3 PBS and incubated overnight at +37 C° to allow for oligomerization of the peptide. Cultured neurons were incubated overnight in the
presence of Aβ1-42 oligomers (0.01 – 1.0 M), and the viability of cells was measured on the following day based on their ability to reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to formazan. MTT was dissolved in water, and 5 μl of solution was added to the cells for a final concentration of 0.36 mg/ml. After incubation (2 h, +37°C) the medium was carefully removed and the blue formazan crystals were dissolved in 100 M DMSO. Absorbance was measured at 540 nm. Viability is given as percentage of untreated cells. There were six parallel wells per treatment condition.

4.2 p75 cleavage reporter gene assay

4.2.1 Plasmids and molecular cloning

To detect proteolytic processing of p75 we developed a reporter gene assay (Fig. 8). Rat p75 cDNA was cloned into pcDNA3 plasmid containing a Gal4/VP16 (GVP) cassette. The GVP domain is a strong transcription-activating domain that consists of the DNA-binding fragment of yeast transcription activator Gal4 fused with herpes simplex virus transcription activating protein 16 (VP16) (Sadowski et al. 1988). Gal4 binds to the yeast upstream activator sequence (UAS), a transcriptional enhancer, which drives expression of neighboring genes (Webster et al. 1988). When α- and γ-secretases cleave p75-GVP, the released p75ICD-GVP can enter the nucleus and activate transcription of a reporter gene, in our case firefly luciferase (Fig 8). Plasmids used in this work were received from Dr. Elizabeth Coulson, University of Queensland, Brisbane, Australia and reporter plasmid containing nine repeats of Gal4-responsive upstream activation sequence (UAS) was obtained from Promega (UAS_Luc2P, pGL4.35). Since some studies have connected p75ICD to apoptosis (Kenchappa et al. 2006), we also developed a C-terminally truncated reporter construct where p75 cDNA was cut at C287 and fused to the Gal4/VP16-containing vector. The resulting EC/TMP7-GVP contains the extracellular and transmembrane domains, and 14 amino acids of the intracellular juxtamembrane domain before the Gal4/VP16 sequence (Fig 8). To produce a single plasmids containing both elements of the assay p75-GVP and EC/TMP7-GVP plasmids were linearized with MunI. The pGL4.35 reporter vector was digested with ClaI and the fragment containing 9xUAS_Luc2P was purified from a gel and inserted into p75GVP vectors by blunt-end cloning after filling in the recessed 3’ ends with Klonef fragment.
Figure 8. p75 cleavage reporter gene assay. Detection of proteolytic processing of p75 is based on a fusion protein construct where Gal4/VP16 (GVP) DNA binding and transcription activating domain is fused to the C-terminus of rat p75 (FLp75-GVP). When FLp75-GVP is proteolytically cleaved, the receptor intracellular domain enters the nucleus where GVP domain can bind to Gal4-responsive upstream activation sequence (UAS) and promote production of a co-transfected reporter protein. C-terminally truncated reporter construct (p75EC/TM) contains only p75 extracellular and transmembrane domains, and 14 amino acids of the intracellular juxtamembrane domain before GVP domain.

4.2.2 Transfections

HEK293T cells were plated on 24-well culture plates for immunoblotting, or on 96-well culture plate with white walls and transparent bottoms (Viewplate, PerkinElmer) for the reporter gene assay. Cells were transfected with 9xUAS_Luc2P and p75-GVP constructs using lipofection (Lipofectamine2000, Invitrogen) when they had reached 50% confluency. For 96-well plates 0.1 g of p75-GVP or EC/TMp75-GVP DNA, and 0.1 g of UAS_Luc2P DNA was incubated with 0.5 l Lipofectamine reagent per well. For cells grown on 24-well plates, the DNA to Lipofectamine ratio was 0.8 g DNA to 2 l of reagent per well. The transfection complexes were allowed to form in DMEM (no additives) for at least 20 minutes before being added to cells. Culture medium was changed to antibiotic-free medium before transfection. Transgene expression was allowed to develop for at least 24 hours before experiments. Efficacy of the transfection protocol was confirmed with fluorescent reporters.
4.2.3 Treatments and detection of luciferase activity

Transfected HEK293T cells were pretreated with the γ-secretase inhibitor DAPT (2 M), or the proteasome inhibitor β-clasto-lactacystin (Lac, 5 M) for 60-90 min. Then the cells were treated with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml). For Western blotting the incubation was stopped after 40 minutes by discarding the medium and by putting the plate on ice. The wells were washed and the cells lysed in NP++. Western blotting was done as described in chapter 4.1.5 using an antibody against p75ICD (Table 5). For the luciferase assay the incubation continued overnight to allow for accumulation of the reporter protein. On the following day the culture medium was discarded and luciferase substrate (One-Glo, Promega) mixed 1:1 with 25 1 DMEM was added to the cells. Luminescence was measured using a sensitive plate reader (Varioskan, Thermo Scientific).

4.3 p75 cleavage reporter mouse

To detect proteolytic processing of p75 in vivo, we designed a knock-in strategy to introduce GVP domain to the C-terminus of mouse p75 receptor. Thus, the expression of p75-GVP reporter protein will be driven by the p75 promoter, which enables natural pattern and levels of reporter receptor expression. The p75-GVP knock-in mouse will be crossed to different Gal4-responsive reporter mouse lines: with LacZ or a fluorescent protein as reporter protein, the mouse will enable accurate temporal and spatial localization of p75 signaling. In the injured CNS, for example, our reporter mouse will allow us to identify the cells where apoptotic signaling takes place. Furthermore, fluorescent reporter protein could enable imaging of p75 signaling in the brain with 2-photon in vivo microscopy in almost real-time. On the other hand, crossing the p75-GVP mouse with a UAS_Luciferase mouse creates sensitive screening platform capable of detecting endogenous p75 signaling in various conditions. Moreover, the mouse may reveal p75 signaling in non-nervous tissue and prove to be a valuable tool for scientists outside the field of neuroscience. In prostate cancer, for example, p75 acts as a tumor suppressor and inhibits metastasis (Krygier and Djakiew 2001). As another example, proteolytic processing of p75 promoted invasiveness of malignant glioma tumors (Wang et al. 2008).
Figure 9: Schematic presentation of the targeting vector used to generate a p75-GVP reporter mouse, and PCR and Southern blot strategies used to screen the embryonic stem cells. Only restriction sites relevant for the generation of the vector are shown. The restriction sites in parenthesis were removed in the final phase to correct the vector. B) Example of PCR screening of ES cells. Clone 3D4 shows two PCR products corresponding to the expected molecular weights from R’ (wt) 3.0 kB and R’(tg) 2.5 kB reactions. C) Southern blot of PCR-positive clones (3D4, 5B5 and 6C1) and negative clone (4B3) shows successful targeting in clones 3D4 and 5B5. D) Finally, p75-GVP mouse will be crossed with reporter mouse expressing reporter gene driven by Gal4-sensitive promoter. FRT = Flippase (Flp) recombination site; 3’UTR = 3’ untranslated region; Myc = Myc epitope tag; V, VI = p75 exons V and VI; UAS = upstream activation sequence, GVP = Gal4/VP16.

4.3.1 Construction of targeting vector

We designed a targeting vector to knock in a Myc epitope tag and GVP domain in frame with p75 exon VI, followed by a sequence encoding last four C-terminal amino acids and a STOP sequence (Fig. 9). Irie et al. (1999) reported that the C-terminal TSPV-motif of p75 interacts with the PDZ domain of FAP-1 (Fas-associated phosphatase-1), which limits apoptotic activity of the receptor. Therefore, we wanted to keep the TSPV motif in the C-terminus of the receptor construct. The targeting vector pFlexible (van der Weyden et al. 2005) was provided by Dr. Jaan-Olle
Figure 10. Sequence of targeting vector used in generation of a p75-GVP knock-in mouse showing p75 exon VI and modifications.

Andressoo, Institute of Biotechnology, University of Helsinki. The vector contains a PGK-driven selection marker puromycin/ΔTK flanked by flippase (Flp) recombination sites (FRT). Homologous arms and the p75 exon VI were amplified from bacterial artificial chromosome (BAC) BMQ412N8 (Source Biosciences) containing the p75 locus and generated from 129S7/SvEv Brd-Hprt b-m2 mouse ES cells. The BAC was transformed into E.Coli and purified by Outi Nikkilä. Vector integration was assessed using restriction analysis with NotI and AgeI, which produced the expected 15 kB fragment (data not shown).

PCR primers were designed to amplify homologous arms and introduce the necessary restriction sites for arm insertion. Homologous arms were designed to be as long as possible while avoiding the long repetitive sequences in the 5’ intron and 3’ untranslated region (UTR). With these conditions, maximal homology was 2.0 kB for the 5’ arm and 3.8 kB for the 3’ arm. Sufficient overhangs were included to enable restriction digestion of the PCR products. All PCR reactions were done with high-
fidelity proofreading polymerases. Correct orientation of the inserted fragment was confirmed with restriction digestions and the targeting vector was sequenced from relevant parts after each ligation. First, p75 exon VI was amplified by PCR. The primers introduced HindIII sites, which were used to insert the fragment to pFlexible. In addition, the R’ primer was designed to delete nucleotides coding the C-terminal TSPV motif and the STOP codon, and to introduce Myc epitope tag, and NheI restriction site required for the next cloning step. Next, the GVP domain was amplified from the p75-GVP vector described in chapter 4.2.1. The primers introduced NheI sites to insert the PCR product after exon VI in the targeting vector. In addition, the R’ primer reintroduced a sequence coding the TSPV motif and the STOP codon. The resulting Gal4/VP16-Myc-PDZ-STOP fragment was then inserted in frame with p75 exon VI in pFlexible with NheI. Thirdly, the 5’ homologous arm was amplified with primers introducing AscI sites in order to insert the arm into pFlexible. PCR was used to flank 3’ homologous arms with NotI sites. This time the PCR product was first inserted into the TOPO vector (ZeroBlunt, Invitrogen), and then into pFlexible using NotI restriction sites.

At this point, however, we noticed that our cloning strategy had deleted the splicing acceptor site preceding exon VI. Therefore, we digested the vector with EcoRI and inserted the fragment into the Bluescript plasmid. This plasmid was then used as a template for corrective PCR, which was designed to amplify the entire 6 kB vector excluding the sequence preceding exon VI we desired to delete (AscI and HindIII sites in parenthesis in figure 9). We then phosphorylated the resulting Bluescript and circularized the vector. Then the fixed EcoRI fragment was inserted into the targeting vector. Although time-consuming, this operation increased the homologous sequence by 320 base pairs as the 5’ arm now included the exon VI, uninterrupted. The entire vector was sequenced and we detected a single nucleotide mismatch in the 3’ homologous arm. The change was, however, outside the 3’UTR and we decided proceed. Figure 10 presents partial sequence of targeting vector showing p75 exon VI and modifications in the receptor C-terminus.

4.3.2 Homologous recombination and screening of embryonic stem cells

The targeting vector was linearized with Clal and delivered to Biocenter Oulu Transgenic Core Facility for electroporation. W4/129S6 embryonic stem cells were grown under selection pressure (puromycin 0.6 mg/ml). DNA from surviving clones
was isolated and sent to us in 70 % EtOH for screening. Figure 9 presents the screening strategy of ES cells. The first round of screening for successful recombination was done with PCR. The forward primer (PCR F’ in Fig. 9) was designed to map upstream of 5’ homology. The reverse primers were designed to map either in the 3’UTR (PCR R’ wt) or in the Gal4/VP16 domain (PCR R’ tg) to produce a 3.0 kb and 2.5 kb PCR products in wildtype and knock-in clones, respectively. Prior to screening, PCR protocol was optimized to produce a wildtype band from mouse DNA. DNA from ES cells was centrifuged (5 min, 14 000 rpm, +4°C) and the pellets were dried at 37 °C for 10 min, and resuspended in 400 1 TE buffer. Four 1 of DNA with unknown concentration was used as a template for PCR reactions. Both R’ primers were included in all PCR reactions to amplify both alleles. PCR products were separated on agarose gel and visualized under UV-light. Out of 200 clones, three were PCR-positive and were analyzed by Southern blotting.

Southern blotting strategy is described in Figure 9. DNA (10 g) from PCR-positive ES cell clones 3D4, 5B5, 6C1; and from PCR-negative clone 4B3 was digested with SpeI (+37°C, overnight). On the following day the digested DNA was precipitated and resuspended in 30 1 loading buffer. DNA was separated by electrophoresis on a 0.7 % agarose-TAE gel (100 V, 3 h), and the gel was quickly imaged under UV light with a ruler and DNA ladder to later identify the size of migrated bands. To allow efficient transfer of DNA and subsequent hybridization, the DNA was denatured by incubating the gel in 0.4 M NaOH (30 min, rocker) and neutralized (1.5 M NaOH, 0.5 M Tris pH 7.0, 30 min, rocker). Southern blotting was carried out in neutral conditions, using the capillary transfer method. Southern stack was assembled in a plastic tray containing 20x saline-sodium citrate (SSC) buffer in the following order: 1) sponge, 2) Whatman paper, 3) the gel, 4) Amersham Hybond-N+ nylon membrane (GE Healthcare), 5) Whatman paper, 6) a stack of paper towels (30 cm), and 7) a glass tray as a weight. On the following day the stack was disassembled and the DNA was immobilized with UV crosslinking. The membrane was rinsed with water, allowed to dry and stored at 4°C.

Detection of hybridization was done using the digoxigenin system (DIG High Prime DNA Labeling and Detection Kit II, Roche). First, the probe used in Southern blotting was designed to hybridize upstream of the 5’ homologous arm and was produced by PCR using BAC BMQ412N8 as a template. The probe (3 g) was denatured (10 min, 98°C) and quickly chilled on ice. Then the probe (in 16
H2O) was mixed with DIG-High Prime reagent mixture (4 μl) containing random primers, all dNTPs, digoxigenin-11-dUTP and Klenow polymerase, and incubated overnight (+37 °C) to produce digoxigenin-labeled probe. The dried nylon membrane was wet in H2O and 2x SSC, and prehybridized in prewarmed DIG Easy-Hyb buffer in a hybridization oven (30 min, 42°C, rotation). The labeled probe was denaturated (98°C, 10 min), cooled on ice, and diluted in 20 ml Easy-Hyb buffer (50 ng/ml). Hybridization was allowed to take place overnight (42°C, rotation). On the following day, the membrane was rinsed twice and washed (2 x 5 min) with washing buffer (2x SSC with 0.1% SDS, RT), followed by stringency washes (2 x 15 min) with warm wash buffer (0.5x SSC with 0.1% SDS, 67.5°C, rotation).

For immunological detection, the membrane was first rinsed with maleic acid wash buffer (0.1 M Maleic acid, 0.15 M NaCl, pH7.5, 0.3% Tween 20) and blocked with the kit blocking solution (30 min). Sheep-anti-digoxigenin antibody conjugated to alkaline phosphatase was diluted in blocking buffer (1:10000), and incubated with the membrane for 30 min. This was followed by washes (2 x 15 min) and equilibration in alkaline conditions (0.1 M Tris-HCl, 0.1 M NaCal, pH 9.5, 5 min). Finally, the chemiluminescent substrate (CSPD) for the alkaline phosphatase was introduced, and the membrane was visualized with a Fuji LAS-3000 Camera (Tamro Medlabs, Vantaa, Finland). The migration of bands was compared to the image taken previously from the gel with the ladder and a ruler. The clones 3D4 and 5B5 revealed the expected 5.5 kB band. Thus, targeting efficacy was only one percent.

4.3.3 Generation of chimeras and removal of puromycin selection cassette

ES cells from clones 3D4 and 5B5 were cultured at GM mouse unit of University of Helsinki. The modified ES cells were aggregated with morula-stage embryos to generate chimeric mice. Chimeras were mated with C57BL6/RCC females and the pups were screened with PCR. Out of nine chimeras chosen for mating one transmitted the mutation to the germline. The heterozygous p75-GVP mice were mated with Caq-FLP mice in order to remove the puromycin selection cassette to produce the final knock-in.
5. RESULTS

5.1 The effects of anticholinesterases on brain neurotrophin signaling

5.1.1 Phosphorylation of hippocampal Trk neurotrophin receptors by cholinesterase inhibitors

To examine if ChEIs could activate brain neurotrophin signaling we tested two chemically different AChE-selective anticholinesterases, donepezil and galantamine in mice. We gave mice a single intraperitoneal injection of donepezil (3.0 mg/kg) or galantamine (3.0-9.0 mg/kg) and analyzed Trk receptor activity 60 minutes later. We assessed the neurotrophin receptor phosphorylation using antibodies raised against the PLCγ1 binding site in the TrkB receptor (Y816) or TrkA receptor (Y794) (Rajagopal et al. 2004, Bath et al. 2008). Both donepezil and galantamine significantly increased (p < 0.001, ANOVA) phosphorylation of a 140 kDa band corresponding to the molecular weight of full-length Trk receptors (Fig 11 A, B). In addition, both antibodies recognized additional bands of unknown identity, which appeared to be regulated by the treatments. These bands have been detected previously in response to antidepressant drugs and G-protein coupled receptor ligands and may be immaturely glycosylated intracellular Trk receptors (Saarelainen et al. 2003, Rajagopal et al. 2004, Rantamaki et al. 2007).

The PLCγ1 binding sites of TrkA and TrkB share significant homology, which makes antibody cross-reactivity possible. Therefore, we tested ChEIs in TrkB.TK mice, which overexpress full-length TrkB cDNA driven by the Thy 1.2 cassette (Koponen et al. 2005). This minigene drives TrkB expression in postnatal neurons of the cortex and hippocampus, which results in increased TrkB signaling and improved improves spatial and associative learning. Moreover, N-terminal FLAG epitope tag in the receptor allows specific pulldown of overexpressed TrkB from the mouse brain. Thus, we treated TrkB.TK mice with donepezil and galantamine, pulled down TrkB receptors with an antibody against the FLAG tag and probed the membranes with phospho-TrkB antibody. Donepezil increased phosphorylation of TrkB (118.1%, p < 0.05, unpaired T-test, Fig 11 C). Galantamine increased TrkB phosphorylation (140.9% of control) but the change was insignificant due to variability (p = 0.367, unpaired T-Test).
Figure 11. The effect of donepezil and galantamine on Trk receptor tyrosine phosphorylation in the mouse hippocampus. Donepezil (DON, 3.0 mg/kg, 60 min, i.p.) and galantamine (GAL, 9.0 mg/kg, 60 min, i.p.) increased the phosphorylation of TrkB tyrosine 816 (A) and TrkA tyrosine 794 (B) in the mouse hippocampus. Donepezil increased Y816 phosphorylation of overexpressed TrkB, pulled down from TrkB.TK mice with an antibody against the FLAG epitope tag (C). The Trk receptor phosphorylation induced by imipramine (30 mg/kg, 60 min, i.p.) and galantamine (3.0 mg/kg, 60 min, i.p.) in wildtype (wt) and TrkB.Y816F knock-in mice with normal (Y816) or mutated (Y816F) TrkB PLCγ1 binding site (D). The number of animals in figures A and B were SAL = 8, DON = 8, GAL = 7, and in figure C: SAL = 5, DON = 4, GAL = 5. *: p < 0.05, ***: p < 0.001 (ANOVA).

To study the contribution of other Trk receptors we turned to the TrkB.Y816F mutant mice, which harbor a tyrosine-to-phenylalanine knock-in mutation in the TrkB PLCγ1 binding site (Minichiello et al. 2002). Consequently, activation of TrkB in these mice is unable to initiate PLCγ signaling, which results in impairment of hippocampal LTP. In the absence of Y816 in these mice, any change in phospho-TrkY816 immunoreactivity must result from phosphorylation of a homologous site in other Trk receptors. We injected galantamine and the antidepressant imipramine to the Y816F mice and analyzed Trk receptor phosphorylation with two different antibodies. The number of animals in the experiment was too low for statistics (n = 2). However, both imipramine and galantamine appeared to increase phospho-TrkY816 despite the absence of the tyrosine in TrkB (Fig. 11 D). Taken together, these data indicate that ChEIs phosphorylate several different Trk receptors in the mouse hippocampus. To discriminate between contributions of different Trk receptors the drugs should be tested in conditional TrkA and TrkB knockouts.
Figure 12. The effect of donepezil and galantamine on Trk downstream signaling. Donepezil (DON, 3.0 mg/kg, 60 min, i.p.) and galantamine (GAL, 9.0 mg/kg, 60 min, i.p.) tended to increase phosphorylation of AKT (p = 0.067) and significantly increased phosphorylation of cAMP response element-binding protein (CREB) in the mouse hippocampus (A, B). Neither drug affected the phosphorylation of MAPK44/42 (C). The number of animals was SAL = 8, DON = 8, GAL = 7. *: p < 0.05 (ANOVA).

In addition, we studied the activity of signaling pathways connected to Trk receptors after ChEi treatment. Donepezil and galantamine significantly increased phosphorylation of the transcription factor CREB (Fig. 12 A). The activity of AKT was increased two-fold whereas the activity of MAPK42/44 was unaltered (Fig. 12 B, C).

5.1.2 Mechanisms of Trk receptor activation induced by anticholinesterases

We hypothesized that the activation of Trk signaling results from target-derived neurotrophin release due to increased cholinergic neurotransmission through nicotinic or muscarinic receptors or both. To study which receptor type mediates the effects of ACh on hippocampal Trk signaling we treated mice with nicotine (1.0 mg/kg, 30 min, i.p.) or oxotremorine (0.1 mg/kg, 30 min, i.p.), a non-selective agonist of muscarinic receptors. Unlike ChEIs, neither agonist significantly increased Trk receptor tyrosine phosphorylation 30 minutes after injection (Fig. 13 A, B). AChEIs, however, increase the effect of ACh on both receptor types and we therefore decided to treat mice with a mixture of the two agonists. Combination of nicotine and oxotremorine induced a two-fold increase in Trk receptor phosphorylation in the
Figure 13. The effect of nicotine and oxotremorine on Trk receptor activity in the mouse hippocampus. Nicotine (NIC, 1.0 mg/kg, 30 min, i.p) and oxotremorine (OXT, 0.1 mg/kg, 30 min, i.p.) had no effect on Trk receptor tyrosine 674/5 phosphorylation when the drugs were given separately (A, B), but increased it significantly when given at the same time (C, D). The number of animals in the first experiment was SAL = 10, NIC = 8, OXT = 8, and in the second experiment SAL = 9 and NIC/OXT = 7. * = p < 0.05 (unpaired t-test).

hippocampus (199.6%, p < 0.023, t-test, Fig. 13 C, D). Thus, the cholinergic activation of hippocampal Trk signaling is unlikely to result from activation of individual ACh receptor subtypes, but requires comprehensive activation of the cholinergic system.

We then went on to study the role of BDNF in ChEI-induced activation of TrkB using heterozygous Bdnf knockout mice. Whereas Bdnf<sup>−/−</sup> mice die soon after birth due to loss of sympathetic neurons, Bdnf<sup>+/−</sup> mice avoid these problems and survive to adulthood (Ernfors et al. 1994, Korte et al. 1995). Importantly, BDNF protein levels in Bdnf<sup>+/−</sup> mice are 50% lower and the mice show marked deficits in hippocampal LTP indicating that BDNF signaling in their brain is significantly perturbed (Korte et al. 1995, Kolbeck et al. 1999). We treated Bdnf<sup>+/−</sup> mice with galantamine (5.0 mg/kg) and studied Trk receptor phosphorylation 60 minutes later. Western blot analysis showed a significant increase in phosphorylation of 145 kDa [treatment: F (1, 8) = 6.221, p = 0.037; Fig 14 A, B] and 105 kDa [treatment: F (1, 8) = 24.00, p = 0.001; Fig. 14 A, C] pTrkB immunoreactive bands in response to galantamine treatment. Notably, galantamine treatment failed to increase phosphorylation of TrkB in
Figure 14. The effect of galantamine on TrkB receptor phosphorylation in Bdnf<sup>−/−</sup> mice. Galantamine (GAL, 5.0 mg/kg, 30 min, i.p.) promoted full-length TrkB receptor Y816 phosphorylation in Bdnf<sup>−/−</sup> mice [F (1, 8) = 6.221, p = 0.037; Fig A, B], and increased the phosphorylation of a 105 kDa phospho-Trk immunoreactive protein in both genotypes [F (1, 8) = 24.00, p = 0.001; Fig. A, C]. The number of animals was three per group.

wildtype mice in this experiment. Regardless, even conservative interpretation of this data suggests that the phosphorylation response to galantamine was present in Bdnf<sup>−/−</sup> mice despite significant deficiency in brain BDNF-TrkB signaling.

Next, we tested donepezil in Sortilin<sup>−/−</sup> mice (Jansen et al. 2007). Sortilin interacts directly with Trk receptors and participates in their anterograde axonal transport to synapses (Vaegter et al. 2011). The amount of TrkB receptors in synaptic fractions prepared from the hippocampus of Sortilin<sup>−/−</sup> mice was significantly lower than in wildtype controls (Vaegter et al. 2011). Moreover, sortilin is reported to interact with the pro-domain of proBDNF intracellularly, and participate in the trafficking of the proneurotrophin to secretory granules, which is a prerequisite for activity-regulated release (Chen et al. 2005). Sortilin knockouts therefore serve as an indirect way to manipulate synaptic neurotrophin signaling.

We injected vehicle or donepezil (3 mg/kg, i.p) to 24 Sort1 wildtype mice and 8 homozygous knockouts and analyzed the activity of hippocampal Trk receptors 30 minutes after injection. Due to the less-than-mendelian reproduction of sortilin knockouts, we needed to use both females and males for the experiment. Therefore, we first asked if gender might affect the response to donepezil treatment. To test the treatment effect in males and females we used a two-way ANOVA. Gender had no effect on Trk receptor activity [Gender (F 1, 20) = 0.03, p = 0.865] or on response to the donepezil treatment [Interaction (F 1, 20) = 0.35, p = 0.560; Fig. 15 A, B]. Thus,
Figure 15. The effect of donepezil on Trk receptor activity in Sortilin1−/− mice. Donepezil treatment (DON, 3.0 mg/kg, 30 min, i.p.) increased hippocampal Trk receptor tyrosine Y674/5 phosphorylation in wildtype male and female mice (A, B). Figure B shows quantified data averaged from six animals per group. Two-way ANOVA revealed a significant treatment effect (p < 0.05) whereas gender did not affect Trk receptor phosphorylation (p = 0.865) or response to donepezil treatment (p = 0.560). Trk phosphorylation response to donepezil treatment was similar in wildtypes and Sortilin1−/− mice (C, D). The bars in figure D are averages of 12 wildtype or four sortilin knockouts per treatment group. Donepezil treatment significantly increased Trk receptor phosphorylation (p = 0.004) whereas genotype did not affect Trk receptor phosphorylation (p = 0.322) or response to treatment (p = 0.903).

we pooled males and females for further analysis. We then proceeded to compare the effect of donepezil on Trk receptors in sortilin knockout and wildtype mice. Donepezil treatment significantly increased Trk signaling [Treatment (F 1, 28) = 9.661, p = 0.004; Fig. 15 C, D] and this was unaffected by knockdown of sortilin [Interaction (F 1, 28) = 0.015, p = 0.903]. Basal Trk receptor phosphorylation in sortilin knockouts was 84.3% of that in wildtype controls, but the difference was statistically insignificant.

Taken together, these experiments suggest that the activation of Trk receptors in response to ChEIs may be independent of synaptic neurotrophin release. Previously, G-protein coupled receptor ligands, such as adenosine and PACAP, were reported to transactivate Trk receptors through Src family kinases (Lee and Chao 2001, Lee et al. 2002). Indeed, transactivation is a plausible mechanism for ChEI-induced Trk receptor phosphorylation. Transactivation takes place in intracellular membranes
(Rajagopal et al. 2004), which could explain why drug-induced Trk receptor phosphorylation was normal despite deficient synaptic targeting of the receptors in Sortilin1−/− mice and decreased BDNF protein levels in Bdnfβ−/− mice. Moreover, similar to G-protein coupled receptor ligands, ChEIs only activate PI3K/AKT pathway, but leave the MAPK/ERK pathway unaffected (Lee and Chao 2001). Neurotrophin binding to Trk receptor, in contrast, would be expected to affect both signaling cascades (Lee and Chao 2001, Huang and Reichardt 2003). Treatment with oxotremorine-M alone, however, failed to increase hippocampal Trk receptor activity arguing against Trk receptor transactivation through muscarinic receptors. Treatment with ChEIs could conceivably result in release of other G-protein coupled receptor ligands, which could eventually activate Trk receptors. Testing these drugs in conditional NGF and BDNF knockouts combined with validated immunoprecipitation strategies for TrkA and TrkB would further elucidate the contribution of neurotrophins in the drug-induced Trk receptor phosphorylation.

5.1.3 Neurotrophin synthesis, protein levels and balance between mature and proneurotrophins are unaffected by repeated galantamine treatment

Next, we wanted to study if the rapid Trk receptor phosphorylation leads to long-term changes in hippocampal neurotrophin synthesis. The antidepressant fluoxetine, for example, rapidly activates TrkB and increases BDNF synthesis when mice are treated for three weeks with the drug (Nibuya et al. 1995, Saarelainen et al. 2003, Rantamaki et al. 2007). We gave mice galantamine (3 mg/kg, i.p.) daily for two weeks and analyzed NGF and BDNF mRNA and protein levels after the treatment. Repeated daily treatment with galantamine, however, failed to affect neurotrophin mRNA or protein levels in the mouse hippocampus (Fig. 16 A, B). Our findings are in line with preceding studies, which showed that chronic treatment with donepezil or galantamine did not increase BDNF, NGF, TrkA or p75 protein levels in the rat brain (Hernandez et al. 2006, Kotani et al. 2008). Chronic treatment with AChEIs is known to result in significant tolerance to drug effects on ACh levels (Sweeney et al. 1989, Hernandez et al. 2006). Since neurotrophin ELISAs may detect both mature and proneurotrophins, we wanted to confirm our findings with Western blotting. Galantamine treatment did not alter the balance between mature and proneurotrophins (Fig. 16 C, D). To confirm that our antibody was able to detect proBDNF we analyzed tissue from knock-in mutant mice expressing cleavage-
resistant proBDNF. We detected a clear BDNF-immunoreactive band of 26 kDa in the knock-in mice confirming that the antibody recognizes proBDNF if the proneurotrophin is present in significant amounts in the brain (Fig. 16 E).

Even though we did not detect long-term effects on neurotrophin signaling, chronic treatment with galantamine, donepezil and galantamine reverse the loss of ChAT positive neurons in the basal forebrain and deficits in the object recognition task in anti-NGF mice (Capsoni et al. 2002; Capsoni et al. 2004). Moreover, rivastigmine and donepezil reversed decreased cortical NGF levels in rats with experimental allergic encephalomyelitis (D'Intino et al. 2005). Furthermore, Leyhe et al. (2008) reported that donepezil treatment may normalize serum BDNF protein levels in patients with AD. These findings suggest that although galantamine did not affect hippocampal neurotrophin synthesis in healthy mice, ChEIs may be able to restore the compromised neurotrophin signaling in various disorders.
Figure 17. The lack of protective effect of galantamine against amyloid toxicity. Dose-dependent toxicity of Aβ1-42 peptide (24 h) in cultured cortical neurons measured with the MTT assay (A). Pretreatment with BDNF for two hours (B) or 24 hours (C) protected neurons from amyloid toxicity and concomitant treatment with Trk kinase inhibitor k252a reversed the effect. Galantamine (GAL) did not affect the viability of neurons treated with Aβ (B, C). The bars show average viability of neurons in six parallel wells compared to untreated controls. ***: p < 0.001, ANOVA and Tukey’s post hoc test.

5.1.4 The lack of galantamine neuroprotection in vitro

According to previous studies, ChEIs protect cultured hippocampal and cortical neurons from various toxins, including glutamate and Aβ in vitro (Takada et al. 2003, Noh et al. 2009). Curiously, these effects were observed in dissociated cultures, which lack cholinergic innervation and thus the main source of ACh. Moreover, neuroprotection requires higher concentration of drugs than required to inhibit AChE suggesting other mechanism of action (Akaike et al. 2010). Antagonists of NACHRs, Src family kinases, PI3K, and janus-associated kinase (JAK2) block the neuroprotective effects (Takada-Takatori et al. 2006). In contrast, the MAPKK inhibitor PD98059 does not affect protection. These findings led us to hypothesize that ChEIs could protect cultured neurons through transactivation of Trk receptors. To test this idea we studied the effect of galantamine on Aβ1-42 toxicity in the presence of the Trk tyrosine kinase inhibitor, k252a. First, we treated cultured rat cortical neurons with Aβ1-42 in different concentrations and measured the amount of viable cells 24 hours later using the MTT assay. Aβ1-42 dose-dependently reduced the capability of neurons to convert tetrazolium dye into formazan (Fig. 17 A). Next, we treated neurons with BDNF or galantamine in doses previously reported to protect neurons in vitro (Takada-Takatori et al. 2006). We pretreated cortical neurons with galantamine for two or 24 hours before Aβ1-42 treatment. According to earlier studies,
24 hour ChEI pretreatment is required for neuroprotection (Takada et al. 2003, Takada-Takatori et al. 2006). Again, Aβ reduced the viability of neurons to 56.0%. BDNF partially rescued the neurons, but not in the presence of k252, indicating that BDNF protects neurons through TrkB activation (Fig. 17 B, C). Galantamine, on the other hand, did not protect neurons. Thus, we were unable to repeat previous findings and could not clarify the role of TrkB in the putative neuroprotective effect of ChEIs.

5.2 The effects of β-amyloid pathology on BDNF-TrkB signaling

Post-mortem studies suggest that BDNF-TrkB signaling is perturbed in patients with AD. Apart from one study (Durany et al. 2000), widespread downregulation of BDNF synthesis and protein levels have been consistently reported in patients with AD (Phillips et al. 1991, Narisawa-Saito et al. 1996, Ferrer et al. 1999, Hock et al. 2000, Peng et al. 2005). Moreover, patients with AD may have less full-length TrkB and increased levels of the truncated TrkB.T1 isoform in the brain (Connor et al. 1996, Ferrer et al. 1999). Based on in vitro studies, TrkB.T1 appears to act as a dominant negative BDNF receptor, because neurotrophin binding to the truncated receptor does not elicit activation of intracellular tyrosine kinase activity (Eide et al. 1996, Haapasalo et al. 2001). Thus, upregulation of TrkB.T1 together with decreased availability of BDNF could disturb TrkB signaling and neuronal plasticity and contribute to memory deficit in AD.

We wanted to study how increased Aβ accumulation and deposition in a mouse model of early-onset familial AD affects brain BDNF-TrkB signaling, and if impaired or enhanced BDNF-TrkB signaling would affect memory deficits induced by amyloid pathology. We used APdE9 transgenic mice expressing human APP with the Swedish mutation (K595N, K596L), and presenilin-1 with exon 9 deletion, two mutations that lead to early-onset familial AD in humans. These mice start to develop amyloid plaques in the hippocampus and cortex at four months of age followed by emergence of memory impairment at 8-12 months of age (Savonenko et al. 2005, Garcia-Alloza et al. 2006). To study the effect of impaired or enhanced BDNF-TrkB signaling on learning and memory in APdE9 mice we crossed these mutant mice with Bdnf+/− mice and with mice overexpressing full-length (TrkB.TK) or truncated TrkB (TrkB.T1). Overexpression of TrkB in these mice is driven by Thy 1.2 expression cassette, which promotes receptor expression in postnatal neurons in the brain. Consequently, TrkB signaling in the cortex and spatial learning are enhanced in
TrkB.TK mice and disrupted in TrkB.T1 mice (Saarelainen et al. 2000b, Koponen et al. 2004). Our collaborators tested the animals in various behavioral assays, and sent us the frozen brain tissue afterwards. We then analyzed protein levels of BDNF, full-length TrkB and TrkB.T1, and TrkB receptor activity from the samples.

5.2.1 Increased cortical BDNF and TrkB.T1 protein levels in APdE9 mutant mice

Table 6 summarizes BDNF, TrkB.T1, full-length TrkB, and phospho-TrkB protein levels in the frontal cortex (FCX), parietal cortex (PCX), temporal cortex (TCX) and the hippocampus (HC) of 13 months old wildtype, TrkB.T1, APdE9, and double transgenic female mice. As expected, the TrkB.T1 protein levels were significantly elevated in mice overexpressing the receptor compared to wildtype controls in all brain areas. However, overexpression of TrkB.T1 was only two-fold, which is significantly less than in original studies, where the amount of TrkB.T1 mRNA was 20-fold higher in TrkB.T1 transgenic mice (Saarelainen et al. 2000b). This could indicate a loss in the copy number of the transgene during this period. Although phosphorylation of TrkB was unaltered, AKT and ERK2 activity in the parietal cortex and AKT and GSK3β in the dentate gyrus were lower in TrkB.T1 mice (II). Together with the behavioral data, these changes indicate that two-fold overexpression of TrkB.T1 has functional consequences.

Notably, TrkB.T1 protein levels were also elevated in cortical samples from APdE9 mutant mice. TrkB.T1 protein levels were highest in TrkB.T1/APdE9 double mutants indicating that APdE9 and TrkB.T1 mutations had additive effects on TrkB.T1 expression. Our collaborators in this study showed in vitro that treatment of cortical and hippocampal neurons with Aβ_{25-35} (25 μM) results a in rapid decline in full-length TrkB protein levels, followed by significant accumulation of TrkB.T1 protein levels in the next two days (II). These data indicate that increased Aβ production and accumulation to plaques may lead to upregulation of TrkB.T1, and could thus explain the earlier clinical findings of elevated TrkB.T1 levels in patients with AD (Connor et al. 1996, Ferrer et al. 1999). Full-length TrkB protein levels, in contrast, were unaltered in APdE9 mice. This finding is in contradiction with decreased TrkB levels in AD patients (Ferrer et al. 1999). The decrease in TrkB immunoreactivity in patients, however, was observed in tangle-bearing neurons, which are absent in APdE9 mice (Ferrer et al. 1999).
Table 6. Levels of BDNF, full length and truncated TrkB, and phosphorylated TrkB in APdE9 and TrkB.T1 overexpressing mice. BDNF levels were analyzed with ELISA and TrkB.FR, TrkB.T1 and TrkB.pY816 levels with Western blotting from 13 month old female mice overexpressing truncated TrkB (TrkB.T1), in APdE9 double transgenic mice expressing human familial Alzheimer’s disease mutations APPswe/PS1dE9 (APP; K595N, K596L) and presenilin-1 exon 9 deletion, or both. All values are given as average +/- SEM normalized to wildtype controls. The effects of genotypes were analyzed using two-way ANOVA and bolded texts indicate significant genotype effects. The number of animals in each group was between 8-12.

<table>
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<th>BDNF</th>
<th>TrkB.T1</th>
<th>APdE9</th>
<th>Both</th>
<th>Significance (2-way ANOVA)</th>
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<tr>
<td>BDNF</td>
<td>100.0 ± 3.3</td>
<td>109.3 ± 6.0</td>
<td><strong>162.9 ± 7.4</strong></td>
<td>152.1 ± 5.3</td>
<td>APdE9: F(1, 45) = 84.92, p &lt; 0.0001</td>
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<td>TrkB.FR</td>
<td>100.0 ± 2.7</td>
<td>106.8 ± 4.4</td>
<td>107.6 ± 6.0</td>
<td>120.2 ± 5.5</td>
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<tr>
<td>TrkB.T1</td>
<td>100.0 ± 8.9</td>
<td><strong>242.4 ± 24.8</strong></td>
<td>157.1 ± 15.4</td>
<td>330.3 ± 31.9</td>
<td>TrkB.T1: F(1,44) = 51.13, p &lt; 0.0001; APdE9: F(1,45) = 10.80, p = 0.002</td>
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<td>pY816/FL</td>
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<td>98.8 ± 6.7</td>
<td>108.9 ± 12.3</td>
<td>97.8 ± 8.5</td>
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<th>Significance (2-way ANOVA)</th>
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<td>BDNF</td>
<td>100.0 ± 7.6</td>
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<td><strong>161.5 ± 13.7</strong></td>
<td>138.9 ± 10.6</td>
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<td>TrkB.FR</td>
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<td><strong>114.9 ± 5.4</strong></td>
<td>118.6 ± 7.5</td>
<td>128.6 ± 6.7</td>
<td>TrkB.T1: F(1,45) = 4.069, p = 0.0497</td>
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<td>289.0 ± 27.1</td>
<td>TrkB.T1: F(1,45) = 48.94, p &lt; 0.0001; APdE9: F(1,45) = 8.141, p = 0.0065</td>
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<tr>
<td>BDNF</td>
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<td><strong>76.5 ± 2.8</strong></td>
<td>111.3 ± 5.7</td>
<td>86.7 ± 5.5</td>
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<td><strong>181.8 ± 15.2</strong></td>
<td>92.6 ± 7.6</td>
<td>164.3 ± 19.6</td>
<td>TrkB.T1: F(1,44) = 28.60, p &lt; 0.0001</td>
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<td>106.7 ± 9.9</td>
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BDNF protein levels in APdE9 mice, on the other hand, were elevated in all brain areas apart from the hippocampus. Our findings agree previous studies on BDNF protein levels in APP mutant mice (Burbach et al. 2004, Szapacs et al. 2004). However, Peng et al. (2009) detected a significant decrease in BDNF mRNA levels in some, but not all, AD mutant mouse lines. According to Peng and co-workers (2009) different mutations in APP and PS1 favor production of different ratios of Aβ_{1-42} and Aβ_{1-40} leading to formation of a variety of Aβ oligomers, which differentially affect BDNF synthesis. Thus, data from different AD mutant mouse lines is not directly comparable. Overexpression of TrkB.T1 decreased BDNF protein levels in the parietal cortex and the hippocampus, and limited the increase induced by APdE9 mutation in the temporal cortex. This was unsurprising because activity-induced BDNF synthesis is known to be reduced in TrkB.T1 mice (Saarelainen et al. 2000a).

Notably, we did not detect elevated BDNF protein levels in the hippocampus, where plaque density in APdE9 mice is lowest (Garcia-Alloza et al. 2006). Moreover, we did not detect increased cortical BDNF levels in young APdE9 mice that did not have visible amyloid plaques (III). Burbach et al. (2004) reported that increased BDNF levels in their APP-Swe mutant mice correlated with amyloid load. Furthermore, they detected BDNF protein in amyloid plaques. In agreement with this finding, our immunohistochemical analysis of aged APdE9 mice revealed BDNF immunoreactivity in Aβ plaques (III). This finding indicates that increased BDNF levels in the cortex of APdE9 mutant mice may reflect accumulation of the protein in amyloid deposits. The exact source of BDNF is unclear, but Burbach et al. (2004) reported that glial cells associated with plaques increased their BDNF synthesis. In our study, however, BDNF mRNA levels were unaltered (III). Notably, some groups have detected BDNF protein in amyloid plaques in patients with AD (Ferrer et al. 1999, Murer et al. 1999). Ferrer et al. (1999) detected strong BDNF immunoreactivity in dystrophic neurites surrounding plaques in AD patients. Apart from localization similarities, however, our findings in APdE9 mouse are in obvious contrast with decreased cortical BDNF protein levels in patients with AD. This discrepancy is an important reminder that the APdE9 mouse is by no means an accurate model of human sporadic AD. Instead, it combines overexpression of two mutant proteins into a model of hyperaccelerated Aβ accumulation. Nevertheless, our study shows that accumulation and deposition of Aβ directly affects BDNF-TrkB signaling in various ways likely to undermine synaptic plasticity.
5.2.2 Impairment of BDNF-TrkB signaling exacerbates memory defects in APdE9 mice

To test how genetic impairment or enhancement of BDNF-TrkB signaling affects memory deficits in APdE9 mice our collaborators tested the double transgenic mutants in the Morris water maze task. ApdE9 mice were significantly impaired on this task, displaying increased latency to escape, longer swim path length, and thigmotaxic behavior as evidenced by tendency to swim along arena walls (II). TrkB.T1 male mice did not differ from wildtype controls, but TrkB.T1/APdE9 double transgenic mice performed worse than APdE9 single mutants did. These results indicate that subtle overexpression of TrkB.T1 does not have a significant effect on learning and memory on its own, but aggravates the memory deficits seen in APdE9 mice. Moreover, Bdnf+/APdE9 double mutants performed worse than single mutants indicating that loss of other Bdnf allele, similarly to overexpression of TrkB.T1, worsened performance of APdE9 mice on the task (III). In contrast, overexpression of TrkB.TK reduced escape latency (II). TrkB.TK/APdE9 double transgenic mice also performed better than APdE9 single mutant mice as evidenced by significantly shorter swim path length and decreased escape latencies. Moreover, TrkB.TK/APdE9 transgenics spent more time in the target area compared to APdE9 mutants. These findings indicate that increased TrkB signaling may counteract memory deficits induced by perturbed APP processing and Aβ generation.

5.3 Characterization of p75 cleavage reporter gene assay

The role of p75 in the adult CNS is still unclear, partially due to the lack of established methods to detect receptor signaling. Moreover, the number of ligands, co-receptors and signaling pathways makes finding a straightforward detection method challenging. Proteolytic processing of the receptor, however, is regarded as common event in p75 signaling in both trophic and apoptotic signaling (chapters 2.1.5 and 2.1.6). The resulting cleavage products, especially the soluble intracellular domain, are difficult to detect and often require overexpression of the receptor combined with proteasome inhibitors (Jung et al. 2003, Kanning et al. 2003). Therefore, we developed a reporter gene assay described in detail in chapter 4.2.1. to detect proteolytic processing of p75.

To show that cells process p75-GVP fusion protein normally we transfected
Figure 18. Detection of proteolytic processing of p75 with reporter gene assay. Treatment of full-length p75-GVP (FLp75-GVP) transfected HEK293T cells with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml, 40 min) resulted in proteolytic processing of the receptor (A). Pretreatment with DAPT (2 μM, 90 min) prevented formation of p75ICD and promoted accumulation of p75CTF. PMA treatment increased luciferase activity in HEK293T cells cotransfected with UAS_Luc2P and FLp75-GVP (B) or C-terminally truncated p75EC/TM-GVP (C). Basal luciferase activity was 20-fold higher in cells transfected with FLp75-GVP than in cells transfected with p75EC/TM-GVP (D). The bars are average of six parallel wells. LAC = clasto-lactacytstin β-lactone.

HEK293T cells with FLp75-GVP. We treated the cells with phorbol 12-myristate 13-acetate (PMA), which activates metalloproteases through protein kinase C, and has been shown to lead to proteolytic processing of p75 (Kanning et al. 2003). PMA (100 ng/ml, 40 min) induced the processing of p75-GVP as indicated by the appearance of p75ICD-immunoreactive bands corresponding to the molecular weights of p75CTF-GVP and p75ICD-GVP. (Fig. 18 A). The production of p75ICD-GVP was blocked by pretreatment with the γ-secretase inhibitor DAPT, which also led to accumulation of p75CTF-GVP. To show that our reporter gene assay is capable of detecting processing of the receptor, we plated the cells on 96-well plates and co-transfected them with p75 fusion proteins and a reporter plasmid. This time the cells were treated overnight with PMA to allow for accumulation of luciferase. PMA increased luciferase activity by approximately two-fold in HEK293T cells transfected with FLp75-GVP (Fig. 18 B) and p75ECD/TM-GVP (Fig. 18 C). These data show that the reporter gene assay can efficiently detect the regulated intramembrane proteolysis of p75 in vitro.

The truncated and full-length constructs, however, differed significantly from each other. We compared signal intensity from the HEK293T cells transfected with either
full-length or truncated construct and found that with similar amount of transfected DNA, the FLp75-GVP resulted in 20-fold higher basal luciferase activity than p75EC/TM-GVP (Fig. 18 D). Several explanations may be suggested. First, the translocation of Gal4/VP16 to the nucleus may be less efficient in the absence of interaction between p75ICD and its intracellular signaling partners. Second, Lac increased luciferase signal from cells transfected with p75EC/TM more efficiently (Fig. 18 B, C), indicating that the GVP domain in the absence of p75ICD may be susceptible to proteasomal degradation. Third, subcellular localization of the constructs may be different. Fourth, overexpression of p75 may lead to constitutive activation (Rabizadeh et al. 1993) and possibly proteolysis of the receptor by an as yet unknown mechanism. Constitutive processing of the overexpressed FLp75-GVP could also explain the loss of assay dynamics: the γ-secretase inhibitor DAPT (2 M) efficiently inhibited the production of p75ICD, but prevented the increase of luciferase activity only in cells expressing p75EC/TM-GVP, but not FLp75-GVP (Fig. 18 A-C). It is conceivable that ongoing constitutive processing of the receptor masked the subtle change induced by the treatment. Careful optimization of expression levels will be necessary for further in vitro experiments.
6. DISCUSSION

The scientific rationale to target neurotrophin signaling for the treatment of AD still exists 30 years after the neurotrophin hypothesis of the disease (Hefti 1983). After decades of NGF research there is hardly any doubt if activation of TrkA receptors would support cholinergic function in AD. Delivery of NGF to the basal forebrain with adeno-associated virus (AAV2-NGF, CERE-110) is currently in phase II clinical trial and, based on preclinical models, this treatment is very likely to have trophic effects on cholinergic neurons (Bishop et al. 2008). Furthermore, the notable plasticity of BFCNs is encouraging for NGF-based therapies: NGF is capable of restoring the cholinergic function in Ts65Dn and anti-NGF mice after life-long atrophy (Cooper et al. 2001, Capsoni et al. 2002). As most of the cholinergic neurons do not actually die in AD, but shrink and lose their phenotype instead, activation of TrkA signaling could potentially restore their function even in late stages of disease (Vogels et al. 1990, Gilmor et al. 1999). The fundamental question, however, is whether activation of TrkA has any significant impact on the progression of AD. Maintenance of cholinergic neurotransmission via TrkA should somehow propagate the survival of neurons in target areas of cholinergic innervation. Otherwise, NGF gene therapy may turn out to be nothing but a highly invasive way to increase cholinergic neurotransmission, an effect that can be achieved with current pharmacotherapies. Curiously, work on the anti-NGF mouse suggests the existence of a vicious cycle where perturbation of NGF action promotes Aβ pathology, which in turn interferes with trophic actions of NGF (Fig. 7). Should such interdependency exist, enhancement of TrkA activity could indeed have benefits beyond the cholinergic system. More preclinical studies on the effects of NGF on Aβ accumulation in mouse models of AD are warranted.

Synaptic failure and synapse loss in AD, on the other hand, suggest molecular mechanisms underlying synaptic plasticity as potential therapeutic targets. The BDNF receptor TrkB is ideally located in degenerating neurons of the cortex and the hippocampus (Yan et al. 1997, Drake et al. 1999). Although these neurons do not depend on BDNF for their survival (Rauskolb et al. 2010), the neurotrophin is capable of protecting them from Aβ toxicity and from lesions in vivo (Nagahara et al. 2009). Moreover, overexpression of BDNF and genetic enhancement of TrkB activity improved learning and memory in mouse models of familial AD (II, Nagahara et al. 2009). Importantly, BDNF reversed synapse loss in the APP mutant
mice (Nagahara et al. 2009). Moreover, 7, 8-dihydroxyflavone, an alleged TrkB agonist, reversed memory deficit in AD mutant mice (Devi and Ohno 2012). When the first signs of memory impairment appear in patients, a significant number of neurons and synapses are already lost (Gomez-Isla et al. 1996, Kordower et al. 2001, Scheff et al. 2006, Scheff et al. 2007). Activation of TrkB receptor at this point could enhance synaptic transmission and protect the remaining synapses and neurons. Ideally, to prevent pathogenesis of the one needs to be able to identify and treat patients at risk of developing dementia years before the onset of symptoms.

Treatment of AD with neurotrophins would require invasive surgery. Moreover, since both cholinergic and non-cholinergic degeneration are widespread in AD, effective treatment with growth factors would require delivery of the peptides to a number of locations in the brain. Poor diffusion of BDNF is very likely to make such an approach unfeasible (Morse et al. 1993). Clearly, activation of Trk receptors with small molecules would be more convenient. Such pharmacological approaches could include molecules that act as Trk receptor agonists; transactivate the receptors, or otherwise potentiate receptor signaling; promote neurotrophin synthesis, release or maturation; or prevent neurotrophin degradation. Ideally, the novel drugs should target both TrkA and TrkB receptors, and activate them independently of neurotrophins and cholinergic neurotransmission. This way drug effect would persist amid degeneration of the cholinergic system and potential perturbations in neurotrophin action in AD (Podlesniy et al. 2006, Bruno et al. 2009, Kichev et al. 2009). The failure of retrograde transport of neurotrophin signaling complexes, however, poses a serious challenge for any therapeutic strategy based on the activation of Trk receptors (Mufson et al. 1995).

Another potential way to target neurotrophin signaling is inhibition of p75 function. Massa et al. (2006) identified p75 ligands, which prevented cell death induced by Aβ in young hippocampal neuron cultures, prevented neurite dystrophy in mature cultures (Yang et al. 2008), and reversed memory deficits in a mouse model of familial AD (Knowles et al. 2013). Indeed, inhibition of p75 signaling may prevent detrimental effects of Aβ on BFCNs, and favor trophic effects of NGF on cholinergic neurons (Sotthibundhu et al. 2008, Knowles et al. 2009). Theoretically, p75 ligands could also prevent detrimental effects of proNGF and Aβ on non-cholinergic neurons. Neurons in the CNS increase p75 expression in response to various traumas, and binding of proNGF to the receptor promotes post-traumatic apoptosis (2.1.5). The
evidence supporting a similar role for p75 in AD is, however, unconvincing. Given the implications this question has on the potential of p75 as target for novel drugs, it is surprising that a simple quantitative PCR approach to analyze receptor mRNA levels in target areas of cholinergic innervation in post-mortem AD patients has not been taken. Moreover, the limited expression of p75 in the mature CNS casts a shadow of doubt on the translatability of in vitro findings that the receptor mediates Aβ-induced neurite dystrophy of cultured hippocampal neurons (Knowles et al. 2009). Connecting synaptic failure, synapse loss, and hippocampal and cortical neuronal degeneration in adult APP mutant mice to p75 signaling would significantly increase confidence in the receptor as target for drug discovery.

The absence of good animal models is a major challenge for predictions of therapeutic efficacy of new drugs. Testing NGF-based therapies in the anti-NGF mouse would be redundant, despite the apparent face validity of the model. Mouse models based on familial mutations in APP or PS1, on the other hand, bear good construct validity, but mimic accurately only a small minority of human AD cases. It may be too much to blame this for the failure of amyloid immunotherapies in clinical trials, but undoubtedly the treatments were successful preclinically in APP-based mouse models (Mullard 2012). Ideally, new treatments should be tested both in APP- and NGF-based animal models. Furthermore, comparative studies of these models could elucidate the interrelationships between NGF-, BDNF- and Aβ signaling in AD. Our findings about BDNF signaling in APdE9 mice should be complemented with analyses of NGF signaling in the same model. Similarly, changes in BDNF-TrkB signaling in anti-NGF mice deserve a closer look.

Understanding the actions of existing drugs on brain neurotrophin signaling is the first step towards development of novel therapies. Memantine, an uncompetitive NMDA receptor antagonist indicated for moderate-to-severe AD, induces widespread BDNF synthesis in the rat and monkey brain (Marvanová et al. 2001, Meisner et al. 2008). Memantine has beneficial effect on cognition, mood, behavior and the ability to perform activities in patients with advanced AD, but the effect is modest in early AD (McShane et al. 2006, Schneider et al. 2011). Whether increased BDNF synthesis plays a role in its method of action is unclear, but memantine has been reported to protect dopaminergic neurotransmission in SIV-infected rhesus monkeys, an effect that could be potentially mediated through TrkB (Meisner et al. 2008). Long-term studies on the effect of memantine on the progression from MCI to AD are
warranted. Additionally, commonly used antidepressant drugs elicit effects on neurotrophin signaling: they activate TrkB receptors in the mouse brain (Saarelainen et al. 2003, Rantamäki et al. 2007), increase BDNF synthesis (Nibuya et al. 1995), enhance synaptic plasticity (Karpova et al. 2011), and reopen developmental-like plasticity in the adult rodent brain (Vetencourt et al. 2008, Karpova et al. 2011). Even though antidepressants are commonly used to treat depression and behavioral symptoms in patients with AD, clinical trials have not been designed to assess their effects on cognitive capabilities or progression of the disease (Henry et al. 2011). As inexpensive, safe, and reasonably well tolerated drugs, antidepressants should be thoroughly studied in APP- and NGF-based mouse models, as well as in clinical trials.

Finally, our findings suggest that ChEIs, the current first-line therapy against AD, activate neurotrophin signaling in the brain. As activators of both TrkA and TrkB, the ChEIs appear to be ideal neurotrophic drugs. However, even though some studies indicate that ChEIs may have modest disease-modifying effects, the consensus from 20 years of clinical experience is that they do not stop progression of the disease (Mori et al. 2006, Russ and Morling 2012). This underlines the importance of resolving where and how ChEIs activate Trk receptors, whether the rapid phosphorylation of Trk receptors sustains in chronic treatment and if it translates into long-term neurotrophic effects, and if ChEIs are indeed neurotrophic drugs in patients with AD. If so, it will be imperative to understand why they are unable to prevent progression of the disease. This will enable the evaluation of neurotrophin-based treatment strategies in general, and facilitate the development of novel, enhanced neurotrophic drugs for AD.
7. SUMMARY AND CONCLUSIONS

1. ChEIs, the current first-line drugs for AD, activate TrkA and TrkB neurotrophin receptors in the mouse hippocampus. Cholinergic activation of Trk receptors requires simultaneous stimulation of nicotinic and muscarinic receptors. Drug-induced Trk receptor phosphorylation is unaltered in Sortilin1−/− mice and Bdnf+− mice despite defects in anterograde transport and synaptic targeting of Trk receptors, and BDNF signaling in these mice.

2. Increased Aβ production and deposition in the APdE9 mouse model of familial AD perturbs BDNF-TrkB signaling indicated by accumulation of BDNF in amyloid plaques and upregulation of dominant negative TrkB.T1. Overexpression of TrkB.T1 or knocking down BDNF expression exacerbates memory impairment in APdE9 mice, while overexpression of TrkB.TK alleviates these deficits. These findings elucidate functional and neurobiological interactions between Aβ pathology and BDNF-TrkB signaling and provide support for TrkB receptor as a potential target for novel pharmacotherapies against AD.

3. p75 cleavage reporter gene assay is a sensitive and efficient method to detect proteolytic processing of the receptor in vitro. Our knock-in mouse could enable accurate temporal and spatial localization of p75 signaling in healthy, aged and injured nervous system and serve as a tool to discover inhibitors of detrimental p75 signaling.
REFERENCES


Dougherty, K.D. & Milner, T.A. (1999) p75(NTR) immunoreactivity in the rat dentate gyrus is mostly within presynaptic profiles but is also found in some astrocytic and postsynaptic profiles. Journal of Comparative Neurology 407, 77-91.


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