EVALUATION OF THE PHYSIOLOGICAL FUNCTIONS OF BLEOMYCIN HYDROLASE IN THE MURINE CNS

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EVALUATION OF THE PHYSIOLOGICAL FUNCTIONS OF BLEOMYCIN HYDROLASE IN THE MURINE CNS

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The overall hypothesis of this thesis project was that bleomycin hydrolase (BLMH) has biologically specific and unique functions in the central nervous system (CNS). BLMH is a multifaceted papain superfamily cysteine protease that has importance in drug metabolism. The physiological functions of this protease are unknown as are factors regulating its expression. Immunohistochemical examination of B6.129Blmh^{tm1Geh}/J null and control animals showed no gross abnormalities; however, marked global astrogliosis was observed in the null aged animals. To define the role of BLMH in the brain, the behavioral phenotype of hybrid [129S6-Blmh^{tm1Geh}/J X B6.129 Blmh^{tm1Geh}/J]F1 null and littermate controls was characterized using multiple behavioral paradigms. Deletion of *Blmh* was found to result in deficits among young animals in water maze probe trials. Retention of target platform location during the probe trials requires both learning and memory as well as sensory and locomoter skills. No overt sensory or motor deficits were noted in *Blmh* null F1 hybrids. The profile of BLMH expression and its regulation in the CNS was studied next. Inducible transcription of BLMH was evaluated in the context of a putative role in the processing of MHC I epitopes. BLMH was found to be differentially regulated in microglia and astrocytes. In microglia, Blmh protein was significantly induced by gamma interferon or tumor necrosis factor α, whereas in astrocytes, no change in protein expression was observed. Treatment of microglial derived cell lines with both gamma interferon and tumor necrosis factor α revealed synergistic effects between the cytokines.

BLMH protein induction was accompanied by increased *Blmh* mRNA. These results suggest that cell specific regulation of BLMH is an important control mechanism for this protease. These data also provide further evidence for a targeted immune related biological function for BLMH. It is concluded that BLMH potentially has multiple unique and biologically important functions within the brain.

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This work is dedicated to Ruth Martha Johnson

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LIST OF ABBREVIATIONS

129S6	129S6/SvEvTac
AD	Alzheimer's disease
ADRC	Alzheimer's Disease Research Center
AEC	3-amino-9-ethylcarbazole
APP	amyloid precursor protein
Aβ	amyloid beta peptide
APC	antigen presenting cell
APOE	apolipoprotein E
B6	C57BL/6J inbred mouse strain
Blh1/Gal6	yeast bleomycin hydrolase; bleomycin hydrolyzing enzyme 1
BLMH	human bleomycin hydrolase gene
Blmh	rodent bleomycin hydrolase gene
BLMH	human bleomycin hydrolase protein
Blmh	rodent bleomycin hydrolase protein
CNS	central nervous system
Cys	cysteine
DAB	diaminobenzidine
DIG	digoxigenin
E64	1-(L-trans-epoxysuccinylleucylamino)-4- guanidinobutane
ED	embryonic day
ERAAP	endoplasmic reticular aminopeptidase associated with antigen presentation
F1	first filial generation
GFAP	glial fibrillary acid protein
HEK	human embryonic kidney
His	histidine
I443V	isoleucine, valine substitution at residue 443
INFγ	interferon gamma
JAK	janus kinase
LTP	long term potentiation
MEF	murine embryonic fibroblast
MHC I	major histocompatability complex class I
MHC II	major histocompatability complex class II
PBS	phosphate-buffered saline
Pep C	bacterial aminopeptidase C
RACE	rapid amplification of cDNA ends
SLC6Δ4	serotonin transporter gene
SNP	single nucleotide polymorphism
SSCP	single stranded comformational polymorphism
STAT	signal transducer and activator of transcription
TK	thymidine kinase
TNFα	tumor necrosis factor alpha
Ycp 1	yeast cysteine proteinase

1 INTRODUCTION

1.1 Summary

Bleomycin hydrolase (BLMH) is a multifaceted protein that has importance in drug metabolism and potentially a role in human disease. BLMH was discovered as an enzymatic activity that metabolically inactivates the chemotherapeutic agent bleomycin (Umezawa et al., 1972). Through this action, BLMH attenuates bleomycin toxicity and may influence susceptibility to potentially fatal bleomycin induced pulmonary fibrosis. To date BLMH remains the only known mammalian enzyme that metabolizes bleomycin. Despite this established role, the physiological functions of BLMH remain unknown. BLMH is a member of the papain superfamily of cysteine proteases (Sebti et al., 1989; Bromme et al., 1996). The protease is a highly conserved cytosolic aminopeptidase with broad substrate specificity that exhibits maximal activity at neutral pH (Enenkel et al., 1993; Chapot-Chartier et al., 1994; Bromme et al., 1996). The crystal structure of BLMH discloses a homohexameric ring barrel enclosing the active sites (Joshua-Tor et al., 1995; O'Farrell et al., 1999). Protease activity appears to be regulated by self-compartmentalization as well as through restriction and positioning of the substrate within the active site by the BLMH C-terminus (Zheng et al., 1998). Deletion of *Blmh* in mice reveals that the protease possesses specific, non-redundant functions beyond terminal degradation of cellular proteins and peptides (Schwartz et al., 1999). Emerging evidence suggests a role for BLMH in dermal integrity and immune function as well as a potential function in the pathogenesis of Alzheimer's disease.

1.2 Bleomycin metabolism and pulmonary fibrosis

Isolated from *Streptomyces verticilus*, bleomycin is a mixture of glycopeptide congeners that differ in their terminal alkylamine groups (Umezawa, 1966). Clinically, the glycopeptide is used as a chemotherapeutic agent for cancers including squamous cell carcinoma, Hodgkin's and non-Hodgkin's lymphoma and testicular tumors (Blum et al., 1973*;* Loehrer et al., 1995; de Wit et al., 1997). The mechanism of action of the agent is principally due to oxidative cleavage of single and double stranded DNA. The cleavage reaction is initiated by a bleomycin-metal-oxygen complex that allows abstraction of a hydrogen atom from the DNA substrate and formation of a C-4' radical (Burger et al., 1979; Giloni et al., 1981; Stubbe et al., 1987; Hecht, 2000). Although bleomycin lacks significant myelotoxicity common to many other chemotherapeutics, tumor resistance and potentially fatal pulmonary fibrosis limit its clinical utility. Pulmonary fibrosis arises from acute pulmonary injury and inflammation, and is characterized by interstitial collagen deposition and compromised pulmonary function (Jules-Elysee and White, 1990). The incidence of bleomycin-induced pulmonary fibrosis is estimated at 1-2% and less serious pulmonary toxicity manifested by radiographic changes and dyspnea is observed in approximately 6-11% of patients (Blum et al., 1973; Williams et al., 1987; O'Sullivan et al., 2003). The molecular basis for this untoward effect is not established, and methodology to screen patients prior to treatment is unavailable. Metabolism of bleomycin by BLMH may be an important factor in protection against pulmonary toxicity as well as resistance of malignant tissue (Umezawa et al., 1972; Lazo and Humphreys, 1983; Sebti et al., 1991; Jani et al., 1992). Deamination of the carboxyamide amino group of the N-terminal β-aminoalanimide of bleomycin by BLMH prevents formation of the active complex. Consequently, deamido bleomycin

possesses significantly less biological activity and, in mice, does not induce pulmonary fibrosis (Umezawa et al., 1974; Huang et al., 1981; Lazo and Humphreys, 1983).

Animal models of bleomycin-induced pulmonary fibrosis result in comparable pathology to that in human patients (Jules-Elysee and White, 1990) and provide a good system for exploring the role of BLMH. In mice, susceptibility to pulmonary fibrosis induced by bleomycin is a heritable trait (Ekinoto et al., 1987; Haston et al., 1996). Bleomycin sensitive C57BL/6 inbred strain of mice exhibit significantly less Blmh activity in lung, but not kidney or liver, than the fibrosis resistant BALB/c mice (Filderman and Lazo, 1991). In addition, strain sensitivity to pulmonary fibrosis is drug specific as shown by the relative resistance of C57BL/6 mice to cyclophosphamide-induced fibrosis in comparison to BALB/c mice (Harrison and Lazo, 1988). Deletion of murine *Blmh* by gene targeting results in a greater than ten fold increased mortality from equivalent dosages of bleomycin compared to wild type controls (Schwartz et al., 1999). Necropsy results reveal loss of normal hematopoetic cells and increased neutrophilic infiltrates in medullary regions of bone and superficial mucosal ulceration and hemorrhage in gut. Non-lethal dosages of bleomycin result in increases in lung hydroxyproline, a measure of collagen content, in Blmh null, but not wild type mice indicating increased susceptibility to pulmonary fibrosis in null animals. In humans, differences in bleomycin sensitivity are also heritable based on frequency of bleomycin induced chromatid breaks in peripheral lymphocytes of healthy volunteers (Cloos et al., 1999). These differences are attributed in part to DNA repair gene X-ray repair cross-complementing 1 (XRCC 1) and, to a lesser extent, *BLMH* (Tuimala et al., 2002). Polymorphisms in XRCC 2 and xenobiotic metabolizing enzymes glutathione-S-transferase M1, T1 and N-acetyltransferase-2 do not affect sensitivity. Collectively, these studies support a vital role for BLMH in protecting nonmalignant tissues from the toxic effects

of bleomycin and suggest that genetic variability in Blmh may influence susceptibility to pulmonary fibrosis.

1.3 Membership in papain superfamily and evolution

BLMH orthologs have been cloned from numerous species ranging from bacteria (pep C) to yeast (blh 1, gal 6, ycp 1) to mammals including humans (Sebti et al., 1987; Neviani et al., 1989; Kambouris et al., 1992; Chapot-Chartier et al., 1993; Bromme et al., 1996; Ferrando et al, 1996). The protein is remarkably conserved displaying 40% identity between yeast and human BLMH, and over 90% identity among mammalian orthologs (Bromme et al., 1996). Based on sensitivity to inhibitors including iodoacetic acid, N-ethylmaleimide and 1-(L-trans-epoxysuccinylleucylamino)-4- guanidinobutane (E64), and conservation of active site residues, BLMH is classified as a cysteine protease, and a member of the papain superfamily (Sebti et al., 1989; Bromme et al., 1996). The MEROPS protease database (Rawlings and Barrett, 2000) recognizes seven cysteine protease clans or groups of evolutionarily related families. The CA clan is the largest containing about half the families including the papain like lysosomal cathepsins, BLMH and the closely related cytoplasmic Ca^{2+} dependent calpains (Barrett and Rawlings, 2001). BLMH, cathepsins and calpains share sequence similarity only in the vicinity of the active site triad defined by the catalytic cysteine 25, histidine 159 and asparagine 177 (numbering based on papain) (Polgar et al., 1987; Berti et al., 1995). Substitution of the yeast Blmh Cys^{102}, the putative catalytic cysteine, or His^{398} abolishes the enzymatic activity confirming the essential role of these conserved amino acids (Pei et al., 1996). These thiol

proteases also share an ancestral tertiary structure characterized by two large globular domains surrounding the active site cleft (Chapman et al., 1997).

At least 12 unique paralogous cysteine proteases of the papain superfamily exist in mammals (Berti et al., 1995). These are believed to have arisen very early in eukaryote evolution through gene duplication (Hughes et al., 1994; Berti et al., 1995). In prokaryotes, BLMH orthologs, which share approximately 38 and 35% identity with the yeast and human protease respectively, are found in at least four distinct bacterial species (Neviani et al., 1989; Chapot-Chartier et al., 1993; Chapot-Chartier et al., 1994; Klein et al., 1997; Winters et al., 2000). These proteases are believed to be part of a complex system that functions in the acquisition of essential amino acids (Chapot-Chartier et al., 1994; Kunji et al., 1996). In *Lactobacillus delbruckii*, *BLMH* like genes, Pep G and orf W, are believe to have arisen by duplication of Pep C, the bacterial *BLMH* ortholog (Klein et al., 1997). Curiously, existence of prokaryotic forms of BLMH is thought to be due to lateral transfer of the eukaryotic gene (Berti et al., 1995). Acquisition of proteases such as pep C may aid in bacterial survival during conditions of nutritional deficiency.

1.4 Gene structure and characterization

Human *BLMH* is encoded by a twelve-exon single copy gene mapped to chromosome 17q11.1-11.2 by linkage analysis and in situ hybridization (Montoya et al., 1997; Ferrando et al., 1997) (GDB accession ID: 3801467; GenBank accession: X92106). The gene structure reflects the small introns and exons common in other papain superfamily members, but differs in intron-exon boundary locations consistent with evolutionary divergence from related thiol proteases

(Montoya et al., 1997, Ferrando et al., 1997). *BLMH* contains least two known polymorphic sites, a CCG-trinucleotide repeat 134 base pairs 5'to the ATG start site and a single base pair substitution A1450G that results in either a valine or isoleucine residue (I443V) (Bromme et al., 1996; Ferrando et al., 1997).

The human *BLMH* promoter has not been well characterized. The cloned 1.2 kb of the 5'untranslated region (GenBank accession: Y14426) is GC rich and lacks a consensus TATA box, but contains a putative AP-1 site (-224 bp) and several consensus Sp1 sites (Ferrando et al., 1997). Although initial surveys of tissue expression patterns found *BLMH* mRNA present in most samples (Bromme et al., 1996; Ferrando et al., 1997), available in situ hybridization and immunohistochemistry studies suggest that RNA and protein expression is not homogeneous (Namba et al., 1999; Takeda et al., 1999; Malherbe et al., 2000). Detailed, reliable expression pattern characterization is currently lacking.

Murine *Blmh*, which lies on the systenic region of chromosome 11(GenBank accession NM178645; EMBL accession Q8BLZ4) (Okazaki et al., 2002), lacks the human –134 CCG trinucleotide repeat polymophism. The putative proximal promoter does, however, display regions of remarkable conservation between mouse and human (69% identity between human –171 to –862 and 97% identity between human –179 and –279) suggesting the presence of important regulatory elements in these regions (GenBank accession AL603842.9).

1.5 Protein activity, structure and regulation

BLMH is an aminopeptidase with a broad specificity for in vitro chromogenic substrates except those initiated by proline (Enenkel et al., 1993; Chapot-Chartier et al., 1994; Bromme et al.,

1996). No or minimal activity is observed with N-terminal protected peptide substrates (Bromme et al., 1996, Koldamova et al., 1998). Unlike the lysosomal cathepsins that are most active at a slightly acidic pH (Turk et al., 1997), BLMH functions optimally at a pH of 7.2 consistent with the lack of signal peptide and cytosolic localization (Bromme et al., 1996; Koldamova et al., 1999; O'Ferrell et al., 1999).

Human BLMH is a 455 amino acid, 52 kD protein with an unusual multimeric structure. Although tetramers and other multimers have been noted (Enenkel et al., 1993; Koldamova et al., 1998), most reports suggest the protease is a hexamer. Crystalization of both the human and yeast protein reveal a ring barrel homohexamer containing a prominent central channel housing the active sites (Joshua-Tor et al., 1995; O' Ferrell et al., 1999). Although the openings to the channel are relatively narrow (22Å), the central region is wider, approximately 45Å in diameter and 30Å in length. Physical sequestration of active sites is seen in other self-compartmentalizing proteases such as the yeast 20S proteasome, the serine protease DegP from *E. coli*, and the tricorn protease from *T. acidophilum* (Vandeputte-Ruttene and Gros, 2002). Sequestration of the catalytic activity prevents indiscriminate proteolysis and permits selective entry of potential protein substrates. Entry to the BLMH central channel through the narrow axial opening would necessitate either peptide or partially unfolded protein substrates (Joshua-Tor et al., 1995). Alternatively, larger molecules could access the active site following structural rearrangement of the monomeric subunits potentially mediated by other macromolecules (Joshua-Tor et al., 1995). Protein partner screening in a yeast two-hybrid system has revealed interactions between BLMH and ubiquitin-conjugating enzyme 9 and the ribosomal proteins L11 and L29 (Koldamova et al., 1998; Koldamova et al., 1999). The physiological significance of these interactions is unknown, but may suggest a role in protein modification and degradation.

Three structural domains are defined in the BLMH monomer: a catalytic domain, a helical domain and a hook domain (Joshua-Tor et al., 1995; O'Ferrell et al., 1999). The catalytic domain displays a typical papain like architecture despite lacking the multiple disulfide bonds common in related proteases. The helical and hook domains mediate interactions between adjacent monomers. These interactions result in tightly associated dimers and, in fact, the hexamer has been described as a trimer of dimers (Joshua-Tor et al., 1995).

The C-terminus of BLMH is an important regulator of enzymatic activity. The penultimate eight residues are absolutely conserved in all known forms of the enzyme (Mata et al., 1997; Koldomova et al., 1998). In the crystal structure, the BLMH C-terminus projects into its active site cleft resembling the alignment of the reversible inhibitor leupeptin within the papain active site (Joshua-Tor et al, 1995). The terminal caboxylate appears to interact with the N-terminus of the peptide substrate positioning it within the active site (Zheng et al., 1998). N-terminally acetylated and nonacetylated peptide substrates bind with a similar K_m, but the acetylated substrate displays a 50 fold decrease in K_{cat} indicating the need for an unprotected N-terminus for proper substrate positioning (Zheng et al., 1998). Surprisingly, C-terminal deletions result in loss of aminopeptidase activity, gain of endopeptidase function and, acquired sensitivity to leupeptin (Mata et al., 1997; Koldomova et al., 1998; Zheng et al., 1998). Thus, the C-terminus appears to function as a regulator of enzymatic activity by limiting access to the active site cleft and positioning of the substrate. Regulation of enzymatic activity by structural features that limit substrate access is common among the cathepsins. For example, cathepsin B possesses a 20 residue occluding loop that restricts the active site and provides, together with two histidines, an anchor for the C-terminal carboxylic group of the substrate making the enzyme a carboxypeptidase (Turk et al., 1997, Nagler et al., 1999). Cathepsin H retains an eight residue

long disulfide bonded section of the propeptide chain that blocks the active site making the enzyme an aminopeptidase (Turk et al., 1997; Vasiljeva et al., 2003).

In addition to lysosomal compartmentalization and physical restriction of substrate access, cathepsin activity is modulated by synthesis of inactive precursor and presence of native inhibitors, the cystatins. BLMH lacks a propeptide, but does undergo cleavage of the terminal amino acid residue by autocatalysis (Joshua-Tor et al., 1995; Zheng et al., 1998). The importance of this processing in the physiological regulation of protease is unknown and no native inhibitors have to date been identified (Takeda et al., 1996).

1.6 Physiological functions

Although papain superfamily members have been widely viewed as mediators of protein catabolism, recent discoveries reveal that these proteases have specific, non-redundant functions. For example, cathepsin K is crucial for normal bone remodeling (Chapman et al., 1997; Saftig et al., 1998), cathepsin L has a role in epidermal homeostasis and regulation of hair follicle morphogenesis and cycling (Roth et al., 2000), and cathepsin S and L are essential for the normal functioning of the immune system (Nakawaga et al., 1998; Nakagawa et al., 1999; Shi et al., 1999). The physiological functions of BLMH remain unknown although recent studies suggest the protease may play a role in dermal integrity and Alzheimer's disease (Montoya et al., 1998; Schwartz et al., 1999; Lefterov et al., 2000).

1.6.1 Yeast

In yeast, the BLMH ortholog Blh1/Gal6 is biochemically and genetically identical to lap 3, one of four characterized aminopeptidase activities against leucine beta-naphthylamide (Trumbly and Bradley, 1983, Enenkel et al., 1993). Although Blh1/Gal6 is non-essential for normal growth (Kambouris et al., 1992; Enenkel et al., 1993), deletion of the protease ablates hydrolyzing activity against acidic chromogenic peptide substrates (Enenkel et al., 1993). *Blh1/Gal6* is a single copy gene mapped to yeast chromosome XIV about 500 bp upstream of the calcium dependent serine protease KEX 2 (Kambouris et al., 1992). The *Blh1/Gal6* gene is induced by the positive galactose regulon transcriptional activator Gal 4 through an upstream activating sequence (UAS) located –150 bp from the ATG start site (Zheng et al., 1997). The transcriptional activator IMP 2, which is involved in glucose derepression of the maltose, galactose, and raffinose utilization pathways, also influences both constitutive and inducible *Blh1/Gal6* expression (Alberti et al., 2003). Surprisingly, Blh1/Gal6 appears to be a negative regulator of galactose regulon at the RNA level (Zheng et al., 1997; Ostergaard et al., 2001). Deletion of Blh1/Gal6 results in a 2-5 fold higher expression of the GAL1, GAL2, GAL7 and MEL1 genes, but not other inducible genes unrelated to the galactose regulon (Zheng et al., 1997). Blh1/Gal6 binds to single stranded DNA and RNA in a sequence independent fashion due to a strong positive electrostatic potential in the central channel (Xu et al., 1994; Zheng et al., 1997), a feature that is not conserved in the human protease (O'Farrell et al., 1999). The physiological significance of this observation is unclear and the mechanism for the action of Blh1/Gal6 on the galactose regulon is unknown.

1.6.2 Mice

Deletion of murine *Blmh* by homologous recombination results in neonatal lethality, reduced body weight and epidermal abnormalities (Schwartz et al., 1999). Although Blmh null mice are viable and fertile, only 65% of the expected number based on a Mendelian distribution survives to weaning. Surviving animals are smaller than littermate controls and often present with generalized scaling and sloughing of the skin over the entire body. In adult null animals, variably penetrant dermatopathology is confined to the tail and characterized by swelling, erythmatosis, necrosis and autoamputation. The tail pathology is inducible by low humidity conditions in a manner similar to rodent ringtail. The pathophysiology of both Blmh tail dermatitis and rodent ringtail are unknown. Gross evaluation of other tissues from null mice revealed no further abnormalities. These findings indicate that in mice, BLMH has non-redundant and physiological important functions.

1.6.3 Humans

BLMH has been genetically associated with increased risk for Alzheimer's disease (AD) (Montoya et al., 1998; Papassotiropoulos et al., 2000). Genotyping of AD cases and controls in two independent populations for the twelfth exon A/G polymorphism (I443V) revealed that the G/G frequency was overrepresented in AD cases (12.7% vs. 6.6%; odds ratio 2.05, P=0.009) (Montoya et al., 1998). When the data were stratified by apolipoprotein (APOE) genotype, the increased risk was found to segregate in the non APOEε4 population. Other genetic studies have failed to discern this association (Farrer et al., 1998; Namba et al., 1999; Prince et al., 2001;

Emahazion et al., 2001). Epidemiological and genetic studies suggest a significant portion of late onset AD is attributable to genetic factors; however, these proposed susceptibility genes probably exert only small to modest effects (Risch et al., 2000). Although over 100 genes have been reported associated with AD, only one, APOE has been consistently confirmed as a susceptibility risk factor. The most likely reasons for inconsistencies between studies are population stratification and heterogeneity (Bertram and Tanzi et al., 2001; Emahazion et al., 2001; Finckh, 2003).

Surprisingly, in a family based association study, the *BLMH* twelfth exon polymorphism, but not the trinucleotide repeat, was found to exhibit modest transmission disequilibrium with autistic disorder (TDT χ^2 5.24, P=0.02) (Kim et al., 2002). Significant transmission disequilibrium was also found with the serotonin transporter gene (SLC6A4) located approximately 15 kb downstream from BLMH (Kim et al., 2002). The role of BLMH in the pathogenesis of AD and other psychiatric disorders remains unclear based on current genetic studies.

BLMH is expressed in the human brain from which it was cloned and also purified to homogeneity (Chang and Abraham, 1996; Ferrando et al., 1996). However, the regional and cellular distribution of BLMH in the central nervous system (CNS) remains to be characterized. Published immunohistochemisty suggest protein expression is primarily neuronal, although in situ hybridization shows the strongest signals originating from clusters of cells within the white matter (Namba et al., 1999; Raina et al., 1999; Malherbe et al., 2000). Currently, immunohistochemistry studies are limited by a lack of reliable antibodies.

Overexpression of BLMH in cell culture appears to influence the processing of the amyloid precursor protein (APP). The amyloid beta (Aβ) peptide is proteolytically derived from

APP and a hallmark for AD pathology. Aβ is also believed to have a causative role in the pathogenesis of the disease (Selkoe, 2000; Hardy and Selkoe, 2002). In CHO and 293 cells, ectopic overexpression of proteolytically active BLMH results in an approximately fifty percent increase in the secretion of Aβ (Lefterov et al., 2000; Lefterov et al., 2001). BLMH is not thought to directly cleave APP to generate Aβ, and the mechanism for the effect is unknown. Aβ secretion is known to be modulated by several proteins such X11 and Fe65, which alter APP stability and subcellular localization (Sastre et al., 1998; Sabo et al., 1999; Kimberly et al., 2001). Consistent with the BLMH overexpression data, treatment of CHO cells with the protease inhibitor E64d results in a significant reduction in Aβ secretion indicating a papain like cysteine protease may modulate APP processing (Figueiredo-Pereira et al., 1999). These results suggest that BLMH or another papain superfamily cysteine protease may affect APP processing through a physiologically relevant process.

BLMH is also a candidate aminopeptidase for the processing of major histocompatability complex class I (MHC I) epitopes (Stolze et al., 2000). MHC I molecules are cell surface peptide receptors and components of an intracellular immune surveillance system (York et al., 1999; Saveanu et al., 2002). Eight to nine residue long peptide epitopes are primarily generated by the ubiquitin-proteasome system in the cytoplasm. These are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP) and transferred to MHC I molecules by a multiprotein loading complex (Lankat-Buttgereit et al., 1999; York et al., 1999; Paulsson et al., 2004). Proteolytic regulation of epitope generation can be modulated by treatment of cells with interferon gamma (INFγ) (Fruh and Yang, 1999).

Emerging evidence suggests that other proteases in addition to the proteasome are involved in production of peptide epitopes (Goldberg et al., 2002; Kessler et al., 2002; Saveanu

et al., 2002). Studies with proteasome inhibitors and N-terminally extended antigenic peptides demonstrate the presence of putative cytosolic aminopeptidases involved in epitope processing (Goldberg et al., 2002; Kessler et al., 2002). Fractionation of cellular extracts capable of trimming N-extended precursors of vesicular stomatitis virus protein has revealed that BLMH is a candidate protease for MHC I epitope generation (Stolze et al., 2000). BLMH has been hypothesized to function downstream of the proteasome in degradation of peptide substrates based on its subcellular localization and activity profile (Zheng et al., 1998). Other aminopeptidases that are postulated to function in MHC I epitope processing include the cytosolic leucine aminopeptidase (Beninga et al., 1998) and puromycin sensitive aminopeptidase (Stolze et al., 2000), and the endoplasmic reticular aminopeptidase associated with antigen presentation (ERAAP) (Serwold et al., 2002). Both leucine aminopeptidase and ERAAP are inducible by INFγ (Beninga et al., 1998; Serwold et al., 2002). It is unknown if BLMH expression is modulated by INFγ.

In the CNS, MHC I molecules may also play a role in normal neuronal development and synaptic plasticity. Although the expression of MHC I in neurons was initially believed to be a mechanism for flagging of electrically silent neurons for culling during infection (Neumann et al., 1995), more recent studies have shown that MHC I molecules are also expressed in neurons that undergo activity dependent changes (Corrveau et al., 1998). During development, spontaneously generated activity coupled with MHC I surface expression is required in the remodeling of retinal axons from each eye into the layers within the lateral geniculate nucleus (Huh et al., 2000). This requirement is evidenced by the significant alteration of this projection in mice lacking β_2-microglobulin (Zijlstra et al., 1990) and/or TAP 1 (Dorfman et al., 1997) and also in mice lacking CD3ζ (Love et al., 1993), a MHC I receptor subunit. β_2-microglobulin and

the TAP 1 transporter are molecules required for the assembly of nearly all cell surface class I MHC molecules (Dorfman et al., 1997). Synaptic plasticity is also altered in the MHC I deficient genetically modified mice. N-methyl-D-aspartate receptor-dependent long-term potentiation is enhanced and long-term depression is absent in the Schaffer collateral CA1 synapse of the hippocampus of the genetically modified adult mice with MHC I expression deficits (Huh et al., 2000). MHC I dependent neuronal development and plasticity could require peptide signals (Rall et al., 1995; Corriveau et al., 1998) and consequently pose an important role for proteases involved in generating antigenic peptides.

BLMH and its orthologs have evolved multiple unique and specific functions from a role in the galactose regulon in yeast to possibly processing of MHC I epitopes in mammals. Further characterization and elucidation of the physiological roles of this protease is not only of academic interest, but also of potential importance to understanding the possible functions BLMH may have in human disease.

1.7 Hypothesis and specific aims

BLMH is a multifaceted cysteine protease with putative specific biological functions in immune surveillance and possibly neurodegeneration. This thesis project has focused on the potential unique physiological functions that BLMH may have in the CNS and has utilized the *Blmh* null mouse as a valuable tool for evaluation of these functions. The hypotheses are that (1) deletion of murine *Blmh* results in alterations in CNS function, (2) genetic polymorphism in the BLMH promoter results in differential expression potentially accounting for increased AD risk with

certain genotypes, and (3) BLMH expression is modulated by factors that alter MHC I epitope processing.

The Specific Aims are: (1) to determine if deletion of the murine *Blmh* affects behavioral phenotype; (2) to evaluate the trinucleotide repeat polymorphisms of the BLMH promoter for differential expression among the polymorphic variants; (3) to determine if *Blmh* expression is altered by induction of the cell stress response or by cytokines known to upregulate MHC I epitope processing.

2 MATERIALS AND METHODS

2.1 *Blmh* null mice: maintenance and genotype determination

All mice were housed four or five to a cage and maintained under controlled conditions with a twelve hour light cycle (lights on at 7 am) and free access to food and water. Hybrid [129S6-Blmh^{tm1Geh}/J X B6.129 Blmh^{tm1Geh}/J]F1 *Blmh* null animals and littermate controls were bred from female 129S6-Blmh^{tm1Geh}/J backcrossed 7-9 generations to 129S6/SvEv (Taconic, Germantown, NY) and male B6.129Blmh^{tm1Geh}/J backcrossed 9-11 generations to C57BL/6J (Jackson Labs, Bar Harbor, Maine). B6.129 Blmh^{tm1Geh}/J null and wild type animals used for CNS immunohistochemistry were bred from male and female heterozygote B6.129 Blmh^{tm1Geh}/J. All experimental procedures were approved by the Animal Care and Use Committee of the University of Pittsburgh.

Genomic DNA for genotyping was isolated from tail clippings taken at weaning (Laird et al., 1991) and further purified with InstaGene Matrix (BioRad, Hercules, CA) per manufacturers instructions. Animals were genotyped by three primer PCR (Schwartz et al., 1999). The forward primer p1 (CACTGTAGCTGTACTCACAC) recognized both the null and wild type alleles. Reverse primer p2 (GCGACAGAGTACCATGTAGG) annealed within exon 3 giving rise to a 0.6 kb amplicon, whereas reverse primer p3 (ATTTGTCACGTCCTGCACGACG) annealed within the targeted deletion neomycin cassette producing a 0.95 kb amplicon. The PCR mixture (25 µl) contained 6 µl InstaGene purified DNA, 2 mM Mg^{2+}, 0.2 mM dNTP, 2 µM p1, 0.8 µM p2, 6.4 µM p3 and 0.5 units of Taq polymerase (Invitrogen, Carlsbad, CA). The thermal cycling reaction was run on a PTC-100TM (MJ Research Inc., Watertown, MA) with 34 cycles

of 92°C for 30s, 65°C for 40s and 72°C for 90s. Amplicons were resolved on a 1% agarose gel and stained with ethidium bromide.

2.2 Animal behavioral studies

Young (less than 8 months) or aged (greater than 14 months) groups of male and female *Blmh* null and wild type littermate controls were tested in a variety of established behavioral paradigms to evaluate locomotion and learning and memory. Most animals were tested in multiple paradigms beginning with rotarod testing and contextual fear conditioning followed by open field testing and evaluation in the water maze.

2.2.1 Rotarod testing

Mice were tested for seven consecutive days on a 3 to 19 rpm accelerating Ugo Basile Model 7650 rotarod apparatus (Ugo Basile, Camerio, Italy). Each animal was scored for time on rotarod defined as latency to fall from the apparatus during the180 s trial.

2.2.2 Open field testing

Locomotion and exploratory behavior were evaluated for each mouse. Each animal was placed into an open field chamber (28 x 28 x 40 cm) (MED Associates, St. Albans, VT) for 10 minutes and the velocity, distance, location and duration of travel bouts, rearing and occurrence

of stereotypical behaviors were recorded. Center was considered the inner 50% of the testing surface. Open field testing was performed in collaboration with Dr. Edda Thiels, of the Department of Neuroscience, University of Pittsburgh.

2.2.3 Contextual fear conditioning and light-dark box testing

Learning to fear both a tone that was paired with a foot shock and the context in which the tone-shock pairing was presented was evaluated using contextual fear conditioning (Paylor et al., 1994). Mice were transferred to the conditioning chamber and after an initial acclimatization period of 2 min, animals were presented with two pairings of a 30 s tone (80dB, 2000 Hz) with a 2 s foot shock (0.75 mA) administered during the last 2s of the tone. Pairings were separated by 2 min and mice were returned to their home cages 30 s after the last shock presentation. The conditioning chamber was custom built at the University of Pittsburgh.

Approximately 24 h later, mice were tested for contextual conditioning. Mice were placed into the conditioning chamber for 5 min and freezing behavior was scored. Freezing was scored manually and defined as absence of movement except respirations. For the cued test, mice were placed into a novel context and scored for freezing for 3 min before and 3 min during presentation of the tone. Contextual fear conditioning testing was performed in collaboration with Dr. Edda Thiels.

Light-dark testing was used to evaluate anxiety, which was reflected by time spent in the darkened chamber. Each animal was placed into a 50 % darkened chamber (28 x 28 x 40 cm) (MED Associates) for 10 minutes and the velocity, distance, location and duration of travel bouts, rearing, occurrence of stereotypical behaviors, and time spent in light and dark were

recorded. Light-dark testing was performed in collaboration with Dr. Edda Thiels, of the Department of Neuroscience, University of Pittsburgh.

2.2.4 Hot plate testing

Pain sensitivity was determined by the hot plate test. The animals were placed on a 25.5 x 25.5 cm surface (Columbus Instruments, Columbus, OH) heated to 52° C for a maximum of 5 min. Pain threshold was accessed by latency to paw licking, shaking and/or jumping. Animals exhibiting significant distress were immediately removed from the heated surface. Hot plate testing was performed in collaboration with Dr. Edda Thiels.

2.2.5 Water maze testing

Spatial learning and memory were evaluated in the Morris water maze (Morris, 1984). The water maze consisted of an 83 cm diameter tank filled with water to a depth of 29 cm obscuring a translucent platform submerged 1 cm below the surface of the water. Visual cues in the testing room and platform location remained fixed for all trials. On five consecutive training days, each animal received four 120 s training trials. Animals that failed to find the submerged platform during the trial were manually placed on the platform for 30 s. Performance during training was evaluated as average latency to find the platform for each of the training days. Probe trials were conducted following completion of training. The platform was removed from the tank and swim pattern was recorded for 60 s using a video tracking system (Chromotrack 3.0, San Diego Instruments, San Diego, CA). Probe trials were scored by time spent in each of the four pool

quadrants during the test. Following the probe trials, visual acuity of each mouse was determined using a visible platform that was exposed 1.5 cm above the level of the water and marked with colored tape. For data analysis, only the results from probe trial 1 were evaluated. Overall, probe trials 1 and 2 yielded similar results. Water maze testing was conducted in collaboration with Dr. Edda Thiels and Dr. Edward Dixon of the Department of Neurological Surgery, University of Pittsburgh.

2.2.6 Statistical analysis of behavioral data

Animal behavioral data were analyzed with Stat View (SAS Institute, Cary, NC). Each group of animals was individually evaluated for trial and genotype effects. If a replicate group of the same sex and age mice was tested on separate dates, then the groups were compared for differences and pooled if no significant differences were found. Male and female groups in the same age category were then compared for gender effects and pooled if no differences were observed. Pooled groups were re-evaluated for trial and genotype effects. Finally, young and aged pooled groups were compared to each other. Three values for velocity (cm/s) from the open field test were discarded as outliers based on Grubb's test (Iglewicz and Hoaglin, 1993); no other values were omitted from any other of the datasets. Data were evaluated by χ^2, two-tailed t-test or two-way repeat measures ANOVA followed by Bonferroni posthoc tests and individual contrasts when appropriate. The differences between means were accepted as significant at the $P < 0.05$ level.

2.3 Immunohistochemistry

2.3.1 Animal perfusions

Each animal was deeply anesthetized by intraperitoneal injection of 125 mg/kg sodium pentobarbitol prior to sequential transcardial perfusion of physiological saline and 4% paraformaldehyde fixative infused at controlled pressure using a peristaltic pump (Cole-Palmer, Vernon Hills, IL). The isolated brain was postfixed for 2 h and placed in phosphate buffered sucrose solution at 4° C overnight.

2.3.2 Immunohistochemistry of free floating sections

For preparation of free floating sections, the brain tissues were serially sectioned in the coronal plane at 35 µm /section using a sliding microtome equipped with a freezing stage (Leitz Physitemp). Sections were sequentially collected in six bins of cryoprotectant and stored at -20° C until processed for immunohistochemistry. Paired null and wild type tissue sections were stained with Nissl or processed for immunohistochemistry using the avidin-biotin modification of the immunoperoxidase procedure. Briefly, tissues were washed four times with 0.1M sodium phosphate buffer to remove cryoprotectant solution. Sections were then transferred to diluted primary antibody (Appendix A) in 10mM sodium phosphate buffer containing 0.3% Triton X-100 and 1% normal donkey serum. Following overnight incubation at 4° C, the tissues were washed three times in 10 mM sodium phosphate buffer followed by 1 h incubation in affinity

purified biotinylated donkey secondary (Jackson ImmunoResearch Laboratories, West Grove, PA). The sections were incubated in ABC complex for 90 min using reagents from the Vector Elite Kit (Vector Laboratories, Burlingame, CA), washed in 10 mM phosphate buffer, and then incubated in a 0.1 M Tris-saline buffer containing diaminobenzidine (DAB) for 10 min. The immunoperoxidase reaction product was visualized by adding H_20_2. Sections were mounted on gelatin coated slides, dried at room temperature, dehydrated in a graded ethanol series, cleared in xylene and coverslipped with Cytoseal60 (Richard Allan Scientific, Kalamazoo, MI).

2.3.3 Immunohistochemistry of paraffin embedded sections

Brain tissues were placed in phosphate-buffered saline (PBS) overnight and then paraffin embedded. For immunohistochemistry, paraffin embedded tissue sections were deparaffinized with Histoclear (National Diagnostics, Atlanta, GA), rehydrated, and treated with 3% hydrogen peroxide (Sigma Chemical Co., St. Louis, MO) for 30 min to quench endogenous peroxidases. Sections were rinsed with PBS and incubated in TNG blocking buffer (DuPot NEN, Boston, MA) for 30 min. After overnight incubation at 4° C with rabbit anti-GFAP antibody (1:500) (Daco, Winnipeg, CA), the sections were rinsed in PBS and then incubated at room temperature for 1 h with biotynilated goat anti-rabbit IgG (1:200, Biogenex, San Ramon, CA). Chromagen reactions were developed with 3-amino-9-ethylcarbazole (AEC, Biogenex) and counterstained with Mayer's hematoxylin.

All sections were visualized and recorded with a Leica DMR microscope equipped with a DC100 digital camera (Leica Microsystems, Wetzlar, Germany). Immunohistochemistry studies

were conducted with the assistance of Dr. Patrick Card of the Department of Neuroscience, Univeristy of Pittsburgh, and the Neuropathology Laboratory at the University of Pittsburgh.

2.4 *BLMH* Polymorphism screen

All exons of the BLMH gene with their flanking intronic boundary sequences as well as 1.2 kB of the 5'upstream region (Ferrando et al., 1998) were PCR amplified and screened for polymorphisms by direct cycle sequencing and single strand conformational polymorphism analysis (SSCP). Each exon and overlapping regions of the 5' upstream sequences were amplified from human genomic DNA from 10 Pittsburgh Alzheimer's Disease Research Center (ADRC) autopsy confirmed cases and 10 ADRC controls (Table 1). Amplicons were sized on ethidium bromide stained 1% agarose gel with a 1 kb ladder molecular size marker (Invitrogen). Confirmation of the identity of the PCR products and screening for polymorphisms were performed by dye labeled terminator direct cycle sequencing using the DNA Sequencing Kit (Applied Biosystems Inc., Foster, CA). Sequencing products were analyzed on an ABI Prism 377 automatic DNA sequencer (Applied Biosystems Inc.). DNA sequences were assembled using Sequencher v. 3.0 (Gene Codes Corp., Ann Arbor, MI).

For SSCP analysis, PCR amplicons were separated on 4-20% TBE polyacrylamide precast gels (NOVEX, San Diego, CA) run on Mighty Small II Vertical Electrophoresis Unit (Hoefer, San Francisco, CA) for 2.5 h at 20 mA with a temperature gradient of -7° to 20°C (Programmable Temperature Controller, Polyscience, Niles, IL). Gels were visualized by staining with SYBR Green II (Molecular Probes, Eugene, OR) for 20 min followed by inspection using the Still Video Imaging System (Eagle Eye II, Stratagene, La Jolla, CA).

Table 1 ***BLMH*** **polymorphism screen primers**

5’	**PRIMER**	**SEQUENCE**	Annealing (°C)	Amplicon (kb)
-1210	FORWARD	GTTCCTTAGAACTGCGATG	58	1178
-33	REVERSE	TTCCAGGGT CCTTGGGC		
-747	FORWARD	GAAGAGCTTGGTTCCCATTCC	58	219
-529	REVERSE	GCCAAAAGGGAGCTCGAG		
-534	FORWARD	TTTGGCTTCTCACAAGGCTTCCTGG	60	201
-334	REVERSE	CGCGCAGGACCGCACAGATGG		
-360	FORWARD	CGGCAGACCATCTGTGCGGTCC	60	197
-164	REVERSE	GGGAGGCTGAGGCTGGAGAAACG		
EXON				
1	FORWARD	CGCCGAGCCGGTTTCCTT (-140)	60	162
	REVERSE	TTGAGTCCCGAGCTGC (+20)		
2	FORWARD	CCAACAGGACTGAATTCG	58	209
	REVERSE	CCACACCTGAGCTCTTCTG		
3	FORWARD	CAGTGCAGTCTATGTGAACC	56	216
	REVERSE	GGCAGGCTATAACTGGAA		
4	FORWARD	CTCTTTAGGTTGAACGCTG	56	189
	REVERSE	CATGCTGAGTTTCAGTTCC		
5	FORWARD	AGAAAAATATGGTGTTATCCCTAAGAAATGC	55	110
	REVERSE	CCTGCTTCATATATTGTACC		
6	FORWARD	GGTAACTGTTGGAGAGTGCT	56	136

	REVERSE	TACCTCCTCCATCATGACG		
7	FORWARD	GATATTCCGAGTGGTGTG	56	184
	REVERSE	CAGTGACTACCAGTGCCA		
8	FORWARD	GTTGCTTGACATTCCTGG	56	177
	REVERSE	AGGAAGTCAATGGGCTG		
9	FORWARD	CAACAACCAGCCCATTGACTTCC	58	257
	REVERSE	GAGATTCATGTCACTGAGGCCCAGC		
10	FORWARD	CAGCTATGACCATGAGTTAG	58	126
	REVERSE	CATACCTTCTCTGAGACAGC		
11	FORWARD	CAGGATGATCAGGATGG	56	82
	REVERSE	TGAGGCTCACCTTTGTGG		
12	FORWARD	TCTCCAGGTTACCTGTGC	56	77
	REVERSE	CTCTTCAGGGACATGCTTC		
	FORWARD	GGAAGCATGTCCCTGAAGAGGTGC	58	160
	REVERSE	CCTGGATCTGTCCTTTGCAGCTACG		

2.5 Cloning and vectors

2.5.1 Construction of pGL3-*BLMH* vectors

1.2 kB of the 5' upstream region of the putative BLMH promoter was amplified with Pfu polymerase (Stratagene, La Jolla, CA) from genomic DNA from ADRC cases and controls who differed in genotype at the –134 bp trinucleotide repeat polymorphism. The primers used were: 5'-GCCTGGTACCGTTCCTTAGAACTGCGATG-3' (-1210 5'*BLMH*) and 5'-GCTAAGCTTTCCA GGGTCCTTGGGC-3' (-33 5' *BLMH*). Amplified fragments were incubated with excess dATP and Taq polymerase (Invitrogen) at 72°C for 10 min to add 3' overhangs and were then ligated into the TA cloning vector pCR 2.1 (Invitrogen) with T4 ligase (Invitrogen). Clones were screened by restriction enzyme digestion with Kpn I and Hind III (New England Biolabs, Beverly, MA) and direct cycle sequencing. Positive clones were subcloned into the pGL3 basic vector (Promega, Madison, WI). The pGL3-5'BLMH plasmids were amplified in DH5α *E. coli* (Invitrogen) and grown on ampicillin-containing LB agar plates. The plasmids were then purified by Wizard miniprep (Promega) and the constructs were verified by double digestion with Kpn I and Hind III (New England Biolabs) as well as by direct cycle sequencing using an ABI 377 (Applied Biosystems Inc.).

2.5.2 Construction of EGFP-*BLMH* vector

Human BLHM cDNA was amplified by long distance PCR with Elongase (Invitrogen) using cDNA kindly provided by Dr. D. Bromme (Mt. Sinai School of Medicine) and the following

primers: 5'-CGAAGCTTGCAGATGAGCAGCTCG-3' (BLMH exon 1) and 5'-CCGGTACCGTATCACTCACTCAGCCAA-3' (BLMH exon 12). The PCR amplicon was cloned into the enhanced green fluorescent protein (EGFP-C_2) vector (Clonetech, Palo Alto, CA) using the Hind III and BamHI restriction endonuclease cleavage sites. The EGFP-BLMH plasmid was amplified in DH5α *E. coli* (Invitrogen) and grown on kanamycin containing LB agar plates. The plasmid was then purified by Wizard miniprep (Promega) and the construct was verified by double digestion and direct cycle sequencing using an ABI 377 (Applied Biosystems Inc.).

2.5.3 Vectors

pGL3 basic vector, containing firefly luciferase lacking any defined promoter elements, pGL3 control vector, with the luciferase construct driven by the SV40 promoter, and the pRL TK vector which expresses renilla luciferase under the control of the thymidine kinase promoter were purchased from Promega. EGFP-C2 was obtained from Clonetech.

2.6 Luciferase assays

Luciferase assays were run using the Dual-Luciferase Reporter Assay System (Promega) per manufacturers directions. Briefly, pGL3 and pRL TK vector concentrations were measured with a DU640 spectrophotometer (Beckman Instruments, Fullerton, CA) and verified by digestion with Hind III (New England Biolabs) and electrophoresis on 1% agarose gel stained with

ethidium bromide. Dilutions of digested pGL3 basic vector of known concentrations were used as standards. DNA concentrations were determined using the Still Video Imaging System (Eagle Eye II, Stratagene). Cells were co-transfected with pGL3 and pRL TK vectors 24 h prior to assaying and were harvested by incubation with passive lysis buffer (Promega). Cell lysate was then mixed with Luciferase Assay Reagent II (Promega) and after a 2 s delay, firefly luciferase luminescence was measured. Stop and Glo (Promega) was then added to quench the firefly luciferase and concomitantly activate the renilla luciferase and renilla luminescence was measured. All luciferase measurements were performed on a Victor 2 multilabel, multiplate reader (Perkin Elmer, Boston, MA) with a dual auto injector system. Luminescence from the pGL3 vectors was normalized to renilla luminescence.

2.7 5'RACE

Total RNA was purified from murine cerebral cortex with Trizol reagent (Invitrogen) per manufacturers instructions. The primer 5'-ACCATCATTTGCAGGGTTCATAAG-3' was used to prime cDNA synthesis from 500 ng of total RNA using reverse transcriptase (Invitrogen). The RNA:DNA hybrids were purified with GLASS MAX spin cartridge (Invitrogen) and dA-tailed with terminal deoxynucleotidyl transferase. Tailed cDNA was amplified by two rounds of PCR. In the first round, the primers were 5'-CAGGTAAGATTGGCTAAACTC-3' (*BLMH* exon 3) and anchor-primer 5'-GGCCACGCGTCGAC TAGTACGGGIIGGGIIGGGIIG-3'. Amplification was performed using the following cycling conditions: 94 °C 1 min, 55 °C 30s and 72 °C 1 min. In the second round of PCR, the primers were 5'-

GTGATTGGCTTGCCCTCCTGG-3' (*BLMH* exon 2) and 5'-GGCCACGCGTCGACTAGTAC-3'. Amplicons from nested PCR were sequenced to identify the transcriptional start sites.

2.8 RNA isolation and Northern blotting

Total RNA was isolated from EOC20 cells using an RNeasy Kit (Qiagen, Valencia, CA). RNA concentrations were measured using a DU640 spectrophotometer (Beckman Instruments). Northern blotting was performed using NorthernMax™ system (Ambion, Austin, TX) according to the manufacturer's instructions. Briefly, 5 µg of total RNA and Digoxigenin (DIG)-labeled RNA Molecular Weight Marker I (Roche Applied Science, Indianapolis, IN) were electrophoresed on 1% denaturing agarose gel containing 2.2 M formaldehyde, transferred to Nytran® SuPerCharge membrane (Schleicher & Schuell, Keene, NH) and UV cross-linked. A 1365-base pair DIG-labeled double-stranded DNA probe was generated by PCR amplification of the cloned BLMH cDNA with primers 5'-CGAAGCTTGCAGATGAGCAGCTCG-3' (*BLMH* exon 1) and 5'-CCGGTACCGTATCACTCACTCAGCCAA-3' (*BLMH* exon 12) using a PCR DIG Probe Synthesis Kit (Roche Applied Science). The probe was hybridized to the membrane overnight in ULTRAhyb™ Ultrasensitive Hybridization Buffer (Ambion), and the membrane was processed using DIG Wash and Block Buffer Set (Roche Applied Bioscience). The hybridized probe and anti-DIG-alkaline phosphatase (AP) Fab fragments (Roche Applied Sciences) complex was visualized on x-ray film (Eastman Kodak Co., Rochester, NY) after incubation of the membrane with CDP-*Star* ultrasensitive chemiluminescent substrate for AP (Roche Applied Sciences). Membranes were reprobed with DIG-labeled actin RNA probe (Roche Applied Sciences) as a loading control. For quantitation of RNA expression levels x-ray films were

scanned by densitometry (SI, Amersham Biosciences, Piscataway, NJ) and analyzed using the ImageQuant software package (version 4.1, Amersham Biosciences).

2.9 Cytokines, protease inhibitors and antibodies

2.9.1 Cytokines and protease inhibitors

Recombinant rat γ interferon and mouse recombinant tumor necrosis factor α were purchased from Calbiochem (La Jolla, CA). Recombinant human γ interferon was purchased from Biosource International (Camarillo, CA). 10 mM MG132, a proteasome inhibitor, in anhydrous DMSO was purchased from Calbiochem.

2.9.2 Antibodies

All antibodies are listed in Appendix A. Rabbit anti rat Blmh was a kind gift of Dr. Atsushi Takeda (Sagami Women's University, Kanagawa, Japan). Chicken anti-human BLMH was produced through Aves Labs (Tigard, Oregon) and the IgY fraction was affinity purified. Briefly, 5 ml IgY fractions were run over recombinant purified His-BLMH crosslinked to an Affi-15 affinity support (BioRad). The column was washed with 100 mM sodium phosphate buffer at pH = 7.2 with increasing concentrations of sodium chloride and then eluted with pH = 2.0 elusion buffer (100 mM glycine buffer with 10% 1,4-dioxane). The affinity purified chicken

anti-BLMH was precipitated with 100% saturated ammonium sulfate, resuspended in PBS and dialyzed against PBS. Rabbit polyclonal anti-heat shock protein 70 (SPA-812) was purchased from Stressgen (Victoria, BC, Canada); rabbit anti-proteasome 20S LMP 7 (PA1-972) was purchased from Affinity BioReagents (Golden, CO); rabbit anti-vinculin (H-300) was purchased from Santa Cruz.

2.10 Cell culture

Blmh wild type and null murine embryonic fibroblasts (MEF) were established in culture from embryonic day (ED) 16-18 embryos and immortalized with Simian virus 40 large tumor antigen by Dr. D. Schwartz, Department of Pharmacology, University of Pittsburgh (Lefterov et al., 2001). MEF as well as HeLa human cervical carcinoma cells (HPV-positive) [American Tissue Culture Collection (ATCC), Manassas, VA] were maintained in Dulbecco's minimal essential medium containing 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin-streptomycin (Invitrogen). Human embryonic kidney (HEK) 293 (ATCC) cells were maintained in minimal essential media containing 1.5 g/L sodium bicarbonate and 10% fetal bovine serum (HyClone). Human neuroblastoma SHSY-5Y (ATCC) was maintained in 1:1 Eagle's minimum essential media: Ham's F-12 with 10% fetal bovine serum (HyClone). LADMAC cells (ATCC), which secrete colony stimulating factor 1, are derived from c-myc transfected murine bone marrow and were maintained in minimal essential media and 10% fetal growth serum. Murine microglial cell line EOC20 (ATCC) was maintained in Dulbecco's minimum essential medium containing 10% fetal bovine serum and 20% LADMAC conditioned media. Primary astrocyte and microglial cultures were isolated from the cerebral cortices of ED21 Sprague-Dawley rats by

Dr. I. Lefterov, Department of Pharmacology, University of Pittsburgh, as previously described (Koldamova et al., 2003) and were maintained in Dulbecco's minimum essential medium containing 10% fetal bovine serum (HyClone). All cultures were grown in a humidified atmosphere of 5% CO_2 at 37 °C.

2.11 Transfections

Cells were transiently transfected using LipofectAMINE PLUS™ (Invitrogen) in serum-containing medium according to the manufacturer's instructions. Briefly, DNA and lipid complexes were incubated for 20 min in OPTIMEM (Invitrogen) and then added to cell cultures. Media containing the DNA-lipid complexes were removed after 3 h and replaced with complete growth media, and cells were harvested after 24 h.

2.12 Western blot

Cells were harvested in 1% NP-40 lysis buffer containing10 µg/ml leupeptin, 10 µg/ml aprotinin, 100 µg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride and centrifuged at 13,000 x *g* to clear the lysates. Protein content was determined by the Bradford method. Total cell lysates (20 µg protein) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were incubated in 5% milk (BioRad), 1% IgG free bovine serum albumin (BSA) (Sigma) blocking solution and probed with primary antibodies overnight at 4° C. Positive antibody reactions were visualized using peroxidase-conjugated secondary antibodies and an

enhanced chemiluminescence detection system (Renaissance, Perkin-Elmer Life Sciences) according to the manufacturer's instructions. Affinity purified anti-β-tubulin (Cedarlane, Hornby, Ontario) was used as a loading control for the Western blots. Protein expression levels were quantified as described for Northern blots.

3 EVALUATION OF THE CNS IN THE *BLMH* NULL MOUSE

3.1 Introduction

The papain superfamily neutral cysteine protease BLMH has been genetically associated with increased risk for Alzheimer's disease (AD) (Montoya et al., 1998; Papassotiropoulos et al., 2000) and shown to alter the processing of the amyloid precursor protein (APP) when ectopically overexpressed (Lefterov et al., 2000; Lefterov et al., 2001). Despite these observations, the distribution of BLMH and physiological functions within the CNS are unknown.

Papain superfamily cysteine proteases are ubiquitous proteases that function not only in general protein degradation but also have roles linked to specific biological processes important for both normal physiology and disease. In the CNS, calcium dependent calpains are activated following excitotoxic and ischemic insults and are implicated in neurodegeneration by their ability to cleave a small targeted set of proteins such as the cytoskeletal protein spectrin and the Ca^{2+}/calmodulin dependent phosphatase calcineurin (Nixon et al., 1994, Chan et al., 1999; Wang et al., 2000; Wu et al., 2004). These proteases are also believed to play roles in signal transduction and possibly synaptic plasticity (Hell et al., 1996; Wechsler et al., 1998; Chan et al., 1999; Sato et al., 2001). Another member of the papain superfamily, cathepsin L has recently been shown to be responsible for converting proenkephalin to the active enkephalin peptide neurotransmitter (Yasothornsrikul et al., 2003). Active enkephalin is involved in regulation of analgesia, behavior and immune cell functions. In cathepsin L knockout mice, active enkephalin levels are significantly reduced and precursor levels are relatively increased (Yasothornsrikul et al., 2003).

AD is a devastating age associated progressive neurodegenerative disease characterized by cognitive decline, deposition of APP protein derived Aβ peptide in extracellular amyloid

plaques, intraneuronal neurofibrillary tangles and neuronal loss. Age is the single largest predictive factor for the onset of sporadic AD, and the occurrence of dementia, of which AD is the most common cause, rises exponentially with age (Jorm et al., 1987, Rocca et al., 1991). Although they fail to fully replicate the human disease, mouse models of AD have proven useful for understanding some aspects of the molecular pathology of the disease (Duff and Rao, 2001; Janus and Westaway, 2001; Dodart, 2002). In addition, normal age related changes in rodents such as changes in locomotion and spacial memory cognitive declines may parallel aging in humans (Janicke et al., 1983; Gower et al., 1993; Ingram and Jucker, 1999; Frick et al., 2000)

In the following studies, effects in the CNS of the targeted deletion of *Blmh* in mice were evaluated by immunohistochemistry and behavioral phenotyping. Male and female young and aged animals were evaluated to access changes related to aging in *Blmh* null mice. Deletion of *Blmh* was shown to result in increased astrogliosis and decreases water maze probe trial performance suggesting that Blmh may have specific and non-redundant functions in the CNS.

3.2 Results

3.2.1 Distribution of Blmh in the murine CNS

The expression and distribution of Blmh in the murine CNS homogenates was determined from snap frozen microscopically dissected brain tissues. Blmh protein was found to be present in all regions of the brain surveyed by Western blotting. Relatively little variation in protein levels were noted among the regions surveyed (Figure 1).

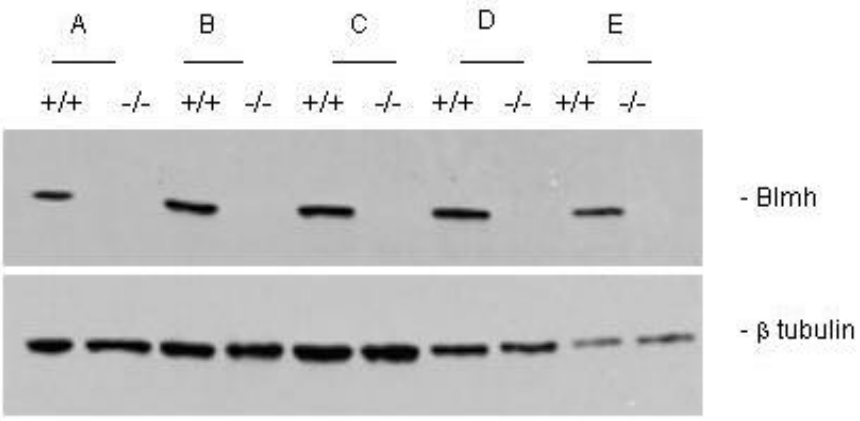


Figure 1 Murine Blmh is expressed throughout the CNS.

Murine brain tissues from (A) hippocampus, (B) cerebral cortex, (C) medial septum, (D) brain stem and (E) cerebellum from wild type (+/+) and *Blmh* null (-/-) mice were microscopically dissected and snap frozen. Tissue was then homogenized, run on sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS PAGE) and probed by Western blot analysis for Blmh with rabbit anti-rat antibody, and then reprobed with anti-β-tubulin as a loading control.

3.2.2 Immunohistochemistry

Brains of twenty month old male B6.129 Blmh^{tm1Geh}/J null and littermate wild type mice were stained with Nissl to detect cell bodies, probed for glial fibrillary acidic protein (GFAP) to identify astrocytes, and for an antigen associated with the complement C3bi receptor (OX42) to identify microglia. *Blmh* null mice and wild type littermate controls were similar in gross appearance of the CNS and Nissl staining (Figure 2). Immunostaining for GFAP revealed that the aged null animals had marked global astrogliosis in comparison to their wild type littermates (Figure 3 A-D). The GFAP stained cells in the null brains appeared hypertrophied and were poorly organized in comparison to those in the brains of the wild type littermates. No obvious significant differences in staining for OX42 were noted between wild type and null animals (data not shown).

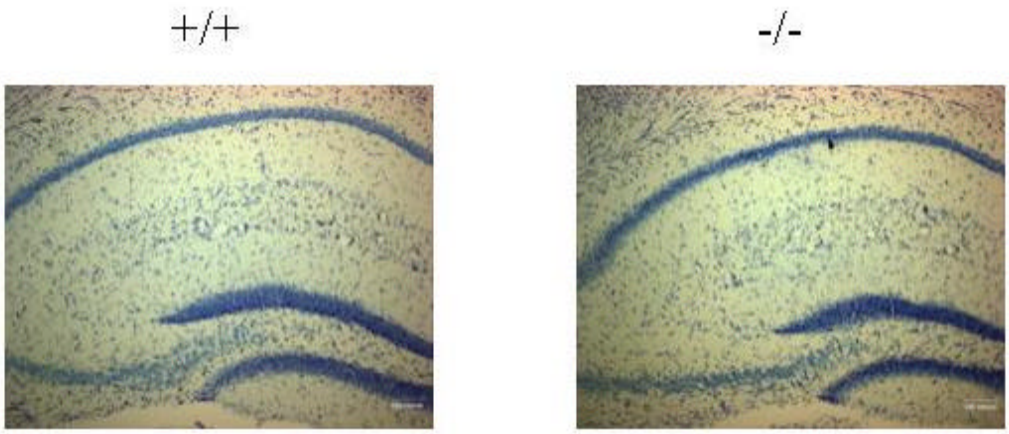


Figure 2 Nissl staining of B6.129 Blmh^{tm1Geh}/J hippocampus

Serially sectioned 4 % paraformaldehyde fixed brain tissue from littermate aged wild type and *Blmh* null B6.129 Blmh^{tm1Geh}/J mice were stained with Nissl. Representative Nissl staining of the hippocampus (n=3 +/+, -/-)(10x). Bar represents 100 microns.

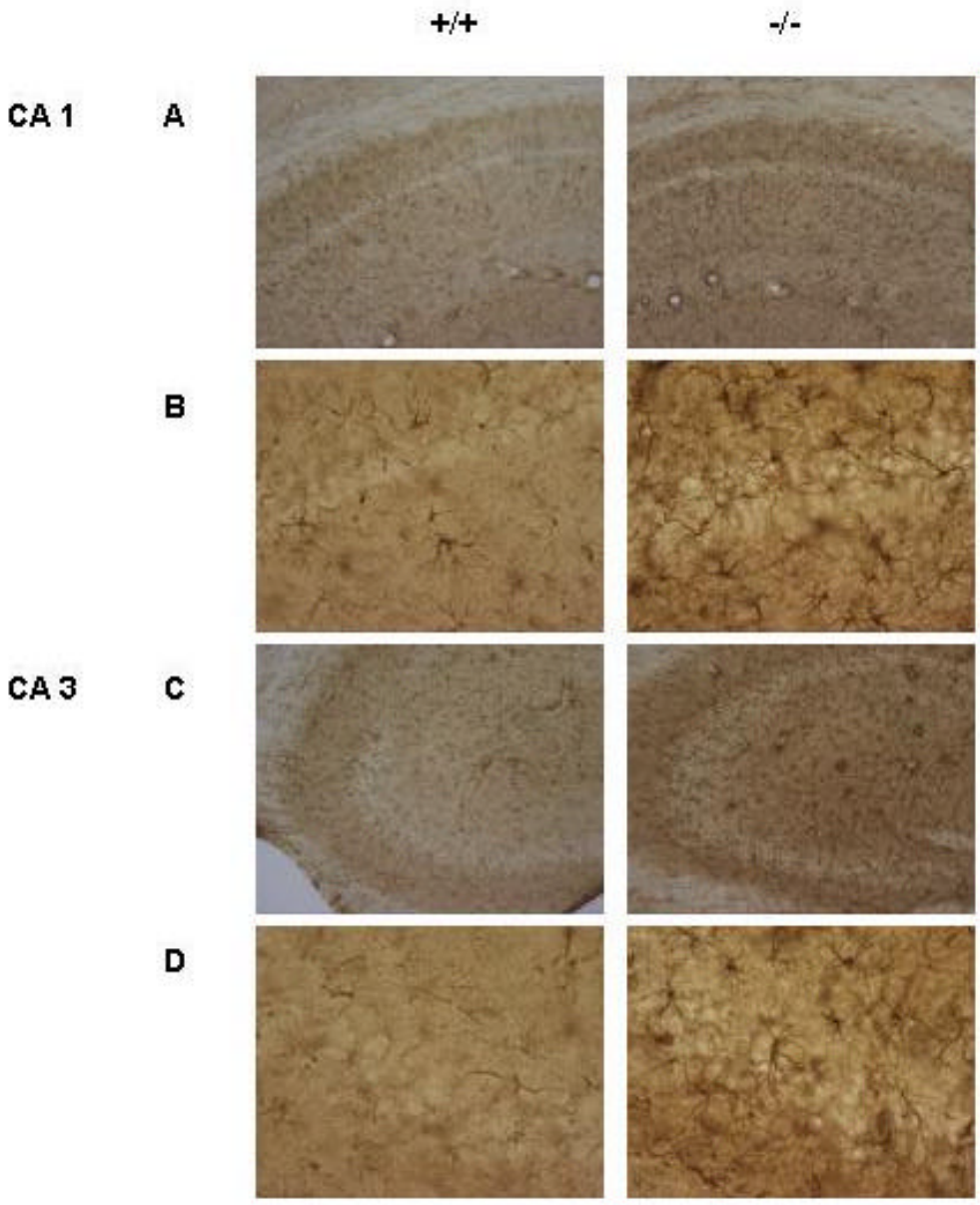


Figure 3 GFAP staining of B6.129 Blmh^{tm1Geh}/J hippocampus

Serially sectioned 4 % paraformaldehyde fixed brain tissue from littermate aged wild type and *Blmh* null B6.129 Blmh^{tm1Geh}/J mice were immunostained with GFAP to detect astrocytes. Representative GFAP staining of A. CA 1 region of hippocampus (10x), B. CA 1 region of hippocampus (40X), C. CA3 region of hippocampus (10x), D. CA3 region of hippocampus (40x) (n=3 +/+, -/-).

3.2.3 [129S6-Blmh^{tm1Geh}/J X B6.129 Blmh^{tm1Geh}/J]F1 hybrid animals

Littermate hybrid *Blmh* null, heterozygote and wild type mice were bred in accordance with the recommendations of the Banbury Conference (Banbury Conference, 1997) to standardize genetic background. Phenotypes of 'knock-out' mice by homologous recombination can vary significantly due to genetic background and the presence of unknown genetic modifiers (Owen et al., 1997; Ingram et al., 1999; Magara et al., 1999; Royle et al., 1999). Although targeted deletions are maintained in inbred strains of mice, these are generally poor subjects for behavioral analysis and other phenotypic characterization due to abnormalities present in the parental strains (Banbury Conference, 1997). Hybrid crosses may eliminate the homozygosity of alleles that complicate the characterization of targeted deletions in inbred strains (Banbury Conference, 1997). In addition, 129B6 F1 hybrid animals in particular have been shown to outperform many inbred strains in the Morris water maze and contextual fear conditioning (Owen et al., 1997).

Genotype distribution of the F1 hybrids did not deviate from the expected Mendelian ratio ($\chi^2 = 4.62$, $P > 0.10$) (Table 2). Null animals often appeared smaller at weaning, but were otherwise indistinguishable in gross appearance from heterozygote and wild type littermates. Body weight at 4-5 months was significantly smaller for null animals (-/- = 30.8 $\pm$ 0.7 g, n = 7; +/+ = 35.9 $\pm$ 0.9 g, n = 8; $P < 0.005$, two-tailed t-test) but this difference did not persist in animals older than 14 months (aged males, 42.2 $\pm$ 1.8 g; aged females 42.2 $\pm$ 1.5 g) and no significant differences were noted among genotypes in the older animals (males, $P = 0.14$; females, $P = 0.10$). In the isogenic 129S6-Blmh^{tm1Geh}/J and the congenic B6.129Blmh^{tm1Geh}/J background, the Blmh targeted deletion resulted in an unusual tail dermatopathology (Schwartz et al., 1999) that was not observed in any of the hybrid mice.

Table 2 Genotype distribution of *Blmh* F1 hybrids.

	+/+		+/-		-/-	
	Observed	Expected*	Observed	Expected*	Observed	Expected*
Male	44	31	56	63	26	32
Female	35	30	61	61	26	31
Total	79	62	117	124	52	62

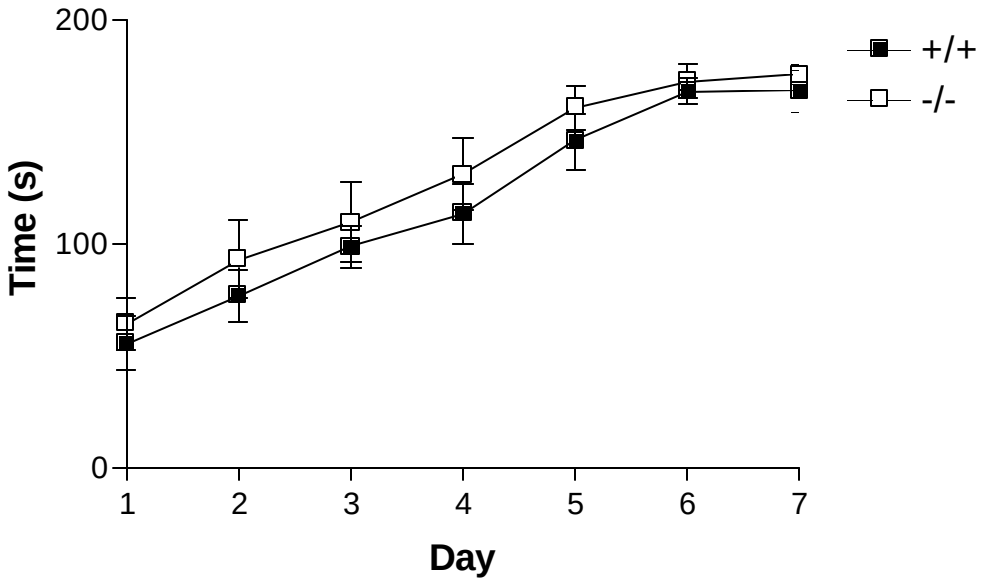
*expected number of animals assuming a 1:2:1 Mendelian distribution

3.2.4 Rotarod test

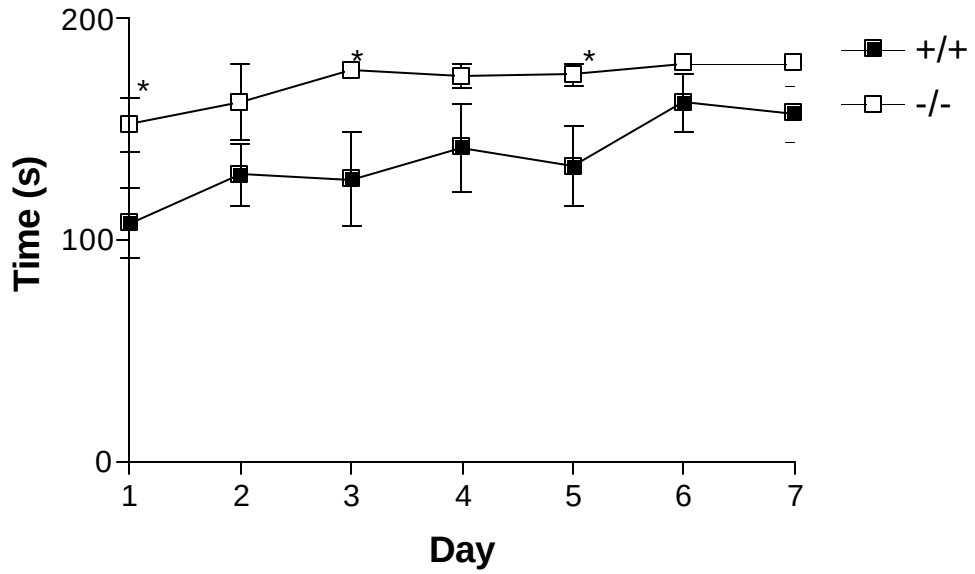
Motor coordination and balance were assessed for null and wild type young and aged [129S6-Blmh^{tm1Geh}/J X B6.129 Blmh^{tm1Geh}/J]F1 hybrid animals mice by rotarod testing. Time spent on the apparatus reported as latency to fall from the rotarod apparatus was measured in seconds and assessed for each animal during each day of testing. At 4-5 months, the performance of both wild type and null male mice significantly improved over the 7 day trial period as noted by the increased time spent on the rotarod over consecutive days (repeat measures ANOVA, $F(6,78) = 41.0$, $P<0.0005$) and did not vary between the genotypes ($F(1,13) = 1.9$, $P = 0.19$) (Figure 4A). Retesting of the same group of male animals at 14-15 months again showed significant improvement over the trial period ($F(6,72) = 3.9$, $P < 0.005$), but surprisingly, significant differences emerged between genotypes ($F(1,12)=6.3$, $P<0.05$) (Figure 4B).

Naïve aged female mice (20-23 months) were evaluated to determine if the differences seen between genotypes in aged males were related to previous rotarod testing. Significant improvement in performance was observed over the trial days ($F(5,50) = 4.6$, $P < 0.005$) and significant differences were noted between genotypes ($F(1,10) = 17.1$, $P < 0.005$) (Figure 4C). Post hoc tests showed that the aged female null mice had significantly longer latency to fall than wild type controls on days 1-4 of testing. Overall performance of aged males and females did not differ ($F(1,52) = 0.8$, $P = 0.37$) indicating that the observed difference in performance was independent of previous rotarod exposure.

A.



B.



C.

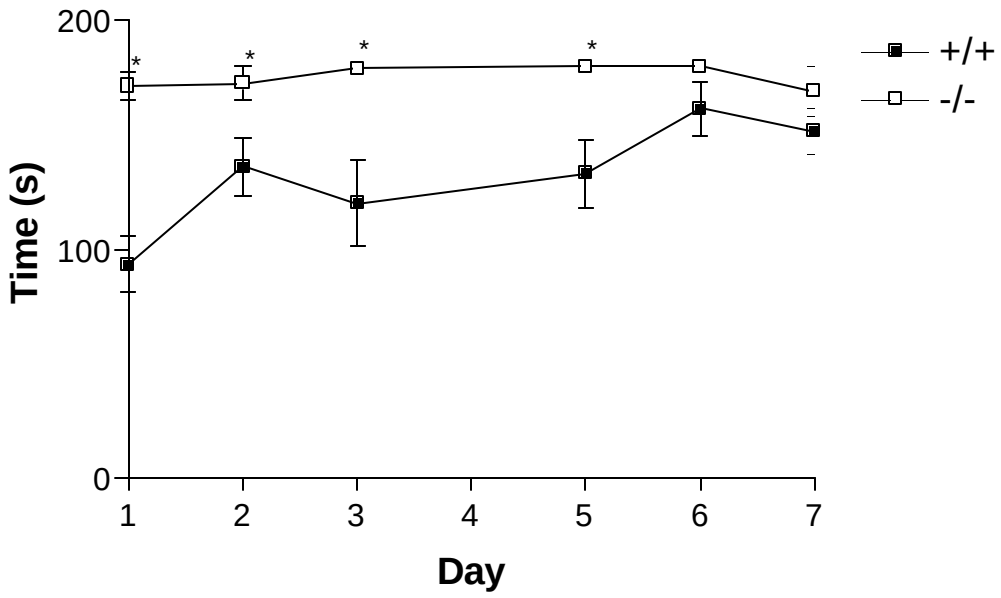


Figure 4 Rotarod testing of *Blmh* null F1 hybrids.

Young and aged F1 hybrid Blmh wild type and null mice were tested over seven days on an accelerating roratod apparatus (3-19 rpm). Latency to fall from the apparatus was measured for each animal during each 180 s trial and represented as mean for each genotype ± SEM. A. Seven day accelerating rotarod test for 4-5 month old young males (+/+ n = 8, -/-n = 7). B. Seven day accelerating rotarod retesting for males at 14-15 months (+/+ n = 7, -/- n = 7). C. Seven day accelerating rotarod test for naïve aged 20-23 month females (+/+ n = 6, -/- n = 6). * Post hoc test $P < 0.05$.

3.2.5 Open field test

Spontaneous exploratory behavior and locomotion were tested using the open field format. No differences between genotypes were observed for any of the parameters tested in any of the groups of animals evaluated (Table 3). Rearing behavior was significantly different between pooled young (4-6 months) and aged mice (20-23 months) ($P<0.05$, t-test); otherwise, young and aged animals performed similarly.

Table 3 Open field testing of *Blmh* null F1 hybrids.

Young and aged F1 hybrid *Blmh* wild type and null mice were individually evaluated for 10 min in an open field apparatus. The velocity, distance, location and duration of travel bouts, rearing and occurrence of stereotypical behavior were recorded and represented in the table as mean ± SEM. Rearing behavior differed significantly between pooled young and aged mice. * $P < 0.05$.

	YOUNG MICE		AGED MICE	
	+/+ (n=15)	-/- (n=18)	+/+ (n=12)	-/- (n=10)
Velocity (cm/s)	34.4 ± 1.5	31.8 ± 0.9	33.4 ± 2.0	33.8 ± 2.9
Time traveled (s)	41.9 ± 3.8	45.1 ± 4.5	32.6 ± 7.1	43.4 ± 8.2
Time at rest (s)	468.2 ± 7.0	462.9 ± 7.4	477.3 ± 14.5	471.5 ± 13.6
Stereotypical behavior (s)	83.2 ± 3.1	85.2 ± 3.4	79.3 ± 5.6	83.7 ± 6.4
Rearing (s)*	22.8 ± 4.9	16.0 ± 3.0	10.0 ± 3.6	9.9 ± 4.0
Percent time in center	14.4 ± 3.3	10.5 ± 1.8	14.7 ± 4.4	16.6 ± 7.4

3.2.6 Contextual fear conditioning

In the contextual fear conditioning paradigm, both young and aged mice displayed significant conditioning to context and tone (young animals, $F(5,170) = 116.6$, $P < 0.0001$; aged animals, $F(5,125) = 60$, $P < 0.0001$). No significant differences due to genotype were observed for either group of pooled animals (young animals, $F(1,34) = 1.3$, $P = 0.26$; aged animals $F(1,25) = 0.8$, $P = 0.37$) or for any of the individually tested groups. In comparison to young animals, aged animals were found to display a higher degree of fear as indicated by freezing behavior in the contextual fear test ($F(1,61) = 77.5$, $P < 0.0001$) (young animals +/+ n = 17, -/- n = 19; aged animals +/+ n = 14, -/- n = 13) (Figure 5). Post-hoc testing revealed the aged animals displayed increased fear in all aspects of contextual fear conditioning compared to young animals.

Animals were also tested in a light-dark box to assess anxiety as indicated by increased time spent in darkness. No differences were noted between genotypes in percent time spent in light for either young (young males: -/- = 11.7 $\pm$ 3.4, n = 8, +/+ = 9.9 + 3.2, n = 7; P = 0.71) or aged males (aged males: -/- = 31.2 $\pm$ 11.7, n = 6, +/+ = 33.3 $\pm$ 14.2, n = 8; P = 0.92). Young and aged males did however significantly differ in the percentage of time spent in the light (young = 10.9 $\pm$ 2.3 n = 15, aged = 32.4 $\pm$ 10.0 n = 14; $P < 0.05$).

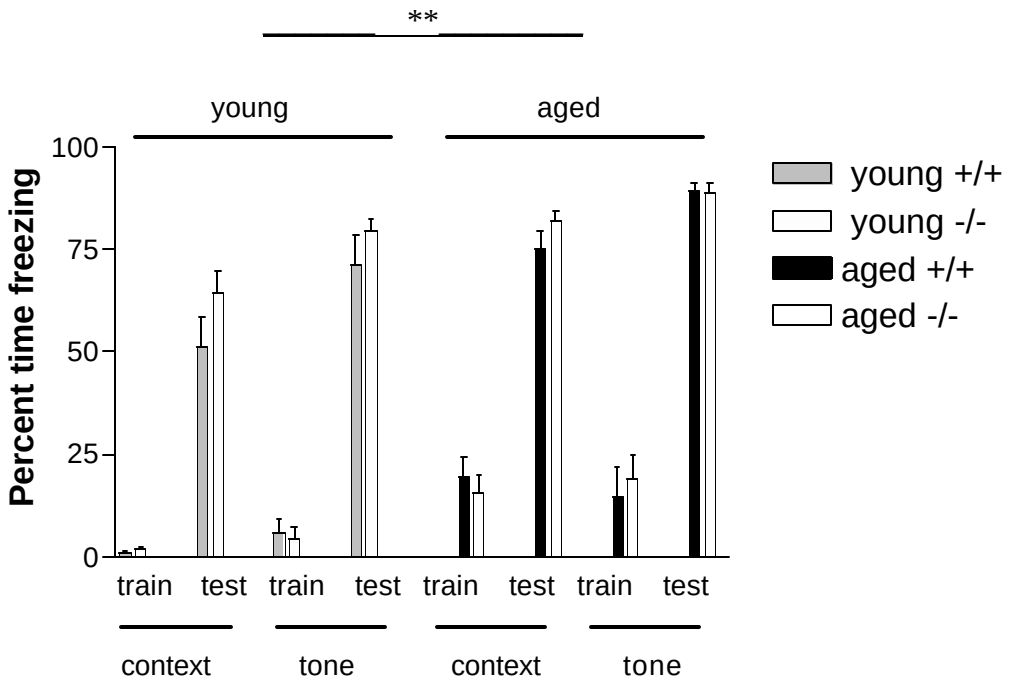


Figure 5 Contextual fear conditioning of *Blmh* null F1 hybrids.

Contextual fear conditioning was evaluated in young and aged F1 hybrid Blmh wild type and null mice. On the first day of testing, animals were exposed for 2 min to a cued context (train-context) and a 30 s tone (train – tone). During the last 2 s of the tone, the animals received a 0.75 mA foot shock. Tone and shock pairing was repeated once after 2 min. Twenty four hours later, the animals were evaluated for freezing when exposed to the original cued context (context) or when exposed to the tone in a novel context (tone). Data are represented as genotype mean $\pm$ SEM. Aged animals showed significantly more freezing than young animals in all components of the test (**P < 0.0001)

3.2.7 Hot plate test

A hot plate test was conducted to evaluate the animals ability to sense uncomfortable stimuli. Latency to paw lick, shake and jumping was evaluated as a measure of pain. In the hot plate test, young wild type animals showed a decreased latency to front paw lick in comparison to nulls ($P<0.0005$, t-test) (Table 4). In fact, in all the parameters tested in the hot plate test, the young null animals showed a tendency toward increased latency that did not reach statistical significance. Aged animals showed a decreased latency for hind shake compared to young animals ($P<0.05$, t-test). No significant differences in latency to front paw lick, hind paw lick or jump were observed between young and aged mice.

Table 4 Hot plate testing for *Blmh* null F1 hybrids.

Latency to behaviors reflecting pain sensation were recorded for young and aged F1 hybrid *Blmh* wild type and null mice during the hot plate test. Times are noted as the mean ± SEM. * P< 0.0005.

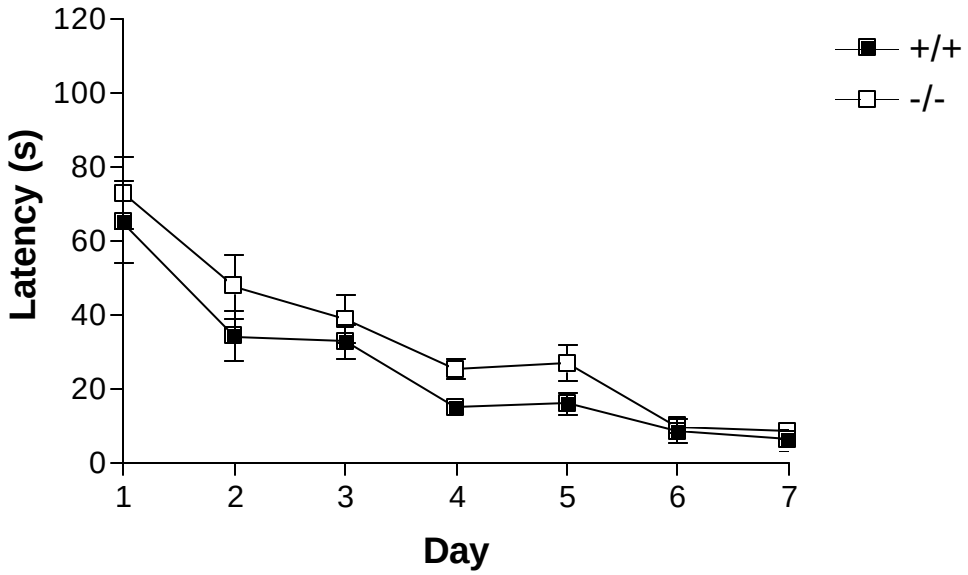
	Young mice		Aged mice	
	+/+ n=17	-/- n=19	+/+ n=13	-/- n=13
Latency to front lick (s)	14.4 ± 0.6*	18.8 ± 0.9*	15.2 ± 1.3	15.4 ± 2.6
Latency to hind lick (s)	29.6 ± 2.2	36.6 ± 2.7	33.5 ± 6.9	24.4 ± 1.9
Latency to hind shake (s)	26.4 ± 1.5	32.2 ± 2.5	21.1 ± 2.0	21.5 ± 2.0
Latency to jump (s)	162.6 ± 12.7	199.7 ± 14.6	170.8 ± 20.0	222.3 ± 20.1

3.2.8 Water maze test

The ability of mice to acquire and recall spatial information was assessed by escape latency from the Morris water maze over a five day testing period. The testing period was followed by two days of visual acuity testing using the water maze with the platform made plainly visible above the water surface. The first group of young male animals tested differed significantly in overall performance from the other two groups of young animals in water maze acquisition results as well as swim speed and thus was not included in the pooled data (data not shown).

Groups of male and female young and aged animals individually displayed significantly improved performance over the trial period as indicated by the decreased escape latencies over consecutive days. Performance did not differ by genotype in any of the groups tested. No significant differences were observed for gender in the young or aged animals; thus, the males and females were pooled. Pooled data also revealed significant improvement in escape latency over the trial period (young animals, $F(6,138) = 31.9$, $P < 0.0005$; aged animals, $F(6,120)=34.1$, $P<0.0005$; repeat measures ANOVA). Water maze acquisition and visible platform testing did not differ between genotypes for either pooled young ($F(1,23) = 3.2$, $P = 0.09$) or aged animals ($F(1,20) = 2.3$, $P = 0.14$) (Figure 6 A and B).

A.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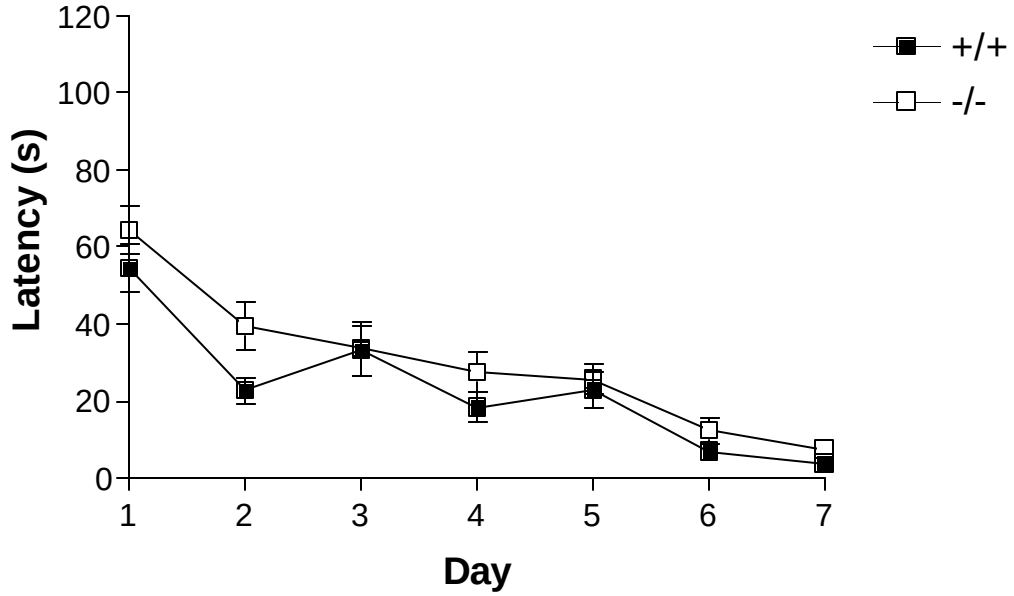
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Figure 6 **Water maze testing - acquisition**

Young and aged F1 hybrid Blmh wild type and null animals were evaluated for learning and vision by water maze testing. On days 1-5, each animal received four 120 s training trials in which the animal was allowed to swim freely about a pool where a submerged hidden platform was located. On days 6 and 7, the same testing procedure was repeated with the platform made visible. Latency to escape from the water was measured for each animal in s and represented as mean $\pm$ SEM. A. Water maze testing for pooled male and female young animals (+/+ n = 12, -/- n = 13). B. Water maze testing for pooled male and female aged animals (+/+ n=10, -/- n=12).

After the five days of the acquisition or learning phase of the water maze testing, each animal was evaluated for retention of the submerged platform location by probe trial testing. The platform was removed from the water tank and each mouse was evaluated for 60 s for the amount of time spent in each of the quadrants of the tank. Memory of the platform location is indicated by percent time spent in the target quadrant. If an animal shows no prefence for the quadrant target over the other three quadrants, this animal would be expected to spend at least 25 % of the probe trial in the target quadrant as a function of chance.

In the probe trials tests, young null animals spent significantly less time in the target quadrant than littermate controls, but still preferred the target quadrant above the other quadrants (+/+ n = 12, -/- n = 13, $P<0.05$) (Figure 7A). This difference was also independently observed in the first group of young males tested (+/+ n = 5, -/- n = 6, $P < 0.05$) (data not shown). Aged wild type and null mice did not display differences in probe trial performance (+/+ n = 10, -/- n = 12, $P=0.54$) (Figure 7B). Swim speeds did not differ by genotype within groups; however, aged animals swam significantly slower than young animals (young +/+ = 22.25 $\pm$ 0.43 cm/s; aged +/+ = 18.72 $\pm$ 1.05 cm/s, $P = 0.0034$). Overall, young wild type animals tended to perform better in the probe trial than aged controls, but the trend did not reach significance (pooled young +/+ percent time in target = 47.07 $\pm$ 2.65 %, pooled aged +/+ = 38.88 $\pm$ 3.68 %, $P = 0.08$).

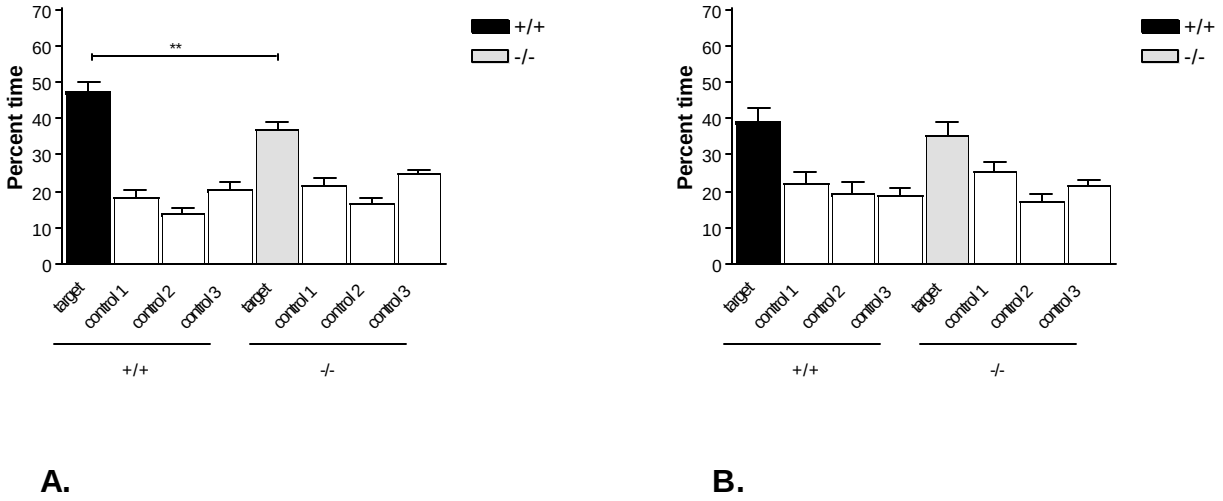


Figure 7 **Water maze testing - probe trials.**

Probes trials were conducted to evaluate retention of the submerged platform location after the 5 days of acquisition training. Young and aged F1 hybrid Blmh wild type and null animals were allowed to swim freely for 60 s in the water tank with the platform removed. Time spent in each quadrant was recorded. Target quadrant represents the quadrant that formerly contained the submerged platform. Young null males performed significantly differently than wild type controls. A. Probe trial testing for pooled young male and female mice. B. Probe trial testing for pooled aged male and female animals.

** $P<0.05$

3.3 Conclusions

Significant differences were observed between animals lacking the *Blmh* gene and littermate controls in both brain immunohistochemistry and a complex spatial memory dependent behavioral task. Immunohistochemical analysis revealed an increased presence of GFAP stained astroglia in the brains of aged null mice. In the water maze probe trials, an indicator of memory retention, young null animals displayed less recollection of the target quadrant than controls. These data suggest a non-redundant physiological role for *Blmh* in the CNS.

Studies characterizing targeted deletions can be confounded by gene redundancy, compensation of lost activity by other functionally similar proteins, genotypic influence of genetic background, disruption or modification of neighboring genes and unintended consequences of expression of selection markers (Bockamp et al., 2002). Although *Blmh* is a single copy gene, some phenotypic features of the *Blmh* knockout may be masked by activity of other functionally similar cytosolic aminopeptidases. F1 hybrids generated from backcrossed isogenic 129S6-Blmh^{tm1Geh}/J and congenic B6.129Blmh^{tm1Geh}/J may represent a better background for studing the null mutation than inbred strains, but generation of F1 hybrid animals is both time consuming and expensive. F1 hybrids also do not overcome the problems associated with flanking genes (Wolfer et al., 2002). For genes linked to the targeted deletion, linkage disequilibrium will exist between the null mutation and flanking sequences that are derived from substrains of 129 inbred mice. These studies cannot rule out the observed phenotypic effects

being related to flanking genes, disruption or modification of neighboring genes or expression of the neomycin selection marker rather than disruption of the *Blmh* gene.

3.3.1 Astrogliosis

Immunohistochemical evaluation of the CNS of aged B6.129 Blmh^{tm1Geh}/J wild and null animals revealed significant global astrogliosis as indicated by increased GFAP staining in null animals, but no obvious differences in the staining for OX42, an activated microglial marker. It is unclear if the increased GFAP staining observed in the null mice resulted in increased proliferation and/or simply the activation and hypertrophy of existing astroglial cells. GFAP is the major intermediate filament in astrocytes and also a specific marker for mature astrocytes (Eng et al, 1971; Eng and Ghirnikar, 1985). The protein is augmented in aging and also in response to all types of CNS injury and changes in neuronal-glial interactions that underlie the astrocyte's primary role in the maintenance of the neural microenvironment (Bjorklund et al., 1985; Newcombe et al., 1986; Hansen et al., 1987; Steward et al., 1991). Global astrogliosis is found in targeted deletions such as the neurofibromatosis type 1 null (Zhu et al., 2001). Astrogliosis is also observed in disease of the human CNS including AIDS dementia, prion associated spongiform encephalopathies and neurodegenerative disease such as Alzheimer's disease and is believed to indicate neuronal injury or dysfunction (Eng and Ghirnikar, 1994). The role of astrogliosis in promoting neuronal healing after injury or disease remains unclear (Ridet et al., 1997; McGraw et al., 2001). A glial scar produced by astrocytosis may be an attempt to isolate an injured area with resulting limited capability for neuronal axon regeneration (Ridet et al.,

1997; McGraw et al., 2001). Alternatively, astrogliosis may play an important role in providing a healing environment for the injured neuron (Janzer and Raff, 1987; Bush et al., 1999; John et al., 2004). The observation that expression of the microglial marker OX42 is not increased in aged *Blmh* null animals indicates that the pathology present in the brains of the null mice at the time of examination did not appear to have a significant inflammatory component. Although Blmh null mice did not appear to have any gross neuronal loss, neuronal injury and subtle loss, or neuronal dysfunction may be present accounting for the observed astrogliosis.

3.3.2 Behavioral phenotype of F1 hybrids

In three independently tested groups of young F1 hybrid [129S6-Blmh^{tm1Geh}/J X B6.129 Blmh^{tm1Geh}/J] animals, deletion of *Blmh* resulted in a modest, but statistically significant decrease in target quadrant retention in the water maze probe trial. These differences were not accounted for by diminished locomotor activity or visual impairment as evidenced by the performance of the young null animals in the rotatod, open field and water maze visible platform tests. In addition, young null animals did not differ significantly in swim speed from wild type controls; however, subtle differences in sensory and motor function cannot be ruled out.

Aged wild type and null animals performed similarly in learning and memory tasks including the water maze acquisition and probe trials, and contextual fear conditioning. The lack of a significant difference in probe trial results between aged null and wild type mice is probably due to a small decline in the performance of the wild type animals with age. Age related decline in complex learning tasks such as the Morris water maze are well documented in multiple strains

of mice (Gower et al., 1993; Frick et al., 2000; Magnusson et al., 2003). Non-cognitive impairment does not appear to account for these changes. Although both water maze and contextual fear conditioning learning and memory are believed to be mediated by hippocampal dependent components, lack of correlated findings in the two paradigms may reflect significant differences in the biology underlying learning in these tasks (Owen et al., 1997). Data from lesion studies has shown that the degree of hippocampal dependency for water maze and contextual fear conditioning differs and implies that different pathways may be involved in learning these tasks (Logue et al., 1997).

The aged Blmh null mice unexpectedly displayed an increased latency to fall from the rotarod in comparison to wild type controls irrespective of prior exposure to the task. Similar differences between null and wild type animals were observed in the 129S6-Blmh^{tm1Geh}/J background (D. Schwartz, personal communication). Other rodent studies have noted age related declines in complex motor tasks including rotarod (Janicke et al., 1983; Shukitt-Hale et al., 1998). The observed results may reflect a combination of significantly larger size of the aged animals compared to the younger animals ($P<0.0005$) and a perhaps a greater decline in strength or other parameters affecting locomotor ability and or balance in wild type animals. The role of BLMH in the neuromuscular system is uncharacterized. The related calpain proteases have significant functions in the neuromuscular system; for example, loss of function genetic mutations in calpain 3 have been identified as the cause of limb-girdle muscular dystrophy 2A (Huang et al., 2001).

Although having a lower body weight in the first few months of life than wild type controls, [129S6-Blmh^{tm1Geh}/J X B6.129 Blmh^{tm1Geh}/J] knockout animals did not display neonatal mortality and dermatopathology observed in the inbred stains with *Blmh* deletion (Schwartz et

al., 1999). It is unknown if the hybrid animals are more resistant to the dermal pathology or if environmental conditions that induce this pathology (Schwartz et al., 1999) were lacking for the duration of the hybrid studies. Overall, the F1 hybrid animals performed well in all behavioral paradigms evaluated consistent with reports of overall hybrid vigor (Owen et al., 1997) and represent a well defined background for the characterization of the *Blmh* deletion.

Aging is a dynamic process and age related behavioral changes do not necessarily correlate linearily with chronological age. In addition, age related changes in rodents display a significant amount of individual variability (Doyere et al., 2000). Age related changes occur as a function of strain (Lhotellier et al, 1989; Ingram et al., 1999) and this study provides baseline data on behavioral changes with aging in the F1 hybrid background. Overall few significant changes were noted between young and aged animals with the most notable difference being the increase in generalized freezing, an expression of fear, in aged mice in contextual fear conditioning. Similar results were noted for aged 22 month old vs 7 month old Winstar rats (Doyere et al., 2000). Other studies have reported significant declines in contextual conditioning in aged rodents observed only with repeated presentation of the context or tone over time (Ward et al., 1999; Houston et al., 1999). The reason for the increased freezing in the aged animals is not known and did not appear to be caused by overall greater anxiety levels as aged animals spent more time in the lighted section of the light-dark box than young animals.

4 EVALUATION OF THE *BLMH* TRINUCLEOTIDE REPEAT POLYMORPHISM

4.1 Introduction

Molecular genetic studies have indicated a possible role for the papain superfamily cysteine protease *BLMH* in neurodegenerative disease. Genetic association studies have reported *BLMH* as a susceptibility locus for AD based on the genotype distribution of a single nucleotide polymorphism (SNP) that results in a conserved I443V substitution (Montoya et al., 1998; Papassotiropoulos et al., 2000). Genotyping of the same SNP has also shown *BLMH* to exhibit modest transmission disequilibrium with autism (Kim et al., 2002). Although the polymorphism is in the conserved BLMH C-terminus which functions in regulation of the enzymatic activity (Zheng et al., 1998), the physiological importance of the I443V substitution is unknown. The location of the functionally significant locus could be the conserved C-terminal SNP or alternatively, another linked locus within *BLMH* or a neighboring gene.

Preliminary examination of promoter regions of papain family cysteine proteases reveals the lack of a classical TATA box and constitutive expression mediated in part by GC rich Sp1 binding sites (Hata et al., 1989; Azizkhan et al., 1993; Shi et al., 1994; Phillipsen et al., 1999; Jean et al., 2002; Yan et al., 2003). Despite the appearance of having a housekeeping gene promoter, transcriptional regulation among the papain superfamily members is an important control mechanism as highlighted by the focal expression pattern and transcriptional induction of some of the cathepsins and calpains (Shi et al., 1994; Rantakokko et al., 1996; Storm van's Gravesande, 2002).

Like other papain superfamily members, the cloned upstream region of *BLMH* lacks common consensus transcriptional regulatory elements, such as a TATA box or CCAAT box and contains several consensus Sp1 binding sites (Ferrando et al, 1997). The putative promoter

region of human *BLMH* also contains a polymorphic trinucleotide repeat –134 bp upstream the ATG start site (Ferrando et al., 1997). The impact of this polymorphic site on *BLMH* transcription is unknown, however the polymorphism lies near a highly conserved region of the 5‘untranslated region that contains several putative Sp1 binding sites and an AP1 consensus site. Polymorphic trinucleotide repeats in the 5’ untranslated regions of other genes have been found associated with neurological disease for example X-linked mental retardation results from hyperexpansion of a CCG repeat in the 5’ untranslated region (UTR) of FMR2 (Gu et al., 1996; Gecz et al. 1996). In addition, small insertions and deletions in putative promoter regions have been shown to be associated with and even cause altered gene expression (Boniotto et al., 1996; Ye et al., 2001). In the human stromelysin 1 gene a single adenosine insertion/deletion results in a run of either 5 or 6 adenosine nucleotides in the 5’untranslated region and is found to mediate differential expression of this gene as shown using a chloramphenicol acetyltransferace reporter assay (Ye et al., 1996).

In the following studies, two additional polymorphic sites in the *BLMH* were identified from a polymorphism screen of the *BLMH* 5’ putative proximal promoter and exonic regions. In addition, the trinucleotide repeat polymorphism was found not to affect basal transcription from the proximal promoter based on results of a luciferace reporter assay system. These finding are discussed in the context of the genetic association of *BLMH* with AD.

4.2 Results

4.2.1 5' RACE and polymorphism screen of the *BLMH* gene and 5' upstream region

5'RACE analysis of murine RNA derived from cerebral cortex revealed two transcriptional start locations at –104 bp (numbering based on human gene) and –53 bp from the ATG start site. This area of the murine 5' untranslated region has 62 % identity with the human gene. The murine transcriptional start site also lies adjacent to a region of DNA from –179 to –279 that is highly conserved with 97 % identity to the human gene.

All twelve exons with their flanking intronic regions and 1.2 kb of the putative proximal promoter were screened for polymorphisms using SSCP and direct cycle sequencing of genomic DNA amplified by PCR from the Pittsburgh Alzheimer's Disease Research Center (ADRC) autopsy confirmed cases (n = 10) and controls (n = 10). The screen identified two novel polymorphic sites. A silent C602G polymorphism was found in exon 6 by differential pattern of migration on the SSCP gel and confirmed by direct cycle sequencing. A CA repeat polymorphism was found at –1156 by direct cycle sequencing with $(CA)_7$ and $(CA)_8$ observed in the screened population (Figure 8). Both mutations were observed in both AD cases and controls.

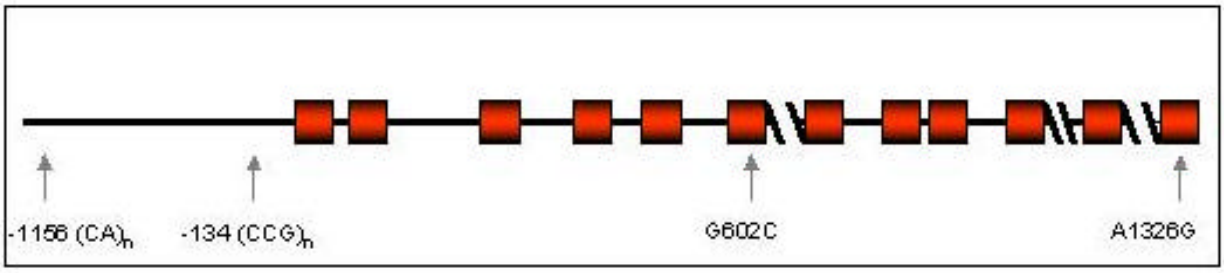


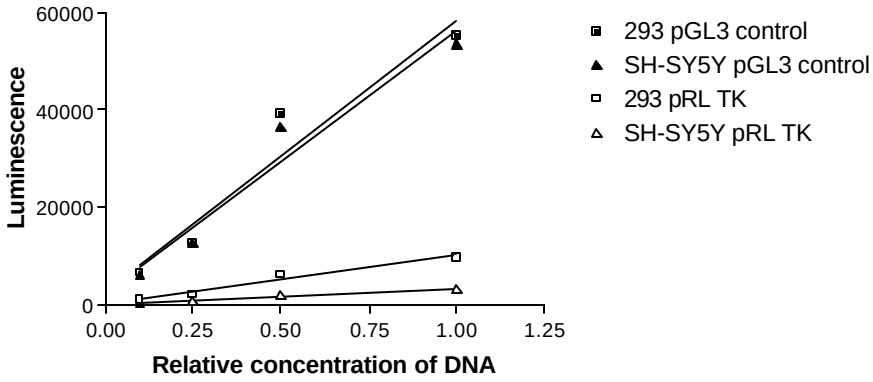
Figure 8 Polymorphic sites in the *BLMH* gene

PCR amplicons from each of the *BLMH* gene exons and flanking 5'untranslated region from AD cases (n = 10) and aged matched controls (n = 10) from the Pittsburgh ADRC were screened by SSCP and direct cycle sequencing for polymorphic sites. Four polymorphic sites were identified. A novel $(CA)_n$ was observed at –1156 from the ATG start site by SSCP and a novel G602C SNP was seen in the sixth exon. A previously described trinucleotide repeat $(CCG)_n$ in the proximal promoter (Ferrando et al., 1997) and the A1326G polymorphism (Bromme et al., 1996) were also observed.

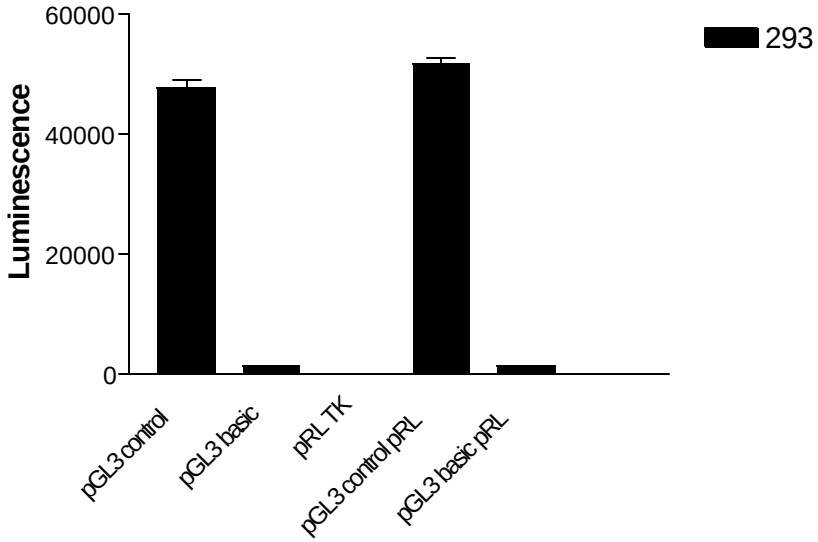
4.2.2 pGL3 5'BLMH luciferase reporter assays

To evaluate the role of the -134 $(CCG)_n$ trinucleotide repeat polymorphism in the basal transcription of *BLMH*, 1.2 kb of the 5' untranslated region containing either the $(CCG)_9$ or $(CCG)_{10}$ polymorphic variants were cloned from human genomic DNA into the pGL3 basic luciferase reporter vector (Promega). Seven to eleven repeats were observed in a Caucasian population of 116 individuals with the allele frequencies of 0.621 for $(CCG)_9$, 0.362 for $(CCG)_{10}$ and less than 0.05 for the $(CCG)_7$ and $(CCG)_{11}$ alleles which were not cloned. Two cells lines, HEK 293 cells and SH-SY5Y neuroblastoma cells, were selected for these assays due being derived from human tissues, with SH-SY5Y cells being brain derived. Ease of transfection also contributed to the selection process. Both cell lines also endogenously express BLMH. Luminescence assays from firefly and renilla luciferase were linear over a 10X dilution of transfected cell lysate and no detectable cross over of signal was seen between the firefly and renilla luciferase (Figure 9 A, B and C). The cloned 1.2 kb 5'untranslated region was found to function effectively as a promoter in the luciferase construct in both cell lines. No significant differences in basal transcription from the $(CCG)_9$ and $(CCG)_{10}$ pGL3 constructs were observed for either the HEK 293 ($P = 0.92$), or SH-SY5Y cells ($P = 0.06$) (Figure 9 D and E).

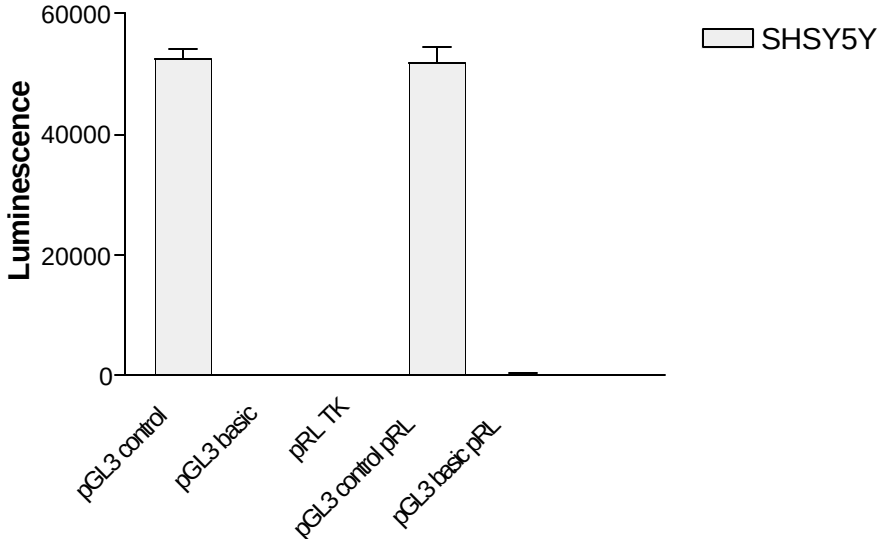
A.


60000
40000
20000
0
Luminescence
0.00
0.25
0.50
0.75
1.00
1.25
Relative concentration of DNA
293 pGL3 control
SH-SY5Y pGL3 control
293 pRL TK
SH-SY5Y pRL TK

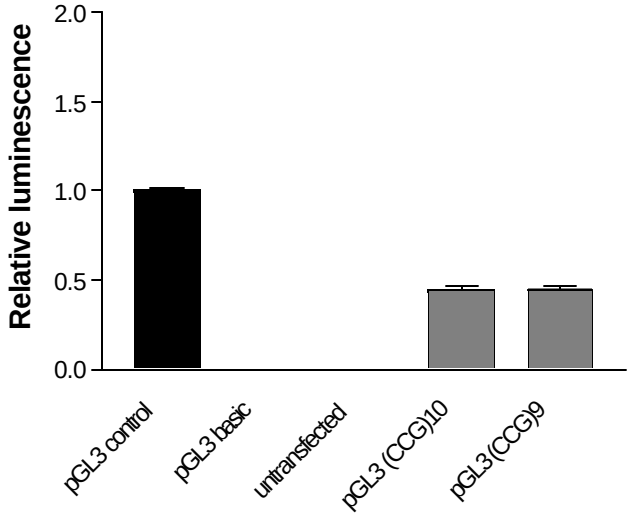
B.


60000
40000
20000
0
Luminescence
293
pGL3 control
pGL3 basic
pRL TK
pGL3 control pRL
pGL3 basic pRL

C.


60000
40000
20000
0
Luminescence
SHSY5Y
pGL3 control
pGL3 basic
pRL TK
pGL3 control pRL
pGL3 basic pRL

D.


2.0
1.5
1.0
0.5
0.0
Relative luminescence
pGL3 control
pGL3 basic
untransfected
pGL3 (CCG)10
pGL3 (CCG)9

E.

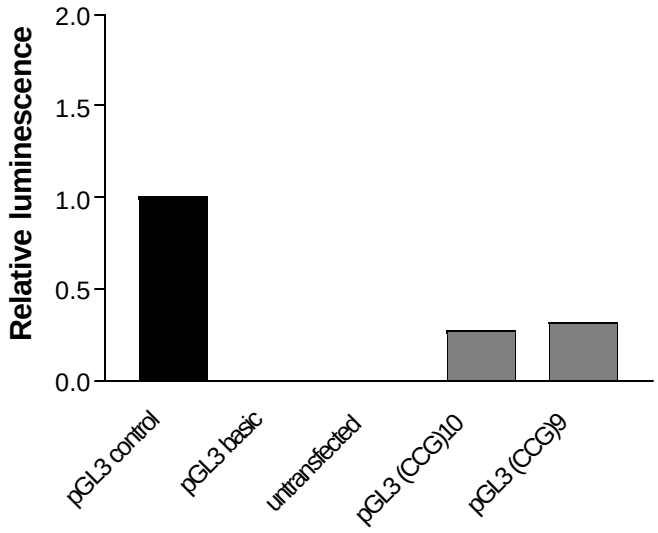


Figure 9 Luciferase reporter assay for *BLMH* trinucleotide repeat polymorphism

Luminescence from a luciferase reporter construct driven by 1.2 kb of the 5' untranslated region of *BLMH* containing a polymorphic trinucleotide repeat was measured in HEK 293 and SH-SY5Y cells. Linearity of the assay was determined by serial dilutions of either cell lysates transfected with the pGL3 control vector containing SV40 promoter elements or pRL TK renilla vector driven by the thymide kinase promoter. Firefly luciferase or renilla luciferase crossover was assayed by single transfections with each of the vectors and compared to the reporter assay dual transfection with the pGL3 and pRL vectors. Data represent mean values and error bars show SEM from 3-6 independent experiments. A. Linearity of assay. B. Control plasmid expression in HEK 293 C. Control plasmid expression in SH-SY5Y. D. HEK 293 relative luminescence for $(CCG)_9$ and $(CCG)_{10}$ pGL3 constructs. E. SH-SY5Y relative luminescence for $(CCG)_9$ and $(CCG)_{10}$ pGL3 constructs. Luminescence for $(CCG)_9$ and $(CCG)_{10}$ pGL3 constructs

were not statistically significantly different for either cell line based on analysis by t-test.

4.2.3 Genetic association study revisited

BLMH was first associated with AD through genotyping at the A1326G loci in the twelfth exon (Montoya et al., 1998). AD confirmed and clinical cases derived from the Pittsburgh ADRC and age matched controls from the same population were screened at the A1326G loci by SSCP of PCR amplicons of the twelfth exon derived from genomic DNA. The linked 5'$(CCG)_n$ repeat is located about 30 kb from the A1326 loci. Pittsburgh ADRC cases and controls were amplified using PCR conditions as described by Ferrando et al. (1997) following 5' ^{32}P labeling of the forward primer. PCR products were electrophoresed on a 7% polyacrylamide gel and visualized by autoradiography. When allele and genotype frequencies from the A1326G BLMH C-terminal SNP were compared with those from the –134 CCG repeat for Pittsburgh ADRC AD cases and controls, only the A1326G locus showed significant evidence of association with AD in the Pittsburgh ADRC population (Table 5). Allele frequencies were not significantly differenct between cases and controls for neither the A1326G BLMH C-terminal SNP or the –134 CCG repeat. Genotype frequencies for the G locus of the A1326G SNP was overrepresented in cases compared to controls (OR=2.28, 95% confidence interval = 1.02 to 5.00, $X^2 = 4.01$, $P < 0.05$). Genotype frequencies were not significantly different for cases and controls for the – 134 CCG repeat (OR=1.43, 95% confidence interval = 0.74 to 2.80, $X^2 = 1.09$, P not significant).

Table 5 BLMH allele frequency and genotype distribution in AD cases and controls

		TOTAL	ALLELE FREQUENCY		GENOTYPE FREQUENCY		
A1326G*			A	G	A/A	A/G	G/G
	CASES	151	0.67	0.33	0.426	0.358	**0.152**
	CONTROLS	124	0.70	0.30	0.468	0.460	**0.073**
$(CCG)_n$**			$(CCG)_9$	$(CCG)_{10}$	$(CCG)_9/(CCG)_9$	$(CCG)_9/(CCG)_{10}$	$(CCG)_{10}/(CCG)_{10}$
	CASES	179	0.63	0.36	0.43	0.41	**0.16**
	CONTROLS	116	0.62	0.36	0.38	0.46	**0.13**

*A1326G allele and genotype distribution was previously published (Montoya et al., 1998)

** Montoya, 1998

4.3 Conclusions

These studies focused on the trinucleotide repeat polymophism of the 5' untranslated region of *BLMH* based on the proximity of the trinucleotide to the putative translational start site and conserved regions of the 5' untranslated area. Although no evidence for expansion of the trinucleotide repeat had been observed, 5' untranslated region trinucleotide repeats may provide important spacing between elements of the promoter. The calmodulin 1 gene has 7 CAG repeats (Toutenhoofd et al., 1998). Expansion of this trinucleotide to 20 or 45 repeats in a luciferase reporter construct does not alter transcriptional strength of the promoter, but complete deletion of the trinucleotide decreases the output of the promoter sequence by 45% (Toutenhoofd et al., 1998).

The cloned 1.2 kb of the 5' untranslated region contains sufficient active promoter elements to drive luciferase expression in both 293 and SH-SY5Y cells. Basal transcription from the proximal promoter containing either the $(CCG)_9$ or $(CCG)_{10}$ allele was not significantly different. These data do not eliminate the possibilities that the trinucleotide repeat could (1) differentially affect transcription from the intact promoter, (2) regulate transcription that is induced, or differentially regulate transcription in another untested cell type.

When comparing the allele distributions of the Pittsburgh ADRC AD cases and controls at the A1326G SNP and trinucleotide repeat, association with AD was only observed with the A1326G SNP. These observations suggest that the trinucleotide repeat polymorphism is not a significant functional locus in AD in the Caucasian Pittsburgh population studied. It is

interesting to note that in the linkage disequilibruim study of BLMH with autism, the trinucleotide repeat polymorphism locus was also found not significant (Kim et al., 2002). Two additional novel polymorphisms were found in *BLMH* by polymorphism screening. A C602G SNP that does not alter the amino acid usage and a CA repeat located –1156 bp from the ATG start site. The allele distribution for the CA repeat polymorphism and C602G SNP remain to be determined in a normal population. Further investigation may help determine if the functional locus for genetic association in AD and auatism is the same, and whether it lies within *BLMH* or another neighboring gene, such as the serotonin transporter gene, which is located about 15 kb downstream of *BLMH* A1326G.

5 BLMH INDUCTION BY INF g

5.1 Introduction

MHC I epitopes are generated primarily through degradation of cellular proteins by the proteasome (Goldberg et al., 2002; Kessler et al., 2002). Additional N-terminal trimming of peptides destined for MHC I presentation occurs through unidentified cytosolic aminopeptidases. MHC I epitope presentation can be modulated by stress proteins (heat shock) and stimulated by interferon gamma (INFγ) (Fruh and Yang, 1999; Srivasta, 2002). MHC I epitope presentation is important for immune system recognition of viruses and tumors as well as potentially having a function in neuronal development and synaptic plasticity (Huh et al., 2000).

Cytosolic stress proteins are chaperones and constituents of the ubiquitin-proteasome system. These proteins participate in the transfer of candidate peptide epitopes into the endoplasmic reticulum for assembly onto MHC I molecules (Srivastava 2002). In response to cellular stresses, the normal complement of cytosolic stress proteins are altered. For example, in the CNS, cytosolic stress protein composition can be altered by insults such as ischemia and trauma and is upregulated in some neurodegenerative diseases (Bonini, 2002; Yenari, 2002).

Proteolytic processing both by the proteasome and putative N-terminal MHC I epitope processing aminopeptidases can be altered by INFγ (Fruh and Yang, 1999; Kessler et al., 2002; Goldberg et al., 2002). Exposure of cells to INFγ can induce the replacement of the normal β subunits present in the proteasome by LMP2, LMP7 and MECL-1 (Gaczynska et al., 1993; Fruh et al., 1994). INFγ treatment also results in the induction of the 11S PA28α/β subunits that can replace one or two of the 19S caps of the 26S proteasome (Groettrup et al., 1996). Following

treatment with INFγ, the resulting modified proteasome assembly, the "immuno-proteasome", displays altered proteolytic activity including stimulated cleavage after hydrophobic, basic and branched chain residues and diminished cleavage following acidic residues (Driscoll et al., 1993; Gaczynska et al., 1993; Aki et al., 1994). The proposed N-terminal MHC I epitope processing aminopeptidases leucine aminopeptidase and ERAAP are also induced by INFγ (Beninga et al., 1998; Serwold et al., 2002). BLMH is a candidate aminopeptidase for cytosolic MHC I epitope processing (Stolze et al., 2000). The protease was identified though its ability to produce correct sized peptides from putative protein substrates.

In the following studies, the induction of BLMH is examined in the context of a potential role in MHC I processing (Stolze et al., 2000). Both heat shock induction and INFγ induction were evaluated as potential mechanisms for BLMH regulation. Accumulating evidence suggests that *BLMH* expression is transcriptionally regulated. In skin, BLMH is expressed strongly in the epidermis, but weakly or not at all in the underlying dermis or muscle layers (Takeda et al., 1999; unpublished data). Increased BLMH expression is observed in some head and neck tumors compared to the surrounding normal tissue (Ferrando et al., 1996) and, differential activity of the protease is found among tissues such as lung and liver (Lazo and Humphreys, 1983; Filderman and Lazo, 1991). In the following experiments, BLMH protein expression was not modified by heat shock induction, but was significantly increased by INFγ in the principle antigen presenting cells of the brain, the microglia.

5.2 Results

5.2.1 Evaluation of Blmh induction by cytosolic stress

Wild type MEF and HEK 293 cells were subjected to 42° C for 1 h or treated with 10 μM MG132, a proteasome inhibitor, for 16 h to evaluate if BLMH can be induced by heat shock or proteasome inhibition. Proteasome inhibition has been shown to result in rapid induction of stress protein chaperones (Bush et al., 1997). Upregulation of the inducible form of heat shock protein 70 (hsp 72) was used as a positive control to show induction of cell stress proteins after heat shock or proteasome inhibition. Although induction of hsp 70 was observed in both MEF and HEK 293 cells 24 h after a 1 h exposure to heat stress, no changes in the expression of BLMH were observed (MEF $P = 0.84$, 293 $P = 0.13$ t-test)(Figure 10 A and B). Proteasome inhibition in HEK 293 cells also resulted in significant induction of hsp 70, and a 30 % decrease in the expression level of BLMH ($P = 0.02$) (Figure 10C).

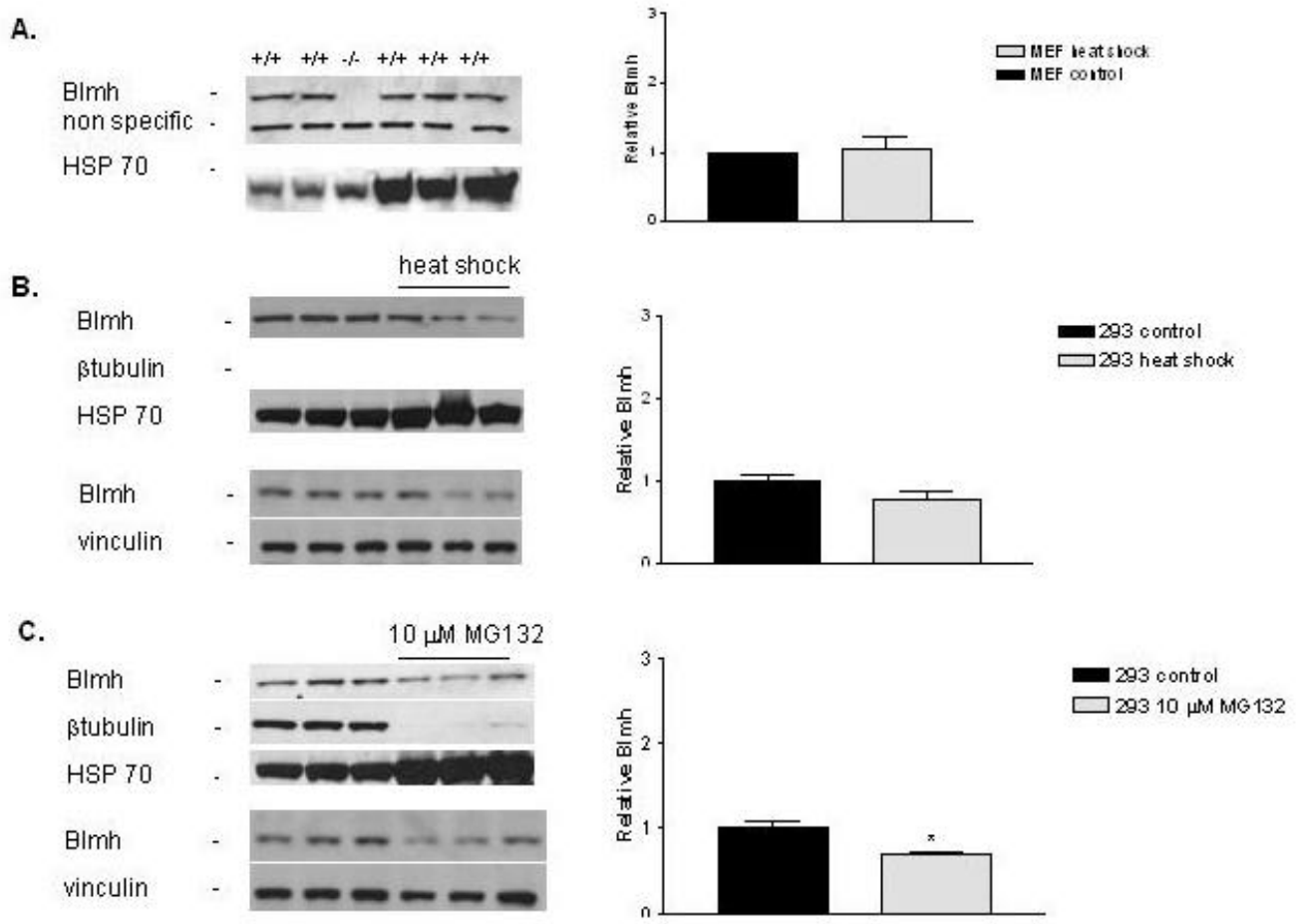


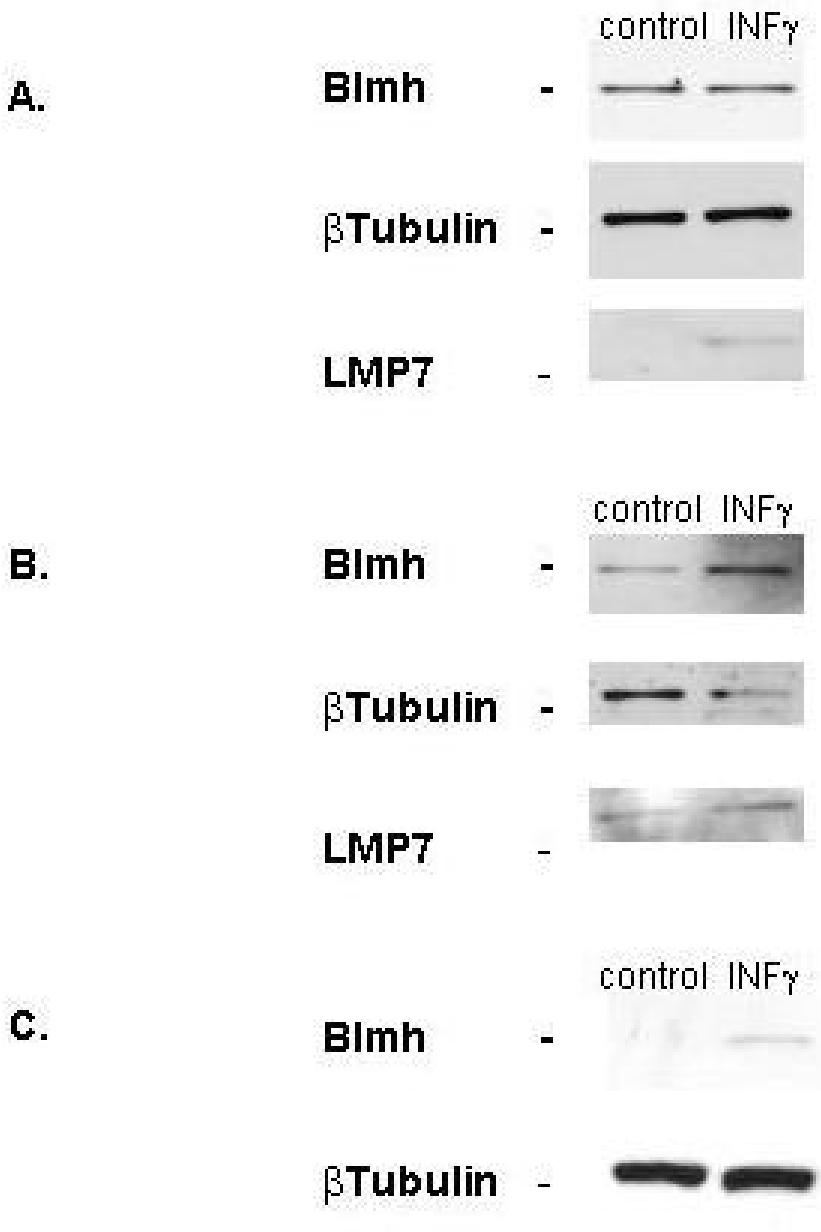
Figure 10 BLMH protein expression is not altered by heat shock induction.

MEF or HEK 293 cells were heat stressed by 1 h incubation at 42°C. After 24 h, cell lysates were harvested and immunoblotted for BLMH with rabbit anti-rat Blmh, and HSP 70. (A) MEF immunoblot following heat shock induction; Blmh genotype indicated by +/+ for wild type and -/- for null. Quantitation to right, data normalized to β tubulin. (B) HEK 293 immunoblot following heat shock induction; repeat immunoblot with vinculin loading control shown in lower panels. HEK 293 cells were further evaluated for cell stress protein induction by treatment for 16 h with 10 μM MG132 or vehicle control (DMSO). Cells were then harvested, lysed and proteins were separated by SDS-PAGE and immunoblotted with rabbit anti-rat Blmh, anti-HSP

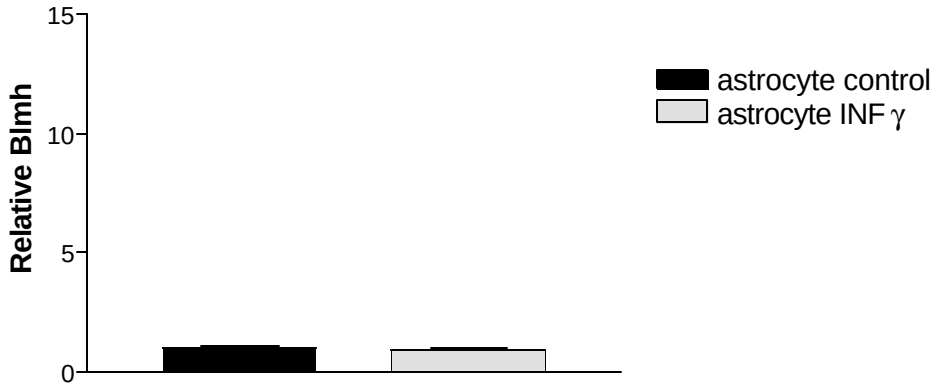
70 and anti-β tubulin. Quantitation to the right, data normalized to vinculin. (C) Immunoblot of cell lysates from HEK 293 cells treated with MG132 or vehicle control; repeat immunoblot with vinculin loading control shown in lower panels. Quantitation to the right, data normalized to vinculin. * $P < 0.05$.

5.2.2 Evaluation of Blmh induction by INFγ in primary astrocytes and microglia

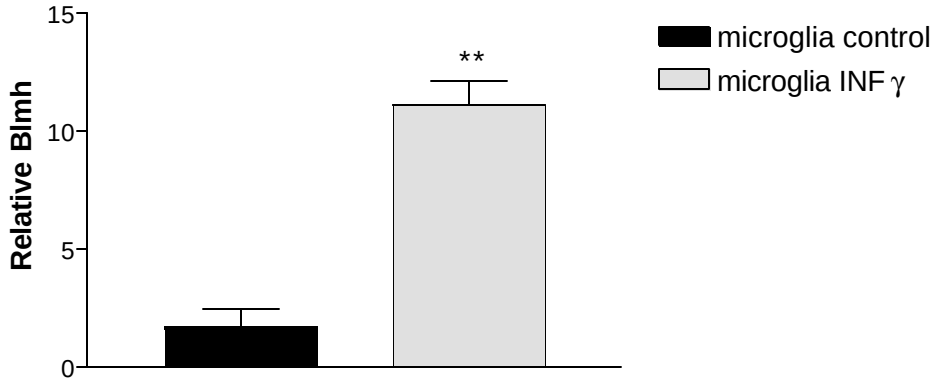
INFγ induction of the Blmh protein was evaluated in astrocytes and microglia. Rat primary astrocyte and microglial cultures were obtained and treated with 300 pg/ml recombinant rat INFγ. Cells were harvested 24 h after treatment and assayed by Western blot for levels of Blmh protein, β tubulin and LMP 7 as a positive control. LMP 7 is induced by INFγ as a component of the immunoproteasome and serves to indicate activity of the cytokine. Although the levels of Blmh were unaltered in the astrocytes ($P = 0.20$), evidence for induction of Blmh was seen in the rat primary microglial cultures ($P < 0.005$) (Figure 11A, B, D, E). LMP 7 induction in both primary astrocytes and microglial cells indicates active cytokine signaling in both cell lines. The microglial cell line EOC20 was obtained from ATCC and used to verify Blmh induction in microglia. Treatment of EOC20 cells with recombinant INFγ resulted in distinct induction of murine Blmh approximately 5 fold over 24 h ($P < 0.01$) (Figure 11 C, F).


control INFγ
A.
Bmh -
βTubulin -
LMP7 -
control INFγ
B.
Bmh -
βTubulin -
LMP7 -
control INFγ
C.
Bmh -
βTubulin -

D. ASTROCYTES



E. MICROGLIA



E. EOC20

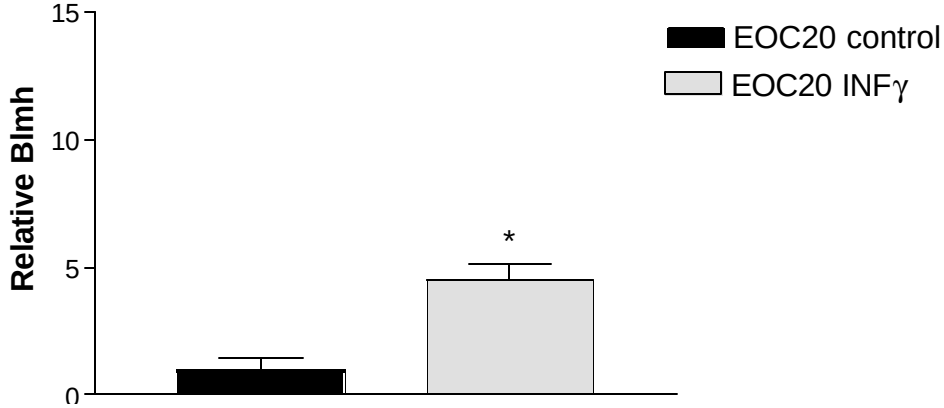
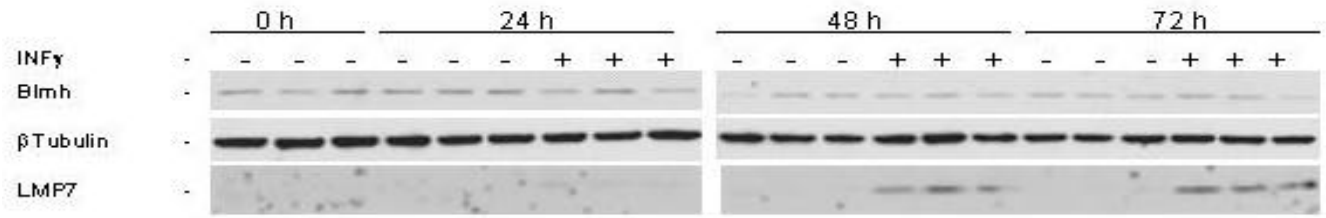


Figure 11 Blmh is induced in microglia by INFg

Primary astroglia, primary microglia and EOC20 murine microglial derived cells were either treated with recombinant INFγ or DMSO as a control and harvested 24 h after treatment. Cell lysates were separated on SDS PAGE, analyzed by Western blot for Blmh, β tubulin or LMP 7. Data shown are representative of 3-9 independent experiments; quantitation grafts represent mean ± SEM. A. Western blot analysis for primary astrocytes. B. Western blot analysis for primary microglia C. Western blot analysis for EOC20 microglial cells. D. Quatitation of astrocyte data, n = 3. E. Quantitation of microglial data, n = 3. F. Quantitation of EOC20 data n = 3. For quantitation, Blmh protein band densitometry was normalized to β-tubulin. *P < 0.01; ** P < 0.005.

Primary astrocytes and EOC20 cells were treated with INFγ and harvested at 0, 24, 48 and 72 h to determine if the kinetics of Blmh induction differed between the two cell lines. Over the time course of the experiment, LMP 7 was clearly induced in approximately ten fold in astroglia, but only very weakly induced in the EOC20 microglial cell line. Furthermore, Blmh was not induced over the 72 h time course in astroglia (Figure 12 A and B), but was induced nearly 10-20 fold in the microglial derived cells (Figure 13 A and B).

A.



B.

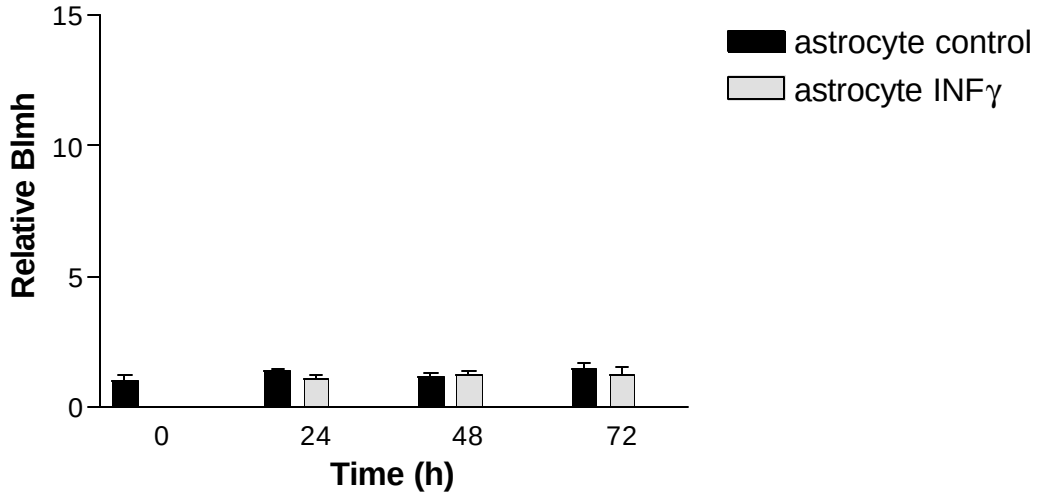
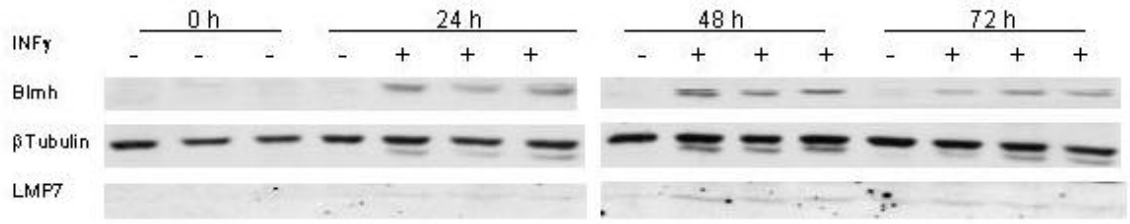


Figure 12 INFγ treatment does not result in induction of Blmh in astrocytes

Primary astrocytes were treated with recombinant murine INFγ and harvested after 24, 48 or 72 h. Cell lysates were separated by SDS PAGE and analyzed by Western blot for Blmh, β tubulin or LMP 7. A. Representative Western blot analysis of astrocyte cell lysates. B. Quantitation of data based on densitometry scan, n = 3; bars represent mean ± SEM. Blmh protein levels are unaffected by treatment with INFγ.

A.



B.

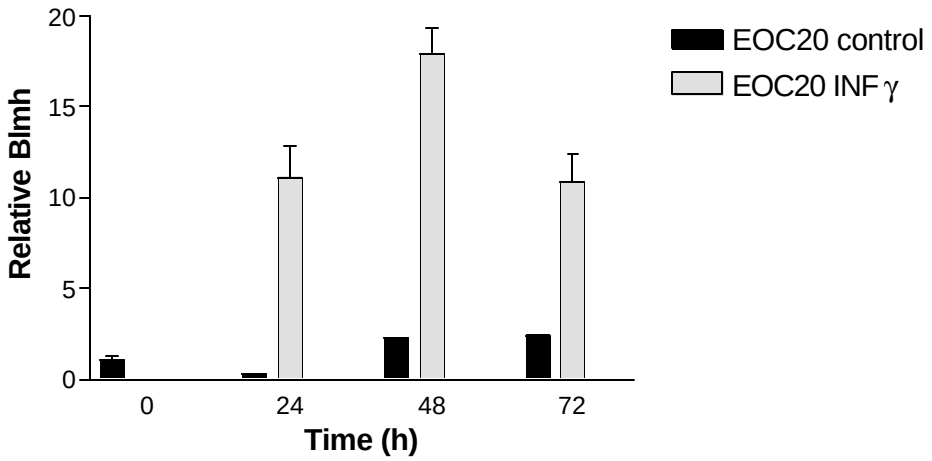


Figure 13 Blmh induction in EOC20 is time dependent

EOC20 cells were treated with recombinant murine INFγ and harvested after 24, 48 or 72 h. Cell lysates were separated by SDS PAGE and analyzed by Western blot for Blmh, β tubulin or LMP 7. A. Representative Western blot analysis of EOC20 cell lysates. B. Quantitation of data based on densitometry scan, n= 6; bars represent mean ± SEM.

5.2.3 Blmh induction by INFγ results in increase mRNA

EOC20 cells were treated with 300 pg/ml INFγ for 0, 6 or 16 h and then harvested for Western blotting to evaluate the early time course of Blmh induction. Induction of Blmh began to emerge as early as 6 h after treatment (Figure 14A). EOC20 cells were treated for 24 h with INFγ after which RNA and protein were harvested from parallel cultures and probed by Northern blot or Western blot for Blmh respectively. At 24 h, *Blmh* RNA levels were found to be substantially increased following treatment with INFγ (Figure 14B).

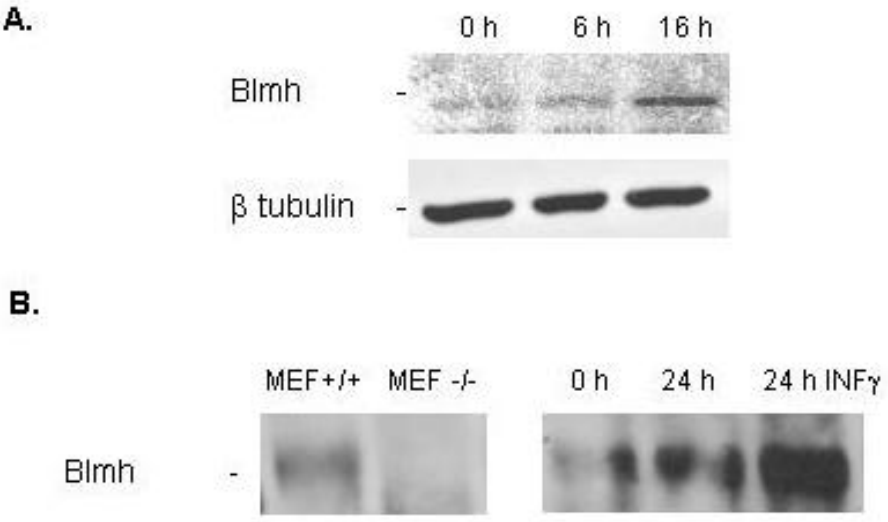


Figure 14 Increased *Blmh* mRNA in response to INFg in EOC20 cells

EOC20 cells were treated with recombinant murine INFγ and harvested at the time points indicated. Cell lysates were separated by SDS PAGE and analyzed by Western blot for Blmh, β tubulin or LMP 7 to determine early induction times. Induction of Blmh was seen as early as 6 h after INFγ treatment. A. Western blot analysis of EOC20 cell lysates. Total RNA was isolated from EOC20 cells before and after INFγ treatment and probed by Northern blot for *Blmh* mRNA with a 1365 bp DIG- labeled double stranded DNA probe. *Blmh* mRNA was increased following INFγ treatment B. Northern blot of total RNA isolated from EOC20 cells.

5.2.4 Evaluation of Blmh induction by TNFα

EOC20 cells were treated with 50 or 100 pg/ml recombinant murine TNFα alone or in combination with 300 pg/ml INFγ for 24 h to determine if Blmh is also induced by TNFα. Western blot analyses of the cell lysates confirmed prior results with INFγ and revealed that Blmh was not only induced by TNFα over 24 h, but that it was induced synergistically with INFγ (Figure 15).

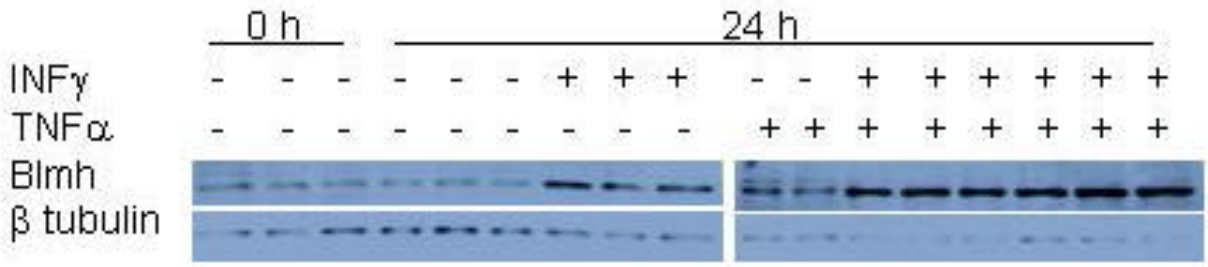


Figure 15 Blmh is induced by TNFa

EOC20 cells were treated for 24 h with INFγ, TNFα or both agents combined (50 pg/ml TNF α or 100 pg/ml TNF α). Cells lysates were separated by SDS PAGE and analyzed by Western blotting for Blmh and β tubulin.

5.2.5 Evaluation of Blmh induction by INFγ in the human HeLa cell line

Induction of the putative MHC I N-terminal epitope trimming aminopeptidase leucine aminopeptidase by INFγ has been shown in the human HeLa cell line (Beninga et al., 1998). HeLa cells were treated for 24 h with 500 pg/ml human recombinant INFγ to determine if BLMH was induced in this human cell line. Cell lysates were assayed by Western blot for induction of LMP 7 as a positive control. No induction of BLMH was observed over 24 h following INFγ treatment, although strong induction of LMP7 was observe. These resulta suggest a cell type specific function for BLMH in immune surveillence (Figure 16).

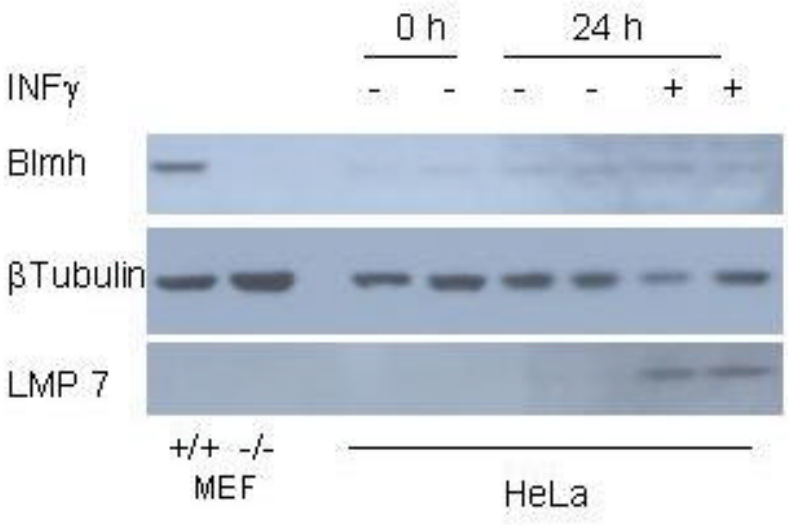


Figure 16 BLMH is not induced in HeLa by INFg

HeLa cells were treated for human recombinant INFγ for 24 h and then cell lysates were harvested and probed by Western blot for BLMH, β tubulin or LMP 7.

5.3 Conclusions

Blmh protein was found to be inducible by INFγ in primary rat microglia and the murine microglial derived cell line EOC20. Blmh was also induced by TNFα alone and synergistically in combination with INFγ in EOC20 cells. Treatment with INFγ also resulted in increased *Blmh* mRNA. Surprisingly, Blmh was not induced in rat primary astrocytes or in the human HeLa cell line although both cell types readily induced the LMP 7 immunoproteasome component. These results suggest a focal immune related function for BLMH that is perhaps limited only to antigen presenting cells (APC). Confirmation of these observations in vivo is important to accertain the physiological relevance of the observed cytokine response. Induction of BLMH in other APC such as macrophages and dendritic cells has not been tested; however, Blmh has been identified as an induced gene head kidney leukocytes from carp treated with Concanavalin A (Yin et al., 1999).

INFγ is a 45kD homodimeric glycosylated cytokine secreted by thymus derived cells and natural killer cells under pathological conditions (Boehm et al., 1997; Billau et al., 1998). INFγ mediates a number of diverse immune related responses including the structural modulation of the proteasome, induction of MHC II expression, and induction of expression of adhesion molecules such as ICAM 4 from endothelial cells (Boehm et al., 1997; Billau et al., 1998; Fruh et al., 1999). The cytokine acts primarily through a ubiquitous receptor and the janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway (Ramana et al., 2002; Kerr et al., 2003). Synergism between INFγ and TNFα is well documented and can

be mediated in part by cooperation between STAT1 and nuclear factor κB (NFκB) (Ohmori et al., 1997; Paludan et al., 2000; Wesemann et al., 2003).

In the normal brain, immune function is quiescent with minimal expression of MHC molecules (Havenith et al., 1998;Wekerle et al., 1986). In addition, the brain parachyma is separated from the blood circulation by the blood-brain barrier (Dermietzel, 1975) limiting access to most circulating antigen presenting cells. In early response to injury and infection, astrocytes and most notably microglia cells gain the capacity to present antigens via MHC molecules. MHC II antigen presentation is associated with the processing of extracellular antigens by proteases in the endolysosomal compartment (Honey and Rudensky, 2003) whereas MHC I molecule antigen presentation requires peptides generated by cytosolic proteolysis (Kessler et al., 2002).

Microglia are derived from monomyeloid lineage and function both in support of normal neuronal activities as well as possessing immunological competence (Streit et al., 2002). Activation of microglia results in conversion from immunologically quiescent to an active phagocytic antigen presenting cell with activity that is modulated by inhibitory factors intrinsic to the CNS (Dickson et al., 1991; Fontana et al., 1992; Streit et al., 2002). Although astrocytes are not immunologically competent, astrocytes, like microglia, respond to INFγ by the expression of MHC molecules as well as the production of proinflammatory cytokines such as TNFα and IL-1 (Chung et al., 1990; Neuman et al., 2001).

The results from this study further support the hypothesis that BLMH has specific biological functions in the CNS and that these functions may be related to a role in immune surveillance. In addition, these studies show for the first time, in mammalian cells, regulation of the cysteine protease BLMH.

6 DISCUSSION

6.1 Summary of conclusions

The studies presented in this thesis were designed to test the central hypothesis that BLMH has unique and important physiological functions in the CNS. Two different approaches were chosen to ask questions regarding the physiological involvement of BLMH in the CNS. First, the immunohistochemical profile of the brains and the behavioral phenotype of mice lacking *Blmh* were compared to wild type controls. Next, basal transcription of BLMH and potential avenues of regulation of BLMH were examined. The overall conclusions from the data presented in the thesis document are consistent with the hypothesis that BLMH has unique and important physiological functions in the CNS.

Immunohistochemical evaluation of the brains of the aged B6.129 $Blmh^{tm1Geh}$/J.animals showed global astrogliosis in null but not wild type animals. The presence of astrogliosis is suggestive of underlying neuronal injury or dysfunction. Although the etiology of the astrogliosis currently remains unknown, the lack of reactive gliosis characterized by the concurrent activation of microglia indicates the abcense of an inflammatory component to the pathology. Activation of astrocytes without microglial activation can occur by paracrine signaling from injured neurons through transforming growth factor alpha (Rabchevsky et al., 1998).

The behavioral studies examined spontaneous activity (open field test), locomotion and balance (rotarod test), pain perception (hot plate test) and learning and memory (contextual fear conditioning and water maze). No differences were observed between null and wild type

animals in the open field testing, but surprisingly, rotarod performance of aged null animals was significantly improved over wild type controls. The reasons for the observed results are unknown, but may reflect differences in attributes such as grip strength and balance that become more apparent with aging. Hot plate test results were significantly different between young null and wild type animals for front paw lick. Null animals showed increased latency to front paw lick and a trend towards increased latency in other parameters although not statistically significant. The relevance of this finding is unclear. Rotarod and hot plate testing results suggest that *Blmh* null animals may have subtle yet uncharacterized differences in functions relating to balance and coordination, and pain perception.

Young male and female null animals spent significantly less time in the target quadrant during water maze probe trials compared to wild type littermates. Although subtle differences in sensory function or locomotion cannot be excluded when evaluating these differences, open field testing, rotarod testing, visual platform trials during the water maze acquisition phase and water maze swim speed assessment indicate that no overt deficits in sensory or locomotor function exist in the null animals. No differences between null and wild type animals performance were observed in contextual fear conditioning paradigm, another test of learning and memory. The discrepancy between the results from the water maze trials and contextual fear conditioning may be due to biological differences underlying learning and memory in these paradigms or could reflect the more demanding conditions of the water maze paradigm.

The underlying reasons for the observed changes in water maze probe trial are unknown, but may indicate deficits in learning and memory consistent with a physiological function for Blmh in the brain. Analysis of CNS of the *Blmh* null mice may be confounded by redundancy in function of proteases, effects of flanking genes and unintended consequences on neighboring

genes. Recent strategies for overcoming some of these problems include backcrossing of hybrids to address the questions raised by linked genes (Wolfer et al., 2002), the removal of selection cassettes by flanking with lox P and conditional knockouts (Brockamp et al., 2002). The proximity of a serotonin transporter gene within 15 kb of human BLMH suggests a systenic localization in the mouse. Although the *Blmh* targeted deletion appears to have occurred as intended, disruption of the serotonin transporter could have obvious unintended consequences in the CNS and may merit further investigation.

Additional CNS characterization of the null animals was hindered by the lack of immunhistochemical localization of Blmh. Although the protein was found distributed throughout the CNS by immunoblot, the regional and cellular distribution of the protease in the CNS is not known. Two reports on BLMH immunohistochemistry in human brain suggest that protein is primarily neuronal, but those reports lack a definitive positive control (Raina et al., 1999; Namba et al., 1999). Attempts at immunohistochemical staining of mouse brains using the same antibodies from the published reports failed to reveal any significant signal from the tissue of wild type animals. The data from Blmh immunohistochemistry would permit further detailed characterization of the murine CNS. For example, one could ask if Blmh expression pattern is consistent with a role in hippocampal mediated learning and memory or, if regions with significant Blmh expression exhibit any neuronal loss or injury in null animals. Furthermore, the cellular distribution of Blmh may also be instructive in better understanding the observed astrogliosis.

The second part of the thesis focused on examination of basal and regulated transcription from the BLMH promoter. Basal transcription was found to be partially mediated by undefined elements in the proximal promoter and unaffected by the –134 bp trinucleotide repeat

polymorphism. More importantly, Blmh protein was shown to undergo significant induction in response to INFγ as well as TNFα in microglial cells. The Blmh induction by INFγ was accompanied by increased mRNA. Surprisingly, Blmh induction was not seen in astrocytes or in HeLa cells even though these cells clearly responded to INFγ by upregulating LMP7. These data suggest that focal inducible regulation of BLMH in microglial cells is a physiological response and further provide support for the notion that BLMH has unique functions in the CNS.

6.2 Future directions

The studies described herein represent only the beginning of an understanding of the physiological functions of the cysteine protease BLMH. The detailed characterization of expression pattern of the BLMH in the CNS and in other organs such as lung and skin, where the protease is believed to play important roles in normal physiology and disease, is imperative. The function of BLMH in the CNS cannot be discussed meaningfully without an understanding of the regional and cellular distribution of the protease.

In further characterization of the *Blmh* null mice, the presence of neuronal loss and injury should be carefully accessed as the underlying pathology for the observed astrogliosis. Additional exploration of the behavioral paradigms may also be fruitful. Assesment of grip strength and balance may help clarify the unsual results of the rotarod testing in which aged null animals outperformed their littermate counterparts. Results from the water maze paradigm can be confounded by subtle sensory and/or motor impairments. One strategy that has been employed to address possible visual impairment is to simplify the visual clues by use of black and white patterns (Lamberty and Gower, 1991; Robinson et al., 2001). Extention of the water maze

paradigm to reversal learning using a moving platform format (Chen et al., 2000) or assessment of retention by probe trial at longer time intervals following the learning phase may also be useful to magnify the small differences in performance already observed. Evaluation of CA 1 long term potentiation (LTP), which is an indicator of synaptic plasticity, could provide evidence for the decreased water maze probe trial performance being related to cognitive deficits. If BLMH is found to function in MHC class I epitope processing, its function may impact neuronal development and plasticity through peptide dependent signaling (Huh et al., 2000). Evidence for BLMH participation in peptide epitope processing in neurons awaits further investigation.

Although 5' untranslated regions of the members of the papain superfamily including *BLMH* lack classical promoter elements, transcriptional regulation may still provide an important means of temporal and spatial control for these proteases. Definition of the *BLMH* promoter will potentially require identification of additional 5' upstream regions from the 1.2 kb that has been cloned. Classical deletion studies with reporter constructs to determine the location of the putative functional promoter elements and gel shift assays to actually identify the transcription factors that bind to these elements will also be needed to define the promoter.

The present history of genetic association studies, particularly with focus on AD, raises questions on the usefulness of this type of analysis. Inconsistencies among these studies may be largely attributable to population stratification and heterogeneity (Bertram and Tanzi et al., 2001; Emahazion et al., 2001; Finckh, 2003). If this is indeed the case, then usage of the proper controls of disease unrelated SNPs for association studies coupled with linkage disequilibrium family and twin studies should allow for correction of the present problems (Kennedy et al, 2003). Genetic factors are believed to play a significant role in pathogenesis of late onset AD and at least four additional loci of magnitude similar or greater to apolipoprotein E are proposed

to exist (Warwick et al., 2000). The significance of the genetic association of *BLMH* with AD is unknown; population based molecular genetic studies that are properly controlled and aknowledge potential interactions between genes may provide some answers to this question.

BLMH may function in antigen presentation of MHC I molecules and consequently may have an important function in protection against viral infections, tumors, and may play a role in peptide mediated MHC I synaptic plasticity and neuronal development (Huh et al., 2000) as well as neurodegeneration (Piehl et al., 2001, Weiner and Selkoe, 2002). In AD, inflammation appears to contribute to the pathogenesis of the disease as illustrated by adminstration of ibuprofen in a transgenic mouse model of AD in which the anti-inflammatory agent resulted in significantly less pathology in treated animals compared to controls (Lim et al., 2000). Surprisingly, anti-inflammatory agents appear to act in part by altering the processing of APP though modulation of the beta-secretase (Sastre et al., 2003). Epidemiological evidence suggests a reduced risk for AD in patients using nonsteroidal anti-inflammatory drugs although effectiveness of non-steroidal use in treatment of the disease remains inconclusive (Aisen et al., 2002).

Basic characterization of the induction of BLMH by cytokines and its function in immune mediated processes should include assessment of BLMH RNA and protein in antigen presenting cells including macrophages, dendritic cells and B cells before and after cytokine stimulation. Once the cell populations in which the protease is induced are defined, other questions such as the activity profile of BLMH against MHC I aminopeptidase targeted peptides and the localization of BLMH can be addressed. If BLMH is involved in MHC epitope processing cytokine induction may result in association of a percentage of the cytoplamic protein with components of the MHC I epitope transport system at the ER surface. An appropriate

immunostaining protocol would need to be developed or subcellular fractionation assays utilized to assess potential changes in localization. Although classical signaling pathways for INFγ and TNFα mediated transcription are known, these pathways remain to be defined for these cytokines in BLMH induction. Finally, many important questions regarding immune function of BLMH in microglia in vivo may be effectively addressed utilizing the null mouse model, and in fact, several research groups with interest in MHC I epitope processing have requested and received breeder pairs for the B6.129Blmh^{tm1Geh}/J animals from our laboratory. Future research on the immune related functions should provide additional understanding of the biological functions of this unique cysteine protease and perhaps shed light on its proposed role in Alzheimer's disease.

APPENDIX A

Antibodies

Table 6 Antibody list

Antibody	Species	Epitope	Application	Dilution
Rat BLMH	Rabbit	Unknown	WB, IC	1:500
Human BLMH	Chicken	Unknown	WB	1:500
HSP70	Rabbit	Multiple	WB	1:1000
LMP7	Rabbit	Multiple	WB	1:1000
β tubulin	Mouse	Single	WB	1:2000
Vinculin	Rabbit	Multiple	WB	1:250

WB, Western blot; IC, immunohistochemistry; IP, immunoprecipitation;

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