BRAIN-DERIVED NEUROTROPHIC FACTOR IN ALZHEIMER'S DISEASE

BRAIN-DERIVED NEUROTROPHIC FACTOR: mRNA AND PROTEIN LEVELS IN NORMAL AND ALZHEIMER'S DISEASED BRAIN

By

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ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disorder of the central nervous system. One pathological characteristic is excessive neuronal loss in specific regions of the brain. Among the areas most severely affected are the basal forebrain cholinergic neurons and their projection regions, the hippocampus and cortex. Neurotrophic factors, particularly the neurotrophins nerve growth factor and brain-derived neurotrophic factor, play an important role in the development, regulation and survival of basal forebrain cholinergic neurons. Furthermore, brain-derived neurotrophic factor regulates the function of hippocampal and cortical neurons. Neurotrophins are synthesized in hippocampus and cortex and retrogradely transported to the basal forebrain. Decreased levels of neurotrophic factors are suspected to be involved in the neurodegenerative changes observed in Alzheimer's disease. We examined autopsied parietal cortex, hippocampus and nucleus basalis of Meynert samples from age- and gender-matched Alzheimer's diseased and neurologically non-impaired individuals using the quantitative technique of competitive RT-PCR. We also examined parietal cortex samples by Western blotting. We demonstrate a 3.4-fold decrease in brain-derived neurotrophic factor mRNA levels in the parietal cortex of patients with Alzheimer's disease compared to controls ($p < 0.004$) but fail to observe changes in BDNF protein levels in that brain region. We also demonstrate, for the first time, BDNF mRNA in the

nucleus basalis of Meynert and report an age-related decline in the levels of BDNF mRNA in both control and AD samples. Using the competitive RT-PCR technique we fail to observe differences in BDNF mRNA levels in the hippocampus between AD and control subjects, conflicting with previous in situ hybridization studies and RNase protection assays. A decrease in brain-derived neurotrophic factor synthesis could have detrimental effects on hippocampal, cortical and basal forebrain cholinergic neurons and may account for their selective vulnerability in Alzheimer's disease.

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LIST OF ABBREVIATIONS

μg	microgram (10^{-6}g)
Aβ	amyloid β-protein
A2M	α-2-macroglobulin
ACh	acetylcholine
AChE	acetylcholinestarase
AD	Alzheimer's disease
apoE	apolipoprotein E
APP	amyloid precursor protein
BDNF	brain-derived neurotrophic factor
BFCN	basal forebrain cholinergic neurons
BSA	bovine serum albumin
cDNA	complementary strand deoxyribonucleic acid
ChAT	choline acetyltransferase
CNS	central nervous system
DNase	deoxyribonuclease
dNTPs	deoxynucleotide triphosphates
ELISA	enzyme-linked immunosorbant assay
fg	femtogram (10^{-15}g)
kDa	kilo-Daltons
KLH	keyhole limpet hemocyanin
mRNA	messenger ribonucleic acid
MuLV	murine leukemia virus
NBM	nucleus basalis of Meynert
NGF	nerve growth factor
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
pg	picogram (10^{-12}g)
PMI	postmortem interval
PS	Presenilin
PVDF	polyvinylidene difluoride
rhBDNF	recombinant human brain-derived neurotrophic factor
RNase	ribonuclease
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
TBS(-T)	tris buffered saline (with Tween-20)
trkA	tropomyosin-related kinase A - high affinity receptor for NGF
trkB	tropomyosin-related kinase B - high affinity receptor for BDNF
trkC	tropomyosin-related kinase C - high affinity receptor for NT-3

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1.0 INTRODUCTION

1.1 Alzheimer's disease

Alzheimer's disease (AD), a neurodegenerative disorder that is positively diagnosed only at postmortem by its characteristic amyloid plaques and neurofibrillary tangles in brain tissue, affects a significant proportion of the population over the age of 65. A 1990 study by Evans reported that in the US 3% of the population between the ages of 65 to 74 are afflicted with AD. This number rises to 19% of the population between the ages of 75 to 84 and to 47% for individuals over the age of 85. Thus, increasing age appears to be the main risk factor associated with AD. The physical characteristics of the disease include a gradual loss of memory, decline in other cognitive functions and a decrease in functional capacity with death normally occurring approximately 10 years after the onset of symptoms.

Ultramicroscopic examination of the AD brain reveals severe neuronal damage and loss of synapses (Terry and Katzman, 1983) associated with senile plaques, which consist of accumulations of cellular debris surrounding a central core of protein referred to as beta-amyloid. Formation of neurofibrillary tangles in the AD brain have been linked to an abnormal accumulation of a microtubule-associated protein, tau, which is normally located in axons. Neuronal loss and damage are most severe in the basal forebrain cholinergic neurons followed by the hippocampus, the amygdala and neocortical regions (Whitehouse et al., 1982; Cuello and Sofroniew, 1984; Etienne et al., 1986; Mann, 1991). The basal

forebrain consists of a group of neurons with projections to the hippocampus and neocortical areas of the brain. Atrophy and loss of basal forebrain cholinergic neurons (BFCN), cells that are critically involved in functions of memory and cognition (Hefti and Weiner, 1986), correlate strongly with the degree of dementia in AD (Coyle et al., 1983).

Alzheimer's disease has historically been categorized into early-onset ("presenile") dementia and late-onset ("senile") dementia (Terry and Katzman, 1983). Early-onset refers to onset of the disease prior to the age of 65, which accounts for approximately 25% of all cases.

Current theories rely heavily on the data generated by genetic analysis of the minority of patients in whom AD is caused by an inherited autosomal dominant trait. To date, five genes have been associated with the development of familial AD: amyloid precursor protein (APP) on chromosome 21 (Goate et al., 1991), presenilin 1 (PS1) on chromosome 14 (Sherrington et al., 1995), presenilin 2 (PS2) on chromosome 1 (Levi-Lahad et al., 1995; Rogaev et al., 1995), apolipoprotein (apo)E on chromosome 19 (Corder et al., 1993), and the recently identified α-2-macroglobulin (A2M) protein on chromosome 12 (Blacker et al., 1998). The first 3 genes are associated with early-onset AD while the apoE and A2M proteins have been associated with both the early and late onset of the disease in patients with and without a family history of AD, and hence the sporadic form of the disease as well.

1.1.1. Genetics of Alzheimer's Disease

1.1.1.1. Amyloid precursor protein

Amyloid precursor protein is a type 1 transmembrane glycoprotein that is widely expressed in brain and platelets. Proteolytic cleavage of APP by enzymes referred to as secretases (α,β, and γ) results in the generation of amyloid β-protein (Aβ), a 39-42 amino acid peptide (Kang et al., 1987). The cleavage of APP by α-secretase, which occurs within the Aβ peptide sequence, prevents the generation of Aβ (see Hutton and Hardy, 1997). Cleavage by β-secretase at the N-terminus leads to the generation of Aβ following cleavage by γ-secretase. The site of cleavage of APP by γ-secretase is the C-terminal of residue 712. If cleavage occurs between residues 712 and 713, the short form of Aβ (Aβ40) is generated. However, if cleavage occurs after residue 714, long Aβ [Aβ42(43)] is generated. This long form of Aβ has been suggested to be amyloidogenic (reviewed by Selkoe, 1998).

Deposition of $A\beta_{42}$ in plaques and in the walls of cerebral blood vessels is a central feature of the pathology of AD (Selkoe, 1994). Missense (Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991; Naruse et al., 1991) and substitution (Levy et al., 1990; Van Broeckhoven et al., 1990; Hendricks et al., 1992) mutations account for six forms associated with the APP gene in familial Alzheimer's disease (FAD). Transgenic mice over-expressing the mutant form of APP develop amyloid deposits, neuritic plaques, synaptic loss and astrocytosis in the hippocampus and cortex (similar areas affected in

humans) that result in memory deficits when compared to their age-matched controls (Games et al., 1995).

1.1.1.2. Presenilins

PS1 and PS2 are highly homologous integral membrane proteins of 52 kDa located mainly in the nuclear envelope, endoplasmic reticulum and golgi apparatus of neurons (Kovacs et al., 1996). The PS1 gene consists of 10 protein coding exons Clark et al., 1995; Cruts at al., 1996) with 6 to 9 transmembrane domains (TM). Naturally occurring endoproteolytic processing results in the production of two proteins of 25 to 28 kDa and 16 to 19 kDa.

At least forty-one different mutations have been reported for the PS1 gene (Hardy, 1997) and all, except one, are missense mutations. Most mutations lie in exons 5 (TM 1-2) and 8 (TM 6-7) resulting in significantly different ages of onset of AD (Cruts et al., 1996). PS1 mutations account for 30-50% of presenile AD (Hutton et al., 1996).

The intron-exon structure of PS2 is very similar to that of PS1 and shares a 67% homology with PS1 at the amino acid level. Identified originally to consist of a missense mutation in a Volga German family (Levy-Lahad et al., 1995a,b) susceptible to AD, a second missense mutation has recently been identified in eight unrelated families (Cruts et al., 1996; Hardy, 1997; Lendon et al., 1997). An unique feature of the PS2 gene mutations is that individuals carrying such mutations may not be affected by them (Levy-Lahad et al., 1995a; Sherrington et al., 1996).

Studies in transgenic mice and transfected cell lines indicate that an interplay between PS and Aβ results in detrimental effects (Scheuner et al., 1996; Li et al., 1997; Citron et al., 1997). Transgenic animals that co-expressed mutant forms of PS1 and APP develop numerous amyloid deposits not seen in the brains from age-matched animals that express mutations in one of the above genes alone (Borchelt et al., 1997). Expression of PS variants with the same mutations observed in AD has been demonstrated to alter C-terminus APP processing resulting in an increased secretion of $A\beta_{42}$ (Lemere et al., 1996).

1.1.1.3. Apolipoprotein E

A glycoprotein consisting of 299 amino acids with a molecular mass of 32 kDa (Mahley, 1988; Weisgraber et al., 1994a,b), ApoE is involved in the transport and metabolism of cholesterol and triglycerides (Weisgraber et al., 1994). It is also involved in nerve regeneration (Handelmann et al., 1992), immunoregulation (Siest et al., 1995) and the activation of several lipolytic enzymes (Siest et al., 1995). ApoE is localized in the cytoplasm of neurons that express low-density lipoprotein receptor-related proteins (LRP - the ApoE binding protein (Rebeck et al., 1993). There are three major isoforms of ApoE (E2, E3 and E4) that are the products of three allelic forms (ε2, ε3 and ε4) (Emi et al., 1988) that in turn give rise to six different genotypes of which ApoE ε3/3 is the most common (Lehtimäki et al., 1990). Epidemiological studies have demonstrated that the ApoE ε4 allele is over-represented in the late-onset form of both familial and sporadic AD (Roses, 1996). Further, the risk of developing AD appears to be allele dose dependent.

Individuals carrying two alleles of the ε4 type have a greater risk of developing AD compared to those with one ε4 allele who in turn are at a greater risk to individuals with a type ε3 or type ε2 allele respectively (Corder et al., 1993).

1.1.1.4. Alpha-2-macroglobulin

A2M, a serum pan-protease inhibitor, has recently been linked to AD based on its ability to break down and clear Aβ from the brain (Blacker et al., 1998). A common genetic mutation in this gene, present in 30% of the population, strongly predisposes the carrier to AD. Unlike the ε4 allele of ApoE, which lowers the age of onset for AD, the A2M mutation appears to increase the risk for both early- and late-onset AD.

1.1.2. Sporadic Alzheimer's Disease

Many factors including gender, environmental, chemical, neurochemical, ApoE, A2M and lifestyle have been implicated in the sporadic onset of AD. Neurochemically, acetylcholine (ACh), a neurotransmitter synthesized by choline acetyltransferase (ChAT) in, and released by the cholinergic system has been shown to play a crucial role in the onset of AD. ACh is synthesized primarily by the basal forebrain cholinergic neurons and is catabolized by acetylcholinesterase (AChE). The receptors for ACh belong to the muscarinic and nicotinic family of receptors. Reductions in ChAT and AChE activities have been reported in the hippocampus, amygdala and neocortex among other areas of subjects with AD (Bowen et al., 1976; Davies and Maloney, 1976; White et al., 1977). The hippocampus and amygdala play a critical role in the formation and maintenance of

memory and cognitive functions. The reduction of ChAT activity, which correlates strongly with memory impairments in AD has been linked to the loss of cholinergic neurons in the basal forebrain (Candy et al., 1983; Arendt et al., 1985). Accumulating evidence suggests that neuronal growth factors referred to as neurotrophic factors are capable of sustaining the growth and differentiation of these cholinergic neurons.

1.2 Neurotrophic Factors

Neurotrophic factors are a large class of molecules that support the growth, differentiation and survival of neurons in both the developing and adult nervous system (Bothwell, 1995). Family members of neurotrophic factors include neurotrophins, neuroproteins, insulin-like growth factors and fibroblast growth factors among others. Of these, the neurotrophins are of particular interest since they have been shown to promote the survival, differentiation and maintenance of cholinergic neurons (Hefti, 1986; Alderson et al., 1990; Rylett and Williams, 1994).

1.2.1 Neurotrophins

Identified in the 1940s, nerve growth factor (NGF), the first and most well characterized member of the neurotrophins, has entered clinical trials as a potential therapeutic agent for treatment of AD and Huntington's disease. Other members of the neurotrophin family include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). These factors are synthesized in many regions of the brain and are important for neuronal survival, function and regulation (Goedert et al.,

1986; Hefti and Weiner, 1986; Ernfors et al., 1990; Maisonpierre et al., 1990; Phillips et al., 1990; Wetmore et al., 1990; Maness et al., 1994; Dugich-Djordjevic et al., 1995). Referred to as target-derived factors, neurotrophins exert their actions by binding to specific tropomyosin-related kinase (trk) receptors located on presynaptic neurons (Bothwell, 1991; Parada et al., 1992; Barbacid, 1994) and being retrogradely transported to the cell body of the neuron. Each member of the neurotrophin family has been linked to a specific trk receptor although cross-talk does exist between many family members. Both NGF and BDNF support the function and survival of the basal forebrain cholinergic neurons (Hefti, 1986; Alderson et al., 1990; Rylett and Williams, 1994) which has led to the suggestion that these factors are important in the etiology of AD. The rest of this introduction will focus on BDNF, the subject of this thesis.

1.2.1.1 Brain-derived neurotrophic factor

1.2.1.1.1. Regional distribution of BDNF within the central nervous system (CNS)

BDNF is a basic protein consisting of two non-covalently linked 13-14 kDa subunits (Rosenfeld et al., 1995). It shares an approximately 50% structural homology with NGF (Maisonpierre et al., 1990) and is highly conserved across species (Phillips et al., 1990). Widespread distribution of BDNF mRNA and protein have been detected in the brain (Phillips et al., 1990; Wetmore et al., 1990; 1991; Gall et al., 1992; Maness et al., 1994; Dugich-Djordjevic et al., 1995; Nawa et al., 1995). Highest levels of BDNF mRNA

have been reported in the hippocampus while lower levels have been detected in the amygdala, piriform, cingulate and entorhinal cortices and neocortex (Ernfors et al., 1990; Hofer et al., 1990; Phillips et al., 1990; Wetmore et al., 1990). BDNF protein largely correlates with the distribution of its mRNA (Altar et al., 1997; Connor et al., 1997; Yan et al., 1997) although some exceptions have been reported (Nawa et al., 1995). Furthermore, mRNA for the high affinity receptors for BDNF, trkB, are expressed by a variety of cells in the CNS and are densely distributed in the brain (Klein et al., 1990; Merlio et al., 1992). The trkB gene encodes two cell surface glycoproteins, gp^{95trkB} and $gp^{145trkB}$ (Klein et al., 1989; Middlemass et al., 1991). The gp^{95trkB} cell surface receptor does not contain the kinase catalytic domain and therefore cannot transduce a signal once activated (Middlemass et al., 1991).

Known for their growth promoting and survival functions, neurotrophins have been classically defined as trophic factors that act by retrograde axonal transport. Synthesized by target areas of the basal forebrain (hippocampus and neocortex) the neurotrophins support the survival of cholinergic cell bodies within the basal forebrain. The basal forebrain cholinergic neurons express receptors for the neurotrophins in high concentrations (Salehi et al., 1996). The receptors synthesized in the cholinergic neuron cell bodies are transported to the axon terminals where they bind BDNF and other neurotrophins and then transport these factors back to the cell body. This has been demonstrated by classical experiments in which transection of the fimbria/fornix, an anatomical structure through which axons of cholinergic neurons project to the

hippocampus, results in decreased expression of cholinergic indices, such as ChAT expression, ACh release and high-affinity choline uptake, and eventually atrophy and cell death (Gage et al., 1986; Hefti, 1986; Williams et al., 1986; Kromer, 1987). Intraventricular administration of NGF and BDNF reduces the axotomy induced cell loss (Hefti, 1986; Kromer, 1987; Murray et al., 1994).

1.2.1.1.2. Actions of BDNF within the CNS

Aside from its conventional target-derived actions in the brain BDNF has been shown to exert its trophic effects via autocrine/paracrine mechanisms (Acheson et al., 1995; 1996) and more recently, via anterograde transport mechanisms as well (Altar et al., 1997; Smith et al., 1997; Kokaia et al., 1998). Cytoplasmic and nuclear BDNF staining within neurons has been observed, suggesting both autocrine activity and retrograde transport (Wetmore et al., 1991). Autocrine loops have been suggested to occur in both the substantia nigra and hippocampus where neurons in these areas have been shown to be responsive to BDNF and where high levels of BDNF mRNA have been localized (Kokaia et al., 1993; Miranda et al., 1993). Cellular localization of BDNF protein within preterminal fiber systems and in areas devoid of BDNF mRNA (eg. striatum) strongly support anterograde axonal transport (Altar et al., 1997; Kokaia et al., 1998).

1.2.1.1.3. Regulation of BDNF within the hippocampus

Neuronal activity within the hippocampus has been shown to regulate both BDNF and NGF (Gall, 1992; Thoenen et al., 1991). The hippocampus receives glutamatergic

innervation from the entorhinal cortex via the angular bundle (Swamson et al., 1987) that terminates in the dentate gyrus. Cell bodies in the dentate gyrus synapse on cells in field CA3 of the hippocampus (that also include fields CA1 and CA2) which in turn synapse on cells in the entorhinal cortex via field CA1 completing a trisynaptic circuit. Activation of the entorhinal cortex induces rapid immediate early gene expression within the hippocampal formation (see Morgan and Curran, 1991). Transynaptic stimulation of hippocampal afferents was observed to increase the level of BDNF mRNA within the rat hippocampus (Falkenberg et al., 1993). Interestingly, stimulation of the entorhinal cortex resulted in the activation of different BDNF promoters within the hippocampus in a differential and dose-dependent manner (Falkenberg et al., 1993). The investigators propose that the upregulation of BDNF mRNA may have been due to specific changes in BDNF-mediated neuronal plasticity in selective regions of the hippocampus that were influenced by glutamatergic afferents from the entorhinal cortex. It is also possible that the dose dependent activation of the different promoters of BDNF was partially a result of recurrent activation of the trisynaptic pathway.

Further evidence of BDNF regulation within the hippocampus was observed following the administration of quisqualate into the entorhinal cortex (Lindefors et al., 1992). This treatment resulted in a 15-, 5- and 17-fold increase in the expression of BDNF mRNA in the ipsilateral granule cells, CA3 and CA1 pyramidal cells, respectively. Similar increases were also observed in the hippocampus contralateral to the injection.

1.2.1.1.4. Regulation of BDNF in the cortex

As with the regulation of BDNF mRNA and protein levels in the hippocampus, most of the data regarding BDNF regulation in the cortex arise from studies in rats and mice following the induction of seizures. In situ hybridization analysis and immunofluorescence labeling in normal rats revealed irregular labeling of neurons in the visual cortical layers II-VI (Schmidt-Kastner et al., 1996). Prominent staining of layers II and VI were observed in the parietal cortex with the middle layers displaying lower staining intensities. However, seizures produced a dramatic increase in the levels of BDNF mRNA throughout the cortical areas including the piriform and entorhinal cortical regions. Strong labeling of neurons in layers II-VI was observed throughout the cortex (Schmidt-Kastner et al., 1996). In adult rat and pig brain, Wetmore and colleagues (1990) reported strong labeling for BDNF mRNA over a subset of pyramidal cells in both the inner and outer pyramidal layers of the cortex.

1.2.1.1.5. Cholinergic regulation of BDNF mRNA

The cholinergic neurons of the basal forebrain project to the hippocampus via the fimbria/fornix pathway. The cholinergic system consists mainly of three groups of cell bodies referred to as the medial septum (MS), the diagonal band of Broca (DB) and the nucleus basalis of Meynert (NBM). The monkey hippocampus has been shown to be primarily innervated by the medial septum and the diagonal band of Broca (Amaral and Cowan, 1980), whereas in the monkey and rat, nucleus basalis of Meynert projects onto

the frontal and parietal cortex (McKinney et al., 1983). Partial transection of the fimbria/fornix in the rat resulted in a reduction of BDNF and NGF mRNA levels in the hippocampus, suggesting that cholinergic input is involved in the regulation of hippocampal BDNF and NGF mRNA (da Penha Berzaghi et al., 1993). Furthermore, administration of pilocarpine, a muscarinic receptor agonist, markedly increased NGF and BDNF mRNA levels in the hippocampus (da Penha Berzaghi et al., 1993). This increase was abolished by the muscarinic antagonist scopolamine. A transient increase in BDNF and NGF mRNA expression in the hippocampus was also observed following quisqualate administration into the medial septal nucleus (Lindefors et al., 1992).

1.2.1.1.6. Trophic action of BDNF within the CNS

While both BDNF and NGF promote the survival and maintenance of neurons in the dorsal root ganglia (Lindsay and Peters, 1984) and basal forebrain (Alderson et al., 1990, 1993; Knusel et al., 1992), BDNF also acts on many other neuronal populations not influenced by NGF. In vitro and in vivo studies have demonstrated that BDNF acts as a survival factor for placode-derived sensory neurons (Lindsay and Rohrer, 1985), motoneurons (Henderson et al., 1993), noradrenergic neurons (Friedman et al., 1993), dopaminergic neurons (Hyman et al., 1991) and retinal ganglion cells (Johnson et al., 1986) among others. This diverse action of BDNF is consistent with its widespread distribution mentioned above. During development, exogenous BDNF has been observed to prevent naturally occurring cell death in vivo (Hofer and Barde, 1988; Hyman et al.,

1991; Oppenheim et al., 1992). Similar exogenous treatment of BDNF increases excitatory post-synaptic currents in visual cortical slices and induces immediate gene expression in hippocampal cultures as well (Ip et al., 1993). In addition, pretreatment of rat cortical cultures with recombinant BDNF has been found to significantly reduce glutamate-induced neurotoxicity (Shimohama et al., 1993).

BDNF has been shown to promote the survival of E17 rat embryo septal cholinergic neurons in culture and increase the number of NGF receptor-bearing cholinergic neurons within the central nervous system (Alderson et al., 1990). Intraventricular administration of recombinant human BDNF (rhBDNF) to adult rats with transections of the fimbria was shown significantly reduce axotomy-induced degenerative changes of the cholinergic cells in the basal forebrain (Knusel et al, 1992). Taken together these studies demonstrate that BDNF may be capable of providing trophic support for cholinergic neurons of the basal forebrain.

1.2.1.2 BDNF and Alzheimer's Disease

In AD, an area that is most severely affected is the basal forebrain neuronal system. Interestingly, the projection areas of the basal forebrain also sustain severe neuronal degeneration. Among these areas are the hippocampus and neocortical association areas. The observation that neurotrophins are capable of supporting these cells led investigators to examine the levels of these factors and their respective receptors in the AD brain.

Using in situ hybridization techniques, BDNF mRNA expression levels have been reported to be decreased in the hippocampus of individuals with AD compared to normal subjects with no history of neurological disease (Phillips et al., 1991; Murray et al., 1994). Further confirmation of these observations was provided by RNase protection assays (Phillips et al., 1991). However, using the reverse transcription-polymerase chain reaction (RT-PCR) method of mRNA detection, Hock and colleagues (1998) failed to observe changes in BDNF mRNA levels in the parietal cortex of subjects with AD, although they did observe a decrease in the mRNA levels of the high affinity NGF receptor, trkA. Utilizing a sensitive enzyme-linked immunosorbant assay (ELISA) to detect protein, Narisawa-Saito and colleagues (1996) reported a significant decrease in the level of BDNF protein in the entorhinal cortex of subjects with AD when compared to controls. However, they failed to observe any changes in the levels of BDNF protein within the dentate gyrus of the AD hippocampus, a direct contradiction to what was expected based on the observations of BDNF mRNA levels (Phillips et al., 1991; Murray et al., 1994). However, in agreement with the results of the mRNA study, immunohistochemical analysis of AD subjects revealed a significant reduction in the level of BDNF-immunoreactivity within the hippocampus and temporal cortex comapred to controls (Connor et al., 1997).

Studies on the high-affinity receptor for BDNF, trkB, have demonstrated decreased levels of both its mRNA and protein in the AD brain when compared to controls (Connor et al., 1996; Salehi et al., 1996; Boissiere et al., 1997). Significant

decreases were observed in the NBM, an area that projects almost exclusively to the cortex. Expression levels of trkA and trkC, the high-affinity receptors for NGF and NT-3 respectively, were also significantly decreased in AD NBM compared to controls (Salehi et al., 1996). Decreased levels of trkB coupled with decreases in both BDNF mRNA and protein in the hippocampus, temporal and entorhinal cortices (Phillips et al., 1991; Murray et al., 1994; Narisawa-Saito et al., 1996; Connor et al., 1997) indicate one possible mechanism by which structures such as the basal forebrain and hippocampus may be compromised in AD. Furthermore, as mentioned, BDNF mRNA expression levels are regulated by activity-dependent mechanisms and therefore neuronal loss and/or dysfunction may adversely affect the basal forebrain cholinergic system. Since neurons from the entorhinal and temporal cortices and the basal forebrain (MS and DB) among other areas, project to the hippocampus, it is feasible that degeneration of neurons within these regions, commonly observed at autopsy, may severely diminish hippocampal function. Also, since cholinergic neurons of the NBM project to cortical structures, decreases in neurotrophin levels in the cortex may adversely affect the nucleus basalis, since such decreases would result in diminished trophic support.

HYPOTHESIS AND OBJECTIVES

Neurons in the basal forebrain cholinergic system are severely affected in AD. Cell culture and lesion studies have demonstrated that neurotrophins are capable of promoting the growth, survival and maintenance of cholinergic neurons. Brain-derived neurotrophic factor is widely expressed in the central nervous system and high-affinity receptors for BDNF have been localized in the basal forebrain cholinergic system. Decreases in BDNF mRNA and protein have been reported in the hippocampus of individuals with AD compared to controls, although one study examining protein levels has failed to observe changes in the dentate gyrus of AD subjects, an area where significant decreases in BDNF mRNA is observed. However, no decreases in BDNF mRNA have been observed in the parietal cortex, although decreased levels of BDNF protein have been reported in the entorhinal and temporal cortices.

We hypothesize that decreases in both mRNA and protein of brain-derived neurotrophic factor in subjects with Alzheimer's disease are more widespread than previously detected. Based on this hypothesis, experiments were designed to detect both BDNF mRNA and protein in the parietal cortex and BDNF mRNA in the hippocampus and nucleus basalis of Meynert. The data obtained was compared to that from neurologically unimpaired age- and gender-matched subjects.

2.0 MATERIALS AND METHODS

2.1 Chemicals and Apparatus

Protein analysis

The pre-stained broad-range molecular weight markers (6.5 - 175 kDa) were obtained from New England Biolabs Inc., Mississauga, Ontario. The 4-20% Tris-HCl linear gradient Ready gels, the DC protein assay kit, bromophenol blue, the Ready Gel Electrophoresis Cell, Mini Trans-Blot Electrophoretic Transfer Cell and the PowerPac 200 power supply systems were purchased from Bio-Rad Laboratories, Mississauga, Ontario. Polyvinylidene difluoride (PVDF) membranes and the enhanced chemiluminescence (ECL™) Western blotting detection system were purchased from Amersham Pharmacia Biotech, Baie d'Urfé, Quebec. Carnation® instant skim milk powder was from Nestlé, Don Mills, Ontario. Goat serum and phenylmethylsulfonyl fluoride (PMSF) were obtained from Gibco BRL, Burlington, Ontario. Aprotinin, bovine serum albumin (BSA) and polyoxyethylene-sorbitan monolaurate (Tween 20) were purchased from Sigma-Aldrich Canada Ltd., Oakville, Ontario. Kodak X-AR Scientific Imaging Film was purchased from Eastman Kodak Company, Rochester, New York, USA. Tris (hydroxymethyl) aminomethane and pepstatin were from Boehringer Mannheim, Laval, Quebec. Sodium chloride was from ACP, Montreal, Quebec. Ethylenediaminetetraacetic acid (EDTA), glycerol, glycine, 2-mercaptoethanol, and sodium lauryl sulphate (SDS) were purchased from BDH Inc., Toronto, Ontario. The Eppendorf microcentrifuge (Model 5415C) was purchased from Brinkmann Instruments, Mississauga, Ontario.

mRNA analysis:

TRIzol® was obtained from Gibco BRL, Burlington, Ontario. The Gene Amp RNA PCR core kit, which included dNTPs, random hexamers, oligo $d(T)_{16}$, RNase inhibitor, MuLV reverse transcriptase, 10X PCR buffer II, magnesium chloride, and AmpliTaq® DNA polymerase and AmpliTaq Gold® DNA polymerase and the GeneAmp PCR system 2400 thermal cycler were purchased from Perkin Elmer, New Jersey, USA. RQ1 RNase-free DNase was from Promega Biotech, Madison, WI, USA. The GeneRuler™ 100 bp DNA ladder was obtained from MBI Fermentas, Flamborough, Ontario. The Polaroid Type 57 instant positive/negative film was purchased from Polaroid Corporation, Cambridge, MA, USA. A model PT 10/35 homogenizer with a PTA 10S generator was purchased from Brinkmann Instruments, Mississauga, Ontario. A Beckman DU-64 spectrophotometer was from Beckman Instruments, Mississauga, Ontario.

2.2 Other Materials

2.2.1 Primers

The primers used in the analysis of BDNF and β-actin mRNA were synthesized by the Central Facility of the Institute for Molecular Biology and Biotechnology (MOBIX) at McMaster University, Hamilton, Ontario.

2.2.2 Antibodies

Antibodies against mature BDNF were obtained from three sources. An affinity-purified rabbit polyclonal antibody raised against a peptide sequence,

HSDPARRGELSVCDSISEWV (amino acids 128-147) mapping at the amino terminus of the mature form of BDNF of human origin (Figure 1) was obtained from Santa Cruz Biotechnology (Cat #sc-546), Santa Cruz, CA, USA. A second anti-BDNF antibody was provided as a generous gift by Dr. David Kaplan (Montreal Neurological Institute, Montreal, Québec). The antibody was raised against amino acids 168-177 of human BDNF corresponding to the sequence EKVPCSKGQL (see Figure 1). The third anti-BDNF antibody was also a generous gift from Dr. Josette Carnahan (Amgen Inc., Thousand Oaks, CA, USA) and is a polyclonal antibody raised in turkey against the entire mature BDNF molecule. A monoclonal anti-β-actin (mouse IgG1 isotype) raised against a slightly modified synthetic β-cytoplasmic actin N-terminal peptide Ac-DDDIAALVIDNGSGK conjugated to KLH was used as the immunogen to prepare an antibody specific to the β-isoform of actin (Sigma-Aldrich Canada Ltd., Oakville, Ontario).

The secondary antibodies used in these experiments were a peroxidase-linked anti-rabbit antibody raised in donkey, a peroxidase-linked anti-mouse also raised in donkey (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec), a peroxidase-linked anti-chicken/turkey IgG (H+L) raised in rabbit (Zymed Laboratories Inc., San Francisco, CA, USA), and a peroxidase conjugated IgG fraction of anti-turkey [H+L] produced in rabbit from Cederlane Laboratories Ltd. (Hornby, Ontario).

Signal peptide

Italics – Santa Cruz anti-BDNF antibody

Bold – Kaplan anti-BDNF antibody

Figure 1: Amino acid sequence of BDNF. Peptide sequences against which the Kaplan and Santa Cruz anti-BDNF antibodies were raised are indicated.

2.3 Tissue samples

2.3.1 Source of human tissue samples

Human postmortem brain tissue samples were provided by the Institute for Brain Aging and Dementia Tissue Repository at the University of California, Irvine, CA, USA. Samples from the parietal cortex, nucleus basalis of Meynert and hippocampus were obtained from subjects with Alzheimer's disease (n = 7 for each area) and control, neurologically unimpaired subjects (n = 7 for each area) stored at -80°C until use. A diagnosis of Alzheimer's disease was confirmed by pathological and clinical criteria (McKhann et al., 1984). Control and AD samples were matched for gender and age.

2.3.2 Age and postmortem delay

Mean ages of subjects in the parietal cortex group that were studied for BDNF mRNA content were 75.86 ± 3.48 (mean ± SEM) years for control subjects and 76.42 ± 3.48 years for the AD group (Table 2). Control subjects in the nucleus basalis group had a mean age of 76.43 ± 2.48 years while those in the AD group had a mean age of 78.14 ± 2.40 years (Table 3). The mean age of control subjects in the hippocampal group studied for BDNF mRNA content was 80.14 ± 3.27 and those in the AD group had a mean age of 81.86 ± 2.74 (Table 4).

The mean age of subjects in the control group for BDNF protein analysis was 75.00 ± 4.11 years and that of the AD group was 75.67 ± 4.01 years. The postmortem

delay time for this set of subjects was 5.90 ± 0.58 and 2.92 ± 0.29 hours for the control and AD groups respectively (Table 1).

Postmortem delay times were 5.92 ± 0.47 (control) and 2.68 ± 0.33 (AD) hours for the parietal cortex group, 6.00 ± 0.46 and 2.54 ± 0.24 hours for control and AD subjects respectively in the nucleus basalis group, and 6.29 ± 0.55 hours for the control and 3.26 ± 0.48 hours for the AD subjects in the hippocampal group (Tables 2, 3 and 4).

2.4 Western Blotting

Western blotting was performed on parietal cortex tissue samples (n = 6 for AD and n = 6 for controls) provided by the Institute for Brain Aging and Dementia Tissue Repository at the University of California, Irvine.

2.4.1 Tissue homogenate preparation

Tissue samples were prepared for Western blotting by homogenizing approximately 0.5 g of tissue in 2.5 ml of homogenization buffer (0.5mM Tris-HCl pH 7.5, 10mM EDTA, 0.5% Tween-20, 2μg/ml aprotinin, 2μg/ml pepstatin, and 100μg/ml PMSF) on ice using a homogenizer model PT 10/35 with a PTA 10S generator. The homogenates were centrifuged at 14,000 X g for 15 minutes in an Eppendorf microcentrifuge (Model 5415C) at 4°C. Protein concentrations were determined using the detergent compatible (DC) protein assay as described by the manufacturer (BioRad Laboratories, Mississauga, Ontario).

2.4.2 Electrophoresis of brain tissue homogenates

Homogenates were prepared for electrophoresis by adding 1/3 volume of 4X Laemmli sample buffer (50mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100mM dithiothreotol, 0.1% bromophenol blue and 5% mercaptoethanol) to each sample and then boiling the samples for 10 minutes. Samples (40 μg) were separated on 4-20% (w/v) Tris-HCl linear gradient resolving gels (Ready Gels) using the Mini-Protean II Ready Gel electrophoresis system and a 1X gel running buffer (3g Tris, 14g glycine, 10 ml 10% SDS). A broad-range prestained molecular weight marker was added to one lane in each gel to gauge the apparent molecular weight of the resolved bands. Each gel consisted of two control and two AD samples, a broad-range molecular weight marker, recombinant human BDNF, and four samples from a control human brain that was not used in the BDNF protein assay. The four samples were run in all assays at concentrations of 20, 40, 60, and 80 μg per lane permitting normalization of the densitometric values across assays. Electrophoresis was performed at 70V for 15 minutes followed by an increase to 120V until the dye ran to approximately 1 cm from the bottom of the gel (a further 60 minutes).

2.4.3 Electrophoretic transfer of proteins

Prior to transfer, the PVDF membrane was incubated in methanol for 10 s and both the gel and the PVDF membrane were placed in transfer buffer at room temperature and permitted to equilabrate for 15 minutes. Proteins were electrophoretically transferred onto PVDF membranes in ice cold transfer buffer (14.42 g glycine, 3.02 g Tris base, 200 ml methanol per litre of water) at 4°C. Transfer proceeded for 2 hrs at 100V.

2.4.4 Blocking of non-specific binding sites

Following the transfer of proteins to PVDF membranes, non-specific binding sites on the membrane were blocked using a variety of blocking solutions. It was finally determined that the most efficient blocking occurred using a 10% goat serum (v/v)/1% BSA (w/v) in TBS (50mM Tris, 119mM NaCl, pH 8.5) solution at room temperature for 1-2 hrs depending on the primary antibody used. Other blocking solutions used included 10% skim milk powder (w/v) in TBS-T (0.2% (v/v) Tween-20) incubated at room temperature, at 4°C, at 37°C and at 60°C, a 2%, and 4% gelatin (w/v) solution in TBS-T and a 3% BSA (w/v) solution in TBS-T.

2.4.5 Primary and secondary antibody immunolabeling

Following blocking, blots were incubated with a i)1:500 or 1:1000 dilution of the Santa Cruz rabbit anti-BDNF antibody in TBS containing blocking solution; ii) 1:500 dilution of the Kaplan anti-BDNF antibody in TBS; or iii) 1:1,000 dilution of a turkey anti-BDNF antibody in TBS. All incubations with the primary antibodies occurred on a rocking platform shaker overnight at 4°C. Different blocking solutions were also attempted with the primary antibody incubation as indicated in the results section of this thesis.

Following incubation with the primary antibodies, the blots were washed twice for 6 minutes each and once for 10 minutes in TBS-T and then incubated in a 1:6,667 (i and ii) dilution of a peroxidase conjugated donkey anti-rabbit secondary antibody in TBS or a 1:80,000 (iii) dilution of a peroxidase conjugated anti-chicken/turkey IgG (H+L) in TBS

on a rocking platform shaker at room temperature for 45 minutes. The blots were washed following secondary antibody incubation as above. The concentrations for the secondary antibodies suggested by the manufacturers yielded very high background and non-specific binding during negative control experiments in which the primary antibody was omitted from the incubation step. Therefore, several of the above-mentioned blocking conditions were used during the secondary antibody incubation. However, as shown in the results section, this procedure did not yield successful results.

2.4.6 Enhanced chemiluminescence

The ECL™ method of antibody detection was used to detect immunoreactive bands on the PVDF membranes. The principle behind the ECL detection system involves the oxidation of luminol, a component of the detection system, by HRP. Immediately following oxidation, the luminol is in an excited state that emits light upon returning to ground state. Enhanced chemiluminescence is achieved by performing the oxidation reaction in the presence of chemical enhancers such as phenols (adapted from manufacturers' protocol/description, Amersham-Pharmacia Biotech). The protocol for detection included mixing sufficient amounts of solutions 1 and 2, supplied by the manufacturer, to cover the protein side of the PVDF membrane and incubating it for 1 minute at room temperature. Excess reagent was drained and the membrane placed between plastic wrap, followed by exposure to Kodak X-AR film for varying time periods.

2.4.7 Densitometry analysis

BDNF immunoreactive bands were analyzed using scanned images of the autoradiographs and the ScionImage software package (Scion Corporation, Frederick, Maryland, USA). Background, as measured from three random areas on the autoradiograph, were averaged and subtracted from each band. The value obtained was then normalized to the density of the β-actin content of each sample prior to comparing the samples between graphs. Density measurements from the different autoradiographs were analyzed by initially standardizing the intensity of the concentration gradients between each autoradiograph and multiplying each band on a given autoradiograph by the derived constant.

2.5 cRT-PCR

2.5.1 RNA isolation

Total cellular RNA was purified from cortical, hippocampal and NBM samples using the TRIzol™ Reagent (GIBCO/BRL) following the manufacturer's protocol. Briefly, 100 mg of frozen tissue was homogenized in 1 mL of TRIzol™ using a homogenizer model PT 10/35 with a PTA 10S generator. The homogenate was subjected to chloroform extraction, isopropanol precipitation, ethanol washes, and resuspension in 30 µl of autoclaved, double-distilled water. Total RNA concentrations and RNA purity were determined by absorbance at 260 and 280 nm using a Beckman DU-64 spectrophotometer. Samples exhibiting an absorbance ratio (260/280) greater than or

equal to 1.7 and exhibiting strong 28S and 18S ribosomal RNA bands on 1% (wt/vol) agarose gels were used for further analysis. RNA samples were stored at -20°C until use.

2.5.2 BDNF deletion standard and amplification primers

The BDNF deletion standard plasmid (generous gift from Hana Friedman, Montreal Neurological Institute, Montreal, Quebec) consists of the rat BDNF sequence (Maisonpierre et al., 1991), containing a deletion of a 120 bp PstI restriction enzyme fragment, inserted into the plasmid pGEM7Z (Promega Biotech, Madison, WI). This insert was transcribed using the T7 promoter and a MEGAscript™ Kit from Ambion (Austin, TX) according to the manufacturer's protocol. The RNA product was quantitated by spectrophotometry and visualized on a 1% (wt/vol) agarose gel.

The primer set used for BDNF transcript analysis amplifies a 278 bp human BDNF fragment and a 158 bp fragment from the BDNF deletion standard. The 5′ primer sequence is 5′- GAG CTG AGC GTG TGT GAC AG - 3′ and the 3′ primer is 5′ - GC AAA AAG AGA ATT GGC TGG CG - 3′ (Figure 2). The primer set used to amplify endogenous β-actin transcripts in the human samples amplifies a 282 bp fragment and consists of the 5′ primer 5′ - TCA TGA AGT GTG ACG TTG ACA TC - 3′ and the 3′ primer 5′ - AGA AGC ATT TGC GGT GGA CGA TG - 3′ (Nakajima-Iijima et al., 1985).

2.5.3 cDNA synthesis

RNA samples were treated with RQ1 RNase-free DNase (0.35 - 0.60 U/10 μg total RNA) at 37°C for 1 hour. For each pair of matched samples, the minimum amount

1	gaattcgggg	ctgccgccgc	cgcgcccggg	cgcacccgcc	cgctcgctgt	cccgcgcacc
61	ccgtagcgcc	tcgggctccc	gggccggaca	gaggagccag	cccggtgcgc	ccctccacct
121	cctgctcggg	gggctttaat	gagacaccca	ccgctgctgt	ggggccggcg	gggagcagca
181	ccgcgacggg	gaccggggct	gggcgctgga	gccagaatcg	gaaccacgat	gtgactccgc
241	cgccggggac	ccgtgaggtt	tgtgtggacc	ccgagttcca	ccaggtgaga	agagtgatga
301	ccatcctttt	ccttactatg	gttatttcat	actttggttg	catgaaggct	gccccc[atga
361	aagaagcaaa	catccgagga	caaggtggct	tggcctaccc	aggtgtgcgg	acccatggga
421	ctctggagag	cgtgaatggg	cccaaggcag	gttcaagagg	cttgacatca	ttggctgaca
481	ctttcgaaca	cgtgatagaa	gagctgttgg	atgaggacca	gaaagttcgg	cccaatgaag
541	aaaacaataa	ggacgcagac	ttgtacacgt	ccagggtgat	gctcagtagt	caagtgcctt
601	tggagcctcc	tcttctcttt	ctgctggagg	aatacaaaaa	ttacctagat	gctgcaaaca
661	tgtccatgag	ggtccggcgc	cactctgacc	ctgcccgccg	agggg**gagctg**	**agcgtgtgtg**
721	**acag**tattag	tgagtgggta	acggcggcag	acaaaaagac	tgcagtggac	atgtcgggcg
781	ggacggtcac	agtccttgaa	aaggtccctg	tatcaaaagg	ccaactgaag	caatacttct
841	acgagaccaa	gtgcaatccc	atgggttaca	caaaagaagg	ctgcaggggc	atagacaaaa
901	ggcattggaa	ctcccagtgc	cgaactaccc	agtcgtacgt	gcgggccctt	accatggata
961	**gcaaaaagag**	**aattggctgg**	**cg**attcataa	ggatagacac	ttcttgtgta	tgtacattga
1021	ccattaaaag	gggaagatag]	tggatttatg	ttgtatagat	tagattatat	tgagacaaaa
1081	attatctatt	tgtatatata	cataacaggg	taaattattc	agttaagaaa	aaaataattt
1141	tatgaactgc	atgtataaat	gaagtttata	cagtacagtg	gttctacaat	ctatttattg
1201	gacatgtcca	tgaccagaag	ggaaacagtc	atttgcgcac	aacttaaaaa	gtctgcatta
1261	cattccttga	taatgttgtg	gtttgttgcc	gttgccaaga	actgaaaaca	taaaaagtta
1321	aaaaaaataa	taaattgcat	gctgcccgaa	ttc		

Figure 2: mRNA sequence of BDNF. The 5′ and 3′ primer sequences are indicated in bold underline. The primer set used for BDNF transcript analysis amplifies a 278 bp human BDNF fragment and a 158 bp fragment from the BDNF deletion standard. The sequence within brackets corresponds to the amino acid sequence in Figure 1.

of RQ1 RNase-free DNase that yielded a clean negative control was used so as to minimize carry-over into the RT and PCR phases of the experiment. The DNase was inactivated by incubating the mixture at 95°C for 10 minutes followed by cooling at 4°C.

A constant amount of human cortical, hippocampal or NBM mRNA and a serial dilution of BDNF deletion standard RNA were reverse transcribed into cDNA in 10 µl reaction mixtures containing 5 mM $MgCl_2$, 1X PCR buffer II (50 mM KCl, 10 mM Tris-HCl pH 8.3), 1 mM dNTPs, 1 U/ml RNase inhibitor, 2.5 µM random hexamers and 2.5 U/ml MuLV reverse transcriptase using the GeneAmp® RNA PCR kit (Perkin Elmer, Norwalk, CT). Each reaction tube contained 1 µg human parietal cortex total RNA and one dilution (750 fg - 75 pg) of BDNF deletion standard RNA. cDNA synthesis was carried out using the GeneAmp PCR system 2400 thermal cycler (Perkin Elmer) at 37°C for 1 hour followed by incubation at 99°C for 5 minutes to terminate the reaction and cooling at 4°C for 5 minutes.

The reverse transcription step for β-actin was identical, with the exception that the BDNF deletion standard RNA was omitted.

2.5.4 PCR amplification

PCR was performed in the GeneAmp PCR system 2400 on the entire 10 µl aliquot of reverse transcription reaction mixture by addition of 40 µl of a PCR reaction mixture containing 2 mM $MgCl_2$, 1X PCR Buffer II, 0.35 µM each of the 3′- and 5′-BDNF primers, and 2.5 U/ml Taq Gold polymerase (Perkin Elmer) in 200 µl MicroAmp tubes. The amplification profile included an initial eleven-minute heat activation step of the Taq

Gold polymerase at 94°C followed by 35 cycles of denaturation at 94°C for 15 seconds, primer annealing at 67°C for 30 seconds, extension at 72°C for 1 minute, and a final extension after the 35 cycles at 72°C for 7 minutes, ending with a 4°C hold cycle.

The PCR reaction mixture for β-actin consisted of identical constituents except for the substitution of 0.35 μM each of the β-actin primers. The amplification profile for β-actin included an initial eleven-minute heat activation step at 94°C followed by 22 cycles of denaturation at 94°C for 30 seconds, primer annealing at 64°C for 45 seconds, extension at 72°C for 1 minute, and a final extension after the 22 cycles at 72°C for 7 minutes terminating with a 4°C hold cycle. In order to determine the optimal number of cycles for noncompetitive β-actin PCR, cDNA was amplified for 15 to 35 cycles in five-cycle increments. Relative comparisons of β-actin mRNA in human samples were performed non-competitively, which required that β-actin quantitation be carried out in the logarithmic phase of amplification. Thus, 22 cycles were chosen for further experiments (Figure 3).

2.5.5 Quantitative analysis

Ten μl of each RT-PCR reaction mixture was electrophoresed in a 1.8% (wt/vol) agarose gel containing 20 μg of ethidium bromide in Tris-borate/EDTA buffer (2.7g Tris, 1.37 g boric acid, 10 mL 0.5M EDTA in 1 L distilled water) (0.5X TBE). A 100 bp ladder (MBI Fermentas) was used as a molecular weight marker. The gels were visualized on a UV trans-illuminator and photographed using Polaroid Type 57 instant positive/negative film. Image analysis was carried out on the photographic negatives

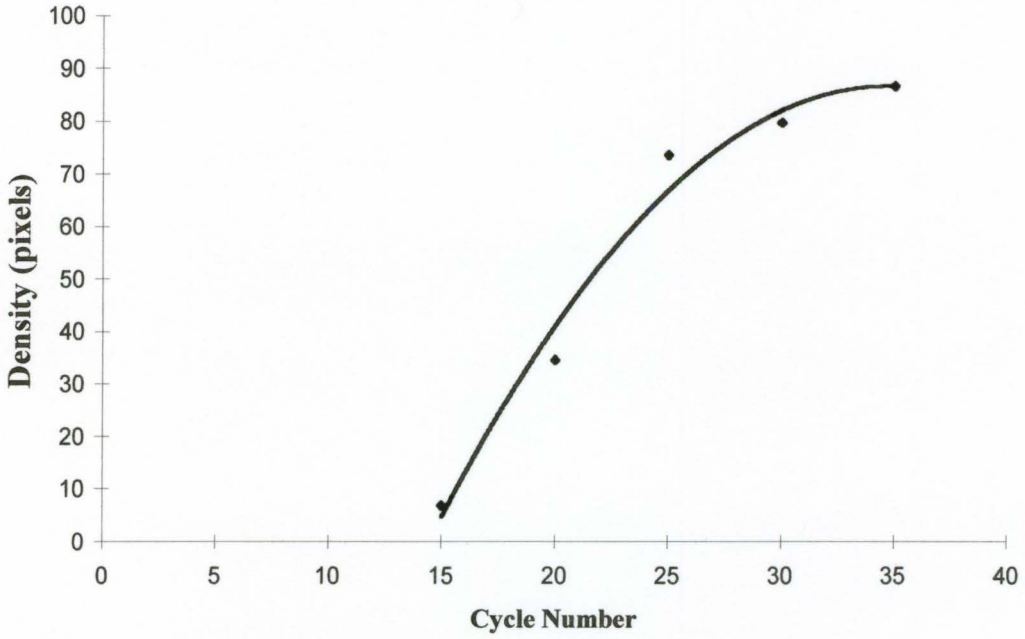

Figure 3: Cycle number optimization for β-actin. To determine the optimal cycle number for β-actin gene amplification, cDNA was amplified for 15 to 35 cycles in five-cycle increments. Twenty-two cycles were chosen for β-actin amplification, as this point lies within the logarithmic phase of amplification.

obtained from each agarose gel using the MCID image analysis system (Brock University, St. Catherines, ON, Canada). Background, obtained by measuring the density of an area representative of the BDNF target in the lane containing no reverse transcriptase, was subtracted from both the BDNF deletion standard and BDNF target pixel density. Since longer DNA sequences incorporate more ethidium bromide stain than shorter sequences, the pixel density of the 158-bp ethidium bromide-stained band was normalized to the 278-bp band by multiplying it by 1.76 (278/158). The log of the ratio of the normalized product band densities was plotted against the log of the concentration of the deletion standard RNA using Excel (Microsoft Corporation, WA, USA), and the point at which the intensities of the two bands were equal (ratio = 1; log 1 = 0) defined the initial amount of BDNF mRNA present in the tissue sample (Figure 4).

2.6 Statistical analysis

Statistical comparisons between control and AD subjects were based on regression analysis and paired-sample t-tests with data shown as mean ± Standard Error of the Mean (SEM). An $\alpha = 0.05$ level of statistical significance was used in all tests. Statistics were calculated using the SPSS for Windows statistical analysis software package (SPSS Inc., Chicago, U.S.A.)

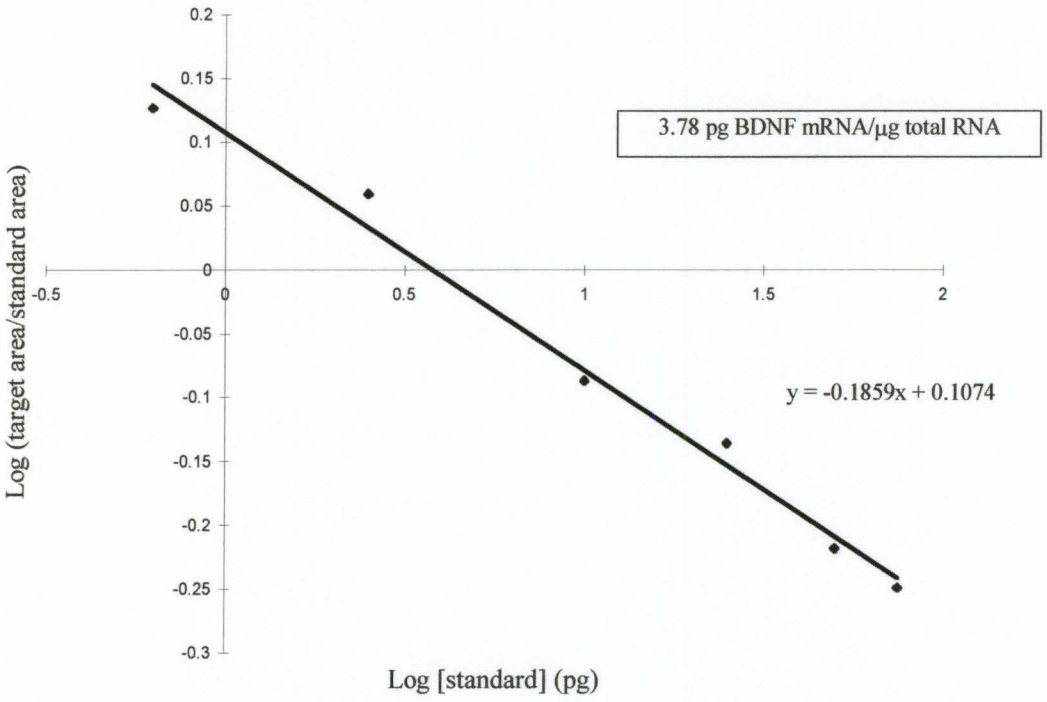

Figure 4: BDNF quantification of a representative AD sample. The log of the ratio of the product bands (density of unkown sample product to deletion standard) was plotted against the log of the concentration of the deletion standard. The point at which the intensities of the two bands were equal (ratio = 1; log 1 = 0) defines the initial amount of BDNF mRNA present in the sample.

3.0 RESULTS

3.1 Samples

3.1.1 Parietal Cortex

Six control and six AD parietal cortex samples were analyzed for their BDNF protein content (Table 1). The average age ± SEM of this group of subjects was 75.00 ± 4.11 years (control) and 75.67 ± 4.01 years (AD). The average postmortem delay times (PMI) were 5.90 ± 0.58 hours for the control and 2.92 ± 0.29 hours for the AD group. Protein yield for control and AD groups did not differ statistically from each other (98.12 ± 2.44 μg/g tissue for control and 95.40 ± 3.02 μg/g tissue for AD, $p = 0.52$). Although the postmortem delay (PMI) times differed significantly ($p < 0.004$) regression analysis revealed no statistically significant differences between total protein yield and PMI ($r = 0.59$ for control and $r = -0.16$ for AD). Additionally, regression analysis also revealed no statistically significant differences between age and protein yield in either subject group ($r = -0.24$ for control and $r = -0.02$ for AD).

Seven control and seven Alzheimer's disease postmortem parietal cortex samples, matched for age and gender (Table 2), were also analyzed for their BDNF mRNA content. The average age ± SEM of the subjects in the control group was 75.86 ± 3.58 years and that of the Alzheimer's disease group was 76.42 ± 3.48 years. No statistically significant differences in age were observed between the two groups ($p= 0.10$). Furthermore, no significant differences were observed in the yield of total RNA extracted from either group

(462.7 ± 20.2 μg/g tissue for control and 420.0 ± 30.1 μg/g tissue for AD samples; p=0.29), or in the integrity of the purified RNA. The average postmortem delay was 5.92 ± 0.47 hours for the control group but only 2.68 ± 0.33 hours for the AD subjects. The difference in postmortem delay between the control and AD groups was statistically significant ($p < 0.002$). However, regression analysis revealed no significant correlation between age and yield of total RNA ($r = 0.22$ for control and $r = 0.70$ for AD; $p = 0.08$ for AD) or postmortem delay and yield of total RNA ($r = 0.08$ for control and $r = 0.37$ for AD). Regression analysis also revealed no statistically significant correlation between BDNF mRNA content and age ($r = -0.39$ for control and $r = -0.10$ for AD) or PMI ($r = -0.29$ for control and $r = -0.37$ for AD).

3.1.2 Nucleus Basalis of Meynert

Seven control and seven Alzheimer's disease postmortem NBM samples, matched for age and gender (Table 3), were also analyzed for their BDNF mRNA content. The average age ± SEM of the subjects in the control group was 76.43 ± 2.48 years and that of the Alzheimer's disease group was 76.71 ± 2.45 years yielding no statistically significant differences between the two groups (p= 0.31). No significant differences were observed in the yield of total RNA extracted from either group (480.81 ± 37.06 μg/g tissue for control and 483.35 ± 40.40 μg/g tissue for AD samples; p=0.96), or in the integrity of the purified RNA. Once again regression analysis revealed no significant correlation between age and yield of total RNA ($r = -0.16$ for control and $r = 0.14$ for AD) or PMI and yield of total RNA ($r = -0.19$ for control and $r = -0.03$ for AD). The average PMI was 6.00 ±

0.46 hours for the control group and 2.54 ± 0.24 hours for the AD subjects resulting in a statistically significant difference between the groups ($p < 0.0006$). Although regression analysis revealed no statistically significant correlation between BDNF mRNA content and PMI ($r = -0.18$ for control and $r = -0.11$ for AD), a significant correlation was observed between BDNF mRNA content and age for both groups of subjects ($r = 0.62$ for control ($p < 0.03$) and $r = 0.64$ for AD ($p < 0.02$)) (Figure 5 and 6).

3.1.3 Hippocampus

As in the previous two regions of the brain, seven control and seven AD postmortem hippocampal samples, matched for age and gender (Table 4), were analyzed for their BDNF mRNA content. The average age ± SEM of the subjects was 80.14 ± 3.27 years and 80.43 ± 3.08 years for the control and AD groups respectively. Once again no statistically significant differences were observed between the control and AD subjects ($p= 0.32$). Although the average age of subjects was higher in both groups compared to those in the parietal cortex and nucleus basalis groups, no significant correlation between BDNF mRNA content and age ($r = 0.03$ for control and $r = 0.06$ for AD) or PMI ($r = -0.18$ for control and $r = -0.16$ for AD) was observed. Although once again a statistically significant difference was observed between the PMI period for the control and AD groups (6.29 ± 0.55 hours and 3.26 ± 0.48 hours respectively; $p < 0.001$) no significant differences were observed in the yield of total RNA extracted (518.22 ± 45.32 μg/g tissue for control and 515 ± 28.94 μg/g tissue for AD; $p = 0.96$).

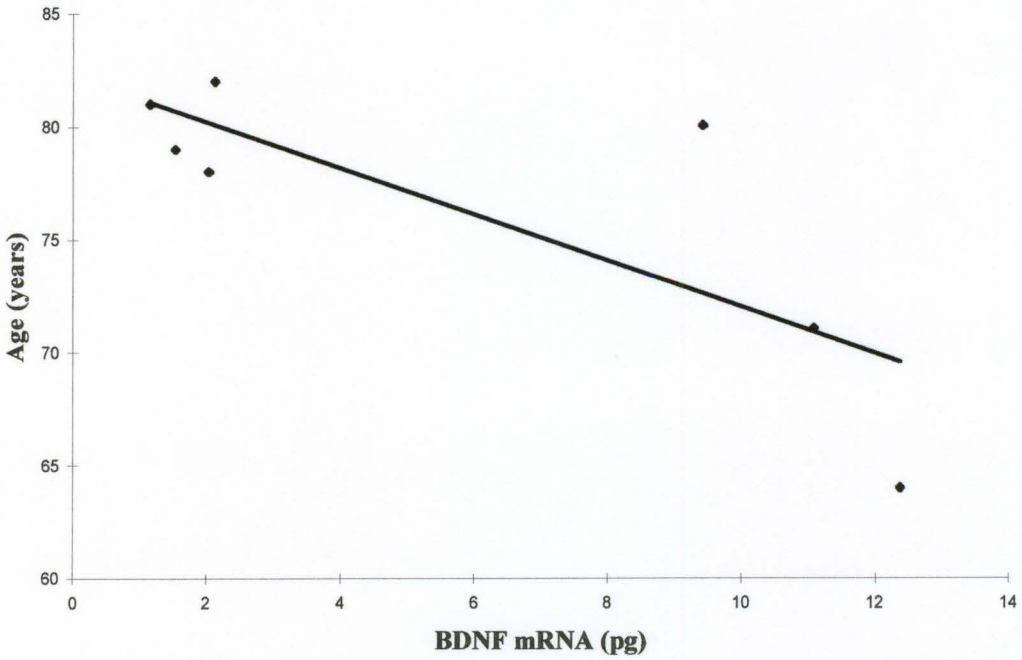

Figure 5: Regression analysis of the correlation between age of subject and BDNF mRNA content in the NBM of control subjects ($r = 0.62$) revealed a significant correlation between these two variables ($p < 0.03$). Each data point represents a different subject in the group.

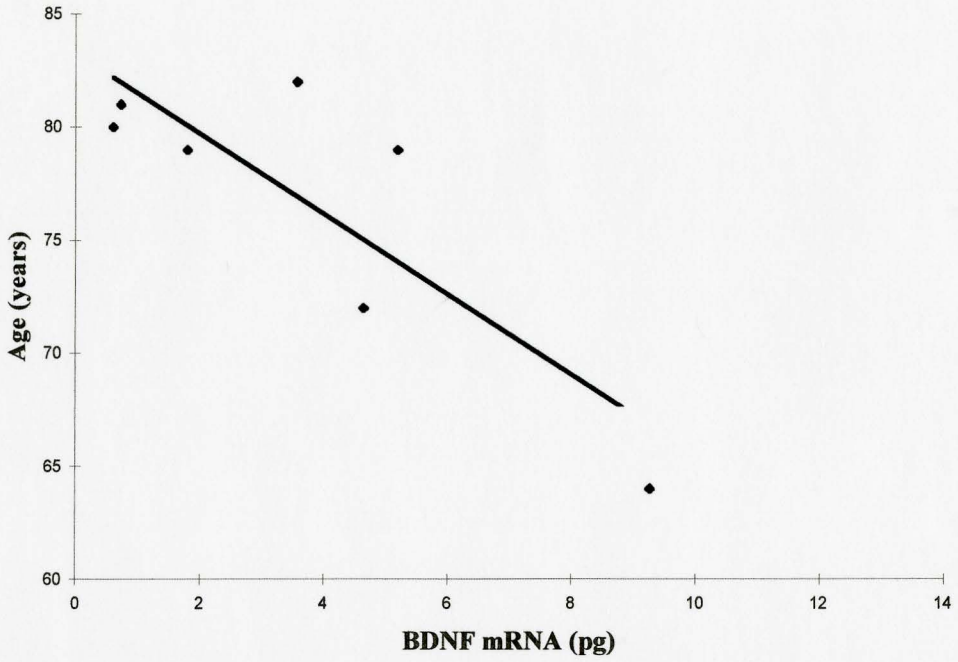

Figure 6: Regression analysis of the correlation between age of subject and BDNF mRNA content in the NBM of AD subjects ($r = 0.64$) revealed a significant correlation between these two variables ($p < 0.02$). Each data point represents a different subject in the group.

3.2 Analysis of RQ1 DNase treatment of samples

A concentration-dependent effect in the use of RQ1 RNase-free DNase was observed during the process of analysis. The manufacturer's protocol recommends the use of 1 unit (U) of RQ1 DNase (1 U/μl) per 10 μg total RNA. The use of the suggested concentration was observed to result in a reduction of BDNF RT-PCR product (Figure 7). Instead, the minimum concentration of RQ1 RNase-free DNase that yielded a clean negative control (a sample containing 1 μg of sample RNA that was not reverse transcribed and should therefore contain no BDNF mRNA) for each pair of matched samples was used so as to minimize carry-over into the RT and PCR phases of the experiments. As such 0.24 to 0.60 U / 10 μg total RNA was used. Using the modified protocol no significant correlation was observed between the amount of RQ1 RNase-free DNase and BDNF mRNA (for control and AD samples respectively; $r = 0.152$ and $r = 0.090$ for parietal cortex, $r = -0.19$ and $r = -0.02$ for nucleus basalis, and $r = -0.20$ and $r = -0.15$ for hippocampus). None of the values differed from control at $p < 0.05$ level.

3.3 Quantitative competitive RT-PCR assay

All cRT-PCR reaction mixtures contained 1μg of total RNA from human postmortem tissue and deletion standard RNA ranging from 0.375 pg to 75 pg per reaction. The range of deletion standard RNA was chosen to encompass the concentrations of endogenous BDNF mRNA in all the tissue samples. The RNA mixtures were reverse transcribed and amplified by PCR. Migration of the cRT-PCR products

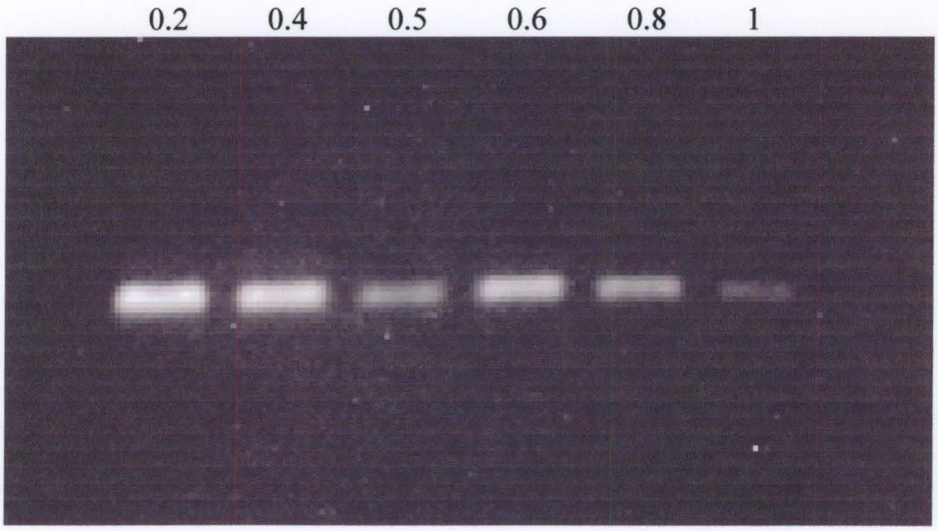

Figure 7: Concentration effect of RQ1 RNase-free DNase. 10 μg of human cortical total RNA was treated with 0.2, 0.4, 0.5, 0.6, 0.8 and 1 unit of RQ1 respectively. The samples were then subjected to non-competitive RT-PCR for 35 cycles. Increasing concentrations of RQ1 RNase-free DNase decreases the amount of BDNF RT-PCR product.

through an ethidium bromide-stained agarose gel demonstrated a 278 bp target RNA band and a 158 bp competitor band (Figure 8).

In order to control for variations in mRNA levels between samples, β-actin gene expression was used as an internal standard for each sample and the value of BDNF mRNA obtained for a given sample was normalized to its respective β-actin mRNA content. The normalized values were used in the calculations below

3.3.1 Parietal Cortex

Analysis of the imaged gels revealed a statistically significant 3.4-fold decrease in BDNF mRNA levels in the parietal cortex of subjects with AD compared to controls ($p < 0.004$). The means (± SEM) of the amounts of BDNF mRNA were 12.11 ± 2.59 pg/μg total RNA and 3.53 ± 0.77 pg/μg total RNA for the control and AD samples, respectively (Figure 9).

3.3.2 Nucleus Basalis of Meynert

Analysis of nucleus basalis of Meynert samples revealed no statistically significant differences between subjects with and without AD ($p = 0.18$). The means (± SEM) of the amounts of BDNF mRNA were 5.67 ± 1.90 pg/μg total RNA for the control group and 3.69 ± 1 15 pg/μg total RNA for subjects with AD (Figure 10). Nevertheless, there appears to be a trend towards decreased levels of BDNF mRNA in AD compared to controls. Furthermore, a significant negative correlation between BDNF mRNA content and age in both groups was also observed.

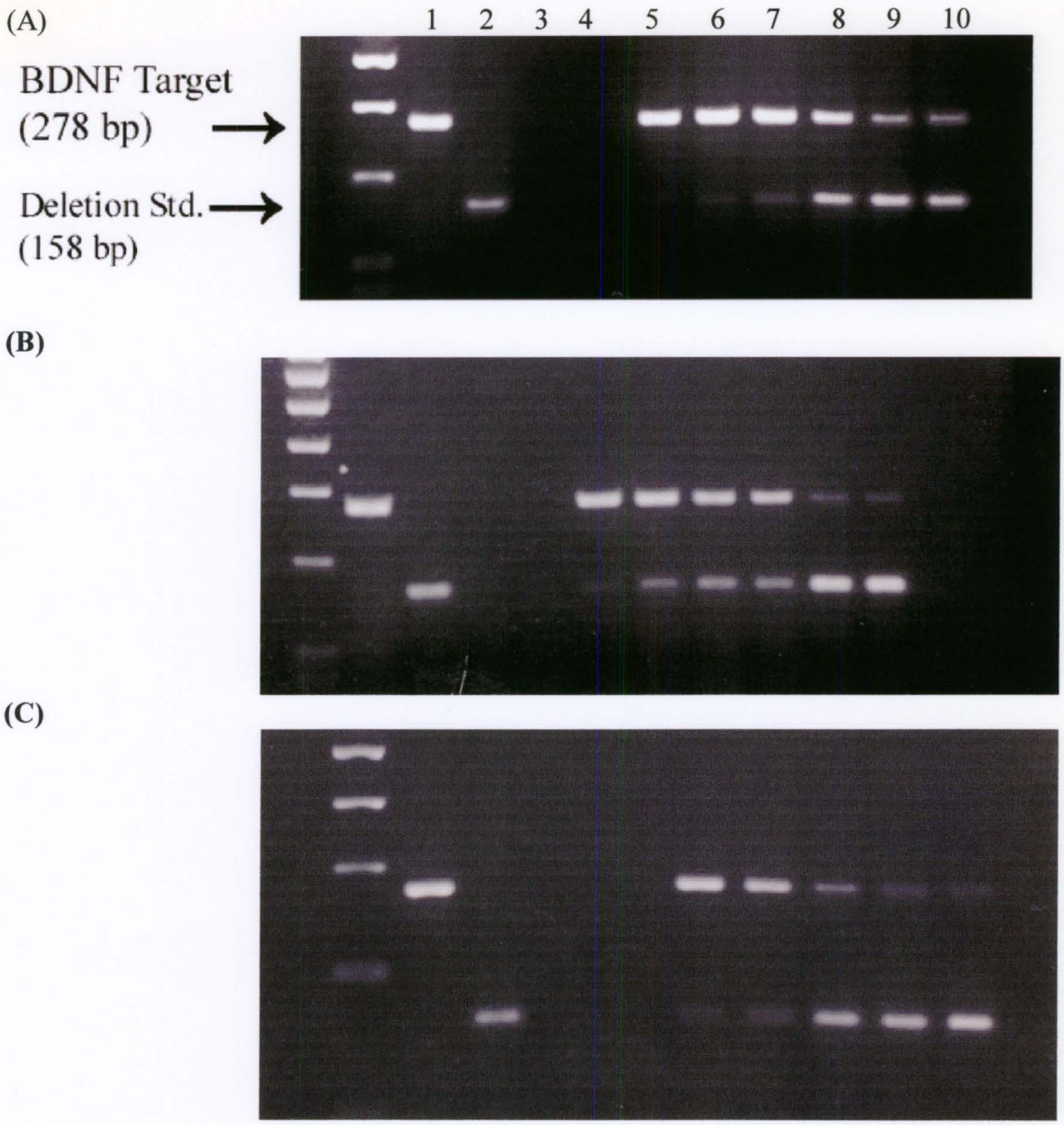

Figure 8: Competitive RT-PCR assay for BDNF in **(A)** parietal cortex, **(B)** hippocampus and **(C)** nucleus basalis of Meynert. Lane 1 contains human cortical or hippocampal mRNA with reverse transcriptase. Lane 2 is the deletion standard with reverse transcriptase and Lane 3 has no nucleic acid added. Lane 4 contains human cortical or hippocampal mRNA without reverse transcriptase. Lanes 5-10 contain BDNF deletion standard (lower band) increasing from 0.375-75pg, with a constant 1μg of human cortical or hippocampal mRNA in each lane. The concentration at the equivalence point, which occurs here between Lanes 8 and 9, (A and B) and Lanes 6 and 7 (C) is determined by regression analysis.

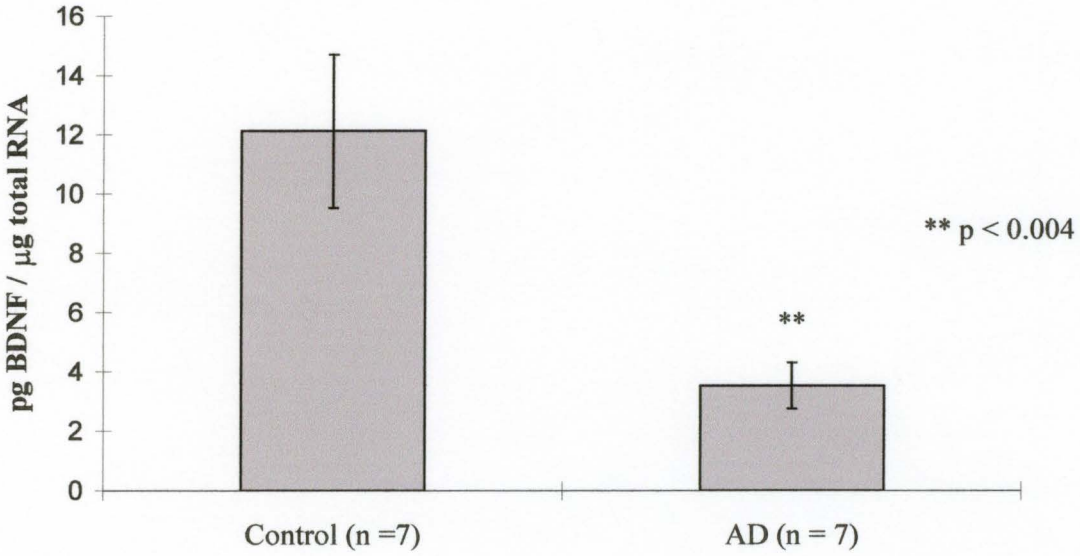

Figure 9: Densitometric analysis of BDNF cRT-PCR products of seven control and seven AD subjects reveals a statistically significant (3.4-fold) decrease in BDNF mRNA levels in the parietal cortex of AD subjects compared to controls ($p < 0.004$)

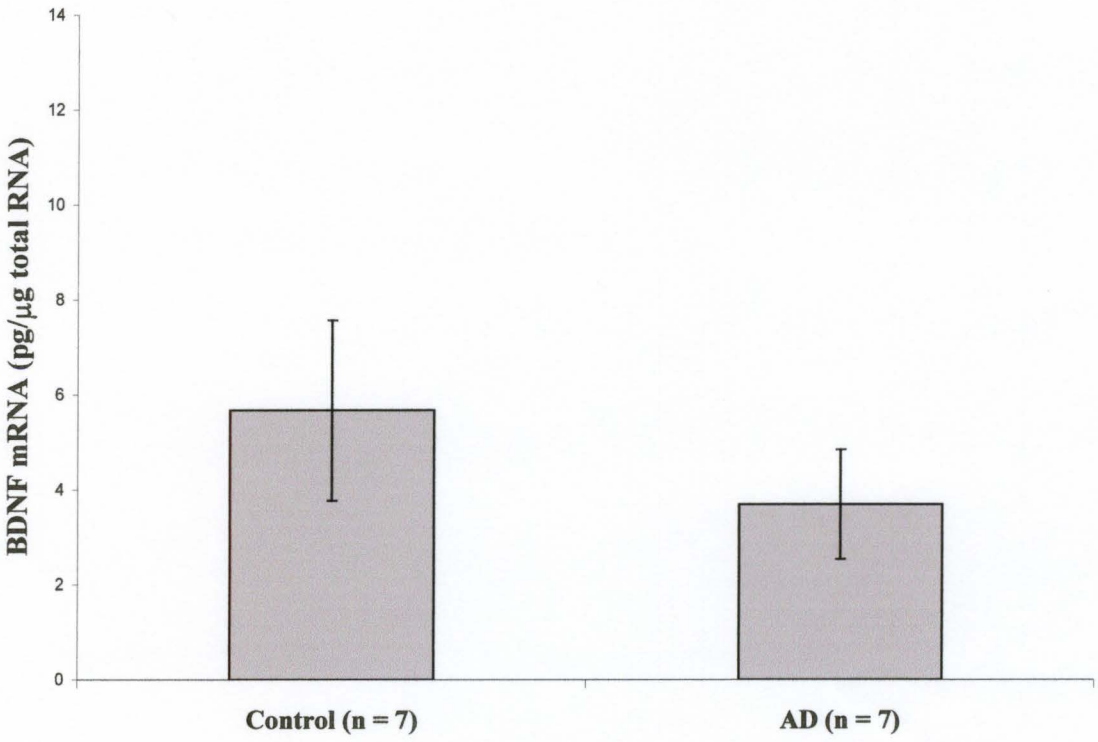

Figure 10: Densitometric analysis of BDNF cRT-PCR products from the nucleus basalis of Meynert (NBM) of seven control and seven AD subjects reveals no statistically significant differences between subjects with and without AD ($p = 0.18$).

3.3.3 Hippocampus

Paired two-sample t-tests for means failed to reveal statistically significant differences in BDNF mRNA content in the hippocampus between control and AD samples ($p = 0.45$). BDNF mRNA content in the control group was 5.25 ± 1.94 pg/μg total RNA and 3.17 ± 1.28 pg/μg in the AD group indicating a trend towards lower levels in the AD group (Figure 11).

3.4 Reproducibility of competitive RT-PCR technique

In order to determine the reproducibility of the competitive RT-PCR procedure used to detect BDNF mRNA in our study, three samples were randomly chosen and the competitive RT-PCR procedure was repeated twice on each. Figure 12 demonstrates the reproducibility for one of the three samples tested. For the first sample, the BDNF mRNA content measured was 1.93 and 1.52 pg (variation of 21.2%) while for the second sample the values were 1.20 and 1.44 pg (variation 16.67%) and 9.53 and 7.89 pg for the third sample (variation 17.2%).

3.5 Detection of BDNF protein in human brain

Five different antibodies raised against either different peptide sequences or the entire sequence of mature human BDNF were tested for suitability for BDNF protein analysis in control and AD human parietal cortex tissue. Using our standard Western blotting conditions, three of these antibodies failed to react specifically with BDNF in human brain samples. One of the antibodies bound non-specifically with the broad-range

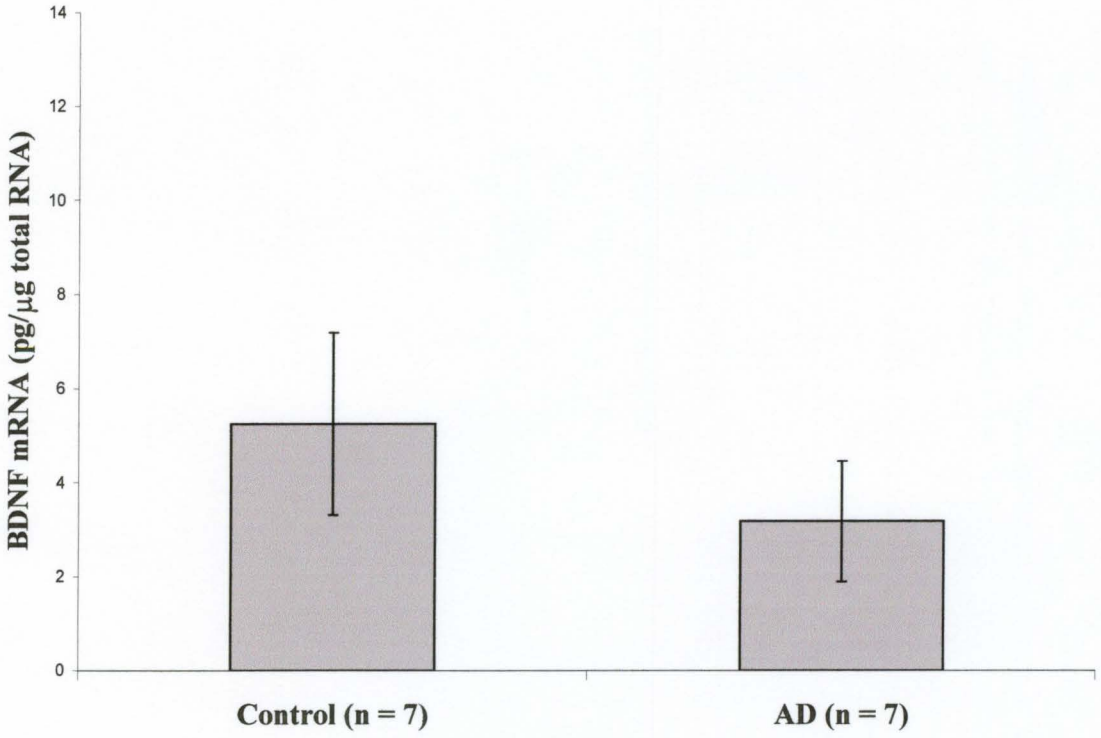

Figure 11: Densitometric analysis of BDNF cRT-PCR products from the hippocampus of seven control and seven AD subjects reveals no statistically significant differences between control and AD subjects ($p = 0.45$).

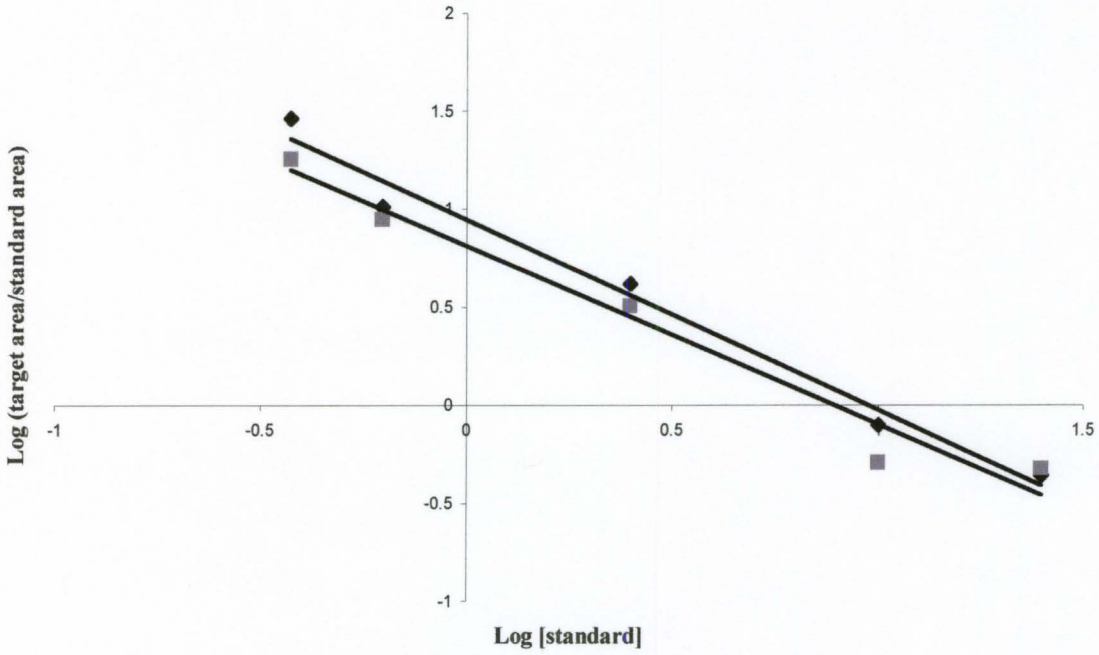

Figure 12: Reproducibility of competitive RT-PCR technique. A randomly chosen sample was subjected to the cRT-PCR method on two separate occasions. The figure illustrates the accuracy of the technique.

molecular weight markers as well, while another failed to bind rhBDNF and did not react with any proteins in the human samples. The two remaining antibodies were used for further analysis.

3.5.1 Santa Cruz anti-BDNF antibody and BDNF-like immunoreactivity

The sc-546 Santa Cruz antibody recognized and bound strongly to rhBDNF as well as to the mature form of BDNF in human samples. The binding to rhBDNF was observed at 13-kDa, the estimated molecular weight of mature BDNF. However, the binding to proteins in human samples occurred at a lower molecular weight of 9.5-kDa (Figure 13). It was also observed that the sc-546 antibody bound to rhBDNF expressed in bacculovirus at a lower than predicted molecular weight of 11-kDa.

It is possible that the 9.5-kDa immunoreactive protein that we see in human parietal cortex samples is not a BDNF-specific protein. As discussed later, although the antibody has been tested for cross-reactivity with other known neurotrophins it is highly probable that the antibody may react with an unknown, non-specific protein or with an unidentified neurotrophin with similar sequence homology. Although this explanation may seem reasonable a more likely one would be that sample degradation as a result of homogenization and storage may account for the migration pattern observed. Another possible explanation is that since we load 40μg of protein on each lane and a very small amount of recombinant BDNF protein the migration characteristics may differ resulting in different molecular weights.

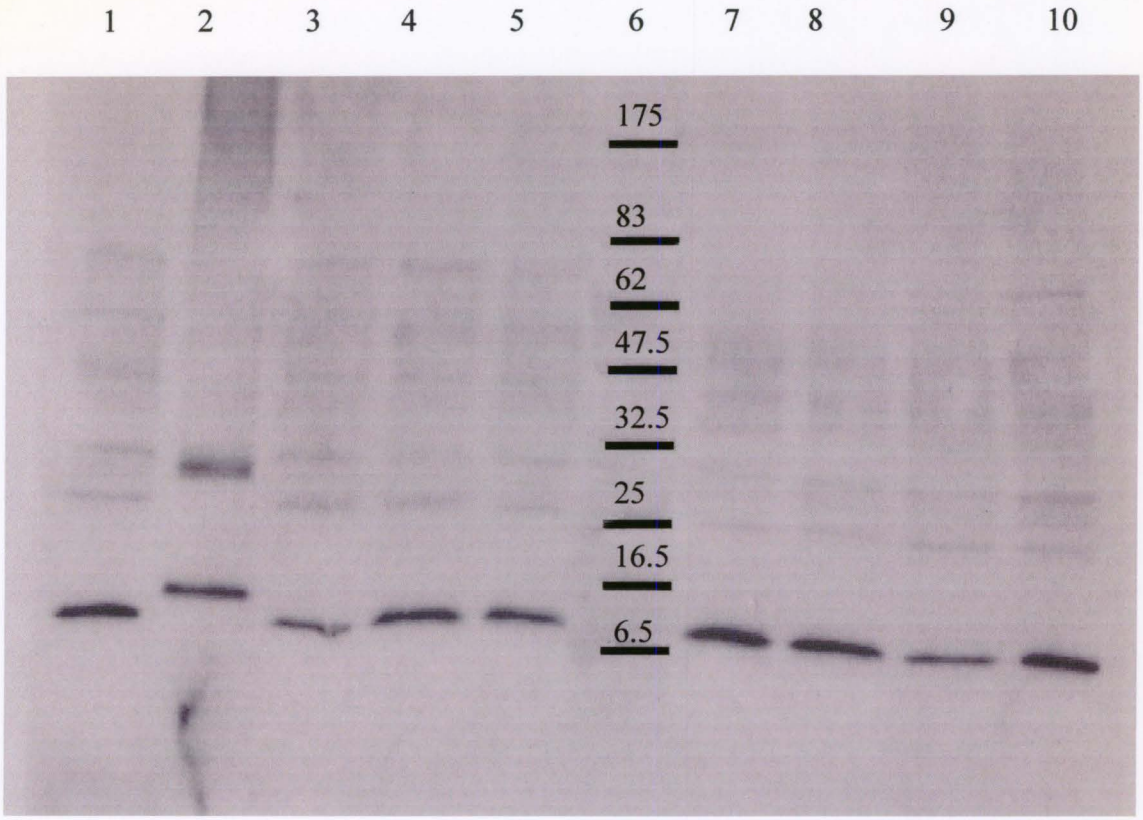

Figure 13: Western blot of BDNF protein from human parietal cortex using the Santa Cruz anti-BDNF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This blot was initially blocked with a 10% goat serum / 1% BSA solution in TBS at room temperature for 2 hours. The primary antibody was incubated at 4°C overnight in blocking solution. Lane 2 is rhBDNF; Lane 6 is a molecular weight standard; Lanes 1, 5, 8, 9 and 10 are control samples while Lanes 3, 4, and 7 are AD samples

It should be noted that blocking conditions played a vital role in the detection of mature BDNF in the human samples. When initially blocked with milk solids (two different concentrations of 5% and 10% (w/v) were used) at room temperature, no mature BDNF was observed in any of the samples of human origin except the rhBDNF. Instead, many high molecular weight immunoreactive bands were observed. When using a 10% milk blocking solution for 2 hours at room temperature followed by primary antibody incubation overnight at 4°C on a rocking platform, immunoreactive bands were detected at molecular weights of 28/29- (doublet), 31.5/32.5-, 36.5/37.5-, 46/53-, 75- and 168-kDa (Figure 14). Often during repetition of these experiments it was observed that the lower molecular weight bands, i.e., those under 35-kDa, did not appear reproducibly even though transfer conditions were similar.

Additionally, it was observed that two of the HRP-conjugated secondary antibodies bound non-specifically with proteins on the membrane. This was identified by the use of negative controls during which all steps of the protocol were carried out without the use of a primary antibody, i.e., blots were incubated in TBS-T instead of a primary antibody in TBS-T and then probed with a HRP-conjugated secondary antibody. As seen in Figure 15, incubation with only the secondary antibody resulted in certain immunoreactive bands been visible at low molecular weights, somewhat similar to those observed with the sc-546 primary antibody following blocking with milk solids. This problem was rectified by the purchase of a new batch of secondary antibodies.

Since blocking with milk solid solutions appeared to inhibit the sc-546 antibody binding to mature BDNF in human samples, alternative blocking conditions were

considered. Attempts to block non-specific binding with a 3% BSA (w/v) solution in TBS-T also proved futile, as the concentrations of the secondary antibody at which the negative control experiments appeared to work (no secondary antibody immunoreactive bands visible) were too low to interact with the primary antibody and failed to reveal any immunoreactive bands. The same was true using a 2% (w/v) gelatin blocking solution in TBS-T followed by a 0.5% gelatin blocking with the secondary antibody.

Employing the techniques used by Connor and colleagues (1997) but using a lower concentration of secondary antibody (1:6,667 instead of 1:1,500) that worked well during negative control experiments (no secondary antibody immunoreactive bands), we were able to detect mature BDNF protein in all human postmortem tissue samples. The protocol required incubation following transfer of proteins to PVDF membrane in a 10% goat serum (v/v) / 1% BSA (w/v) solution in TBS (no Tween-20) for 1-2 hours followed by incubation with the sc-546 antibody at a concentration of 0.1 mg/ml in blocking solution overnight at 4°C on a rocking shaker. The membranes were washed in TBS-T and then incubated in the HRP-conjugated secondary antibody at a concentration of 1:6,667 in TBS. Following incubation for one minute in the ECL™ mixture and exposure to autoradiographic film, BDNF immunoreactive bands were visible at the mature molecular mass of 13-kDa in the rhBDNF sample lane and at 9.5-kDa in the lanes containing human brain tissue homogenates (Figure 13). However, no quantitative measurements were possible using this antibody since the purchase of a second vial of sc-546 antibody from a new lot resulted in non-specific binding with the broad- range molecular weight markers and other proteins in the human samples.

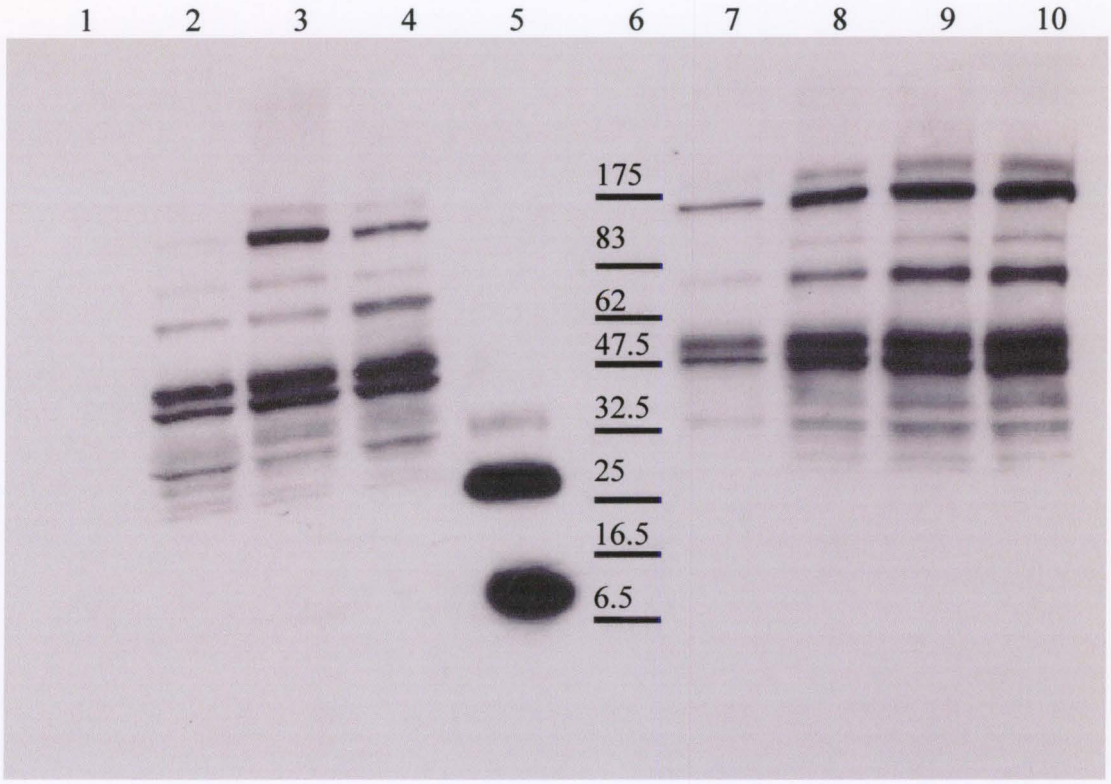

Figure 14: Western blot of BDNF protein using the Santa Cruz anti-BDNF antibody. The blot was initially blocked with a 10% (w/v) milk blocking solution at room temperature for two hours Lane 5 is rhBDNF; Lane 6 is a molecular weight standard; Lanes 2 and 4 are AD samples while Lane 3,is a control sample. Lanes 7-10 represent a protein gradient ranging from 40-100 μg of a standard brain sample.

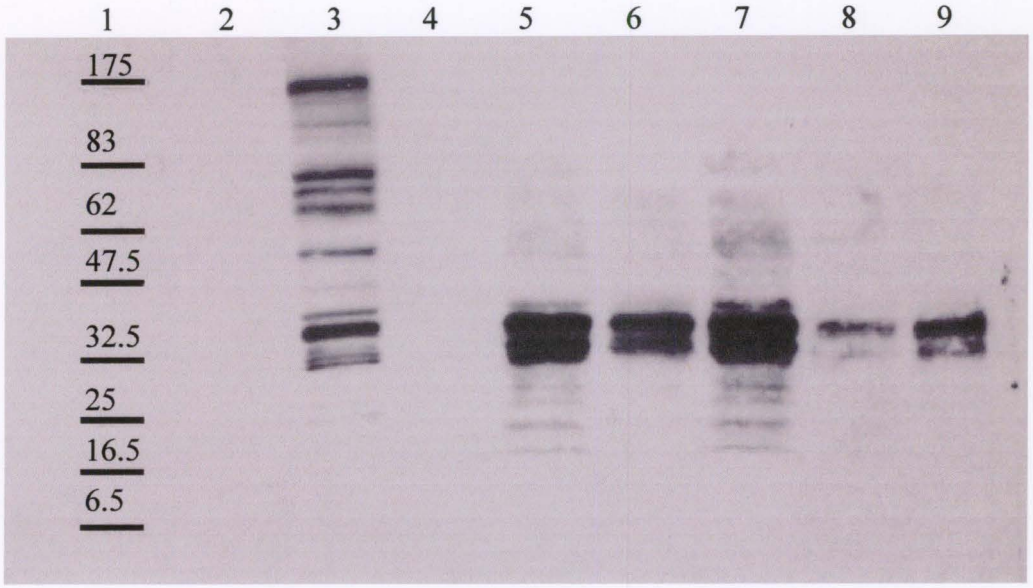

Figure 15: Western blot of a negative control experiment using a HRP-conjugated anti-rabbit secondary antibody raised in donkey. In this experiment, all steps of the protocol were carried out without the use of the primary antibody. The bands seen on this blot represent non-specific binding by the secondary antibody. Lane 1 is a molecular weight marker; Lane 3 is a mouse brain sample and Lanes 5 – 9 are human brain samples.

3.5.2 Turkey anti-BDNF antibody and BDNF-like immunoreactivity

A non-commercial antibody raised against the entire mature BDNF molecule (a gift from Dr. Josette Carnahan, Amgen Inc., Thousand Oaks, CA, USA) was also used to identify BDNF protein in human samples. Unfortunately both the primary and secondary (Cedarlane) antibodies had expired and, despite many efforts, no data was obtained using this anti-BDNF antibody.

3.5.3 Kaplan anti-BDNF antibody and BDNF-like immunoreactivity

The Kaplan affinity-purified anti-BDNF antibody, raised against a peptide downstream (Figure 1) from that used for the Santa Cruz anti-BDNF antibody, bound strongly to recombinant human BDNF but displayed different binding characteristics than those observed with the sc-546 anti-sera in human samples.

The protocol used with this antibody was similar to that described for that of sc-546 with the exception that the primary antibody was diluted in TBS instead of blocking solution. Blocking solution appeared to inhibit the antibody binding. Since the secondary antibody used for immunodetection was identical to that used with sc-546, the issues encountered regarding negative control experiments in the previous section applied to the Kaplan anti-BDNF antibody as well. As before, the use of a new batch of secondary antibody resolved the non-specific secondary antibody immunoreaction. Conditions that produced quantifiable results included blocking the membrane for one hour in a 10% goat serum / 1% BSA solution in TBS on a rocking platform at RT followed by overnight

incubation at 4°C in 1.1 μg/ml primary antibody solution in TBS on a rocking platform. Following washes in TBS-T, the secondary antibody was added at a dilution of 1:6,667 and incubated at RT for 1 hour.

In human brain samples, this antibody immunoreacted strongly with a protein at a molecular weight of 35-kDa, weaker with a protein at 50-kDa and, in some samples, to one at 67-kDa. Interestingly, immunoreaction with the 67-kDa protein was visible only in samples that displayed lower immunoreactivity with the 35-kDa protein (Figure 16). At present it is not possible to distinctively identify any of these three immunoreactive bands as being BDNF precursors. However, as discussed later, a BDNF-immunoreactive protein demonstrating a molecular weight of 30 kDa has been identified in the rat brain and a 35-kDa protein in Shwann cells in culture. As briefly described in section 3.5.1, it is possible that none of these bands are BDNF proteins. However, the deduced molecular weight of proBDNF (27.5-kDa) together with a consensus sequence for N-glycosylation upstream from the start site of mature BDNF may support the existence of the 35-kDa imunoreactive protein. Deglycosylation of the parietal cortex samples with the PNGaseF enzyme prior to electrophoresis may assist in supporting this hypothesis.

3.6 Quantification of BDNF-immunoreactive signals in human samples

In order to compare BDNF immunoreactivity between samples and between autoradiographs it was important that a standard procedure be used. A control human parietal cortex sample that was not used in the experimental sample pool served as a reference sample. This sample was loaded on each gel in concentrations of 20, 40, 60 and

80 μg in order to produce a concentration curve to which the experimental samples could be compared. In addition to immunodetection using anti-BDNF antibodies, the cytoskeletal protein β-actin was used as a measure to gauge the linearity of immunodetection and to allow normalization between samples. The pixel values obtained from the reference samples were graphed to obtain a regression line against which the density values of the experimental samples were compared (Figure 17). This was done to determine whether the data obtained was within the linear range of the film, and all experimental samples fell within this range. In order to be able to compare samples from different blots, the autoradiograph with the highest density value was used as a reference to standardize the other blots. This was done by first normalizing the values of the reference samples for both BDNF-immunoreactivity and β-actin on each autoradiograph to the autoradiograph with the highest density value. The values obtained from normalization were then used as multiplication factors to normalize the BDNF-immunoreactive and β-actin values obtained for each experimental sample.

The normalized densitometry value (BDNF-like immunoreactivity) for each sample was then divided by its normalized β-actin content and compared between groups.

Analysis of BDNF-like immunoreactivity in the parietal cortex using the Kaplan anti-BDNF antibody revealed no statistically significant differences between control and AD subjects of the 35-kDa protein ($p = 0.71$) (Figure 18). However, caution must be used when interpreting these results as they were obtained using a single antibody. This

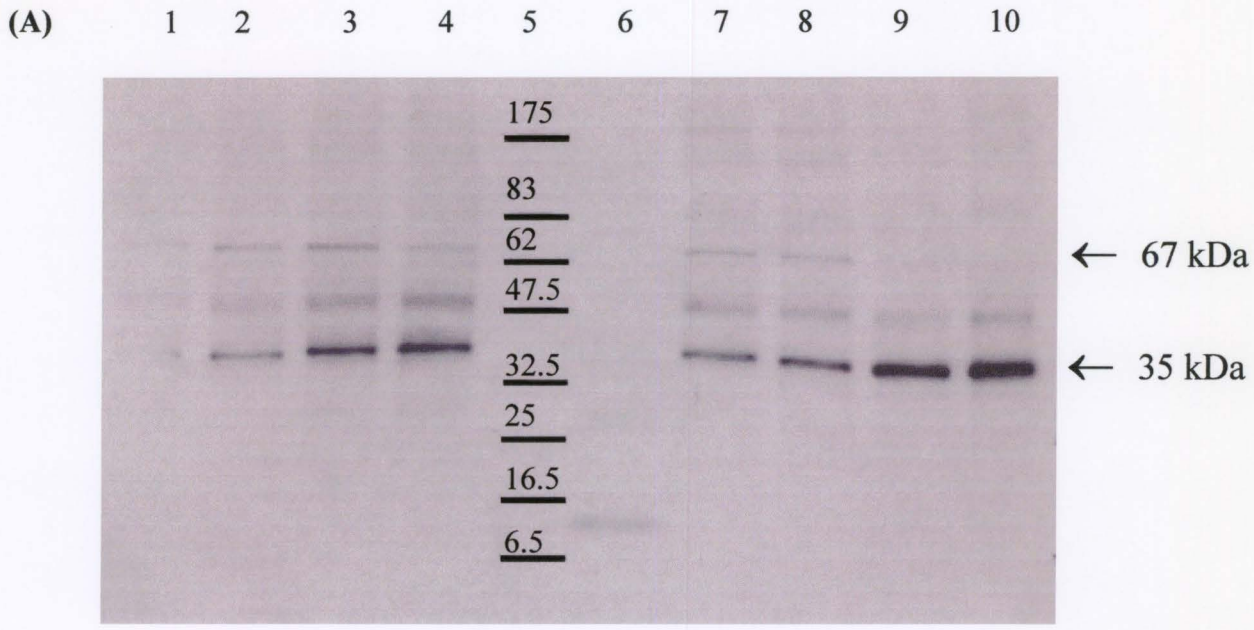

Figure 16:. Western blot of BDNF protein from human parietal cortex using antibodies from Dr. David Kaplan, MNI. Lanes 1-4 are a gradient from 20-80 μg protein of a standard brain sample; lane 5 is a molecular weight standard; lane 6 is rBDNF; lanes 7 and 10 are AD samples, while lanes 8 and 9 are control samples.

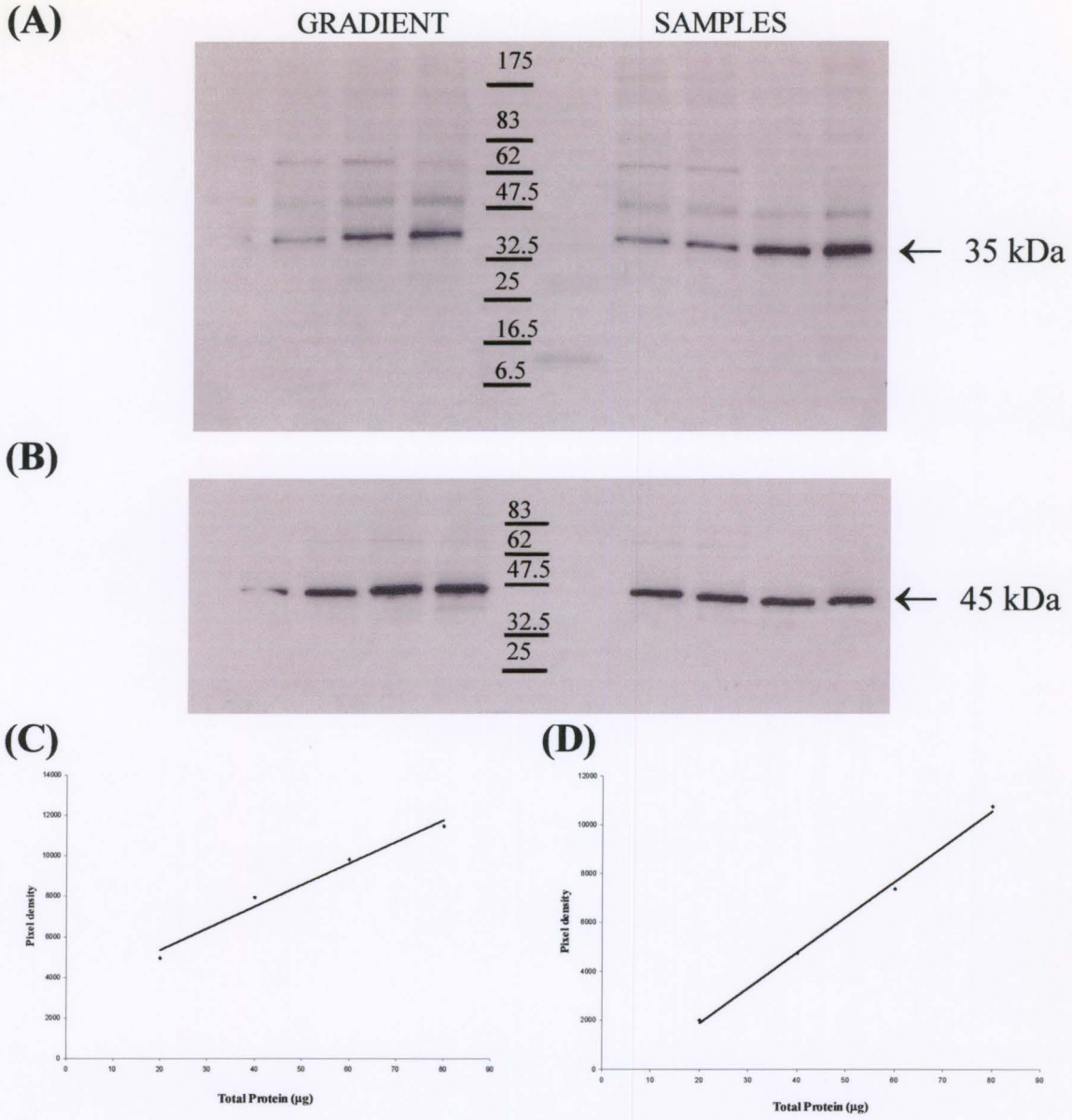

Figure 17A Representative quantitative Western blot of BDNF protein in normal and AD tissue as in Fig. 3A.

Figure 17B Beta-actin immunoreactivity from the same blot.

Figure 17C Graph of pixel values of beta-actin protein versus amount of total protein loaded on the gel, showing linearity of the Western blotting and ECL detection system used.

Figure 17D Graph of pixel values of BDNF protein versus amount of total protein loaded on the gel, showing linearity of the Western blotting and ECL detection system used.

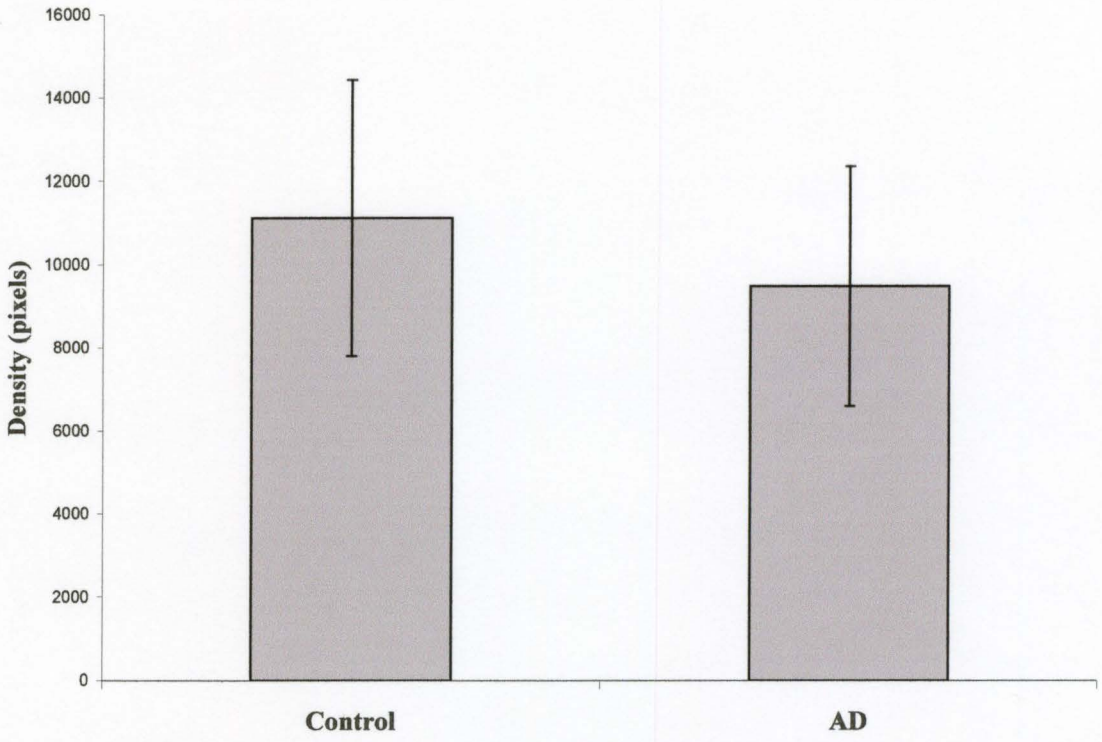

Figure 18: Analysis of the 35-kDa protein detected using the Kaplan anti-BDNF antibody revealed no statistically significant differences between control and AD subjects ($p = 0.29$).

antibody fails to recognize mature BDNF (13-kDa) in human samples, although it does recognize and bind to recombinant human BDNF.

4.0 DISCUSSION

The results of the present investigation demonstrate quantitation of BDNF mRNA in human postmortem brain tissue. Using the sensitive technique of competitive RT-PCR we measured levels of BDNF mRNA in neurologically normal human brain tissue samples and in those from age- and gender- matched subjects with Alzheimer's disease. We also examined relative levels of BDNF-like immunoreactivity, using Western blotting, in the parietal cortex of a subset of subjects analyzed in the mRNA study.

We demonstrate a 3.4-fold decrease of BDNF mRNA in the parietal cortex of patients with AD (12.11 ± 2.59 pg/μg total RNA in control tissue vs. 3.53 ± 0.77 pg/μg total RNA in AD (Figure 9). We also demonstrate, for the first time, BDNF mRNA in the nucleus basalis of Meynert where we observe a statistically significant age-related decline in the amount of BDNF mRNA in both control and AD subjects. However, in contrast to other studies (Phillips et al., 1990; Murray et al., 1994), we fail to observe any differences in the levels of BDNF mRNA in the hippocampus of subjects with AD compared to controls. Furthermore, we do not observe any changes in the level of BDNF-like immunoreactivity in the parietal cortex of individuals with AD, in whom we reported a decrease in the levels of BDNF mRNA, when compared to control subjects.

4.1 BDNF in the parietal cortex

Previous studies attempting to examine the levels of BDNF mRNA in the parietal cortex of subjects with AD failed to observe any differences when compared to controls (Hock et al., 1998). However, closer examination of their data does demonstrate a trend

towards decreased levels in their AD samples. The non-competitive RT-PCR technique employed by Hock and colleagues, although a sensitive tool for mRNA analysis, embodies some disadvantages. Their method requires that mRNA detection remain within the logarithmic phase of amplification. Using cRT-PCR, a highly sensitive technique, it is unnecessary to remain within the logarithmic phase because the BDNF mRNA in the sample of interest competes with a known concentration of competitor (Gause and Adamovicz, 1994; St. Amand et al., 1996). Using our cRT-PCR conditions we routinely detect as little as 250 fg BDNF mRNA/μg total RNA in human brain and 10 fg BDNF mRNA/μg total RNA in mouse brain (Yu and Fahnestock, unpublished data). Previous analysis of BDNF mRNA levels in humans reported 0.57 ± 0.06 pg/10 μg of total RNA in the hippocampus, a 200-fold lesser amount than we detect in the parietal cortex (Phillips et al., 1991). Hofer and colleagues (1992), examining the regional distribution of BDNF mNRA in the adult mouse brain, where two BDNF mRNA transcripts have been reported, detected a total of approximately 3.36 pg/mg wet weight of tissue for cortical samples. Transforming our data to correspond to the Hofer study we detect a comparable amount of BDNF mRNA (5.60 pg/mg wet weight of tissue) in the human parietal cortex.

The cholinergic neurons of the basal forebrain innervate a variety of regions in the brain and in turn receive neurotrophic support from many of these connections via retrograde axonal transport. The neurons of the basal forebrain are localized in three nuclei; the medial septum (MS), the diagonal band of Broca (DB) and the nucleus basalis of Meynert (NBM). These neurons project to the rest of the cortex via two major

cholinergic pathways. The monkey hippocampus has been shown to be primarily innervated by the MS and the vertical limb of the DB, whereas in monkey and rat, cells in the NBM and the horizontal limb of the DB project onto the amygdala and neocortex (Amaral and Cowan, 1980; McKinney et al., 1983; McGeer et al., 1984). As our study is the first to demonstrate a decrease in the levels of BDNF mRNA in the AD cortex, we were interested in inquiring whether this decrease in mRNA would translate to the protein level as well. If it did, it could be hypothesized that the decrease in BDNF protein would directly compromise cholinergic neurons in the horizontal limb of the DB and the NBM.

Using an antibody raised against a ten-peptide sequence of mature BDNF (Kaplan anti-BDNF antibody) we were able to identify a 35-kDa immunoreactive band in parietal cortex samples from human brain. Statistical analysis of the amount of 35-kDa immunoreactive protein did not reveal a significant difference between control and AD subjects. Although a mature BDNF immunoreactive protein was observed migrating at 9.5-kDa in human samples using the commercially obtained Santa Cruz anti-BDNF antibody (sc-546), no analysis of protein measures was possible due to poor antibody standards (different antibody batch to the one that immunoreacted with the 9.5-kDa protein in human samples).

The 35-kDa immunoreactive band seen in the cortical samples using the Kaplan antibody is similar to the 35-kDa precursor observed in Schwann cells isolated from newborn rat sciatic nerve using the sc-546 antibody (Marcinkiewicz et al., 1998). The ability of two anti-BDNF antibodies, raised against different peptide sequences of mature BDNF, to identify proteins of similar molecular weights from human and mouse samples,

strongly supports our hypothesis that the 35-kDa immunoreactive protein observed in human cortical samples is a BDNF precursor molecule. In the rat brain, pro-proteins of 30- and 43-kDa were observed in addition to a 14-kDa protein using BDNF-specific antibodies (Katoh-Semba et al., 1997). It is possible that the 35-kDa BDNF-immunoreactive protein observed in human samples corresponds to the 30-kDa BDNF-immunoreactive protein observed by Katoh-Semba and colleagues (1997). The differences in molecular weights may be attributable in part to the different concentration of the polyacrylamide gels used in the experiments (4-20% gradient gel in our experiments, and an Affi-Gel 10 used in the Katoh-Semba study). In human hippocampal and temporal cortical samples, in addition to a 14-kDa protein, Connor and colleagues (1997) reported a 28-kDa immunoreactive band using a polyclonal antibody (Amgen) directed against the BDNF polypeptide. Recently, a type I membrane-bound proteinase referred to as subtilisin-kexin-isozyme-1 (SKI-1) which is highly conserved among human, rat and mouse has been shown to generate a 28-kDa product from the 32-kDa BDNF precursor produced by vaccina virus recombinants (Seidah et al., 1999). These results further support the probability that the 35-kDa immunoreactive band obtained using the Kaplan anti-BDNF antibody is most likely an unprocessed precursor molecule of BDNF. It is also possible that the 67-kDa immunopositive band visualized with this antibody may be an unprocessed prepro-BDNF molecule since increased levels are observed in samples that demonstrate weak immunoreactivity with the 35-kDa protein.

As mentioned, decreases in BDNF mRNA have also been reported in the AD hippocampus. However, conflicting results have emerged regarding its protein where

decreased levels have been reported from immunohistochemical analysis (Connor et al., 1997) but no significant reductions in the dentate gyrus were observed using enzyme-linked immunosorbant assays (Narisawa-Saito et al., 1996). Our observations of decreased mRNA in the parietal cortex of AD subjects compared to matched controls adds further controversy to the issue since we do not observe statistically significant differences in protein levels from the same tissue. However, it is possible that the 35-kDa immunoreactive protein that we observe with the Kaplan anti-BDNF antibody is not BDNF specific. The possibility that the antibody may interact non-specifically with a protein of high concentration cannot be eliminated. This would account for the conflicting results observed.

Ferrer and colleagues (1999) have recently reported decreased BDNF protein levels (23%) in the frontal cortex of subjects with AD when compared to controls. Strong BDNF immunoreactivity was observed in dystrophic neurites surrounding senile plaques suggesting that decreases in BDNF protein could be the result of impaired BDNF transport and release (Ferrer et al., 1999). However, it is also possible that the strong BDNF immunoreactivity observed in dystrophic neurites may contribute to elevated levels of unusable BDNF in AD samples probed by Western blotting. Although we too observe slightly decreased (11%) levels of BDNF protein in the AD brain it is unlikely, given the 3.4-fold decrease in the levels of BDNF mRNA in these parietal cortex samples, that we and Ferrer et al., (1999) should observe such small changes in protein levels. Since preparation of samples for analysis by Western blotting results in the inclusion of all components of tissue being homogenized, it is likely that high concentrations of BDNF, as

observed by immunohistochemistry surrounding senile plaques, produce an unusable source in the AD brain that is detected by Western blotting.

The lack of statistically significant differences in levels of the 35-kDa BDNF-immunoreactive protein between control and AD subjects in our study may also be due to deficiencies in BDNF transport that result in increased amounts of BDNF protein being detected in the cortex. Studies on the levels of the high-affinity BDNF receptor, trkB, have demonstrated decreased levels in NBM (Salehi et al., 1996), an area from which cholinergic neurons project exclusively to the cortex, and in the frontal cortex of subjects with AD (Ferrer et al., 1999). As a result, it is possible that although there may be a decrease in the production of BDNF protein in the AD parietal cortex, no differences are observed when compared to controls because the protein that is produced is not retrogradely transported to the basal forebrain and is therefore detected in cortical samples.

It is also possible that an increase in the truncated, non-catalytic form of the trkB receptor plays a significant role in the discrepancy between the levels of BDNF mRNA and protein in the AD brain. Decreased full-length trkB expression together with increased truncated trkB expression was observed in the frontal cortex of patients with AD (Ferrer et al., 1999). BDNF bound to truncated receptors that are abundant in glial, neuronal and non-neuronal cells may present an unusable source of BDNF protein that can be detected by Western blotting techniques.

BDNF protein in cortical structures is produced in an activity-dependent manner. As in the hippocampus, protein production remains low until requirements for increased

levels, often following noxious stimuli, are received at which time there is a rapid increase in protein (Zafra et al., 1990; Ernfors et al., 1991; Kokaia et al., 1998). In the rat, up-regulation of BDNF protein was observed in the visual cortex between postnatal days 15-20 (P15-P20), a period that coincides with the increase of electrical input to the cortex that takes place at the time of eye opening (Rossi et al., 1999). Monocular deprivation, a classical experimental model for the study of activity-dependent plasticity, results in decreased levels of BDNF protein exclusively in the visual cortex (Rossi et al., 1999). These results suggest an alternate possibility that although there is a significant decrease in BDNF mRNA levels in AD, both in the hippocampus (Phillips et al., 1991; Murray et al., 1994) and parietal cortex (this study), it is probable that the protein produced in these areas of the brain (in the diseased state) by activity dependent mechanisms is sufficient to maintain and fulfill the requirements of the system. This hypothesis is supported by observations in the hippocampus, a region that is highly regulated by activity, where no changes in the levels of BDNF protein were observed between AD and control subjects using ELISAs (Narisawa-Saito et al., 1996).

It is possible that in the normal adult brain requirements for BDNF protein in certain brain regions are low and therefore the protein required is supplied by a small number of the population of cells that express BDNF. It is also possible that cytoplasmic storage and release rates may differ in different brain regions. A dose-dependent regulation of the different BDNF promoters in the rat hippocampus (Falkenberg et al., 1993) strongly supports this idea. Cloning of the rat BDNF gene resulted in the identification of a complex gene structure with four short 5′ exons and one 3′ exon

encoding the mature BDNF protein (Timmusk et al., 1993). A separate promoter has been identified upstream of each exon and alternative use of these promoters associated with differential splicing results in four BDNF mRNAs with different 5′ untranslated exons and a common exon encoding the mature part of the BDNF protein. Therefore, it is possible that activity dependent mechanisms result in differential regulation of the BDNF gene in different areas of the brain.

Subject characteristics and methods employed may play a significant role in the variation of results between different studies. Ferrer and colleagues (1999) have demonstrated that postmortem delay (PMI) plays a significant role in BDNF protein analysis where delays in tissue processing result in a loss of BDNF. A decay of 15% in BDNF immunoreactivity was observed in the brains of rats maintained at 4°C for 24 hours (maximum time period studied) prior to freezing at -80°C (Ferrer et al., 1999). In a similar study using rats, Narisawa-Saito and colleagues (1996) failed to observe any changes in neurotrophin levels in samples stored up to six hours at room temperature prior to freezing following death. Postmortem interval times for subjects used in the present study were 5.90 ± 0.58 and 2.92 ± 0.29 hours for the control and AD groups respectively. These times were similar to those of subjects used in the Narisawa-Saito et al., study (1996) where no decreases in BDNF protein levels were observed in the dentate gyrus (of the hippocampus) of subjects with AD compared to controls. Subjects used by Connor and colleagues (1997), who found a significant decrease in BDNF protein in the hippocampus of those with AD, had PMIs of 12 ± 1.9 (control) and 16.3 ± 4.5 (AD)

hours with a range between 2 - 47 hours. Although no statistically significant correlation was observed between the mean number of BDNF-immunoreactive neurons and PMI in the AD brain it is possible that a small subject number (3) with low PMIs contributed to this result. Although Ferrer and collaborators (1999) also used a subject pool with PMIs similar to ours and they report differences in BDNF immunoreactivity in cortical and hippocampal neurons in subjects with AD compared to controls, no statistical analysis or evidence for hippocampal results were provided.

Preparation of samples for Western blotting have also been shown to result in variability of protein detection. Ketoh-Semba and colleagues (1997) discovered that BDNF binds to membranes or intracellular particles and can be solubilized only in the presence of guanidine hydrochloride. When samples were homogenized with 100 mM phosphate buffer (pH 7.2) all the BDNF was recovered from precipitates obtained by centrifugation at 46,000 g. When 2 M guanidine hydrochloride was added to the phosphate buffer prior to homogenization and subsequent centrifugation at 46,000 g, all the BDNF was recovered from the supernatant (Katoh-Semba et al., 1997). Immunohistochemical analysis revealed that BDNF appeared to be bound to intranuclear particles. The substitution of 0.32 M sucrose in phosphate buffer also resulted in all the BDNF protein being extracted from the precipitate albeit at different speeds of centrifugation. However, the other studies examining BDNF protein levels by Western blotting or ELISAs, including ours, have used homogenization buffers lacking guanidine hydrochloride (Narisawa-Saito et al., 1996; Connor et al., 1997; Ferrer et al., 1999). It is

possible that the variation in homogenization buffers and the lack of guanidine hydrochloride could account for the variability of results observed.

Another possible confounding variable lies in the antibodies directed against BDNF. Although most of the antibodies used have been determined not to cross-react with other known neurotrophins, cross-reaction with previously unidentified members of the neurotrophin family or with unknown proteins containing similar epitopes cannot be excluded (Schmidt-Kastner et al., 1996). Furthermore, reactivity of protein samples with secondary antibodies (negative control experiments) as observed in this and other studies (Connor et al., 1997) could also account for some of the variability observed. Moreover, as identified in this thesis, non-specific protein blocking solutions could play a major role in antibody specific interactions. Inadequate blocking of non-specific proteins may result in antibodies binding to proteins with high concentrations rather than to those specific to the antibody. Furthermore, although less likely, it is also possible that proteins in blocking solutions may bind non-specifically to proteins of interest, thereby inhibiting the binding of antibodies to those proteins. It should be noted that prior to using the blocking solution proposed by Connor and colleagues (10% goat serum / 1% BSA in TBS), together with high background we failed to observe any mature BDNF-immunoreactivity (14-kDa) on Western blots of our brain homogenates.

4.2 BDNF in the hippocampus

Numerous studies have demonstrated that BDNF expression (as well as other neurotrophins) is most abundant in the hippocampus, which is a major target region of

basal forebrain cholinergic neurons (Ernfors et al., 1990; Hofer et al., 1990; Maisonpierre et al., 1990; Phillips et al., 1990; Wetmore et al, 1990). Studies in humans examining the levels of BDNF mRNA and protein in normal and diseased states have demonstrated conflicting results. Decreased levels of BDNF mRNA have been reported in the hippocampus of individuals with AD compared to neurologically unimpaired subjects (Phillips et al., 1991, Murray et al., 1994). However, using the competitive RT-PCR method of mRNA detection we fail to observe differences in BDNF mRNA content in the hippocampus of AD and control subjects. However, we believe that the results we observe are due to the fact that hippocampal tissue used in our experiments were obtained from the entire region of the hippocampal formation. As observed from in situ hybridization studies (Murray et al., 1994) regions such as areas CA1, CA2 and subiculum have been shown to contain very low or no detectable levels of BDNF mRNA. Since no definite identification of sample area is obtainable for the tissue used in our study, it is highly probable that sections for some subjects were obtained from areas displaying low to no BDNF mRNA. This would justify the low levels of BDNF mRNA we observed for the control hippocampal samples (5.25 ± 1.94 pg/μg total RNA) compared to those we reported in the parietal cortex (12.11 ± 2.59 pg/μg total RNA). Using RNase protection assays Phillips and collaborators reported 0.57 ± 0.06 pg/10 μg of total RNA for controls and 0.29 ± 0.04 pg/10 μg of total RNA for AD subjects. Although values obtained by us appear to be 100-fold higher that those in the Phillips study (1991), it should be noted that the competitive RT-PCR method employed in our experiments is much more sensitive

than the method used by Phillips et al., (1991). Quantitative analysis performed using Northern blotting (by including a known amount of sense transcripts to all samples at the time of tissue homogenization) demonstrate a total of approximately 4 pg of BDNF mRNA per milligram wet weight of tissue in mouse brain (Hofer et al., 1990). Converting our human hippocampus data we obtain 2.72 pg BDNF per milligram wet weight of tissue, a number similar to mouse brain but considerably lower to what we obtained for parietal cortex samples (5.60 pg/mg wet weight tissue).

Possible explanations for these discrepancies have been discussed in the parietal cortex section of this discussion and include PMI, preparation techniques and detection methods. As evident from the slight modification of the non-competitive RT-PCR technique used by Hock and colleagues (1998) to the more sensitive competitive RT-PCR method used in our investigations variability in the different detection methods also play a vital role. Furthermore, the different RNA and protein extraction procedures and solutions used may also contribute to variability in results.

4.3 BDNF in the nucleus basalis of Meynert

Consistent with the widespread distribution of BDNF mRNA in the brain we now demonstrate, for the first time, BDNF mRNA in the basal forebrain. Competitive RT-PCR analysis of BDNF mRNA in this area failed to detect differences in BDNF mRNA levels between AD and control subjects (5.67 ± 1.90 pg/μg total RNA for the control group and 3.69 ± 1.15 pg/μg total RNA for subjects with AD).

Support for the observation of BDNF mRNA in the basal forebrain stems from hippocampal lesion studies that fail to destroy cholinergic neurons that project to it. Since basal forebrain cholinergic neurons have been shown to depend upon neurotrophic support, destruction of projection areas of the cholinergic system would be expected to result in degeneration and death of the forebrain cholinergic neurons. Surprisingly, excitotoxic ablation of the entire hippocampus failed to produce cell death of medial septal cholinergic neurons (Sofroniew et al., 1990; Kordower et al., 1992). This indicates that the basal forebrain neurons are either not solely dependent on neurotrophic factors for survival or can obtain trophic support from other sources after target neurons are lost (Sofroniew et al., 1990). Using in situ hybridization techniques, Lauterborn and colleagues (1991) demonstrated that NGF mRNA was expressed by cells within the basal forebrain. Further analysis combining colorimetric and isotopic in situ hybridization techniques revealed that basal forebrain GABAergic neurons were the principle source of NGF-producing cells (Lauterborn et al., 1995). We now show the presence of BDNF mRNA in the basal forebrain, although the cellular origin of this factor is unclear.

The functional role of BDNF in the basal forebrain is also unclear. It is possible that this neurotrophin is acting on basal forebrain cholinergic cells in an autocrine manner similar to that observed in dorsal root ganglion neurons (DRGs). In an attempt to understand their apparent lack of dependence on exogenous growth factors, Acheson and colleagues (1995) treated cultured adult DRGs with anti-sense oligonucleotides targeted against BDNF. DRG neurons express mRNA encoding both BDNF (Ernfors et al., 1990) and the high-affinity BDNF receptor, trkB (Klien et al., 1991; Soppett et al., 1991).

When treated with anti-sense oligonucleotides, expression of BDNF protein was reduced by 80% and neuronal survival by 35%. The neurons were rescued by exogenous BDNF or NT-3, but not by other growth factors. Similar results were obtained with single-neuron microcultures in which cultures derived from BDNF mutant mice were unaffected by anti-sense treatment, strongly supporting an autocrine role for BDNF in mediating the survival of adult DRG neurons (Acheson et al., 1995).

Although previous *in situ* hybridization analysis of coronal brain sections from rat have failed to identify or not examined BDNF mRNA in the NBM in normal animals or following seizures (Phillips et al., 1990; Schmidt-Kastner et al., 1996) it is probable that our technique of competitive RT-PCR is more sensitive than those previously used. As referred to in the previous section of this discussion, using the competitive RT-PCR technique we were able to detect one hundred-fold more BDNF mRNA in the hippocampus of normal subjects than was detected using an RNase protection assay (Phillips et al., 1991). Furthermore, the parietal cortex results presented in this thesis demonstrate an approximately two hundred-fold higher concentration of BDNF mRNA than was detected in the hippocampus using the RNase protection assays.

Although our technique of mRNA detection is highly sensitive we cannot localize the source of BDNF mRNA in the NBM using this method. Interestingly, following seizure induction in adult rats, Schmidt-Kastner and coworkers (1996) were able to visualize BDNF mRNA in the striatum, a structure that was previously reported to be devoid of BDNF mRNA. The cellular characterization of the BDNF-positive neurons within the striatum indicated that they included GABAergic neurons, the same cell type in

which NGF mRNA was localized in the NBM (Lauterborn et al., 1991) suggesting that GABAergic cells in the basal forebrain may be the source of BDNF mRNA.

Our finding of BDNF mRNA in the NBM and the localization of trkB receptors on cholinergic neurons in the basal forebrain (Boissiere et al., 1997) suggest an autocrine/paracrine mode of action for BDNF within this region of the brain. We believe that BDNF mRNA in the NBM is expressed by GABAergic neurons. Three lines of evidence lend support to this claim: as demonstrated by competitive RT-PCR, no differences in BDNF mRNA levels are observed between AD and control subjects (this study) even though cholinergic neurons are severely affected and degenerate in the AD brain; the Lauterbon study described above; and the fact that BDNF mRNA has been detected in GABAergic neurons in the striatum of rats following the induction of seizures (Schmidt-Kastner et al., 1996). If BDNF is synthesized in GABAergic neurons it is likely that its trophic actions on NBM neurons are exerted via paracrine mechanisms. Since cholinergic neurons in the NBM co-express receptors for NGF and BDNF (Salehi et al., 1996), the paracrine interactions afforded by both these neurotrophins may play a vital role in the function and survival of cholinergic neurons. It is also possible that some of the BDNF produced in this area of the brain may be anterogradely transported to the projection areas of the NBM.

Although we failed to observe a statistically significant difference in the level of BDNF mRNA between control and AD subjects, regression analysis revealed a statistically significant reduction of BDNF mRNA content with age in both the control and AD groups of subjects. Hence, it appears that differential regulation of the BDNF gene within the

nucleus basalis occurs with increasing age since we fail to observe such differences in the parietal cortex and hippocampus of control subjects. Therefore, it appears that during normal aging neurons in the NBM may be compromised by low levels of "endogenous" BDNF protein (as opposed to retrogradely transported BDNF) produced by cells within the structure. Additionally, since the cholinergic neurons in the NBM are severely affected in AD it is possible that the lower levels of BDNF mRNA in this structure accompanying age poses more detrimental consequences on this population of cells that depend, in part, on neurotrophins for support and survival.

The age-related decline in BDNF mRNA raises another interesting issue. Studies have demonstrated that levels of BDNF mRNA and protein regulate the expression of the high-affinity BDNF receptor, trkB (Ferrer et al., 1998). Studies examining the levels of trkB in the NBM report a 47% reduction in AD when compared to controls (Salehi et al., 1996). It should be noted however, that the mean ages of the AD and control groups were 60.5 and 59.7 years respectively, notably lower than those used in this thesis. Another study by Boissiere and coworkers (1997) using in situ hybridization coupled with immunohistochemistry failed to observe any changes in the levels of trkB mRNA in the NBM. Interestingly, the age group of subjects in the Boissiere study was similar to that of ours with a mean age of 79 years for control subjects and 87 years for subjects with AD. Therefore, it is possible that as the levels of BDNF mRNA decline with increasing age, and since BDNF regulates the levels of the trkB receptor, a further decline in the expression of the trkB receptors might occur. A further observation made by these

researchers was that cells expressing trkB were localized to cholinergic neurons of the NBM indicating their dependence on neurotrophins for support.

The observation that the basal levels of BDNF mRNA in the NBM decline with age (this study) and that trkB receptor levels are decreased in AD (Salehi et al., 1996) demonstrates an unwelcome combination for patients with Alzheimer's disease. Together with the decreases in BDNF mRNA in the hippocampus (Phillips et al., 1991; Murray et al., 1994) and parietal cortex (this study) and decline in BDNF protein levels in the hippocampus, temporal (Connor et al., 1997), frontal (Ferrer et al., 1999), and entorhinal cortices (Narisawa-Saito et al., 1996), an overall loss of neuroprotection afforded by BDNF may contribute to the progressive neuronal atrophy of the basal forebrain cholinergic cells and hippocampal neurons associated with Alzheimer's disease.

FUTURE DIRECTIONS

In this thesis we observe decreases in BDNF mRNA but not BDNF-like immunoreactivity in the parietal cortex of subjects with AD. Studies should be carried out to conclusively determine whether BDNF-like immunoreactivity levels are, in fact, unaffected in the AD parietal cortex. The obstacle in conducting such experiments rests on the antibodies. The main aim should be to find antibodies that would permit successful detection and reproducibility. The *sc-546* Santa Cruz anti-BDNF antibody appears to be the most likely candidate. Additionally, antibodies raised against precursor molecules of BDNF or the Kaplan antibody could be used to examine the parietal cortex samples and also to determine whether the decreases in BDNF protein observed in the frontal cortex of AD subjects (Ferrer et al., 1999) are a result of deficient processing or synthesis.

The detection of BDNF mRNA in the nucleus basalis of Meynert entertains interesting possibilities for future investigation. The neuronal localization of BDNF-producing cells in the NBM could be investigated using double-label immunohistochemistry for GABA (as hypothesized in this thesis), ACh and BDNF. Results from such studies would demonstrate the origin of BDNF in the nucleus basalis neurons.

Although our hippocampal data fails to demonstrate decreases in BDNF mRNA levels in the AD brain compared to control we have now obtained samples that would permit us to analyze the dentate gyrus. These are hippocampal slices from which dentate gyrus can be dissected and RNA subjected to cRT-PCR.

It would also be interesting to examine BDNF mRNA and protein in areas of the brain that are relatively unaffected in AD to determine whether BDNF is differentially regulated compared to areas that are susceptible to neuronal degeneration. The area most fascinating would be the BDNF-responsive dopaminergic nigro-striatal system, an area that is severely affected in Parkinson's disease but relatively untouched in AD.

CONCLUSIONS

1. A significant (3.4-fold) decrease in BDNF mRNA levels was observed in the parietal cortex of AD subjects compared to controls.

2. We demonstrate, for the first time, BDNF mRNA in the nucleus basalis of Meynert. However, no difference in BDNF mRNA between AD and controls was observed.

3. An age-related decline in levels of BDNF mRNA in the NBM of both control and AD subjects was observed.

4. No difference in the level of the 35-kDa BDNF immunoreactive protein was observed in the parietal cortex of AD subjects compared to controls.

5. Although no difference in the levels of BDNF mRNA in the hippocampus was detected between AD and control subjects, we believe that this was due to the area of the tissue samples (entire hippocampus as opposed to the dentate gyrus).

Table 1. Parietal cortex samples used for Western blotting

Group, Autopsy no.	Age (years)	Gender	Postmortem delay (h)
Controls			
UCI 019-98	84	F	N/A
UCI 030-94	87	F	6.50
UCI 007-96	64	M	7.00
UCI 021-94	64	M	4.50
UCI 033-96	71	M	4.50
UCI 031-97	80	M	7.00
Mean ± SEM	75.00 ± 4.11		5.90 ± 0.58
AD			
UCI 028-94	84	F	2.75
UCI 050-97	88	F	4.25
UCI 031-95	64	M	2.50
UCI 023-94	66	M	3.00
UCI 047-94	72	M	2.25
UCI 008-95	80	M	2.75
Mean ± SEM	75.67 ± 4.01		2.92 ± 0.29

Table 2. Parietal cortex samples used for quantitative competitive RT-PCR

Group, Autopsy no.	Age (years)	Gender	Postmortem delay (h)
Controls			
UCI 019-98	84	F	N/A
UCI 030-94	87	F	6.50
UCI 037-94	81	F	6.00
UCI 007-96	64	M	7.00
UCI 021-94	64	M	4.50
UCI 033-96	71	M	4.50
UCI 031-97	80	M	7.00
Mean ± SEM	75.86 ± 3.58		5.92 ± 0.47
AD			
UCI 028-94	84	F	2.75
UCI 050-97	88	F	4.25
UCI 023-97	81	F	1.30
UCI 031-95	64	M	2.50
UCI 023-94	66	M	3.00
UCI 047-94	72	M	2.25
UCI 008-95	80	M	2.75
Mean ± SEM	76.43 ± 3.48		2.68 ± 0.33

Table 3. Nucleus Basalis of Meynert samples used for quantitative competitive RT-PCR

Group, Autopsy no.	Age (years)	Gender	Postmortem delay (h)
Controls			
UCI 034-96	79	F	4.25
UCI 037-94	81	F	6.00
UCI 019-97	82	F	7.25
UCI 007-96	64	M	7.00
UCI 033-96	71	M	4.50
UCI 025-97	78	M	6.00
UCI 031-97	80	M	7.00
Mean ± SEM	76.43 ± 2.48		6.00 ± 0.46
AD			
UCI 049-97	79	F	3.25
UCI 023-97	81	F	1.30
UCI 004-96	82	F	3.00
UCI 031-95	64	M	2.50
UCI 047-94	72	M	2.25
UCI 018-97	79	M	2.75
UCI 008-95	80	M	2.75
Mean ± SEM	76.71 ± 2.45		2.54 ± 0.24

Table 4. Hippocampal samples used for quantitative competitive RT-PCR

Group, Autopsy no.	Age (years)	Gender	Postmortem delay (h)
Controls			
UCI 034-96	79	F	4.25
UCI 037-94	81	F	6.00
UCI 019-97	82	M	7.25
UCI 030-94	87	M	6.50
UCI 053-97	93	M	8.00
UCI 012-95	68	M	7.50
UCI 033-96	71	M	4.50
Mean ± SEM	80.14 ± 3.27		6.29 ± 0.55
AD			
UCI 049-97	79	F	3.25
UCI 023-97	81	F	1.30
UCI 004-96	82	F	3.00
UCI 050-97	88	F	4.25
UCI 016-97	92	F	3.75
UCI 005-94	69	M	5.00
UCI 047-94	72	M	2.25
Mean ± SEM	80.43 ± 3.08		3.26 ± 0.48

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