“In the attitude of silence the soul finds the path in a clearer light, and what is elusive and deceptive resolves itself into crystal clearness. Our life is a long and arduous quest after Truth.”

Mahatma Gandhi, 1869-1948

University of Alberta

Interactions between Amyloid Precursor Protein and Prion Protein Impact Cell Adhesion and Apoptosis in the Developing Zebrafish

by

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A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Master of Science

in

Molecular Biology and Genetics

Biological Sciences

Fall 2011

Edmonton, Alberta

I would like to dedicate this works to my beautiful wife Amanda. Without your support, understanding, and love this work would never have been completed. I thank you from the bottom of my heart for helping me accomplish this goal.

ABSTRACT

We report here that knockdown of either amyloid precursor protein (APP) or prion protein (PrP) in the zebrafish produces an overt dose-dependent phenotype characterized by systemic morphological defects and CNS cell death. We report findings that the zebrafish *PrP-1* gene genetically interacts with one, but not the other, zebrafish APP gene paralog. The interaction proves specific to these genes and human and mouse mRNAs can rescue the observed phenotypes, highlighting conserved functions. We find apoptotic labeling increased and aggregating ability of cells decreased when either of these genes is substantially knocked down, or sub-effective knock down of each gene is combined. Using a cell mixing paradigm we further provide evidence that the interaction occurs in a cell autonomous fashion. Our study is the first to report an *in vivo* interaction between these two genes, which could prove invaluable to the design and screening of therapeutics.

ACKNOWLEDGEMENTS

I would like to thank my supervisor W. Ted Allison and all the members of the Allison lab past and present, your input and help with experiments proved invaluable. I would like to thank all the members of the Centre for Prion and Protein Folding Diseases, you provided a positive, energetic, and nurturing environment for which I am very grateful. I would like to thank my committee members Ted, Andrew and David, your guidance helped make this project an exciting and fruitful endeavour. I would like to thank my parents for always being there and keeping me on track when I strayed. Lastly, I would like to thank my finance Amanda, you love and support has made this whole thing possible.

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Abbreviations

AD: Alzheimer Disease

APP: Amyloid Precursor Protein

Aβ42: Amyloid Beta 42

Aβ: Amyloid Beta

BACE: Beta-site APP Cleaving Enzyme

PSEN1: Presenilin 1

PSEN2: Presenilin 2

Aph-1: Anterior Pharynx-Defective 1

ApoE: Apolipoprotein E

CLU: Clusterin

CR1: Complement Receptor 1

PCALM1: Phosphatidylinositol binding clathrin assembly protein 1

LTP: Long-Term Potentiation

KO: Knock-Out

APLP-1: Amyloid Precursor Like Protein 1

APLP-2: Amyloid Precursor Like Protein 2

EGF: Epidermal Growth Factor

sAPP: Soluble Amyloid Precursor Protein

AICD: Amyloid Precursor Protein Intracellular Domain

GFLD: Growth factor like domain

CuBD: Copper binding domain

KPI: Kunitz-type Protease Inhibitor

CAPPD: Central Amyloid Precursor Protein Domain

CNS: Central Nervous System

ADAM: A Disintegrin and Metalloproteinase

NCAM: Neural Cell Adhesion Molecule

NOGO-66: Neurite Outgrowth Inhibitor 66

JIP: JNK Interacting Protein

BIP: Binding Immunoglobulin Protein

HSP90: Heat Shock Protein 90

BPI: Bactericidal Permeability Increasing Protein

JNK: c-Jun N-Terminal Kinase

LRP: Lipoprotein Receptor Related Protein

CDK5: Cyclin Dependent Kinase 5

JNK3: c-Jun N-Terminal Kinase 3

GSK3B: Glycogen-Synthase Kinase B

ABL: Abelson murine leukemia viral oncogene homolog

PIN1: Peptidyl-Prolyl cis/trans Isomerase 1

Tip60: TAT Interactive Protein 60

UK: United Kingdom

CJD: Creutzfeldt-Jakob disease

GSS: Gerstmann-Straussler-Scheinker syndrome

FFI: Fatal Familial Insomnia

BSE: Bovine Spongiform Encephalopathy

CWD: Chronic Wasting Disease

PrP: Prion Protein

PrP^{c}: Cellular Prion Protein

PrP^{sc}: Scrapie Prion Protein

ORF: Open Reading Frame

GPI: Glycosylphosphatidylinositol

BAX: BCL-2 Associated X Protein

TNF-α: Tumor Necrosis Factor α

OR: Octapeptide Repeat

SOD1: Superoxide Dismutase 1

L1CAM: Neural Cell Adhesion Molecule L1

HSP60: Heat Shock Protein 60

CNS: Central Nervous System

RNA: Ribonucleic Acid

MO: Morpholino Oligonucleotide

RT-PCR: Reverse-Transcription Polymerase Chain Reaction

GFAP: Glial Fibrillary Acidic Protein

GFP: Green Fluorescent Protein

TSE: Transmissible Spongiform Encephalopathies

fAD: Familial Alzheimer Disease

DNA: Deoxyribonucleic Acid

cDNA: complementary DNA

mRNA: Messenger RNA

PBS: Phosphate Buffered Saline

RT: Room Termperature

NGS: Normal Goat Serum

SD: Standard Deviation

SE: Standard Error

RFP: Red Fluorescent Protein

ANOVA: Analysis of Variance

HSD: Honestly Significantly Different

HPF: Hours Post Fertilization

PCA: Protein-Fragment Complementation Assay

CHAPTER ONE

Etiology of Alzheimer Disease:

Alzheimer Disease (AD) first characterized by German psychiatrist and neuropathologist Alois Alzheimer in 1906 [1] has become an ever increasing problem in today's aging society. Increasing age is the number one risk factor for development of AD [2]. The prevalence of this slow, sporadic, and invariably fatal neurodegenerative disease was estimated to be 1.6% in 2000 both overall and in the 65–74 age group, with the rate increasing to 19% in the 75–84 group and to 42% in the greater than 84 age group [3]. In Canada alone there will be over a million people afflicted with AD by 2038 representing 1 out of approximately every 40 Canadians [4]. The estimated cumulative economic costs of this disease in Canada alone will top 800 billion dollar by 2038 [4] and in the USA the economic costs of Alzheimer disease may have already reached 100 billion dollars per year [5]. In light of these staggering numbers regarding the prevalence and economic costs of Alzheimer disease every effort must be made to increase our understanding of this disease and provide a means of creating therapeutics to halt or at least delay disease progression.

AD is clinically characterized by a sporadic onset followed by progressive decline in memory and other cognitive functions eventually leading to death of the patient [2]. At present definitive AD can only be diagnosed by the post-mortem presence of pathological hallmarks of AD; senile plaques, neurofibrillary tangles, and pronounced neuronal death within the brain [2, 6-8]. The disease is hypothesized to be initiated by a pathological aggregation of a small portion of the

endogenous Amyloid Precursor Protein (APP) termed amyloid beta 42 (Aβ42) [9-11]. Aβ peptides of 40 or 42 amino acids in length are endogenously formed through sequential cleavage of APP via beta and gamma secretases, the former an aspartic protease dubbed the beta-site APP cleaving enzyme (BACE) [12], the latter a multi-enzyme complex comprised of Presenilin 1 (PSEN1), Presenilin 2 (PSEN2), anterior pharynx-defective 1 (Aph-1), and Nicastrin [13-16]. The hypothesis that Aβ42 peptides specifically precipitate disease arose from observations that 5-10% of AD cases are familial and cosegregate with mutations in the *APP* [17], *PSEN1* [18], or *PSEN2* [19] genes that serve to increase either production of both Aβ peptides or increase the ratio of Aβ42 to Aβ40 peptides [17-21]. At present 23 missense mutations sufficient to cause AD have been identified in APP, all mutations to date are positioned in or near the Aβ coding exons (exons 16 and 17) and appear to influence APP cleavage [22, 23]. Large increases in Aβ production can be attributed to mutations near the β cleavage site [20], while mutations in amino acids 716 and 717 do not influence total Aβ concentration, they increase the proportion of Aβ42 to 40 [21]. To date 178 different mutations related to AD in have been identified in *PSEN1*, and 14 mutations detected in *PSEN2* [22]. *PSENs* have been shown to be critically involved in the γ-secretase dependent cleavage of APP [13]. Mutations in *PSENs* have been shown to disrupt the location preference of this proteolytic cleavage, resulting in an increased Aβ42/Aβ40 ratio by either increasing Aβ42 as shown in plasma and fibroblast media of *PSEN* mutation carriers [24, 25] or by decreasing Aβ40 levels [26, 27]. Yet further evidence for the involvement of APP in AD

comes from studies on persons afflicted with Down syndrome. Pathological hallmarks of AD are exceedingly common in a large percentage of all Down syndrome patients that survive past 35 years of age [28, 29]. This is believed to be due to the extra copy of chromosome 21 present in these patients, giving these patients 3 copies of the APP gene [30], and increased levels of Aβ42 [7, 29]. The strongest genetic risk factor for late onset or sporadic AD is variability in the Apoliprotein E (*ApoE)* allele thought to be responsible for proteolysis of Aβ42 aggregates [31-33]. The *ApoE2* allele appears protective against AD while the *ApoE4* allele increases risk for AD via a decreased ability to bind and catalyze the breakdown of amyloid [31, 33]. Recently, three novel risk genes have also been identified; Clusterin (*CLU)*, Complement Receptor 1 (*CR1)* and Phosphatidylinositol binding clathrin assembly protein 1 (*PICALM1)* although their exact roles in AD are unclear at this time [34, 35].

Hypothesized Functions of the Amyloid Precursor Protein:

Despite APP lying at the central position of a major neurodegenerative disease very little is known about the biological functions of the protein. Decreased neurite outgrowth and neuronal viability can be attributed to a loss of APP *in vitro* [36], while over-expression of APP promotes neuronal outgrowth [37]. Subtle phenotypes including smaller size, reactive gliosis, reduced forelimb grip strength and reduced locomotor activity have been observed when APP was completely knocked out in mice [38]. It was later shown that memory deficits, impairments in long-term potentiation (LTP), and reduced synaptic density were

also present in these knock-out (KO) mice [39]. The corollary in which injection of soluble APP was able to increase learning and memory in rats was also observed [40]. Systemic knock down of APP in adult mice using siRNA led to no overt phenotype, but mice displayed reduced scores on the Y-maze indicating an impairment of spatial working memory [41]. When intraocular injections of siRNA against APP were done a significant decrease in retinal ganglion cell synaptic activity occurred [42]. It was later shown that APP was involved in modulating synaptic transmission and was neuroprotective during ischemia and other excitotoxic injuries [43-45]. The lack of an overt or lethal physiological phenotype in mice was thought to be due to redundancies between APP and the two APP like proteins (APLP1/2) and in alignment with this notion mice lacking APP and APLP2 produce motorneuron axons that bypass their targets and do not form correct synapses [46]. Mice lacking all three homologues of APP (APP and APPL1/2) die prematurely and show a high number of over migrated neurons and cortical dysplasias similar to the pathology of cobblestone (type 2) lissencephaly [47]. Like mice, subtle behavioural phenotypes can be observed in APPL (homologue of APP) KO flies, but they remain fertile and viable [48]. Analysis of the neuromuscular junctions in these KO flies revealed neuronal abnormalities such as reduced synapse number [49], and reduced synapse bouton formation [50]. Disruptions in axonal transport can also be induced by either deletion or over-expression of APPL in flies [50, 51]. When dsRNA was used to down regulate the APP homologue Apl-1 in *C.elegans* they developed normally with the exception of disruption in pharyngeal pumping mimicking a phenotype due to

a lack of Amyloid beta A4 precursor protein-binding family B member 1 (Fe65) [52]. Recent work involving knocking down the zebrafish APP homologue *APPb* suggests that APP knock down leads to disruptions and a widening of collagen type II, alpha-1a (col2α1a) expression (labels the developing notochord) due to abberant convergent-extension movements in the developing embryo [53].

The Role of APP in Development:

APP possibly acts as a contact receptor in neuronal growth cones which may help facilitate neuronal growth [46]. During prenatal development APP is expressed at high levels [54-56] where it has been shown to act as a co-factor with Epidermal Growth Factor (EGF) to stimulate proliferation [57]. APP expression is also closely linked to regions that undergo high levels of synaptic formation and modification [58, 59]. It is further expressed in radial glial cells where it may play a role in guiding neurons to their proper locations [60]. Mice when raised in enriched environments show increased levels of APP and increased synaptic plasticity compared to mice raised in comparatively un-stimulating conditions providing evidence for APP's synaptogenic functions [61]. In mice models of ischemia and traumatic brain injury APP was quickly up-regulated and anterogradely transported to synaptic sites after insult where its levels were highly correlated with levels of synaptogenesis [62, 63]. Multiple lines of evidence point to APPs involvement in cellular adhesion to other cells, substrates, extracellular matrix components, and glial cells as well [64-69]. APP possibly facilitates this intercellular adhesion though formation of homodimers across cells or through

heterodimers using various other portions of the APP peptide such as the heparin sulphate proteoglycan binding region [68]. It is through this adhesion APP that is believed to regulate the proliferation of cells [46, 57, 70] and tissue morphogenesis [71] as APP has been shown to promote adhesion in migratory cells and play a neuritogenic role in stationary, immature neurons [36, 37, 65, 72-76]. During development APP may also be important in morphogenesis as an increase in the velocity of growth cone migration has been noted upon Fe65 binding to the cytoplasmic tail of APP [66].

The Structure of the Amyloid Precursor Protein:

The structure of the human APP protein may provide clues to functional properties of the protein and may help provide explanations for any deleterious effects noted when the APP protein is knocked down. In humans there exist three forms of APP; the shortest isoform of 695 amino acids (APP 695) is found mainly within neurons of the Central Nervous System (CNS) [78]. The two longer isoforms of 751 and 770 amino acids reside primarily in the periphery and contain a peptide region analogous to a Kunitz-type protease inhibitor (KPI domain) [79-81]. Structurally the APP protein can be divided into multiple domains with specific functions such as neurite outgrowth, cell adhesion, and synaptogenesis linked to individual domains. At the N terminal side of the protein lies the E1 domain comprised of the growth factor like domain (GFLD) and the copper binding domain (CuBD) [82] (Fig.1-1). The GFLD domain seems to be necessary for inducing neurite outgrowth and other growth-promoting properties ascribed to

APP [76]. The GFLD also contains a heparin sulfate proteoglycan binding domain that may facilitate APP's role in cell adhesion [83]. The CuBD, as its name implies, has been shown to bind copper ions, possibly contributing to metal homeostasis within the brain [84, 85]. C-terminal to the E1 domain is the E2 domain comprised of the RERMS sequence and the carbohydrate central APP domain (CAPPD) (Fig.1-1). The RERMS sequence is also likely involved in the neuronal growth promoting properties of APP [70, 86, 87]. The E2 domain as a whole has been shown to interact with matrix proteins and facilitates the formation of homodimers that possibly contribute to APP's role in cell adhesion [68]. Adjacent to the E2 domain are the linker and transmembrane regions of APP that house the pathologically infamous Aβ region of the protein (Fig.1-1). Apart from its role in Alzheimer Disease, Aβ has been shown to regulate ion channel function [88, 89] and possibly inhibit synaptic function [90](Fig.1-1). G-protein receptor signalling [91], kinase mediated signalling [92, 93], calcium signalling [94], gene transcription regulation [95, 96], and apoptotic [97-99] roles have all been ascribed to the most C-terminal portion of the APP protein (the APP intracellular domain or AICD) (Fig 1-1). An extremely well conserved YENPTY sequence within this domain has been shown to interact with Fe65, JIP, Mint, and may be influential in regulating Clathrin-mediated endocytosis [100, 101]. Overall, many cellular functions identified for extracellular portions of the APP protein like neurite outgrowth, synaptogenesis, and apoptosis have also been linked to intracellular domains, possibly indicating the protein facilitates the

transduction of extracellular signals to intracellular signalling pathways (rev in [82]).

Interactors with the Amyloid Precursor Protein:

Another route to examine the possible functions of the APP protein is to look at proteins that interact directly with APP. If proteins with known signalling roles are shown to physically interact with APP we can hypothesize that APP is also involved to some extent in those same signalling pathways. As expected, many of the proteins known to cleave APP such as BACE-1, and members of the A Disintegrin And Metalloproteinase (ADAM) (10/17) proteases were found to associate strongly with APP [102]. The ectodomain of APP has mainly been shown to interact with proteins that play a role in cell adhesion such as Heparin [103], Collagen [104], Laminin [105], Laminin receptor protein [102], F-spondin [106] and the extracellular protein Fibulin [107]. In spots of cellular adhesion and along the surface of axons immunohistochemistry shows APP also co-localizes and interacts with Integrins [108, 109]. Interactions with Neural Cell Adhesion Molecules (NCAMs) [110], Neurofascin [110], and Contactin [102, 110] round out APPs preponderance of interactions with adhesion molecules. Not only has binding of APP to F-spondin been implicated in cell adhesion it has been shown to subsequently influence the cleavage of APP and Aβ release [106]. Aβ processing and release may also be influenced via binding of APP to the Neurite Outgrowth Inhibitor (NOGO)-66 receptor [111]. There is evidence for APP associating with heat shock proteins like the Binding Immunoglobulin Protein

(BIP) [110], Heat Shock Protein 90 (HSP90) [112] and the promiscuous 14-3-3 protein [112]. Interestingly, Aβ itself (40 and 42) was also shown to bind to the N-terminal side of APP [113], this binding has subsequently been shown to influence both the dimerization state of APP and the neurotoxicity of Aβ peptides [114, 115]. There is evidence for the cytoplasmic or AICD of APP binding to numerous proteins including; Go [116], Bactericidal Permeability Increasing Protein (BPI) [117], Fe65 [118], Mint/ XII [118], JNK Interacting Protein 1 (JIP-1) [119], c-Jun N-Terminal Kinase (JNK) [101, 120], Shc/Grb2 [92, 93], and kinesin light chain [51, 121]. Once bound to APP, Fe65 may facilitate a link to the lipoprotein receptor related protein (LRP) which can then modulate APPs endocytic trafficking and proteolytic processing [122]. Multiple enzymes like Cyclin Dependent Kinase 5 (CDK5), JNK3, and Glycogen Synthase Kinase 3B (GSK3B) and the tyrosine kinase Abelson Murine Lukemia Viral Oncogene Homolog 1 (ABL) are able to phosphorylate APP at Thr668 [95, 123-126]. Phosphorylation at this residue seems to reduce the affinity APP has for Fe65 [126-128], but increases its affinity for Peptidyl-Prolyl cis/trans Isomerase 1 (PIN1) [129] an isomerase that modulates the interactions of phosphorylated proteins [130]. Indirect interactions have been observed for APP and Notch and Numb, APP overexpression leads to a parallel overexpression of Notch and the corollary phenotype seen when Notch alone is overexpressed [131]. Some propose that the AICD of APP actually acts as a transcription factor [95, 96] and can regulate the levels of itself (APP) [132], TAT Interactive Protein 60 (Tip60) [133], Neprilysin [134], (GSK3B) [99], and interestingly the prion protein (*Prnp*)

[135]. It seems illogical that the AICD of APP would act to stimulate both its production and degradation by neprilysin at the same time, but perhaps adaptor proteins function to regulate this interaction.

Etiology of Prion Diseases:

Prionopathies represent another class of slow, sporadic, progressive, and invariably fatal neurodegenerative diseases caused by a host encoded, endogenous protein. In humans prionopathies take the form of Creutzfeldt-Jakob disease (CJD), German-Straussler-Scheinker syndrome (GSS), Kuru, and fatal familial insomnia (FFI). Various animal species can also be infected with disease as evidenced by scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk, mink encephalopathy, and the rare feline spongiform encephalopathy in cats [136]. Due to the transmissible nature of prion diseases, with transmission between species possible [137, 138], detection of disease in domestic animal species can have huge implications economically. It is estimated that the total economic cost of the BSE epidemic in the UK was 4.2 billion pounds [139]. Likewise, when BSE was detected in Alberta, the Canadian cattle industry lost a reported 7 billion dollars [140]. More recently Canadian and American governments have become increasingly concerned about prion diseases as CWD has been observed spreading in deer and elk populations across the western United States and Canada [141, 142].

The first cases of human prion disease, Creutzfeldt-Jakob disease (CJD), were characterized by Creutzfeld and Jakob in the 1920s and set apart from other neurodegenerative diseases by their insidious onset, long incubation period, and pathological features of spongiform changes, astrogliosis, and substantial neuronal loss [143-145]. It was later found that during disease progression there was a pathological aggregation of an aberrant conformer of the endogenous cellular prion protein (PrP^{c}) termed PrP scrapie (PrP^{sc}), leading to the hypothesis that the prion protein was the central cause of the disease [146]. Similar to AD, support for the prion proteins role in disease came from investigations of familial forms of prionopathies. Mutations in the open reading frame (ORF) of the *Prnp* gene lead to familial forms of CJD which account for approximately 10-15% of all CJD cases [145]. Mutations in codons 178 [147], 183 [148] and 200 [149-151] all cosegregate with genetic CJD, with mutations in codons 102 [152], 105 [153-155], 117 [156, 157], and 198 [158-160] cosegreating with inherited forms of GSS. FFI was later shown to be attributable to a mutation in codon 178 (D178N) in the open reading frame of *Prnp* [161]. These mutations may act to destabilize the protein and making an aberrant conformer of PrP (PrP^{sc}) more likely as they are all clustered in the highly organized C-terminal globular domain [162, 163]. Codon 129 has also proved to be important in the development of prion disease as there is a strong correlation between homozygosity at codon 129 and susceptibility to sporadic forms of CJD [164]. Most CJD patients are homozygous for either methionine or valine at codon 129 [165], and this homozygosity is linked to a decreased age of disease onset [166]. It is hypothesized that

homozygosity at codon 129 facilitates the homodimerization of PrP peptides leading to an increased spreading of disease within the host [164]. To date, transmission of Prion disease from animal sources [167], somatic mutations of the *Prnp* gene, and spontaneous conversion of PrP^{c} into the aberrant conformation (PrP^{sc}) have all been hypothesized to be responsible for sporadic CJD [168-170]. To date no solid evidence has been presented that links sporadic CJD with transmission of PrP^{sc} from animal sources [171-174]. It is therefore possible that the prion protein naturally possesses a low propensity to misfold, thereby seeding disease within the host or somatic mutations in the *Prnp* gene gained throughout life may be responsible for the PrP^{c} to PrP^{sc} conversion and development of sporadic CJD [145].

Hypothesized Functions of the Prion Protein:

The cellular prion protein also occupies a central position in a major neurodegerative disease, yet its normal biological functions too remain relatively unclear. PrP^{c} is a glycophosphatidylinositol (GPI)-anchored extracellular membrane protein that has been shown abundant in neurons and glial cells of the developing and mature mammalian nervous system [175, 176]. Aside from expression data PrP^{c} may be linked to neurogenesis through evidence it positively regulates neuronal precursor proliferation [177, 178] and axonal growth [179]. Similar to *App* KO mice, *Prnp* KO mice display no overt phenotype, but myriad subtle phenotypes have been reported. PrP may be involved in sleep regulation as aside from the D178N mutation leading to FFI, deletion of the *Prnp* gene in mice

has been shown to disrupt their circadian rhythms and sleep wake cycles [180, 181]. Disruptions in energy metabolism have also been observed in *Prnp* KO mice; reduced numbers of mitochondria were present per cell and disruption in the physiology of the mitochondria have been noted [182]. PrP^c has been implicated in neuronal signal transduction and synaptogenesis as binding of Aβ oligomers to PrP has been shown to robustly decrease LTP and synaptic plasticity [183, 184]. An interaction between PrP^c and the NR2D subunits of glutamate receptors may also influence synaptic plasticity and signal transduction [185]. This interaction may be responsible for the increased sensitivity to seizure inducing drugs observed in *Prnp* KO animals [186, 187].

When cells derived from *Prnp* knock-out mice are challenged with serum deprivation they are more likely than PrP expressing cells to undergo apoptosis, suggesting PrP also plays an anti-apoptotic role [191]. Further evidence for this role comes from studies in which the pro-apoptotic molecules BCL-2 Associated X Protein (Bax) or Tumor Necrosis Factor (TNF-α) have been over-expressed in human neuronal cells, PrP^c co-expression was able to decreased the rate of apoptotic induction [192]. In rodent models of ischemia overexpression of PrP^c was shown to reduced infarct size [193] while in mouse models of ischemic brain injury *Prnp* knockout mice displayed significantly increased infarct volumes when compared with wild-type mice [194, 195]. These results are possibly due to enhanced levels of activated caspase-3 brought about by an impairment of the anti-apoptotic phosphatidylinositol 3-kinase/Akt pathway observed in these mice

[195, 196]. Arguing against PrPs anti-apoptotic role, one study found that over-expression of PrP^c actually transformed MCF-7 cells that were originally sensitive to apoptotic induction by TNF-α into cells resistant to TNF-α [197], hence PrP's role in cytoprotection remains open to debate.

Evidence for the formation of PrP^c homodimers and PrPs binding to adhesion molecules like NCAMs and E-cadherins implicate the protein in cell adhesion [188-190]. Recently, it has been shown in zebrafish that the prion homologue *PrP-1* plays a critical role in promoting cellular adhesion during embryonic development [190]. Knock down of *PrP-1* induced a failure of epiboly formation and development arrest at the shield stage [190]. Immunohistochemistry of these knock down fish showed that e-Cadherin localization was disrupted indicating *PrP-1* facilitates the proper localization of cell adhesion molecules during development [190].

Structure of the Prion Protein:

Much work has focused on elucidating the structure of the native prion protein and perhaps this structural information can give clues as to what the biological functions of this protein are. The prion protein is first translated into a large precursor protein that is proteolytically cleaved in the endoplasmic reticulum and golgi complex [200] (Fig.2A). Following cleavage of the 22 amino acid signal peptides PrP may be N-glycosylated and a GPI-anchor is attached [201]. The proteins mature form is around 208-209 amino acids in length and in

mammals the majority is exported to the cell surface [201] (Fig.2A). Aspargine residues are found at AAs 181 and 197 representing two places within the PrP sequence that glycans may be attached [202] (Fig.2A). The entire plethora of non-, mono-, and di-glycosylated forms of PrP^c have been observed in the normal human brain [203, 204]. Structurally the prion protein can be split into amino terminal and carboxy terminal domains [201]. Nuclear magnetic resonance analysis indicates the amino terminal domain of mature PrP^c is around 100 amino acids long and generally adopts a flexible, random coil sequence [205](Fig. 2B). The carboxy terminal domain of PrP is also around 100 amino acids long, but structurally adopts a globular conformation [199](Fig. 2B). The first region within the amino terminal domain is the octapeptide repeat (OR) region where repeating sequences of PHGGSWGQ have been shown to bind copper and other divalent metal ions [206, 207]. Changes in the number of these repeat regions within PrP^c can drastically alter the infective and neurodegenerative properties of PrP^c, an increasing number of repeats drastically decreases the age of onset and increases the severity of disease [208]. C terminal to the OR is the hydrophobic region. This region may be important in conferring PrPs anti-apoptotic functions as well as modulating its interactions with SOD1 [209]. Development of GSS is well correlated with mutations within this region that may disrupt these protective functions [152-154]. The entire N-terminal region is bordered by two positive charge clusters termed charge cluster 1 (CC1) and charge cluster 2 (CC2) [145] that possibly play a role in internalization and recycling of PrP^c from the cell surface [210, 211].

The carboxy domain of mouse PrP^C is arranged in three alpha helices that correspond to amino acids 144–154 (H1), 173–194 (H2), and 200–228 (H3) [199](Fig. 2B). β-strands formed by amino acids 128-131 and 161-164 create antiparallel β-sheets that intersperse these helices [199]. Linking helices H2 and H3 together is a single disulfide bond formed between cysteine residues 179 and 214 [199](Fig 2B). Lastly, amino acids 229-230 form a short flexible carboxy-terminal domain [212]. Four mutations associated with familiar prion diseases are in the hydrophobic core of this region: V180I, F198S, V203I and V210I [163]. These mutations may aid the development of familial prion disease by decreasing the stability of PrP^c and allowing misfolding into the pathogenic PrP^{sc} isoform more likely [163]. The structure of the globular half of human PrP^C is common to various other mammals, as expected from a high degree of sequence identity [213-215]. Although the size and identity of the PrP nucleotide sequence in non-mammalian species can vary considerably between species the major structural features of the protein remain incredibly conserved [216].

Interactors with the Prion Protein:

A number of interactome studies have been completed against the endogenous prion protein [217-219] with a few common "groups" of interactors found. We can hypothesize that if many proteins from within a similar group interact with the prion protein that PrP is somehow involved in these processes. Multiple cell adhesion proteins have been shown to interact with PrP, including; NCAMs [189, 217], Neural Cell Adhesion Molecule L1 (L1CAMs) [217, 218],

Laminin [201, 220], Laminin receptor precursor (LRP) [218, 221], Heparin sulphate proteoglycans [201], and Contactin [217]. Heat shock proteins such as BIP [201, 217], HSP90 [218], and Heat Shock Protein 60 (HSP60) [201] are also shown to associate with PrP^c. Cleavage proteins such as members of the ADAM [217] family of proteases and BACE-1 [222] have been shown to interact physically with PrP^c. PrP^c was subsequently shown to actually decrease BACE-1 activity on APP [222]. Interactions with Vitronectin may influence PrPs ability to increase neuronal precursor proliferation and axonal growth [179]. A well characterized interaction with the NR2D subunit of glutamate receptors may further facilitate PrPs ability to influence synaptic plasticity and transmission [185]. Evidence for PrPs anti-apototic role comes from identification of its interaction with the Bcl-2 family of proteins where it was shown to inhibit apoptosis [223]. PrP has also been shown to associate with and activate Super Oxide Dismutase 1 (SOD1) conferring increased oxidative protection to cells [209].

Evidence for an Interaction Between APP and PrP:

APP and PrP are both membrane bound proteins; APP via its transmembrane domain and PrP through its GPI anchor. The expression of both APP and PrP is up-regulated during neuronal development and well correlated with levels of synaptogenesis and neurogenesis [54, 58-60, 175, 176], indicating both proteins play a role in growth promotion. In adulthood the expression of both proteins overlaps to a large degree as each is widely expressed throughout neurons and glia

of the brain [54, 58, 175] and each protein is particularly concentrated at synapses [45, 224]. There is evidence for both proteins involvement in neuronal maintenance, as they protect neurons from apoptosis [43, 45, 194-196, 225]. Structurally and functionally it seems that both proteins have a role in metal homeostasis within the brain; APP binds metal ions though its CuBD while PrP has a well-defined octarepeat region shown to bind copper and other metal ions [77, 206, 207]. Interactome studies implicate the proteins not only in the neuronal maintenance mentioned above but also in cell adhesion. NCAMs [110, 189, 219], Heparin sulphates [102, 201], Laminin [102, 145], Laminin receptor protein [102, 217, 218], Fe65 [112, 217], and Contactin [102, 110, 217] are all shown to bind both proteins. Cell adhesion by these proteins may also come about from their propensity to form homodimers, if formed between cells they could serve to facilitate neuronal adhesion during development [68, 188]. Interactome studies further indicate that APP and PrP may actually physically associate themselves [110, 217, 219].

Why the Zebrafish Model Organism:

To examine the in vivo roles of the APP and PrP proteins and any possible interactions between them we turned to the zebrafish model organism. The zebrafish has become a powerful model for genetic investigations. On a daily basis zebrafish produce a large number of transparent embryos that rapidly develop an intact vertebrate neural architecture. These embryos develop external to the mother allowing for isolation and manipulation of individual embryos

within each brood. The large brood size, rapid development of the CNS and relative ease of gene knock down allows for very large scale screening of disruptions in physiology brought about by genetic manipulations. The high identity found between many zebrafish proteins and proteins of higher mammals further makes the zebrafish an excellent candidate to probe the in vivo function of many genes.

APP and PrP in the Zebrafish:

Zebrafish possess two copies of the APP gene termed *APPa* and *APPb*. Both proteins show ~70% identity to each other and ~70% identity to human APP, this identity increases to ~90-100% in the transmembrane and intracellular regions of the protein [226]. Defects in synaptic bouton formation and disrupted convergent-extension movement phenotypes observed in flies and zebrafish as a result of APP(L/b) knock down are able to be partially rescued by injection of human APP mRNA indicating a high level of conservation between the species [48, 53]. Prior zebrafish expression experiments show that both APP mRNAs are expressed throughout the CNS from very early time points (2.5hpf) and this expression continues as the fish mature [226]. Recent studies by Joshi *et al* (2010) have shown that knockdown of the *APPb* gene produces a phenotype in the zebrafish comprised of disruptions in notochord development and symmetry indicating aberrant convergent-extension movements. Bioinformatics, expression analysis, and these recently characterized knockdown results suggest that the zebrafish is an excellent candidate for *App* down-regulation experiments.

Zebrafish also possess two homologues of the mammalian prion protein termed *PrP-1* and *PrP-2*. Despite their expanded size both PrP homologues display an impressive conservation of all features previously described for the human and mouse prion proteins [227]. Amino acids 1-23 correlate to a putative signal peptide, and amino acids 48-332 contain a long stretch of repeats analogous to the OR domain [227]. A central hydrophobic domain lies within amino acids 379-395, and two cysteine residues capable of forming an intramolecular disulfide bond reside at residues 463 and 554 [227]. Putative N-glycosylation sites can be found on two asparagine residues (amino acids 367 and 445), and it has been shown that N-glycosylation indeed occurs on zebrafish prion proteins [227, 228]. As assessed via RNA in situ hybridization ubiquitous distribution of *PrP-1* transcripts can be observed as early as midblastula stages (2.5hpf) [190] and continue throughout early embryonic development [190]. Conversely, *PrP-2* transcripts remain relatively low during embryonic development, then become prominent at later developmental time points [190]. Based on bioinformatics, expression analysis and work of Malaga-Trillo *et al* (2009) it is clear that the zebrafish is also an excellent model in which to study down regulation of the *PrP-1* gene.

Techniques used in the Zebrafish:

In the current study, we investigated the *in vivo* roles of each *App* gene in zebrafish using morpholino oligonucleotide (MO) technology to specifically

knock down the *App* and *PrP* genes from the one cell stage of zebrafish development (Fig 1-3a). We chose to design splice-blocking MOs against the zebrafish *APPa* and *APPb* genes so we could easily test their efficacy via RT-PCR (Fig1-3b). Against the *PrP-1* gene we employed a previously published and validated translation blocking MO [190] (Fig 1-3a). We initially quantified the effects of gene knockdown based on visual inspection of the physiology of the fish and quantification of the apoptotic levels with the different gene knock downs. Concurrent to *App* knock down we knocked down levels other genes believed to interact in some physical or functional way with *App*, namely *PrP-1*. Aside from the inherent advantages of using zebrafish (transparency, speed of development, ease of genetic manipulation) one major advantage to this morpholino based system stems from the fact that we are able to titrate levels of translation disruption, effectively modulating levels of each given gene product within the fish. This allows for gene levels to be knocked down to levels that by themselves do not induce any overt phenotype. By combining this sub-effective knock down with sub-effective knock down of genes hypothesized to interact with our gene of interest, we can conclude functional interactions occur when sub-effective doses of two morpholinos "synergize" to produce a phenotype, much like creating a compound heterozygous mouse. Quantification of this interaction was based initially on visual inspection of the physiology of the fish and quantification of apoptotic induction. We then examined to what extent knock down of these genes impacts cell adhesion using a well characterized aggregation assay [190].

To probe if APP and PrP are interacting across cells we will employ a variant of the aggregation assay mentioned above, the cell mixing assay [229]. Cells from different treatments will be mixed together and the ability of those cells to interact and adhere to one another will be assessed. The assay will be performed on zebrafish cells in which APP and PrP levels have been reduced, over-expressed via injection of mRNAs, or human APP and mouse PrP have been over-expressed.

Hypothesis:

In summation, the field has shown that knockout of the amyloid precursor protein in mice has minimal physiological repercussions [38]. Mild phenotypes have been reported that suggest APP has some role in neuronal maintenance [36], synaptogenesis [45, 49, 50, 54, 61], neurogenesis [56, 57, 230], metal homeostasis [84], and cell adhesion [67-69]. APP has been shown to interact physically with a number of proteins involved in processes like neuronal growth, synaptogenesis, and cell adhesion, strengthening the hypothesis that these are the proteins normal functions [102, 110, 112]. In zebrafish it has been shown that knockdown of the *APPb* gene within the fish has deleterious consequences in development as shown by disrupted convergent-extension movements [53].

Not unlike APP, knockout of the prion protein in mice was shown to produce no substantial physiological phenotype. The mice develop normally, but did

display subtle signs of neuronal perturbation implicating the prion protein in apoptotic protection [192, 223], neurogenesis [177, 231], and cellular adhesion [189, 190, 218]. Structural analysis further indicates PrPs role in cell adhesion and implicates the protein in metal homoestasis [206]. Multiple cell adhesion molecules are shown to interact with PrP along with molecules involved in neuronal protection [145, 189, 217, 218]. In zebrafish knockdown of the *PrP-1* gene resulted in severe defects in cellular adhesion and an overt phenotype characterized by developmental arrest at the shield stage [190].

Based on interactome studies showcasing interactors common to both APP and PrP and the similar functions of; neurogenesis, apoptotic protection, metal binding, and cell adhesion ascribed to both proteins we formulated the hypothesis that these two proteins lie within a common genetic pathway. Analysis of interactomes highlighting cell adhesion molecules as common interactors [102, 110, 217, 218], that PrP and APP may physically interact [110, 217, 219], and recent reports of high affinity binding of Aβ42 to PrP [183, 184, 232, 233] furthered our hypothesis to include that not only do APP and PrP lie in a common genetic pathway but these proteins interact in a non-cell autonomous fashion to mediate cell adhesion during development.

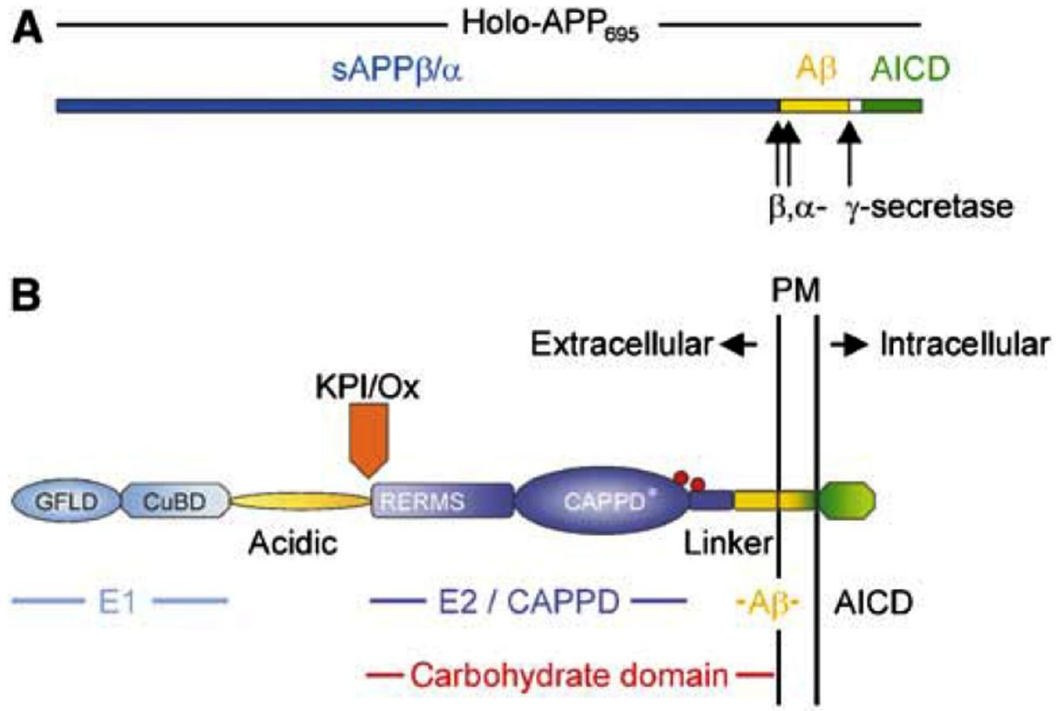

Figure 1-1: The Structure of the Amyloid Precursor Protein. Adapted from Reinhard *et al* (2005) [77].

A) Representation of the human APP695 protein. Proteolysis at the α or β secretase cleavage sites releases the soluble APP fraction (sAPP). Further processing by the γ-secretase complex releases the amyloid precursor protein intracellular domain (AICD) and the amyloid beta (Aβ) portions of the protein.

B) Different domains of APP. From the N-terminal side of the protein the E1 region is comprised of the growth factor-like domain (GFLD) and the copper binding domain (CuBD). The acidic domain links this E1 region to the more carboxy terminal carbohydrate domain and provides a site where the Kunitz-type protease inhibitor domain (KPI) is inserted in the longer forms of APP (751 and 770). An Ox2 sequence is also inserted here in the longest (770aa) isoform of APP. The carbohydrate domain can be further subdivided into the E2 region, also known as the central APP domain (CAPPD), which contains the RERMS sequence, and another linker domain. The transmembrane and intracellular domains of APP follow this linker domain. The Aβ portion of APP is contained within the linker and transmembrane domains.

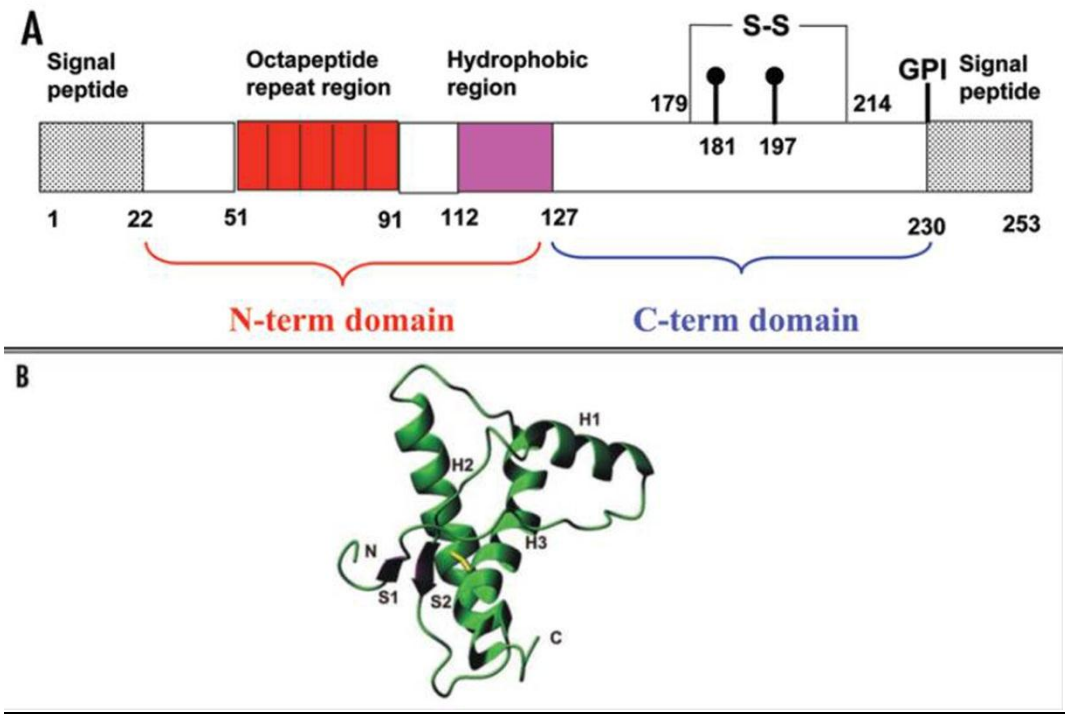

Figure 1-2. The Structure of the Prion Protein. Adapted from Pastore and Zagari (2007) [198], and Reik *et al* (1996) [199].

A) Schematic of the human PrP sequence. Within the N-terminal domain a signal sequence comprises amino acids 1-22, an octarepeat region is contained within amino acids 51-91, and a hydrophobic region lies within amino acids 112-127. Contained within the more C-terminal domain are the glycosylation sites as indicated by Asn181 and Asn197, and the cysteine bridge as Cys179 is covalently bound to Cys214.

(B) Ribbon diagram of the secondary structure of the C-terminal domain of mouse PrP. N and C represent the N and C terminus, H denotes Helices, and S1 and S2 label the antiparallel β-sheets.

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CHAPTER TWO*

*This Chapter in its entirety has been prepared for submission to PNAS under the title: "Interactions between Amyloid Precursor Protein and Prion Protein During Cell Adhesion and CNS Development" Authors: Darcy M Kaiser, Hao Wang, W.Ted Allison.

INTRODUCTION:

APP is a highly conserved type I transmembrane protein that liberates Aβ peptides into the extracellular space when it is sequentially cleaved by β- and γ-secretases [1]. These Aβ peptides can aggregate into amyloids and are thought to be the initiating pathological event in Alzheimer Disease [1, 2]. Cellular prion protein (PrP^{C}) is a conserved GPI- anchored membrane protein that, when misfolded into an aberrant conformation (PrP^{Sc}), is able to recruit and convert normal PrP proteins, thus initiating the pathological events in Creutzfeldt-Jakob disease, Scrapie, and Bovine Spongiform Encephalopathy [3].

Intense interest has recently focused on a high-affinity interaction between PrP^{C} and oligomerized Aβ [4-6], though the physiological effects of this interaction on synaptogenesis and long-term potentiation have been controversial. Considering the possible biochemical interaction between APP holoprotein and PrP^{C} suggested via high-throughput interactomics [7-9], and striking parallels in their cellular locations [10, 11] and functions [12, 13], we hypothesized that APP and PrP may be in a common genetic pathway. We examined the functional significance and conservation of this hypothetical interaction via concerted disruption of these genes in zebrafish. Conservation of interactions implicates a fundamental biological importance, and the tractable gene manipulation and accessible development of the zebrafish system allows insights into genetic interactions that can prove influential in the design of therapeutics.

Zebrafish has two paralogs of APP and two paralogs of PrP. Zebrafish APP paralogs show ~70% identity to human APP, escalating to ~90-100% identity in the regions that encode amyloidogenic, transmembrane and intracellular domains [10]. The two zebrafish paralogs of PrP share a striking degree of conservation of protein domains with mammalian PrP, though they differ in overall length [11]. The ability of mammalian homologues to replace zebrafish APP or PrP genes argues for an impressive conservation of function [14, 15].

We show here that *APPa, APPb* or *PrP-1* knockdown causes a phenotype early in CNS development, characterized by apoptosis and disrupted cell adhesion. *APPa* and *APPb* are able to replace each other, appearing functionally redundant during zebrafish development. *APPa* shows a genetic interaction with *PrP-1*, whereas *APPb* does not. Similarly, *APPa* interacts with *PrP-1*, but not with *PrP-2*. Cell death and disrupted cell adhesion are evident outcomes of the *APPa* and *PrP-1* genetic interaction. Finally, our results demonstrate that mRNAs encoding mouse PrP or human APP are able to replace their zebrafish orthologs in the cellular processes that require this APP-PrP interaction. These conserved interactions are of great interest because the mechanisms that transduce APP and/or PrP dysfunction are attractive as therapeutic targets in various neurodegenerative diseases.

RESULTS:

***APPa, APPb, or PrP-1* knock down in zebrafish produces morphological defects and CNS cell death**. *APPa* mRNA splicing was disrupted in wild type zebrafish by injecting a splice blocking morpholino (MO) designed to bind the exon-intron boundary between exon 2 and intron 2-3 (Fig.2-12A). For *APPb* the site chosen was the exon intron boundary between exon 3 and intron 3-4(Fig.2-12A). These sites lie immediately 5' to a highly conserved region of the genes that are included in all splice forms. Following determination of an effective MO dose, ascertained via a dose response graph as seen in Fig. 2-1A and represents a dose that led to more than 50% of fish displaying a phenotype (CNS cell death). We confirmed by RT-PCR that the *APPa* MO led to retention of intron 2-3 and sequencing revealed this intron contained 5 in-frame stop codons (Fig. 2-1B) (Fig.2-12B). The larger PCR product present within the gel likely represents a non-specific product, but sequencing of this band has not been carried out. Likewise, RT-PCR of *APPb* MO injected fish showed retention of intron 3-4 in mature mRNA and sequencing of this mRNA revealed the presence of 3 in frame stop codons (Fig. 2-2B)(Fig.2-12B). Delivery of *APPa* or *APPb* MO at high doses resulted in fish displaying a neurodevelopmental phenotype comprised of overt physiological malformations; cranial edema, reduced body size, improper CNS development and structure, and the presence of apoptotic CNS cell death (Fig. 2-3C,E,2-5). During the initial screen fish showing morphological deficits along with visually apparent cell death at 24hpf were then classified as

"necrotic/malformed" (Fig. 2-3C,E, 2-1A). At low *APPa* MO doses of 0.5ng and 0.75ng a small percentage (~30-40%, N=3 trials, >50fish/treatment/trial, 250 fish total) of fish displayed a mild phenotype consisting of slight body and CNS malformations, whereas at a dose of 1.0ng, 86% (N=8 trials, >25 fish/treatment/trial, 450 total) of living fish displayed a phenotype of body and CNS malformations, with the majority of those also displaying overt CNS cell death (Fig. 2-3C, 2-A).

Fish injected with increasing doses of MO against *APPb* showed an increased occurrence of a neurodevelopmental phenotype that closely mimicked the phenotype apparent from *APPa* knock down (Fig. 2-3C,G). Fish injected with low doses of the *APPb* MO (1ng) showed no apparent phenotype, while at the "effective dose" of 2.5ng the percentage of fish displaying a malformed CNS with cell death rose to 73% (N=6 trials, >25fish/treatment/trial, 375 total)) (Fig. 2-2). For the remainder of the APP MO injection experiments fish that were morphologically disrupted but had no detectable cell death were classified as "mild" and fish that were both morphologically disrupted and had obvious cell death were classified as "severe" (Fig. 2-3). To further confirm specificity of the *APPa* and *APPb* MOs, fish were co-injected with 200pg of cognate zebrafish mRNA. Injection of *APPa* mRNA alone did not cause any significant phenotype (Fig. 2-1C, N=4 trials, >25fish/treatment/trial, 127 total) while co-injection of *APPa* mRNA along with the *APPa* MO significantly increased the percentage of "normal" fish compared to those that only received the *APPa* MO (79±12% vs

24±24%, N=4 trials, >25fish/treatment/trial, 150 total, P<0.05) (Fig. 2-1). The percentage of fish that displayed a "severe" phenotype was also significantly reduced to 8±12% (N=4 trials, >25fish/treatment/trial, 150 total. P<0.05) in fish receiving both the *APPa* MO and mRNA opposed to 45±45% (N=4 trials, >25fish/treatment/trial, 151 total) of those that received the *APPa* MO only (Fig. 2-1C). Co-injection of *APPb* mRNA also significantly increased the percentage of "normal" fish compared to those that only received the *APPb* MO (99±4% vs 3±1% N=3 trials, >25fish/treatment/trial, 109 total, P<0.01) (Fig. 2-2C). The percentage of fish that displayed a "severe" phenotype was also significantly reduced in fish receiving both the *APPb* MO and mRNA opposed to those that received the *APPb* MO only (0±2% vs 96±3% N=3 trials, >25fish/treatment/trial, 109 total, P<0.01) (Fig. 2-2C). Apart from the disruptions in early peripheral and CNS development due *APPa* or *APPb* MO injection, disruptions in vasculature and overall body size became apparent at later time points (Fig. 2-4). At 3dpf blood was observed pooling in the heads in a small number of fish: when total body length was examined at 7dpf fish were noted to be 35-60% (N=5) smaller than fish injected with the standard control MO (Fig. 2-4). The reduced body size mimics post-embryonic effects seen in mice when APP protein is knocked out.

To expand on prior work by Magala-Trillo *et al* (2009) indicating knock down of the zebrafish *PrP-1* gene resulted in a developmental phenotype we used an identical translation blocking MO and followed fish for 24hpf [5]. We observed a phenotype similar to that observed with our APP MO's (Fig. 2-3K)

Fish injected with the low dose (0.5ng) of *PrP-1* MO presented with a delay in development, some slight CNS malformations, and at higher doses (1ng) began to show signs of apoptotic cell death (Fig. 2-3K,2-5).

***APPa* and *APPb* are redundant in early development**. The *APPa* and *APPb* proteins share a high degree of identity (~70%) and overlap in expression [6]. To test the hypothesis that *APPa* and *APPb* are redundant, doses of MOs that disrupt splicing of *APPa* and *APPb* were reduced to levels that produced little observable phenotype (sub-effective doses; 0.5ng *APPa*, 1.0ng *APPb*) (Fig. 2-6B,C). When these subeffective doses of *APPa* and *APPb* were co-injected there was a significant increase in the percentage of fish displaying a severe phenotype including both morphological malformations and CNS cell death to 73±37% vs 7±3% and 3±5% (N=3 trials, >20fish/treatment/trial, 140 total) when either MO was injected alone (N=3 trials, >25fish/treatment/trial, 307 total) (Fig. 2-6D,E). mRNA from one paralog was shown to rescue the phenotype caused by knock down of the other paralog ($P<0.05$), thus *APPa* mRNA was able to effectively rescue the phenotype caused by a knock down of the *APPb* mRNA and *vice versa* (Fig. 2-6F,G).

***APPa, APPb,* or *PrP-1* knock down in zebrafish leads to an increased activation of Caspase-3**. APP and PrP have been hypothesized to have anti-apoptotic roles (reviewed in [9], [8]). The cell death in fish injected with the *APPa*, *APPb*, or *PrP-1* MOs strengthened this hypothesis (Fig. 2-3). To probe the

nature of the cell death in more detail, fish were fixed and stained with an anti-activated caspase 3 antibody (Fig. 2-5). Prominent labeling was apparent in both the periphery and the CNS, especially in the mid and forebrain regions, of fish injected with effective doses of *APPa*, *APPb*, and *PrP-1* MOs. To quantify the staining five fish from each experimental group were staged and the number of caspase-3 positive cells above the yolk sac extension of the fish counted as per Aamar and Dawid (2008) [20] (Fig. 2-5). Fish injected with the *APPa* MO showed a significant ($P<0.05$) increase in the average number of caspase positive cells (135.2±33, N=5) as did fish injected with the *APPb* or *PrP-1* MOs (228±41 and 78±50 respectively, N=5, $P<0.05$) (Fig. 2-5I) when compared to control injected fish (23.2±10, N=5) (Fig. 2-5A,I). Caspase staining was also performed and counted when sub-effective doses of the *APPa* and *APPb* morpholinos were combined and, again, an increase in activated caspase 3 labeling was observed compared to control fish (data not shown).

***APPa* genetically interacts with *PrP-1*, but *APPb* does not.** Based on the similarity between the *APPa, APPb* and *PrP-1* knockdown phenotypes and prior reports in the literature indicating that APP and PrP physically interact [10-12] (see Introduction) we tested the hypothesis that there are functional redundancies or genetic interactions between APP and PrP. When sub-effective doses of the *APPa* (0.5ng) and *PrP-1* (0.5ng) MOs were injected alone a number of fish displayed morphological malformations (63±38%, 8±7% respectively, N=4 trials, >25fish/treatment/trial, 262 total), but only 2±3% displayed any signs of CNS cell

death (Fig. 2-7A-E). When these were co-injected there was a significant ($P<0.01$) increase in the percentage of fish displaying both peripheral and CNS malformations along with cell death (99±3%, N=4 trials, >25fish/treatment/trial, 235 total) (Fig. 2-7A-E). Anti-activated caspase-3 labeling and counting further confirmed that apoptotic cell death was significantly ($P<0.01$) increased in fish injected with a combination of sub-effective doses of the *APPa* and *PrP-1* MOs (Fig. 2-8). Due to the redundancies between *APPa* and *APPb* identified above, a functional interaction study was also conducted with *PrP-1* and *APPb*. Sub-effective doses of the MOs (0.5ng *PrP-1* and 1.0ng *APPb*) were combined and injected into the fish. Both the *PrP-1* MO and the *APPb* MO when injected at sub-effective levels, resulted in few fish displaying CNS malformations (4±1% and 6±5% N=3 trials, >25fish/treatment/trial, 249 total), but no cell death within the CNS was observed (Fig. 2-7G,H,J). When sub-effective doses of these two MOs were combined there was no significant change in the percentage of fish displaying CNS malformations (16±19%, N=3 trials, >25fish/treatment/trial, 133 total), and no significant increase in the percentage of fish displaying CNS cell death was observed (Fig. 2-7F,J). We tested if the genetic interaction of *PrP-1* with *APPa*, and not with *APPb*, was an artifact of our MO reagents affecting different exons or of different spatiotemporal expression domains between *APPa* and *APPb*. As expected, concerted injection of *APPa* mRNA was able to rescue the *APPa-PrP-1* knockdown phenotype (Fig. 2-7K, $P<0.05$); in contrast injection of *APPb* mRNA was not (Fig. 2-7K). Thus expression of *APPa* from mRNA, likely representing ectopic over-expression, was able to rescue the phenotype

whereas *APPb* mRNA was not (Fig. 2-7K), eliminating these alternate hypotheses.

We similarly tested the specificity of our approach and genetic interactions in regards to PrP. Concerted mRNA injections, in a background of *APPa* & *PrP-1* knockdown, demonstrated that 100 pg of *PrP-1* mRNA could rescue the phenotype ($P<0.05$) (Fig. 2-7L). In contrast, 100pg of *PrP-2* or Shadoo-1 (*Sho-1)* mRNAs were not able to rescue the observed phenotype (Fig. 2-7K,L). Thus the *APPa* interacts with *PrP-1*, but not with the related prion family members *PrP-2* or *Sho-1*. Based on high structural similarity between zebrafish and mouse PrP and prior reports indicating a partial rescue of zebrafish *PrP-1* knock down with mouse PrP mRNA [5], a rescue using mouse PrP was attempted and in line with prior work a partial rescue occurred (Fig. 2-7L). The percentage of normal fish was significantly ($P<0.05$) increased when 100pg of mouse PrP was co-injected relative to injections that contained *Sho-1* or *APPb* mRNA, the instance of a severe phenotype was also reduced, but not to a significant extent (Fig. 2-7L).

***APPa* and *PrP-1* mediate cell adhesion**. To test the hypothesis that APP and PrP play a role in cell adhesion *APPa* and *PrP-1* MOs were injected and the aggregating ability of cells was examined [5]. Cells from embryos that had been injected with MO solutions and dextran dyes were first dissociated (Fig. 2-9E,F), incubated, and the number of cells present in aggregates (10 or more cells in physical contact) was compared to the number of cells alone in solution on a GE

IN cell 2000 machine. Sub-effective knock down of *APPa* or *PrP-1* had little effect on the aggregating ability of cells, but when sub-effective doses were combined a significant ($P<0.01$) decrease in the number of cells in aggregates was observed (Fig. 2-9G). Effective doses of *APPa* knock down (1ng) or *PrP-1* knock down (1ng) alone each led to a reduction in the number of cells present in aggregates compared to the number of cells dissociated and alone in solution, although *APPa* knock down reduced aggregation to a greater extent (Fig. 2-10).

Human APP is able to replace zebrafish APP during *APPa* and *PrP-1* joint knockdown. Human APP shows a striking degree of similarity to both the zebrafish APP proteins (~70% identity in both instances) and, as such, rescue experiments were carried out using wild-type human APP mRNA, and human APP mRNA harboring the Swe (K670N/M671L) and Ind (V717F) mutations associated with familial AD [21, 22]. Both mRNAs were able to efficiently ($P<0.05$) rescue the observable phenotype caused by co-injection of sub-effective doses of *APPa* and *PrP-1* MOs (Fig. 2-11) and the amount of activated Caspase 3 labeling above the yolk sac extension (Fig. 2-11). No significant difference was found between wildtype and mutant human APP in regard to their ability to rescue the APP-PrP phenotype, contrasting a role for these mutations in zebrafish development reported previously [4]. For these experiments transgenic *GFAP*:GFP (green fluorescence production from the glial fibrillary acidic protein promoter) were employed to better visualize the CNS following *APPa* and *PrP-1* knock down and rescue (Fig. 2-11F-I). Under fluorescence it was noted that when

sub-effective doses of MO were combined and injected there was a clear lack of GFP expression in regions of the zebrafish CNS (Fig. 2-11F-I). Control MO injections confirm that the GFAP:GFP transgenic fish harbored no intrinsic susceptibility to MO injection as these fish developed normally (Fig. 2-11F).

CONCLUSIONS:

Alzheimer Disease (AD) and Transmissible Spongiform Encephalopathies (TSEs) represent insidious, slow and inevitably fatal neurodegenerative diseases. Myriad similarities exist between their endpoints and histopathologies, and their antemortem differential diagnoses remains challenging [16, 17]. Both diseases present in sporadic and familial forms, whereas TSEs are differentiated from AD in that they can also present in infectious forms (though see [18] suggesting that even this distinction might best be considered a matter of degree). Pathologically, these AD and TSEs share hallmarks of disease progression: short toxic protein oligomers that form into extracellular plaques containing both PrP^{Sc} and Aβ, early loss of dendritic spines and synaptic plasticity associated with learning deficits, tau hyperphosphorylation and neurofibrillary tangles, dysfunction in metal homeostasis, gliosis, neuronal apoptosis and dementia [19-22]. As expected from these similarities, differential gene expression points to overlaps in AD and TSE disease endpoints [23, 24].

Our study represents an attempt to uncover putative genetic and biochemical relationships between disease effectors early in neuropathological progression,

before the endstage commonalities described above. In the recent past, the onset of these diseases were viewed as fundamentally different entities, though prescient suggestions identified the likelihood of discovering important commonalities [16]. We briefly summarize the linkages between AD and TSEs into three lines of evidence below, two of which remain controversial and the third being nascent and awaiting confirmation. The data in the current manuscript speak to a highly conserved and thus important interaction between PrP^{C} and APP, effecting cell adhesion and neuron survival, which we interpret below as support for these biochemical and/or genetic interaction nodes.

Firstly, a vibrant literature suggests that PrP^{C} can act as a receptor for oligomerized Aβ, the disease-associated cleavage product of APP [4, 5, 6]. The result of such binding was argued to influence synaptic plasticity and perhaps excitotoxicity [5, 6, 25, 26]. Indeed both PrP^{C} and Aβ may interact to mediate toxicity via regulation of NMDA receptors [27]. The data sets from these groups are contentious regarding the effects of Aβ binding to PrP^{C} on LTP and learning [6, 25, 26], though all groups have used a battery of techniques to repeatedly confirm high affinity binding of PrP^{C} to Aβ oligomers. The relevance of this data to PrP^{C} interactions with APP holoprotein remains unexplored.

Secondly, human genetics has frequently, though not consistently, described a controversial association of the *Prnp* locus with risk for AD. In particular, the PrP^{M129V} genotype that is protective in various TSEs in a heterozygous state has

been found to be associated significantly with AD in several past and recent studies [28-31]. This association is not supported in all populations, which may be understandable in light of a multigenic risk factor for a late onset disease. Mechanistically, it has been shown that PrP^{M129V} mutations modulate BACE (β secretase) cleavage of APP and thus effect levels of Aβ42 associated with increased AD risk [32, 33]. Conversely, genes associated with familial AD or associated with risk for sporadic AD have been identified as risk loci for TSEs. One emerging candidate in this regard is AIDA-1, an APP-binding protein associated with excitotoxicity [34] that was identified as a risk loci for BSE [35]. Further, a well-documented AD risk-associated locus, APOE-E4, has also been shown to be linked with risk for sporadic TSEs, though delayed onset of inherited forms of TSEs in humans with $Prnp^{P102L}$ is also observed [36]. P102L is within the region where Aβ oligomers bind PrP^{C} [5]. Thus human genetics tentatively suggests links between these diseases.

Finally, proteomic analyses have suggested that APP and PrP interact biochemically *in vivo*, though it cannot yet be excluded that intermediary binding partners are required. Protein interactomes of APP and PrP^{C} each independently annotate high-quality data that make APP and PrP^{C} likely interactors in rodent brains and cell culture paradigms [9, 37, 38]. This is consistent with APP and PrP^{C}, representing Type I transmembrane and GPI-anchored proteins respectively, both being localized to the external leaf of cell membranes, at synapses and within lipid rafts. These interactomes remain to be validated with conventional

biochemical techniques such as co-immunoprecipitations. These independent interactome studies also identify several protein interactors that APP and PrP^C have in common, including APLP1, NCAM, integrins and contactins, supporting the validity of the biochemical interaction and a common role for APP and PrP^C proteins in modulating cell adhesion. Interestingly, APP sits at a central node of a network that includes some of these genes and others that are differentially expressed during *PrP-2* knockdown in zebrafish [39].

Overall, then, identifying functional interactions between APP and PrP has substantial implications for AD and TSE research. We sought to validate and expand upon putative APP-PrP interactions via an independent method. Our concerted *in vivo* APP and PrP knockdown, combined with an mRNA replacement strategy, revealed that APP and PrP have a genetic interdependence in zebrafish. We interpret this to mean that APP and PrP interactions are highly conserved through evolutionary time, and thus important and worthy of further detailed study. The interaction we report underpins cell adhesion and CNS apoptosis, and appears to be entirely relevant to mammalian orthologues, in-so-much that human APP and murine PrP can replace zebrafish orthologues in our neurodevelopmental assays. Our control experiments use mRNA to replace the cognate disrupted gene, and knockdown specificity is further verified by the limitation of the interactions to only one pair of APP and PrP paralogs (*APPa* genetically interacts with *PrP-1*, but not *PrP-2*; *PrP-1* does not interact with *APPb*). Our experiments underline additional commonalities between APP and

PrP^C, including the contrast between a lack of early phenotypes when these genes are disrupted in mice, but an apparent necessity for these proteins during early development of zebrafish. The reasons for such a difference remain speculative, especially considering the ability of mammalian orthologues to rescue normal zebrafish development, but may include the rapidity of zebrafish development (days instead of weeks to form a functioning CNS) creating greater sensitivity to disrupted cell adhesion. Alternatively, there could be redundancies built into murine development that circumvent APP and PrP loss, though this is not supported by our demonstration that the APP orthologues are redundant in early zebrafish development.

In sum, our comparison between APP and PrP paralogs has identified a conserved and specific niche role for APP-PrP interactions that are required for vertebrate CNS development, and the effects of disrupting this interaction are not the result of generalized decrements in neurodevelopmental integrity. Future work will compare these orthologs and paralogs to inspire hypotheses regarding amino acids and domains that are required and sufficient for the APP-PrP interaction. In this regard, expression of human APP was able to rescue the PrP-APP knockdown phenotype, whereas familial AD mutations of human APP reduced this ability, though not to a level of statistical significance ($p=0.084$) in the doses and assay design we deployed. These fAD mutations are associated with increased generation of $A\beta_{42}$, and thus may be taken as tentative support for PrP regulation of BACE or interactions of PrP^C with Aβ before or after its endoproteolytic

release from APP. Further comparing APPa or human APP with APPb, of which only the latter did not functionally interact with PrP in our assay, suggests regions of interest for further study. APPa and human APP are more similar to each other than to APPb in only one contiguous location greater than three amino acids - the amino-terminal end of Aβ, i.e. the site of BACE cleavage. The impressive conservation of the APP intracellular domain between human and zebrafish paralogs suggests that a conserved function of APP includes endoproteolytic cleavage towards intracellular signalling, and disruption of this processing via differences of BACE cleavage would then be expected to be consequential, consistent with a role for PrP^{C} in modulating BACE function [32, 33].

Our data indicate a synergistic interaction between APP and PrP in mediating cell adhesion. It is not yet clear from our data if this disruption in cell adhesion is a primary or contributing cause of the developmental malformations and increased CNS apoptosis that we observed when these genes are both disrupted. APP and PrP both individually have known roles in cell adhesion [15, 40-42], though this is the first data that demonstrates an interaction of these proteins towards this function. PrP and APP are both abundant at synapses, with enrichment in the pre - synaptic compartments [43-46]. Thus our data invoke an untested hypothesis that APP and PrP interact between cells and/or at synapses, such that alterations in folding or endoproteolytic processing in either protein might influence both proteins in ways that are influential on disease precipitation and spreading. Our data imply that interactions between APP and PrP holoproteins or cleavage

products might be consequential to synaptic plasticity and excitotoxicity through additional mechanisms beyond those that are robustly debated in the literature now [4-6, 47]. The latter have recently been influential in design of therapeutic interventions [47], and thus the results we present herein advocate pursuit of additional APP – PrP interactions.

The genetic interactions we describe in the current work do not allow us to discriminate amongst the three nodes of APP-PrP interaction highlighted above, none of which are mutually exclusive. Our work establishes an *in vivo* paradigm for testing epistatic genetic interactions and assessing the effects of protein variants tentatively associated with disease risk or familial disease inheritance. Establishing linkages between AD and TSEs in the early stages of disease process are expected to be influential on developing diagnostic and intervention strategies.

MATERIALS AND METHODS:

Morpholino Injections: Five antisense morpholino oligonucleotides obtained from Gene Tools were used during these experiments A splice-blocking morpholino designed to specifically bind the exon-intron boundary of exon 2-intron 2 of the zebrafish *APPa* mRNA (5'-TAG TGT TGC TTC ACC TCC TGG CAG T-3'), A splice-blocking morpholino designed to specifically bind the exon-intron boundary of exon 3-intron 3 of the zebrafish *APPb* mRNA (5'-CAC ACA CAT ACA TAC CCA GGC AAC G-3'), and a previously published [5]

translation blocking mopholino designed to specifically bind the 5' UTR of zebrafish *PrP-1* mRNA (5'-TGA GCA GAG AGT GCT GCG GGA GAG A-3'). A standard negative control morpholino was obtained from Gene Tools (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3'). All morpholino injection solutions also contained a standardized dose (3ng) of p53 morpholino (5'-GCG CCA TTG CTT TGC AAG AAT TG-3') to counteract off-target effects of morpholino injection [44]. Injection solutions were made using 1.0uL of 1M KCL, 2.5uL of 0.25% Phenol red, 1.2uL of 25mg/ml p53 MO stock, and gene-specific morpholino to effective (10ug for *APPa*, 10ug for *PrP-1*, 25ug for *APPb*) or sub-effective (5ug for *APPa*, 5ug for *PrP-1*, 10ug for *APPb*) concentrations, mRNAs as appropriate, and nuclease-free water to 10uL. One cell embryos were staged on agarose plates, and injection volume calibrated to 1nl using an ocular micrometer immediately prior to injection. Zebrafish injected with MOs and control MOs were staged and screened at 24hpf based on body morphology and presence of CNS cell death.

Animal Husbandry : Zebrafish were maintained at 28.5^{o}C in standard conditions (Westerfield, 2000) [44]. All zebrafish husbandry and experimentation were done under a protocol approved by the University of Alberta Animal Care and use committee. Wild-type (AB) strains were used for all experiments with the exception of rescue experiments using human APPs in which GFAP:GFP transgenic fish were also used.

mRNA rescue experiments: *APPa*, *APPb*, *PrP-1*, *PrP-2* and *Sho-1* cDNAs from wild type zebrafish were cloned into a PCS2+ vector (primers in Table 1) and linearized with NOTI. mRNA was produced using the mMessage SP6 kit (Ambion Cat# AM1340). Control mRNA was produced by engineering stop codons immediately adjacent to the first coding exon into zebrafish *APPa* and *APPb* mRNA via the Quickchange™ site directed mutagenesis kit following the manufacturer's instructions and primers listed in Table 2-1. Human APP wt and human APP mt (swe/ind mutations) were provided by the Westaway lab, cloned into PCS2+ vector, linearized with NOT1 and transcribed for 3hrs using the mMessage SP6 kit (Ambion Cat# AM1340).All mRNAs were quantified using a GE NanoVue, electrophoresed on a 1% nuclease free agarose gel to check purity, and stored at -80 until use. mRNA was then dissolved in injection solution without morpholino and optimal dosage determined using a dose response curve. For rescue experiments mRNA was dissolved in the appropriate morpholino containing injection solution and injected along with the morpholino. Zebrafish were staged and screened at 24hpf based on body morphology and appearance of CNS cell death. Observer was blinded to treatment groups during screening of all phenotypes.

Reverse Transcription-Polymerase Chain Reaction: mRNA was extracted and purified from 24hpf embryos in RNAlater (Qiagen Cat# 76104) using the RNeasy kit (Qiagen Cat# 74104). *APPa* morpholino efficacy was determined using primers designed to bind intron 2 and exon 8 of zebrafish *APPa* (Table 1). *APPb*

morpholino efficacy was determined using primers designed to bind exon 3 and exon 6 of zebrafish *APPb* (Table 2-1). For all RT reactions PCR was also run on extracted mRNA with standard Taq polymerase in place of reverse transcriptase to confirm absence of genomic DNA contamination.

Table 2-1. Primers used for RT-PCR, to amplify zf CDNA, and create APPa/b stop codon mRNAs:

	Forward Primer (5'->3')	Reverse Primer (5'->3')
Amplify APPa CDNA	AGAAGCATGCGGTCGAGGGA	GTGACGGTGCTCCATCAGTTG
Amplify APPb CDNA	CAGCCATGGGTATAGACCGCA	TTAGTTCTGCATTTGCTCAAAGA
Amplify PrP1 CDNA	CAAAATGGGGGAGTTATGCAAAC	CATTAAGTGGTACTAAAAAGCATAG
Amplify PrP2 CDNA	ATGGGTCGCTTAACAATACTATTG	TGAGAATGTCAGTGTAGAAGGGA
Amplify Sho1 CDNA	ATCCAGAATGAACAGGGCAGTC	CTCAAGGGGCAAAGTGCATCAT
Confirm APPa MO	GAGCTCGAGGATGAACACTA	ACAGCGGCGCTCTCAGACT
Confirm APPb MO	AGCCTGTCAGCATCCAGAAC	CACCGTCTTCATCGTTGTCC
Engineer APPa S3X:E5X	CTAGAAGCATGCGGTAGAGGTAGCTCTTCATA TTAC	GTAATATGAAGAGCTACCTCTACCGCATGCTT CTAG
Engineer APPa 14_15insT	CATGCGGTCGAGGGATGCTCTTCATATTAC	GTAATATGAAGAGCATCCCTCGACCGCATG
Engineer APPb M3X:V7X	GCTCAGCCATGGGTTAAGACCGCACGTGATTCCT GCTTTTAATG	CATTAAAAGCAGGAATCACGTGCGGTCTTAACCC ATGGCTGAGC
Engineer APPb Del8A	GCTCAGCCATGGGTTAGACCGCACGG	CCGTGCGGTCTAACCCATGGCTGAGC

Immunohistochemistry: 24hpf zebrafish were fixed in 4% paraformaldehyde in PBS (phosphate buffered saline, pH 7.4) with 5% sucrose overnight and rinsed for 20min in PBS with 5% sucrose (3 times), washed 5 min in water with 1% Tween, washed 7min in -20^{o}C acetone, blocked for 1hour at RT in PBS with 10% normal

goat serum (NGS), incubated in primary antibody (1:500 anti-activated Caspase 3 (BD Biosciences Cat #559565) in PBS with 1% Tween 1% DMSO 1% Triton with 2% NGS overnight at 4^{o}C. Samples were then washed for 20min in PBS containing 1% Tween, 1% DMSO, and 1% Triton twice then incubated overnight in secondary antibody (1:1000 dilution of anti-Rabbit-AlexaFluor-488 or -555, or anti-Mouse-AlexaFluor-488, Invitrogen cat#s A21441, A31572, A21202, A31570) in PBS with 1% Tween 1% DMSO 1% Triton and 2% NGS, and washed for 20min in PBS containing 1% Tween, 1% DMSO, and 1% Triton (3 times). After final wash zebrafish were placed in anti-fade mounting media [45] (Invitrogen cat# S36937) and visualized on Nikon 90i microscope. Caspase positive cells were imaged and quantified above the yolk sac extension of the fish as per Aamar and Dawid (2008) [20].

Aggregation Assays: One cell zebrafish embryos were staged and injected with morpholino solutions as above but containing 2.5uL of Dextran 488 or 555 (Invitrogen Cat#s, D1817, D22910) in place of phenol red. Embryos were incubated at 28.5^{o}C until dome stage was reached. Embryos were then manually dechorinated and placed on agarose plates containing E3 media with 1:200 Penicillin/Streptomycin antibiotic. Yolk was manually removed from the cells using insect pins and yolk free cells were placed in a 1.5mL microcentrifuge tube containing 1mL Ca2+ free Ringers solution (116 mM NaCl, 2.9 mM KCl, 5.0 mM HEPES, pH 7.2). Cells were dissociated by pipetting repeatedly with a 200uL pipette for 30 seconds. A portion of cells were kept at this point to check that

dissociation had occurred. Cells were centrifuged at 550xg for 30 seconds and supernatant removed. 1mL High Ca ringers solution (116 mM NaCl, 2.9 mM KCl, 10 mM CaCl2, 5 mM HEPES, pH 7.2) was then added to cells and cells were incubated at 28.5^{o}C for 1hr in a standard 1.5mL microcentrifuge tube. **For experiments in which sub-effective doses of MO were used:** 200ul of solution was aliquoted in triplicate into wells of 96 well plate (BD Falcon) and imaged on a GE IN cell 2000 analyzer. Exposure was manually adjusted and 9 images (fields) per well were taken at 20x. Analysis of images was done using the GE investigator software package; exposure and contrast were equalized across wells, and the mean size of a single cell calculated. Any continuous fluorescent signal 3x larger than a single cell was then defined as an aggregate. Multi-level object linking was used to determine the total number of cells within these aggregates along with the total number of cells in the well. Data was then manually processed in Microsoft Excel; to avoid ambiguous determination of aggregates only aggregates containing more than 10 cells in direct physical contact were counted and this number in proportion to the total number of cells in the well was used to calculate the % of cells in aggregates**. For experiments in which effective doses of MO were used:** Using a transfer pipette, three aliquots were placed on a standard glass slide and three pictures of each drop were taken at random locations at 10x using a Nikon i90 microscope and QImaging RETIGA 2000R Camera. Cells were manually counted in Photoshop with experimenter blind to treatment groups. A clump was defined as 3 or more cells in direct physical

contact and single cells were defined as cells not in physical contact with any other cells.

Image manipulation: All images were taken on Nikon 90i microscope using QImaging RETIGA 2000R camera or automatically on the GE IN Cell 2000 at 20x. Images were rotated, cropped and brightness adjusted in Photoshop CS5. No other alterations were made.

Statistics: All statistics were performed in SPSS 17.0 using one way ANOVA with Bonferroni correction and Tukey post hoc tests, or Mann-Whitney U test. Significance was set to 0.05 (*) or 0.01 (**).

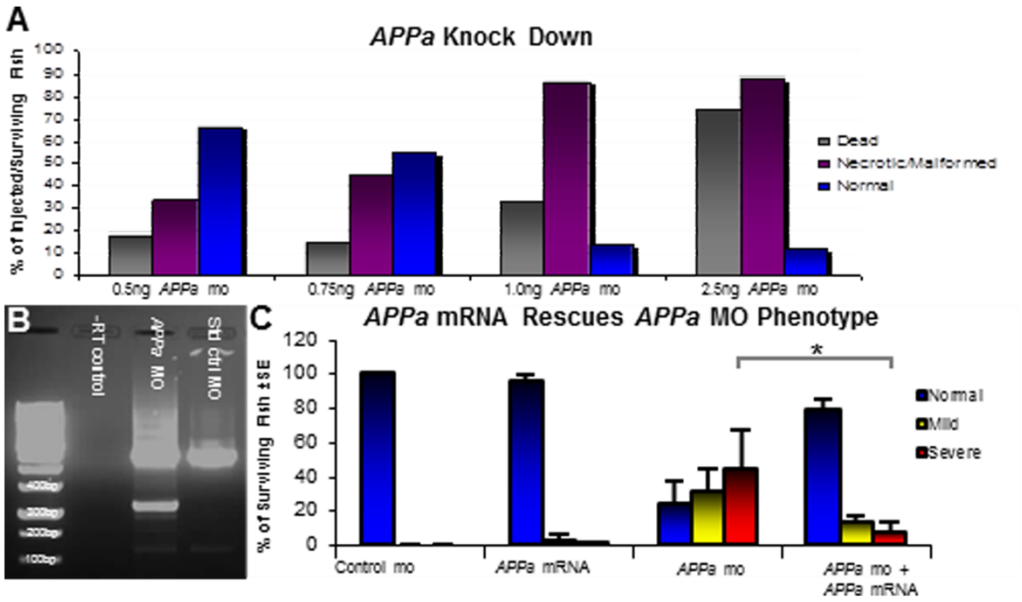

Figure 2-1. *APPa* mo injection leads to a dose-dependent disruption in *APPa* mRNA processing.

(A) One cell stage embryos were injected with increasing doses of *APPa* MO and screened based on presence of morphological malformations and CNS cell death.

(B) mRNA was extracted from fish injected with *APPa* MO, an equivalent dose of control MO, and subjected to RT-PCR. Fish injected with *APPa* MO show a band at ~300bp corresponding to mRNA with intron 2-3 retained. This band is absent in when fish are injected with the control MO, or standard Taq is used in place of RT enzyme. Sequencing of the band confirmed the retention of intron 2-3 in mature mRNA. For placement of primers see Fig.1-3A.

(C) Embryos were injected with the *APPa* MO alone and with 200pg of cognate *APPa* mRNA. The percentage of fish displaying a "severe" phenotype was drastically reduced and the number of "normal" fish significantly increased upon inclusion of the mRNA. *=P<0.05.

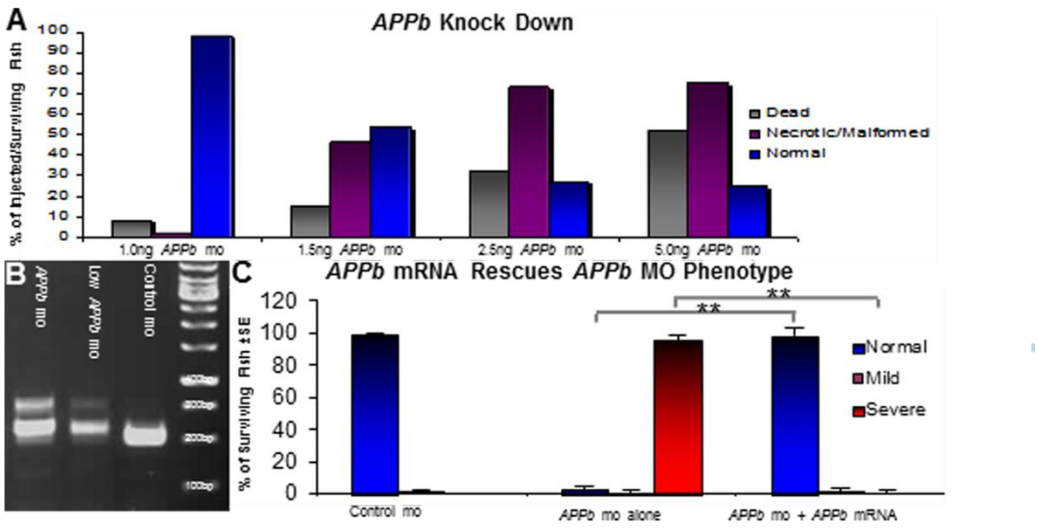

Figure 2-2. *APPb* MO injection leads to a dose-dependent disruption in *APPb* mRNA processing.

(A) One cell stage embryos were injected with increasing doses of *APPb* MO and screened based on presence of morphological malformations and CNS cell death.

(B) mRNA was extracted from fish injected with 2.5ng *APPb* MO, 1.0ng *APPb* MO, an equivalent dose of control MO, and subjected to RT-PCR. Fish injected with 2.5ng of the *APPb* MO show a band at ~300bp corresponding to retention of intron 3-4 in mRNA. This band is reduced when the dose of the MO is reduced, and absent when fish are injected with the control MO. Sequencing of the band confirmed the retention of intron 3-4 in mature mRNA. For placement of Primers see Fig. 1-3A.

(C) Embryos were injected with the *APPb* MO alone and with 200pg of cognate *APPb* mRNA. The instance of fish displaying a "severe" phenotype was significantly reduced and the number of "normal" fish significantly increased upon inclusion of the mRNA. *=P<0.05.

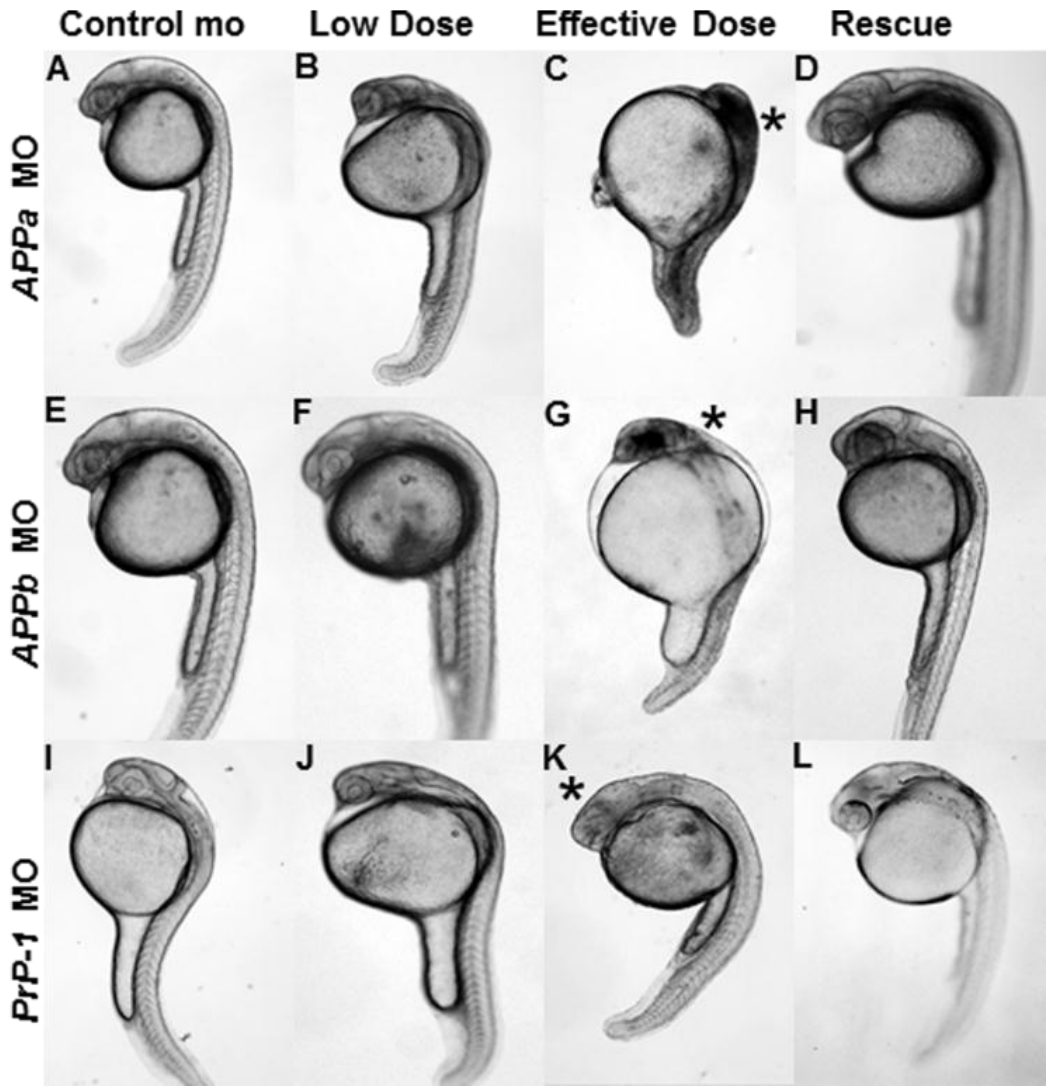

Figure 2-3. Knock down of *APPa, APPb,* or *PrP-1* in zebrafish results in small size, edema, morphological abnormalities, improper CNS formation and death of CNS tissues.

One cell stage embryos were injected with a morpholino (MO) designed to disrupt translation of endogenous *APPa* (B-D), *APPb* (F-H), or *PrP-1* (J-K) mRNA.

(A,E,I) Standard control MO at levels equivalent to an "effective dose" fail to induce any CNS cell death or disruptions in morphology of the fish.

(B,F,J) Sub-Effective doses (0.5ng, 1.0ng, 0.5ng) of *APPa, APPb, or PrP-1* MOs lead to mild changes in overall fish morphology, but no death of CNS tissues.

(C,G,K) Empirically determined effective doses (1.0ng *APPa*, 2.5ng *APPb*, 1.0ng *PrP-1*) lead to severe alterations in peripheral and CNS morphology and death of CNS tissues (*).

(D,H) The phenotype due to injection of an effective dose of *APPa* or *APPb* MO is rescued by co-injection of 200pg of cognate *APPa* or *APPb* mRNA(Fig. 2-1,2-2). All fish staged, screened and imaged at 24hpf.

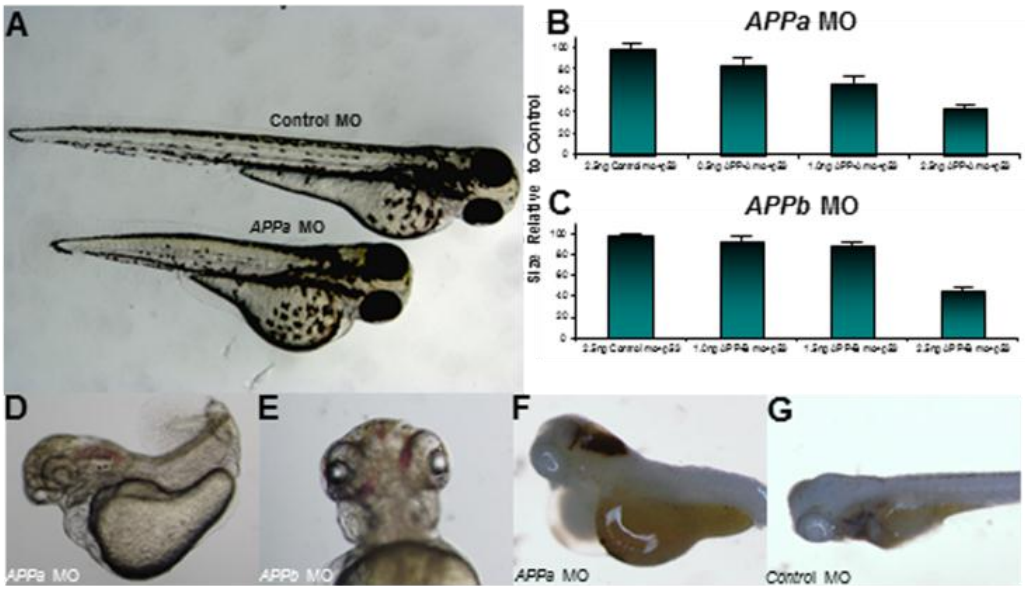

Figure 2-4. APP knock down in zebrafish leads to reduced body size and vasculature abnormalities.
(A) 7 dpf sibling zebrafish injected with an *APPa* MO (bottom A) or a equivalent dose of control MO (top A)
(B,C) 7dpf fish were measured using an ocular micrometer and size of fish injected with either *APPa* (B) or *APPb* (C) MOs relative to those injected with a control MO determined.
(D, E) 3 dpf fish injected with *APPa* (D) or *APPb* (E) MO show significant blood pooling in the head region.
(F,G) 3 dpf fish injected with an *APPa* MO or control mo were subjected to fixation and staining with O-Diansidine to better visualize vasculature abnormalities.

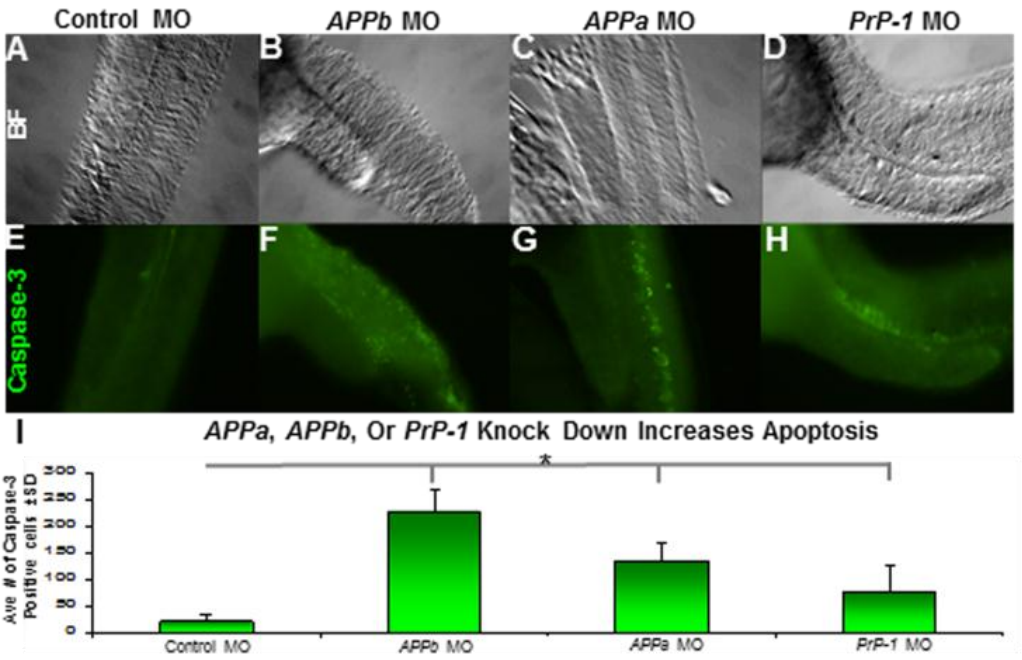

Figure 2-5. Apoptosis levels are increased when *APPa, APPb,* or *PrP-1* mRNA are disrupted.

(A-D) Brightfield images were taken at 24hpf of the area above the yolk sac extension of fish injected with effective doses of Control (A), *APPb* (B), *APPa* (C) and *PrP-1* (D) MOs.

(E-H) Compared to control fish (E) prominent anti-activated caspase-3 staining is apparent in *APPb* (F), *APPa* (G), and *PrP-1* (H) MO injected fish when staining of areas in A-D is viewed under fluorescence.

(I) Number of caspase-3 positive cells were counted above the yolk sac extension in fish injected with effective doses of control, *APPb*, *APPa*, and *PrP-1* MOs. N=5. *=P<0.05.

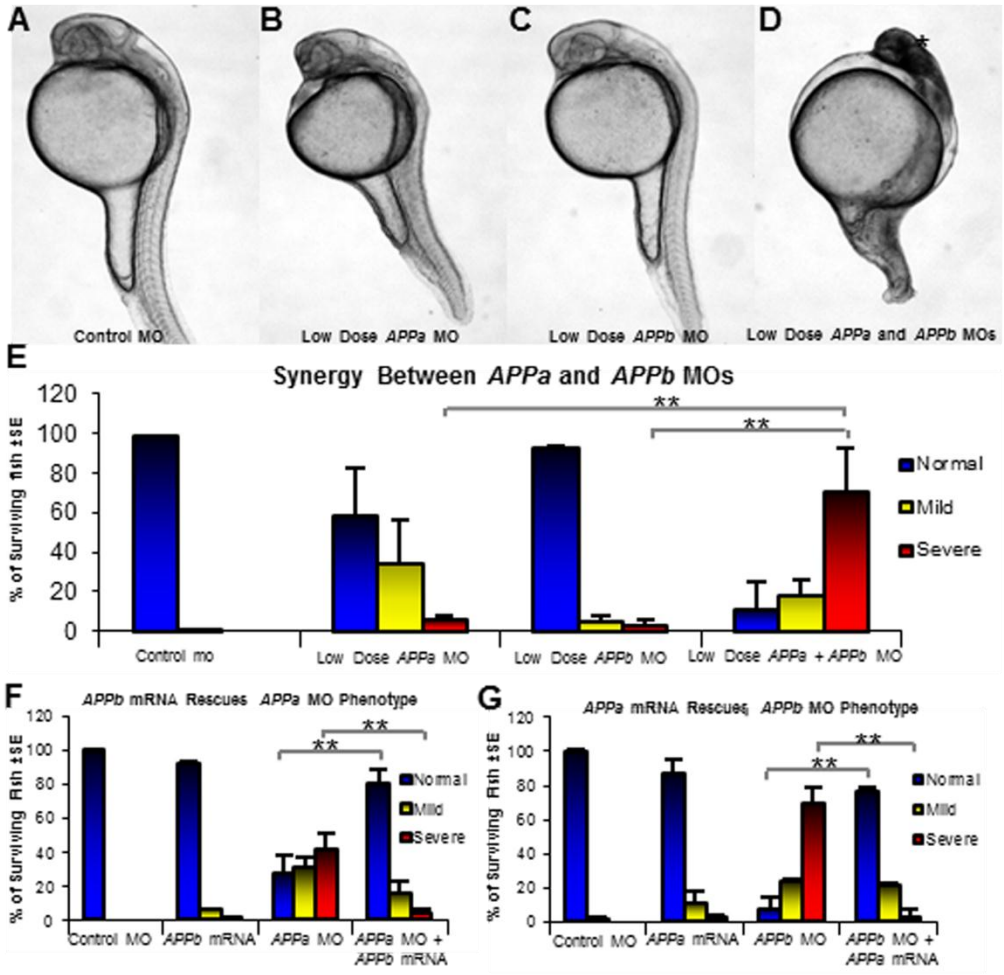

Figure 2-6. *APPa* and *APPb* show redundancy in the zebrafish.
(A-D) Embryos were injected with sub-effective doses of *APPa* (B) and *APPb* (C) MOs. These doses produced no phenotype in the fish compared to control MO (A,B,C). When sub-effective doses of *APPa* and *APPb* MO were combined and injected into one cell stage embryos a strong phenotype emerged consisting of morphological malformations and death of tissues within the CNS(*) (D).
(E) Injection of sub-effective doses of both MOs produced a significant decrease in the number of normal fish and a significant increase in number of fish displaying CNS cell death. *=P<0.05.
(F,G) Fish were injected with an effective dose of one MO along with cognate mRNA from the other paralog to see if rescue of the phenotype occurred. *APPb* mRNA was able to effectively alleviate the phenotype caused by injection of the *APPa* MO (F) and vice versa (G). *=P<0.05.

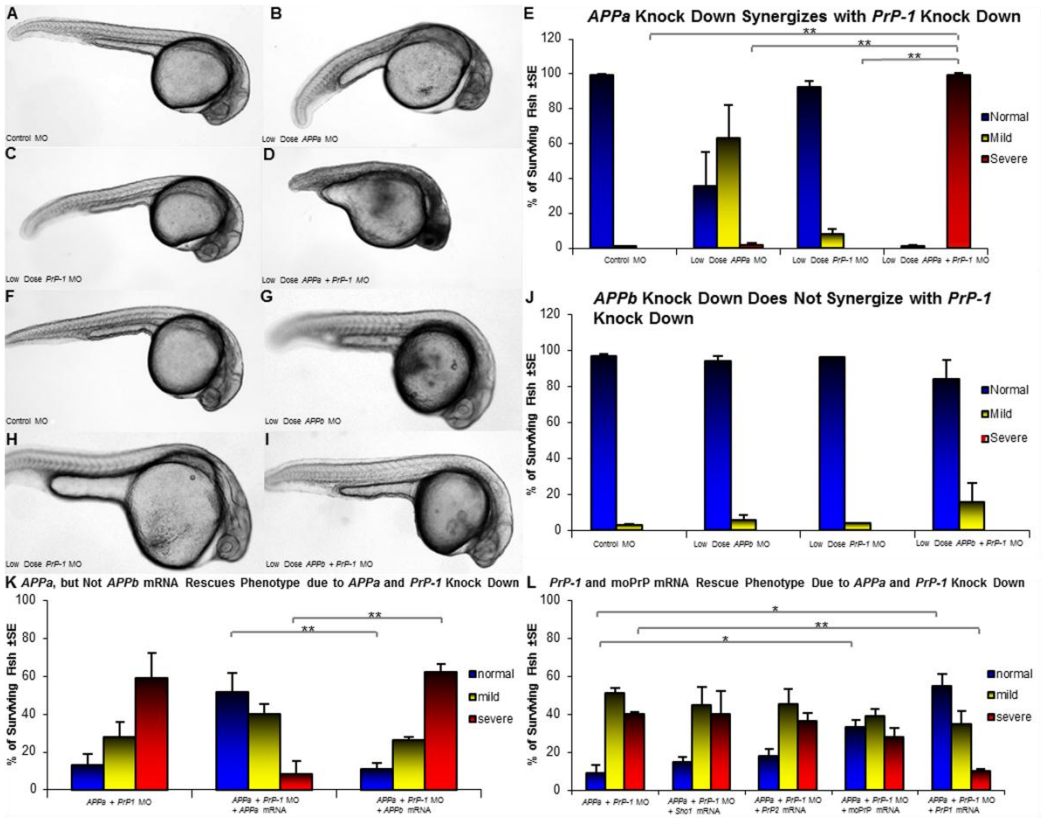

Figure 2-7. *APPa*, but not *APPb* functionally interacts with *PrP-1* in the zebrafish.
(A-E) A combination of sub-effective doses of *APPa* and *PrP-1* MOs "synergize" to produce an overt phenotype in the fish. Fish injected with a control MO (A), a sub-effective dose of *APPa* (B), or a sub-effective dose of *PrP-1* (C) MO fail to display any signs of CNS cell death or disruptions in development. When sub-effective doses of *APPa* and *PrP-1* are combined and injected a "severe" phenotype emerges comprised of prominent morphological disruptions and an overt appearance of cell death within the CNS (D). The % of "normal" fish is significantly reduced, and the % of fish displaying cell death within the CNS (severe) is significantly increased when sub-effective doses of *APPa* and *PrP-1* MOs are combined (E). *=P<0.05.
(F-J) A combination of sub-effective doses of *APPb* and *PrP-1* MOs do not synergize to produce a phenotype. Fish injected with a control MO (F), a sub-effective dose of *APPb* MO (G), a sub-effective dose of *PrP-1* MO (H) or a combination of the two (I) do not display any malformations or signs of cell death within the CNS. When Sub-effective doses of *APPb* and *PrP-1* MOs are combined there is no significant increase in the number of fish showing developmental abnormalities or cell death within the CNS (J).
(K) *APPa* mRNA is able to alleviate the phenotype caused by co-injection of sub-effective doses of *APPa* and *PrP-1* MOs. Cognate *APPa* mRNA significantly increased the % of normal fish and significantly reduced the % of fish displaying a severe phenotype. Cognate *APPb* mRNA at an equivalent dose failed to reduce the % of fish displaying a phenotype. *=P<0.05.
(L) Cognate *PrP-1* and mouse PrP mRNAs are able to alleviate the phenotype produced by co-injection of *APPa* and *PrP-1* MOs while

Sho-1 and *PrP-2* mRNAs are not. Cognate *PrP-1* mRNA alleviated to a significant extent the phenotype produced by injection of subeffective doses of *APPa* and *PrP-1* MOs while *Sho-1* and *PrP-2* mRNA was unable to reduce the % of fish displaying a phenotype. Mouse PrP mRNA was able to partially rescue the phenotype as it increased the number of "normal" fish and decreased the number of fish displaying a "severe" phenotype. *=$P<0.05$.

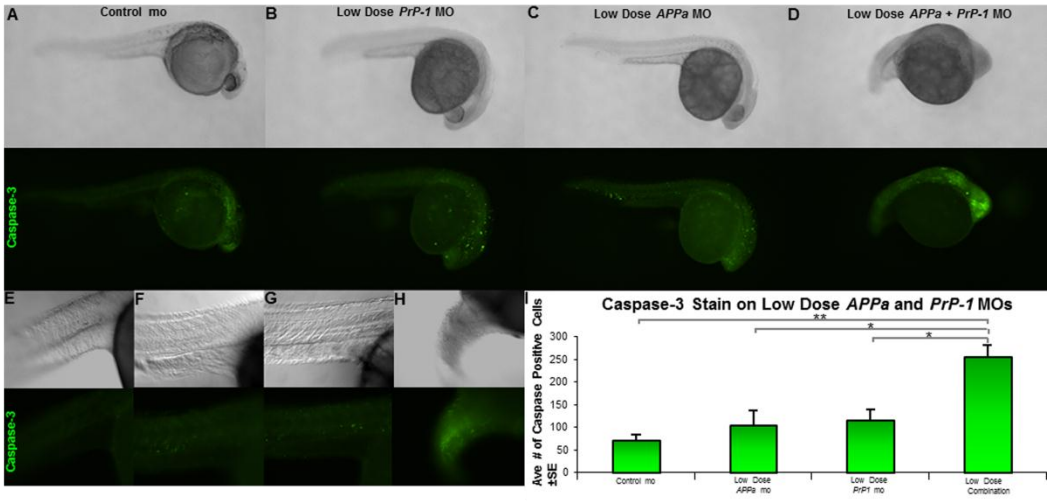

Figure 2-8. Apoptosis is increased when *APPa* and *PrP-1* levels are reduced.
(A-H) 24 hpf fish injected with a control MO (A,E), low dose (sub-effective) *PrP-1* MO (B,F), low dose (sub-effective) *APPa* MO (C,G), or a combination of sub-effective *APPa* and *PrP-1* MOs (D,H) were fixed and stained with an anti-activated caspase-3 antibody (green). Activated caspase-3 labeling was slightly increased in when sub-effective *PrP-1* or *APPa* MOs were injected alone (B,F,C,G) and significantly increased when they were combined in one injection solution (D,H).
(I) Fish were staged and screened for presence of activated caspase-3 positive cells above the yolk sac extension of the fish. N=5.
** = $P<0.01$, * = $P<0.05$.

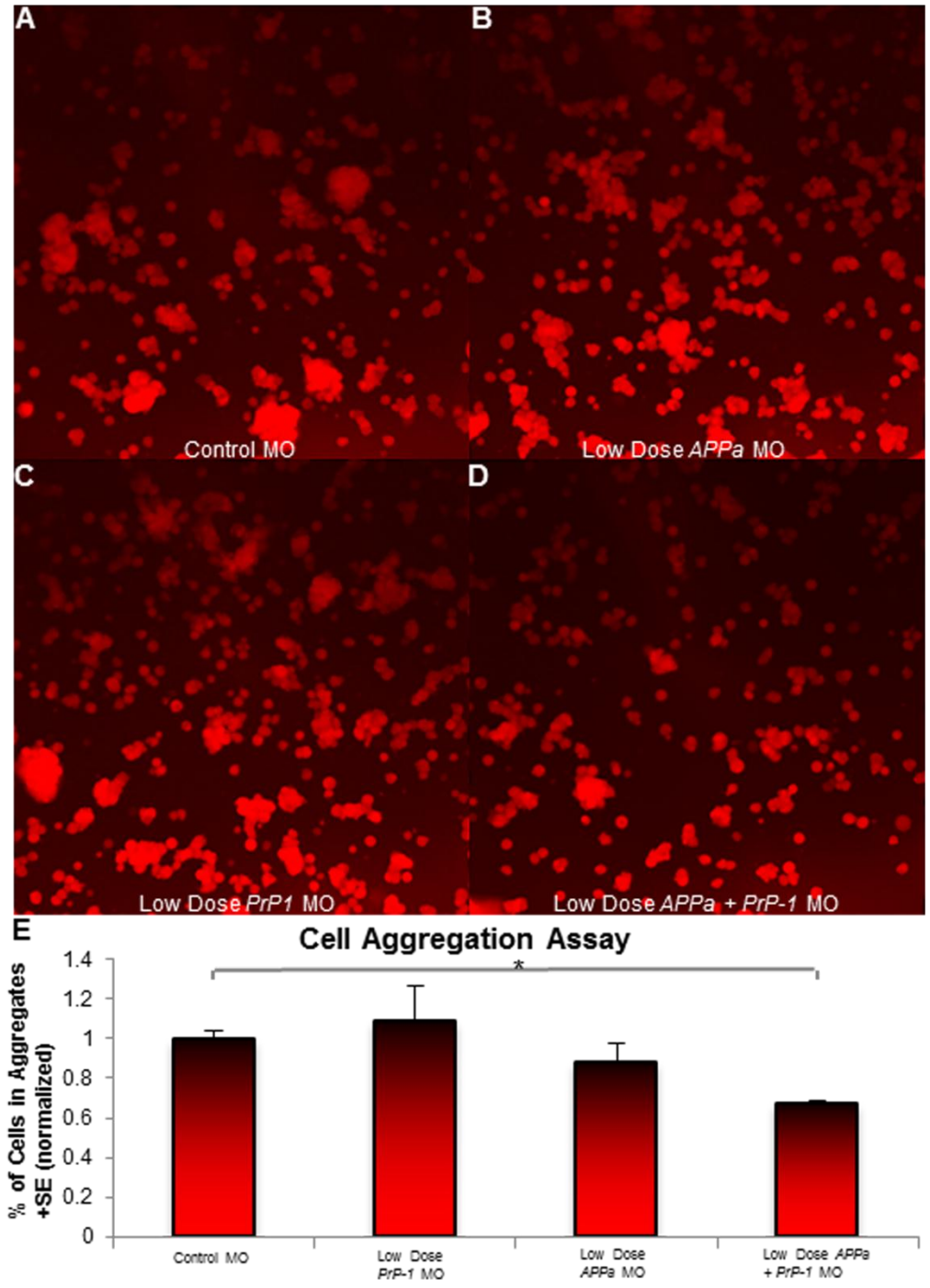

Figure 2-9. Sub-Effective Knock Down of *APPa* and *PrP-1* reduces aggregating ability of cells.

(A-D) Cells from embryos injected with control MO (A), low dose of *APPa* MO (B), low dose of *PrP-1* MO (C), or a combination of the two sub-effective MOs (D) were harvested at 6hpf and subjected to an aggregation assay.

(E) The ability of these cells to form aggregates (10 or more cells in direct physical contact) rather than stay alone in solution after dissociation and incubation was quantified. N=4. ~20000 cells counted.*=P<0.05.

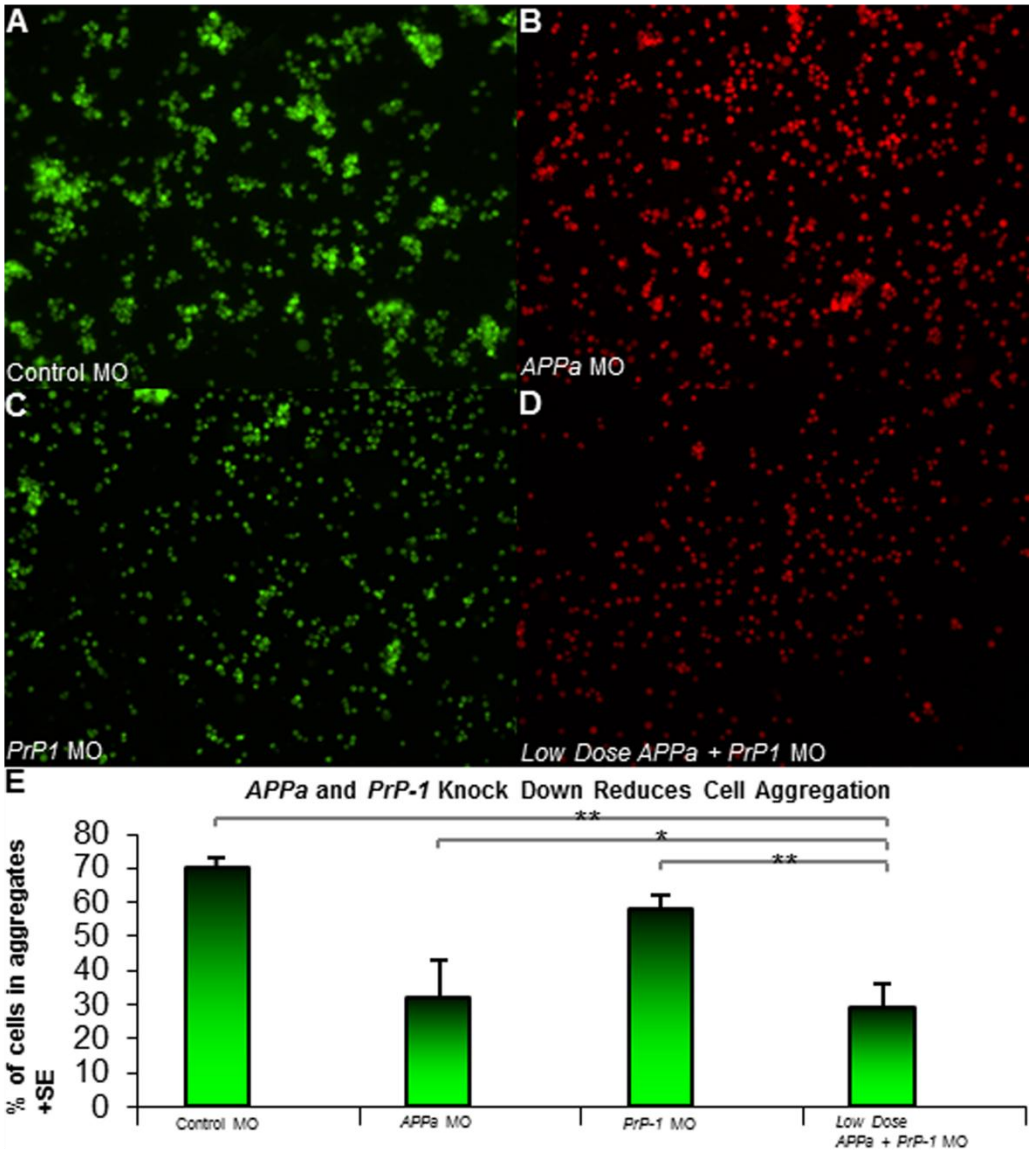

Figure 2-10. Effective Knock Down of *APPa* and *PrP-1* reduces aggregating ability of cells.

(A-D,E) Cells from embryos injected with control MO (A), effective dose of *APPa* MO (B), effective dose of *PrP-1* MO (C), or a combination of the two sub-effective MOs (D) were harvested at 6hpf and subjected to an aggregation assay. The ability of these cells to form aggregates (3 or more cells in direct physical contact) rather than stay alone in solution after dissociation and incubation was quantified (E). N=10. ~5000 cells counted.*=P<0.05.

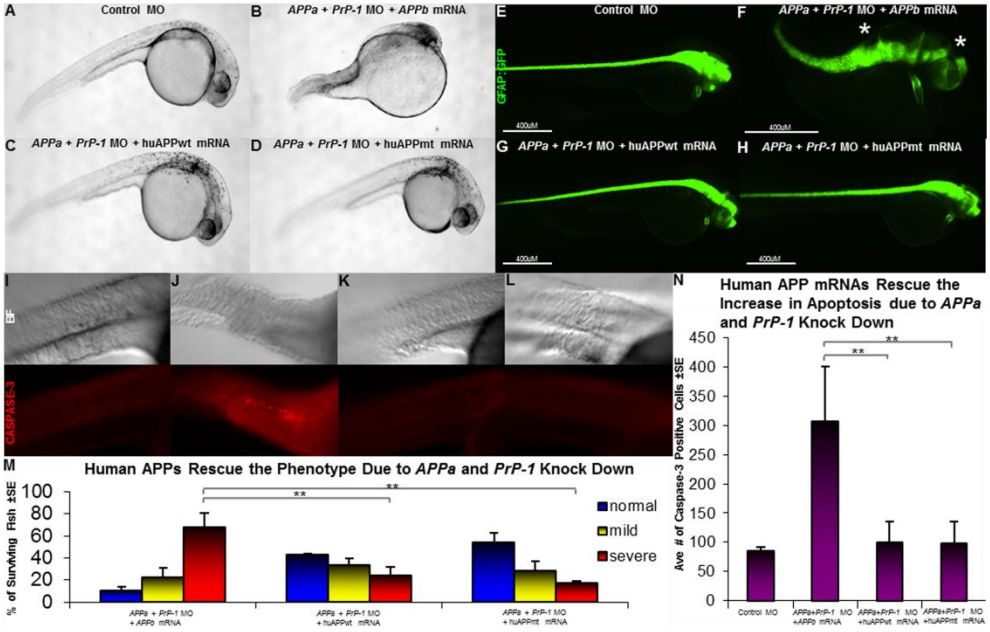

Figure 2-11. Human APP is able to rescue the phenotype caused by *APPa* and *PrP-1* knock down.

(A-D,M) Injection of control MO results in no observable phenotype within the fish (A), Injection of low dose *APPa* MO in combination with low dose *PrP-1* MO and *APPb* mRNA leads to severe disruptions in physiology and readily apparent cell death in the CNS of the fish (B). When 200pg of either wild-type human APP mRNA or human APP mRNA harboring swe (K670N/M671L) and Ind (V717F) mutations injected along with the sub-effective doses of *APPa* and *PrP-1* MOs the disrupted physiology and presence of cell death within the CNS of the fish is significantly reduced (C,D,M). *=P<0.05.

(E-H) Fish stably transgenic for a GFAP (glial fibrillary acidic protein promoter) :GFP transgene were injected with above mentioned MOs to better visualize CNS disruptions. When injected with the combination of sub-effective *APPa* and *PrP-1* MOs (with *APPb* mRNA) a noticeable lack of GFP was apparent along portions of the CNS (F)(*). The lack of GFP in the CNS was abolished upon inclusion of wt or mt human APP mRNA (G,H).

(I-L,N) Brightfield, and anti-activated caspase-3 (red) images of the yolk sac extension taken at 24hpf of fish injected with MOs in (A-D).

The number of caspase positive cells above the yolk sac extension was then counted and quantified (N). N=5. *=P<0.05.

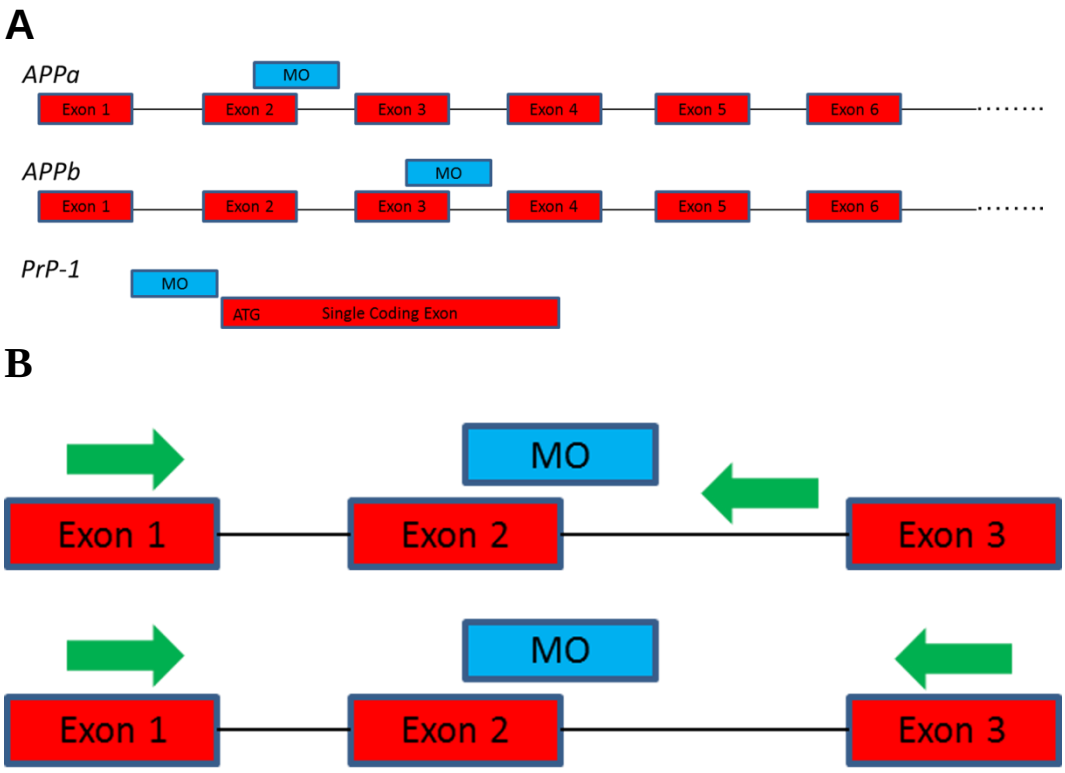

Figure 2-12. Morpholinos designed against zebrafish *APPa* and *APPb*.

A) Placement of morpholinos designed against *APPa*, *APPb*, and location of previously published translation blocking MO against *PrP-1*. Splice blocking MOs are designed early in the APP genes, before any highly conserved regions of the gene, and span exon-intron sites. MO against *PrP-1* sits on the 5' UTR and blocks translation of the single *PrP-1* coding exon.

B) Primer locations (in green) when using RT-PCR to test MO efficacy. In the first instance a forward primer sits on exon one and a second reverse primer sits in the intron that the MO binds. An effective MO will cause retention of this intron and give a PCR product. In the second instance primers sit on exons that span the MO binding site and will produce larger PCR products (containing the retained intron) if the MO is effective.

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CHAPTER THREE

INTRODUCTION:

Familial Crutzfeldt Jacob disease (CJD) caused by mutations in the open reading frame of *Prnp* can clinically mimic a wide array of neurodegenerative diseases including Alzheimer disease (AD) [1]. It has also been noted in epidemiological studies that familial clustering of sporadic CJD is correlated with other types of neurodegenerative disease, the most common of these is AD [2, 3]. valine homozygosity at *PRNP* codon 129 was first shown to be associated with poorer cognitive performance scores in the elderly [4] and later a significant genetic association was found between homozygosity at *PRNP* codon 129 and early onset AD [5, 6]. Although contentious (see [7]) epidemiological findings suggest a link between CJD and AD at the genetic level.

Pathologically, up-regulation of the prion protein (PrP^c) has also been found in neural tissues from persons afflicted with various neurodegenerative diseases [8, 9]. In AD PrP^c immunoreactivity is observed throughout the periphery and also surrounding amyloid-beta (Aβ) plaques in the brain [10]. The precise reason for this PrP^c up-regulation is unclear, but studies overexpressing PrP^c found it inhibited the β-secretase (BACE) cleavage of APP and reduced amyloid beta formation, while depletion of PrP^c facilitated an increase in Aβ load [11]. Aside from indications PrP may be involved in Alzheimer disease process pathological features of AD such as Aβ plaques and hyperphosphorylated Tau protein have also been observed in the brains of persons afflicted with Gerstmann-Straussler-Scheinker (GSS) and variant CJD [12-14]. Thus, APP and PrP are also tentatively

connected by their involvement in CJD and AD pathology and their common interactor BACE.

Expression analysis indicates that PrP^c is broadly expressed in the CNS and highly regulated during development [15, 16]. When this expression is probed at the ultrastructural level multiple studies find PrP^c expression localized in the synaptic boutons where it is mainly presynaptic [17-19]. This finding is compatible with multiple findings that PrP is involved in synaptogenesis and may mediate synaptic ion channel function [20-22]. APP is similarly broadly expressed in the adult brain and its expression too is highly regulated during development [23]. APP expression during development is also concentrated at the synapses where it has been shown to help synaptogenesis and neurogenesis proceed [24, 25]. APP expression, like PrP expression, has been linked mainly to pre-synaptic sites [24]. In the specific case of the septum, levels of *Prnp* mRNA and choline acetyltransferase were found to increase in parallel during development [26]. *App* expression was also shown to follow the maturation of cholinergic neurons where APP facilitated an increased response to glutamate [27]. Perhaps an interaction between APP and PrP takes place within these cholinergic neurons and its disruption during AD disease progression renders these neurons so highly susceptible to degeneration.

Various groups have shown evidence that a direct physical interaction between APP and PrP is likely: by cross-linking proteins in the mouse cortex Bai

et al (2008) provided evidence for a mouse APP PrP^c interaction *in vivo*, Yehiely et al (1997), and Schmitt Ulms et al (2004) provide evidence to a similar effect [28-30]. There is also evidence for an interaction between the mouse APP homologue Amyloid Precursor Like Protein1 (APLP1) and PrP^c possibly contributing to neuronal survival and outgrowth [29, 31]. Interactomes performed against APP and PrP find 12 proteins that interact with PrP are also confirmed interactors with APP; APLP1, BACE-1, Contactin, Laminin, Laminin receptor protein, BIP, HSP90, 14-3-3, Clusterin, NCAM, Fe65, and GFAP with five more regarded as putative interactors [32]. Furthermore, of proteins that have been shown to interact with the cleavage product of APP, amyloid beta (Aβ), six also appear to interact with PrP; MAPT, PDIA3, CLU, C1QA, CALR, and APOE [32]. Recently multiple lines of evidence point to a direct interaction between amyloid beta fibrils and PrP^c [33-36]. Although the precise outcome of the interaction has been hotly debated, all groups provide strong evidence that there is a direct physical interaction between Aβ oligomers and PrP^c. Fresh Aβ42 was shown to also bind PrP^c, albeit not at the level of oligomerized Aβ42, but an interaction was still noted [33]. An impressive array of proteins have been shown to interact with both APP and PrP suggesting that functionally these two proteins are also intertwined.

Results from our prior studies indicate a genetic interaction between *APPa* and *PrP-1* in the developing zebrafish. This interaction is shown to mediate apoptosis and influence cellular adhesion. Although the study found a genetic

interaction between the proteins, the precise nature of the interaction remains unclear. One hypothesis is that the proteins physically interact to mediate these functions, while another hypothesis is that signals from both proteins converge onto a common pathway(s) that serve(s) to mediate apoptotic induction and cell adhesion. To begin to address this larger hypothesis, hypotheses generated regarding the autonomy of the interaction must first be resolved as an interaction between these proteins is plausible both within and between cells. In this study we will utilize the zebrafish model organism and employ a cell mixing assay [37] in an attempt to determine if the interaction noted between *APPa* and *PrP-1* mediates cellular adhesion in a cell autonomous or non-cell autonomous fashion.

RESULTS:

Knock down of *APPa* and *PrP-1* does not influence the mixing ability of cells. Single cell stage embryos were injected with effective (1.0ng *APPa*, 1.0ng *PrP-1*) doses of morpholino (MO) or a standard control MO (1.0ng) along with alexafluor 488 or 555 dyes. After dome stage was reached (~6hpf) cells were manually removed from the yolk sacs and dissociated in a calcium free Ringers solution. Quantification of cells at this point revealed that 92±4% of cells were completely dissociated and free in solution. Cells collected from embryos injected with one MO (*APPa*, *PrP-1*, or Control) along with one dextran dye (488 or 555) were then mixed with dissociated cells that had been injected with a different MO and different dextran dye. This combination of cells was then allowed to incubate and subsequently aliquoted into a 96 well plate. Cells were analyzed by robotic

microscopy and processed with the corresponding software. The degree of cell mixing was calculated by averaging the percent of GFP fluorescent cells within a given well, then counting the number of aggregates (10 or more cells in direct physical contact) containing a percentage of GFP fluorescing cells that significantly deviated (more than 2 standard deviations) from this average percentage.

There was no statistical difference (P>0.05) observed in the "mixing" of cells between any of the groups injected with our various MOs (Fig. 3-1). 60.2±10.3% of aggregates comprised of GFP and RFP fluorescing control MO injected cells contained a proportion of GFP cells more than 2 standard deviations (SD) away from the mean % of GFP fluorescing cells (51.2%-52.1%) within that well (homogeneous aggregates) (Fig 3-1). When cells in which an *APPa* MO had been injected were mixed with control MO injected cells 51.0±13.7% of aggregates exhibited a percentage of GFP fluorescing cells greater than 2 SD away from the mean % of GFP fluorescing cells within that well. 59.2±11.9% of aggregates comprised of *PrP-1* MO injected cells and control MO injected cells were considered homogeneous. Lastly, when *APPa* MO injected cells were mixed with *PrP-1* MO injected cells 47.2±5.3% of aggregates exhibited this homogeneity. The largest difference observed was between the control MO + control MO group and the *APPa* MO + *PrP-1* MO group, with the *APPa* MO + *PrP-1* MO group actually showing an increased level of mixing (Fig 3-1). The results however were not significant based on one way ANOVA with Tukey HSD (P=0.492).

Injection of zebrafish *APPa* or *PrP-1* mRNA does not influence the mixing ability of cells. The corollary experiment was also performed with cells that had been injected with cognate zebrafish *APPa*, *PrP-1*, or control mRNA (*APPa* with stop codons engineered immediately adjacent to the first coding exon), dissociated, mixed, and allowed to aggregate together. There was no statistical difference (P>0.05) observed in the "mixing" of cells between any of the groups injected with these various mRNAs (Fig. 3-2). When cells in which 200pg of cognate zebrafish *APPa* mRNA had been injected were mixed with 200pg of control mRNA injected cells 70.6±20.3% of aggregates exhibited a percentage of GFP fluorescing cells greater than 2 SD away from the mean % of GFP fluorescing cells within that well. 79.2±9.2% of aggregates comprised of *PrP-1* mRNA injected cells (100pg) and control mRNA injected cells were considered homogeneous. When the combination of *APPa* mRNA injected cells were mixed with *PrP-1* mRNA injected cells 73.9±1.6% of aggregates exhibited homogeneous sorting. The largest difference observed was between the *APPa* mRNA + control mRNA group and the *PrP-1* mRNA + control mRNA group, but based on one way ANOVA with Tukey HSD test the difference was insignificant (P=0.811).

Injection of human APP and mouse PrP mRNAs does not influence the mixing ability of cells. In the final experiment the mixing paradigm was repeated using zebrafish cells that had been injected with human APP or mouse PrP mRNA. Similar to the preceding experiment there was no statistical difference (P>0.05) observed in the mixing ability of cells (Fig.3-3). When cells injected

with 200 pg of human APP mRNA were mixed with control mRNA (200pg) injected cells 77.5±3.5% of aggregates showed a % of GFP fluorescing cells that significantly deviated from the mean % of GFP fluorescing cells within that well. 66.1±12.0% of the aggregates comprised of cells injected with mouse PrP mRNA (100pg) and control mRNA injected cells were considered homogeneous. Lastly, when cells injected with human mRNA were mixed with cells injected with mouse PrP mRNA 70.0±14.1% of aggregates were homogeneous. Again, none of the groups deviated from one another to any significant extent, the largest difference observed was between the human APP mRNA + control mRNA and mouse PrP + control mRNA groups with a significance of P=0.613.

CONCLUSIONS:

The results from the first experiment revealed that knock down of zebrafish *APPa* in one set of cells and zebrafish *PrP-1* in another set of cells does not influence those cells ability to adhere to one another. The MOs used in this experiment have been previously validated via PCR, and rescue experiments have proved successful suggesting that the results obtained are not merely due to a lack of effective knock down reagents (see Chapter 2, Fig.2-1, and [38]). *APPa* or *PrP-1* knock down cells were just as prone to adhere to cells injected with a control MO as they were to cells in which the converse gene had been knocked down. These results indicate that any interaction that takes place between *APPa* and *PrP-1* that influences cell adhesion takes place in a cell autonomous manner and not between cells.

The corollary experiment in which zebrafish *APPa* and *PrP-1* proteins were overexpressed by injection of mRNA also failed to show any statistical difference between groups in the degree of cell mixing. When cells that had been injected with 200pg of *APPa* mRNA were mixed with cells injected with 100pg of *PrP-1* mRNA no increase in the propensity for cells to adhere to one another was observed. The mRNA reagents used in this study have been validated by their effectiveness in rescuing the effects of *APPa* or *PrP-1* knock down, but subsequent western blot and immunohistochemical analysis has failed to show any increase in protein levels upon injection of these mRNAs. It therefore remains possible that a lack of mRNA translation within the zebrafish embryo is responsible for these results.

When the experimental paradigm was tried a third time by injecting human APP mRNA in one set of cells and mouse PrP mRNA in another set of cells the degree of cell mixing also remained the same relative to controls. Similar to the aforementioned zebrafish mRNAs these mRNAs have only been validated by their ability to rescue the phenotype due to knock down of zebrafish *APPa* or *PrP-1* genes, and in the case of mouse PrP the amelioration of the *PrP-1* knock down phenotype can be considered a matter of degree (see Chapter 2, Fig.2-2). Western blot analysis and immunohistochemical analysis has also failed to show any increase in human APP or mouse PrP protein levels upon injection of these mRNAs. Therefore, a lack of mRNA translation could theoretically be responsible for the data contained within this experiment as well.

The data presented does not argue against a physical interaction between *APPa* and *PrP-1* it provides support against the hypothesis that these two proteins interact between cells. Based on expression patterns this is perhaps the most plausible result as APP and PrP are found concentrated at synaptic sites where they are both found on the same pre-synaptic side of the synapse [17-19, 24, 25, 39]. One study that provides evidence for an interaction between APP and PrP involves cross-linking proteins from within specific microdomains surrounding PrP [30]. APP was found among this group of proteins closely associated with PrP on membranes the same cell supporting the hypothesis that an interaction occurring between APP and PrP would likely occur in these microdomains in a cell autonomus fashion [30].

Evidence presented supporting the conclusion that these proteins do not interact across cells will facilitate future research into the domains required and cellular locations of the interaction. Experimental investigations into the interaction can henceforth be conducted within the same zebrafish cells making experiments far more controlled and robust than experiments dependent on the interaction across cells. Taken together with structural information regarding APP and PrP one can also begin to more realistically model the hypothesized interaction taking place within zebrafish cells. Activation or repression of specific pathways within these cells can now also more easily be investigated. The relative ease of zebrafish transgenesis should allow researchers to drive expression of these genes (over-expression) within the same set of cells and begin to examine

what specific pathways are being activated or repressed by this APP:PrP interaction. If indeed over-expression of both genes led to a unique or exacerbated physiological phenotype rescue experiments could be utilized in which MOs against known interactors are used to deduce specific pathways responsible for the effect.

Further investigation into the interaction is still required as aside from a potential lack of mRNA translation a multitude of reasons may account for the lack of increased or decrease cell mixing when cells in which *APPa* and *PrP-1* levels have been reduced (or enhanced via mRNA injection) are mixed together. Genes critical to the interaction may not be expressed at the developmental time points we are analyzing. Phenotypically the greatest effect on development when *APPa* and *PrP-1* levels are reduced is observed at 24hpf, hence these experiments done at only 6hpf may be missing some vital interaction partners. The specific experiments utilized here cannot be performed at these later time points so various other methodologies must be employed. One possible technique highly amenable to the zebrafish is cell transplantation. Cells in which *PrP-1* MO have been injected along with dextran dyes could be removed from blastula stage embryos and transplanted into zebrafish in which *APPa* MO has been systemically injected. Observations regarding the adhesion abilities of these *PrP-1* knock-down cells when bordered with *APPa* knock-down cells during different developmental stages may provide insights into the interaction not possible with the methods contained herein.

Although the evidence presented here indicates a cell autonomous interaction between *APPa* and *PrP-1* mediating cell adhesion, a non-cell autonomous interaction may still take place affecting other cellular functions. The previously noted effect on apoptosis could theoretically result from interactions between *APPa* and *PrP-1* between cells. Similar to the aforementioned experiments, cell transplantation could be used to assess what effects on cellular apoptosis are induced by placing cells with reduced *PrP-1* directly adjacent to cells in which *APPa* levels have been reduced (or over-expressed via mRNA injection). Transgenesis could also be used to over-express *APPa* and *PrP-1* in the same and adjacent sets of cells and compare levels of apoptotic resistance to various apoptotic molecules.

MATERIALS and METHODS:

Morpholino Injections: Three antisense morpholino oligonucleotides obtained from Gene Tools were used during these experiments A splice-blocking morpholino designed to specifically bind the exon-intron boundary of exon 2-intron 2 of the zebrafish *APPa* mRNA (5' TAG TGT TGC TTC ACC TCC TGG CAG T 3'), a previously published [38] translation blocking mopholino designed to specifically bind the 5' UTR of zebrafish *PrP-1* mRNA (5' GCA TAA CTC CCC CAT TTT GGT CCA T 3'), and a standard negative control morpholino obtained from Gene Tools (5' CCT CTT ACC TCA GTT ACA ATT TAT A 3'). All morpholino injection solutions also contained a standardized dose (3ng) of p53 morpholino (5' GCG CCA TTG CTT TGC AAG AAT TG 3') to counteract

any off-target effects of morpholino injection [40]. Injection solutions were made using 1.0uL of 1M KCL, 2.5uL of 0.25% Phenol red, 1.2uL of 25mg/ml p53 MO stock, and gene-specific morpholino to effective (10ug for *APPa*, 10ug for *PrP-1*, 25ug for *APPb*) concentrations, and nuclease-free water to 10uL. One cell embryos were staged on agarose plates, and injection volume calibrated to 1nl using an ocular micrometer immediately prior to injection.

mRNA Injections: *APPa*, *PrP-1*, cDNAs from wild type zebrafish were cloned into a PCS2+ vector (primers in Table 2-1) and linearized with NOT1. Control mRNA was produced by engineering stop codons immediately adjacent to the first coding exon into zebrafish *APPa* mRNA via the Quickchange™ site directed mutagenesis kit using the manufacturer's instructions and primers listed in Table 2-1. Sequencing confirmed successful induction of stop codons. mRNA was produced using the mMessage SP6 kit (Ambion Cat# AM1340). Human APP wt and human APP mt (swe/ind mutations) were provided by the Westaway lab, cloned into PCS2+ vector, linearized with NOTI and transcribed for 3hrs using the mMessage SP6 kit (Ambion Cat# AM1340). mRNA was quantified using a GE NanoVue, electrophoresed on a 1% nuclease free agarose gel to check purity, and stored at -80 until use. mRNA was then dissolved in injection solution without morpholino and optimal dosage determined using a dose response curve. One cell embryos were staged on an agarose plate and injection volume calibrated to 1nL using and ocular micrometer immediately prior to injection.

Cell Mixing Assays: One cell zebrafish embryos were staged and injected with morpholino solutions as mentioned previously (see page 3-8) but containing 2.5uL of 1000x dilute Dextran alexafluor 488 (GFP) or 555 (RFP) (Invitrogen Cat#s, D1817, D22910) in place of phenol red. Embryos were incubated for ~6hrs at 28.5^{o}C until dome stage was reached. Embryos were then manually dechorinated and placed on agarose plates containing 10ml E3 media with 50ul (1:200) Penicillin/Streptomycin antibiotic. Yolk was manually removed from the cells using insect pins and yolk free cells were placed in a 1.5mL microcentrifuge tube containing 1mL Ca^{2+} free Ringers solution (116 mM NaCl, 2.9 mM KCl, 5.0 mM HEPES, pH 7.2). Cells were dissociated by pipetting constantly with a 200uL pipette for 30 seconds. A portion of cells were kept at this point to check that dissociation had occurred. Cells were centrifuged at 550xg for 30 seconds and supernatant removed. 600uL High Ca ringers solution (116 mM NaCl, 2.9 mM KCl, 10 mM CaCl2, 5 mM HEPES, pH 7.2) was then added to cells. 200ul Aliquots from different treatments were then mixed in a 1.5ml microcentrifuge tube and cells incubated at 28.5^{o}C for 1hr. 200ul aliquots were then placed in wells of a 96 well plate (BD falcon) and imaged on a GE IN CELL Analyzer 2000 machine. Exposure and brightness were manually adjusted and 18 images/well were taken at 20x magnification (9 on each fluorescent channel).

Cell Mixing Data Analysis: Images were analyzed in GE Investigator software. Images were first pre-processed to equalize brightness and contrast both between wells and between the RFO (555) and GFP (488) channels. The average size of a

single cell was then calculated. The two fluorescent channels were merged into one image and target segmentation function used to define individual cells and aggregates of cells according to size (aggregates defined as overlapping fluorescent signals 3x as large as a single cell). Once all aggregates had been determined the image was again split into two channels and the number of RFP (555) or GFP (488) fluorescent cells within these previously determined aggregates was counted. The total numbers of RFP and GFP expressing cells within a well were also counted. The data was transferred to Microsoft Excel and for statistical reasons a threshold was set to only consider aggregates that contained 10 or more cells. The percentage of RFP (555) and GFP (488) cells within each aggregate containing more than 10 cells was then calculated. Using data collected by sampling the 9 fields within each given well a mean percentage and standard deviation of 488 fluorescing cells within each well was calculated. Aggregates that contained a percentage of GFP (488) fluorescing cells greater or less than 2 standard deviations away from the mean percentage of GFP (488) fluorescing cells within that well were counted as “homogeneous”. Aggregates that contained a percentage of GFP (488) fluorescing cells within 2 standard deviations of the mean of GFP (488) fluorescing cells within that well were counted as “mixed”. The percentage of “homogeneous” aggregates within a well was then determined.

Figures: All images shown were acquired by the GE IN cell 2000 machine at 20x magnification. All images were re-sized in Photoshop CS5, but are otherwise unaltered.

Statistics: All statistics were performed using the SPSS 17 software. Groups were compared using ANVOA with Bonferroni correction and Tukey post hoc tests and significance set to 0.05.

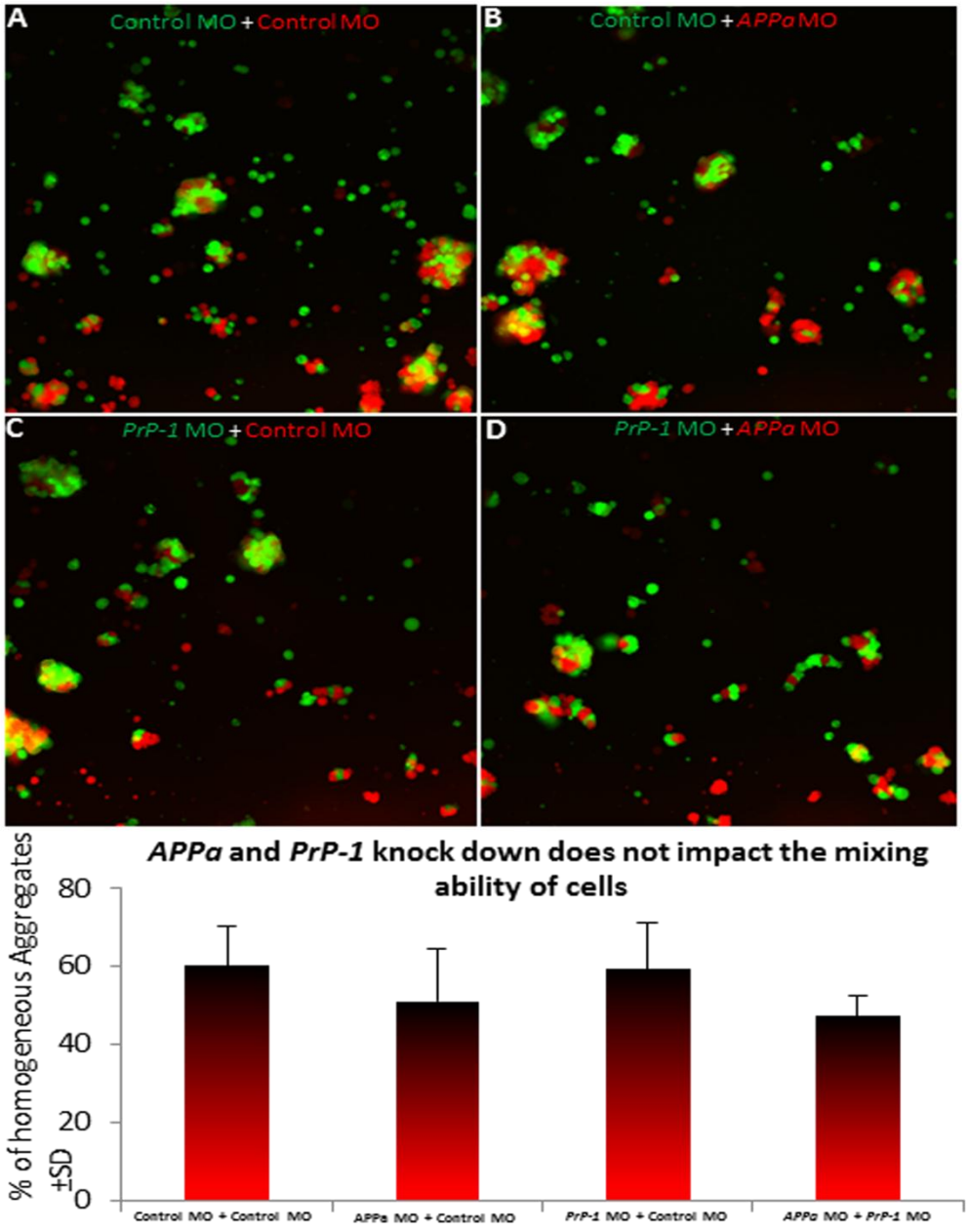

Figure 3-1. Knock down of *APPa* and *PrP-1* does not influence the mixing ability of cells.
A) Zebrafish cells that had been injected with a control MO (red) were mixed with zebrafish cells injected with an identical control MO (green).
B) Cells in which *APPa* MO had been injected (red) were mixed with cells in which a control MO had been injected (green).
C) Cells injected with a *PrP-1* MO (green) were mixed with cells injected with a control MO (red).
D) Cells injected with an *APPa* MO (red) were mixed with cells injected with a *PrP-1* MO (green).
E) The number of aggregates (10 or more cells) that contained a % of GFP fluorescing cells greater than 2 standard deviations away from the mean % of GFP fluorescing cells in that well was quantified. No groups show any significant difference (P>0.05).

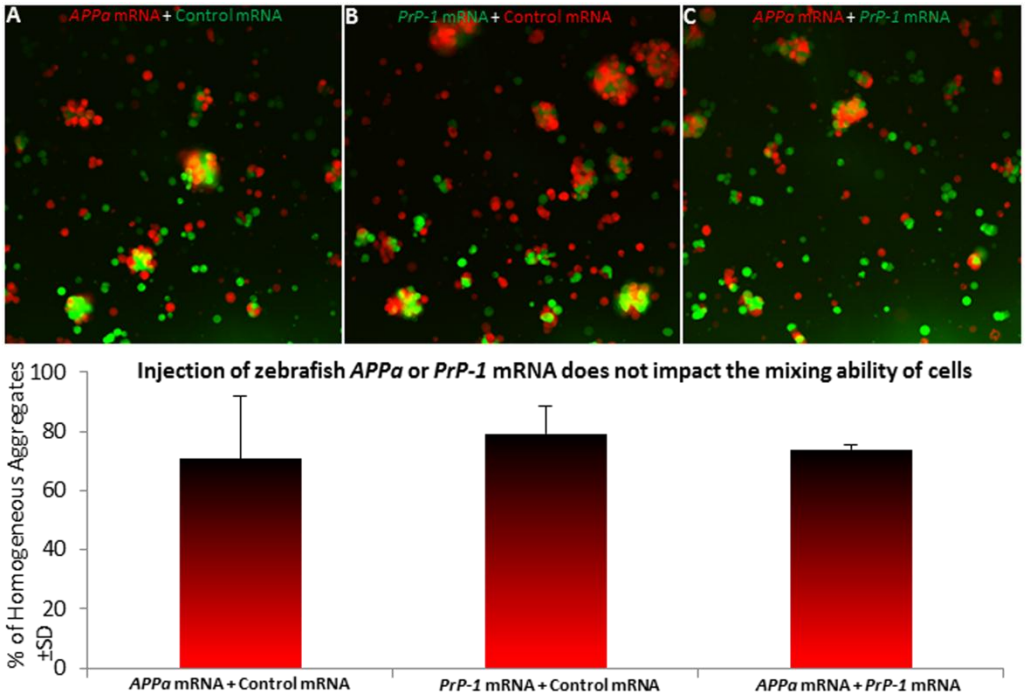

Figure 3-2. Injection of zebrafish *APPa* or *PrP-1* mRNA does not influence the mixing ability of cells.
A) Zebrafish cells that had been injected with cognate *APPa* mRNA (red) were mixed with zebrafish cells injected with a control mRNA (green).
B) Cells in which *PrP-1* mRNA had been injected (green) were mixed with cells in which a control mRNA had been injected (red).
C) Cells injected with *APPa* mRNA (red) were mixed with cells injected with *PrP-1* mRNA (green).
D) The number of aggregates (10 or more cells) that contained a % of GFP fluorescing cells greater than 2 standard deviations away from the mean % of GFP fluorescing cells in that well was quantified. No groups show any significant difference (P>0.05).

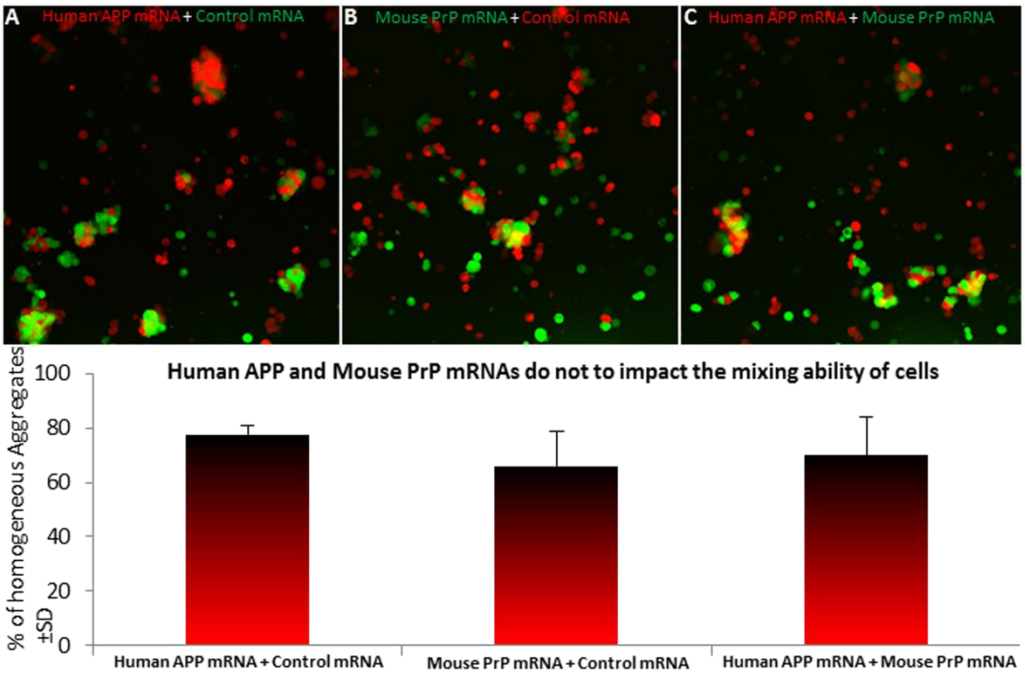

Figure 3-3. Injection of human APP and mouse PrP mRNAs does not influence the mixing ability of cells.
A) Zebrafish cells that had been injected with human *APP* mRNA (red) were mixed with zebrafish cells injected with a control mRNA (green).
B) Cells in which mouse *PrP* mRNA had been injected (green) were mixed with cells in which a control mRNA had been injected (red).
C) Cells injected with human APP mRNA (red) were mixed with cells injected with mouse PrP mRNA (green).
D) The number of aggregates (10 or more cells) that contained a % of GFP fluorescing cells greater than 2 standard deviations away from the mean % of GFP fluorescing cells in that well was quantified. No groups show any significant difference ($P>0.05$).

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CHAPTER FOUR

CONCLUSIONS and FUTURE DIRECTIONS:

Our Study is the first to provide evidence that knock down of *APPa* protein within the zebrafish has deleterious consequences in CNS development, maintenance, and cellular aggregation. We show apoptosis is increased when *APPa*, *APPb*, or *PrP-1* genes are knocked down, or sub-effective knock down of *APPa* and *PrP-1* is combined. We are the first to provide evidence for a specific *in vivo* genetic interaction between the *APPa* and *PrP-1* genes that may play a role in cellular apoptosis and adhesion. We show that human APP orthologs and mouse PrP orthologs are able to replace *APPa* and *PrP-1* in this genetic interaction highlighting the conservation of genes inherent to the zebrafish model. By employing our cell mixing paradigm we are able to show that not only do these genes genetically interact, but they do so in a cell autonomous fashion.

Based on the time course observed (cell death most apparent at 24hpf) and the observation of apoptotic induction it appears *APPa* knock down induces death of already differentiated neurons. Prior reports indicate that APP may have an anti-apoptotic role in neurons [1-3] and by reducing levels of *APPa* in the zebrafish we are possibly eliminating this repression of cell death and inducing apoptosis. *APPa* could potentially sequester some pro-apoptotic protein or activate some anti-apoptotic protein. A genetic strategy could prove useful in answering this question as sub-effective knockdown of *APPa* could be combined with sub-effective knockdown of known anti-apoptotic proteins such as BCL-XL and BCL-2 to see if a synergistic effect on cell death occurred, or levels of pro-

apoptotic molecules could be assessed via immunohistochemistry following *APPa* knockdown.

In the time following our MO design, *APPb* knockdown with distinct MO reagents has been shown to affect zebrafish development including convergent-extension movements associated with cell adhesion [4]. Our results lie in line with this conclusion, as our *APPb* knock down fish presented with systemic apoptotic induction (Fig.2-5), and reduced cell adhesion (Fig.2-10). However, our data contrasts that of Joshi et al, (2009) where *APPa* MO had little effect on development. We attribute this difference to the dose and type of morpholino reagents used, which are designed very differently in the two studies. These differential results may be due to complete blockage of *APPa* translation activating some compensatory pathway that is not activated by our splice-blocking MOs. Western blots presented in the manuscript do not seem to differentiate between *APPa* and *APPb* proteins so it also remains possible that their western blot results only show reductions in *APPb* protein levels and that their *APPa* translation blocking MO was ineffective.

In 2009 Malaga-Trillo *et al* reported a phenotype when zebrafish are injected with a translation blocking MO against *PrP-1* mRNA. At high doses this MO caused embryonic arrest at the shield stage (6hpf), which was reversible by co-injection of zebrafish *PrP-1* mRNA [5]. Western blots by Malaga-Trillo et al, 2009 further confirmed effective *PrP-1* knock down using this MO. We chose to

look at zebrafish embryos injected with a lower dose of this *PrP-1* MO at a different developmental stage and observed that not only does reduced levels of *PrP-1* effect early embryonic development it precipitates a CNS wide induction of apoptosis (Fig.2-3,2-5). Our cellular aggregations assays also corroborate results from Malaga-Trillo et al (2009) indicating that when *PrP-1* levels are reduced there is a reduction in zebrafish embryonic cells ability to adhere to one another (Fig.2-10). As PrP is a GPI anchored protein with a propensity to form homodimers [6], it is possible these homodimers form across cells and aid cells in adhering to one another. By reducing levels of *PrP-1* we may be reducing the number of these homodimers and effectively reducing cellular adhesion throughout the CNS. PrP has also been shown to bind multiple cell adhesion molecules [7-9] and perhaps when *PrP-1* levels are reduced these interactors are unable to function in an adhesion role leading to disruption of migration and adhesion of CNS cells. There is a fairly narrow list of adhesion proteins known to interact with PrP so via MO injection one could begin to genetically test these adhesion proteins for an inability to synergize with *PrP-1* knockdown. If knockdown of an adhesion protein was unable to further reduce adhesion in the context of *PrP-1* knockdown it would be a good indication that that adhesion proteins is working through an interaction with *PrP-1*. If knockdown of all known interacting adhesion proteins were to synergize with *PrP-1* knockdown it would lend support to the hypothesis that *PrP-1* homodimers are contributing to cell adhesion. The experiment may contain a large degree of background interference

due to the promiscuous nature of most adhesion proteins, but perhaps a significant effect could be discerned.

The expression of zebrafish *APPa* and *APPb* mRNAs overlaps to a large degree [10]. Both are apparent in the CNS as early as 2.5hrs and their expression patterns overlap to a large extent as the fish matures [10]. The *APPa* and *APPb* genes exhibit a large degree of sequence identity showing approximately 80% identity at the nucleotide level. Our genetic tests indicate synergy between knockdowns (Fig.2-6), and the ability of mRNA from one APP to alleviate the phenotype caused by lack of the other APP (Fig.2-6). Taken together we concluded there is significant functional overlap between the two APP genes within the fish. At specific time points beyond the scope of our investigation the expression of *APPa* and *APPb* do seem to deviate from one another [10]. It would be useful to examine what effects knock down of *APPa* and *APPb* have at these developmental time points and if physiological defects can be attributed to one gene or the other. If specific phenotypes became apparent one could then attribute these effects to interactions with APP in specific cells of the zebrafish or possibly regions of the one gene that is not shared with the other. Findings of either nature would further help to elucidate functions of specific portions of the APP gene.

Our genetic testing revealed a significant interaction between the zebrafish *APPa* and *PrP-1* genes. When sub-effective doses of MO that produced no phenotype on their own were combined there was an emergence of a very strong

developmental phenotype. This phenotype was comprised of small body size, malformed CNS structures, and systemic induction of apoptosis (Fig.2-7,2-8). Although *APPa* and *APPb* are largely able to replace each other during early zebrafish development (Fig.2-6), this appears not true in the context of reduced *PrP1* (Fig.2-7). The interaction also proves specific to *PrP-1* as cognate *PrP-2* mRNA was unable to reduce the phenotype observed by combined sub-effective knock down of *APPa* and *PrP-1* (Fig.2-7). These data form the first evidence of a genetic PrP:APP interaction *in vivo*, and confirm the specificity of our reagents with a battery of rescue experiments showing cognate *PrP-1* or *APPa* mRNA is able to efficiently rescue this synergistic phenotype while cognate mRNAs of a similar nature (*APPb, PrP-2* and *Sho-1*) are not able to alleviate the phenotype. The precise nature of the interaction cannot be ascertained from studies disrupting mRNA transcription alone as three possibilities are present; the gene products from *APPa* could directly physically interact with *PrP-1* gene products and the signalling cascade mediated by this physical interaction could play a role in neuronal development or maintenance, *APPa* and *PrP-1* signals individually could converge on the same signalling cascade with the endpoint of this cascade having some effect on neuronal cell viability, or both options could theoretically occur. Based on interactome studies cell adhesion proteins shown to interact with both APP and PrP include NCAM1, L1CAM, Neurofascin, and Contactin [9, 11, 12]. These interactors common to both proteins may bring to light to the normal role of these proteins in cell adhesion. Rescue experiments using mRNA derived from the aforementioned proteins may provide insight into which pathway may be

affected by the sub-effective knock down of both genes. Microarrays on fish injected with *APPa* and *PrP-1* MOs could also help decipher pathways common to both genes that may be involved in precipitating these phenotypes.

The finding that *APPa* and not *APPb* functionally interacts with *PrP-1* immediately brings to light many interesting and testable questions about the interaction. It first must be determined if the lack of interaction between *PrP1* and *APPb* is due to some physical aspect of the *APPb* protein that is not shared with *APPa*, or if some spatial restriction prevents *APPb* from coming into contact with *PrP-1*. Based on expression patterns [10] it seems unlikely that a tissue specific spatial restriction is the reason for the lack of interaction, as in the CNS *APPb* expression overlaps to a high degree the expression of *APPa* [10]. Rescue experiments with cognate *APPb* mRNA also provide evidence that a tissue specific spatial restriction is not the reason there is no interaction between *APPb* and *PrP-1* as *APPb* mRNA when directly injected into one cell embryos, which should theoretically permeate all cells of the embryo thus negating specific expression, is unable to rescue the phenotype. There remains however the possibility that *APPa* and *APPb* are restricted to different intracellular compartments that preclude the interaction between *APPb* and *PrP-1*, future work involving elucidating the intracellular distribution of *APPa* and *APPb* in the zebrafish would help satisfy this argument. Future co-localization experiments would also strengthen our findings regarding the specificity of the *PrP-1* interaction with *APPa.* If a physical interaction were occurring different deletion

mutants of *APPa* in domains not shared with *APPb* could potentially provide a physical site of *PrP-1* interaction on the *APPa* protein. As the *APPa* protein within the fish shares such a high degree of homology to mammalian APP and rescue experiments proved successful with human mRNAs these results could likely translate well into to a mouse model or human cell culture model.

It would be interesting to determine if there are any subsets of neurons that are more susceptible to apoptosis following a reduction in APP or PrP within the CNS. One attractive candidate is the cholinergic neurons as PrP^c and APP expression closely follow the maturation of these neurons and choline acetyltransferase levels [13, 14]. Perhaps an interaction between APP and PrP within these cholinergic neurons plays some pivotal role and during AD progression this interaction is disrupted rendering these neurons highly susceptible to degeneration. This subset of neurons could be stained with specific antibodies, along with an activate caspase 3 antibody, and one could then determine the average number of caspase 3 positive cells within this cholinergic subset. Elucidation of a specific subset of neurons highly sensitive to perturbations in the APP:PrP interaction may provide an environment in which to better focus on answering questions of the interactions cellular significance.

Prior reports [4] indicated that the Swedish mutant form of human APP has a different biological activity *in vivo* than wild type APP. In our study both human mRNAs (wt and mt) were able to efficiently rescue both the observable phenotype

caused by co-injection of sub-effective doses of *APPa* and *PrP-1* MOs (fig 5) and the amount of activated caspase 3 labeling above the yolk sac extension (fig 5). Technical variables such as doses of mRNA may account for this discrepancy or factors such as the extra Ind mutation in APP, and that we were reversing a phenotype due to *APPa* and *PrP-1* not *APPb* alone. As *APPb* was shown to lack interaction with *PrP-1*, it is possible that the mutant form of human APP used is able to replace some specific functions of *APPa* that are not inherent to *APPb*. Overall our work points to a maintenance or anti-apoptotic role for the *APPa* and *APPb* genes as described previously [15, 16] and shows that the biological activity of human APP harboring the Swe and Ind mutations is nearly equivalent to that of wild type human APP in terms of replacing *APPa* in zebrafish.

Although evidence has been published that indicates a physical interaction between APP and PrP, no research up to this point had been conducted regarding the nature of this physical interaction. We attempted to gain some insight in the nature of the interaction by conducting a variation of the aggregation assay we call the cell mixing assay [17]. In this assay we first examined the ability of cells with reduced *APPa* or *PrP-1* levels to aggregate to one another. Our results indicated that knock down of *APPa* in one set of cells and *PrP-1* knock down in a second set of cells does not influence the ability of those cells to adhere to one another. Cells in which *APPa* levels had been reduced were just as likely to interact with cells injected with a control MO as cells in which *PrP-1* levels had been reduced. The corollary experiment was also conducted in which cognate

zebrafish *APPa* and *PrP-1* were over-expressed via injection of mRNA. Similar results were obtained using this method, over-expression of *APPa* in one set of cells made no impact on those cells ability to adhere to other cells in which a control mRNA or cognate zebrafish *PrP-1* mRNA had been injected. There was no increased adherence when cells over-expressing *APPa* were mixed with cells over-expressing *PrP-1*. Along a similar vein when the experiment was tried using human APP and mouse PrP mRNAs no difference in adherence ability of cells was noted. These results support the hypothesis that the interaction between these two proteins occurs on or within the same cell. This analysis does not provide evidence for or against a physical interaction between these two proteins hence further investigation into this hypothesis is required. Co-Immunoprecipitations done with *APPa* and *PrP-1* specific antibodies would provide evidence for or against a physical interaction between these two proteins. Likewise, Immunohistochemistry could be used to determine localization of PrP and APP within the cell and on cell membranes possibly providing support for or against a physical interaction. Chip based protein-protein interaction studies would be yet another way to examine the binding partners of PrP and APP proteins [18]. A study of this type would not only give information regarding any interactions between APP and PrP, but could also be used to confirm or refute binding partners found through other methods.

Multiple technologies have become recently available that would facilitate the investigation into the interaction between APP and PrP. The ease of zebrafish

transgenesis along with protein-fragment complementation assay (PCA) technology could be used in live zebrafish to examine to what extent APP and PrP interact when expressed in the same vs adjacent cells. PCAs are based on each of two binding partners containing to one rationally designed fragments of a reporter protein [19]. Separately the fusion proteins do not fluoresce, but when the binding partners come into contact the two fragments of the reporter can associate and leading to a detectable fluorescence signal [19]. A portion of GFP could be engineered into the zebrafish PrP1 protein and the other portion could be engineered into the zebrafish APPa protein. By comparing the fluorescence when these two fusion proteins are both expressed under the control of the same or a single promoter (ie Enolase 2) vs intensities when one is expressed under the neuronal Enolase 2 promoter and one under the control of the glial fibrillary acidic protein (GFAP) promoter one could get a relative idea of the interaction between these proteins within and between cells. The opsin promoters could also be used to drive expression of APPa-split GFP in one set of photoreceptors and PrP1-split GFP in an adjacent set of photoreceptors. When compared to GFP levels when both APPa-split GFP and PrP1-split GFP are expressed in the same photoreceptors one could get an indication of the level of APP:PrP interaction within and between cells.

This work began with the hypothesis that APP and PrP interact in some fashion within the zebrafish. Remarkably fruitful experiments that followed uncovered three novel, deleterious phenotypes due to *APPa*, *APPb*, and *PrP-1*

knock down in the zebrafish that involved apoptotic induction and reduced cellular adhesion. A surprising genetic interaction between *APPa* and *PrP-1* revealed itself and further experiments support the notion that this interaction occurs in a cell autonomous fashion. A multitude of experiments still lie ahead, but hope remains that one day these results can prove useful in the design or screening of therapeutics to help people who are and will be afflicted with these terrible conditions.

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