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REGULATION OF BACE1 PROMOTER ACTIVITY BY NUCLEAR FACTOR-κB

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REGULATION OF BACE1 PROMOTER ACTIVITY BY NUCLEAR FACTOR-κB

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Approved by the Supervisory Committee

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To my husband Nigel, our daughters Kelley and Teagan and to the memory of my grandmother Tressa May Haley Zimmer.

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REGULATION OF BACE1 PROMOTER ACTIVITY BY NUCLEAR FACTOR-κB

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The brains of Alzheimer's disease (AD) patients display cerebrovascular and parenchymal deposits of β-amyloid peptides, which are derived by proteolytic processing of the amyloid precursor protein (APP). The β-site APP-cleaving enzyme 1 (BACE1) is required for the generation of β-amyloid peptides. The NF-κB binding DNA consensus sequence in the BACE1 promoter upstream of the gene's transcription start site suggests a role for NF-κB in the expression of neuronal brain BACE1. Failure of activation of NF-κB responses to stress in the aged and disregulation of NF-κB in the AD brain may result in part in altered NF-κB regulation of BACE1 and alterations in cell specific BACE1 transcription and β-amyloid protein processing.

We identified a number of putative NF-κB transcription factor binding sites on the rat BACE1 promoter. The effects of NF-κB binding to the "primary" NF-κB binding site of the BACE1 promoter were stimulatory for activated astrocytic cells and repressive for neuronal cells. Age-associated perturbations of NF-κB activation may result in increasingly aberrant regulation of β-amyloid processing by BACE1 via changes in the cellular levels of different NF-κB protein subunits and cumulative increases in astrocytically-derived β-amyloid. We confirmed the observation that in

PC12 cells the overall activity of NF-κB and BACE1 was significantly different depending on the apoptotic initiators: H_2O_2 or β-amyloid. Our results are consistent with feedback mechanisms involving β-amyloid exposure overriding NF-κB regulation of BACE1 over time, a source of negative feedback, consistent with observed neuropathologies. It is also likely that a series of transcription factor binding events determine which NF-κB binding sites are operant and this may explain the observed cell specificity of BACE1 regulation. Our observation that BACE1 expression was affected by both soluble and aggregated insulin, coupled with our previous observations that both aggregated $A\beta_{1\text{-}42}$ and $A\beta_{42\text{-}1}$ affected BACE1 expression, strongly suggest that the process of protein aggregation displayed by proteins sharing specific structural characteristics (i.e. zinc stabilized hexamers) may have pathological significance. It is tempting to hypothesize that insulin, and/or other similarly structured proteins, have effects on BACE1 activity that may play a role in the establishment and/or progression of Alzheimer's disease.

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LIST OF ABBREVIATIONS

A4-CT	N-terminus of beta-amyloid
$A\beta_{1-42}$	amyloid beta fragment 1-42
AD	Alzheimer's disease
AP1	activator protein
APP	amyloid precursor protein
APPsw	Swedish mutation in APP
BACE1	Beta-site APP-cleaving enzyme 1
BPR	wild type rat BACE promoter
BPRΔNF-κB$^-$	mutant rat BACE promoter vector
CREB	cAMP response element binding protein
DAB	3, 3'-diaminobenzidine hydrochloride
DHS	donor horse serum
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DS	double stranded
ECL	chemiluminescence lighting system
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assays
ER	endoplasmic reticulum
ERE	estrogen responsive element

FAD	familial early-onset AD
FCS	fetal calf serum
FTIR	Fourier transformed infrared spectroscopy
GFAP	glial fibrillary acidic protein
GPI	lycosylphosphatidylinositol membrane anchor
GRE	glucocorticoid responsive element
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNF3-β	hepatocyte nuclear factor 3 beta
HPLC	high performance liquid chromatography
ICC	immunocytochemistry
IGF	insulin-like growth factors
IKK	inhibitor kappa B kinase
IL-1β	interleukin-1 beta
lacZ	beta galactosidase reporter gene
NF-κB	nuclear factor-kappaB
NGF	nerve growth factor
PBS	phosphate-buffered saline
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PrP	prion protein
PS1	presenilin 1
PSGL-1	P-selectin glycoprotein ligand-1

PSN	penicillin streptomycin neomycin
RA	retinoic acid
ROS	reactive oxygen species
SEM	standard error of the mean
SP1	stimulating protein 1
SS	single stranded
ST6Gal I	Beta-galactoside alpha 2,6-sialyltransferase
STAT1	signal transducer and activator of transcription 1
TBI	traumatic brain injury
TBS	tris buffered saline
TE	tris-EDTA
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor alpha
TSS	transcription start site
YY1	yin-yang 1

CHAPTER ONE: INTRODUCTION

BETA SITE AMYLOID PRECURSOR PROTEIN CLEAVING ENZYME

Amyloid Precursor Protein (APP) Processing

Dr. Alois Alzheimer, a German physician, first described what is now known as Alzheimer's Disease (AD) in 1906. He observed at autopsy of the 51-year-old woman, Auguste D., who had been admitted to an asylum for "delirium and frenzied jealousy of her husband", two abnormal structures in the brain that are now recognized as hallmarks of AD—amyloid plaques and neurofibrillary tangles (Tanzi and Bertram, 2005). AD is an irreversible, progressive brain disease that slowly destroys memory and thinking skills, eventually even the ability to carry out the simplest tasks. The greatest known risk for developing AD is increasing age; the number of individuals with AD doubles every five years beyond the age of 65. In the year 2003 more than 4 million older Americans had AD, resulting in a annual national cost of caring of approximately $100 billion. This number is expected to quadruple by the year 2050 as more people live into their 80s and 90s. As many as 10 percent of people 65 years of age and older have AD, and nearly 50 percent of people 85 and older have the disease (http://www.alzheimers.org/. 2005, http://www.nia.nih.gov/. 2005). Most AD is sporadic in nature (90-95%) with the remaining 5-10% presenting as familial early-onset AD (FAD). In FAD the disease

develops before age 65 and as early as 35 years of age. It is caused by gene mutations on chromosomes 1, 14, 19 or 21 (http://www.alzheimers.org/ 2005). In addition, a significant proportion of traumatic brain injury (TBI) patients develop extracellular β-amyloid deposits as early as 2 hours after injury (Ikonomovic *et al*., 2004) and AD 10 to 30 years after the TBI event (Jellinger *et al*., 2001).

The pathogenic processing of the APP into the β-amyloid protein gives rise to β-amyloid plaques in the brains of AD patients. The APP protein is processed by α-, β- and γ-secretase (Figure 1). In the α-secretase pathway APP is initially cleaved by α-secretase after amino acid 687 within the β-amyloid sequence. This cleavage precludes the generation of β-amyloid peptides. The APP-α formed is released into the extracellular space and the remaining P3-CT fragment undergoes a second proteolytic cleavage by γ-secretase at amino acid 711-713 to liberate the variable C-terminus of the P3 fragment and the C-terminal region of the APP molecule. Alternatively, initial cleavage of the APP molecule by β-secretase after amino acid 671 liberates APP-β and exposes the N-terminus of β-amyloid (A4-CT). After a second proteolytic cleavage by γ-secretase; the variable C-terminus of β-amyloid (including the neuropathogenic forms: $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$) is generated.

BACE1 Non-Pathogenic Expression

The enzymatic activity of β-secretase is a prerequisite for the generation of β-amyloid peptides. The β-secretase or β-site APP-cleaving enzyme 1 (BACE1) is a

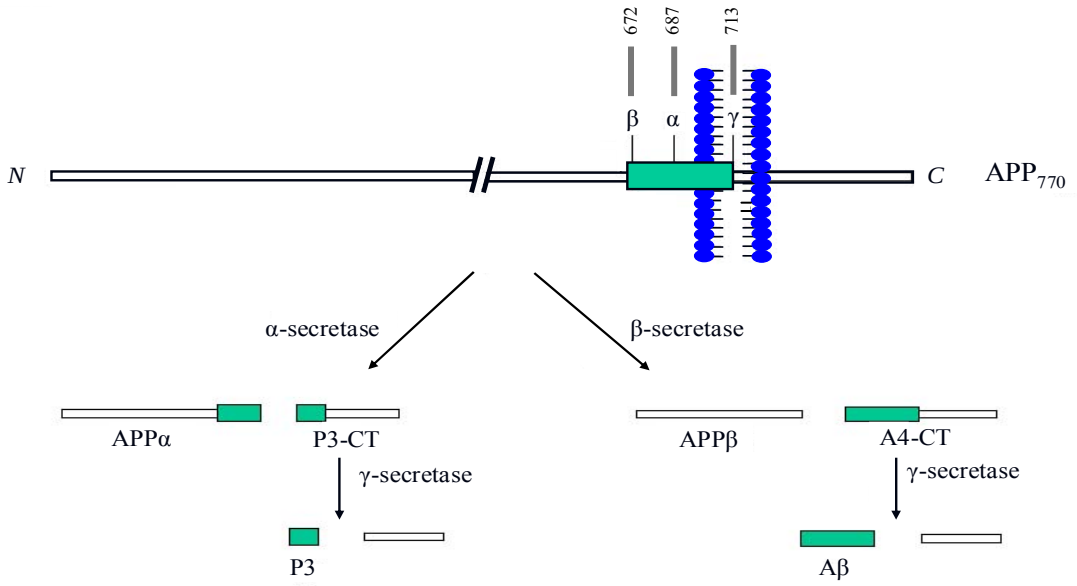


FIGURE 1. Map of APP Processing. In the α-secretase pathway APP is initially cleaved by α-secretase after amino acid 687 within the β-amyloid sequence. This cleavage precludes the generation of β-amyloid peptides. The APP-α formed is released into the extracellular space and the remaining P3-CT fragment undergoes a second proteolytic cleavage by γ-secretase at amino acid 711-713 to liberate the variable C-terminus of the P3 fragment and the C-terminal region of the APP molecule. Alternatively, initial cleavage of the APP molecule by β-secretase after amino acid 671 liberates APP-β and exposes the N-terminus of β-amyloid (A4-CT). After a second proteolytic cleavage by γ- secretase; the variable C-terminus of β-amyloid (including $A\beta_{1\text{-}42}$) is generated (Wilson *et al.*, 1999).

class I transmembrane protein consisting of an NH2-terminal protease domain, a connecting strand, a transmembrane region and a cytosolic domain. A signal peptide governs the insertion of BACE1 into the endoplasmic reticulum (ER) membrane. Four complex N-glycosylations and foldings prepare the pro-form of the enzyme for export from the ER *(*Sinha *et al*., 1999*;* Haniu *et al*., 2000; Charlwood *et al*., 2001). As BACE1 reaches the Golgi apparatus, the pro-peptide is removed by the action of furin or other pro-protein convertases (Bennett *et al*., 2000; Benjannet *et al.*, 2001; Creemers *et al*., 2001; Shi *et al.*, 2001). BACE1 is localized to the same intracellular sites as APP in the late Golgi, in the early endosomal compartment and on the cell surface (Hussain *et al*., 1999; Vassar *et al*., 1999; Capell *et al*., 2000; Huse *et al*., 2000; Creemers *et al*., 2001; Walter *et al*., 2001; Yan *et al*., 2001). The pro-enzymatic form of BACE1 is functional (Benjannet *et al*., 2001; Creemers *et al*., 2001; Shi *et al*., 2001); the activity of BACE1 may be regulated by lumenal acidification. The isolated enzyme has a pH optimum of approximately 4.5 and is virtually inactive at pH values of 6.0 and above (Vassar *et al*., 1999; Lin *et al*., 2000). It cleaves APP on the lumenal side of the membrane and its activity is the rate-limiting step in $A\beta_{1\text{-}42}$ production (Vassar, 2001). BACE1 is phosphorylated at serine 498 by casin kinase I within the cytoplasmic domain. Phosphorylation status affects the cellular localization of BACE1 (Walter *et al*., 2001).

BACE1 mRNA and protein are expressed at high levels in the brain and at lower levels in most peripheral organs with the exception of the pancreas, where very high BACE1 mRNA levels but low protein levels are reported (Yan *et al*., 1999;

Rossner *et al*., 2001). Despite its wide distribution and organ specific levels of expression, the function of BACE1 outside of the CNS remains largely undefined. It is not known which specific BACE1 promoter regions are responsible for the brain-specific expression of BACE1. BACE1 is primarily expressed by neurons in the brain of mice (Bigl *et al*., 2000), rats (Rossner *et al*., 2001) and humans (Fukumoto *et al*., 2002; Li *et al*., 2004; Sun *et al*., 2002) and the over expression of BACE1 has been shown to increase the generation of β-amyloid peptides, whereas inhibition of β-secretase activity by BACE1 antisense oligonucleotides reduces β-amyloid levels (Vassar *et al*., 1999). BACE2 is a homolog of BACE1. The BACE2 may be important in Down syndrome pathology as the gene is located on chromosome 21 and has been implicated in the pathogenesis of AD (Sun *et al*., 2005; Chou, 2004). However, the function of BACE2 in β-amyloid generation is controversial and will not be addressed here.

BACE1 Pathogenic Expression

There is increased expression of BACE1 in the brains of AD patients (Fukumoto *et al*., 2002; Holsinger *et al*., 2002). In AD patients with the Swedish mutation in APP (APPsw), there is a selective increase in BACE1 cleavage products, of which subsequent cleavage by γ-secretase leads to β-amyloid formation. The APP wild-type peptide BACE1 cleavage site is KTEEISEVKMDAE whereas in the APPsw the BACE1 cleavage site is KTEEIVNLDAE (Vassar, 2001). Increased levels of BACE1 have also been detected in brains of patients with sporadic non-familial forms of AD (Yang *et al*.,

2003; Tyler *et al*., 2002). The increase in BACE1 levels in AD may be due to widespread reduction of other proteins. Thus, BACE1 expression increases may be a reaction to the disease process. Alternatively, increased brain specific BACE1 expression resulting in increased Aβ production may be causally involved in sporadic AD (Yang *et al*., 2003).

Emerging evidence indicates an interaction between BACE1 and presenilin 1 (PS1). The γ-secretase complexes include PS1 which binds preferentially to the glycosylated immature BACE1 and is thought to be implicated in BACE1 maturation (Hebert *et al*., 2003). In both humans and mice it is well documented that PS1 mutations increase $A\beta_{1\text{-}42}$ production (Citron *et al*., 1997; Petanceska *et al*., 2000). Interestingly PS1 mutations in AD brain have also been shown to alter both γ-and β-secretase activities (Russo *et al*., 2000). However, in the brains of different transgenic mouse strains with β-amyloid plaque pathology, there are no changes in BACE1 mRNA (Bigl *et al*., 2000; Irizarry *et al*., 2001), protein concentrations or enzymatic activities (Gau *et al*., 2002; Rossner *et al*., 2001). The Tg2576 mice are a commonly utilized transgenic animal model of the Swedish form of AD. Transgenic Tg2576 mice that overexpress human amyloid precursor protein carry the double mutation K670N-M671L. In brains of aged Tg2576 mice, those astrocytes in close proximity to β-amyloid plaques present express BACE1 protein (Rossner *et al*., 2001). This astrocytic BACE1 expression is not a specific response to β-amyloid plaque formation but is also present in experimental paradigms of chronic gliosis *in vivo* and in AD brain (Hartlage-Rubsamen *et al*., 2003).

To identify elements which drive tissue- or cell type-specific BACE1 expression, Lange-Dohna, *et al*. (2003) cloned and sequenced a 1.5 kb fragment of the rat BACE1 promoter and generated BACE1 promoter-luciferase reporter site-directed substitution mutant and deletion constructs. The basal activity of these promoter constructs was highest in neuronal cell lines, somewhat lower in rat primary neurons, astrocytic and microglial cultures, very low in hepatocytes and almost absent in fibroblasts and in the monocyte-macrophage cell line RAW264.7 (Lange-Dohna *et al*., 2003). A preliminary analysis of promoter activities of the different deletion mutants was consistent with the presence of both activators and suppressors of BACE1 transcription in the cloned promoter region (Figure 2A, B).

The rat BACE1 promoter and 5′ UTR contains several putative binding sites for transcription factors, activator protein (AP1), AP2, one GC box, and ten stimulating protein (SP)1 binding sites upstream of the transcription start site (TSS) zinc finger transcription factors GATA, hepatocyte nuclear factor 3 beta (HNF3-β), Yin-yang 1(YY1), AP1, AP2, cAMP response element binding protein (CREB), estrogen responsive element (ERE), glucocorticoid responsive element (GRE), "GC" box, nuclear factor (NF)-κB, signal transducer and activator of transcription (STAT)1, SP1, metal-regulatory elements, and possible Zeste binding sites, which are conserved among rat, mouse and human (Sambamurti *et al*., 2004a, b; Lange-Dohna *et al*., 2003). BACE1 displays the characteristics of a housekeeping gene including the absence of characteristic "CAAT" and "TATA" boxes within 1.5 kb of the TSS. The proportion of

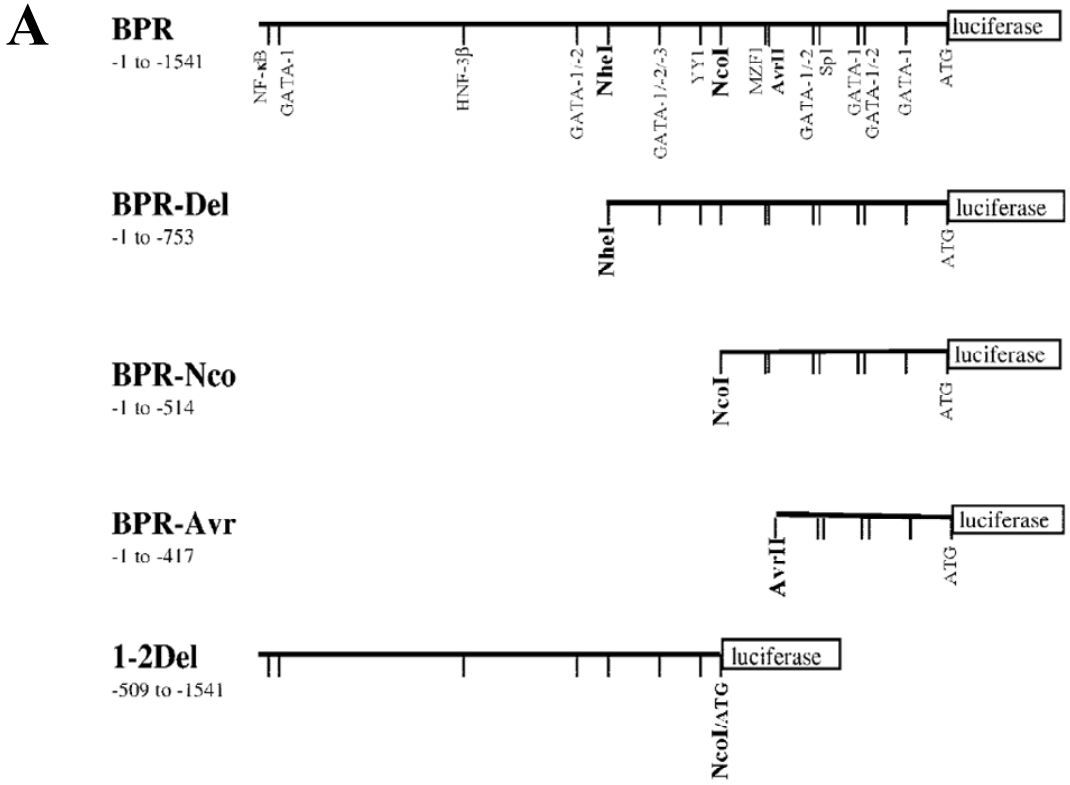


FIGURE 2. A: Schematic Presentation of the BACE1 Promoter Deletion Mutants Constructs. The promoter activity of the BPR-Del deletion mutant construct was composed of bases -1 to -753. The BPR-Nco deletion mutant construct was composed of bases -1 to -514. The BPR-Avr deletion mutant construct was composed of bases -1 to -417. 1–2Del deletion mutant construct was composed of bases -515 to -1541 (Lange-Dohna *et al*., 2003).

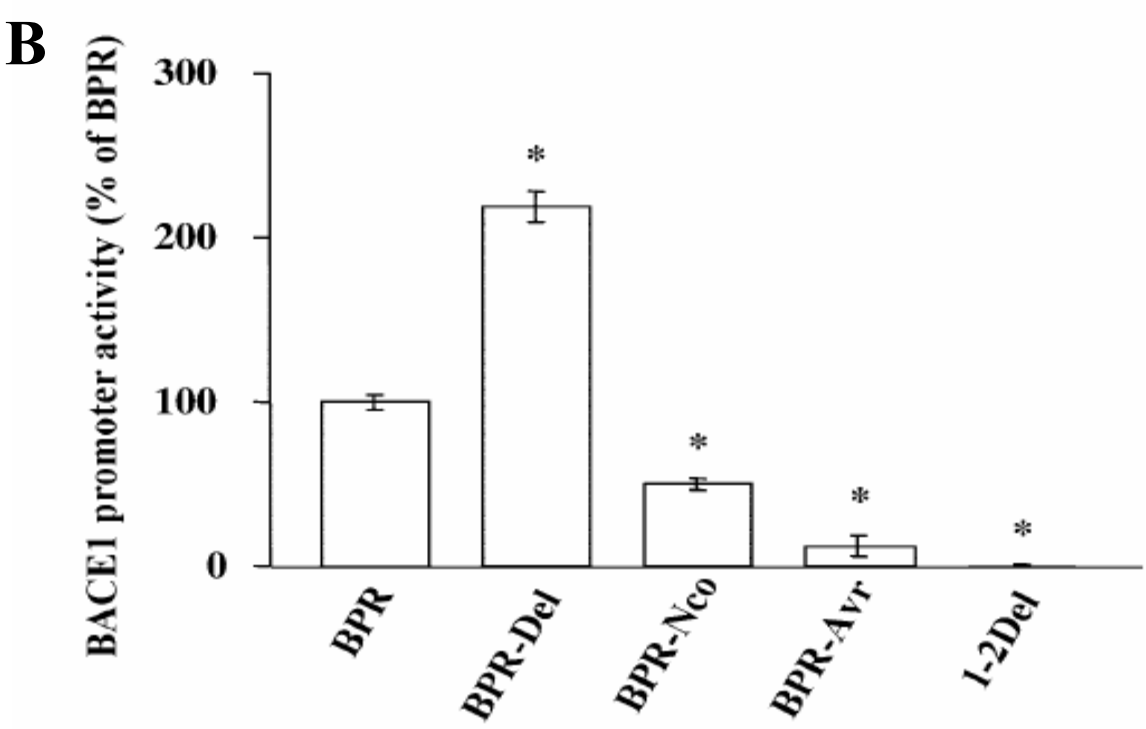


FIGURE 2. B: The Activity of These Deletion Mutants in PC12 Cells. The highest luciferase reporter activity was observed in the BPR-Del deletion mutant composed of bp -1 to -753, followed by the complete 1.5-kb promoter construct (pGl3-BPR). The BPR-Nco deletion mutant from base -1 to -514 displayed significantly less activity than pGl3-BPR, suggesting the presence of activators of BACE1 transcription between bp -514 and -753. The promoter activity of the BPR-Avr construct (-1 to -417) was further reduced. The 1-2Del construct (-748 to -1541) did not display promoter activity. The columns represent mean values (± SEM; n = 6) of one of five identical experiments. *Differences compared to the activity of the BPR construct are statistically significant at $P < 0.05$ (Lange-Dohna *et al*., 2003).

GC enrichment varies between 14% and 90% along the length of the sequence, reaching maximum levels at the 3′ end. Three putative GATA-1 sites were found in the 5′ UTR at locations homologous to those found in the rat promoter (Lange-Dohna *et al*., 2003). It has been shown that deletion of two of the GATA-1 sites in the rat sequence will increase expression. An active SP1 site has been identified and characterized in the human *BACE* promoter (Christensen *et al*., 2004). Four of the five NF-κB sites predicted in the human *BACE* promoter appear in a segment of the triple direct repeat, suggesting a possible regulatory role for these repeats. BACE1 activity has been shown to be regulated by several components of ordered lipid microdomains (lipid rafts), including caveolins (Ikezu *et al*., 1998; Nishiyama *et al*., 1999) and GPI anchored proteins (Sambamurti *et al*., 1999).

It is unlikely that BACE1 evolved primarily to generate potentially pathogenic β-amyloid peptides. It has been reported, for example, to possibly be involved in muscle metabolism (Vattemi *et al*., 2003, 2001). BACE1 is involved in the cleavage and secretion of β-galactoside α2, 6-sialyltransferase (ST6Gal I) a type II membrane trans-Golgi network protein. It is highly expressed in the liver and is expressed in most other tissues to some extent. Increase of ST6Gal I secretion in the early hepatopathological conditions is attributed mainly to up-regulation of BACE1 mRNA transcription in the liver (Kitazume *et al*., 2004). Lichtenthaler *et al.* (2003) reported that BACE1 cleaves P-selectin glycoprotein ligand-1 (PSGL-1), which mediates leukocyte trafficking, suggesting a possible function of BACE1 in the immune system. Cleavage of PSGL-1 by

BACE1 may be an important down-regulation mechanism for controlling leukocyte migration during acute inflammation. Wong *et al*. (2005) found that the β subunits of voltage-gated sodium channels are sequentially processed by BACE1 and γ-secretase. The low density lipoprotein receptor-related protein, a multifunctional endocytic and signaling receptor, has also been suggested to be a BACE1 substrate (von Arnim *et al*., 2005).

DNA transfection analyses have revealed the presence of both positive and negative regulatory elements in the human *BACE1* promoter (Sambamurti *et al*., 1999). They report that the regions from −3765 to −2975 and −2062 to −1056 appear to have a negative regulatory function; the −2975 to −2062 region exerts a positive influence on expression. NF-κB sites can be found in both positive and negative regulatory regions, but not all potential sites are necessarily functional, as has been demonstrated for the APP promoter (Motonaga *et al*., 2002). Thus, NF-κB may function to both up-regulate or down-regulate *BACE1* expression (Ge *et al*., 2004). The studies described here have characterized the role of NF-κB in rat BACE1 promoter expression. Taking advantage of a suitable promoter reporter construct, Lange-Dohna *et al*. found that deleting the NF-κB binding site increased BACE1 expression 220% (BPR-Del vs. wt; Figure 2B).

It is of interest to note that the key regulatory elements present in the promoters of genes with relevance to protein processing and neurotransmitter function in AD display wild-type suppressor activity. For example, NF-κB is a suppressor of the choline acetyltransferase gene in brain (Toliver-Kinsky *et al*., 2000). This is consistent with the

hypothesis that Alzheimer's pathology reflects in part a transcriptional failure induced by impaired transcription factor function or altered protein and DNA binding equilibrium. Such transcriptional perturbations may contribute to the reported increase in BACE1 protein levels and enzymatic activity in the AD brain (Fukumoto *et al*., 2002; Holsinger *et al*., 2002). Based on published evidence on transcription factor activation in the AD brain and our preliminary results, NF-κB is a good candidate. Transgenic mice, which display β-amyloid pathology, can lose their capacity to generate β-amyloid peptides and to form β-amyloid plaques when crossed with homozygous BACE1 knock-out mice (Cai *et al*., 2001; Luo *et al*., 2001). The observation that these mice display a normal phenotype indicates that targeting BACE1 may be a useful therapeutic strategy (Roberds *et al*., 2001; Citron, 2002; Vassar, 2001; Roggo, 2002; Ghosh *et al*., 2002; Tounge and Reynolds, 2003). Here we will identify the role of a transcription factor, NF-κB, which modulates BACE1 expression.

NF-κB FUNCTION

NF-κB was originally identified by it's binding an enhancer element in the first intron of the immunoglobin κ light chain gene in B cells. The transcription factor NF-κB belongs to a family of homo- and hetero-dimeric proteins related by a conserved ~300 residue NH_2–terminal Rel/homology domain that includes p50, p65 (or RelA), p52 (or p49), c-Rel and RelB proteins (Bours *et al*., 1990; Schmid *et al*., 1991; Bours *et al*.,

1993; Nolan *et al*., 1991; Ruben *et al*., 1991). Heterodimerization of NF-κB proteins produces species with different intrinsic DNA-binding specificities (p52, p50) and transactivation properties (p65, c-Rel), an important functional distinction (Baeuerle, 1991; Chen *et al*., 1998; Siebenlist *et al*., 1994). The most commonly described active subunit combination (Flohe *et al*., 1997) is the p50/p65 heterodimer, present in cytoplasm bound to an inhibitory Iκ-B subunit. There are several inhibitory Iκ-B proteins (Iκ-Bα, Iκ-Bβ, Iκ-Bγ, Iκ-Bζ and Bcl-3). Upon stimulation, Iκ-B is phosphorylated, ubiquitinated and degraded (Ghosh and Baltimore, 1990; Liu *et al*., 1993), exposing nuclear localization signals on NF-κB proteins that allow translocation to the nucleus for DNA-binding. NF-κB is stimulated by oxidative stress or receptor ligands via increased Iκ-B degradation and NF-κB nuclear translocation but also via an independent pathway involving Bcl-3 (Gozal *et al*., 1998; Zhang *et al*., 1998; Qiu *et al*., 2001).

Bcl-3, of the Iκ-B family, functions in the nucleus by drawing NF-κB p50 or p52 homodimers away from NF-κB binding sites in promoters (Bours *et al*., 1993; Franzoso *et al*., 1993; Fujita *et al*., 1993; Nolan *et al*., 1993; Bundy and McKeithan, 1997). Thus, Bcl-3 can inhibit p50 or p52 homodimer binding to promoter sites (Nolan *et al*., 1993; Siebenlist *et al*., 1994; Franzoso *et al*., 1993; Zhang *et al*., 1998) allowing cRel/p50 or p65/p50 binding and activation (Heissmeyer *et al*., 1999; Qiu *et al*., 2001). In addition Bcl-3 contains a transactivation domain and may actively participate in transcriptional activation (Chen and Greene, 2004).

There is a generic NF-κB DNA consensus binding sequence of 10 bp, 5'-GGGRNNYYCC-3' (G = guanine, R = purine, N = any nucleotide, Y = pyrimidine, C = cytosine), that encompasses multiple sequences to which different NF-κB proteins can bind. Distinct NF-κB subunit combinations bind preferentially to different DNA sequences, an important factor in NF-κB transcriptional regulation (Perkins *et al*., 1992; Toliver-Kinsky *et al*., 2000; Glasgow *et al*., 2000; Qiu *et al*., 2004, 2001). As determined by MOTIF search sequence homology of the NF-κB binding sequence in the BACE1 promoter indicates that the following subunit binding affinities exist: 92% c-Rel, 86% p65, 83% p65/p50, and 80% p50.

NF-κB, BACE1 AND OXIDATIVE STRESS

Constitutive NF-κB activity in the brain is localized to neurons and synaptic terminals (Kaltschmidt *et al*., 1994; Schmidt-Ulrich *et al*., 1996; Bhakar *et al*., 2002; Lilienbaum and Isreal, 2003). NF-κB activation is regulated via both intrinsic and extrinsic stimuli, including interleukin-1β (IL-1β), tumor necrosis factor (TNF-α), H_2O_2, and hypoxic or ischemic insults (Koong *et al*., 1994a; 1994b; Tamatani *et al*., 1999; Schmidt *et al*., 1995a; Schmidt *et al*., 1995b; Clemens *et al*., 1997a; Clemens *et al*., 1997b; Salminen *et al*., 1995), in the cortex after traumatic brain injury (Yang *et al*., 1995), and in the limbic structures after seizures (Rong and Baudry, 1996). Activation of NF-κB can be either a pro-apoptotic (Kessler *et al*., 1993; Lin *et al*., 1995; Grilli *et al*.,

1996; Clemens *et al*., 1997a; Pizzi *et al*., 2002) or an anti-apoptotic event (Beg and Baltimore, 1996; Van Antwerp *et al*., 1996; Barger and Mattson, 1996a; Barger and Mattson, 1996b; Goodman and Mattson, 1996; Taglialatela *et al*., 1997b). The role of NF-κB in apoptosis in the central nervous system is not fully understood. It is neuro-protective against oxidative insults (Blondeau *et al*., 2001) but when persistently active may lead to neuronal death (Clemens *et al*., 1998).

Dramatic changes in NF-κB occur with age, with tissue-specific increases and decreases in NF-κB activity (reviewed by Giardina and Hubbard, 2002). NF-κB subunit distribution, specifically c-Rel levels, may be altered. Based on sequence homology, c-Rel is the primary subunit responsible for the repressive action of NF-κB in the BACE1 promoter. We postulate that the age and AD-related increase in BACE1 expression takes place despite an increase in NF-κB activity (normal repressive function) due to a shift in availability of the different NF-κB subunits. Pizzi *et al*. (2002, 2005) reported a NF-κB subunit specific response to the modulators of cell viability, IL-1β and glutamate. IL-1β activated the p50, p65, and c-Rel subunits of NF-κB, whereas glutamate activated only the p50 and p65 proteins thus, c-Rel was essential for IL-1β-dependent cell survival.

Aging is associated with oxidative stress (Ames, 2003; Li and Holbrook, 2003; Golden *et al*., 2002). High concentrations of iron, copper, and zinc have been located in amyloid in AD brains. Cu^{2+} and Zn^{2+} bind β-amyloid, inducing aggregation and give rise to reactive oxygen species (Maynard *et al*., 2005). Several lines of evidence suggest that enhanced oxidative stress is involved with either the pathogenesis and/or the progression

of AD (Multhaup *et al*., 2002). Markers of oxidative stress are readily found in AD brains: increased protein carbonyl formation (Smith *et al*., 1991), protein nitration (Good *et al*., 1996; Smith *et al*., 1997), lipid peroxidation (Balazs and Leon, 1994; Lovell *et al*., 1997; Hensley *et al*., 1994; Subbarao *et al*., 1990; Sayre *et al*., 1997) and DNA oxidation (Mecocci *et al*., 1994; Nunomura *et al*., 1999a; Nunomura *et al*., 1999b). Oxidative stress results in increased expression and activity of BACE1 (Tamagno *et al*., 2002) and has been shown to increase the vulnerability of APPsw bearing cells to apoptosis. Oxidative stress results in the accumulation of β-amyloid in cells which in turn promotes further oxidative stress (Misonou *et al*., 2000). Therefore β-amyloid can induce oxidative stress creating a self-propagating cycle of oxidative stress and β-amyloid production (Pappolla and Ogden-Epker, 2000). Both β-amyloid and hydrogen peroxide induce oxidative stress and cell cytotoxicity in SK-N-SH-SY5Y cells (Olivieri *et al*., 2003).

The oxidative processes associated with β-amyloid are: (1) the direct interaction of β-amyloid aggregates with neuronal membranes; (2) the induction of radicals including nitric oxide, through microglial activation; and/or (3) the radicalization of β-amyloid. Exposure of neurons (PC12 cells or rat primary cortical neurons) to high concentrations of β-amyloid can induce rapid membrane disintegration and the breakdown of membranous structures (Behl *et al*., 1994a). Furthermore, it has been shown that: (1) antioxidants and the H_2O_2-detoxifying enzyme catalase are neuroprotective against β-amyloid toxicity; (2) an elevation of intracellular H_2O_2

correlates with β-amyloid neurotoxicity with lipid peroxidation occurring as the final step in these cellular systems (Behl *et al*., 1994b). Therefore, H_2O_2 is one mediator of β-amyloid toxicity. H_2O_2 can also function as an inducer of the activity of NF-κB. The activity of NF-κB is also increased by β-amyloid (Behl *et al*., 1994b) and other fibril-forming peptides (Lezoualc'h and Behl, 1998), another feature that amyloidogenic peptides share (Figure 3). Bales *et al*. (1998, 1999) suggest that β-amyloid-induced neurotoxicity and astrocyte activation may be mediated by NF-κB and that alterations in NF-κB-directed gene expression may contribute to both the neurodegeneration and inflammatory response which occurs in AD.

Although the BACE1 promoter sequence contains several putative cytokine binding sites, cell culture studies have shown that *BACE1* mRNA production is not increased by treating neuronal or astrocytoma cell cultures with TNF-α, IL-1β, IL-6, transforming growth factor-beta (TGF-β), or several other cytokines (Satoh and Kuroda, 2000). β-amyloid "activation" of glia is an early and critical pathogenic event in the development of AD (Bales *et al*., 1999). An upregulation of both Iκ-Bα mRNA and protein, which occurs in cortical neurons exposed to β-amyloid, may be responsible for retaining NF-κB in the cytoplasm accounting for the observed decrease in activated NF-κB (Bales *et al*., 1998). This hypothesis is supported by the observation that pretreatment of cortical cultures with an antisense oligonucleotide to Iκ-Bα mRNA is neuroprotective. In contrast, exposure of rat primary astroglial cultures to β-amyloid results in a concentration- and time-dependent activation of NF-κB with subsequent upregulation of

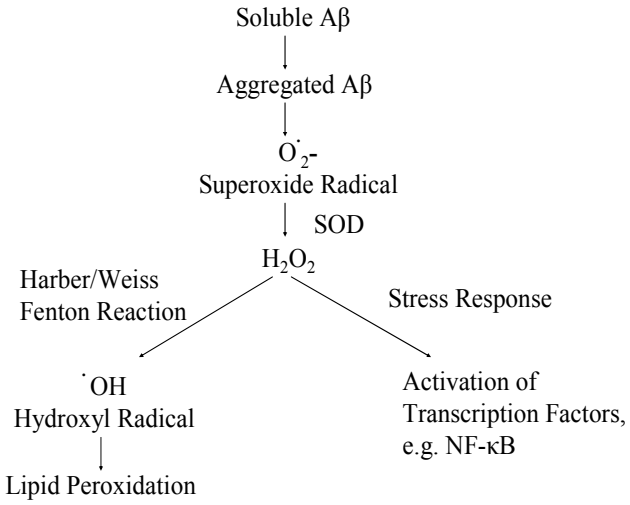


FIGURE 3. Oxidative Stress Induction by Aβ. Model for the induction of oxidative stress by aggregated β-amyloid: hydrogen peroxide as the mediator of β-amyloid toxicity and as an inducer of a stress response. Aggregated β-amyloid can cause the intracellular accumulation of hydrogen peroxide and related peroxides. This may lead to the formation of hydroxyl radicals via the Fenton reaction and ultimately to the peroxidation of membrane lipids and cell death (Behl *et al*., 1994b). In addition, hydrogen peroxide can stimulate the activation of transcription factors such as NF-κB (Behl, 1997).

IL-1β and IL-6. Hartlage-Rubsamen *et al*. (2003) have shown that BACE1 protein is expressed by reactive astrocytes in close proximity to β-amyloid plaques in the brains of aged transgenic Tg2576 mice and AD patients. They report additional studies wherein using six different experimental strategies to activate brain glial cells, there was no detectable expression of BACE1 protein by activated microglial cells of the amoeboid or ramified phenotype in any of the lesion paradigms studied. In contrast, BACE1 expression by reactive astrocytes was evident in delayed and chronic, but not in acute, models of gliosis. The exact mechanism of β-amyloid induced neurotoxicity is unclear; however neurotoxicity is directly correlated to the aggregation state of the β-amyloid peptide used, and the culture conditions employed (Pike *et al*., 1991).

Exposure of cultured neurons to β-amyloid has been associated with the induction of free radical formation either directly by the β-amyloid peptide itself or as a result of cellular free radical generation. Attenuation of β-amyloid-induced neurotoxicity, however, by either antioxidants or Ca^{2+} ion channel blockers / Ca^{2+} chelators has met with limited success indicating that β-amyloid-induced neurotoxicity is not mediated solely by any one of these mechanisms but perhaps by several mechanisms acting in concert.

Astrocytes may play a significant role in beta-amyloid production under pathological conditions and may be more important under physiological conditions. β-amyloid induced neurotoxicity as well as astrocyte activation may be mediated by the NF-κB family of proteins, and thus alterations in NF-κB-directed gene expression may

contribute to both the neurodegeneration and inflammatory response which occurs in Alzheimer's disease.

SUMMARY

The brains of Alzheimer's disease patients display cerebrovascular and parenchymal deposits of β-amyloid peptides, which are derived by proteolytic processing of the amyloid precursor protein. The β-site APP-cleaving enzyme is required for the generation of β-amyloid peptides. The NF-κB binding DNA consensus sequence in the BACE1 promoter upstream of the gene's transcription start site suggests a role for NF-κB in the expression of neuronal brain BACE1 (Lange-Dohna *et al*., 2003). Aberrant activation NF-κB responses to stress occur in the aged and AD brain. This results in an alteration of NF-κB regulation of BACE1 which may account for alterations in cell specific BACE1 transcription and processing of β-amyloid protein.

HYPOTHESIS

Our long-term goal is to characterize the signal transduction pathways responsible for increased levels of β-amyloid associated with AD. Our working hypothesis is that the transcription factor Nuclear Factor-kappa B differentially regulates BACE1 transcription in a cell specific manner. Specifically, that NF-κB represses BACE1 transcription in neurons and stimulates BACE1 transcription in activated

astrocytes. A corollary is that the NF-κB regulation of BACE1 is altered by β-amyloid effects on astrocytes and by extension in AD. In addition neuronal and astrocytic BACE1 expression is altered by hexameric metal containing peptides. Activation of NF-κB responses to stress has been shown to be perturbed in the aged and AD brain. Thus, alteration of NF-κB regulation of BACE1 may account for increases in BACE1 transcription and processing of β-amyloid protein.

CHAPTER TWO: METHODS

MOTIF SEARCH

A Motif search was conducted by searching with a DNA query sequence, in this instance the full length rat BACE1 promoter region (Lange-Dohna *et al*., 2003) against Trans-acting factor library. The server supports the TRANSFAC database which collects eukaryotic cis-acting regulatory DNA elements and trans-acting factors. The server allows a search of a nucleotide sequence against the TRANSFAC database using a program named TRANSFACFind based on the dynamic programming algorithm written in ICR, Kyoto University. TRANSFAC database contains several types of sequence motifs. In this service TFMATRIX (TRANSFAC binding site distribution matrix) library is searched. TFMATRIX contains matrix data of several organisms (shown in BF lines and represented at the first character of the record in ID line, for example V$XXXX_01 for Vertebrates) (Heinemeyer *et al*., 1999). The search was conducted using all species available, from bacteria to human and with a 40% homology cut off.

CELL CULTURE

PC12 Cells

The rat pheochromocytoma PC12 is a well established neuronal cell model when differentiated by nerve growth factor (NGF)-treatment yields neuronal phenotype

wherein the cells become elongated, form aggregates, and display enhanced neuritogenesis. First established in 1976 by Greene and Tischler from a rat adrenal tumor, PC12 have been commonly used as a model of sympathetic neurons as a result of their ability to acquire a sympathetic neuronal phenotype upon exposure to NGF (Greene and Tischler, 1976). Sympathetic neurons and chromaffin cells are derived from a common neural crest-derived progenitor (Greene, 1978). During development, the presence of NGF in the absence of glucocorticoids promotes the differentiation of progenitors into sympathetic neurons.

The de-differentiated state of PC12 cells partially returns them to a condition similar to their progenitor ancestors, restoring their responsiveness to NGF. NGF induces morphological changes, such as the extension of neurites and the upregulation of those enzymes involved in catecholamine biosynthesis, such as tyrosine hydroxylase (Tischler and Greene, 1975; Hatanaka, 1981; Greene *et al*., 1984). In addition, differentiated PC12 cells are electrically excitable and are capable of synthesis, storage, and release of dopamine, norepinephrine, enhanced APP production (Rossner *et al*., 1998a; Rossner *et al*., 1998b; Cosgaya *et al*., 1996) and enhanced choline acetyltransferase activity (Toliver-Kinsky *et al*., 2000).

PC12 cultures obtain from Lloyd Greene, Columbia University, were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 5% (v/v) fetal calf serum (FCS: JRH Biosciences), 5% (v/v) donor horse serum (DHS: JRH Biosciences), and a 1% (v/v) penicillin streptomycin neomycin (PSN: Gibco BRL) antibiotic mixture. Cells were

grown in a humidified cell incubator at 37°C under a 5% CO_2 atmosphere (37°C, 95% O_2, 5% CO_2). Stock cultures were grown in 75cm^2 tissue culture flasks, fed three times weekly, and divided once a week using vigorous shaking to dislodge cells from flasks. For all experiments, cells were seeded at a final density of 1.8 x 10^5 cells/35mm^2 well/6-well plate and allowed to attach overnight. Cells were re-fed with complete media supplemented with 50-100ng/mL NGF (Perez-Polo laboratory isolated from male mouse submaxillary salivary glands, Perez-Polo and Shooter 1975) for 24-120 hours. NGF-induced PC12 differentiation was confirmed visually by phase contrast microscopy.

SK-N-SH-SY5Y Cells

The human neuroblastoma SK-N-SH-SY5Y cell line is a well established neuronal model characterized by a retinoic acid (RA: Sigma-Aldrich)-differentiated neuronal phenotype wherein the cells become elongated, slender, display enhanced neuritogenesis, and enhanced APP expression with an increase in the neuronal APP transcript APP695 mRNA (Konig *et al*., 1990; Murray and Igwe, 2003).

SK-N-SH-SY5Y cultures obtained from June Biedler, Sloan Kettering Cancer Institute, were maintained in HAM F12 (Gibco Invitrogen Corporation) supplemented with 15% (v/v) FCS, and a 1% (v/v) PSN antibiotic mixture. Cells were grown in a humidified incubator (37°C, 95% O_2, 2% CO_2). For all experiments, cells were seeded at a density of 6 x 10^5 cells/ /35mm^2 well/6-well plate and allowed to attach overnight. Cells

were re-fed with complete media supplemented with 10μM RA and incubated for 5 days. RA-induced differentiation was confirmed visually by phase contrast microscopy.

C6 Rat Glial Cells

The rat astrocytoma cell strain, C6, is a well established glial cell model. The C6 cell line was cloned from a rat glial tumor induced by N-nitrosomethylurea by Benda *et al*. (1968) after a series of alternate culture and animal passages. C6 cells have a documented functional NF-κB system with the ability to respond to H_2O_2, Aβ, LPS, IL-1β and tumor necrosis factor alpha (TNFα) exposure (Farrell *et al*., 1987; Nomura, 2001; Grobben *et al*., 2002; Lee *et al*., 2002). In particular exogenous application of β-amyloid ($A\beta_{25-35}$) significantly elevated levels of reactive oxygen species (ROS) in C6 astroglioma cells (Lee *et al*., 2002).

C6 cultures obtained from Jean DeVellis, UCLA, were grown in Dulbecco's modified Eagle's medium (DMEM)/HAM F-12 medium (1:1) (Gibco Invitrogen Corporation), 10% FCS, and a 1% (v/v) PSN antibiotic mixture. Cells were maintained in a humidified incubator (37°C, 95% O_2, 5% CO_2). Stock cultures were grown in 75cm^2 tissue culture flasks, fed three times weekly, and divided once a week using Puck's EDTA to dislodge cells from flasks. When treated with TNFα, a final concentration of 1μg/μl was added to each well of a six well plate of cells (TNFα: Calbiochem, Tumor Necrosis Factor-α Mouse, Recombinant, *E.coli.)*. For all experiments, cells were seeded at a density of 1.8 x 10^5 cells /35mm^2 well/6-well plate and allowed to attach overnight.

Primary Neonatal Rat Astrocytes

Primary cultures of cortical astroglial cells were prepared from newborn (postnatal day 3) Sprague-Dawley rats as previously described (Hutton and Perez-Polo, 1995). After careful removal of meninges, the cortices were dissociated by passage through 5ml pipette followed by filtration through a 60 micron nylon monofilament mesh. The medium consisted of DMEM/HAM F-12 medium (1:1), 10% FBS, and a 1% (v/v) PSN antibiotic mixture. When cells were treated with phorbol 12-myristate 13-acetate (PMA: Promega, Madison WI), 20µl/2ml of 5µM stock was added. PMA is a phorbol ester that affects PKCs by mimicking diacylglycerol, a natural ligand and activator of protein kinase C (PKC), (Newton, 1995; Chiloeches *et al*., 1999; Acs *et al*., 1997; Blumberg, 1980; Jacobson *et al*., 1975). Cells were plated at a density of 1 x 10^5 live cells/cm^2 in 75cm^2 flasks and allowed to grow to confluence prior to shake-off. Loosely attached oligodendroglial and microglial cells were removed by a 72 hour shake-off in a rotary shaking incubator set at 250 rpm at 37 °C. Culture medium was changed every 3 days.

Adult Rat, Guinea Pig and Mouse Astrocytes

Primary cultures of cortical astroglial cells were prepared from female or male adult (3 weeks-6 months) Sprague-Dawley rats, Hartley guinea pigs and Swiss Webster mice as previously described (Wyss-Coray *et al*., 2003). Guinea pigs express the human sequence and have high activity of the β-secretase pathway. Additionally, higher amounts

of brain tissue and CSF samples that can be collected. Guinea pig β-amyloid peptides form higher molecular weight structures such as oligomers, which are reported to be involved in the suppression of long-term potentiation and that have the possibility of β-amyloid plaque formation in a more chronic process than in APP-transgenic mice. Thus guinea pigs represent a more physiological model to examine APP processing and β-amyloid plaque formation *in vivo* (Beck *et al*., 2003). Additionally, APP processing in guinea pig primary neuronal cultures has been shown to be similar to APP processing in cultures of human origin (Holzer *et al*., 2000). TNF-α is one of the many cytokines released by microglia located in the vicinity of the amyloid plaque.

After careful removal of meninges, the cortices were dissociated in Ca^{2+}- and Mg^{2+}-free HBSS with 0.25% trypsin, 1 mM EDTA and 0.04 mg/ml bovine pancreatic DNase I for adult cortices, for 30 minutes at 37°C on a rotary shaker at 150 rpm. The medium consisted of DMEM/HAM F-12 medium (1:1), 10% FBS, and a 1% (v/v) PSN. Cells were plated at a density of 1 x 10^5 live cells/cm^2 in 75cm^2 flasks and allowed to grow to confluence prior to shake-off. Loosely attached oligodendroglial and microglial cells were removed by a 72 hour shake-off in a rotary shaking incubator set at 250 rpm at 37 °C. Culture medium was changed every 3 days of cultivation. When cells were treated with PMA, 20µl/2ml of 5µM stock was added. When cells were treated with TNF-α, 20µl/2ml of 1µg/ml stock was added. Astrocyte purity was determined by Western Blot using antibodies specific for glial fibrillary acidic protein (Santa Cruz Biotechnology).

MUTANT PROMOTER CONSTRUCTION

The construct containing the wild type rat BACE promoter (BPR) was obtained from Dr. Rossner, University of Leipzig, Germany (Lande-Dohna *et al*., 2003). Previously Lange-Dohna *et al*. (2003) cloned a 1.5-kb segment of the sequence upstream of the translational start codon of the rat BACE1 amplified from a GenomeWalker Kit (Clontech; Palo Alto, CA), using the specific reverse primer 5'-GGCAGGCAGCATTCCCGAGC-3' and the nested primer 5'-CCACCCATAGCAGGAGCCAG-3'. The sequences were retrieved from GenBank (accession number AF190727). The DNA fragment created was sequenced (Toplab). From this sequence, a specific forward primer, 5'-ATGCTAAGCTTAGATCTATGGTGGCTGGATTTTATCG- 3', was selected, which contains a restriction site for BglII. This site and the NcoI site around the translational start codon were used to clone the fragment into the pGL3-basic vector (Promega) to create the BPR-pGL3 construct driving the expression of firefly luciferase. The pGL3 – basic vector lacks a promoter. We mutated the rat BACE1 NF-κB sequence by site-directed mutagenesis leaving the remainder of the promoter intact.

The BPRΔNF-κB$^-$ containing vector was constructed by the Sealy Center for Molecular Science Recombinant DNA Laboratory (University of Texas Medical Branch, Galveston, TX). The NF-κB mutation was created by replacing the NF-κB consensus sequence nucleotides within the BACE1 promoter fragment (-1521) AGGGCTTTCCA (-1511) with an 11-bp sequence (-1521) AGATCTA ATCA (-1511) that contains a unique

BglII restriction site. This replacement mutagenesis was performed using a PCR-based strategy and the following specific forward primer 5'-ATGCTAAGCTTAGATCTATGGTGGCTGGATTTTATCG- 3', was selected, which contains a restriction site for BglII. The BACE1 mutated promoter was subcloned into pGL3 basic. The construct was confirmed by DNA sequence analysis. The mutated fragment (BPRΔNF-κB-, underlined) was cloned in the same site of the NF-κB wild type sequence in the vector as the wild-type (BPR) construct and then used in transfections.

PROMOTER ACTIVITY ASSAYS

Cells were plated in 6 well plates (16mm^2) at 9x10^{4}cells/ml and allowed to attach overnight. When cells were differentiated they were incubated with complete medium supplemented with 50-100ng/mL Renin-free β-NGF for PC12 or 10μM RA for SK-N-SH-SY5Y for 24 hours. Neuronal cells were transfected 3:1 (wt/wt) liposome: DNA ratio using DMRIE-C cationic liposome reagent (Invitrogen) and OPTI-MEM reduced-serum medium (GibcoBRL) and glial cells were similarly transfected at 10:1 (wt/wt) liposome: DNA ratio according to manufacturer's instructions for 5 hours, after which ½ final volume was replaced with complete medium with NGF or RA supplemented for neuronal cultures. Glial cells were used either nontreated or treated with TNFα or PMA a commonly used activator of PKC. Transfected cells were collected by scraping with cell scrapers (Corning) after 48 hrs by rinsing with phosphate-buffered saline (PBS) and lysed in 100-200μl of Passive Lysis Buffer (Promega). Lysates were kept on ice, briefly

vortexed, cleared by microcentrifugation (12,000g, 1 min), transferred to microfuge tubes, and stored -80°C. Firefly and Renilla Luciferase activities in 20 µl of lysate were assayed using either the Dual Luciferase Assay System (Promega) or the Single Luciferase Assay System (Promega). A minimum of 4 six-well plates (35mm^2 well) per treatment were used per each of three separate experiments.

IMMUNOCYTOCHEMISTRY

PC12, SK-N-SH-SY5Y or C6 cells transfected 3:1 (wt/wt) liposome: DNA ratio for neurons or 10:1 for C6 cells were plated in 6-well plates at 9 x 10^4 cells /ml/16mm^2 well and allowed to attach overnight on poly-D-lysine pre-coated cover slips and then half of the neuronal cultures were differentiated with 100ng/mL NGF for PC12 or 10µM RA for SK-N-SH-SY5Y for 24 hours. 4% paraformaldehyde warmed to 37°C was dropped gently into the culture medium over the cells, adding approximately the same volume as culture medium for 5 minutes at room temperature. Medium was removed and 1ml full strength paraformaldehyde was added for 10 minutes at room temperature then rinsed 3X with PBS. Endogenous peroxidase activity was quenched with 500µl 3% H_2O_2 then washed with 1 ml PBS. Non-specific binding sites were blocked by 20 minutes incubation at room temperature in 500µl 20% normal goat serum. Cells were incubated with 500µl primary antibody to BACE1 at 1:100 (Chemicon International or Affina Immuntechnik) then washed with PBS. Cells were incubated with 500µl link antibody (LSAB2 kit, DAKO), washed with 1ml PBS, incubated with 500µl streptavidin conjugate

(LSAB2 kit, DAKO), washed with 1ml PBS and developed with 500µl DAB (Sigma FAST DAB). Cover slips were washed with 1ml PBS, air dried, dehydrated in a series of EtOH and xylene and mounted in Permount (Fisher Scientific).

H_2O_2 TREATMENT OF CELLS

Twenty-four hours prior to H_2O_2-treatment, C6, adult astrocytes, PC12 or SK-N-SH-SY5Y cells were plated at a density of 9 $x10^4$ (C6, adult astrocytes and PC12) or 3 $x10^5$ (SK-N-SH-SY5Y) cells per $35mm^2$ well/6-well dish in RPMI 1640 medium supplemented with 5% FBS, 5% DHS, and 1% PSN (PC12) or HAM F-12 supplemented with 15% (v/v) FBS, and 1% PSN (SK-N-SH-SY5Y) or DMEM/HAM F-12 medium (1:1), 10% FCS, and a 1% (v/v) PSN (C6 and adult astrocytes) in humidified air (37°C, 95% O_2, 5% CO_2).

Cells were differentiated, transfected and allowed to recover for 24 hours. To treat cells with H_2O_2, dilutions of fresh H_2O_2 (30%) were made in PBS and were added directly to the cells. The cells were incubated in H_2O_2 for 40 minutes at room temperature. The cells were allowed to recover in NGF or RA supplemented complete media for the specified times. 24 hour after treatment, the cultures were harvested for luciferase measurements, and Western blot assay. A minimum of 4 six-well plates ($35mm^2$ well) per treatment were used per each of three separate experiments.

$A\beta_{1-42}$ TREATMENT OF CELLS

Twenty-four hours prior to $A\beta_{1-42}$ (American Peptide Company, Sunnyvale, CA) exposure C6 rat glioma, adult astrocyte, PC12 or SK-N-SH-SY5Y cells were plated at a density of 1.8 $x10^5$ cells per $35mm^2$ well/6 well dish in DMEM/HAM F-12 medium (1:1) medium supplemented with 10% FBS, and 1% PSN (C6 and adult astrocytes), RPMI 1640 supplemented with 5% (v/v) FBS, 5% (v/v) DHS, and a 1% (v/v) PSN antibiotic mixture (PC12) or HAM F-12 supplemented with 15% (v/v) FBS, and 1% PSN (SK-N-SH-SY5Y) in humidified air (37°C, 95% O_2, 5% CO_2). Neuronal cells were differentiated, transfected and allowed to recover for 24 hours. Recombinant human $A\beta_{1-42}$, was then added at a final concentration of 0.1, 1, 10 and 50μM with the reverse $A\beta_{42-1}$ peptide or recombinant human insulin (Gibco Invitrogen) as control. The $A\beta_{1-42}$ and insulin control were used soluble, freshly prepared at 100μM in 200mM HEPES pH8.5 or aggregated by aging the fresh preparation at 37°C under a 5% CO_2 atmosphere for 5-7 days. The cells were incubated in $A\beta_{1-42}$ in a humidified incubator (37°C, 95% O_2, 5% CO_2). The cells were allowed to recover in supplemented complete media for the specified times. 24-144 hour after treatment, the cultures were harvested for luciferase measurements and Western blot assay. A minimum of 4 six-well plates ($35mm^2$ well) per treatment were used per each of three separate experiments.

INSULIN TREATMENT OF CELLS

PC12 were plated at a density of 9 x10^4 cells per 35mm^2 well/6-well plate in RPMI 1640 supplemented with 5% (v/v) FBS, 5% (v/v) DHS, and a 1% (v/v) PSN antibiotic mixture in humidified air (37°C, 95% O_2, 5% CO_2). Cells were then given fresh complete media supplemented with 50-100ng/mL NGF for 5 days. To treat cells recombinant human insulin, insulin (Gibco) added at final concentrations of 1, and 10μM directly to the cells. Insulin was used soluble, or aggregated by aging the fresh preparation at 37°C under a 5% CO_2 atmosphere for 5-7 days. The cells were incubated with insulin in a humidified cell incubator at 37°C under a 5% CO_2 atmosphere. The cells were allowed to recover in supplemented complete media for the specified times. 24-96 hour after treatment, the cultures were harvested for luciferase measurements and Western blot assay. A minimum of 4 six-well plates (35mm^2 well) per treatment were used per each of three separate experiments.

R-FLURBIPROFEN TREATMENT OF CELLS

Flurbiprofen is a racemic nonsteroidal anti-inflammatory drug known to be anticarcinogenic. R-flurbiprofen is not a COX inhibitor at therapeutically relevant concentrations. R-flurbiprofen is antinociceptive and anti-inflammatory. It is believed to function upstream of the dissociation of the NF-κB-I-κB complex (Scheuren *et al*., 1998). It has been shown to inhibit NF-κB activation, LPS-induced nuclear translocation

of NF-κB and NF-κB dependent gene expression (Tegeder *et al*., 2001; Morihara *et al*., 2002). Primary cultures of guinea pig astroglial cells were exposed to R-flurbiprofen in PBS at varying concentrations for 24 hours. 24 hours after treatment, the cultures were harvested for protein extraction, and Western blot assay. A minimum of 4 six-well ($35mm^2$ well) plates per treatment were used per each of three separate experiments for luciferase activity measurements and Western blot analysis.

DECOY TREATMENT OF CELLS

Chemically modified complementary single-stranded "decoy" oligonucleotides containing one phosphothiorate group on both 5' and 3' end were synthesized by Sigma-Genosys (Woodlands, TX).

NF-κB BACE1 sequence: 5'-TTATCGAGGGCTTTCCACGCCC-3'.

Transcription factor binding sequences within the oligonucleotide is underlined. The single stranded oligonucleotides were annealed in sterile TE buffer (1 mM EDTA, 50 mM NaCl, 10 mM Tris, pH 7.5) at a stock concentration of 6 nmol/μl. Decoy oligonucleotide solution was added directly to the culture medium in the well to a final concentration of 1.6μg /μl.

NF-κB EXPRESSION VECTORS

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAD, NIH: pRSV-NK-κB2 (p49), pRSV-NK-κB1 (p50), and pRSV-RelA (p65) NF-κB from Dr. Gary Nabel and Dr. Neil Perkins (Gorman *et al*., 1983; Schmid *et al*., 1991; Perkins *et al*., 1992; Duckett *et al*., 1993). These expression vectors contain cDNAs from human p49, p50, and RelA/p65 subunits of NF-κB under the transcriptional control of the RSV long terminal repeat. Cells were transfected as described above. 24 hours after treatment, the cultures were harvested for protein extraction, luciferase measurements and Western blot assay. A minimum of 4 six-well (35mm^2 well) plates per treatment were used per each of three separate experiments for luciferase activity measurements and Western blot analysis.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

Probe preparation: As described in (Glasgow *et al*., 2000; Toliver-Kinsky *et al*., 2000; Qiu *et al*., 2001) DS and SS oligonucleotides were purchased and complementary strands reconstituted in Tris-EDTA (TE) buffer to a concentration of 1μg/μl. Oligonucleotides encompassing the IgG-κB enhancer sequence (GGGACTTTCC) and the BACE1 promoter NF-κB sequence (AGGGCTTTCCA) were used as probes and 5' labeled with γ-^{32}P-ATP and T4 polynucleotide kinase. Equal amounts of complementary strands were mixed together, heated above the Tm for 15 minutes and slowly cooled. A

30 µg aliquot of recombinant p50 and p65 protein obtained from Norbert K. Herzog, UTMB, Galveston Texas, were preincubated in a reaction buffer (5 mM HEPES, pH 7.9, 4% glycerol, 0.2 mM EDTA, 20 mM KCl, 0.4 mM MgCl2, 2 mM DTT, and 0.4 mM PMSF) and 2µg poly dI-dC . Approximately 2 pmoles of oligonucleotide to be labeled were incubated with 20 µCi of ^{32}P-ATP (4500 Ci/mmole) and 10 units of T4 polynucleotide kinase in buffer. Reactions were terminated by the addition of 2M ammonium acetate and subsequent incubation at 70ºC for 10 minutes. Samples were cooled to room temperature, 12µg of total yeast RNA added (to facilitate precipitation), and precipitated by addition of 3X of EtOH followed by centrifugation. DNA pellets were reconstituted in buffer, extracted by phenol/chloroform, and centrifuged. The upper aqueous layer was removed and precipitated and the radioactive pellet was dried, resuspended in 100µl TE buffer, and stored at -20ºC.

WESTERN BLOT ANALYSIS

20µg of cytoplasmic proteins were boiled for 5 minutes in an equal volume of sample buffer, and loaded onto a polyacrylamide gel. Samples were separated by electrophoresis in Tris-glycine buffer at 4ºC at 45 mAmp or on ice at 100-200V. Proteins were then transferred to an Immobilon-P® membrane at 4ºC at 25V, or on ice at 50V in transfer buffer. Membranes were subsequently incubated for 1 hour in primary antibody diluted in blocking buffer in Tris buffered saline (TBS)-Tween, and then washed 2X for 15 minutes in TBS-Tween. Primary antibodies were specific and diluted 1:1,000. The

anti-BACE1 antibody was obtained from Chemicon International. Membranes were incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG (Biorad), diluted 1:3000 in blocking buffer, for 1 hour, and then washed in 2 X TBS-Tween for 15 minutes each wash. Peroxidase activity was detected using the Amersham enhanced chemiluminescence lighting system (ECL).

STATISTICAL ANALYSES

Comparisons of the difference in means of two groups (±SEM) were carried out using the Student's t-test. Comparisons were two tailed. All statistical tests were carried out using InStat software. $P < 0.05$ was accepted as significant. Mean values (±SEM) for each experiment with more than two groups were calculated using one-way ANOVA. The Dunnett or Tukey-Kramer multiple comparisons test was used to determine p values.

VERTEBRATE ANIMALS

Description of Protocol

All procedures involving animals had been approved by the Animal Care and Use Committee at UTMB (Protocol # 91-02-020). Timed pregnant Sprague-Dawley rats were purchased from a commercial breeder (Harlan, Indianapolis, Indiana). Mothers were housed individually and fed a standard laboratory diet. Pups were spontaneously vaginally delivered and the day of birth defined as post-natal day 0 (P0). Pups were left

with their mother until taken for culture and checked daily for general good health. Pups of both genders were used in equal numbers. Primary cultures of cortical astroglial cells were prepared from newborn (postnatal day 3) Sprague-Dawley rats as previously described (Hutton and Perez-Polo, 1995). Adult animals of equal numbers of both genders were used (3 weeks-6 months) Sprague-Dawley rats (Harlan, Indianapolis, IN), Hartley guinea pigs (Charles Rivers Laboratories,Wilmington, MA), and Swiss Webster mice (Harlan, Houston, TX) were obtained and primary cultures of cortical astroglial cells were prepared as previously described (Wyss-Coray *et al*., 2003).

Justification

We used Sprague-Dawley rats, Swiss Webster mice and Hartley guinea pigs. Animals were sacrificed and brains dissected promptly for culture. All animal protocols, number of animals, and paradigms were approved by the UTMB Animal Care and Use Committee. The number of animals requested was determined by power analysis and our experience in a number of projects where similar measurements have been made (Rossner *et al*., 1994, 1995a, b, 1997a, b, c; Toliver-Kinsky *et al*., 1997; Wortwein *et al*., 1998; Yu *et al*., 1995, 1996). Non-mammalian species do not have similar enough central nervous system to serve as an adequate model for the human aging brain. These animals are particularly useful for studies looking at rat promoter function, as the cloned and sequenced BACE1 promoter is rat.

Veterinary Care

Professional veterinary care is available from 2 staff veterinarians and 3 staff veterinary technicians. The University of Texas Medical Branch (UTMB) operates to comply with the USDA Animal Welfare Act (Public Law 89-544) as amended by PL91-579 (1970), PL94-279 (1976), and 45 CFR37618 (6-3-80); Health Research Extension Act of 1985 (Public Law 99-158); follows the Public Health Service Policy on Humane Care and Use of Laboratory Animals (revised September 1986); and the Guide for the Care and Use of Laboratory Animals DHEW (NIH) 85-23 revised 1985. UTMB is a registered Research Facility under the Animal Welfare Act. It has a current Letter of Assurance on file with the Office of Protection from Research Risks, in compliance with NIH policy. The Animal Care Center is AAALAC-certified and under the direction of a Doctor of Veterinary Medicine, staffed by veterinarians with training and experience in laboratory animal medicine and surgery, clinical care and diagnostic pathology.

Euthanasia

The rats/mice/guinea pigs were euthanized by overdose of pentobarbital and death confirmed by decapitation after anesthesia. This method is approved by the Panel on Euthanasia of the American Veterinary Medical Association.

CHAPTER THREE: RESULTS

MOTIF SEARCH YEILDS NINE PUTATIVE NF-κB SITES

Screening with an 85% homology cut off, identified one NF-κB site in the rat BACE1 promoter as was previously reported by Lange-Dohna (2003). We refer to this 85% homology identified site as the "primary" NF-κB site. We next screened for additional NF-κB sites by reducing the homology. Using an 80% homology cut-off a total of nine putative NF-κB sites were identified (Figure 4). The various sites had overlapping homologies, yielding ten potential different subunit binding sites of which five had homology to the c-Rel binding consensus sequence, two to the p50 binding consensus sequence, two to the p65 binding consensus sequence and six to the p50/p65 binding consensus sequences. Sequence homology of the primary NF-κB binding sequence in the BACE1 promoter indicates that the following subunit binding affinities existed: 92% c-Rel, 86% p65, 83% p65/p50, and 80% p50. These results indicated that there were multiple potential sites for NF-κB to affect BACE1 promoter expression.

BACE1 DETECTED BY ICC AND WESTERN BLOT ANALYSES

We assessed whether rat and human BACE1 promoters are expressed similarly in the rodent PC12 and human SK-N-SH-SY5Y neuronal cell lines by immunocytochemistry that the cell lines are capable with and without

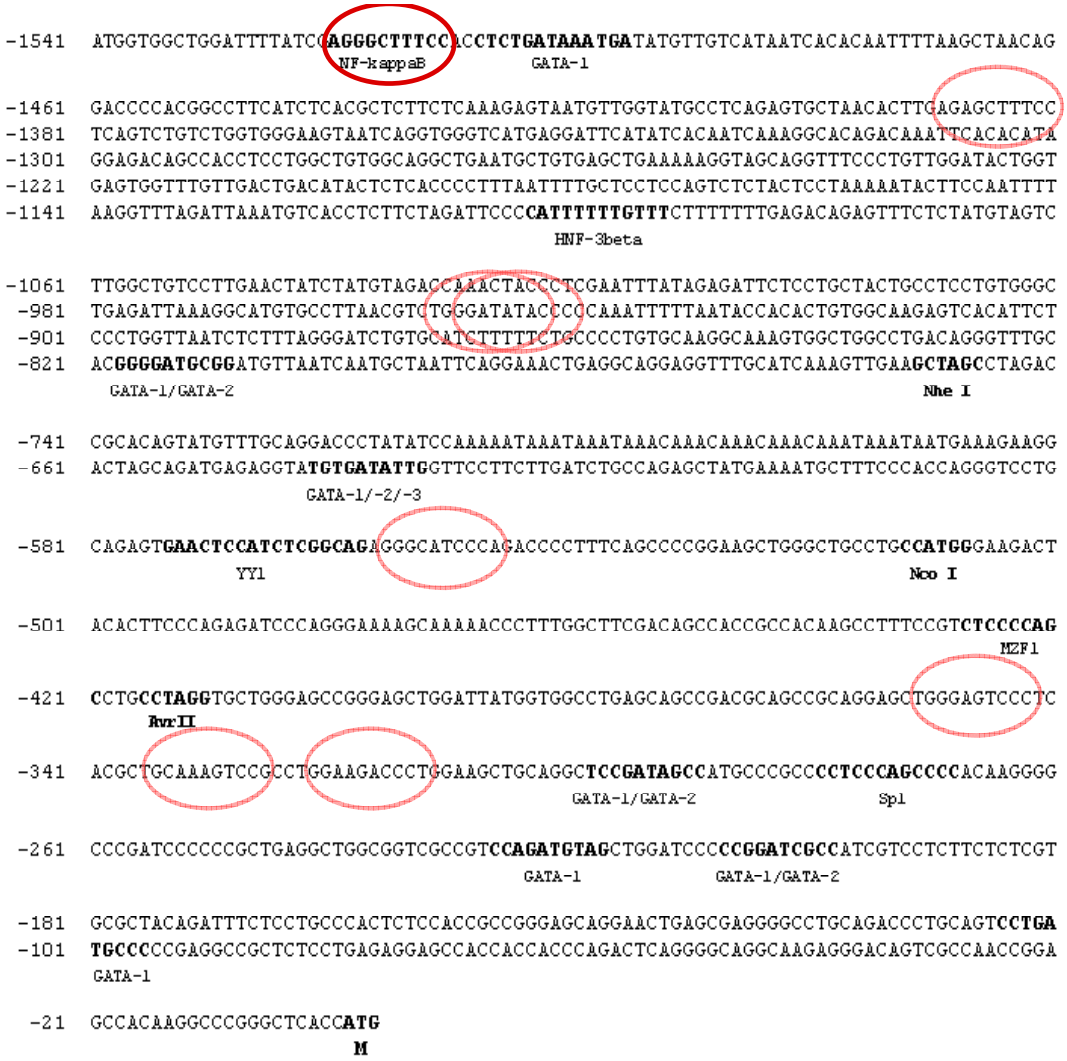


FIGURE 4. Motif Screen: 80% Homology Limit. Sequence of a 1.5-kb fragment upstream of the start codon of the rat BACE1 gene along with restriction sites and putative transcription factor binding sites as marked in bold (Lange-Dohna *et al*., 2003). Motif Screen with an 80% homology cut off; nine putative NF-κB sites were identified in the rat BACE1 promoter as indicated by the ovals. The "primary" site detected at a homology cut off of 85% was mutated in these studies is circled in darker red.

differentiation to express BACE1 (Figure 5A). Figure 5A illustrates that PC12 cells differentiated (+NGF:1-2) and non-differentiated (-NGF: 3-4), SK-N-SH-SY5Y cells differentiated (+RA: 5-6) and non-differentiated (-RA: 7-8) were capable of expressing BACE1 as detected by DAB staining (brown precipitate) using two different BACE1 antibodies which recognize different BACE1 domains (Chemicon: transmembrane and cytoplasmic domains amino acids 458-501 and Affina: end of the catalytic domain amino acids 440-454) (Chemicon: 1, 3 and Affina: 2, 4). Also illustrated are C6 astrocytic cells stained for BACE1 using the Chemicon antibody. The Western blots in Figure 5B illustrates that BACE1 protein was also detected in PC12 non-differentiated (-NGF), differentiated (+NGF), SK-N-SH-SY5Y non-differentiated (-RA) and differentiated (+RA), C6 and neonatal rat, adult rat, mouse and guinea pig hippocampus astrocytic cell lysates using the Chemicon BACE1 antibody.

BACE1 PROMOTER SPECIFICALLY BOUND SELECTIVE NF-κB SUBUNIT PROTEINS: EMSA

To determine if the BACE1 NF-κB site was capable of binding NF-κB subunit proteins we performed electrophoretic mobility shift assays (EMSAs) using the oligonucleotides displaying the IgG-κB enhancer sequence (control: GGGACTTTCC) and the rat BACE1 promoter NF-κB sequence ("primary" site: AGGGCTTTCCA) as probes for control and wild type rat NF-κB binding consensus sequences respectively

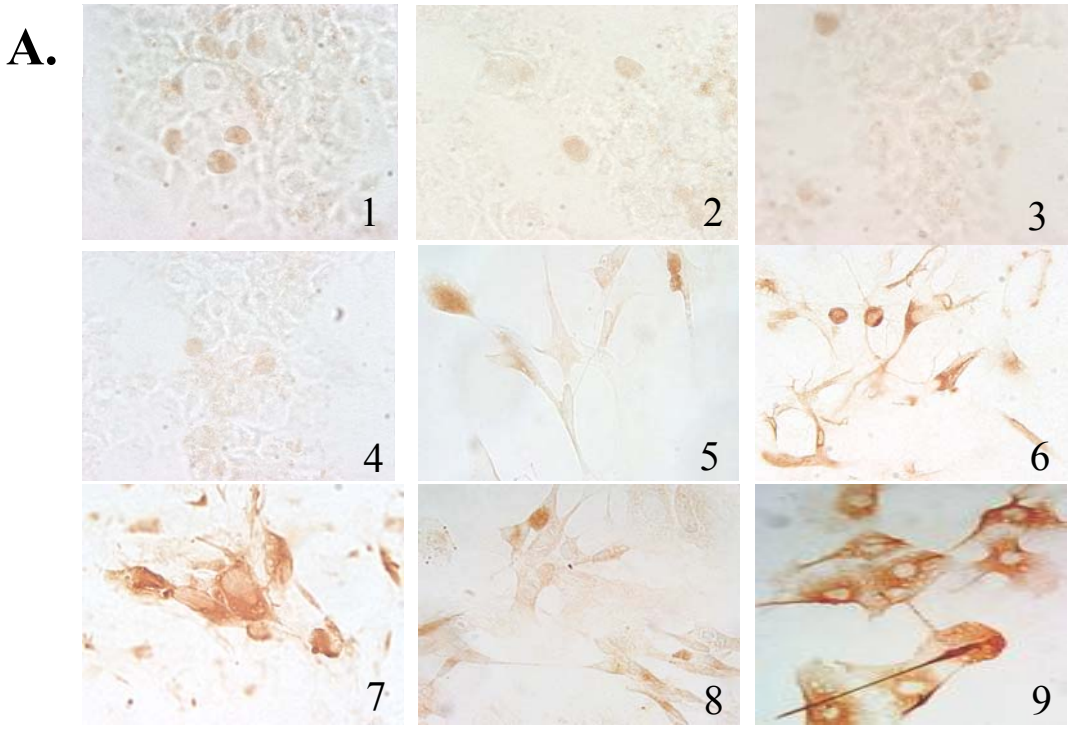


FIGURE 5.A. Immunocytochemistry. Immunocytochemistry in rat neuronal PC12 (1-4), human neuronal SK-N-SH-SY5Y (5-8) and rat glioma C6 (9) cells. All demonstrated endogenous BACE1 activity as detected by brown DAB precipitate. BACE1 immunoreactivity is detectable in both the neuronal cell lines in differentiated (1-2 +NGF or 5-6 +RA) and nondifferentiated (3-4 -NGF or 7-8 -RA) states. (Chemicon antibody: 1, 3, 5, 6, 9 and Affina antibody: 2, 4, 7, 8).

B.

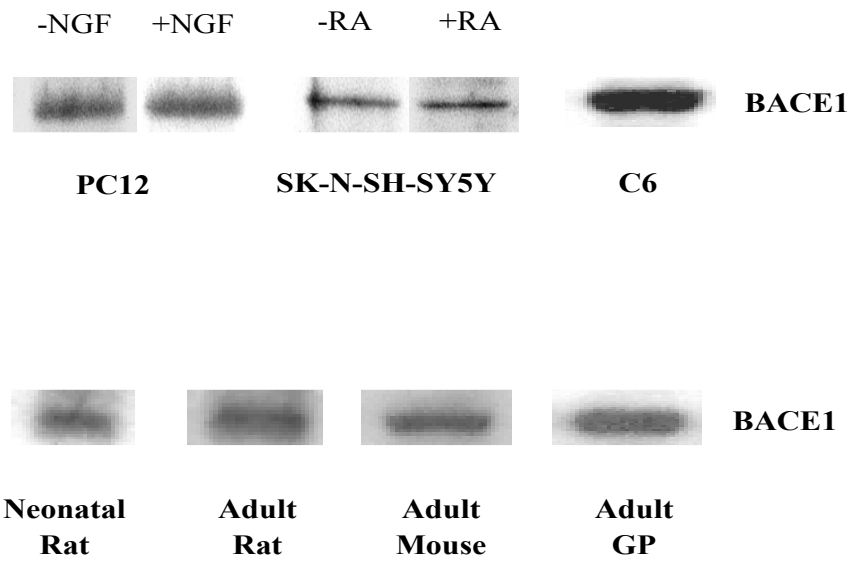


FIGURE 5.B. Western Blot Analyses. Western blot analyses indicated that endogenous BACE1 was present in all cell types analyzed. BACE1 protein was detected by Western blot analysis using Chemicon BACE1 antibody in non-differentiated (-) and differentiated (+) rat PC12, and human SK-N-SH-SY5Y, and the glial cells from the rat glioma cell line C6 and primary neonatal rat, adult rat, mouse and guinea pig (GP) hippocampus astrocytic cells.

together with isolated recombinant NF-κB proteins (Figure 6). As illustrated in Figure 6, the gel was loaded as follows: lanes 1 and 5 probe alone; lanes 2 and 6 p65 recombinant protein; lanes 3 and 7 p50 and p65 recombinant proteins; lanes 4 and 8 p50 recombinant protein. These results indicated that the NF-κB site located in the BACE1 promoter was a functional site as it had the ability to bind recombinant NF-κB subunits.

BACE1 PROMOTER CONSTRUCT EXPRESSION

In order to generate a NF-κB site substitution mutant promoter to analyze the effect of the primary NF-κB site, the promoter containing the wild type rat BACE promoter (BPR) was obtained from Dr. Rossner (Lange-Dohna *et al*., 2003). Lange-Dohna *et al*., cloned and sequenced a 1.5-kb promoter fragment upstream of the ATG start-codon of the BACE1 coding sequence. The BACE1 promoter contains several putative transcription factor binding sites, all conserved among rat, mouse, and human. The fragment was cloned into the pGL3-Basic vector (Figure 7; Promega; Mannheim, Germany) to create the BPR-pGL3 construct driving the expression of firefly luciferase (Figure 8).

We transfected the pGL3-Basic and pGL3-Control vectors into both C6 and PC12 cells. As expected, no luciferase activity was seen in cells transfected with the pGL3-Basic vector since it lacks a promoter. In contrast, good expression was seen in cells transfected with the pGL3-Control since this vector contains a functional SV40 promoter (Figure 9). We next transfected the pGL3-Control vector into both primary neonatal rat

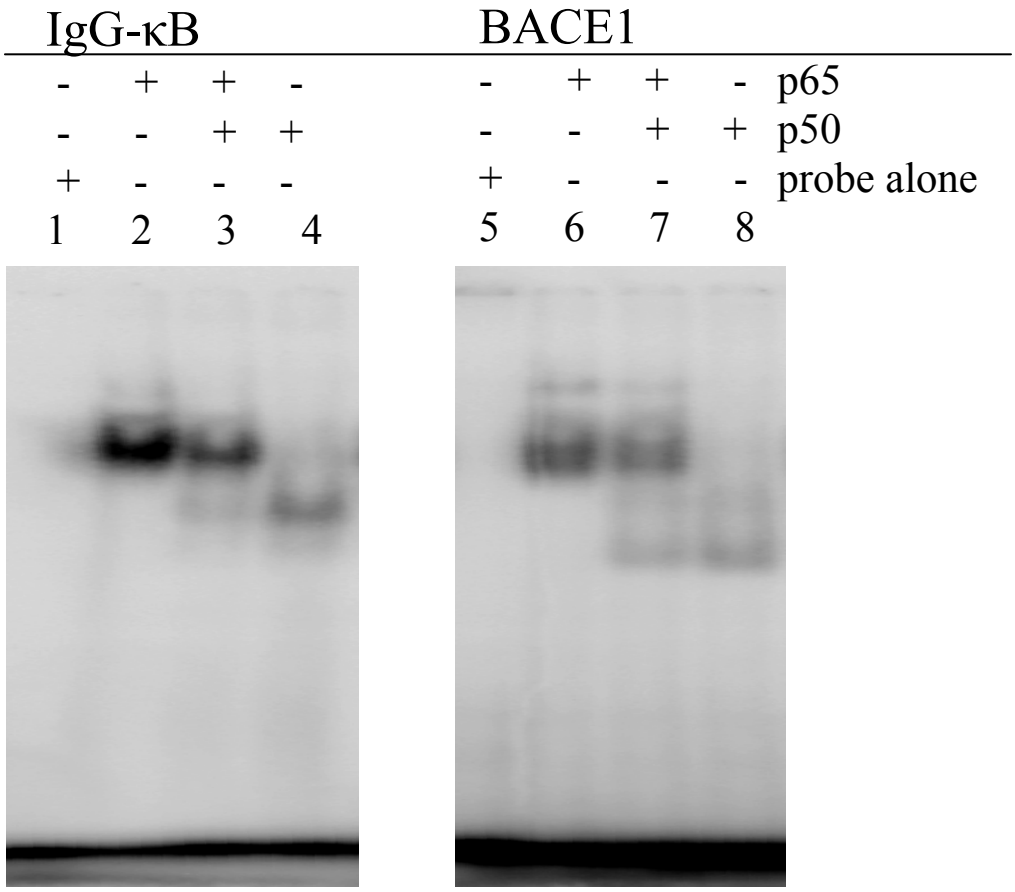


FIGURE 6. EMSA. Electrophoretic mobility shift assays using specific oligonucleotides, IgG-κB enhancer sequence (control: GGGACTTTCC) and the BACE1 promoter NF-κB sequence ("primary" site: AGGGCTTTCCA) were used as probes for control (IgG-κB) and wild type NF-κB (BACE1) binding consensus sequences combined with isolated recombinant NF-κB proteins to determine if the BACE1 NF-κB site was capable of binding NF-κB. The gel was loaded as follows: lanes 1 and 5 probe alone; lanes 2 and 6 p65; lanes 3 and 7 p50/p65; lanes 4 and 8 p50.

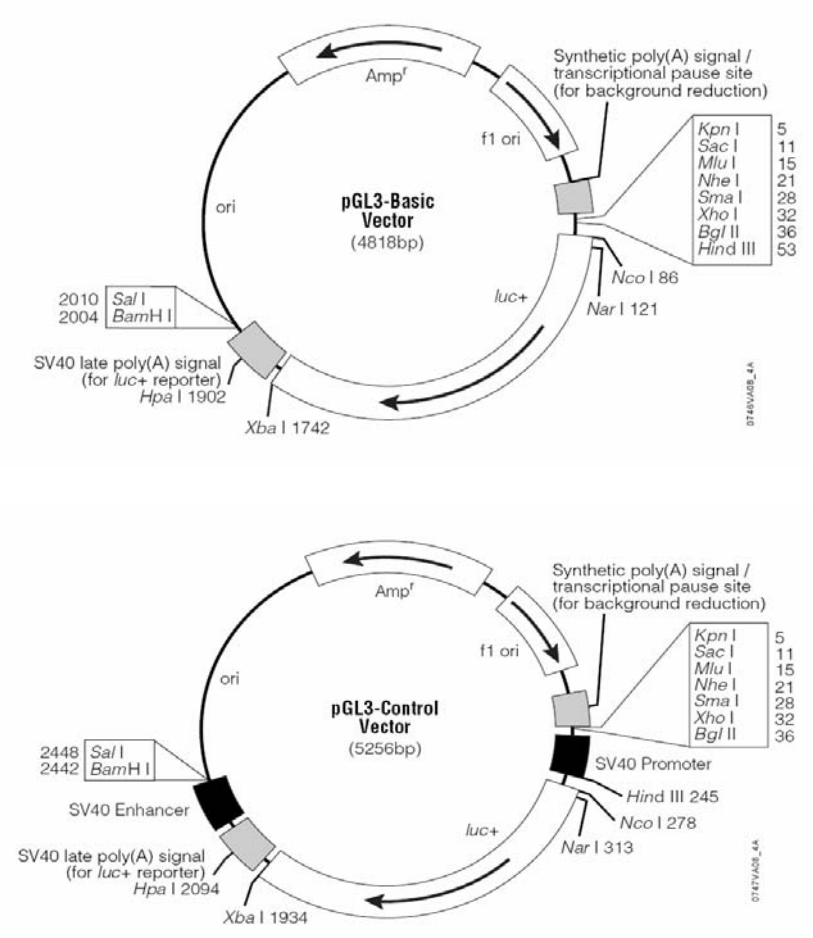


FIGURE 7. Basic and Control Vector. pGl3-Basic vector and pGl3-Control vector circle map. Additional description: luc+, cDNA encoding the modified firefly luciferase; Ampr, gene conferring ampicillin resistance in E. coli; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in E. coli. Arrows within luc+ and the Ampr gene indicate the direction of transcription; the arrow in the f1 ori indicates the direction of ssDNA strand synthesis.

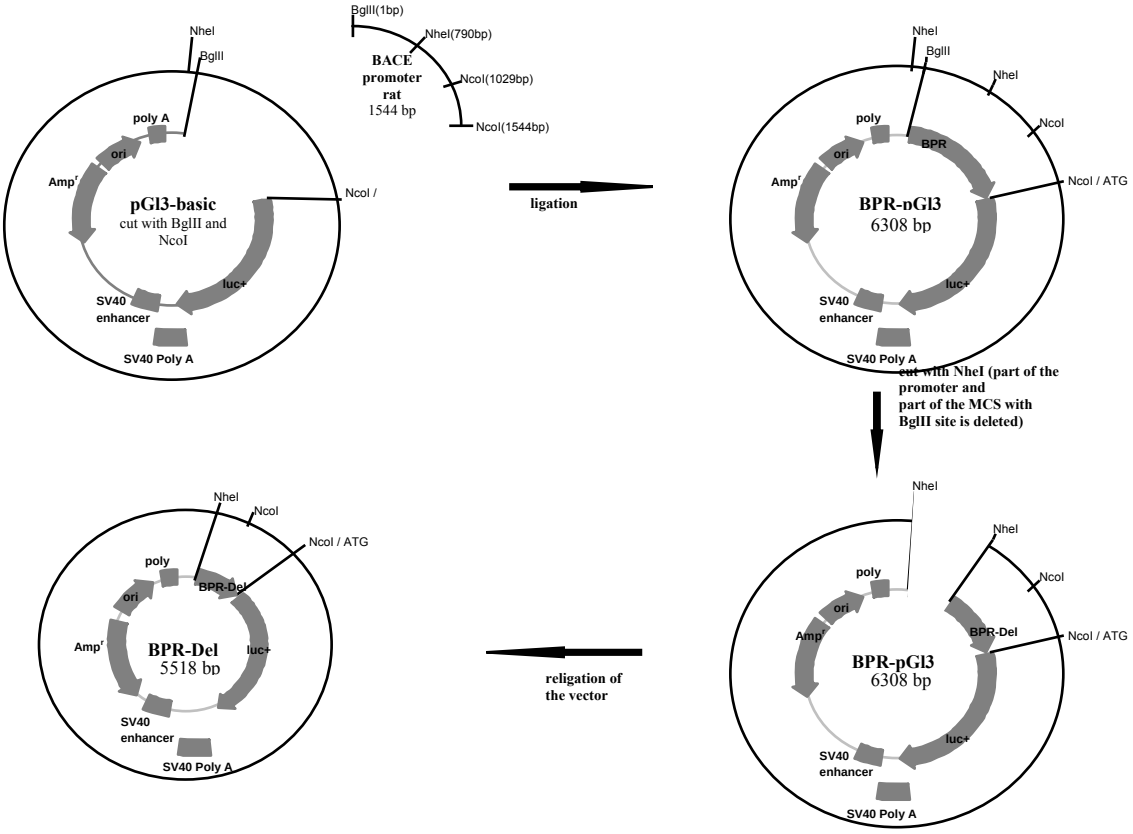


FIGURE 8. BPR and BPRNF-κB⁻ Vector Constructs. BPR and BPRΔNF-κB⁻.site-substitution vector construct construction. From the BACE1 rat promoter sequence, a specific forward primer, 5'ATGCTAAGCTTAGATCTATGGTGGCTGGATTTTATCG-3', was selected, which contains a restriction site for BglII. This site and the NcoI site around the translational start codon were used to clone the fragment into the pGL3-basic vector (Promega; Mannheim, Germany) to create the BPR-pGL3 construct driving the expression of firefly luciferase. Deletions of the 1.5-kb promoter fragment were carried out by cutting BPR-pGL3 with NheI with religation of the vector to create BPR-Del (753 base pairs [bp]).

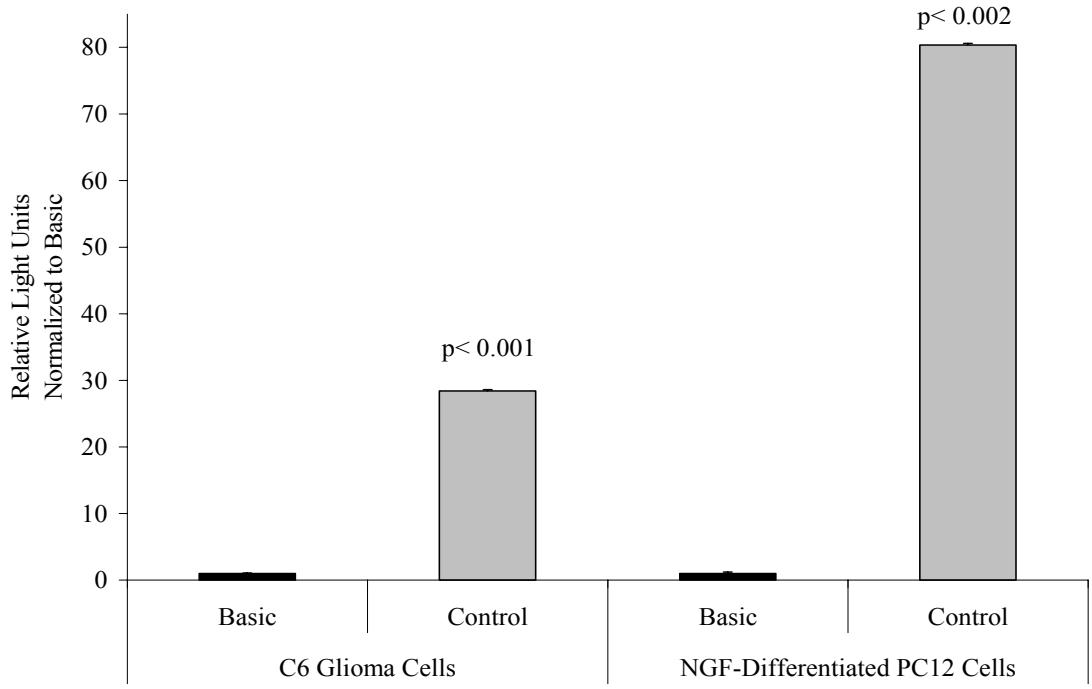


FIGURE 9. pGL3-Basic and pGL3-Control Vector Transfection: NGF-Differentiated PC12 and C6. pGL3-Basic and pGL3-Control vector transfection: Luciferase activity of pGL3-Basic and pGL3-Control vectors after transient transfection into NGF-differentiated neural (PC12) and glia (C6) cells. Note the high activity of the pGL3-Control functional SV40 promoter vector in both PC12 and C6 cells and the negligible activity of the pGL3-Basic promtoerless vector. Statistics are pGL3-Basic compared to pGL3-Control within cell type. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

and adult guinea pig astrocytic and PC12 cells with the resulting strong expression of luciferase as driven by the pGL3-Control SV40 vector indicating that primary cells (neonatal or adult) transfect and express vectors as well as if not better than cell lines (Figure 10).

We generated BACE1 promoter constructs each containing a substitution mutation of the individual primary NF-κB binding site in order to perform luciferase reporter assays of transfected differentiated rat PC12, C6, astrocytes, guinea pig astrocytes and human SK-N-SH-SY5Y human neuroblastoma cells. We mutated the rat BACE1 NF-κB sequence (-1521) AGGGCTTTCCA (-1511) by site-directed mutagenesis to yeild: (-1521) AGATCTA ATCA (-1511) (Figure 11). The mutated fragment (BPRΔNF-κB$^-$ underlined) was cloned at the same site of the pGL3-Basic vector as the wild type (BPR) construct.

We optimized PC12 (Figure 12) and SK-N-SH-SY5Y transfections with the reporter construct (Figure 13). PC12 cells were plated at a previously optimized density of 9 x 10^4 cells per ml per 6 well plate (Lange-Dohna *et al*., 2003). Culture conditions varied by NGF differentiation status, DNA to DMRIE-C ratio (1:3 or 1:2) and the duration of cellular exposure to DNA: DMRIE-C mixture (5 vs. 24 hours). At 24 hours after transfection, cells were harvested in 100μl of lysis buffer (Promega Luciferase Assay kit). Samples were then processed for luciferase activity. There was a significant increase in BACE1 expression in PC12 cells, differentiated with NGF, using a DNA: DMRIE-C ratio of 1:3, allowing the transfection medium to remain on the cells

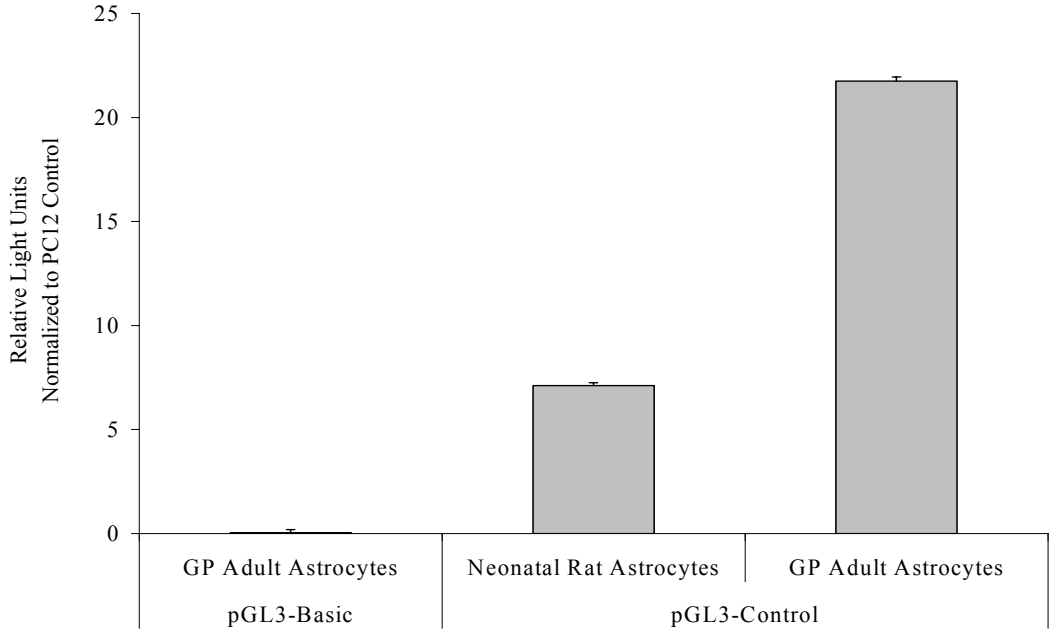
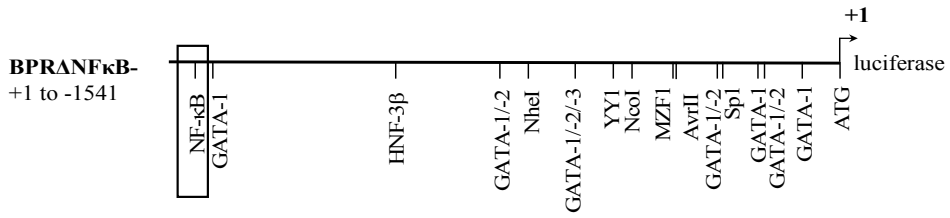


FIGURE 10. pGL3-Basic and pGL3-Control Vector Transfection: Neonatal Rat Primary and Adult Guinea Pig Astrocytic Cells. pGL3-Basic and pGL3-Control vector transfection: Luciferase activity of pGL3-Control vectors after transient transfection into glia (primary neonatal rat or primary adult guinea pig) cells. Note the high activity of the SV40 promoter containing pGL3-Control vector in both primary neonatal rat and adult guinea pig astrocytic cells and the lack of expression in the promoterless pGL3-Basic construct. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.



Wildtype:

(-1521) AGG GCT TTC CA (-1511)

Mutant:

(-1521) AG<u>A</u> <u>T</u>CT <u>AAT</u> CA (-1511)

FIGURE 11. BPRΔNF-κB⁻ Construct. BPRΔNF-κB⁻ site-substitution construct. Schematic presentation of the wild type rat BACE1 promoter construct driving the expression of firefly luciferase. Exact site substitutions utilized to generate the mutant construct BPRΔNF-κB⁻ are indicated by underline. Note the significant number of other transcription factor binding sites present on the rat BACE1 promoter.

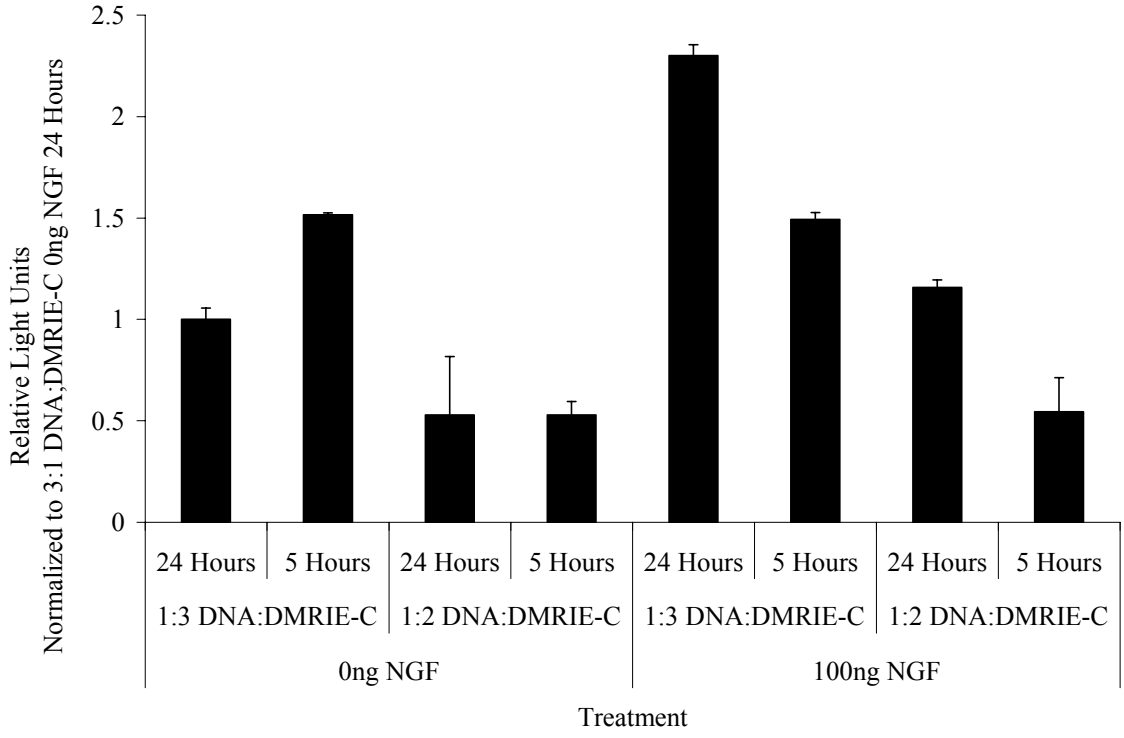


FIGURE 12. Transfection Optimization: PC12. Optimized PC12 transient transfections with the wild type BPR reporter construct. PC12 cells were plated at a previously optimized density of 9 x 10^4 cells per ml per 6 well plate (Lange-Dohna *et al*., 2003). Culture conditions varied by NGF differentiation status, DNA to DMRIE-C ratio (1:3 or 1:2) and the duration of cellular exposure to DNA: DMRIE-C mixture (5 vs. 24 hours). There was expression BACE1 in PC12 cells, differentiated with NGF, using a DNA: DMRIE-C ratio of 1:3, when allowing the transfection medium to remain on the cells for 24 hours (fifth column). Results are $n \geq 6$ per group, repeated a minimum of three separate experiments.

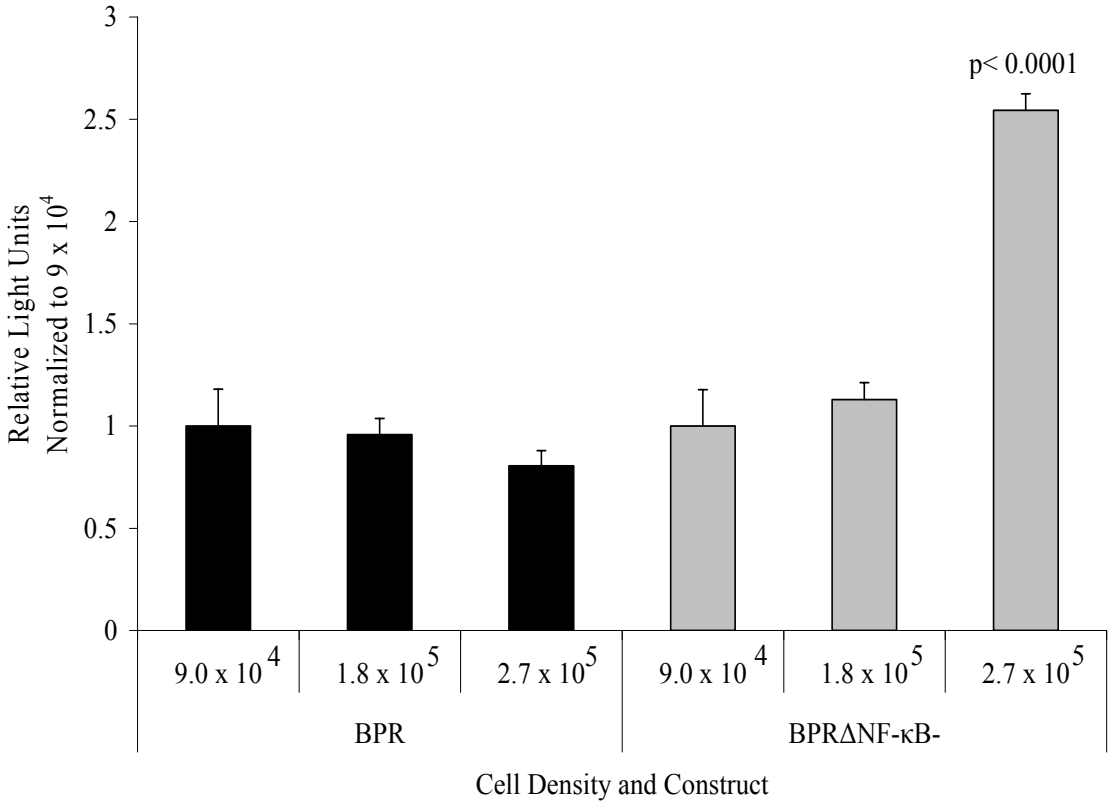


FIGURE 13. Transfection Optimization: RA-Differentiated SK-N-SH-SY5Y. Optimized SK-N-SH-SY5Y transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$. RA-differentiated SK-N-SH-SY5Y cells were plated at different densities: 9.0 x 10^4, 1.8 and 2.5 x 10^5 cells per ml per 6 well plate. Statistical comparison is between constructs within cell density groups. The optimal density for BACE1 expression was determined to be 2.7 x 10^5 cells per ml per 6-well plate (sixth column). Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments.

for 24 hours (Figure 12 fifth column). NGF-differentiated PC12 cells displayed a more neuronal-like phenotype than non-differentiated cells in terms of cell elongation, aggregation, and enhanced neuritogenesis. As the most BACE1 expression was obtained with differentiated PC12 cells, all other cell lines utilized in the studies were also differentiated for consistency. Therefore SK-N-SH-SY5Y cell culture transfection optimization used cells RA-differentiated for five days in culture prior to transfection. Culture conditions optimized for PC12 cells were used with regards to differentiation, DNA: DMRIE-C ratio and length of incubation with transfection medium.

A literature review indicated that the different efficiencies of transfections of SK-N-SH-SY5Y cells appeared to be mostly dependent on cell density. Figure 13 illustrates that the optimal density for BACE1 expression was determined to be 2.7×10^5 cells per ml per 6-well plate. C6 cells were also optimized for efficient transfection based on cell density with optimal density determined to be 1.8×10^5 cells per 2ml or per 9×10^4 cells per ml per 6-well plate (Figure 14). Further literature review indicated that the different efficiencies of transfections of C6 cells appeared to be mostly dependent on DNA to transfectant ratios. Figure 15 illustrates that the optimal DNA: DMRIE-C ratio for BACE1 expression was determined to be in C6 cells to be 1:3. We also explored the use of another transfectant NeuroPorter. Figure 16 illustrates PC12 cells optimized for cell density and NeuroPorter transfectant to DNA ratio simultaneously. Figure 17 illustrates the results obtained with NeuroPorter and C6 cells. Results indicated that NeuroPorter was a less efficient transfectant than DMRIE-C. As we obtained better transfection rates

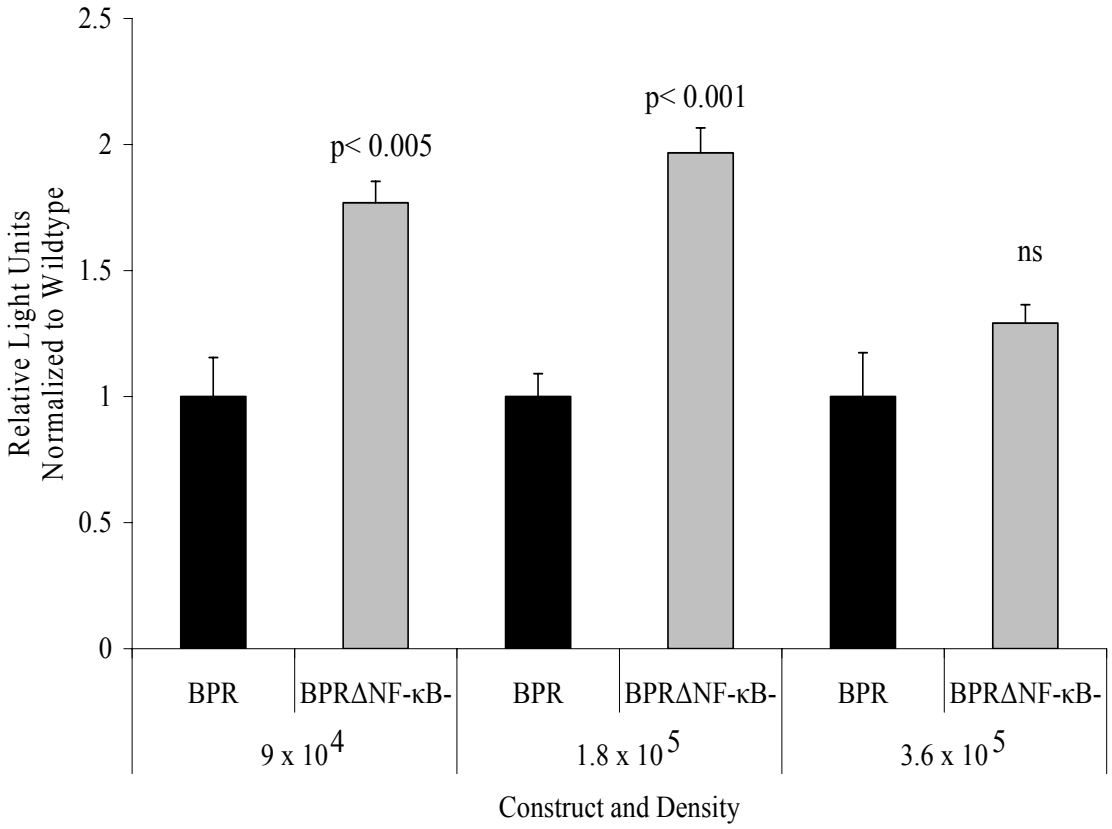


FIGURE 14. Transfection Optimization: C6 -cell Density. Optimized C6 transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$: cell density. C6 cells were plated at different densities: 9.0 x 10^4, 1.8 and 3.6 x 10^5 cells per 2ml per 6 well plate. Statistical comparisons between constructs are within a density group. The optimal density for BACE1 expression was determined to be 1.8 x 10^5 cells per 2 ml per 6-well plate (fourth column). Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. ns: not significant.

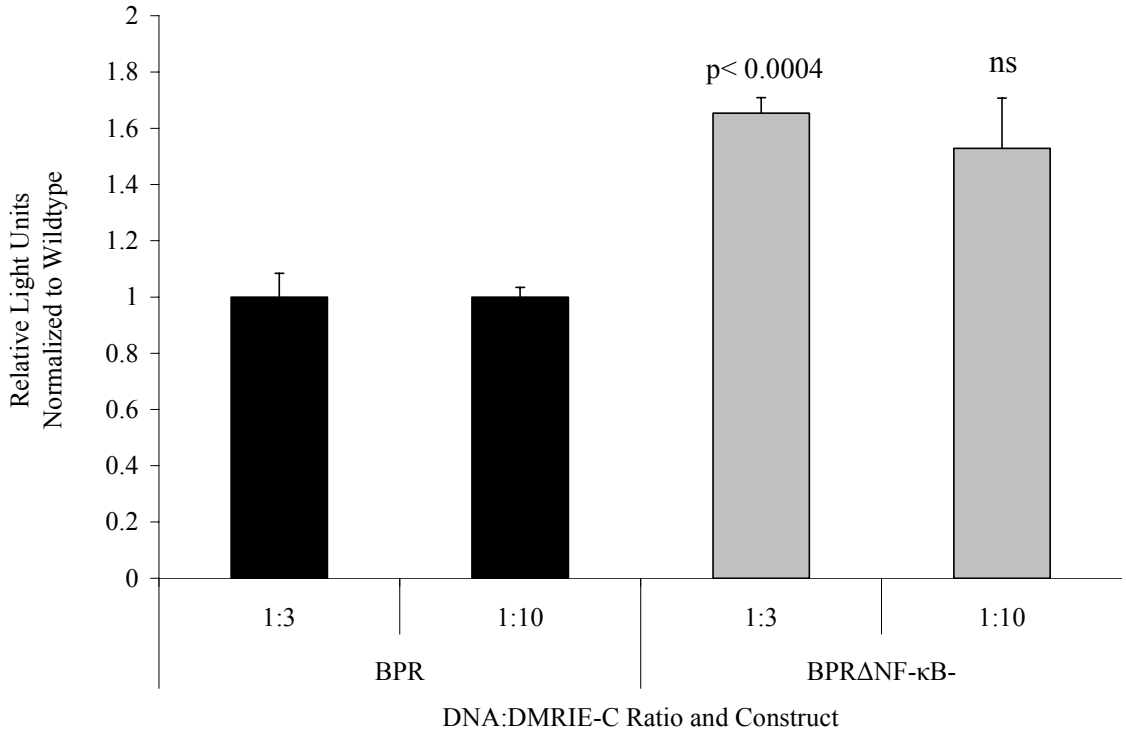


FIGURE 15. Transfection Optimization: C6 -Transfectant:DNA Ratio. Optimized C6 transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$: DNA to transfectant ratio. C6 cells were plated at 9.0 x 10^4 cells per ml per 6 well plate. Culture conditions did vary by DNA to DMRIE-C ratio (1:3 and 1:10). The duration of cellular exposure to DNA: DMRIE-C mixture was 24 hours. Statistical comparisons are between constructs within a ratio grouping. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments.

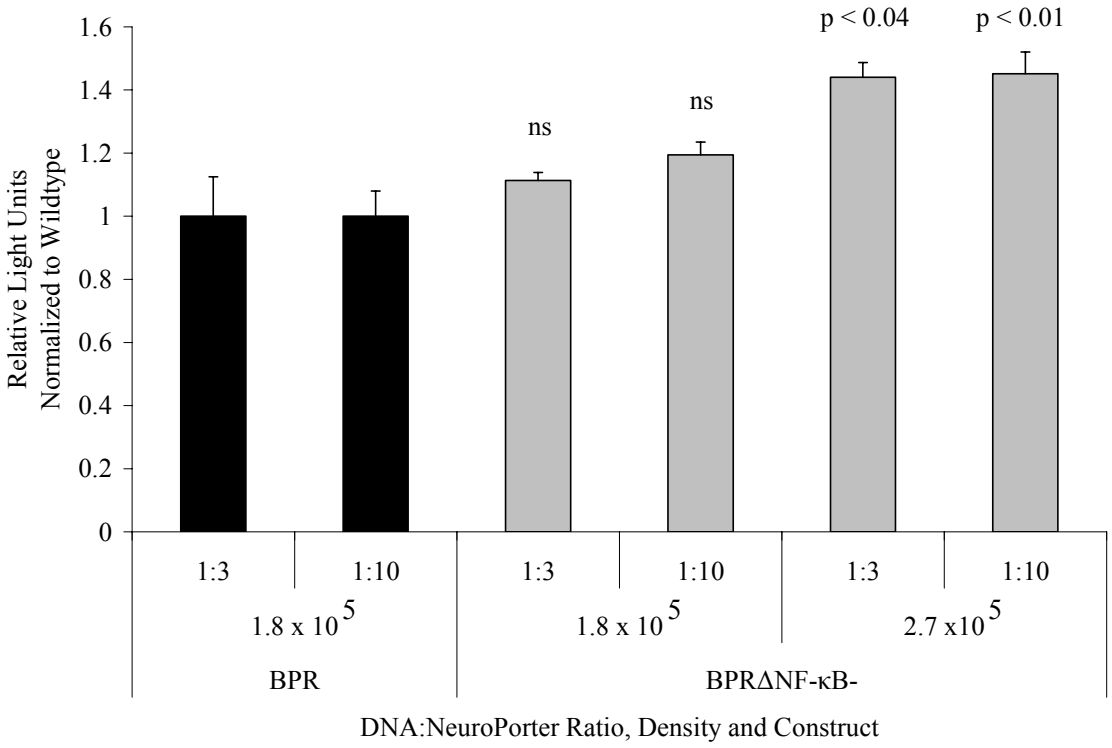


FIGURE 16. Transfection Optimization: NGF-Differentiated PC12 –NeuroPorter. Optimized PC12 transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$: NeuroPorter transfectant to DNA ratio, cell density and transfectant. PC12 cells were plated at 1.8 x 10^5 or 2.7 x 10^5 cells per 2 ml per 6 well plate and NGF-differentiated. Culture conditions did vary by DNA to NeuroPorter ratio (1:3 and 1:10). The duration of cellular exposure to DNA: NeuroPorter mixture was 24 hours. Statistical comparisons are within DNA: transfectant groups compared to wild type. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. ns: not significant.

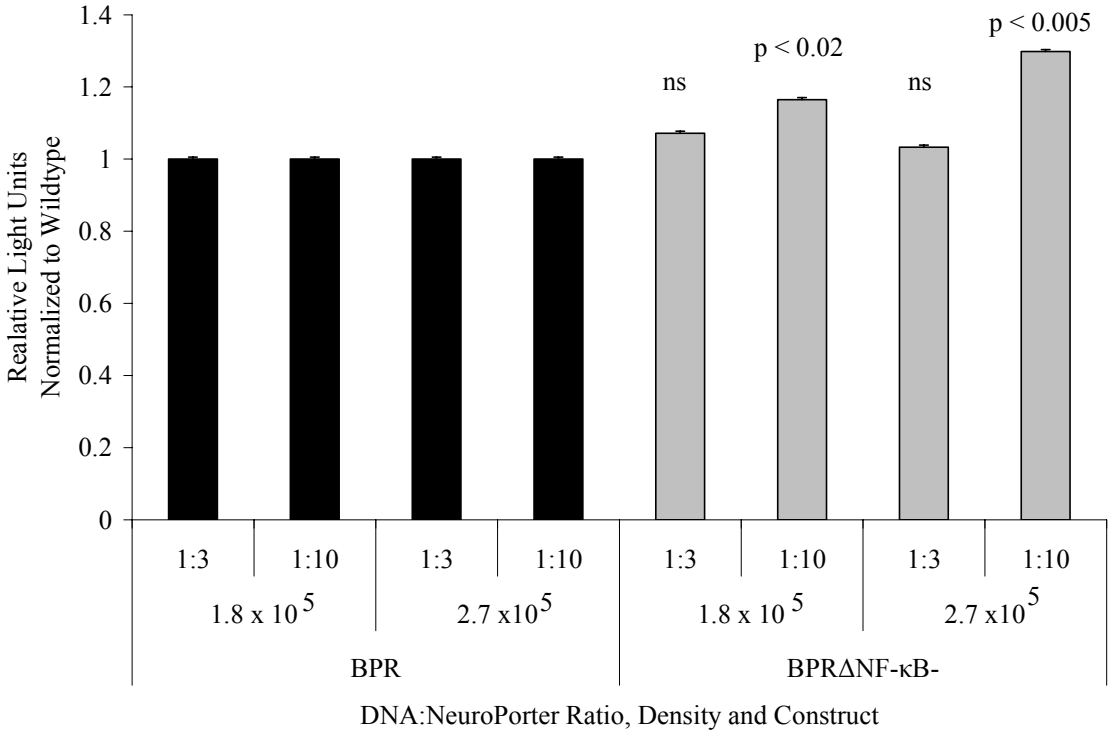


FIGURE 17. Transfection Optimization: C6 –NeuroPorter. Optimized C6 transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$: NeuroPorter transfectant to DNA ratio, cell density and transfectant. C6 cells were plated at different densities: 1.8 x 10^5 or 2.7 x 10^5 cells per 2 ml per 6 well plate. Culture conditions did vary by DNA to NeuroPorter ratio (1:3 and 1:10). The duration of cellular exposure to DNA: NeuroPorter mixture was 24 hours. Statistical comparisons are within DNA: transfectant groups and cell density and are compared to wild type. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. ns: not significant.

in primary glial cells using a DNA to transfectant ratio of 1:10 (data not shown), this ratio was also used for consistency with the glial cell line C6. We carried out transfections under optimal conditions, using DMRIE-C as the transfectant, NGF-differentiated PC12, RA- differentiated SK-N-SH-SY5Y cells (Figure 18: PC12, and SK-N-SH-SY5Y), C6 (Figure 19), neonatal primary rat astrocytes (Figure 20) and TNFα treated adult guinea pig hippocampal astrocytic cells (Figure 21) with the two reporter constructs. There was a statistically significant increase in luciferase activity in the mutated promoter lacking the NF-κB binding site consistent with a suppressor function for the NF-κB site in the rat BACE1 promoter in neurons and non-activated astrocytes (Figure 18: PC12, and SK-N-SH-SY5Y; data not shown). There was a statistically significant decrease in luciferase activities in the mutated promoter lacking the NF-κB binding site that confirmed an activator function of the NF-κB site in the rat BACE1 promoter in glia (Figures 19-21). BACE1 expression significantly differed between constructs at the level of $p<0.0001$ in C6 cells and $p<0.00005$ in TNFα treated adult guinea pig hippocampal astrocytes.

Activation of astrocytes triggers significant changes in phenotype also reflected in gene expression patterns. In an attempt to evaluate activated astrocytes, we exposed neonatal rat astrocytes to PMA, which is known to activate cells via the PKC pathway. Figure 22 displays glial fibrillary acidic protein (GFAP) protein levels evaluated by Western blot assays in cells exposed to the vehicle alone, dimethyl sulfoxide (DMSO) or vehicle and PMA. There was an increase in GFAP levels in the vehicle alone and vehicle plus PMA treatment groups. DMSO itself is a potent neuroprotective chemical.

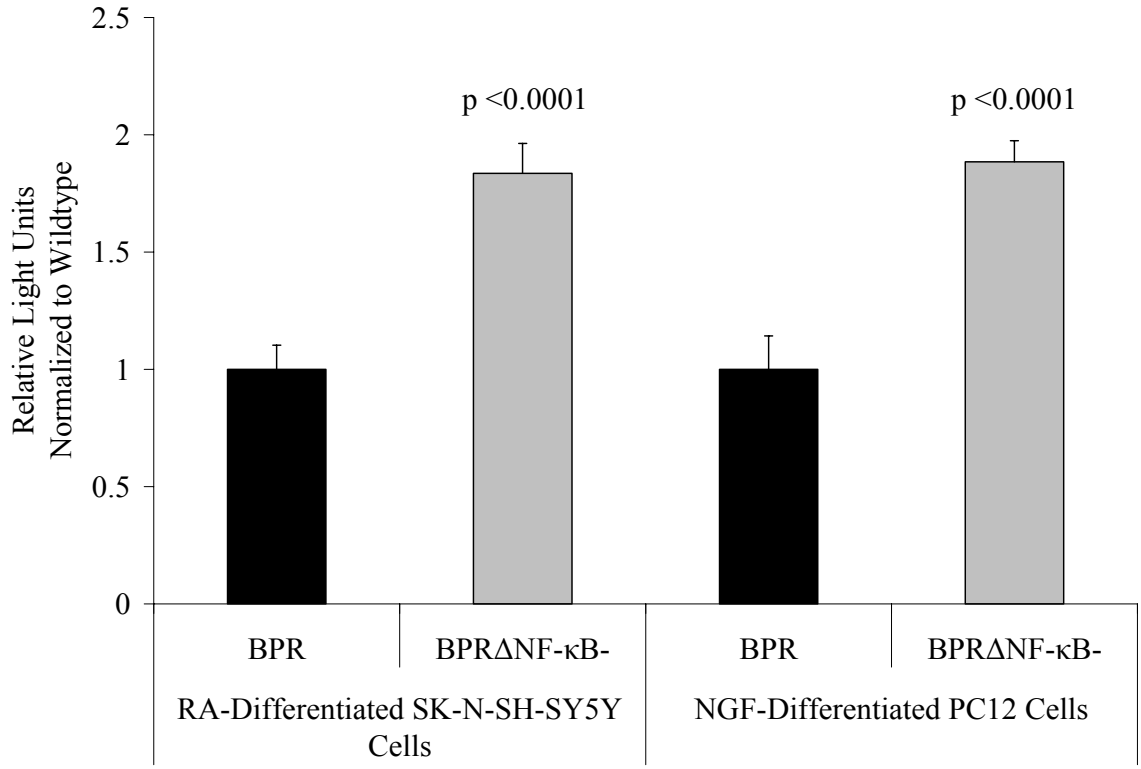


FIGURE 18. Neuronal Transfection: PC12 and SK-N-SH-SY5Y. Transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions, using NGF-differentiated PC12, RA- differentiated SK-N-SH-SY5Y cells. There was a statistically significant increase in luciferase activity in the mutated promoter lacking the NF-κB binding site consistent with a suppressor function for the NF-κB site in the rat BACE1 promoter in neurons (PC12, and SK-N-SH-SY5Y). BACE1 expression significantly differed between constructs within cell type at the level of $p<0.0001$. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

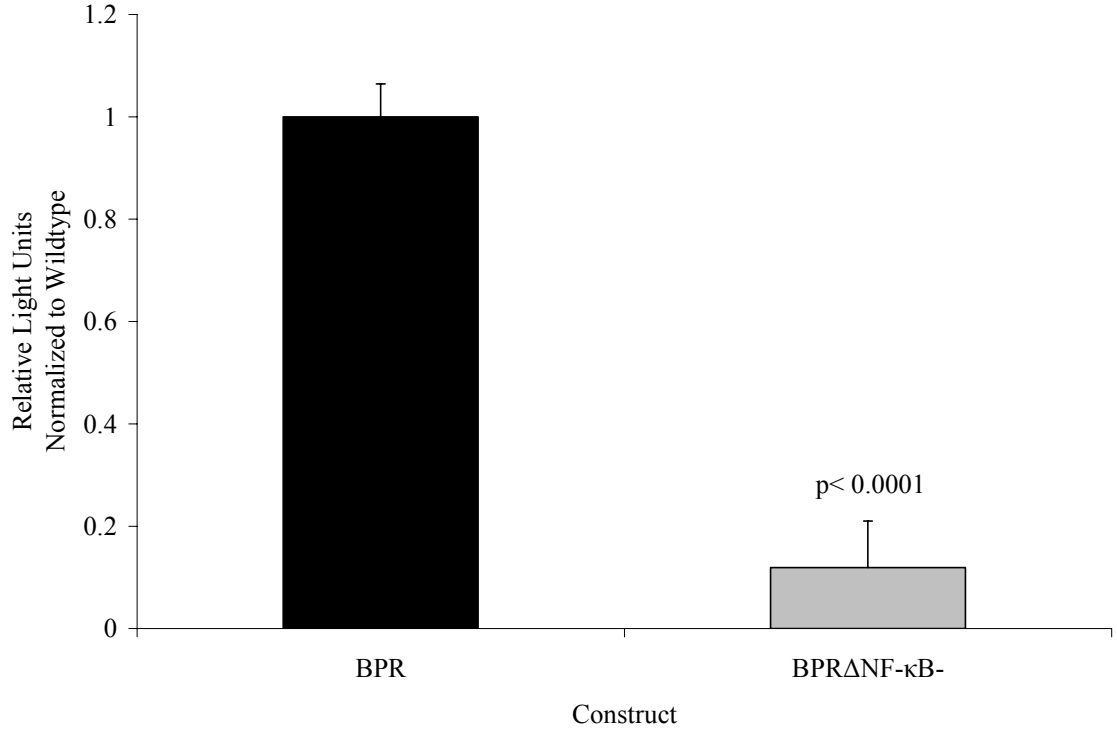


FIGURE 19. Glial Transfection: C6. Transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions, using C6 cells. There was a statistically significant decrease in luciferase activities in the mutated promoter lacking the NF-κB binding site that confirms an activator function of the NF-κB site in the rat BACE1 promoter in glia. BACE1 expression significantly differed between constructs at the level of $p< 0.0001$. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

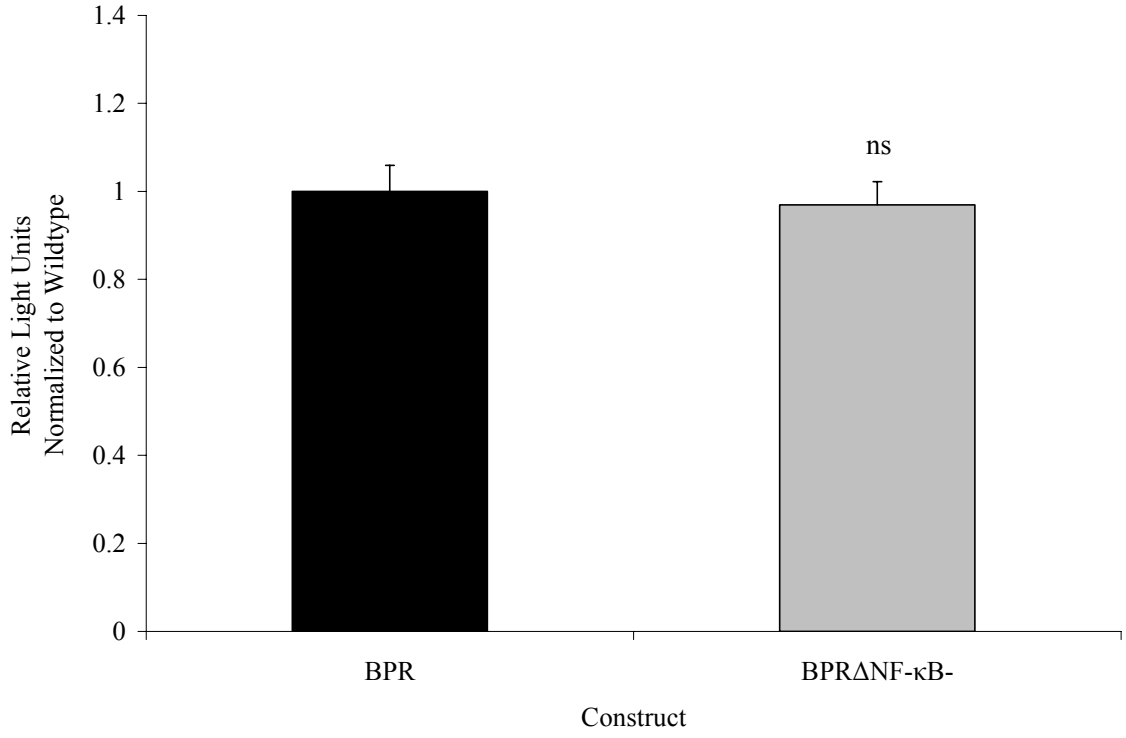


FIGURE 20. Glial Transfection: Neonatal Rat Primary Astrocytic Cells. Transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions, using neonatal rat primary astrocytic cells. There were no statistically significant effects on luciferase activities in the mutated promoter lacking the NF-κB binding. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. ns: not significant.

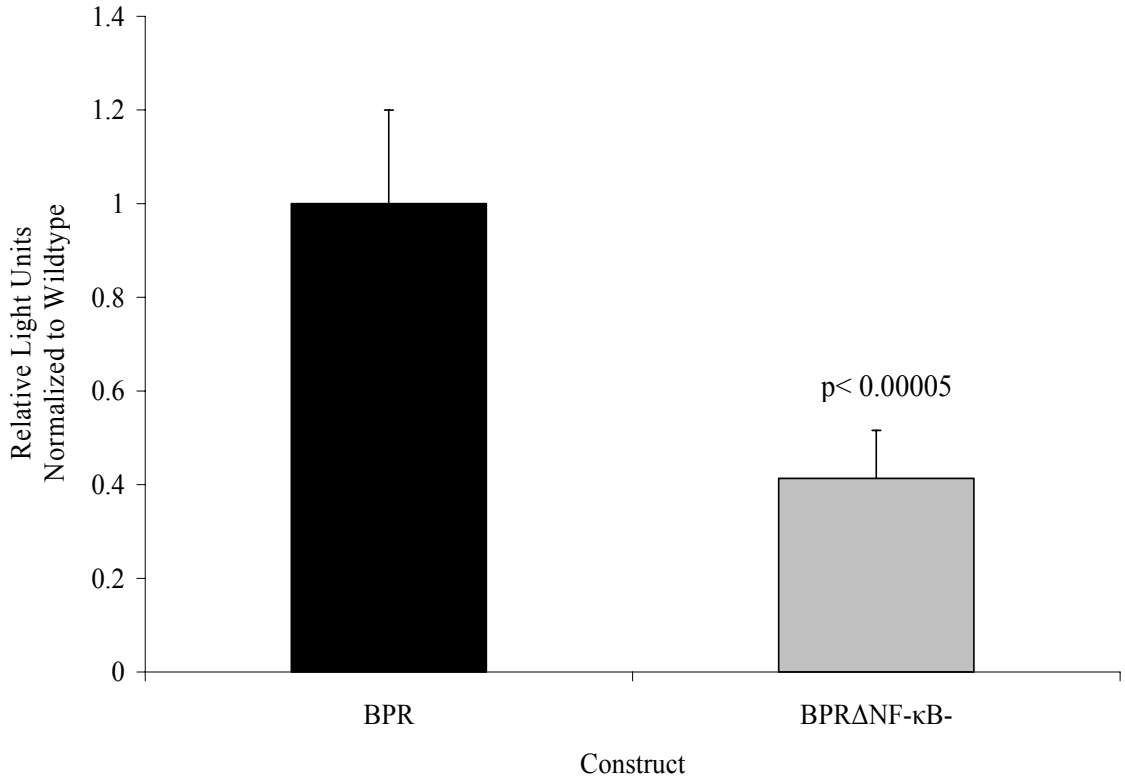


FIGURE 21. Glial Transfection: Adult Guinea Pig Primary Astrocytic Cells. Transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB^{-} under optimal conditions, using TNFα treated adult guinea pig hippocampal astrocytes. There was a statistically significant decrease in luciferase activities in the mutated promoter lacking the NF-κB binding site that confirms an activator function of the NF-κB site in the rat BACE1 promoter in glia. BACE1 expression significantly differed between constructs at the level of $p<0.00005$. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

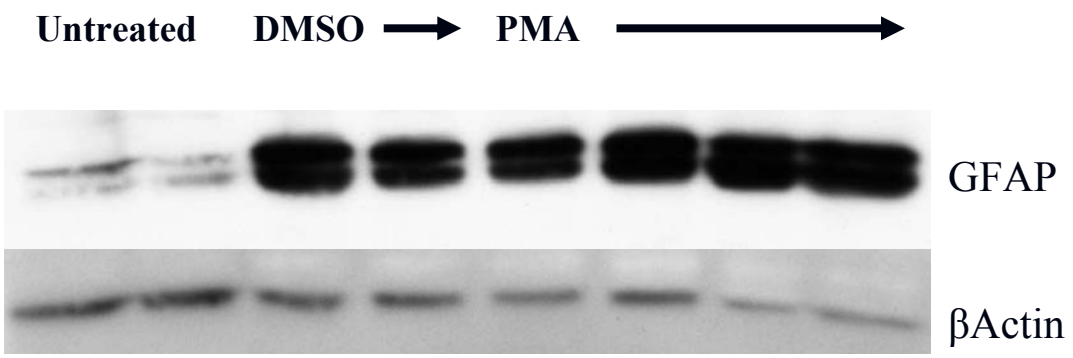


FIGURE 22. GFAP Western Blot PMA Exposed Neonatal Rat Primary Astrocytic Cells. Glia Fibrillary Acidic Acid (GFAP) Western blot 50nM PMA exposed neonatal rat primary astrocytes. Blot is intentionally overexposed to illustrate the low relative levels of GFAP in non-treated cells (untreated) as compared to either DMSO or DMSO/PMA treated cells. Note the significant increase in GFAP protein in either the DMSO alone or the DMSO/PMA treated groups. Actin was included as a loading control.

DMSO is also known to act as an inducer of cellular differentiation and as a free radical scavenger and radioprotectant and these qualities may explain the increased GFAP levels seen with the DMSO alone and DMSO/PMA treatment. When PMA-exposed C6 cells were transfected with the two reporter constructs there was no difference in BACE1 expression in contrast to the significant decrease in promoter expression seen in the DMSO alone groups (Figure 23). Similarly there was no difference in BACE expression in 14 day old ("aged") cultures of neonatal rat primary astrocytic cells (Figure 24). Finally neonatal rat primary astrocytic cells were transfected with the BPR and BPRΔNF-κB$^-$ constructs and were evaluated by Western blot for BACE1 expression following no treatment, DMSO alone, PMA single exposure prior to transfection and to PMA exposure both pre- and post- transfection. Figure 25 demonstrates that there were no significant effects of PMA or vehicle exposure on BACE1 expression with the exception being a significant increase ($p < 0.0001$) was seen with the mutated promoter lacking the NF-κB binding following pre- and post transfection exposure to 50nM PMA. In contrast to the results obtained with adult guinea pig hippocampal astrocytic primary cultures, BACE1 expression was not significantly higher in the cells transfected with BPR as compared to cells transfected with BPRΔNF-κB$^-$. We did not pursue further the use of PMA, as it is known to stimulate PKC pathway. PKC pathway activation favors the alpha site cleavage of APP over the beta site cleavage (Etcheberrigaray *et al*., 2004). Therefore while PMA or DMSO "activated" GFAP expression PMA favors the APP cleavage pathway that is a competitor of the BACE1 pathway, invalidating our use of this model.

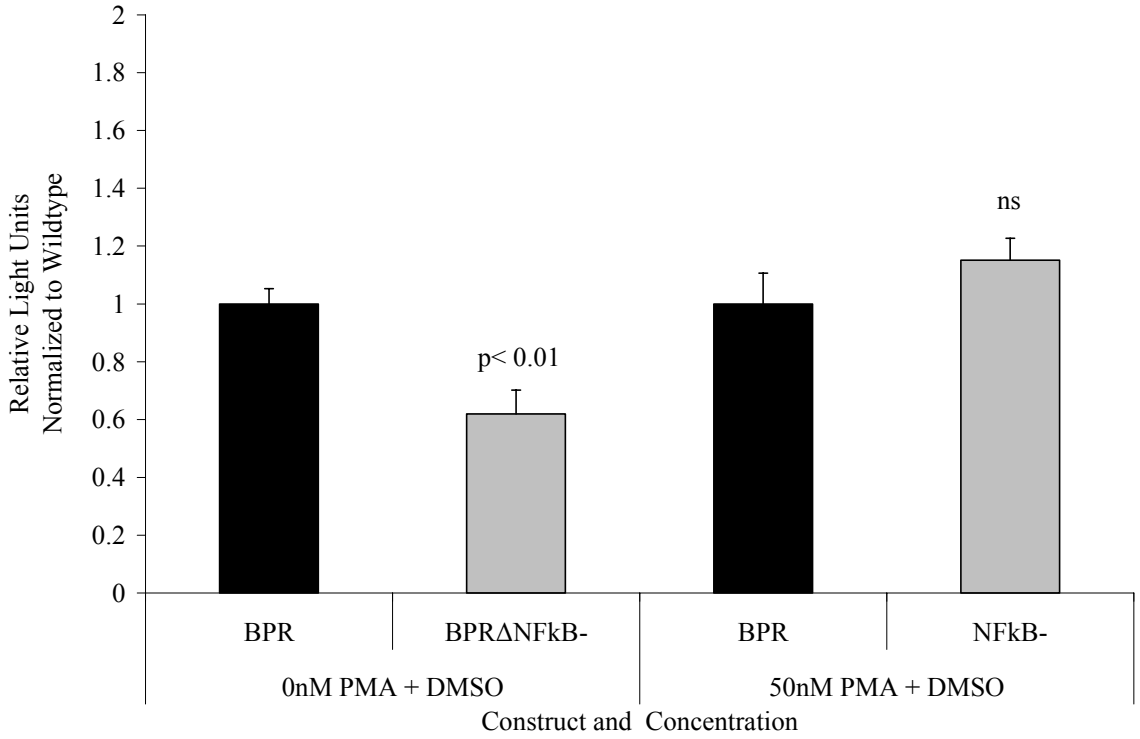


FIGURE 23. BACE1 Promoter Vector Expression After PMA Treatment: C6. Transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions, using rat glioma C6 cells following PMA treatment. There were no statistically significant effects on luciferase activities in the mutated promoter lacking the NF-κB binding regardless of PMA exposure. Statistical comparisons were made within PMA treatment groups. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. ns: not significant.

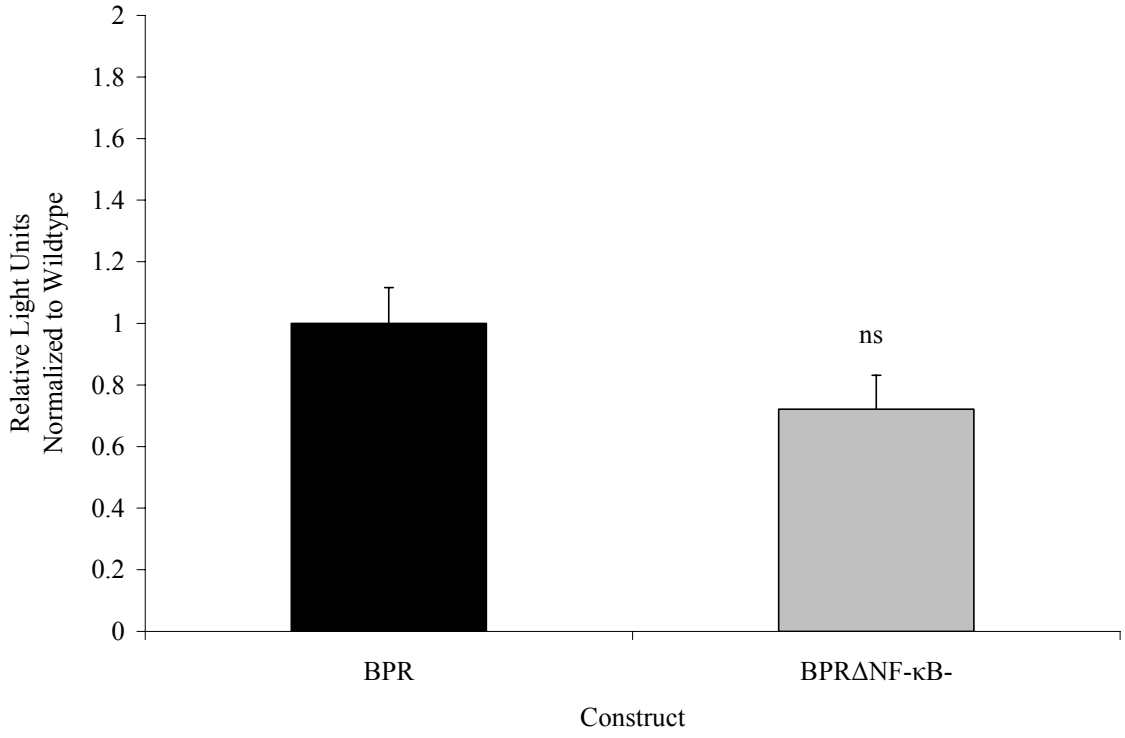


FIGURE 24. BACE1 Promoter Vector Expression After PMA Treatments: "Aged" Neonatal Rat Primary Astrocytic Cells. Transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions, using "aged" neonatal rat primary astrocytic cells. There were no statistically significant effects on luciferase activities in the mutated promoter lacking the NF-κB binding. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. ns: not significant.

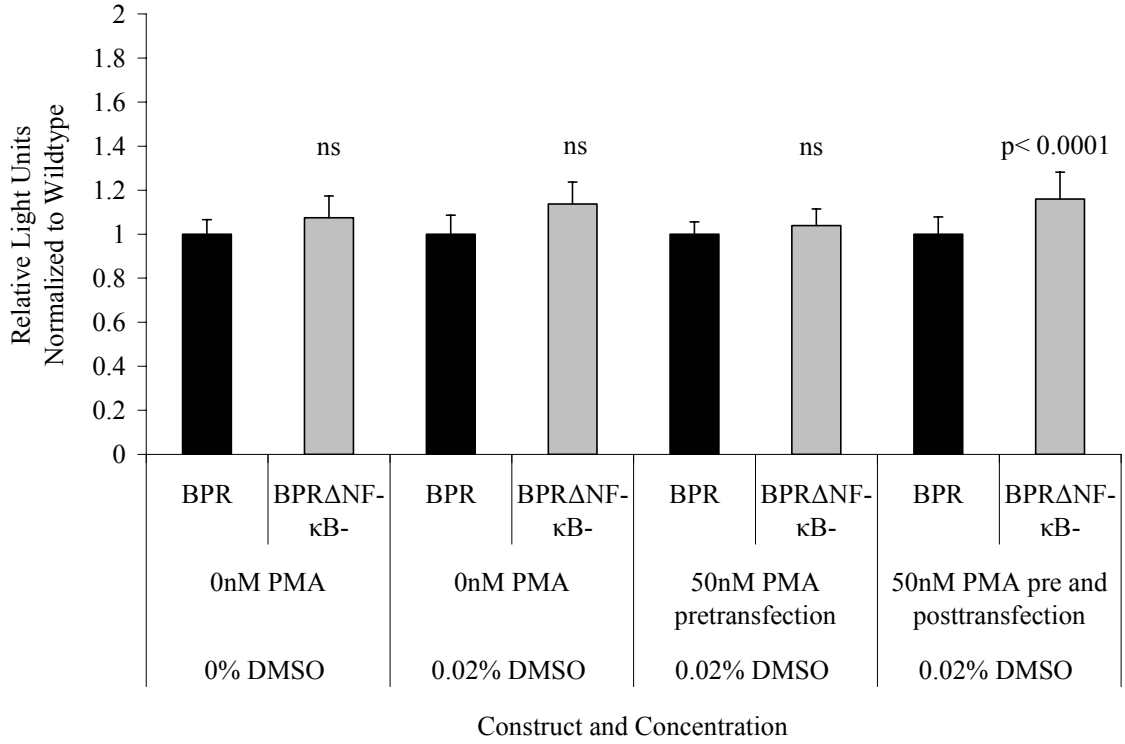


FIGURE 25. BACE1 Promoter Vector Expression After PMA Treatments: Neonatal Rat Primary Astrocytic Cells. Transient transfections with two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions, using neonatal rat primary astrocytic cells. A statistically significant increase ($p< 0.0001$) in luciferase activities was seen with the mutated promoter lacking the NF-κB binding following pre- and post transfection exposure to 50nM PMA. Statistical comparisons were made within PMA treatment groups. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

BACE1 PROMOTER CONSTRUCT SPECIFICALLY BOUND SELECTIVE NF-κB SUBUNIT PROTEINS: LUCIFERASE ACTIVITY

In order to assess the role of combinations of NF-κB protein subunits in the activation of NF-κB-driven regulation of BACE1, one day NGF-differentiated PC12 cells were transfected with wild type BPR or mutant BPRΔNF-κB$^-$ constructs and two at a time of the individual constructs expressing NF-κB subunits p52, p65 or c-Rel. There were also no significant differences in the measured endogenous levels of p50, p52 or p65 protein by Western blot analysis (data not shown). There was a statistically significant increase, confirming an activator role, in BACE1 driven luciferase expression only in cells transfected with the BPRΔNF-κB$^-$ reporter construct and the p52 and c-Rel expressing constructs as compared to control lacZ transfected with wild type BPR or BPRΔNF-κB$^-$ or cells transfected with wild type BPR reporter construct (Figure 26). This suggested that the specific NF-κB subunit combination of p52 and c-Rel in NGF-differentiated PC12 cells was responsible for the repressive action of NF-κB on the rat BACE1 promoter.

In order to assess the role of homodimers vs. heterodimers in astrocytic cells, C6 cells were transfected with BPR or BPRΔNF-κB$^-$ constructs and two at a time of the individual constructs expressing NF-κB subunits p52, p65 or c-Rel. There was a statistically significant increase in BACE1 driven luciferase expression in cells

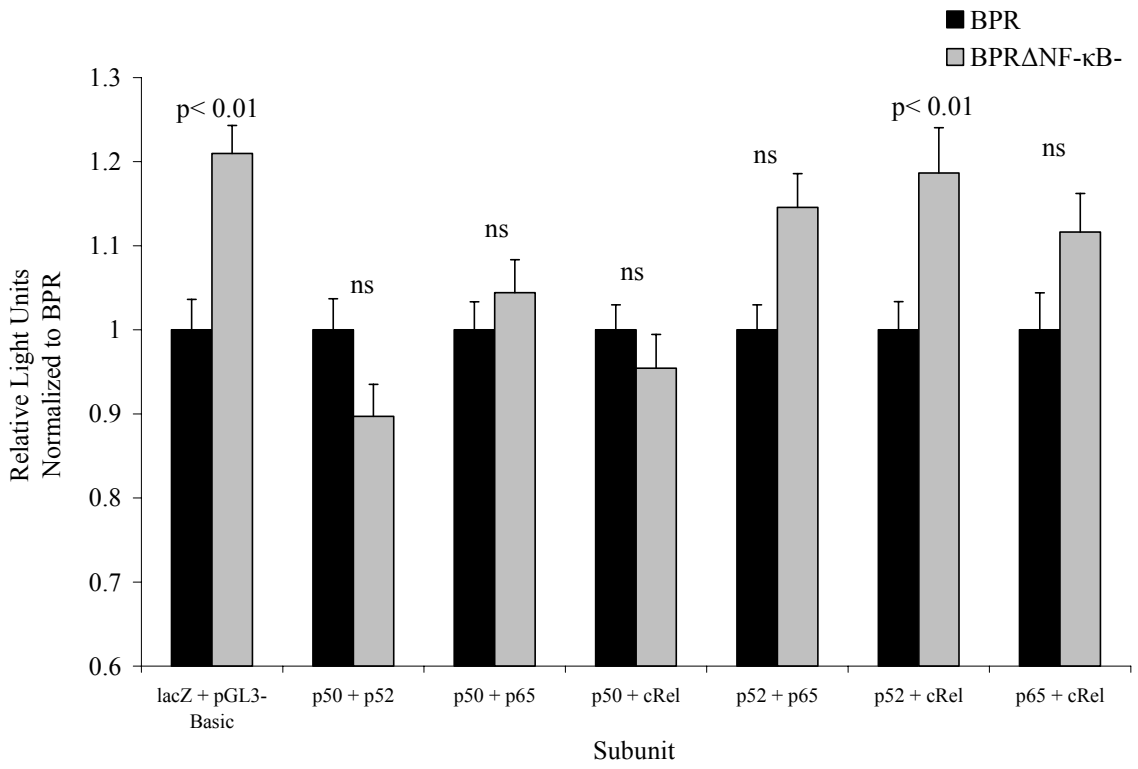


FIGURE 26. BACE1 Promoter Vector Expression with Multiple NF-κB Subunit Transfections: PC12. PC12 were transiently transfected with BPR and two of the individual NF-κB subunits p52, p65 or c-Rel with a resultant statistically significant increase in BACE1 driven luciferase expression being present only in those cells transfected with NF-κB /BPRΔNF-κB$^-$ ($p< 0.01$) and p52 and c-Rel ($p< 0.01$) as compared to the control NF-κB /BPRΔNF-κB$^-$ co-transfected with lacZ and pGL3-Basic. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. Statistical comparisons made within NF-κB subunit groupings. ns: not significant.

transfected with the BPR reporter construct and the p50 and p65 expressing constructs as compared to control lacZ transfected with wild type BPR or BPRΔNF-κB$^-$ or cells transfected with wild type BPR reporter construct (Figure 27). This suggested that the specific NF-κB subunit combination of p50 and p65 in C6 cells was responsible for the activating action of NF-κB on the rat BACE1 promoter.

In order to further assess the role of homodimers vs. heterodimers in astrocytic cells, adult guinea pig hippocampal astrocytic cells were transfected with wild type BPR or mutant BPRΔNF-κB$^-$ constructs and two at a time of the individual constructs expressing NF-κB subunits p50, p52, p65 or c-Rel (Figure 28, Table 1). There was a statistically significant increase in BACE1 driven luciferase expression being present only in those cells transfected with the BPR reporter construct and the p52 and p65 expressing constructs as compared to control lacZ transfected with wild type BPR or BPRΔNF-κB$^-$ or cells transfected with wild type BPR reporter construct. This suggested that the specific NF-κB subunit combination of p52 and p65 in adult guinea pig hippocampal astrocytic cells was responsible for the activating action of NF-κB on the rat BACE1 promoter.

While control triple transfections of wild type BPR or mutant BPRΔNF-κB$^-$ together with a lacZ expressing construct and the pGL3-Basic construct, confirmed the appearance of a suppressor role for NF-κB in the BACE1 promoter in neurons and a activator role in astrocytes, cotransfections with constructs expressing the individual NF

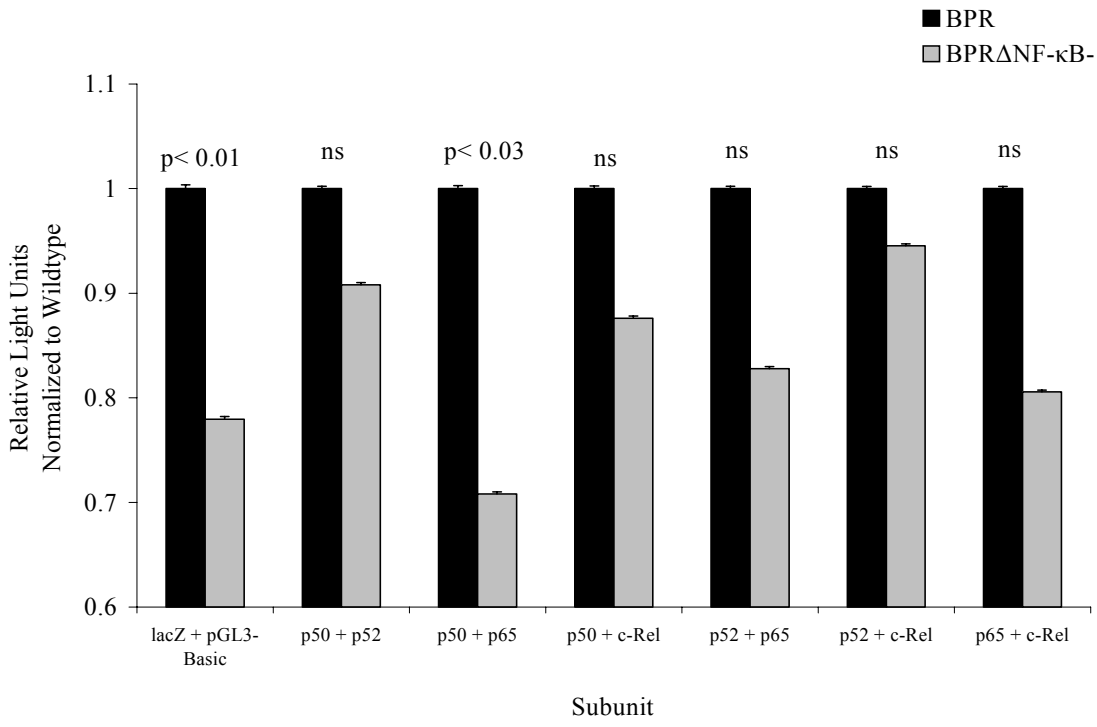


FIGURE 27. BACE1 Promoter Vector Expression with Multiple NF-κB Subunit Transfections: C6. C6 were transiently transfected with BPR or BPRΔNFκB- and pairs of the individual NF-κB subunits p50, p52, p65 or c-Rel with a resultant statistically significant decrease in BACE1 driven luciferase expression being present only in those cells transfected with NF-κB /BPRΔNF-κB$^-$ ($p< 0.01$) and p50 and p65 ($p< 0.03$) as compared to the control NF-κB /BPRΔNF-κB$^-$ co-transfected with lacZ and pGL3-Basic. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. Statistical comparisons made within NF-κB subunit groupings. ns: not significant.

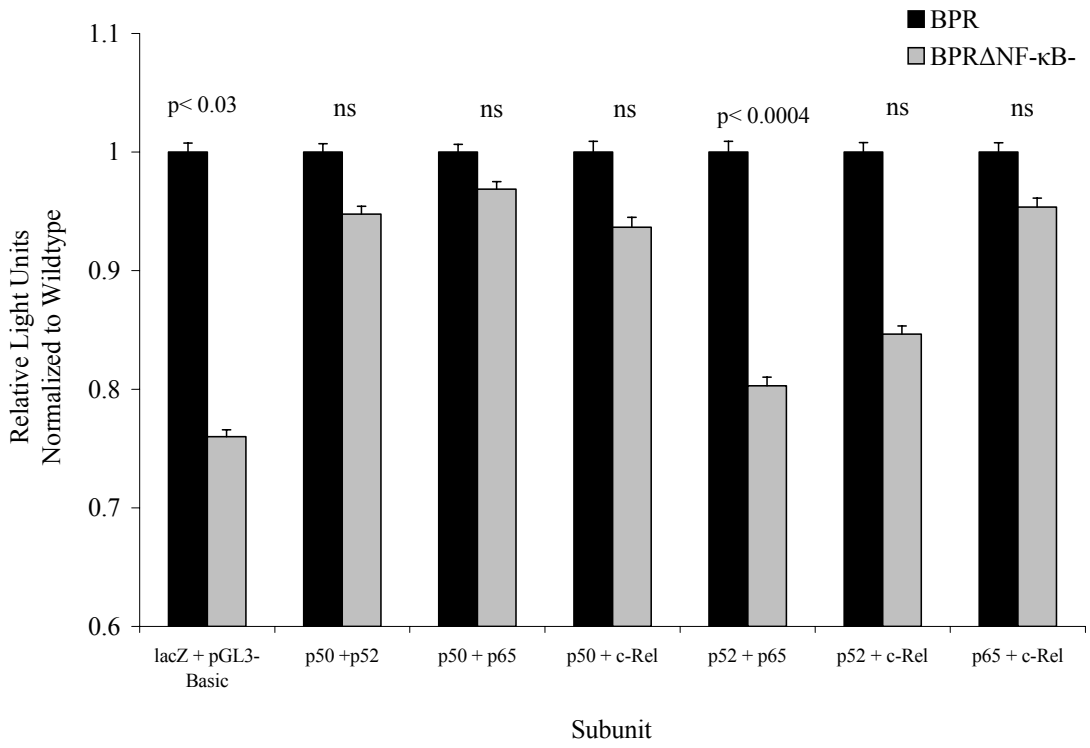


FIGURE 28. BACE1 Promoter Vector Expression with Multiple NF-κB Subunit Transfections: Adult Guinea Pig Primary Astrocytic Cells. TNFα-treated guinea pig hippocampal astrocytic cells were transiently transfected with BPR or BPRΔNFκB- and pairs of the individual NF-κB subunits p50, p52, p65 or c-Rel. There was a statistically significant decrease in BACE1 driven luciferase expression being present only in those cells transfected with NF-κB /BPRΔNF-κB$^-$ ($p< 0.03$) and p52 and p65 ($p< 0.0004$) as compared to the control NF-κB /BPRΔNF-κB$^-$ co-transfected with lacZ and pGL3-Basic. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. Statistical comparisons made within NF-κB subunit groupings. ns: not significant.

	p50/p52	p50/p65	p50/c-Rel	p52/p65	p52/c-Rel	p65/c-Rel
PC12	ns	ns	ns	ns	Repression	ns
C6	ns	Activation	ns	ns	ns	ns
GP	ns	ns	ns	Activation	ns	ns

TABLE 1: **Cell Type and NF-κB Subunit Dimer Binding Specificity.**

BACE1 promoter construct displays specific binding of selective NF-κB subunit proteins. PC12, C6 and guinea pig hippocampal astrocytic cells were transfected with BPR and two of the individual NF-κB subunits p50, p52, p65 or c-Rel with a resultant statistically significant alteration in BACE1 driven luciferase expression being present only in those cells transfected with NF-κB /BPRΔNF-κB$^-$ and specific subunit combinations as compared to the control NF-κB /BPRΔNF-κB$^-$ co-transfected with lacZ and Basic. GP: guinea pig hippocampal astrocytic cells, ns: not significant.

-κB protein subunits resulted in an overall decrease in observed luciferase levels. There were also no significant differences in the measured endogenous levels of p50, p52 or p65 protein by Western blot analysis (data not shown). However, all transfections with individual constructs showed to varying degrees that transfections with the mutant BPRΔNF-κB$^-$ reporter construct displayed higher activity in neuronal cells and lower activity in glial cells as assayed by luciferase activity.

BACE1 PROMOTER CONSTRUCT EXPRESSION WAS ALTERED AFTER ACUTE H_2O_2 EXPOSURE

In order to assess the role of oxidative stress, a known activator of NF-κB, NGF-differentiated PC12, RA-differentiated SK-N-SH-SY5Y cells, TNFα treated adult guinea pig hippocampal astrocytes and C6 cells were transfected with either BPR or BPRΔNF-κB$^-$, and allowed to recover for 24 hours. Following recovery cells were exposed to freshly diluted H_2O_2 in PBS at varying concentrations for 40 minutes at room temperature. The medium was removed; the cells re-fed and allowed a recovery period of 48 hours prior to harvesting and luciferase activity determination.

BACE1 promoter construct expression following acute exposure to H_2O_2 showed no significant change in NGF-differentiated PC12 (Figure 29), RA-differentiated SK-N-SH-SY5Y (Figure 30) or in C6 cells (Figure 31). The decreased amounts of expression observed at 100μM H_2O_2 and above may be attributed to the cytotoxic effects of H_2O_2 as determined by personal observation of floating-detached cells. Adult guinea pig

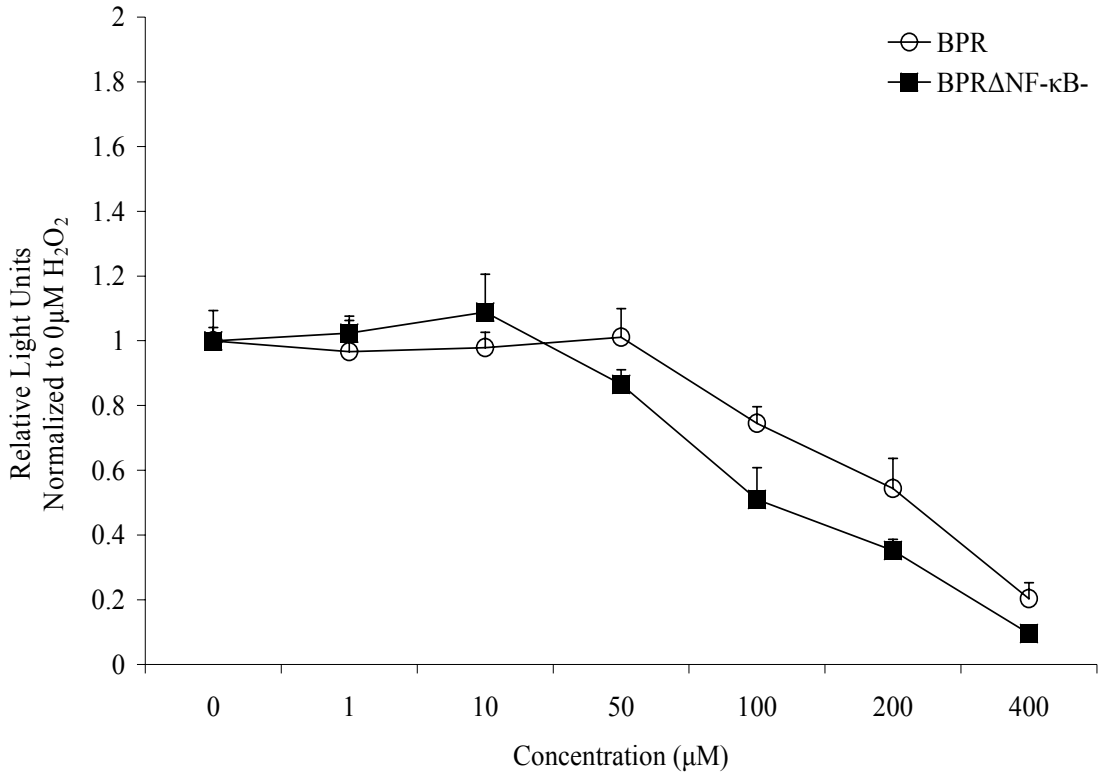


FIGURE 29. BACE1 Promoter Vector Expression After H_2O_2 Treatment: NGF-Differentiated PC12. Transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions and exposed to H_2O_2 using NGF-differentiated PC12 cells. When NGF-differentiated PC12 cell transfected and exposed to increasing concentrations of H_2O_2 there was no significant difference between the BPR or BPRΔNFκB- transfected cells in BACE1 expression. The decreased expression observed at 100μM H_2O_2 and above may be attributed to the cytotoxic effects of H_2O_2. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments.

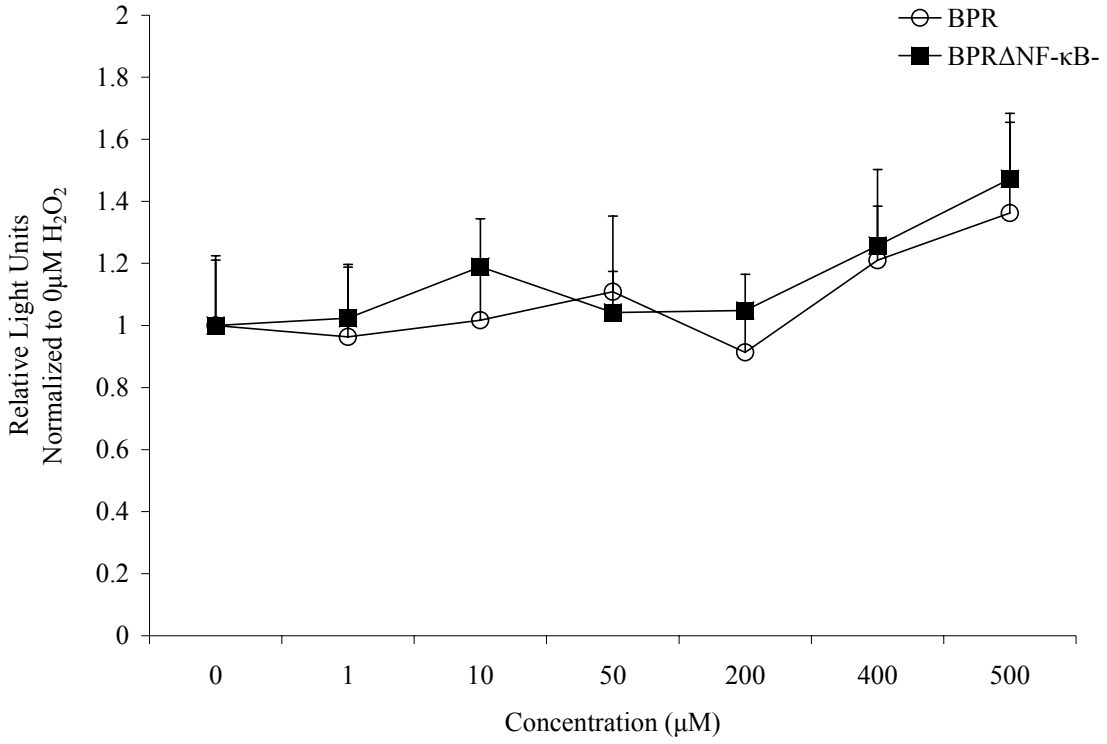


FIGURE 30. BACE1 Promoter Vector Expression After H_2O_2 Treatment: SK-N-SH-SY5Y. Transient transfections of RA-differentiated SK-N-SH-SY5Y cells with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions and exposed to H_2O_2. In RA-differentiated SK-N-SH-SY5Y cells BACE1 expression was unaltered after acute exposure to H_2O_2. It was our observation that SK-N-SH-SY5Y cells appear to be more resistant to injury than other differentiated cell lines, thus the decreased expression observed at 100μM H_2O_2 and above cytotoxic effects of H_2O_2 in PC12 cells was not observed in RA-differentiated SK-N-SH-SY5Y cells. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments.

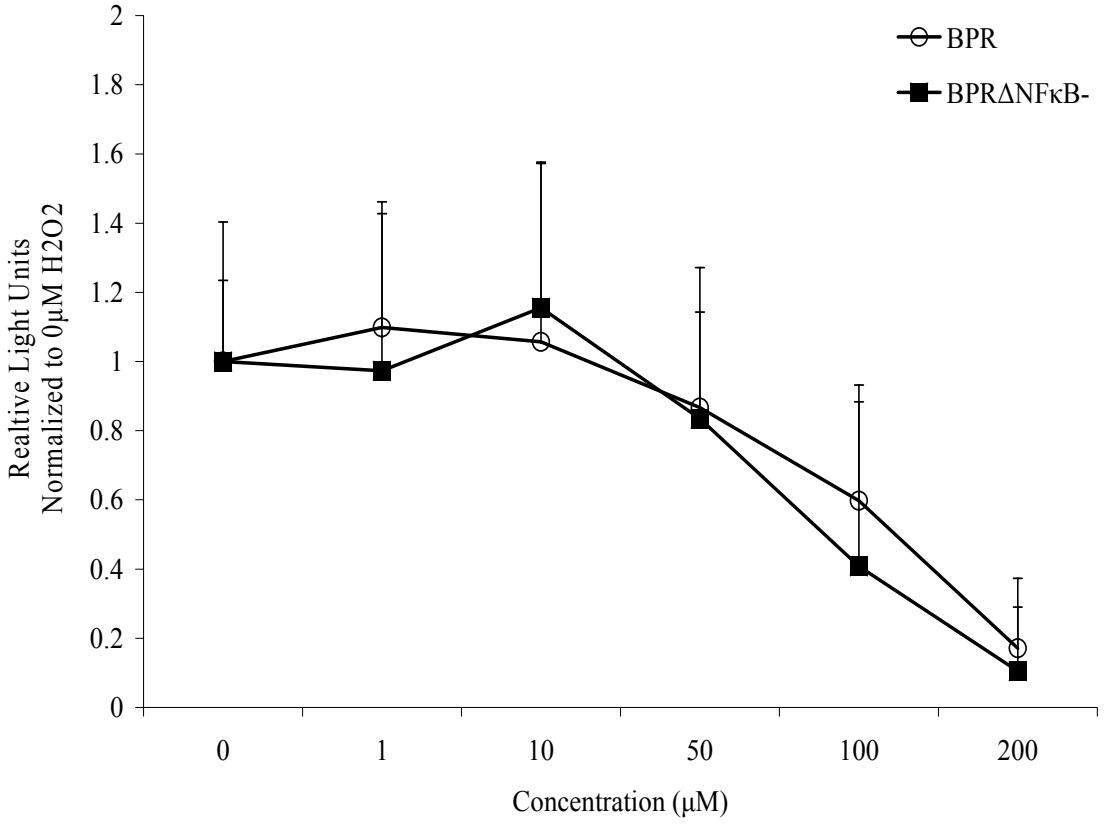


FIGURE 31. BACE1 Promoter Vector Expression After H_2O_2 Treatment: C6.

Transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions and exposed to H_2O_2 using C6 cells. C6 cells BACE1 expression was unaltered after acute exposure to H_2O_2. The decreased amounts of expression observed at 50μM H_2O_2 and above may be attributed to the cytotoxic effects of H_2O_2 . Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments.

hippocampal astrocytes cells BACE1 expression was altered after acute exposure to H_2O_2 (Figure 32). In the non-activated state the astrocytes behave as neurons, but when exposed to activating levels of H_2O_2 (50-100μM), the BPRΔNF-κB- transfected cells displayed reduced BACE1 vector promoter expression as compared to those cells transfected with the functional NF-κB site, indicating that the NF-κB site in astrocytes functioned to activate BACE1 expression. At levels above 200μM H_2O_2 a cytotoxic effect was seen as indicated by the reduced vector expression in all cells transfected (except SK-N-SH-SY5Y cells).

BACE1 PROMOTER CONSTRUCT EXPRESSION AS ALTERED AFTER ACUTE AB_{1-42} EXPOSURE

In order to determine if activation, or repression, of BACE1 and the subsequent changes in β-amyloid levels themselves affect BACE1 expression, the effects of exposure of cells transfected with wild type BPR and mutant BPRΔNF-κB$^-$ constructs to β-amyloid were determined. PC12 cells NGF-differentiated for five days and primary neonatal rat astrocytic cells were transfected with either BPR or BPRΔNF-κB$^-$ and allowed to recover for 24 hours. Following recovery cells were exposed to freshly diluted soluble or aggregated $Aβ_{1-42}$ or $Aβ_{42-1}$ in 200mM HEPES pH 8.5 at varying concentrations (10pM-50μM) for 24 hours then harvested and luciferase activity measured. Depending on which construct, concentration or β-amyloid orientation used, there was an effect on luciferase activity, as measured with these BACE1 promoter

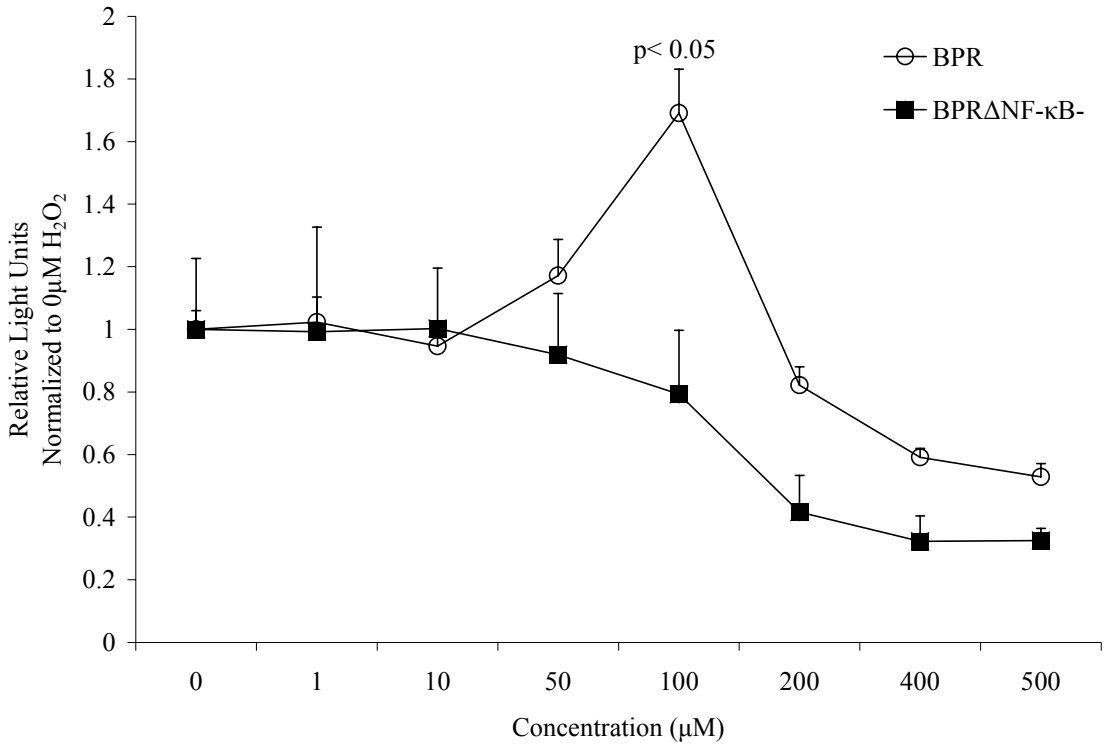


FIGURE 32. BACE1 Promoter Vector Expression After H_2O_2 Treatment: Adult Guinea Pig Primary Astrocytic Cells. Transient transfections with the two reporter constructs BPR and BPRΔNF-κB$^-$ under optimal conditions and exposed to H_2O_2 using adult guinea pig primary hippocampal astrocytic cells. BACE1 expression was significantly increased ($p< 0.05$ BPR vs BPRΔNF-κB$^-$) in those cells exposed to 100μM of H_2O_2. The decreased expression observed at 200μM H_2O_2 and above may be attributed to the cytotoxic effects of H_2O_2. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

expression vectors in PC12 cells (Figures 33-35) or in primary neonatal rat astrocytes (Figures 36-37).

In PC12 cells exposed to 10pM, 50pM, 100pM or 1nM soluble $A\beta_{1-42}$ or $A\beta_{42-1}$ the BPRΔNF-κB$^-$ transfected displayed consistently higher levels of luciferase activity particularly at lower concentrations tested (Figure 33). BPRΔNF-κB$^-$ transfected cells always expressed higher levels of BACE1 promoter expression regardless of concentration or orientation of β-amyloid. When transfected PC12 cells were exposed to aggregated (Figure 34) 50nM $A\beta_{1-42}$ or $A\beta_{42-1}$ no significant differences between treated and untreated were detected. When endogenous BACE1 levels were assessed following exposure to 10pM, 50pM, 100pM or 1nM $A\beta_{1-42}$ or $A\beta_{42-1}$ no observable differences in BACE1 expression were detected (data not shown). Thus we believe that the large increase in BACE1 activity seen at exposure to lower concentrations 10pM, 50pM, 100pM or 1nM $A\beta_{1-42}$ or $A\beta_{42-1}$ in cells transfected with BPRΔNF-κB$^-$ were a result of the absence of normal feedback mechanisms which can not be measured by use of vector reporter constructs and do not reflect a true endogenous response in BACE1 levels to the presence of $A\beta_{1-42}$. Of note was that treatment with either $A\beta_{1-42}$ or $A\beta_{42-1}$ resulted in the same cellular response, indicating a potential lack of Aβ specificity.

When cells were exposed to 100nM soluble Aβ, there were no significant increases in BACE1 promoter expression in those cells transfected with BPRΔNF-κB$^-$ and treated with the soluble$A\beta_{1-42}$ peptide, although there was a significant increase in BACE1 promoter reporter vector expression with $A\beta_{42-1}$ treatment. When PC12 cells

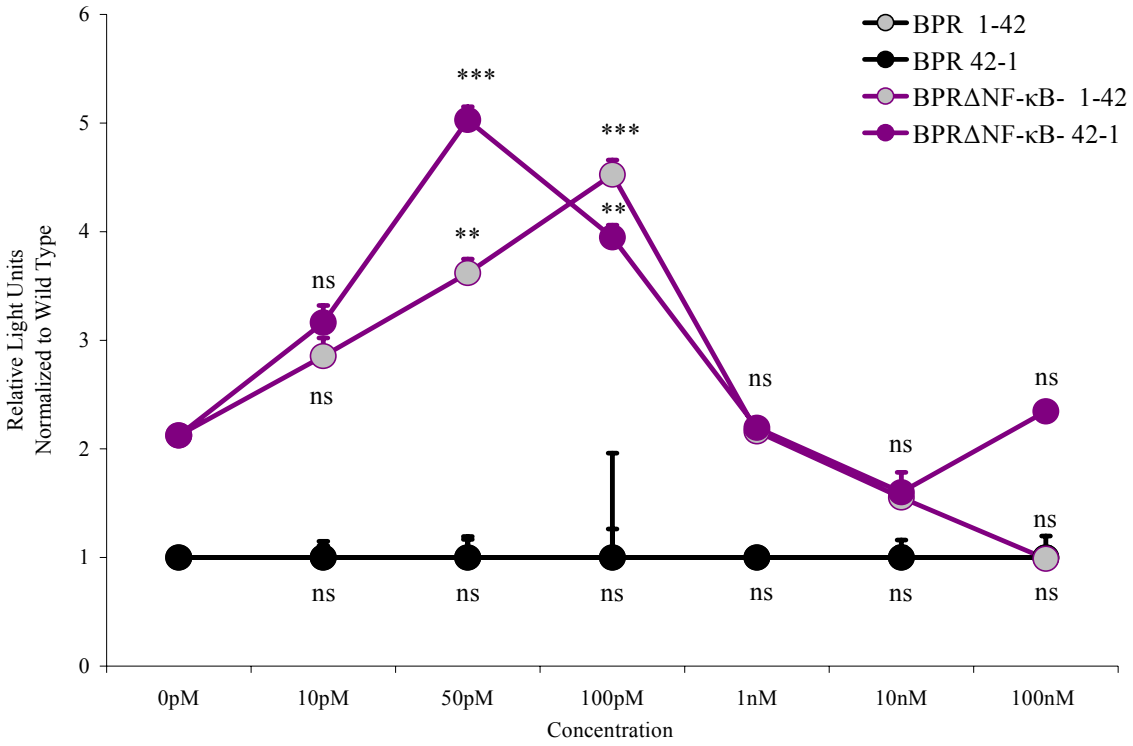


FIGURE 33. BACE1 Promoter Vector Expression After Soluble 10pM-100nM Aβ Treatment: NGF-differentiated PC12. Transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions using PC12 cells. NGF-differentiated PC12 cells were transfected with BPR or BPRΔNFκB- and exposed to soluble 10pM-100nM $A\beta_{1\text{-}42}$ and $_{42\text{-}1}$. There were no statistically significant differences in BACE1 driven luciferase expression within either group between those cells exposed to $A\beta_{1\text{-}42}$ and $A\beta_{42\text{-}1}$. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. *** $p< 0.0001$, ** $p< 0.01$, ns: not significant.

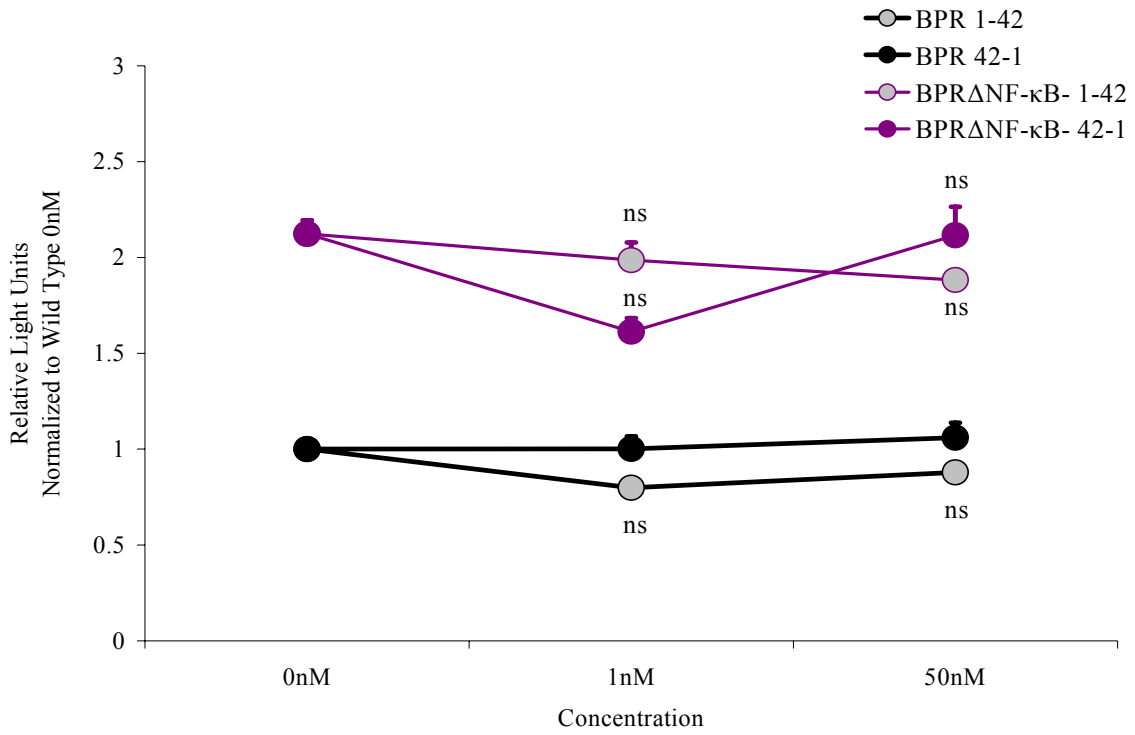


FIGURE 34. BACE1 Promoter Vector Expression After Aggregated 1nM-50nM Aβ Treatment: NGF-Differentiated PC12. NGF-differentiated PC12 were transiently transfected with BPR or BPRΔNFκB- and exposed to aggregated 1nM-50nM $A\beta_{1\text{-}42}$ and $A\beta_{42\text{-}1}$. There was a statistically significant increase ($p< 0.01$) in BACE1 driven luciferase expression in all cells transfected with the mutant BPRΔNF-κB$^-$ and exposed to $A\beta_{1\text{-}42}$ and $A\beta_{42\text{-}1}$. $A\beta_{1\text{-}42}$ and $A\beta_{42\text{-}1}$ exposure did not significantly affect luciferase activity within either group (wild type or mutant) regardless of orientation or concentration. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

were exposed to concentrations of $A\beta_{1-42}$ greater then 100nM there was the same pattern of expression as seen in the non-treated cells (Figure 35 A and B).

Neonatal primary rat astrocytic cells (Figure 36) transfected with BPR or BPRΔNF-κB$^-$ and exposed to soluble 1nM or 50nM $A\beta_{1-42}$ showed a significant increase in BACE1 vector expression in those cells transfected with the mutant construct. No such effect was seen in cells exposed to $A\beta_{42-1.}$ A significant increase in BACE1 vector expression in those cells transfected with the mutant construct was seen only with 1nM when aggregated $A\beta_{1-42}$ was used (Figure 37). When endogenous BACE1 levels were assessed following exposure to soluble 1nM or 50nM $A\beta_{1-42}$ no observable differences in BACE1 expression were detected (data not shown). Thus we believe that the increase in BACE1 activity seen in cells transfected with BPRΔNF-κB$^-$ were a result of the absence of normal feedback mechanisms which can not be measured by use of vector reporter constructs and do not reflect a true endogenous response in BACE1 levels to the presence of $A\beta_{1-42}$.

BACE1 PROMOTER CONSTRUCT EXPRESSION AS ALTERED AFTER CHRONIC AB_{1-42} EXPOSURE

In order to evaluate the effect of time of exposure to Aβ on the expression of BACE1 promoter vectors 5 day NGF-differentiated PC12 cells were transfected with either BPR or BPRΔNF-κB$^-$ and allowed to recover for 24 hours. Following recovery cells were exposed to freshly diluted 10μM of $A\beta_{1-42}$ or $A\beta_{42-1}$ (data not shown for $A\beta_{42-1}$)

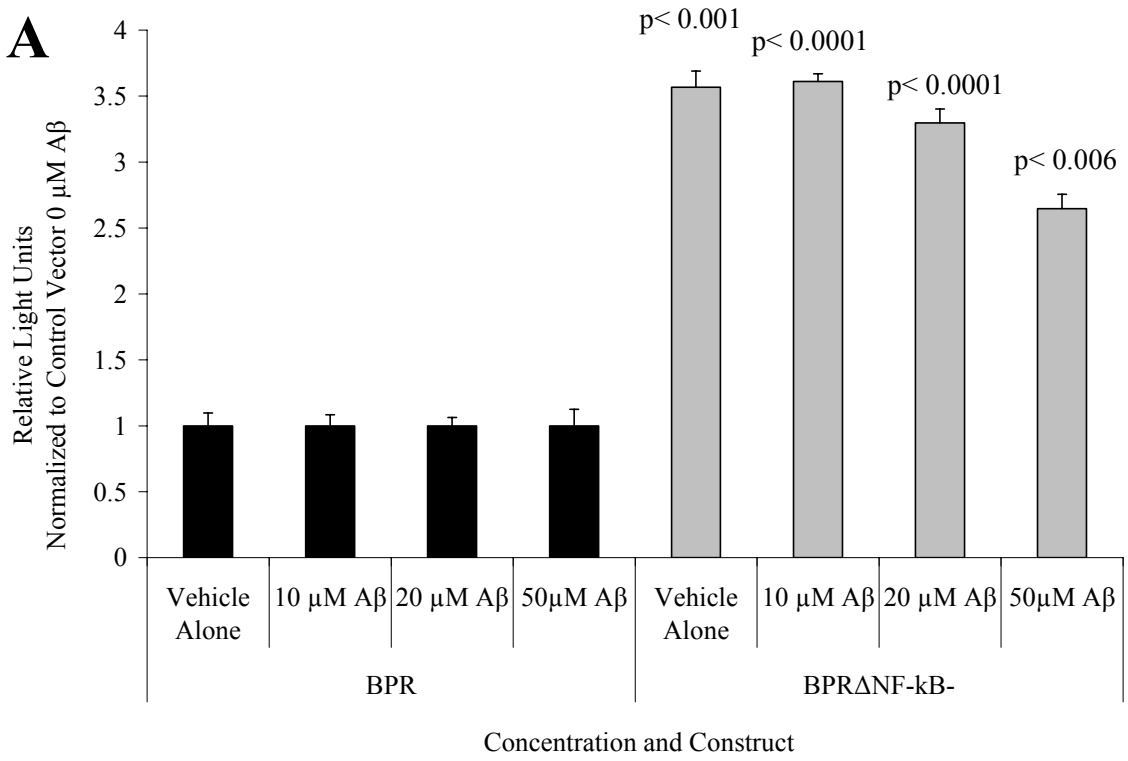


FIGURE 35.A. BACE1 Promoter Vector Expression After Soluble 10µM-50µM Aβ Treatment: NGF-Differentiated PC12. NGF-differentiated PC12 cells were transiently transfected with BPR or BPRΔNFκB- and exposed to soluble 10µM-50µM $A\beta_{1\text{-}42}$. **A.** There was a statistically significant increase ($p< 0.001$) in BACE1 driven luciferase expression in all cells transfected with the mutant BPRΔNF-κB^- and exposed to $A\beta_{1\text{-}42}$. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

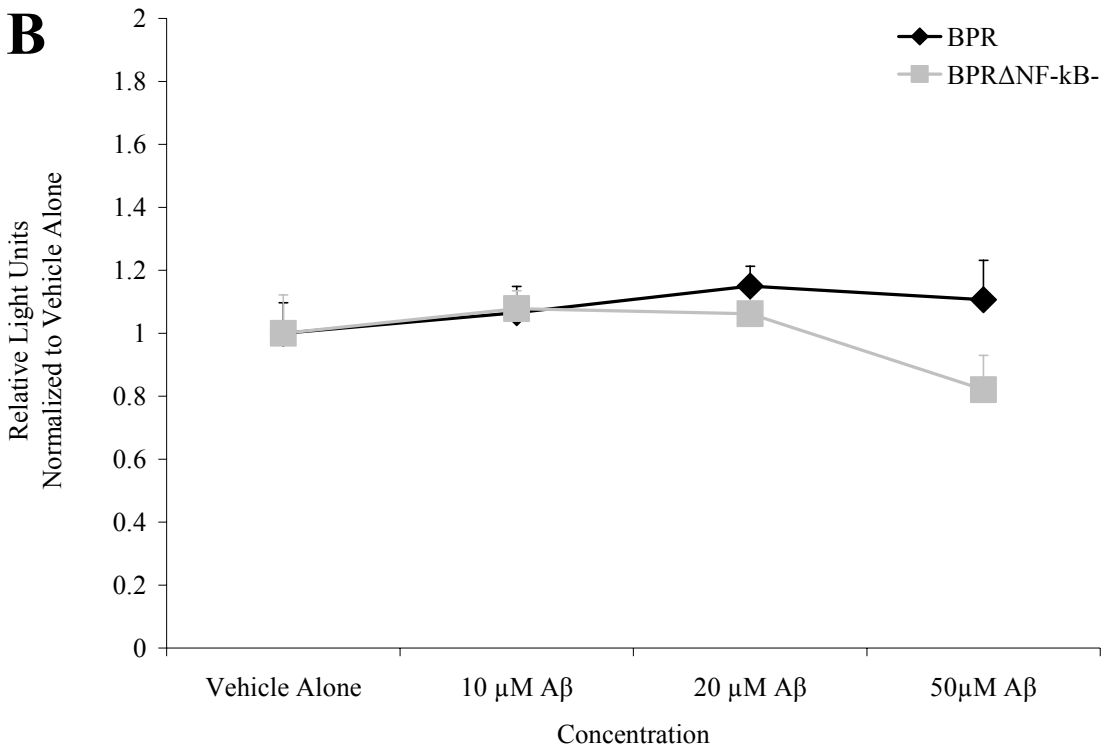


FIGURE 35.B. BACE1 Promoter Vector Expression After Soluble 10μM-50μM Aβ Treatment: NGF-Differentiated PC12. NGF-differentiated PC12 cells were transiently transfected with BPR or BPRΔNFκB- and exposed to soluble 10μM-50μM $A\beta_{1\text{-}42}$. **B.** $A\beta_{1\text{-}42}$ exposure did not significantly affect luciferase activity within either group (BPR or mutant) regardless of concentration. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

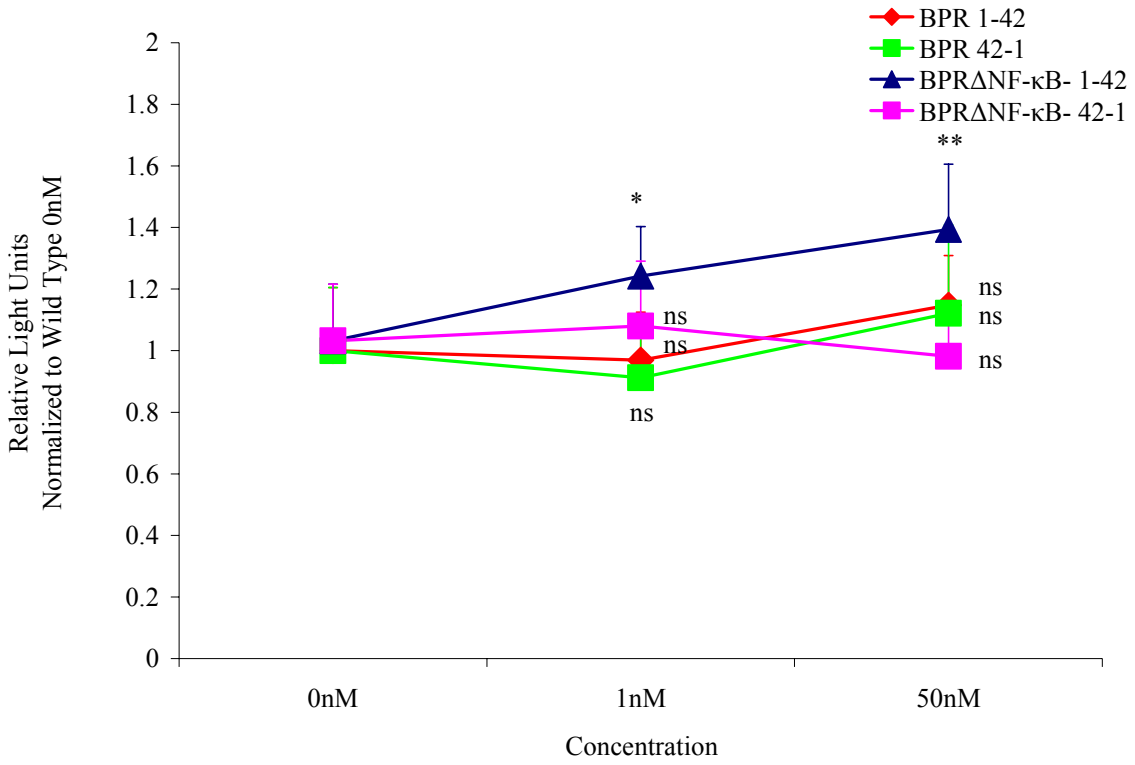


FIGURE 36. BACE1 Promoter Vector Expression After Soluble 1nM-50nM Aβ Treatment: Neonatal Rat Primary Astrocytic Cells. Neonatal rat primary astrocytic cells were transiently transfected with BPR or BPRΔNFκB- and exposed to soluble 1nM-50nM $A\beta_{1\text{-}42}$ and $A\beta_{42\text{-}1}$. There was a statistically significant increase ($p< 0.02$) in BACE1 driven luciferase expression in cells transfected with the mutant BPRΔNF-κB^{-} and exposed to1nM and 50nM $A\beta_{1\text{-}42}$. $A\beta_{42\text{-}1}$ exposure did not significantly affect luciferase activity within either group (wild type or mutant) regardless of concentration. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ** $p< 0.003$, * $p< 0.02$, ns: not significant.

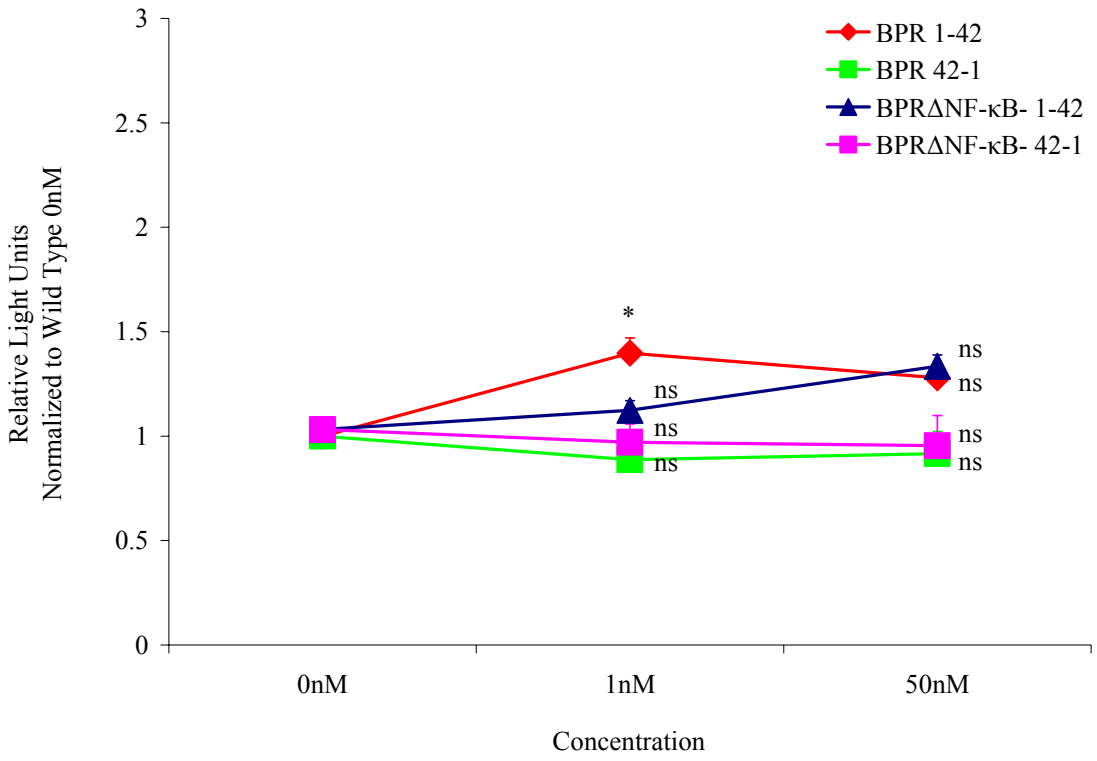


FIGURE 37. BACE1 Promoter Vector Expression After Aggregated 1nM-50nM Aβ Treatment: Neonatal Rat Primary Astrocytic Cells. Neonatal rat primary astrocytic cells were transiently transfected with BPR or BPRΔNFκB- and exposed to aggregated 1nM-50nM $A\beta_{1\text{-}42}$ and $A\beta_{42\text{-}1}$. There was a statistically significant increase ($p<0.02$) in BACE1 driven luciferase expression in cells transfected with the mutant BPRΔNF-κB^- and exposed to 1nM $A\beta_{1\text{-}42}$ but not at other concentrations tested. $A\beta_{42\text{-}1}$ exposure did not significantly affect luciferase activity within either group (wild type or mutant) regardless of concentration. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. * $p< 0.02$, ns: not significant.

in 200mM HEPES pH 8.5 for times ranging from 24-96 hours then harvested and luciferase activity measured (Figure 38) and endogenous BACE1 protein levels examined by Western blot assay (data not shown). When exposed to 10μM Aβ for 24-96 hours there was a statistically significant increase in luciferase activity in the mutated promoter lacking the NF-κB binding as compared to wild type. We failed to detect any differences between groups treated and nontreated regardless of duration of exposure to $A\beta_{1-42}$. We believe that the unaltered pattern of increase in BACE1 activity seen in cells transfected with BPRΔNF-κB^- were a result of the absence of normal feedback mechanisms which can not be measured by use of vector reporter constructs and do not reflect a true endogenous response in BACE1 levels to the presence of $A\beta_{1-42}$.

BACE1 ENDOGENOUS PROTEIN LEVELS AS ALTERED FOLLOWING AB_{1-42} EXPOSURE

While useful in their own right, reporter constructs lack participation in feedback loops typical of biological systems. In order to evaluate the effects of β-amyloid on the endogenous BACE1 promoter, NGF-differentiated PC12, RA-differentiated SK-N-SH-SY5Y cells, TNFα treated C6 cells, mouse and rat adult astrocytic cells and adult guinea pig hippocampal astrocytes were exposed to soluble or aggregated $A\beta_{1-42}$ or $A\beta_{42-1}$ in 200mM HEPES pH 8.5 at varying concentrations (0.01μM-10μM) for 24-144 hours, and endogenous BACE1 protein levels examined by Western blot assay (Figures 40-56). Figure 39 shows PC12 cells exposed to soluble 10μM $A\beta_{1-42}$ showed a biphasic increase

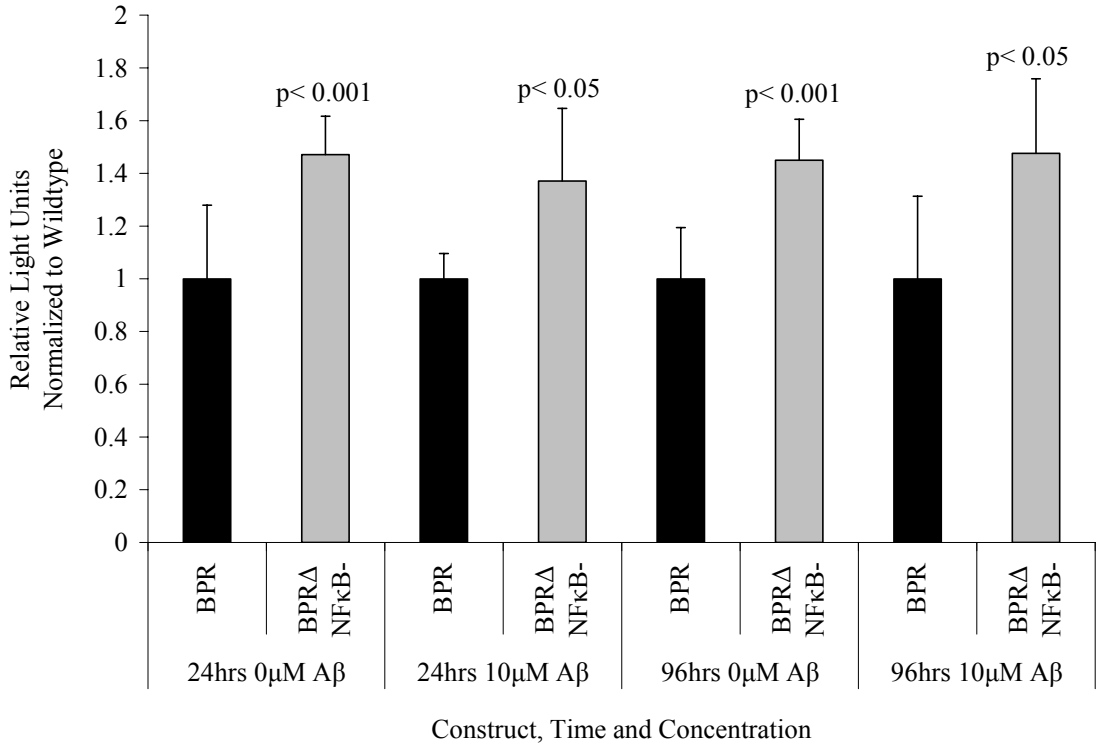


FIGURE 38. BACE1 Promoter Vector Expression 24 + 96 Hours After Soluble 10μm Aβ Treatment: NGF-Differentiated-PC12. Transient transfections with the two reporter constructs wild type BPR and mutant BPRΔNF-κB$^-$ under optimal conditions using NGF-differentiated PC12 cells. Cells were transfected with BPR or BPRΔNFκB- and exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 24-96 hours. There was a statistically significant increase ($p< 0.05$) in BACE1 driven luciferase expression in those cells transfected with the mutant BPRΔNF-κB$^-$ regardless of exposure to $A\beta_{1\text{-}42}$. $A\beta_{42\text{-}1}$ exposure did not significantly affect luciferase activity within either group (wild type or mutant) regardless of concentration or duration of exposure. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

in BACE1 expression with time as the variable with a significant increase in BACE1 expression was seen with a 10μM $A\beta_{1-42}$ exposure at 48 ($p< 0.002$) and 96 ($p< 0.0001$) hours but not at 24 or 72 hours after exposure.

Endogenous BACE1 expression measured at 24 or 96 hours after soluble $A\beta_{1-42}$ or $A\beta_{42-1}$ revealed no significant effect at 24 hours regardless of concentration or orientation, at 96 hours a significant increase was seen with both the 10μM $A\beta_{1-42}$ ($p< 0.01$) and 10μM $A\beta_{42-1}$ ($p< 0.006$) (Figure 40). When the experiment was conducted using aggregated $A\beta_{1-42}$ or $A\beta_{42-1}$, a significant increase in BACE1 protein was seen at 24 hours with only the 10μM concentration of either $A\beta_{1-42}$ ($p< 0.01$) or $A\beta_{42-1}$ ($p< 0.002$). No statistically significant changes were observed at 24 hours at 1μM $A\beta_{1-42}$ or at any of the 96 hour time points tested (10μM $A\beta_{1-42}$ or $A\beta_{42-1}$).

When PC12 cells were exposed to soluble 0.01-10μM for 72 hours an interesting pattern emerged. At 1μM, 5μM and 10μM soluble $A\beta_{1-42}$ there were no significant effects on BACE1 expression (Figure 41). However at the lower concentrations tested 0.01μM ($p< 0.0002$) and 0.1μM ($p< 0.003$) a significant decrease in BACE1 expression was observed. At 48 hours both soluble and aggregated $A\beta_{1-42}$ at either 1μM soluble $p< 0.02$ or aggregated $p< 0.01$; or 10μM soluble $p< 0.002$ or aggregated $p< 0.0005$ resulted in a significant increase in endogenous BACE1 protein levels (Figure 42). In an attempt to prevent the aggregation of Aβ in cultures allowed to incubate over periods greater then24 hours daily changes of media were conducted. BACE1 protein levels were

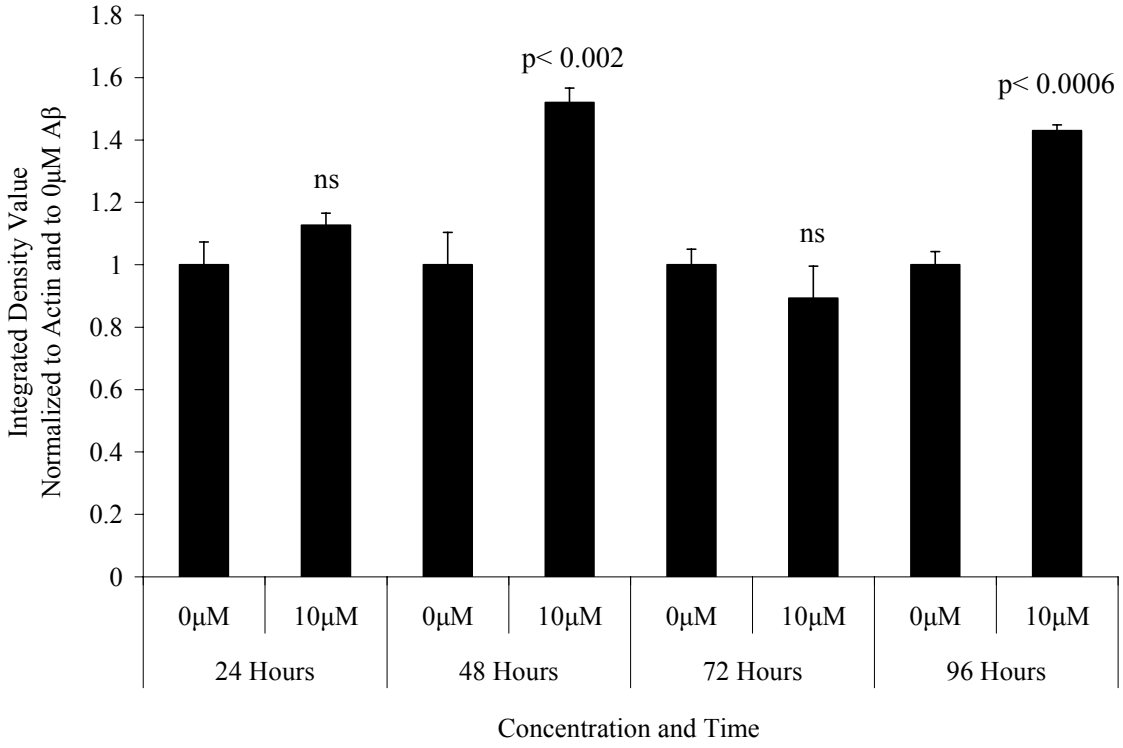


FIGURE 39. Effect of Time of Exposure to Soluble 10μM Aβ on Endogenous BACE1 Expression: NGF-Differentiated-PC12. Endogenous BACE1 expression after 24-96 hour 10μM soluble β-amyloid treatment using NGF-differentiated PC12 cells. Cells were plated, differentiated and exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 24-96 hours. There was a statistically significant increase ($p< 0.002$) in BACE1 expression in those cells exposed to 10μM $A\beta_{1\text{-}42}$ for 48 and 96 hours as compared to 0μM within each time group. $A\beta_{1\text{-}42}$ exposure did not significantly affect BACE1 protein levels at the 24 or 72 hour time point. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

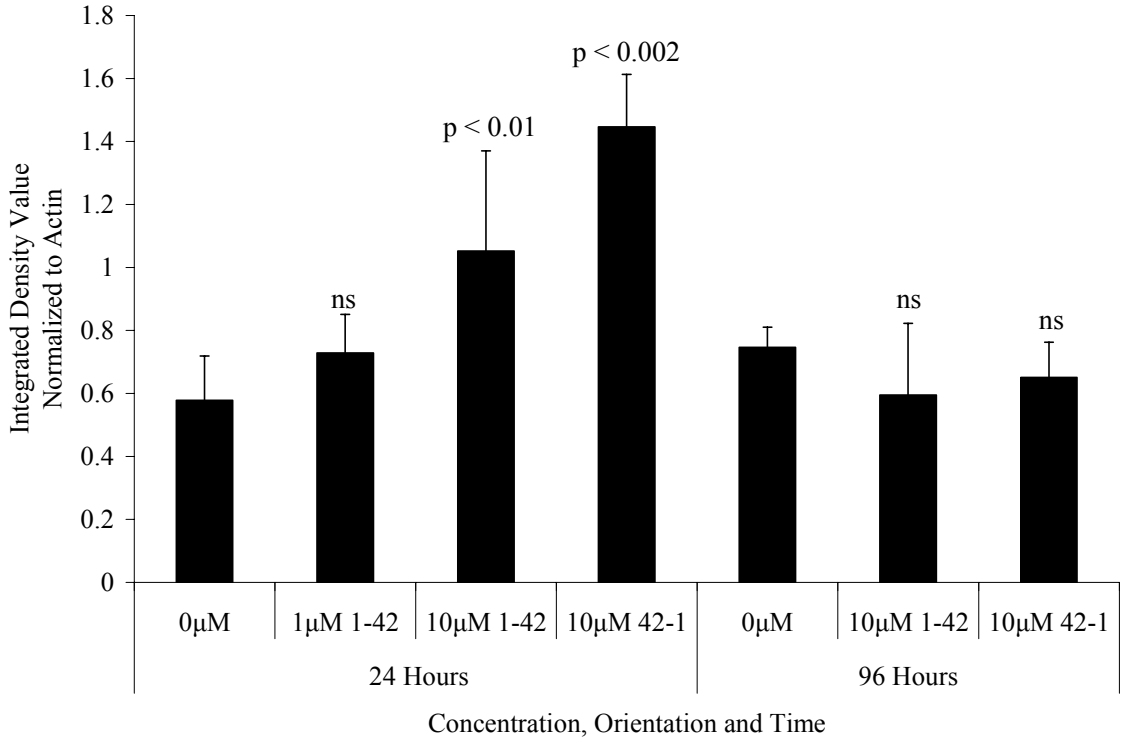


FIGURE 40. Endogenous BACE1 Protein Levels 24 + 96 Hours After Aggregated 1-10μM Aβ Treatment: NGF-Differentiated-PC12. Endogenous BACE1 expression after 24-96 hour 1-10μM aggregated β-amyloid treatment using NGF-differentiated PC12 cells. Cells were plated, differentiated and exposed to aggregated 1-10μM $A\beta_{1\text{-}42}$ for 24-96 hours. There was a statistically significant increase ($p< 0.01$) in BACE1 expression in those cells exposed to 10μM $A\beta_{1\text{-}42}$ and 10μM $A\beta_{42\text{-}1}$ for 24 hours. No effect was seen at 24 hours with exposure to 1μM $A\beta_{1\text{-}42}$. $A\beta_{1\text{-}42}$ or $A\beta_{42\text{-}1}$ exposure did not significantly affect BACE1 protein levels at the 96 hour time point. Statistical comparisons made to 0μM within each time grouping. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

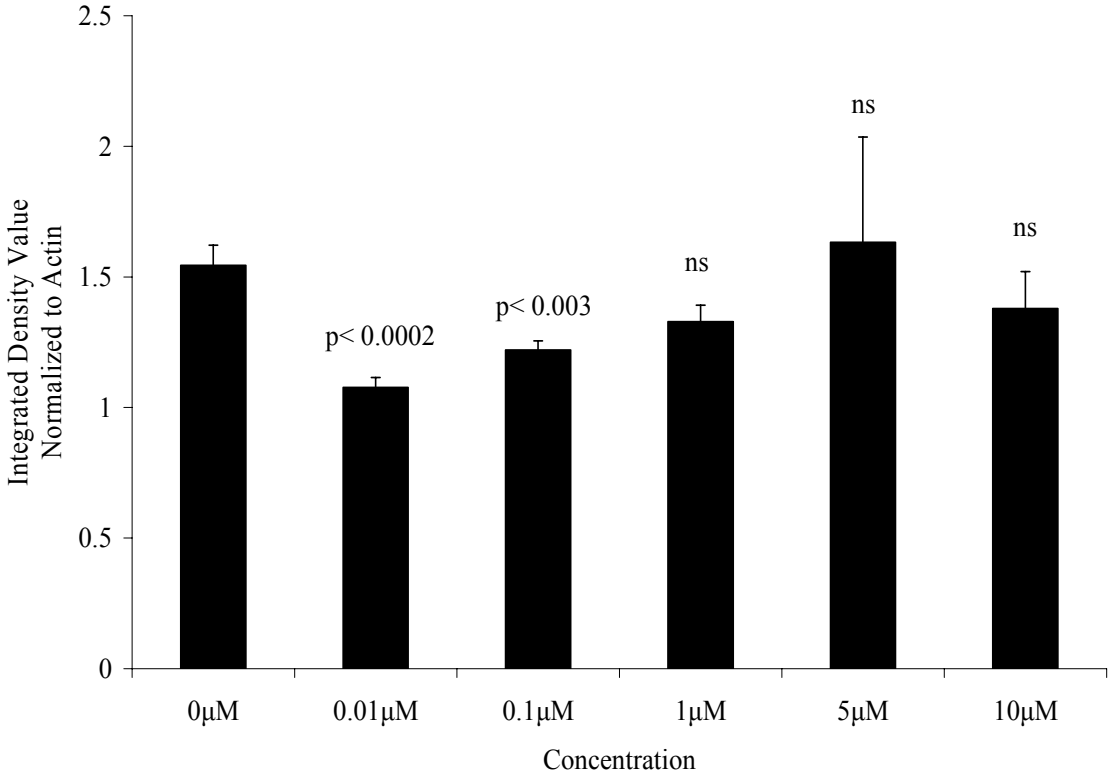


FIGURE 41. Endogenous BACE1 Protein Levels 72 Hours After Soluble 0.01-10μM Aβ Treatment: NGF-Differentiated-PC12. PC12 cells were plated, differentiated and exposed to aggregated 0.01 -10μM $A\beta_{1-42}$ for 72 hours. There was a statistically significant decrease ($p< 0.003$) in BACE1 expression in those cells exposed to 0.01μM and 0.1μM $A\beta_{1-42}$ for 72 hours. No effect was seen at 72 hours with exposure to 1 -10μM $A\beta_{1-42}$. Statistical comparisons made to 0μM. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

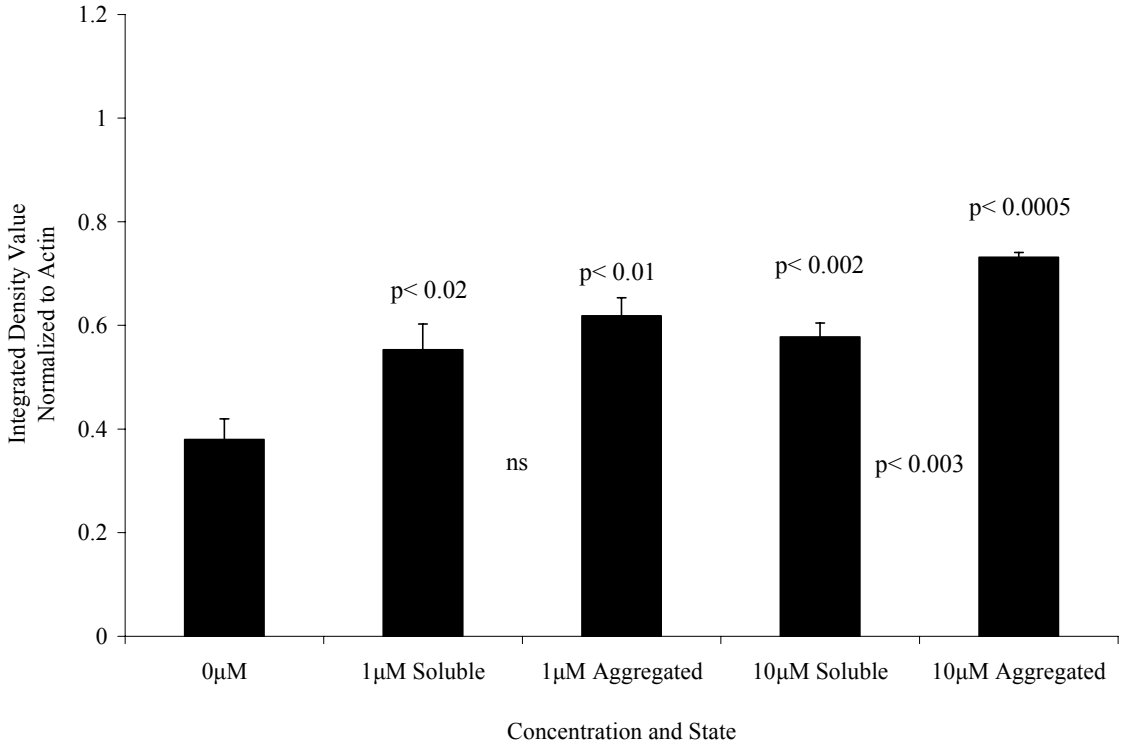


FIGURE 42. Endogenous BACE1 Protein Levels 48 Hours After Soluble or Aggregated 1-10μM Aβ Treatment: NGF-Differentiated PC12. Cells were plated, differentiated and exposed to soluble or aggregated 1-10μM $A\beta_{1-42}$ for 72 hours. There was a statistically significant increase ($p< 0.01$) in BACE1 expression in those cells exposed to either soluble or aggregated 1-10μM $A\beta_{1-42}$ for 48 hours. No difference in BACE1 was observed between soluble and aggregated following exposure to 1μM $A\beta_{1-42}$. A significant increase ($p< 0.003$) in BACE1 expression was observed when the cells were exposed to aggregated 10μM $A\beta_{1-42}$ as compared to soluble 10μM $A\beta_{1-42}$. Statistical comparisons made to 0μM. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

elevated following daily media change in 5 day NGF-differentiated PC12 cells exposed to 0μM soluble $A\beta_{1-42}$; at both 72 and 96 hours after exposure a significant increase in BACE1 protein was observed: 72 hours $p< 0.0002$ and 96 hours $p< 0.02$ (Figure 43). Figure 44 illustrates altered BACE1 protein in cells exposed to 10μM $A\beta_{1-42}$ without a daily change of medium. Seventy-two hour exposure revealed a significant decrease at $p< 0.001$ and 96 hours a significant increase at $p< 0.0001$.

Endogenous BACE1 protein levels were evaluated in 5 day RA-differentiated SK-N-SH-SY5Y cells in response to Aβ exposure. Figure 45 summarizes 24-120 hour exposure to 10μM soluble $A\beta_{1-42}$. A significant increase in BACE1 was observed at all time points 24-96 hours: 24 hours $p< 0.003$, 72 hours $p< 0.0001$, and 96 hours $p< 0.0001$. At 120 hours a significant decrease in BACE1 was seen $p< 0.0001$ in response to soluble $A\beta_{1-42}$ exposure. The 72 hour time point was further examined using a dilution series of $A\beta_{1-42}$. Figure 46 demonstrates that at all concentrations of soluble $A\beta_{1-42}$ tested, except 0.1μM there was a significant increase in BACE1 expression (0.1, 5 and 10μM $p< 0.0001$).

SK-N-SH-SY5Y cells were plated in duplicate and incubated with 10μM $A\beta_{1-42}$ or $A\beta_{42-1.}$ The medium was changed daily in one set of wells but remained unchanged in the duplicate set. After 72 hours the medium was collected from the unchanged set, the cells were harvested and BACE1 protein levels measured. Figure 47 summarizes the results and illustrates a significant increase in BACE1 expression in those cells treated with 10μM $A\beta_{1-42}$ compared to those treated 10μM $A\beta_{42-1}$ with regardless of whether the

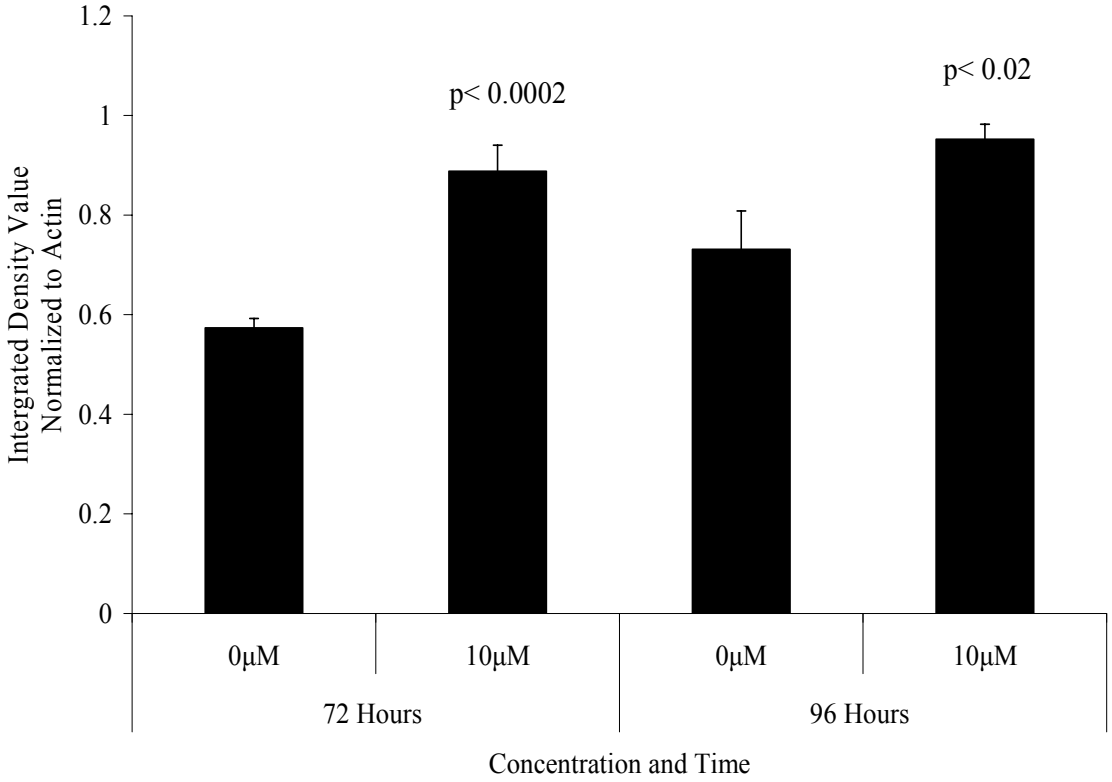


FIGURE 43. Endogenous BACE1 Protein Levels 72-96 Hours After Soluble 10μM Aβ Treatment with Daily Media Change: NGF-Differentiated PC12. Cells were plated, differentiated and exposed to soluble 1-10μM $A\beta_{1\text{-}42}$ for 72-96 hours. There was a statistically significant increase ($p< 0.02$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1\text{-}42}$ for both 72 and 96 hours. Statistical comparisons made to 0μM within a time group. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

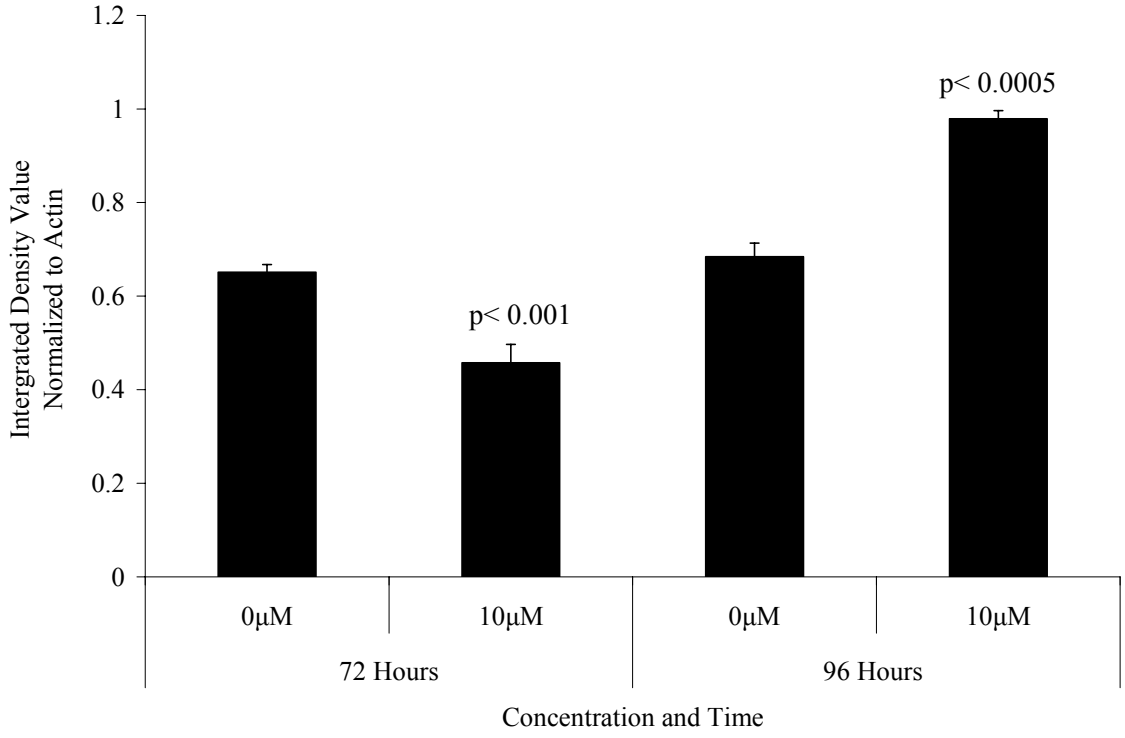


FIGURE 44. Endogenous BACE1 Protein Levels 72-96 Hours After Soluble 10μM Aβ Treatment without Daily Media Change: NGF-Differentiated PC12. Cells were plated, differentiated and exposed to soluble 1-10μM $A\beta_{1\text{-}42}$ for 72-96 hours. There was a statistically significant increase ($p< 0.0005$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 96 hours. There was a statistically significant decrease ($p< 0.001$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 72 hours. Statistical comparisons were made within a time group. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

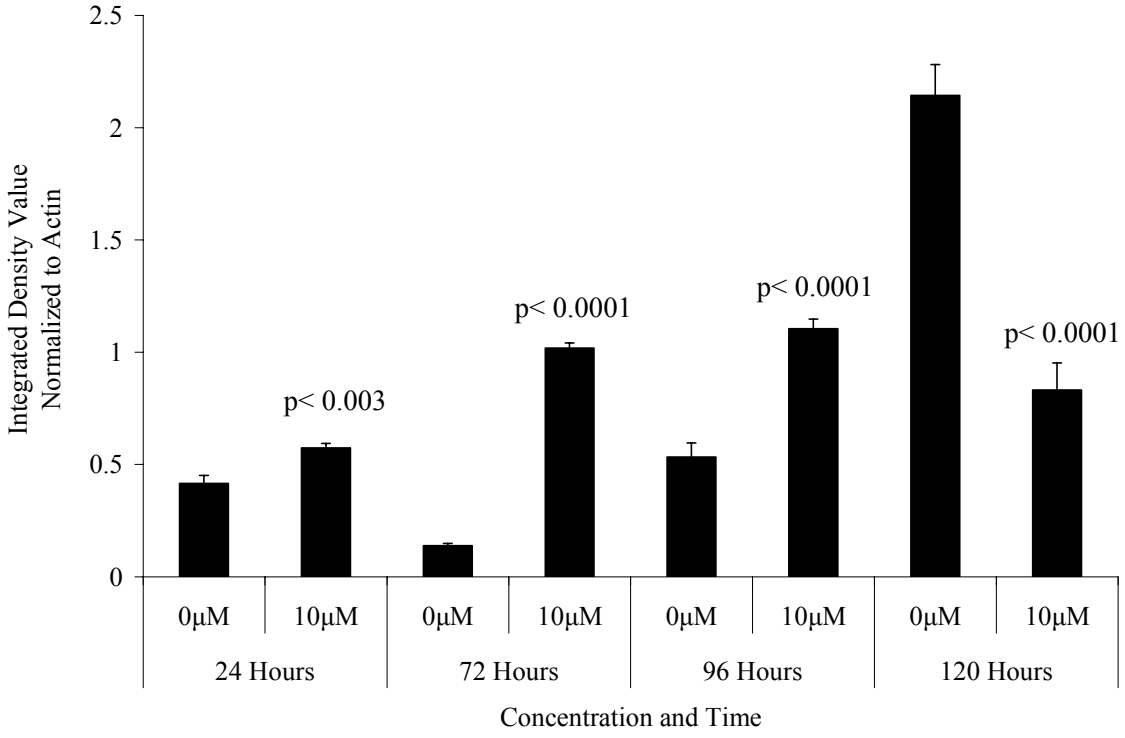


FIGURE 45. Endogenous BACE1 Protein Levels 24-120 Hours After Soluble 10μM Aβ Treatment: SK-N-SH-SY5Y. Cells were plated, differentiated and exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 24-120 hours. There was a statistically significant increase ($p< 0.003$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 24, 72 and 96 hours. There was a statistically significant decrease ($p< 0.0001$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 120 hours. Statistical comparisons were made within a time group. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

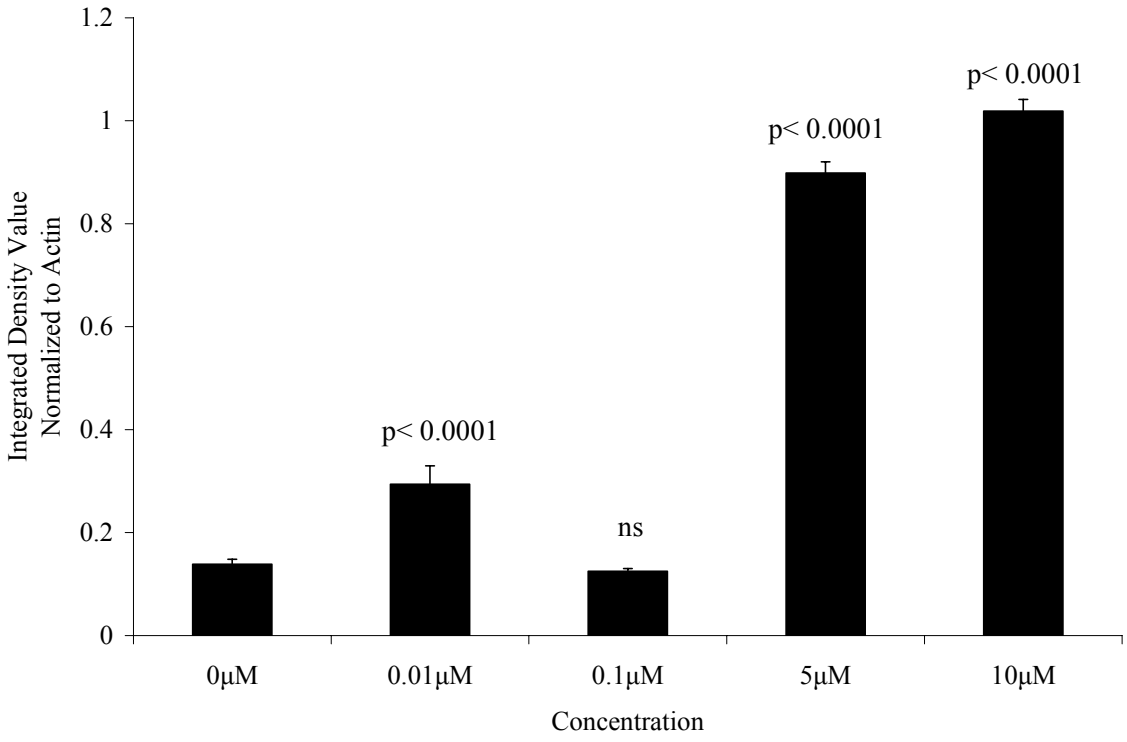


FIGURE 46. Endogenous BACE1 Protein Levels 72 Hours After Soluble 10μM Aβ Treatment: SK-N-SH-SY5Y. Cells were plated, differentiated and exposed to soluble 0.01-10μM $A\beta_{1-42}$ for 72 hours. There was a statistically significant increase ($p< 0.0001$) in BACE1 expression in those cells exposed to soluble 0.01-10μM $A\beta_{1-42}$ for 72 hours with the exception of those cells exposed to soluble 0.1μM $A\beta_{1-42}$ for 72 hours. . Statistical comparisons were made to 0μM. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

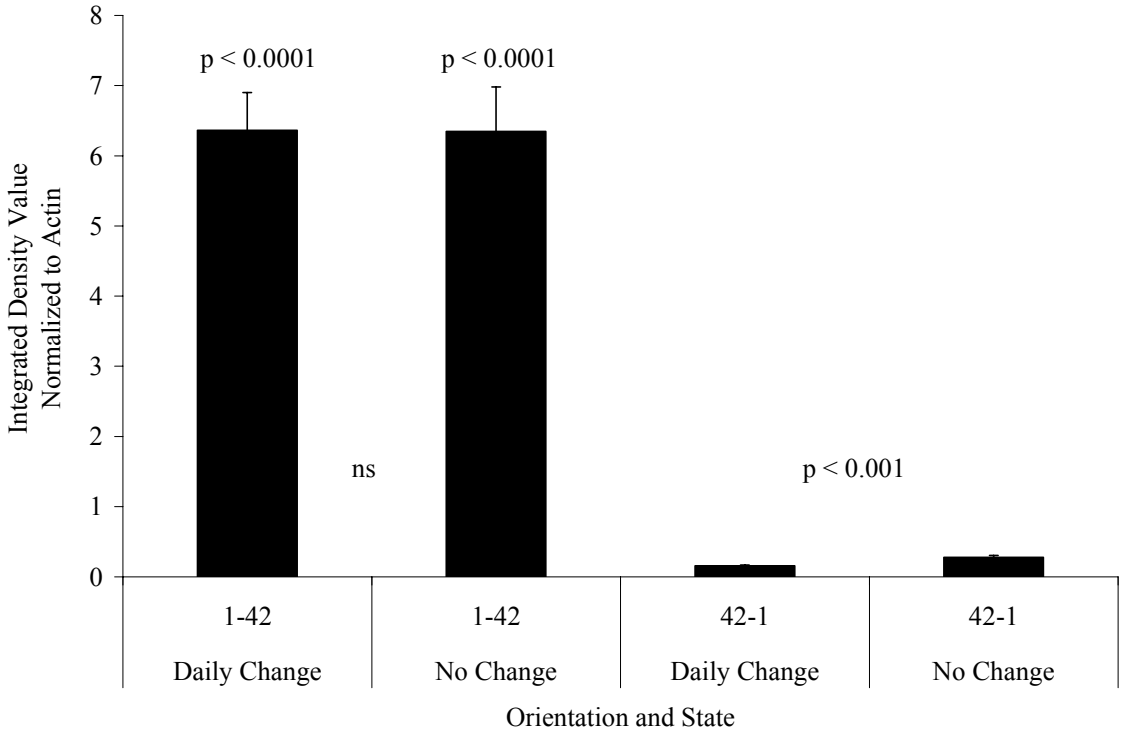


FIGURE 47. Endogenous BACE1 Protein Levels 72 Hours After Soluble 10μM Aβ Treatment With or Without Daily Media Change: SK-N-SH- SY5Y. Cells were plated, differentiated and exposed to soluble 10μM $A\beta_{1\text{-}42}$ or 10μM $A\beta_{42\text{-}1}$ for 72 hours. There was a statistically significant increase ($p< 0.0001$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 72 hours regardless of media change. There was a statistically significant decrease ($p< 0.001$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{42\text{-}1}$ for 72 hours regardless of media change, with a significant increase seen with no media change as compared to daily change. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

medium was changed or not. Despite low levels of BACE1 protein detected in those cells exposed to 10μM $A\beta_{42-1}$, there were statistically significant increases in BACE1 protein levels in those cells that did not have a medium change when compared to the cultures with a daily medium change. In untreated SK-N-SH-SY5Y cells incubated for 96 hours there was no significant difference in BACE1 protein levels between cells with a daily medium change and those in which medium was unchanged (Figure 48). In cells treated with 10μM Aβ with a daily medium change, regardless of orientation $A\beta_{1-42}$ or $A\beta_{42-1}$, there was a significant increase ($p< 0.0001$ and $p< 0.0003$ respectively) in BACE1 expression. Additionally when there was no exchange of medium at 10μM $A\beta_{1-42}$ there was a significant increase in BACE1 protein levels ($p< 0.0002$). Caution must be used when interpreting these results as a daily change of media would serve to create a different environment in regard to factors normally found in culture conditioned media. Therefore the results may be due to the aggregation state of Aβ or the removal of the conditioned media. Our assessments of endogenous BACE1 levels in response to exposure to β-amyloid using neuronal cell models, suggests that NF-κB was but one component of the complex regulatory machinery responsible for the regulation of BACE1 and the eventual fate of β-amyloid.

Rat glioma C6 cells were exposed to varying concentrations of $A\beta_{1-42}$ and harvested at 72 hours. As illustrated in Figure 49 concentrations of (0.01- 5μM) but not 10μM resulted in a significant ($p< 0.006$, $p< 0.007$, $p< 0.02$, and $p< 0.001$ respectively) decrease in BACE1 protein levels compared to untreated controls. C6 cells were exposed

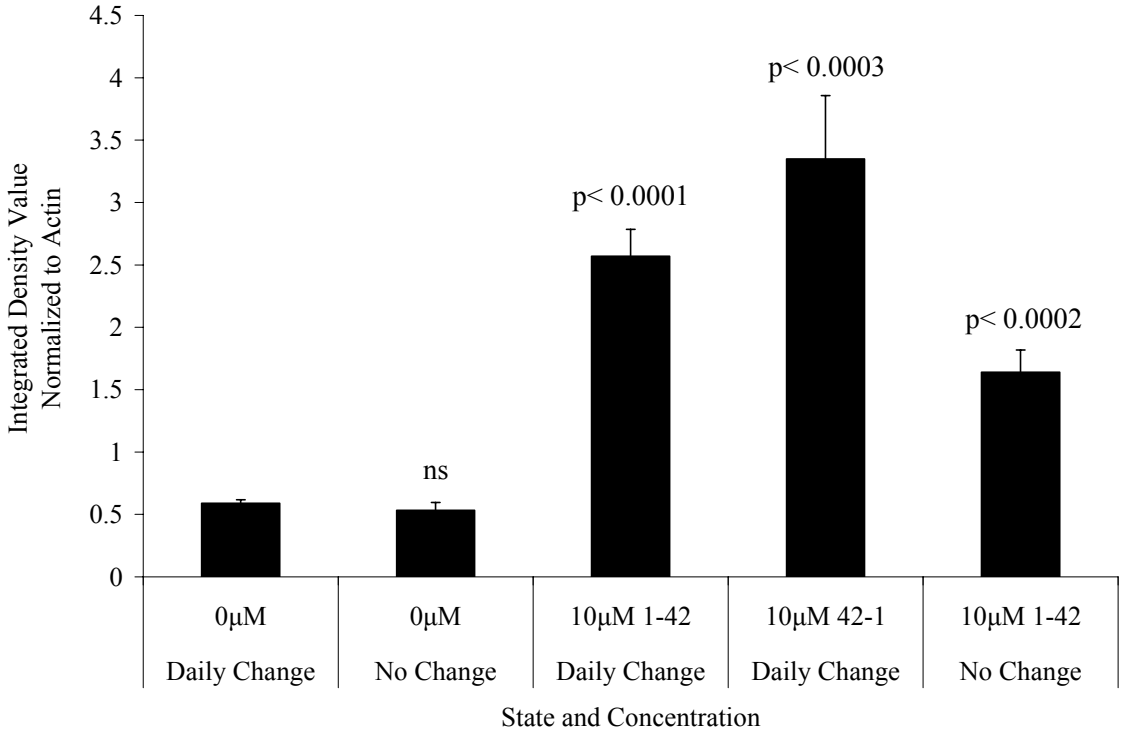


FIGURE 48. Endogenous BACE1 Protein Levels 96 Hours After Soluble 10μM Aβ Treatment: SK-N-SH-SY5Y. Cells were plated, differentiated and exposed to soluble 10μM $A\beta_{1\text{-}42}$ or 10μM $A\beta_{42\text{-}1}$ for 96 hours. There was a statistically significant increase ($p< 0.0002$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 96 hours regardless of media change or orientation. Note the significant increase in BACE1 expression was seen with daily media change with soluble 10μM $A\beta_{42\text{-}1}$. Statistical comparisons are made to 0μM of respective treatment. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

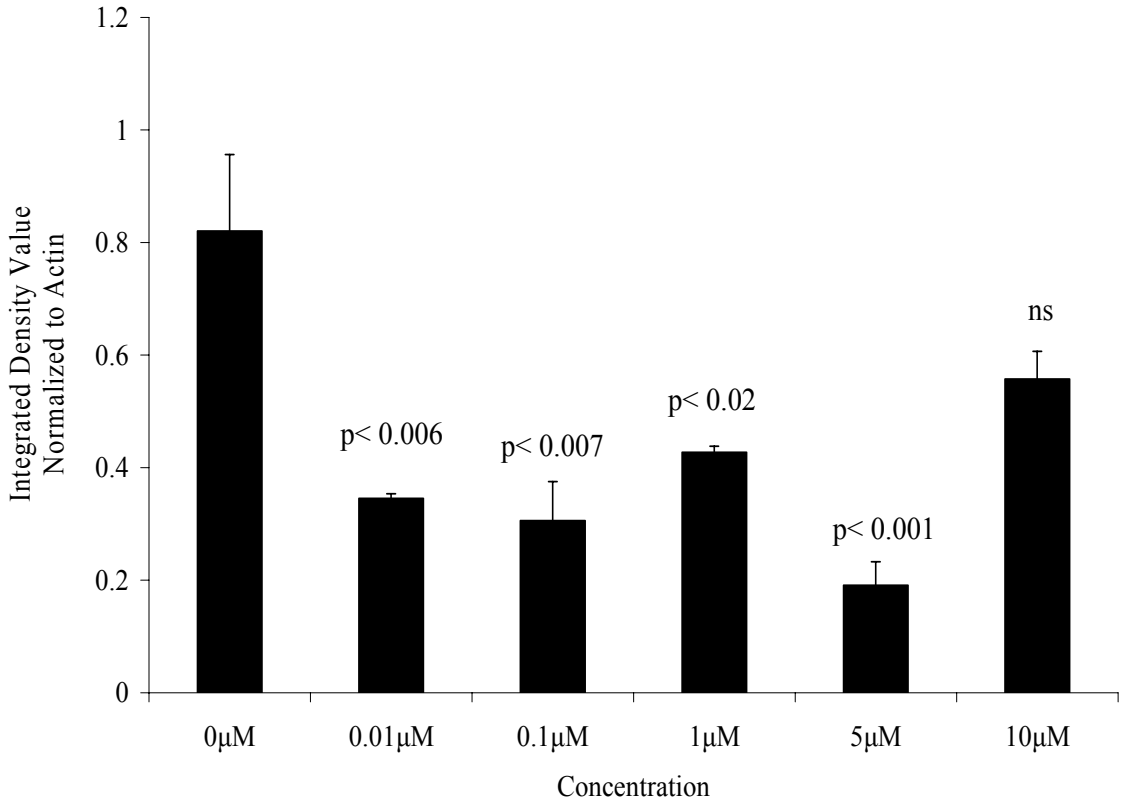


FIGURE 49. Endogenous BACE1 Protein Levels 72 Hours After Soluble 0.01μM-10μM Aβ Treatment: C6. Cells were plated and exposed to soluble 0.01μM -10μM $A\beta_{1-42}$ for 72 hours. There was a statistically significant decrease ($p< 0.02$) in BACE1 expression in those cells exposed to soluble 0.01μM -5μM $A\beta_{1-42}$ for 72 hours. No significant differences were seen with exposure to soluble 10μM $A\beta_{42-1.}$ Statistical comparisons are made to 0μM. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

to TNFα, and treated with 10μM $A\beta_{1-42}$ with or without medium exchange for 96 hours. Under both conditions there was a significant increase ($p< 0.0001$) in BACE1 protein levels compared to the 0μM $A\beta_{1-42}$ control (Figure 50).

Figure 51 shows that there were no significant alterations in BACE1 protein levels in primary adult mouse astrocytic cells exposed to 10μM $A\beta_{1-42}$ for 72 or 144 hours. Primary adult rat astrocytic cells were exposed to 10μM $A\beta_{1-42}$ for 24 or 96 hours. Cells were harvested and analyzed for BACE1 protein levels. A significant decrease was observed at all time points except 72 hours (Figure 52: 24 hours $p< 0.03$, 48 hours $p< 0.00006$ and 96 hours $p< 0.02$).

Primary adult guinea pig hippocampal astrocytic cells were incubated with TNFα and exposed to 10μM $A\beta_{1-42}$ for 72 - 96 hours. Figure 53 illustrates a significant increase in BACE1 protein levels at both 72 ($p< 0.0001$) and 96 ($p< 0.00001$) hour time points. In addition these cells were incubated with a dilution series of $A\beta_{1-42}$ for varying amounts of time. At 24 hours after incubation (Figure 54A) a significant increase in BACE1 protein levels was observed at 0.01μM ($p< 0.0002$) and 0.1μM ($p< 0.006$), however a significant decrease at 1μM ($p< 0.004$) and no significant changes were observed at 0.005μM, 5μM or 10μM. At 48 hours, Figure 54A, significant increases in BACE1 protein levels were observed at 0.001μM ($p< 0.0001$), 0.01μM ($p< 0.005$), 0.1μM ($p< 0.0001$), 1μM ($p\leq 0.0001$) and 5μM ($p< 0.0002$). No significant changes were seen at 48 hours with exposure to 0.005 or 10μM $A\beta_{1-42.}$ BACE1 protein levels were further evaluated at 72 hours after $A\beta_{1-42}$ exposure (Figure 54B). Decreased levels of BACE1 protein were

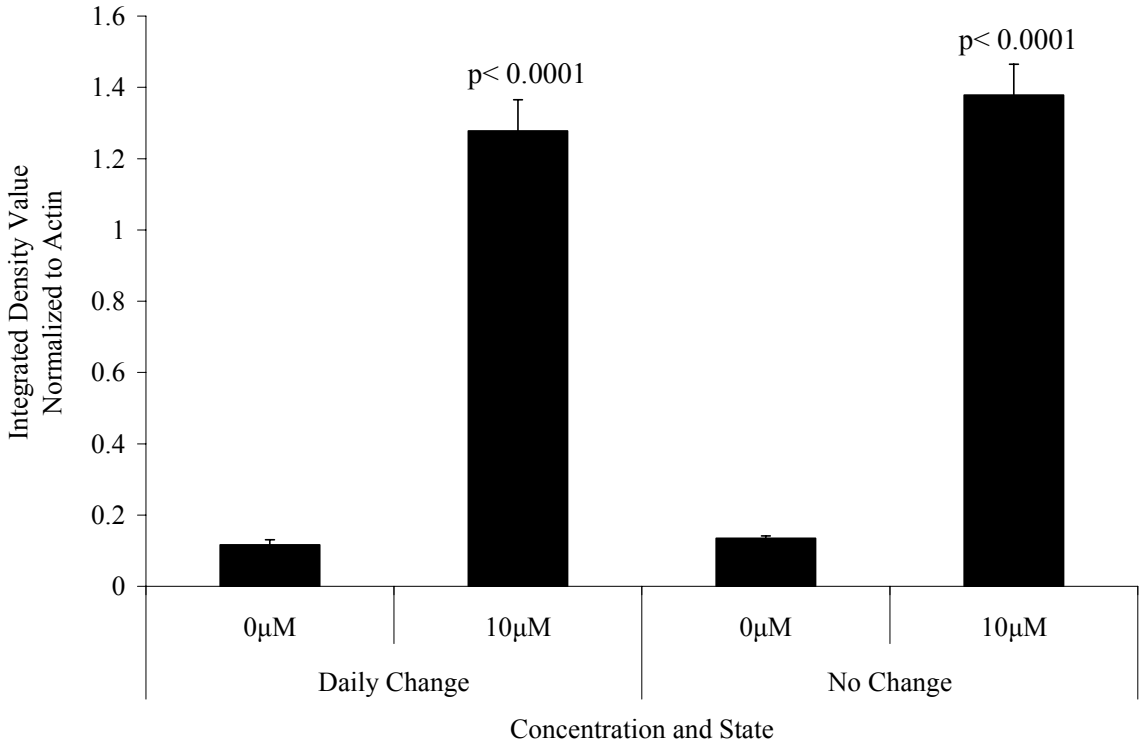


FIGURE 50. Endogenous BACE1 Protein Levels 96 Hours After Soluble 10μM Aβ Treatment: TNFα-Treated C6. Cells were plated, treated with TNFα and exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 96 hours. There was a statistically significant increase ($p< 0.0001$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 96 hours regardless of media change. Statistical comparisons are made to 0μM of respective treatment. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

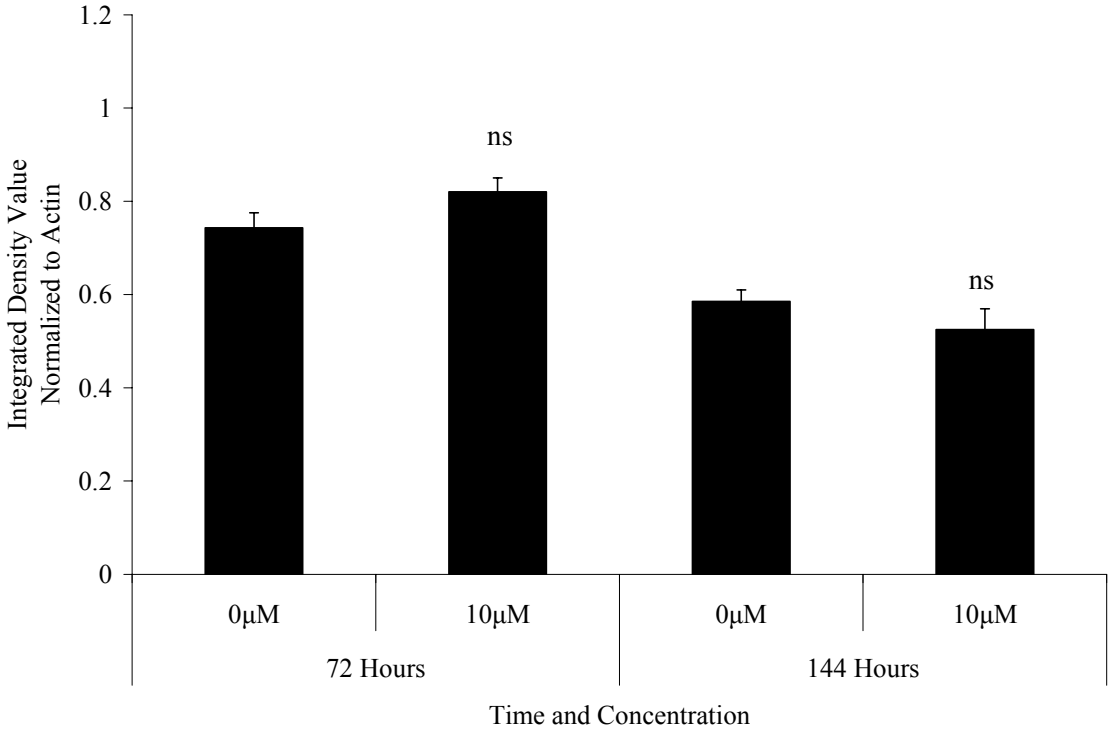


FIGURE 51. Endogenous BACE1 Protein Levels 72-144 Hours After Soluble 10μM Aβ Treatment: Primary Adult Mouse Astrocytic Cells. Cells were plated and exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 72-144 hours. There were no statistically significant changes in BACE1 expression regardless of duration of exposure. Statistical comparisons are made to 0μM at respective time. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

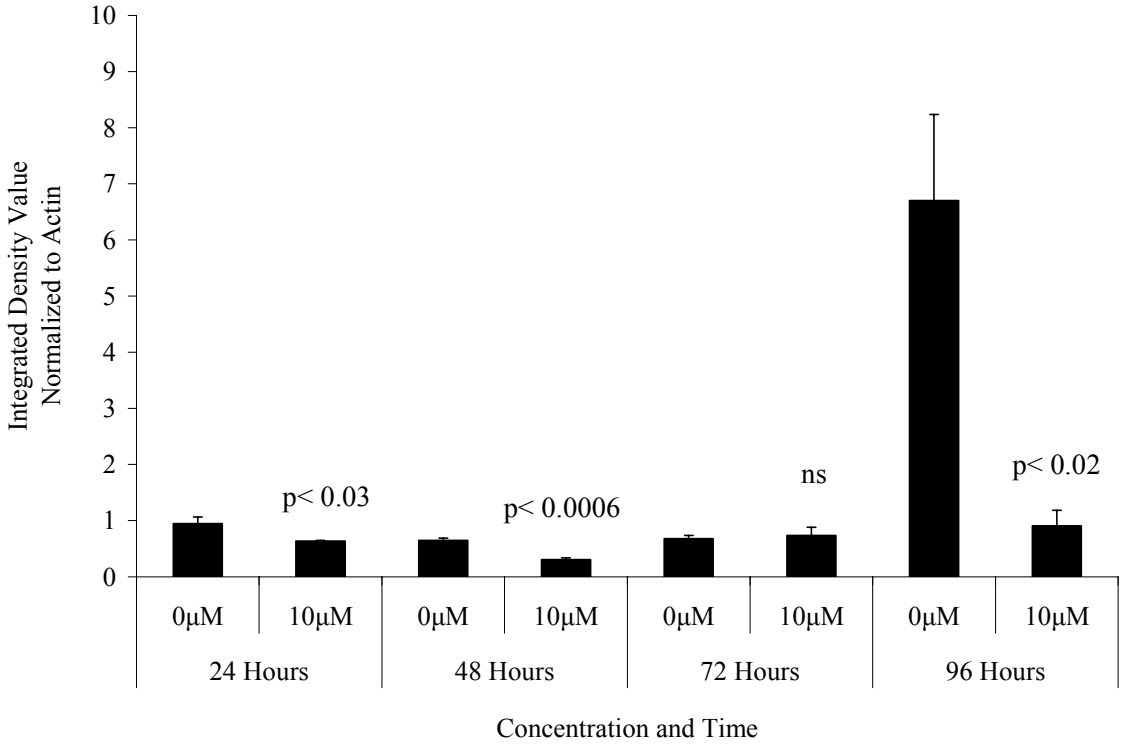


FIGURE 52. Endogenous BACE1 Protein Levels 24-96 Hours After Soluble 10μM Aβ Treatment: Primary Adult Rat Astrocytic Cells. Cells were plated and exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 24-96 hours. There was a statistically significant decrease ($p< 0.02$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1\text{-}42}$ for 24-48 and 96 hours. No significant differences were seen with exposure to soluble 10μM $A\beta_{42\text{-}1}$ at 72 hours. Statistical comparisons made to 0μM within a time grouping. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

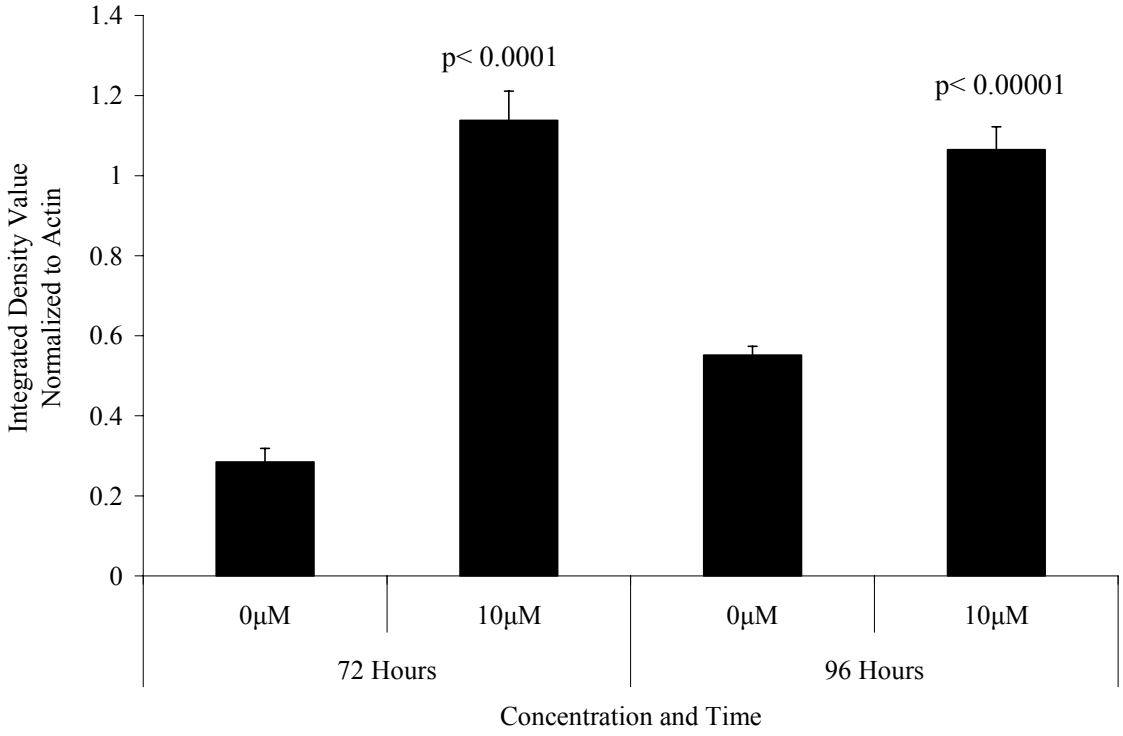


FIGURE 53. Endogenous BACE1 Protein Levels 72-96 Hours After Soluble 10μM Aβ Treatment: TNFα Primary Adult Guinea Pig Astrocytic Cells. Cells were plated and exposed to TNFα and treated with soluble 10μM $A\beta_{1-42}$ for 72-96 hours. There was a statistically significant increase ($p< 0.00001$) in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1-42}$ for 72 and 96 hours. Statistical comparisons made to 0μM within time grouping. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

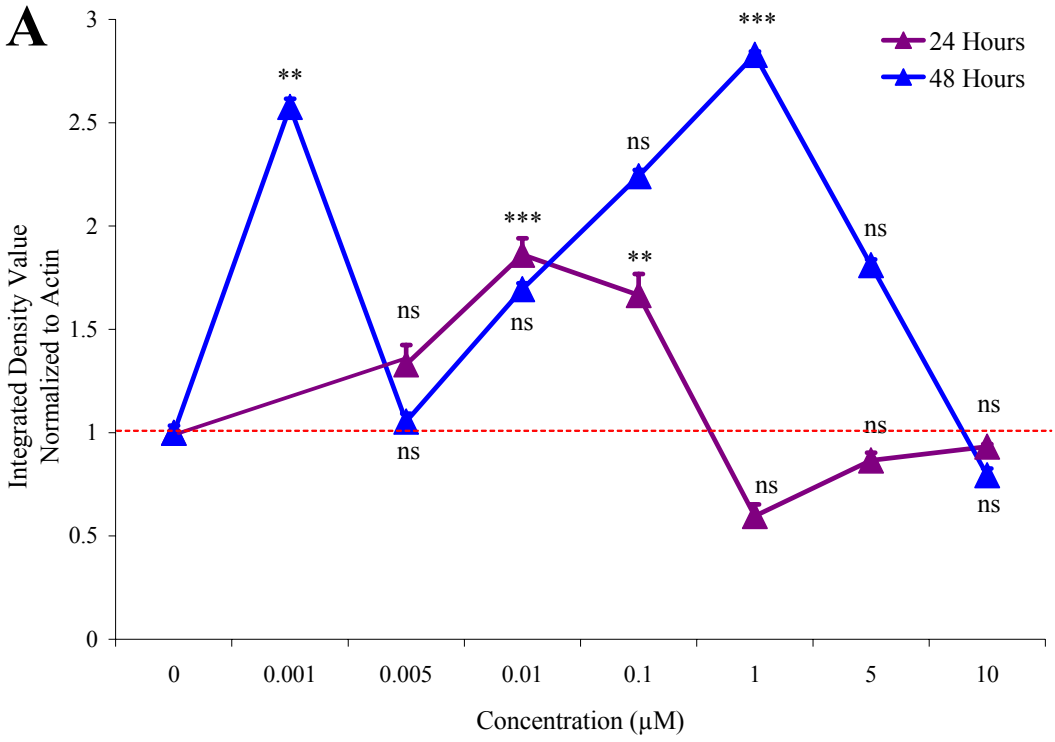


FIGURE 54.A. Endogenous BACE1 Protein Levels 24-48 Hours After Soluble 0.005-10µM Aβ Treatment: Primary Adult Guinea Pig Astrocytic Cells. Cells were plated, treated with TNFα. and exposed to soluble 10µM $A\beta_{1-42}$ for 24-48 hours. There was a biphasic increase and decrease in BACE1 expression in those cells exposed to soluble 10µM $A\beta_{1-42}$ for 24-48 hours. Note that the overall pattern was an increase in BACE1 expression at 24 and 48 hours. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. *** $p< 0.0001$, ** $p< 0.001$ ns: not significant.

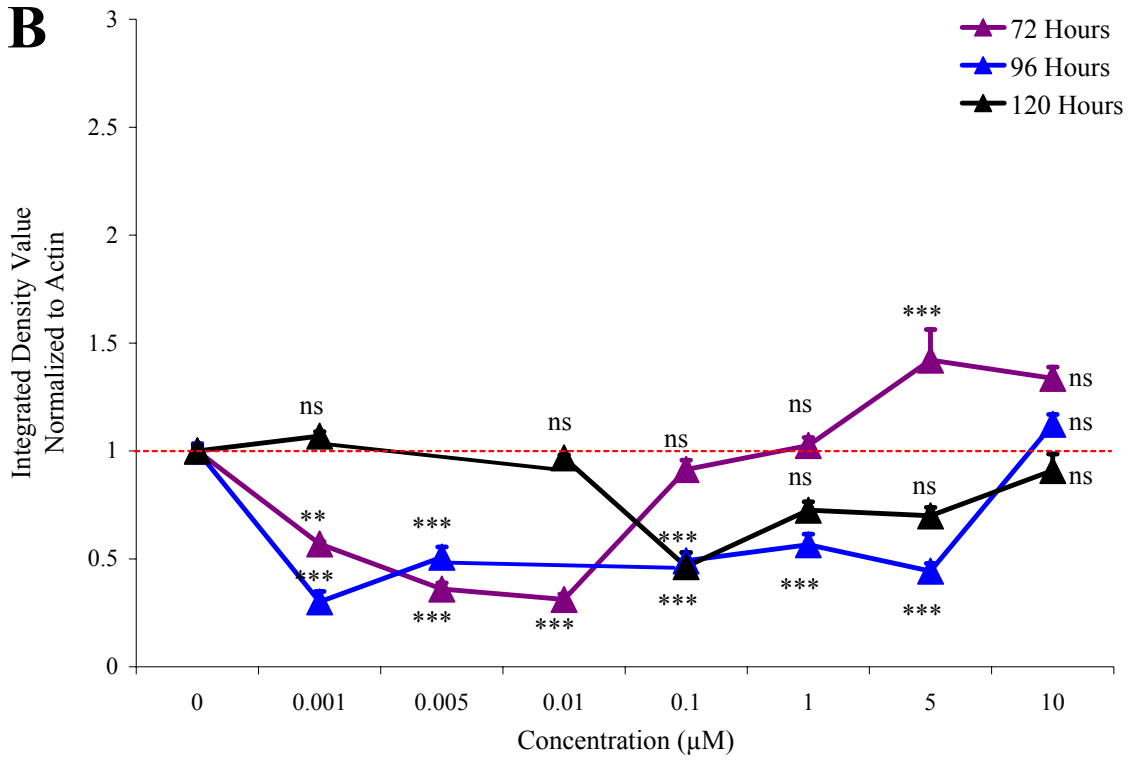


FIGURE 54.B. Endogenous BACE1 Protein Levels 72-120 Hours After Soluble 0.005-10μM Aβ Treatment: Primary Adult Guinea Pig Astrocytic Cells. Cells were plated, treated with TNFα. and exposed to soluble 10μM $A\beta_{1-42}$ for 72-120 hours. There was a biphasic increase and decrease in BACE1 expression in those cells exposed to soluble 10μM $A\beta_{1-42}$ for 72-120 hours. Note that there was an overall decrease in BACE1 expression at all concentrations tested for 96-120 hours. At 72 hours there was an initial decrease then an increase in BACE expression with increasing concentration. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. *** p< 0.0001, ** p< 0.001 ns: not significant.

observed at 0.001μM, 0.005μM and 0.01μM $A\beta_{1\text{-}42}$ ($p< 0.0004$, $p<0.0007$, and $p< 0.0008$ respectively). No changes were observed at 0.1μM, 1μM or 5μM $A\beta_{1\text{-}42}$ and an increase were seen at 10μM $A\beta_{1\text{-}42}$ ($p< 0.0008$).

A significant decrease in BACE1 protein levels was observed at 96 hours after exposure for all concentrations tested except 10μM $A\beta_{1\text{-}42.}$ Figure 54B summarizes the results: 0.001μM, 0.005μM, 0.1μM, 1μM and 5μM, all $p< 0.0001$. This pattern of decreased BACE1 protein was also observed at 120 hours. Figure 54B shows clearly that there was a significant decrease at 0.1μM ($p<0.0006$), 1μM ($p< 0.003$), and 5μM ($p< 0.002$). No significant changes were observed with exposure to 0.001μM, 0.01μM or 10μM $A\beta_{1\text{-}42}$. These results are consistent with a biphasic response by astrocytes over time and concentration. At lower concentrations and for shorter exposures there is an increase in BACE1 protein in these cells. This may be reflective of the initial and mid-stages of response to the deposition of a plaque. That is BACE1 expression has been shown to be increased in astrocytes that surround the plaques in AD patients and in mouse models such as the Tg2576 mouse (Hartlage-Rubsamen *et al*., 2003). However with increased duration of exposure, there was a decrease in expression regardless of concentration of β-amyloid. This was representative of negative-feedback regulation.

BACE1 ENDOGENOUS PROTEIN LEVELS AS ALTERED AFTER INSULIN EXPOSURE

To explore the specificity of these effects of Aβ exposure we determined how the presence of a second similarly sized and state (soluble or aggregated) protein might affect BACE1 expression. Five day NGF-differentiated PC12 cells were exposed to either soluble insulin or aggregated "aged" diluted insulin at varying concentrations (1-10μM) for 24 to 96 hours then harvested and endogenous BACE1 protein levels examined by Western blot assay (Figure 55).

At 24 hours there was no statistically significant change in BACE1 protein levels with 1μM soluble or aggregated insulin. A significant increase in BACE1 protein was observed with either 10μM soluble ($p< 0.001$) or 10μM aggregated ($p< 0.0001$) insulin. No alterations were observed at 48 hours incubation with 10μM soluble or aggregated insulin (data not shown). At 72 hours, a significant decrease in BACE1 ($p< 0.007$) protein levels was observed with10μM aggregated insulin with no significant changes seen with 10μM soluble insulin as compared to control (data not shown). Exposure for 96 hours to aggregated insulin at 1μM or 10 μM resulted in significant decreases in BACE1 protein levels ($p< 0.002$ and $p< 0.01$ respectively). No significant changes were observed with soluble insulin. The results that BACE1 expression was affected by both soluble and aggregated insulin, coupled with our previous observations regarding both aggregated $A\beta_{1-42}$ and $A\beta_{42-1,}$ would strongly suggest that BACE1 protein levels may be capable of responding to a number of peptides.

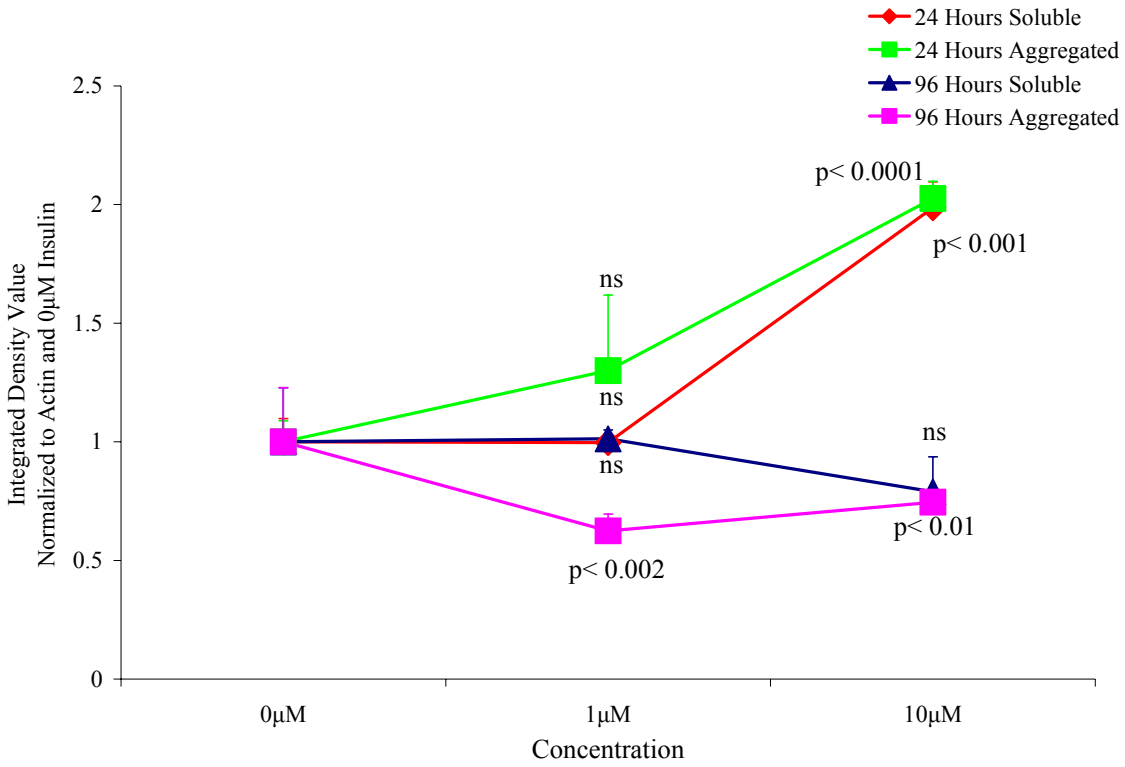


FIGURE 55. Endogenous BACE1 Protein Levels 24-96 Hours After 1-10µm Soluble and Aggregated Insulin Treatment: NGF-Differentiated PC12. There was a statistically significant increase ($p < 0.001$) in endogenous BACE1 in cells exposed to 10µM soluble or aggregated for 24 hours as compared to those cells exposed to an equivalent volume of vehicle. There was a statistically significant decrease ($p < 0.01$) in endogenous BACE1 in cells exposed to 1µM or 10µM aggregated for 96 hours as compared to those cells not exposed to insulin but maintained in culture for 24 hours and treated with an equivalent volume of vehicle or exposed to 1µM or 10µM soluble insulin. Statistical comparisons made within time and state grouping. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. ns: not significant.

IκBα ENDOGENOUS PROTEIN LEVELS AS ALTERED AFTER AB_{1-42} OR INSULIN EXPOSURE

To explore the role of NF-κB in regulating BACE1 protein levels after Aβ or insulin exposure we determined the cytoplasmic levels of the NF-κB regulatory protein Iκ-Bα. Due to its regulatory role IκBα is typically located in the cytoplasm and is responsible for retaining NF-κB in an inactive state. Following insult or injury Iκ-Bα is targeted for degradation and NF-κB is translocated to the nucleus where it may affect transcription. There was a significant decrease ($p< 0.0001$) in cytoplasmic Iκ-Bα in PC12 cells exposed to 1μM soluble $A\beta_{1-42}$ for 48 hours (Figure 56). There was a significant decrease ($p< 0.001$) in endogenous Iκ-Bα in PC12 cells exposed to 10μM soluble $A\beta_{1-42}$ for 72 hours (Figure 57). There was a significant decrease ($p< 0.001$) in endogenous Iκ-Bα in primary adult guinea pig astrocytic cells exposed to 0.001 or 0.01μM soluble $A\beta_{1-42}$ for 48 hours (Figure 58). There was a significant increase ($p< 0.05$) in endogenous Iκ-Bα in cells exposed to 0.001 μM soluble $A\beta_{1-42}$ for 72 hours (Figure 59). There were no statistically significant changes in endogenous Iκ-Bα in cells exposed to 10μM soluble insulin for 24, 48 or 72 hours (Figure 60). There was a statistically significant decrease ($p<0.001$) in endogenous Iκ-Bα in cells exposed to 10μM aggregated insulin for 48 hours (Figure 61). The results that cytoplasmic Iκ-Bα protein levels was affected by soluble $A\beta_{1-42}$ at 48 hours coupled with the increase in BACE1 protein levels at the same time point would strongly suggest that BACE1 protein levels are modulated by the translocation of NF-κB into the nucleus and regulating BACE1 transcription. This effect

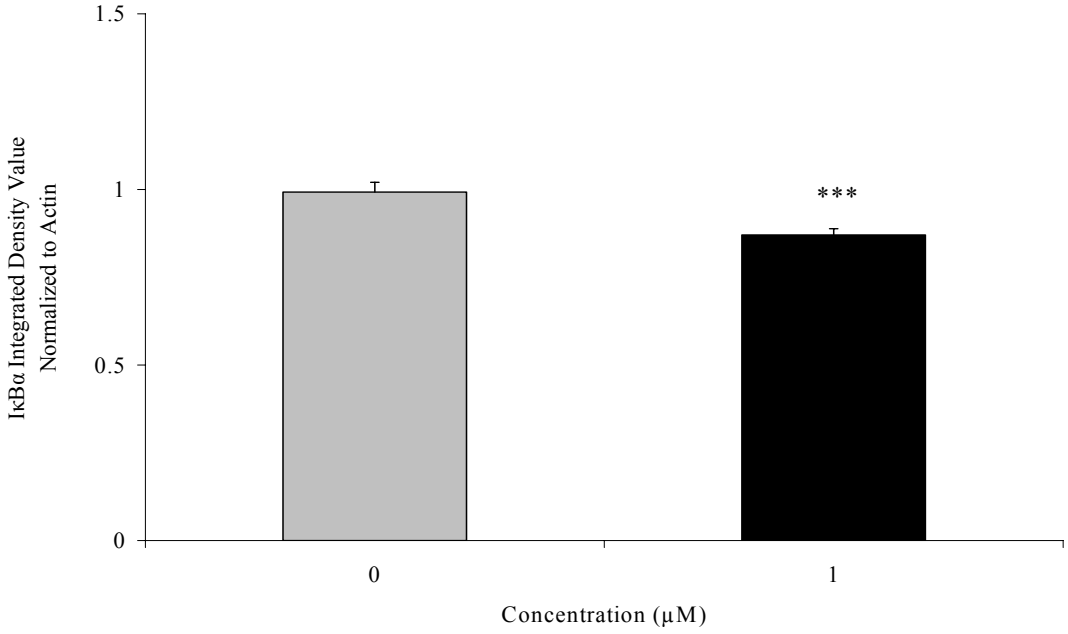


FIGURE 56. Endogenous Iκ-Bα Protein Levels 48 Hours After 1μM Soluble $A\beta_{1-42}$ Treatment: NGF-Differentiated PC12 Cells. There was a statistically significant decrease ($p< 0.0001$) in endogenous Iκ-Bα in cells exposed to 1μM soluble $A\beta_{1-42}$ for 48 hours as compared to those cells exposed to an equivalent volume of vehicle. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

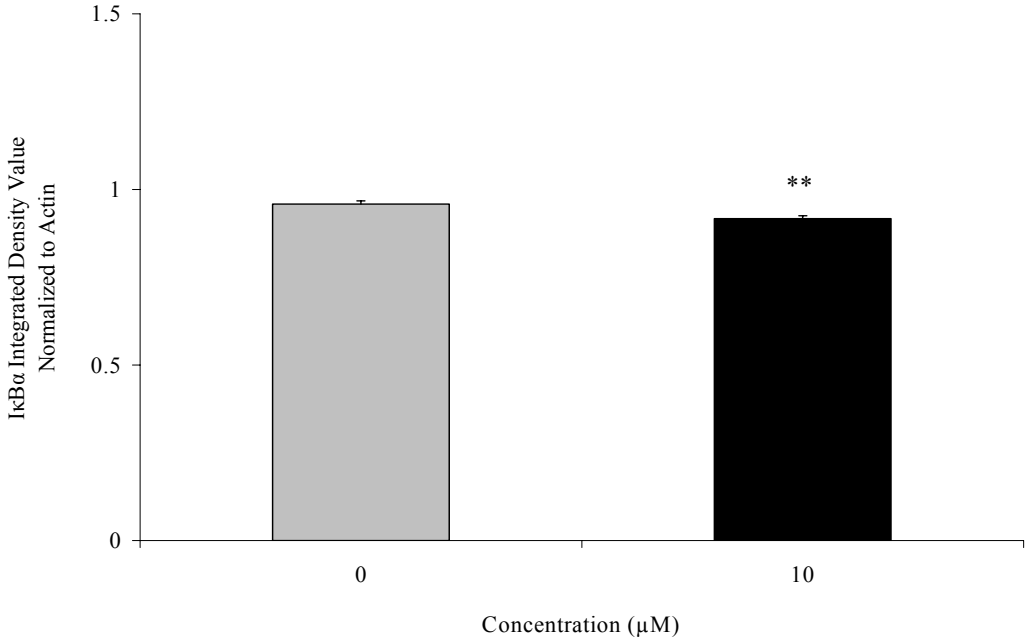


FIGURE 57. Endogenous Iκ-Bα Protein Levels 72 Hours After 10μM Soluble $A\beta_{1-42}$ Treatment: NGF-Differentiated PC12 Cells. There was a statistically significant decrease ($p< 0.001$) in endogenous Iκ-Bα in cells exposed to 10μM soluble $A\beta_{1-42}$ for 72 hours as compared to those cells exposed to an equivalent volume of vehicle. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

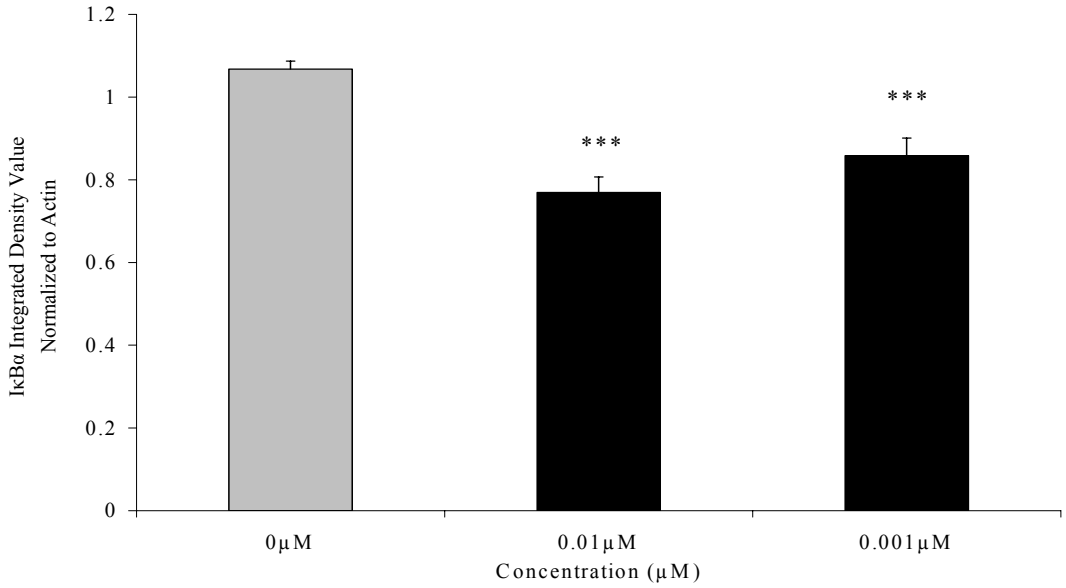


FIGURE 58. Endogenous Iκ-Bα Protein Levels 48 Hours After 0.01 and 0.001μM Soluble $A\beta_{1-42}$ Treatment: Primary Adult Guinea Pig Astrocytic Cells. There was a statistically significant decrease ($p < 0.001$) in endogenous Iκ-Bα in cells exposed to 0.001 or 0.01μM soluble $A\beta_{1-42}$ for 48 hours as compared to those cells exposed to an equivalent volume of vehicle. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments.

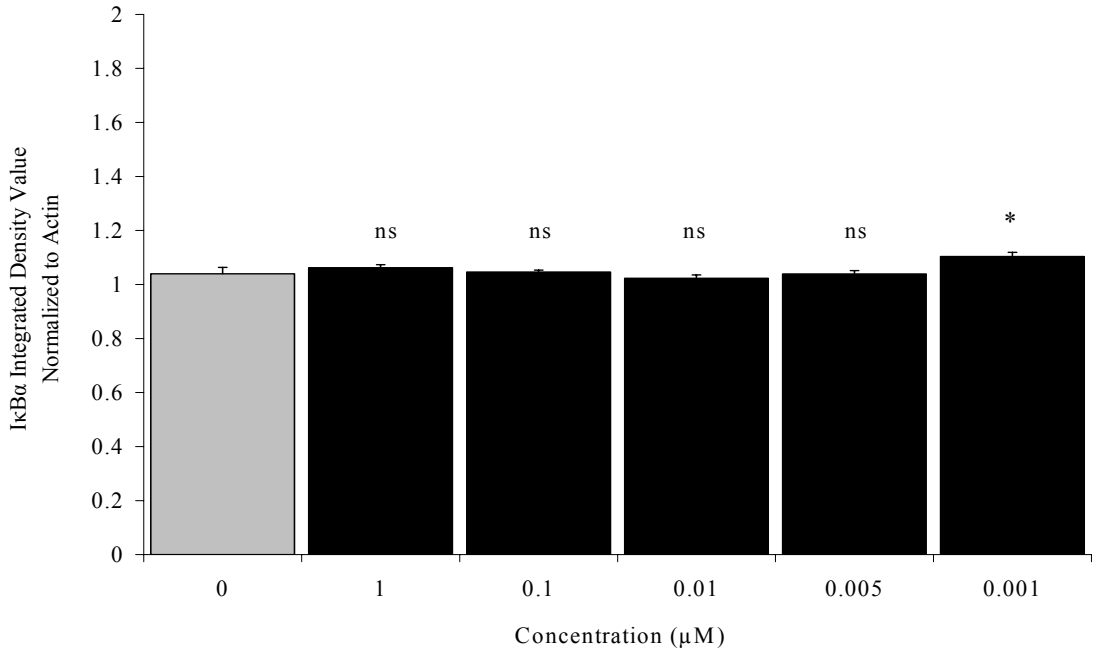


FIGURE 59. Endogenous Iκ-Bα Protein Levels 72 Hours After 0.01 and 0.001μM Soluble $A\beta_{1-42}$ Treatment: Primary Adult Guinea Pig Astrocytic Cells. There was a statistically significant increase ($p< 0.05$) in endogenous Iκ-Bα in cells exposed to 0.001 μM soluble $A\beta_{1-42}$ for 72 hours as compared to those cells exposed to an equivalent volume of vehicle. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

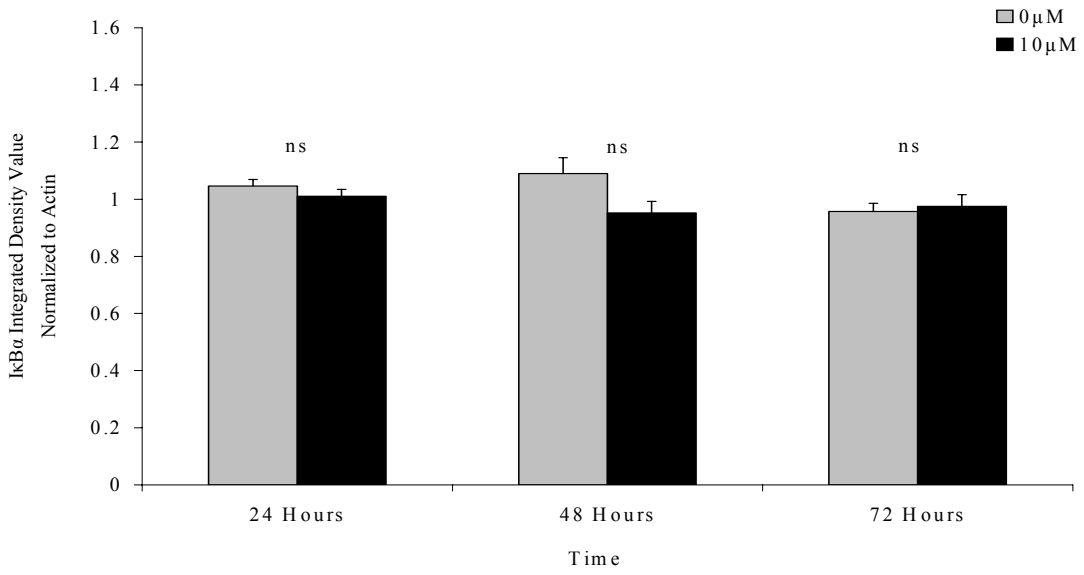


FIGURE 60. Endogenous IκBα Protein Levels 24, 48 and 72 Hours After 10µM Soluble Insulin Treatment: NGF-Differentiated PC12 Cells. There were no statistically significant changes in endogenous IκBα in cells exposed to 10µM soluble insulin for 24, 48 or 72 hours as compared to those cells exposed to an equivalent volume of vehicle. Results are mean ± SEM, n ≥ 6 per group, repeated a minimum of three separate experiments. ns: not significant.

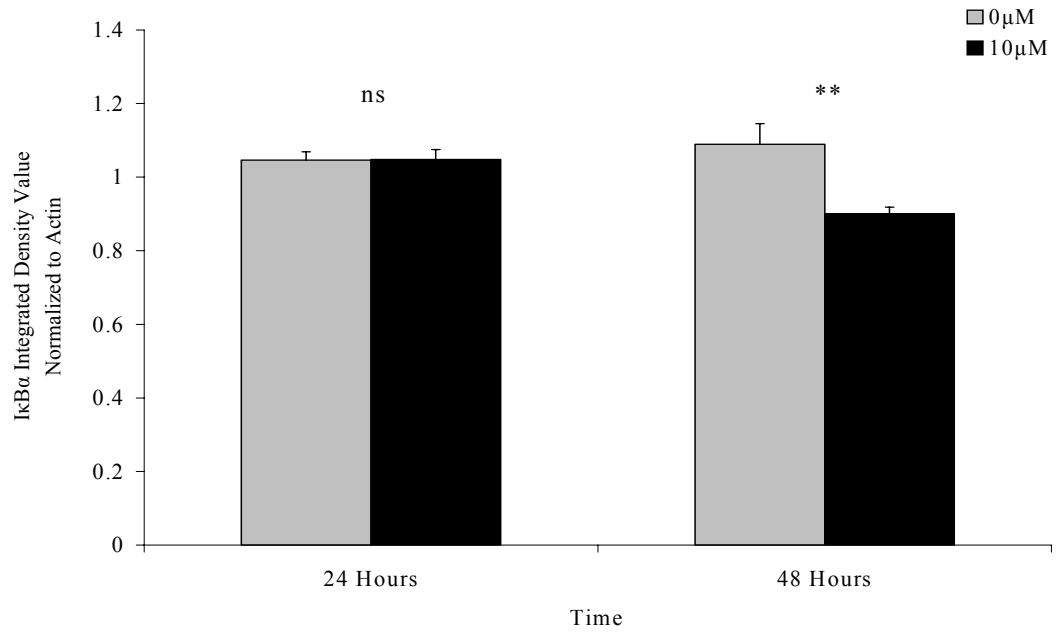


FIGURE 61. Endogenous IκBα Protein Levels 24 and 48 Hours After 10µM Aggregated Insulin Treatment: NGF-Differentiated PC12 Cells. There was a statistically significant decrease ($p<0.001$) in endogenous IκBα in cells exposed to 10µM aggregated insulin for 48 hours as compared to those cells exposed to an equivalent volume of vehicle. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

is no longer seen at 72 hours. Cytoplasmic levels of Iκ-Bα protein do not correlate with changes in BACE1 protein levels following either soluble or aggregated insulin exposure suggesting that NF-κB is not involved in the regulation of BACE1 expression.

BACE1 ENDOGENOUS PROTEIN LEVELS AS ALTERED AFTER R-FLUBIPROFEN EXPOSURE

To explore the role of NF-κB in regulating BACE1 protein levels we sought to block NF-κB by use of the chemical inhibitor R-flurbiprofen. Flurbiprofen is a racemic nonsteroidal anti-inflammatory drug known to be anticarcinogenic. R-flurbiprofen is not a COX inhibitor at therapeutically relevant concentrations. R-flurbiprofen is antinociceptive and anti-inflammatory. It is believed to function upstream of the dissociation of the NF-κB-I-κB complex (Scheuren at al. 1998). It has been shown to inhibit NF-κB activation, LPS-induced nuclear translocation of NF-κB and NF-κB dependent gene expression (Tegeder *et al*., 2001; Morihara *et al*., 2002). Primary adult guinea pig astrocytic cells exposed to R-flurbiprofen resulted in a statistically significant increase (Figure 62, $p<0.01$) in BACE1 protein when treated at 0.1μM, 1.0μM, 100μM or 500μM. BACE1 protein levels remained at untreated levels when exposed to 10μM. These results indicate that the NF-κB inhibitor R-flurbiprofen blocked the repressive action of NF-κB in non-Aβ exposed primary adult guinea pig astrocytic cells resulting in increased levels of BACE1 protein.

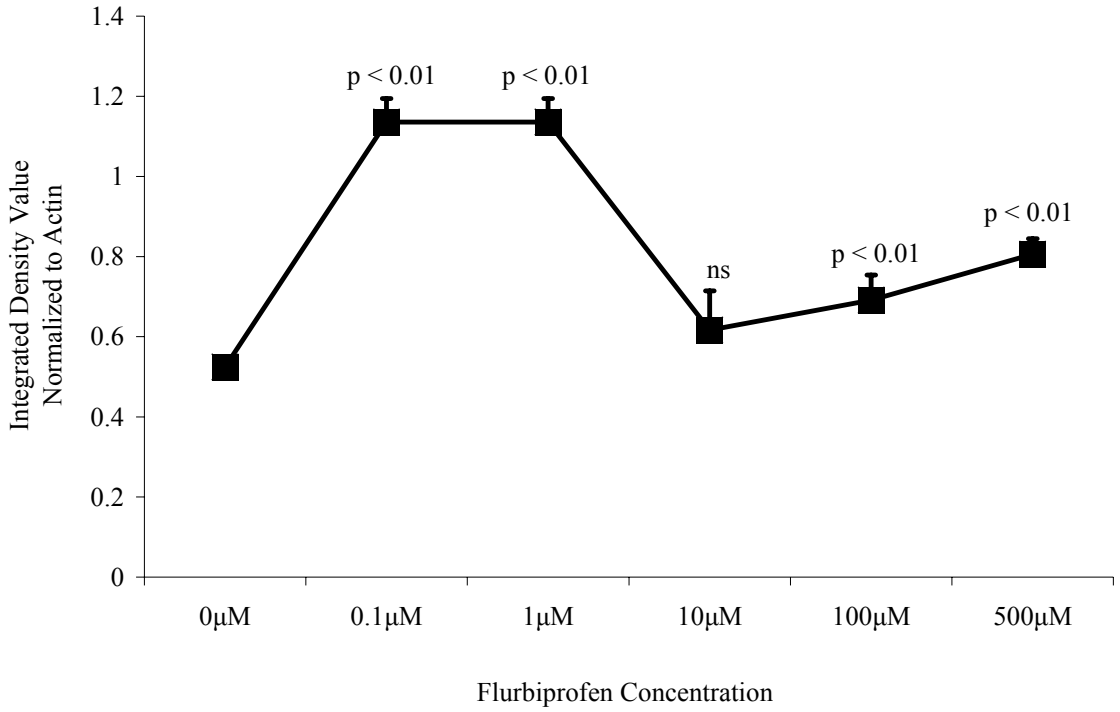


FIGURE 62. Endogenous BACE1 Protein Levels 24 Hours After 0-100μM R-Flurbiprofen Treatment: Primary Adult Guinea Pig Astrocytic Cells. There was a statistically significant increase ($p<0.01$) in endogenous BACE1 in cells exposed to 0.1, 1 or 100, 500μM R-flurbiprofen for 24 hours as compared to those cells exposed to an equivalent volume of vehicle. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

BACE1 ENDOGENOUS PROTEIN LEVELS AS ALTERED AFTER DECOY TREATMENT

To explore the role of NF-κB in regulating BACE1 protein levels we sought to block NF-κB by use of the oligonucleotide specific "decoy" inhibitors. It has been reported that sequence-specific inhibition of transcription factor activation can be accomplished with synthetic double stranded (ds) phosphothiorate oligonucleotides (decoys) containing a NF-κB consensus sequence, which acts as a decoy *cis* element to bind the transcription factors and block the activation of cognate genes (Tomita *et al*., 1998; Yu *et al*., 1999). We applied decoy oligonucleotide to a final concentration of 1.6μg /μl directly to the culture medium, with or without soluble $A\beta_{1-42}$. A significant increase in BACE1 protein was seen with decoy treatment both in the presence and absence of 10μM soluble $A\beta_{1-42}$ (Figure 63). These results indicate that the decoys blocked the repressive action of NF-κB responsible for regulation of BACE1 protein levels resulting in increased BACE1 protein levels.

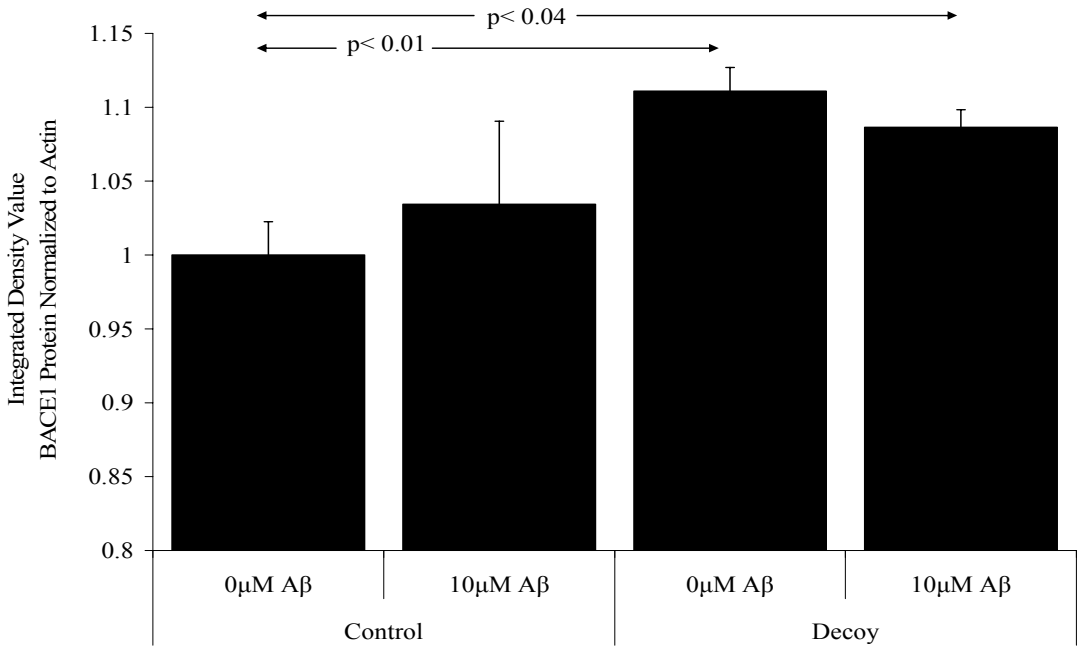


FIGURE 63. Endogenous BACE1 Protein Levels 24 Hours After 10μM Aβ with or without Decoy Co-Treatment: NGF-Differentiated PC12 Cells. There was a statistically significant increase ($p<0.04$ and $p<0.01$) in endogenous BACE1 in cells exposed to 10μM Aβ and decoy or decoy for 24 hours as compared to those cells exposed to an equivalent volume of vehicle or 10μM Aβ. Results are mean ± SEM, $n \geq 6$ per group, repeated a minimum of three separate experiments. ns: not significant.

CHAPTER FOUR: DISCUSSION

The brains of AD patients display cerebrovascular and parenchymal deposits of β-amyloid peptides, which are derived by proteolytic processing of the amyloid precursor protein. The β-site APP-cleaving enzyme is required for the generation of β-amyloid peptides. The NF-κB binding DNA consensus sequence in the BACE1 promoter upstream of the gene's transcription start site suggests a role for NF-κB in the expression of neuronal brain BACE1 (Lange-Dohna *et al*., 2003). Aberrant activation of NF-κB responses to stress occurs in the aged and AD brain. This may then result in a modification of NF-κB regulation of BACE1 accounting for alterations in cell specific BACE1 transcription and processing of β-amyloid protein.

Our long-term goal was to characterize the signal transduction pathways responsible for increased levels of β-amyloid associated with AD. Our hypothesis was that the transcription factor NF-κB regulation of BACE1 transcription is altered in AD. This alteration of NF-κB regulation of BACE1 accounts for increases in BACE1 transcription and processing of β-amyloid protein in a cell specific manner. Given the reported age and AD-associated increases in oxidative stress and NF-κB basal levels, we also asked if β-amyloid exposure has an effect on BACE1 expression. Since β-amyloid plaque deposition is selectively stimulated by chronic stressors targeted at glial cells (Burbach *et al*., 2004; Nagele *et al*., 2003; Apelt and Schliebs 2001; Overmyer *et al*.,

2000; El Khoury *et al*., 1998; Patel *et al*., 1996); we carefully evaluated the effects of length of time of exposure and glial activation on amyloid β regulation of BACE1.

BACE1 is expressed strongly in neuronal tissues and to a much lesser extent in peripheral organs (Vassar *et al*., 1999). BACE1-immunoreactive GFAP positive astrocytes are observed in proximity to β-amyloid plaques in the brains of aged Tg2576 mice and Alzheimer's disease patients (Hartlage-Rubsamen *et al.,* 2003).

IMMUNOCYTOCHEMISTRY AND WESTERN BLOT ANALYSIS IDENTIFIED BACE1 PROTEIN IN CELLS STUDIED

To characterize the expression of BACE1 using cell culture based systems we verified the presence of BACE1 expression in the cell lines and primary cells that we planned to employ. This was done by either immunocytochemistry, Western blot analysis or both. We utilized for our studies a number of well established cell lines and also developed primary cultures from both neonatal and adult animals. The cell lines used were rat pheochromocytoma (PC12), human neuroblastoma SK-N-SH-SY5Y and rat glioma C6 cell lines. We established primary glial cultures from postnatal day 3 rat pups (cortex), postnatal day 9 rat pups (hippocampus), adult mouse (cortex), adult rat (hippocampus) and adult guinea pig (hippocampus).

PC12 is a well established neuronal cell model. When differentiated by nerve growth factor the cells develop a neuronal phenotype wherein they become elongated, form aggregates, and display enhanced neuritogenesis. We also used the human

neuroblastoma SK-N-SH-SY5Y cell line that is a well established neuronal model characterized by a retinoic acid-differentiated neuronal phenotype wherein the cells become elongated, slender, display enhanced neuritogenesis, and enhanced APP expression with an increase in the neuronal APP transcript APP695 mRNA (Konig *et al*., 1990; Murray and Igwe 2003).

The C6 cell line was cloned from a rat glial tumor induced by N-nitrosomethylurea by Benda *et al*.(1968) after a series of alternate culture and animal passages. C6 cells have a documented functional NF-κB system with the ability to respond to H_2O_2, Aβ, LPS, IL-1β and TNFα exposure (Farrell *et al*., 1987; Nomura 2001; Grobben *et al*., 2002; Lee *et al*., 2002). In particular exogenous application of β-amyloid (Aβ25-35) significantly elevates levels of reactive oxygen species in C6 astroglioma cells (Lee *et al.,* 2002).

Guinea pigs express the human sequence β-amyloid peptides and represent a more physiological relevant model to examine APP processing and β-amyloid plaque formation *in vivo* (Beck *et al*., 2003). Additionally, APP processing in guinea pig primary neuronal cultures has been shown to be similar to APP processing in cultures of human origin (Holzer *et al.,* 2000). All cells used displayed BACE1 protein expression. Interestingly not all cells in any given culture expressed BACE1 at the same time. This might indicate an intricate system of BACE1 protein regulation.

MOTIF SCREEN YIELDS NINE PUTATIVE ADDITIONAL NF-κB SITES

Lange-Dohna *et al*., 2003 cloned and sequenced the rat BACE1 promoter. It has been shown to contain a single NF-κB binding site if the homology score is set greater than 85% (-1521 to -1512). We conducted a motif search with the level of homology set to >80% and identified an additional nine potential sites. These NF-κB sites may be "secondary" in nature to the original NF-κB site identified. Our studies indicate that the "primary" NF-κB site may not be solely responsible for the NF-κB regulation of BACE1 promoter activity, rather the different affinities of these sites for the different NF-κB protein dimers could account for the observed differences in NF-κB regulatory activity demonstrated for different cell types; i.e. neurons and glia vs. activated glia and species tested.

Further studies herein described clearly demonstrate an effect of the "primary" NF-κB site which differs from the other rat BACE1 promoter NF-κB sites. The human promoter contains at least five NF-κB binding sites including one at position -1521 which directly correlates to the "primary" NF-κB binding site studied in the experiments describe within this work (Sambamurti *et al*., 2004).

BACE1 PROMOTER SPECIFICALLY BOUND WITH SELECTIVE NF-κB SUBUNIT PROTEINS: EMSA

We verified that the rat primary NF-κB binding (-1521 to -1512, >85% homology) site binds NF-κB and is a functional site. EMSA using BACE1-promoter specific oligonucleotides sequences of a control IgG-κB site known to preferentially bind p65/p50 NF-κB and wild type BACE1 NF-κB binding consensus sequences specific oligonucleotides bound isolated recombinant NF-κB proteins p50 and p65. These results support the hypothesis that the NF-κB site located in the BACE1 promoter is a functional site because it has the ability to bind NF-κB subunits p50 and p65.

BACE1 PROMOTER SPECIFICALLY BOUND WITH SELECTIVE NF-κB SUBUNIT PROTEINS: LUCIFERASE ACTIVITY

When one day NGF-differentiated PC12, and TNFα activated C6 and adult primary guinea pig hippocampus astrocytes were transfected with either BPR or BPRΔNF-κB^{-} and pairs of individual NF-κB subunits p50, p52, p65 or c-Rel unique subunit binding patterns for each cell type were revealed. The triple transfection in NGF-differentiated PC12 cells showed that the combination of p52/c-Rel stimulated BACE1 promoter neuronal activation. In C6 cells p50/p65 and p52/p65 in adult primary guinea pig hippocampus astrocyte cells significantly reduced BACE1 promoter activation as measured by luciferase activity. Binding p50/c-Rel was functional in neurons and non-

activated astrocytes while p50 or p52/p65 was functional in glia. Recent studies have shown that the p65/p50 dimer activates genes coding for proteins with pro-inflammatory properties, while c-Rel/p50 dimer activates genes coding for Bcl-x_L protein that prevent cell death (Qiu *et al.*, 2001; Pizzi *et al*., 2002). Table 2A illustrates the DNA sequences which correlate to specific NF-κB subunits; Table 2B illustrates the DNA binding sequences which correlate to the NF-κB subunits binding sites of the BACE1 promoter, the specificity of each site and the effect of mutating the sequence had on various cells studied; Table 2C summaries the pathway of NF-κB activation resulting in altered BACE1 protein levels.

BACE1 PROMOTER CONSTRUCT EXPRESSION

To better understand the activity of the BACE1 promoter in the different cell types of the central nervous system, a BACE1 promoter/luciferase reporter construct was expressed in a number of cell lines and primary cell cultures. This confirms earlier *in vivo* experimental lesion studies (Hartlage-Rubsamen *et al.,* 2003; Rossner *et al.,* 2001) and our own immunocytochemistry and Western blot analyses. The rat BACE1 promoter was transcriptionally active in human neuronal cells, thus allowing the use of this construct in this and other human cell lines. As a control for transfectability of different cell types, all cells were also transfected with an SV40-driven luciferase expression vector pGL3-Control and the promoterless vector pGL3-Basic. Because the SV40 promoter activity may differ slightly among the different cell types, this control can only give an estimate

Nuclear Factor kappa B consensus sequences		p50		p65		NFκB: p50/p65		cRel	
1	GGGAAACCCC -> GGGGTTTCCC	90	80	84	70	90	82	85	66
2	GGGAATTTCC -> GGAAATTCCC	82	80	100	80	100	87	96	72
3	GGGAATCCCC -> GGGGATTCCC	100	89	89	80	95	89	87	71
4	GGGAAGCCCC -> GGGGCTTCCC	90	82	84	69	92	81	86	63
5	GGGAGTCCCC -> GGGGACTCCC	90	79	78	75	86	82	80	68
6	GGGAGCCCCC -> GGGGGCTCCC	80	70	74	64	80	74	77	61
7	GGGAGTTTCC -> GGAAACTCCC	72	70	95	69	93	79	94	65
8	GGGAGTCCCC -> GGGGACTCCC	90	79	78	75	86	82	80	68
9	GGGAGGTCCC -> GGGACCTCCC	74	72	76	76	88	85	76	74
10	GGGACATTCC -> GGAATGTCCC	64	63	86	64	91	74	91	62
11	GGGACTTTCC -> GGAAAGTCCC	73	72	95	69	97	79	95	65
12	GGGACTCCCC -> GGGGAGTCCC	92	80	78	75	87	86	80	70
13	GGGACTCTCC -> GGAGAGTCCC	81	71	83	66	87	75	82	68
14	GGGACCCTCC -> GGAGGGTCCC	71	62	72	62	78	69	75	66
15	GGGACGTCCC -> GGGACGTCCC	74	74	76	76	89	89	76	76
16	GGGATATTCC -> GGAATATCCC	64	61	85	65	88	76	90	66
17	GGGGAACCCC -> GGGGTTCCCC	88	88	72	68	79	78	71	70
18	GGGATTTTCC -> GGAAAATCCC	73	70	94	70	95	81	94	68
19	GGGGATTTCC -> GGAAATCCCC	91	78	97	68	95	77	100	59
20	GGGGACCCCC -> GGGGGTCCCC	88	88	72	66	78	76	71	66
21	GGGGGACCCC -> GGGGTCCCCC	79	79	63	61	72	71	67	64
22	GGGGACTTCC -> GGAAGTCCCC	82	68	86	63	87	71	92	57
23	GGGGAAAGCC -> GGCTTTCCCC	74	67	66	55	72	63	71	53
24	GGGGCTGTCC -> GGACAGCCCC	76	69	80	50	82	61	85	48
25	GGGTTTCCCC -> GGGGAAACCC	84	79	68	67	78	75	71	66
26	GGGCTTTCCC -> GGGAAAGCCC	80	76	79	70	85	82	78	66
27	GGAAAGGCCT -> AGGCCTTTCC	61	56	81	45	76	58	85	38
28	GGGACTACCC -> GGGTAGTCCC	82	75	75	74	86	83	74	71
29	GGGCGTTTCC -> GGAAACGCCC	71	65	88	57	85	68	90	52
30	GGGTTTTTCC -> GGAAAAACCC	72	64	87	58	86	70	91	56
31	GGGAATTCCC -> GGGAATTCCC	91	91	92	92	99	99	84	84
32	GGGGAATTCC -> GGAATTCCCC	82	69	88	62	89	72	95	57

TABLE 2.A. DNA Binding Sequences and NF-κB Subunit Binding Specificity.

NF-κB concensus sequences and the percent homology to specific NF-κB subunits as determined by Motif search. Homologies greater then 80% are highlighted.

BACE1 Promoter NFkB Sequences		Subunits				Cells Affected	BACE1
		NFκB "p50/p65"	p65	c-Rel	p50		
TGGGATATACCC		+					
AGGGACAGTCGCCA		+					
TGCAAAGTCCGC		+		+			
AGGGCTTTCC	MUTATED MUTATED MUTATED	++	++	++		GP C6 PC12	↑ ↑ ↓
GGGATATACCC		+	+	+			
GGGCATCCCA					+		
TGGGAGTCCC					+		
AGAGCTTTCC				+			
GGAAGACCCT				+			

TABLE 2.B. BACE1 NF-κB Subunit Binding Specificity.

Rat BACE1 promoter NF-κB binding sites and the subunits which a motif search with the level of homology set to >80% are predicted to bind at those sites. The mutant (BPRΔNF-κB^{-}) binding of p50/p65 resulted in an increase in BACE1 promoter construct expression in adult guinea pig hippocampal astrocytic cells, binding of p65 resulted in an increase in BACE1 promoter construct expression in C6 cells, and binding to c-Rel resulted in a decrease in BACE1 promoter construct expression in PC12 cells.

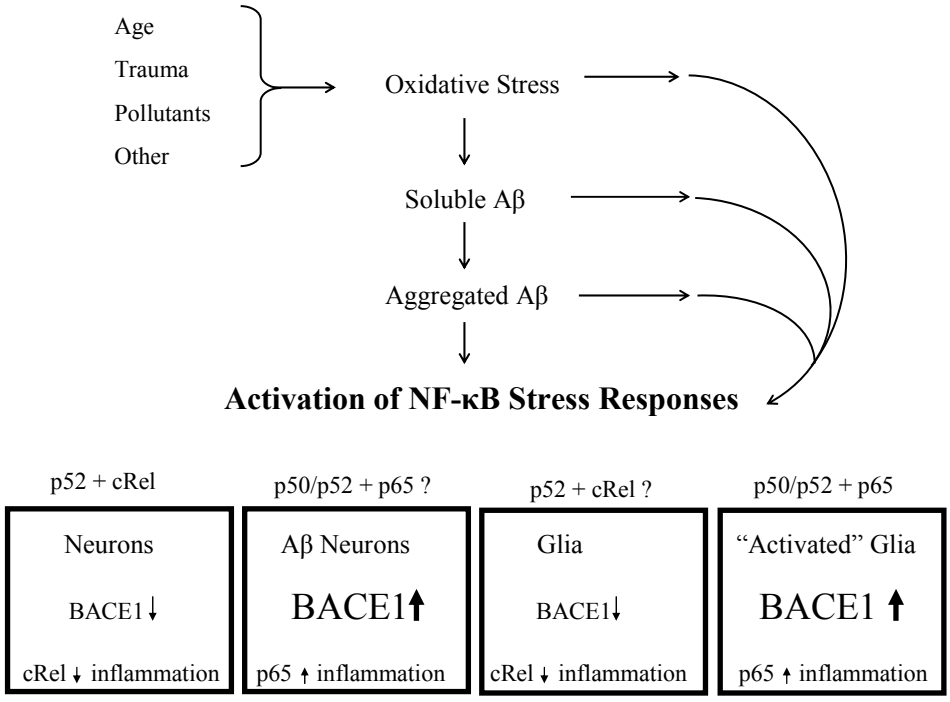


TABLE 2.C. NF-κB Pathway Activation Affecting BACE1 Protein Levels.

NF-κB pathway activation resulting from age, trauma pollutants as well as other stimulants results in oxidative stress. Oxidative stress in turn triggers soluble Aβ production. Soluble Aβ can readily form aggregates. All three, oxidative stress directly as well as soluble and aggregated Aβ have been shown to activate the NF-κB pathway. In resting non-stressed neurons as well as non-stressed (GFAP negative) astrocytes the actions of p52 and c-Rel are proposed to be responsible in part for suppressing BACE1 expression. In Aβ exposed neurons and activated (GFAP positive) astrocytes p65 and p50 or p52 are proposed to be responsible in part for increased levels of BACE1 protein.

of cell transfectability. We noted that all cell types used were transfected with the use of our optimized protocol. Our lowest levels of transfection based on luciferase activity measure following pGL3-Control transfection occurred in C6 cells, with the high levels detected in primary astrocytes and the highest levels using the adult guinea pig hippocampal astrocytic cells.

The differences in BACE1 promoter activity among the different cell types was consistent with the BACE1 mRNA levels reported for these tissues and our own observations of BACE1 mRNA expression in these different cell types. This indicated that our results reflect the specific BACE1 promoter activity present in each cell type. A caveat in these studies and in all promoter-reporter analyses is the absence of feedback effects or of regulatory effects due to "concerted" action of multiple promoter modifications or transcription factors. That is, one only tests whether the studied transcription factor binding site, here a NF-κB binding site, plays a role in regulation. Thus, we conducted analyses of BACE1 endogenous protein levels and the use of β-amyloid exposure over time to better mimic the physiological events that take place during AD development and progression.

To identify the role of the NF-κB site of the BACE1 promoter, the primary NF-κB restriction site present in the wild-type promoter was used to generate a site substitution mutant and the resultant promoter activity was measured using luciferase reporter assays with values reported as relative light units normalized to wild type BPR. For optimization of the transfection protocol, we used rat PC12 cells given the rat origin

of the cloned promoter. Following PC12 optimization we analyzed the promoter activity in a number of cell types. The levels of promoter activity displayed by the mutant suggested that the NF-κB site present on the BACE promoter performed differentially depending on the cell type assayed. The absence of the NF-κB site in the rat BACE1 promoter in neuronal cells resulted in significant increases in BACE1 expression, which suggested an inhibitory role for NF-κB in these cells. In contrast the absence of the NF-κB site in non-neuronal activated glial cells led to significant decreases in BACE1 promoter expression, which indicated an activating role for NF-κB in activated glial cells both the C6 cell line and adult primary guinea pig hippocampus astrocyte cells.

In the healthy state, astrocytes do not express BACE1, but once "activated" (expressing GFAP) in the AD patient brain, astrocytes express BACE1 (Hartlage-Rubsamen *et al*., 2003). We attempted to mimic this "activation" by treatment of the C6 or primary cell cultures with PMA or TNFα. We did not pursue the use of PMA, as while it did "activate" the cells, as determined by increased levels of GFAP, it is also known to activate PKC pathway. This in turn favors the alpha site cleavage of APP over the beta site cleavage (Etcheberrigaray *et al*., 2004). Therefore while PMA does "activate" GFAP expression it also favors the APP cleavage pathway that is in opposition to the BACE1 pathway, invalidating our use of this model. In addition we were unable to separate the actions of PMA from possible influence of the vehicle DMSO.

It is possible that a deregulation of NF-κB or the reported increases in NF-κB basal activity in aged rodent brain or in the brains of AD patients may be in part responsible for the inappropriate expression of BACE1 in activated astrocytes surrounding β-amyloid plaques (Toliver-Kinsky *et al*., 2002). That is, increased NF-κB binding in aged or AD brain may selectively reflect increases in active p50/p65 relative to c-Rel/p50 moieties. This is consistent with the hypothesis that Alzheimer's pathology reflects a transcriptional failure induced by decreased or otherwise impaired transcription factor function, an imbalance of protein: DNA binding equilibrium, or a shift in active transcription factor moieties. Such transcriptional aberrations may contribute to the reported increases in BACE1 protein levels and enzymatic activity observed in the AD brain (Fukumoto *et al*., 2004; Holsinger *et al*., 2002).

BACE1 PROMOTER CONSTRUCT EXPRESSION WAS ALTERED FOLLOWING ACUTE H_2O_2 EXPOSURE

Neuronal cells (differentiated PC12 or SK-N-SH-SY5Y) and C6 glial cells exposed to sub-lethal to lethal doses of H_2O_2 showed no differences in BACE1 promoter driven luciferase or endogenous BACE1 expression. However when adult guinea pig hippocampus astrocyte cells exposed to sub-lethal to lethal doses of H_2O_2 showed significant increases in BACE1 promoter driven luciferase BACE1 in those cells transfected with BPR relative to BPRΔNF-κB$^-$ transfected cells and exposed to 100μM of H_2O_2. We believe that this supports the hypothesis that once "activated" by H_2O_2; glial

cells express higher levels of BACE1 due to selective NF-κB activation given that guinea pig brain astrocytes represents a more relevant tissue for the study of β-amyloid deposition and BACE1 regulation.

Guinea pigs express the human sequence and have high activity of the β -secretase pathway. Additionally, the larger amounts of brain tissue and cerebral spinal fluid samples that can be collected alleviate the analysis of APP and fragments thereof are a benefit. Guinea pig β-amyloid peptides form higher molecular structures such as oligomers, which are reported to be involved in the suppression of long-term potentiation and that have the potential of β-amyloid plaque formation in a more chronic process than in APP-transgenic mice. Thus guinea pigs represent a more physiological model to examine APP processing and β-amyloid plaque formation *in vivo* (Beck *et al*., 2003). Additionally, APP processing in guinea pig primary neuronal cultures has been shown to be similar to APP processing in cultures of human origin (Holzer *et al*., 2000).The lack of increased levels of BACE1 promoter activity seen at concentrations above 100μM of H_2O_2 we believe reflects of the cytotoxic nature of H_2O_2 at these concentrations.

BACE1 PROMOTER CONSTRUCT EXPRESSION WAS ALTERED FOLLOWING AB_{1-42} OR AB_{42-1} EXPOSURE

Dependent upon which construct, concentration, state and orientation of β-amyloid was used there were effects on BACE1 promoter vector activity in PC12 cells. At 24 hours after exposure to $A\beta_{1-42}$ at concentrations ranging from pM to μM for both

wild type (BPR) and mutant (BPRΔNF-κB$^-$) promoter reporter construct, a significant increase in BACE1 promoter vector expression was observed. This pattern mimics the expression pattern seen when no $A\beta_{1\text{-}42}$ was present. Two main questions arise from these results. First, why the cells do not respond to $A\beta_{1\text{-}42}$ and second, why the cells seem to respond to $A\beta_{42\text{-}1}$ the reverse peptide intended as a negative control. The first question was addressed by incubating the transfected cells with $A\beta_{1\text{-}42}$ for longer periods of time. When PC12 cells were exposed to 10μM β-amyloid for 24-96 hours, there were no changes in luciferase activity.

It has been reported that only β-amyloid aggregates bind to the PC12 cell surface and that internalization of $A\beta_{1\text{-}42}$ does not occur until 72 hours after incubation with β-amyloid (Bateman *et al*., 2004). Progressive increases in the different β-amyloid moieties from individual protein species to soluble toxic complexes and insoluble plaques act as cumulative chronic events associated with aging. These events are unlikely to be reflected in acute localized increases over the time frames assayed by *in vitro* models of β-amyloid synthesis and processing in the central nervous system. Thus, concentration effects in the near term may be hard to reconcile with low level chronic effects over time periods of many years duration. In addition, the narrow confines of brain architecture suggest highly localized pockets of high concentrations of extracellular proteins consistent with levels used here and in the general literature (Feng and Zhang 2004; Keil *et al*., 2004; Song *et al*., 2004; Hayashi *et al*., 2004). While we acknowledge that the 10μM doses used are large, the effects are significant and likely to

yield biologically relevant outcomes over time. Lower concentrations were also tested but not found to specifically (AB_{1-42} or $_{42-1}$) significantly perturb outcomes during the time frames here assayed. Larger doses have also been used (25μM) to evaluate the effects of melatonin on β-amyloid-induced apoptosis in PC12 cells (Feng and Zhang 2004). PC12 cells have been treated with extracellular 10nM $A\beta_{1-42}$ to evaluate Aβ induced changes in nitric oxide production and mitochondrial activity leading to apoptosis (Keil *et al*., 2004). PC12 cells treated with 50μM Aβ for 72 hours, showed Bcl-2 protection from β-amyloid-induced cell death and reduced NF-κB and p38 MAP kinase activation (Song *et al*., 2004). Reduced hydroxyl radical generation, an initiator of lipid peroxidation, has been reported after treatment of PC12 cells with 400μM β-amyloid (Hayashi *et al*., 2004). The long delay required for β-amyloid cellular internalization may reflect a requirement for secondary intracellular processes that facilitate internalization once specific threshold levels are reached not dissimilar from the molecular events responsible certain circadian nuclear activation cycles.

Exposure of cells to picomolar concentrations results in aberrant cholinergic neurotransmission (Pedersen *et al*., 1996; Kar *et al*., 1996; Kelly *et al*., 1996). Others have also noted that both fresh (soluble) and aged (aggregated) preparations of β-amyloid have similar effects in PC12 cells consistent with our findings (Jang and Surh 2003). It is estimated that under biological conditions the concentration encountered is nM (Harper and Lansbury 1997). However critical concentrations for aggregation have been reported to be 10-40μM which is the speculated local plaque concentration levels (Harper and

Lansbury 1997). Additionally local transient intracellular concentrations may reach the μM level (Harper and Lansbury 1997).

ENDOGENOUS BACE1 PROTEIN LEVELS WERE ALTERED FOLLOWING AB_{1-42} EXPOSURE

Endogenous BACE1 protein levels were evaluated by Western blot analyses following exposure of cells to $A\beta_{1-42}$ or $A\beta_{42-1}$. At 24 hours exposure to 10μM soluble $A\beta_{1-42}$ or $A\beta_{42-1}$ did not result in significant changes in BACE1 protein levels. When evaluated at 96 hours exposure a significant decrease in BACE1 protein levels was seen with both $A\beta_{1-42}$ and $A\beta_{42-1}$. These endogenous results support what the hypothesis we developed from our vector studies that BACE is down regulated in neuronal cells (such as differentiated PC12 cells) when the innate NF-κB systems are activated (Table 3). However when PC12 cells were treated with 10μM aggregated $A\beta_{1-42}$ or $A\beta_{42-1}$ after 24 hours there was a significant increase in BACE1 protein, with no significant increases or decreases seen at 96 hours after exposure or with exposure to 1μM aggregated $A\beta_{1-42}$ or $A\beta_{42-1}$. Again this raised the questions we have asked before, is it a time issue or is the state of the peptide that is critical. It has been reported that it takes 72 hours for $A\beta_{1-42}$ to be internalized by PC12 cells. Therefore we further evaluated the 72 hour time point with a series of concentrations of soluble 0.01-10μM $A\beta_{1-42}$. At 72 hours a significant decrease in BACE1 protein levels is seen at only the lower concentrations of soluble $A\beta_{1-42}$ tested. Significant increases were seen only with 0.01 and 0.1μM $A\beta_{1-42}$. When

	Amyloid β				Insulin	
	1-42		42-1			
Hours	Soluble	Aggregated	Soluble	Aggregated	Soluble	Aggregated
24	ns	↑	ns	↑	↑	↑
48	↑	↑			ns	ns
72	↓				ns	↓
96	↑	ns	↑	ns	ns	↓

TABLE 3. Endogenous BACE1 Protein Levels After Protein Exposure.

Endogenous BACE1 Expression in 5 Day Differentiated PC12 Cells Exposed to 10μM Aβ or insulin. BACE1 protein levels increased when exposed for 24 hours to aggregated β-amyloid$_{1-42}$, β-amyloid$_{42-1}$ and both soluble and aggregated insulin. BACE1 protein levels increase when exposed for 48 hours to aggregated β-amyloid$_{1-42}$, β-amyloid$_{42-1}$ and a trend towards a significant increase was seen with both soluble and aggregated insulin (data not shown). At 72 hours BACE1 protein levels decreased when soluble β-amyloid$_{1-42}$ and aggregated insulin. BACE1 protein levels increase when exposed for 96 hours to soluble β-amyloid$_{1-42}$, β-amyloid$_{42-1}$ and a decrease was seen with aggregated insulin.

evaluated at 48 hours with soluble or aggregated 1-10μM $A\beta_{1-42}$ a significant increase in BACE1 protein is seen in both the soluble and aggregated 1-10μM $A\beta_{1-42}$. Similarly when soluble 10μM $A\beta_{1-42}$ was placed on cultures and the media was changed daily for a total of 72 or 96 hours, to avoid the development of aggregates forming in the dish, significant increases in endogenous BACE1 protein levels were seen. However when the experiment was repeated without a daily change of media; there was a significant reduction in BACE1 at 72 hours with a significant increase observed at 96 hours. We take these results to suggest that the state of the peptide in combination with the dose and duration of exposure are critical determinants for BACE1 protein levels. With higher doses of 1-10μM $A\beta_{1-42}$ increases in BACE1 protein levels are seen, an increase that continues with longer exposure times while the state of the peptide (soluble versus aggregated) becomes less important than its concentration. It is possible that the NF-κB systems respond to lower doses and for shorter times of exposure (72 hours), but become saturated when the exposure is extended, if the concentration of Aβ is high enough saturation may occur after shorter exposure. This is similar to anti-oxidant regulatory systems where the enzymes glutathione and catalase behave as high affinity, low capacity or low affinity, high capacity respectively in the detoxification process. Thus, our assessments of endogenous BACE1 levels in response to exposure to β-amyloid using the PC12 cell model, suggests that NF-κB is but one component of the complex regulatory machinery responsible for the regulation of BACE1 and the eventual fate of β-amyloid.

When we evaluated endogenous BACE1 protein levels in SK-N-SH-SY5Y cells, a similar pattern to that seen in PC12 was observed. At 24, 72 and 96 hours after exposure to soluble 10μM $A\beta_{1-42,}$ a significant increase in BACE1 protein levels was seen, only after 120 hours exposure was a statistically significant decrease seen. Again, a dose response assessment at 72 hours resulted overall in elevated levels of BACE1 protein. Daily changes of media for 72 or 96 hours all resulted in increased levels of BACE1 protein. Of note is that after 72 hours 10μM $A\beta_{42-1}$ had no effect on BACE1 protein levels, but a significant increase was seen after 96 hours, even with a daily media change. We believe that this again speaks to the presence of an aggregated peptide for a long duration being perhaps the most critical factor affecting BACE1 protein levels.

C6 cells evaluated for endogenous levels of BACE1 protein at 72 hours following exposure to a series of $A\beta_{1-42}$ concentration demonstrated a pattern similar to that observed in with the expression vector studies, with all but the highest dose (10μM), producing a statistically significant decrease in BACE1 protein levels. However when these cells were activated by TNFα, an established trigger of inflammation, there was a significant increase in BACE1 expression in the presence of 10μM $A\beta_{1-42,}$ regardless of media change.

In AD, the inflammatory response is mainly localized to the vicinity of amyloid plaques. Cytokines, such as Interleukin-1 (IL-1), Interleukin-6 (IL-6), TNFα and Transforming Growth Factor beta (TGF-β) are involved in this inflammatory process. Although their expression is induced by the presence of β-amyloid peptide, these

cytokines are also able to promote the accumulation of β-amyloid peptide. IL-1, IL-6, TNFα and TGF-β should be considered as key components in the progression of AD (Cacquevel *et al*., 2004). TNFα has been shown to stimulate γ-secretase-mediated cleavage of amyloid precursor protein through a JNK-dependent MAPK pathway (Liao *et al*., 2004). Thus attempted activation of rat astrocytes as a way of increasing β-amyloid is confounded by the stimulatory effects on α- and γ-secretase.

When we evaluated endogenous BACE1 protein levels in adult mouse astrocytes we did not observe any changes in BACE1 protein with exposure to soluble 10μM $A\beta_{1-42}$ even for as long as 144 hours (6 days). This was disappointing given the availability of many transgenic mice for study in the field of Alzheimer's disease. However it should be noted that these cell culture studies required a large number of mice and a prolonged time in culture before the cells reached confluence. It may well be that present methods used for the isolation and culture of adult mouse astrocytes need improvement. It may also be that the concentration of $A\beta_{1-42}$ tested was not appropriate. Again, the aged mouse brain does not display the β-amyloid deposition properties observed in the guinea pig. An interesting observation is that it would appear that there might be some concern about extrapolating results to the AD condition obtained with transgenic mice given our *in vitro* mouse and guinea pig findings.

Adult primary rat astrocytes gave us a pattern of endogenous BACE1 protein expression very similar to that observed with the C6 cell line. Namely, when exposed to

soluble 10μm $A\beta_{1-42}$ for 24-96 hours, a statistically significant decrease in BACE1 protein levels was seen at most time points tested.

When adult primary guinea pig hippocampus culture cells were treated with TNFα prior to exposure to 10μm $A\beta_{1-42}$ there was a significant increase in BACE1 protein at both 72 and 96 hours. We believe that treatment with TNFα more closely resembles the environment that the astrocyte is exposed to *in vivo*; a plaque that is surrounded by a mixture of cytokines and other chemicals. In one post mortem analysis of AD patient brains, proteomic analysis of senile plaques identified over 480 proteins which co-isolated with the plaques (Liao *et al*., 2004). Microglia, which infiltrate the parenchyma surrounding the plaque, induce neurodegeneration primarily by secreting nitric oxide (NO), TNFα, and hydrogen peroxide (Twig *et al.*, 2005).

Chromogranin A (CGA) has recently been identified as an endogenous component of the neurodegenerative plaques of AD. It has been shown to stimulate microglial secretion of both NO and TNFα (Twig *et al*., 2005; Lechner *et al*., 2004). Thus we carried out dose response and time course with non-TNFα treated adult guinea pig astrocytes. BACE1 expression was affected by both time and dose. Biphasic responses were observed, at low doses there was an initial increase in BACE1 followed by a decrease with prolonged exposure. However at higher β-amyloid doses an initial decrease was followed by an increase and at late time points a decrease in BACE1 protein levels. These results are consistent with there being a biphasic response by astrocytes over time and concentration. Low concentrations for shorter exposure times

result in an increase in BACE1 protein in these cells. This may be reflective of the initial and mid-stages of response to the deposition of a plaque. That is BACE1 expression has been shown to be increased in astrocytes that surround the plaques in AD patients and in mouse models such as the Tg2576 mouse (Hartlage-Rubsamen *et al*., 2003). However with increased duration of exposure, there is a decrease in expression regardless of concentration of β-amyloid. This represents negative-feedback regulation.

Expression of the NF-κB inhibitory proteins, Iκ-Bα and A20, leads to a negative-feedback response that terminates activation of NF-κB. NF-κB present in cytoplasm is bound to an inhibitory Iκ-B subunit. There are several inhibitory Iκ-B proteins (IκBα, IκBβ, IκBγ and Bcl-3). Upon cellular stimulation by trauma or stress, Iκ-B is phosphorylated, ubiquitinated and degraded (Ghosh and Baltimore, 1990; Liu *et al*., 1993), exposing nuclear localization signals on NF-κB proteins that allow their translocation to the nucleus for DNA-binding. NF-κB is stimulated by oxidative stress or receptor ligands via increased Iκ-B degradation and NF-κB nuclear translocation but also via an independent pathway involving Bcl-3 (Gozal *et al*., 1998; Zhang *et al*., 1998; Qiu *et al*., 2001). Each different NF-κB complex has slightly different affinities for each specific DNA binding sequence (Zandi and Karin, 1999).

One of the genes activated by NF-κB is the gene encoding Iκ-Bα. Thus newly synthesized Iκ-Bα enters the nucleus and binds to NF-κB. The complex is then relocated to the cytoplasm by chromosome region maintenance 1 (CRM1)-dependent nuclear export (Sun *et al*., 1993; Arenzana-Seisdedos *et al.,* 1995; Rodriquez *et al*.,

1999). A second regulatory loop for NF-κB is A20 which acts as an Iκ-Bα activator and thus an NF-κB inhibitor (Krikos *et al*., 1992, Lipniacki *et al*., 2004). A20 is a cytoplasmic protein that contains an N-terminal ovarian tumor (OTU) domain and seven novel zinc finger structures with a characteristic Cys-Xaa_{2-4}-Cys-Xaa_{11}-Cys-Xaa_2-Cys motif in its C-terminal domain 3 and 4. Overexpression studies originally showed that A20 can inhibit TNFα-mediated cell death in addition to NF-κB activation (Heyninck and Beyaert, 2005). The proposed mechanism of NF-κB inhibition by A20 is via alteration of the ubiquitination status of receptor-interacting protein (RIP) following TNFα stimulation though probably not restricted to the TNFα pathway. The factors regulating the speed and longevity of the transcriptional response remain to be fully determined. Elegant studies on the kinetics of NF-κB signaling and transcription have been conducted by Nelson *et al*., 2002. Their studies employed living cell models, fluorescently tagged p65 and Iκ-Bα, utilizing the nuclear export inhibitor, lyptomycin B, suggest that normal and post-induction repression of NF-κB-dependent transcription occur even when nuclear export of NF-κB is inhibited. Their studies suggest that other factors are likely to be involved in NF-κB negative feedback mechanisms. NF-κB constitutes a dynamic and versatile system, showing multiple cell-to-cell as well as cell-type to cell-type variations, and is regulated to a significant degree by multiple events involving bidirectional trafficking between the cytoplasm and the nucleus during pathway activation (Nelson *et al*., 2002; Schooley *et al*., 2003; Nelson *et al*., 2004a; 2004b; Lipniacki *et al*., 2004).

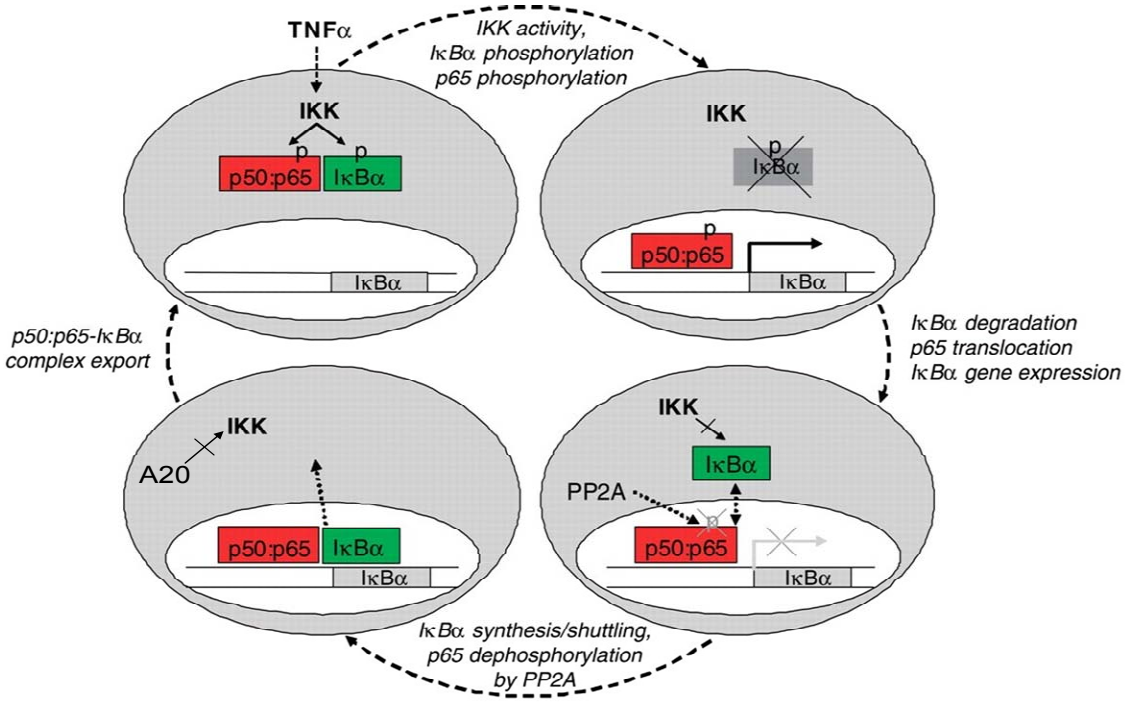


Figure 64. Oscillations in NF-κB localization. Schematic diagram illustrating the potential mechanism for repeated oscillations in NF-κB (p65/RelA) N-C localization (modified from Nelson, *et al*., 2004b). NF-κB dimers are sequestered in the cytoplasm of unstimulated cells by binding to Iκ-Bα proteins. NF-κB –activating stimuli activate the inhibitor kappa B kinase (IKK) signalosome that phosphorylates Iκ-Bα and NF-κB. Phosphorylated Iκ-Bα proteins are ubiquitinated and degraded, liberating NF-B dimers to translocate to the nucleus and regulate target gene transcription. Iκ-Bα is a transcriptional target for NF-κB, creating a negative feedback loop. Newly synthesized free Iκ-Bα binds to nuclear NF-κB, leading to export of the complex to the cytoplasm. This complex, but not free Iκ-Bα, is the target for Iκ-Bα phosphorylation by IKK.

BACE1 ENDOGENOUS PROTEIN LEVELS WERE ALTERED FOLLOWING INSULIN EXPOSURE

Because of the response we observed in BACE1 expression both vector driven and endogenous to the control peptide, we evaluated the response of BACE1 in PC12 cells exposed to a peptide of similar length to $A\beta_{1\text{-}42}$. We wished to determine if BACE1 expression would be altered only by $A\beta_{1\text{-}42}$ or $A\beta_{42\text{-}1}$ as we have demonstrated or by the presence in the cellular environment of a similarly sized and/or aggregated peptide. In an attempt to address this we exposed PC12 cells to 1μM or 10μM soluble or aggregated insulin for 24-96 hours. Insulin is similar in size to Aβ (42AA) and is a small helical protein hormone consisting of two polypeptides, chain A (21 residues) and chain B (30 residues). The polypeptides are linked together by two interchain disulfide bridges. Insulin will readily form amyloid fibrils which much like β-amyloid are detectable by Fourier transformed infrared spectroscopy (FTIR), electron microscopy, High Performance Liquid Chromatography (HPLC), electrophoresis, Congo red staining and thioflavin T (Bouchard *et al*., 2000; Nilsson and Dobson, 2003; Zhoa *et al*., 2004). Insulin is similar to Aβ in that it is a zinc stabilized protein and its disregulation is also associated with a long term chronic disease- diabetes.

At 24 hours, 1μM soluble or aggregated insulin had no effect on BACE1 protein levels. However at 10μM both forms caused a statistically significant increase in BACE1 protein levels. No significant changes were observed at 48 hours and a statistically significant decrease was observed with aggregated 10μM at 72 hours and aggregated

1μM and 10μM at 96 hours. The results that BACE1 expression was affected by both soluble and aggregated insulin, coupled with our previous observations regarding both aggregated $A\beta_{1\text{-}42}$ and $A\beta_{42\text{-}1,}$ would strongly suggest that BACE1 protein levels are capable of responding to a number of peptides. While it does not clearly answer the issue of soluble being more or less toxic than aggregated it does suggest that the presence of similar sized peptides to $A\beta_{1\text{-}42}$ or $A\beta_{42\text{-}1}$ may be of importance to BACE1 protein levels. Alternatively this may indicate that insulin exerts an effect on BACE1 protein levels but other similarly sized peptides may not. Protein presentation including misfolding may be an important factor in disease progression. In scrapie-infected transgenic mice expressing prion protein (PrP) lacking the lycosylphosphatidylinositol (GPI) membrane anchor, abnormal protease-resistant PrPres was deposited as amyloid plaques, rather than the usual nonamyloid form of PrPres. The removal of the removal of the entire second and third a helices was not as important as lack of the GPI group and the carbohydrates for PrP amyloid formation in vivo (Chesebro *et al*., 2005). Additionally cholesterol- and glycosphingolipid-rich microdomains, called ‘‘lipid rafts,’’ are suggested to initiate and promote the pathophysiology of Alzheimer’s disease by serving as a platform for generation, aggregation, or degradation of Aβ (Yu *et al*., 2005). Location, presentation as well as size and perhaps metal status could all potentially contribute to a proteins ability to trigger BACE1 upregulation.

It is well established that insulin has the ability to mimic insulin-like growth factors (IGFs) and this may be the source of the effect. This is consistent with the

finding that there is evidence for a link between diabetes mellitus and AD (Zhu *et al*., 2005; de la Monte and Wands, 2005). Insulin has recently been reported to protect against amyloid beta-peptide toxicity in brain mitochondria of diabetic rats (Moreira *et al*., 2005). Indeed it has been proposed that Alzheimer's disease may be a "type 3" form of diabetes (Steen *et al*., 2005).

CNS insulin resistance is characteristic of AD. However it is unknown whether this represents a local disease process, or complication/extension of peripheral insulin resistance. Current studies have focused on decreases in insulin, insulin-like growth factor type I (IGF-I) and IGF-II protein levels and their receptors. There is evidence that decreased glucose utilization and deficient energy metabolism occur early in the course of AD and suggests a role for impaired insulin signaling in the pathogenesis of AD via changes in the cellular levels of different NF-κB protein subunits. Given that in their soluble state both insulin and β-amyloid are zinc stabilized hexamers, it is tempting to suggest that oligomization events may play a role in the β-amyloid toxicity and subsequent neuropathology. Aggregation of peptides of these sizes (insulin 51 residues, Aβ 42 residues) are likely to result from misfolding events that trigger very slow recruitment into aggregated states. Approximately 20 proteins readily form amyloid structures (Bouchard *et al.*, 2000), additionally 25% of the known protein domains display some form of misfolding, the specific dynamics of the process for β-amyloid, or insulin for that matter, are not well established. To our knowledge this is the first reported evaluation of insulin's effect on BACE1 protein levels. It is tempting to

hypothesize that insulin and it's interactions with BACE may play a role in the progression of Alzheimer's disease.

Iκ-Bα ENDOGENOUS PROTEIN LEVELS WERE ALTERED AFTER AB_{1-42} NOT INSULIN EXPOSURE

As previously described, the expression of the NF-κB inhibitory protein, Iκ-Bα, leads to a negative-feedback response which in turn terminates the activation of NF-κB. NF-κB present in cytoplasm is bound to an inhibitory Iκ-B subunit. There are several inhibitory Iκ-B proteins (IκBα, IκBβ, IκBγ and Bcl-3). Upon cellular stimulation by trauma or stress, Iκ-B is phosphorylated, ubiquitinated and degraded (Ghosh and Baltimore, 1990; Liu *et al*., 1993), exposing nuclear localization signals on NF-κB proteins that allow their translocation to the nucleus for DNA-binding. NF-κB is stimulated by oxidative stress or receptor ligands via increased Iκ-B degradation and NF-κB nuclear translocation but also via an independent pathway involving Bcl-3 (Gozal *et al*., 1998; Zhang *et al*., 1998; Qiu *et al*., 2001). Each different NF-κB complex has slightly different affinities for each specific DNA binding sequence (Zandi and Karin, 1999).

One of the genes activated by NF-κB is the gene encoding Iκ-Bα. Thus newly synthesized Iκ-Bα enters the nucleus and binds to NF-κB. Thus decreased levels of cytoplasmic Iκ-Bα indicated that 48 hours after $A\beta_{1-42}$ exposure the regulatory pathway(s) were indeed activated. No cytoplasmic Iκ-Bα alterations that corresponded

to simultaneous variations in BACE1 protein were observed at any time point evaluated when cells were exposed to either soluble or aggregated insulin suggesting that insulin activation of BACE1 does not occur through a NF-κB mediated pathway.

BACE1 ENDOGENOUS PROTEIN LEVELS WERE ALTERED AFTER R-FLURBIPRFEN AND DECOY EXPOSURE

To explore the role of NF-κB in regulating BACE1 protein levels we sought to block NF-κB by use of the chemical inhibitor R-flurbiprofen or by use of the oligonucleotide specific "decoy" inhibitors. Flurbiprofen is a racemic nonsteroidal anti-inflammatory drug known to be anticarcinogenic. R-flurbiprofen is not a COX inhibitor at therapeutically relevant concentrations. R-flurbiprofen is antinociceptive and anti-inflammatory. It is believed to function upstream of the dissociation of the NF-κB-I-κB complex (Scheuren at al. 1998). It has been shown to inhibit NF-κB activation, LPS-induced nuclear translocation of NF-κB and NF-κB dependent gene expression (Tegeder *et al*., 2001; Morihara *et al*., 2002). Another approach to blocking the action of NF-κB is by the use of decoy oligonucleotides. It has been reported that sequence-specific inhibition of transcription factor activation can be accomplished with synthetic double stranded (ds) phosphothiorate oligonucleotides (decoys) containing a NF-κB consensus sequence, which acts as a decoy *cis* element to bind the transcription factors and block the activation of cognate genes (Tomita *et al*., 1998; Yu *et al*., 1999). Both treatments,

R-flurbiprofen and decoy application resulted in the significant increase in BACE1 expression in our astrocytic cell model(R-flubiprofen) guinea pig astrocytic cells and in our neuronal cell model (decoys) NGF-differentiated PC12 cells. In the absence of stimulation by either $A\beta_{1-42}$ or insulin, we have demonstrated that the NF-κB site on the rat BACE1 promoter serves to repress BACE1 protein levels. Thus if the action of NF-κB was blocked it would be anticipated that an increase in BACE1 protein would be observed as seen in these studies.

SUMMARY

The neurodegeneration that occurs in both familial early onset and especially sporadic AD is associated with a number of characteristic histopathological, molecular, and biochemical abnormalities. These include cell loss, abundant intercellular neurofibrillary tangles and dystrophic neurites, extracellular β-amyloid deposits, increased activation of pro-death genes and signaling pathways, impaired energy metabolism/mitochondrial function, and evidence of chronic oxidative stress.

We have identified a number of putative NF-κB transcription factor binding sites on the rat BACE1 promoter. The effects of the NF-κB binding to the "primary" NF-κB binding site of the BACE1 promoter were stimulatory for astrocytic cells and repressive for neuronal cells reflecting the different NF-κB subunit binding properties. Age-associated perturbations of NF-κB activation may result in increasingly aberrant

regulation of β-amyloid processing by BACE1 via changes in the cellular levels of the different NF-κB protein subunits and cumulative increases in astrocytic-derived β-amyloid. We confirmed the observation by others that in PC12 cells the overall activity of NF-κB (Macdonald *et al*., 2003) and BACE1 was significantly different depending on the apoptotic initiator i.e. H_2O_2 or β-amyloid. Our results are consistent with feedback mechanisms involving β-amyloid exposure overriding NF-κB activation of BACE1 overtime, a source of negative feedback, consistent with observed neuropathologies. It is also likely that a series of transcription factor binding events determine which NF-κB binding sites are operant and this may explain the observed cell specificity of BACE1 regulation. Further studies, relying on chromatin immunoprecipitation (ChIP) would be useful to further define the role of the different NF-κB sites and their interaction with other factors known to be involved in the BACE1 promoter regulation, for example, the recruitment of NF-κB by SP-1 (Liu *et al*., 2004; Zhou *et al*., 2004; Lee *et al*., 2005; Christensen *et al*., 2004) or other transcription factors such as YY-1 (Lu *et al*., 1994; Shi *et al*., 2004; Sepulveda *et al*., 2004).

Abundant reactive microglia and astrocytes surround the β-amyloid plaques in the AD brain (Itagaki *et al*., 1989; Miyazono *et al*., 1991; McGeer and Rogers, 1992; McGeer and McGeer, 1995; Cotman *et al*., 1996; Hartlage-Rubsamen *et al.*, 2003). β-amyloid a major component of AD plaques has been shown to stimulate an NF-κB-dependent pathway required for cytokine, such as TNFα, production (Combs *et al*., 2001). It has been demonstrated in vitro that TNFα stimulation of neuronal cell lines

leads to increased expression of inducible nitric oxide synthase, a known trigger for oxidative stress, and subsequent apoptosis (Ogura *et al*., 1997; Heneka *et al*., 1998). Not surprisingly a number of studies have demonstrated that β-amyloid fibril stimulation increases microglial/monocytic TNFα production (Klegeris *et al*., 1997; Galimberti *et al*., 1999; Combs *et al*., 2000; Yates *et al*., 2000) and increased levels of TNFα have been reported in brains and plasma of AD patients (Fillit *et al*., 1991; Bruunsgaard *et al*., 1999; Tarkowski *et al*., 1999). Given our results we believe that TNFα may play a role in BACE1 promoter regulation as in addition to NF-κB effects on TNFα production, TNFα has been shown to significantly enhanced Sp1/DNA binding (Pazdrak *et al*., 2004) adding another layer of influence to the highly complex BACE1 regulatory system.

Finally our results that BACE1 expression was affected by both soluble and aggregated insulin, coupled with our previous observations that both aggregated $A\beta_{1\text{-}42}$ or $A\beta_{42}$ affected BACE1 expression, would strongly suggest that the process of protein aggregation displayed by proteins sharing specific structural characteristics (i.e. zinc stabilized hexamers) may have pathological significance. To our knowledge this is the first reported evaluation of insulin's effect on BACE1 protein levels. It is tempting to hypothesize that insulin, and/or other similarly structured proteins, have effects on BACE1 activity that may play a role in the establishment and/or progression of AD.

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VITA

Krystyn Zimmer Bourne was born on April 23, 1964 in Milan Italy of American parents John Charles Zimmer, Jr. and Mary Susan Ryan Zimmer. On September 21, 1996 Krystyn married Nigel Bourne. They currently reside in League City, Texas and have two daughters, Kelley Doll and Teagan Bourne.

After graduating in 1982 from Our Lady of the Elms High School, Akron, Ohio, Krystyn then entered Bowling Green State University, Bowling Green, Ohio, where she received the degree of Bachelor of Science in 1986. In August 1986, Krystyn began working toward a Master of Science degree at Bowling Green State University, Bowling Green, Ohio. Her thesis was titled *Morphological Characterization of Two Newly Isolated Mutations, a Female Sterile and an Embryonic Lethal, in Drosophila melanogaster.* Krystyn earned a Master's of Science in August 1988.

Following completion of her Master's degree, Krystyn worked as a research assistant in a variety of biomedical research laboratories.

In 2001 Krystyn entered the Cell Biology Ph.D. Graduate Program of UTMB Graduate School of Biomedical Sciences at Galveston, Texas.

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