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The Role of Lipoproteins/cholesterol in Genomic Instability and Chromosome Mis-segregation in Alzheimer's and Cardiovascular Disease

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The Role of Lipoproteins/Cholesterol in Genomic Instability and Chromosome Mis-segregation in Alzheimer's and Cardiovascular Disease

by

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DEDICATION

Dedicated to Toni, the light of my life.

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ABSTRACT

Several lines of evidence link Alzheimer's disease (AD) to atherosclerosis (CVD), including that elevated low density lipoprotein (LDL)-cholesterol is a common risk factor. Development of genomic instability could also link the two diseases. Previous fluorescence in situ hybridization (FISH) analyses revealed a clonal expansion of aneuploid smooth muscle cells underlying atherosclerotic plaques. Likewise, cellular and mouse models of AD revealed tau-dependent mitotic defects and subsequent aneuploidy partly resulting from amyloid-beta (Aβ) interference with microtubule (MT) stability, and specific MT motors function. Moreover, AD patients develop aneuploid/hyperploid cells in brain and peripheral tissues, implicating similar mechanism that may lead to apoptosis and neurodegeneration.

This dissertation tested the hypothesis that elevated lipoproteins and cholesterol may contribute to genomic instability in AD and CVD and showed that: (1) treatment with oxidized LDL (OX-LDL), LDL and water soluble cholesterol, but not high density lipoprotein (HDL), induced chromosome mis-segregation, including trisomy and tetrasomy 12, 21, and 7 in human epithelial

cells (h-TERT-HME1), primary aortic smooth muscle cells, fibroblasts, mouse splenocytes and neural precursors; (2) LDL-induced aneuploidy may depend on a functional LDL receptor (LDLR), but not amyloid precursor protein (APP) gene; (3) fibroblasts and brain cells of patient with the mutation in the Niemann-Pick C1 gene (NPC1) characterized by impaired intracellular cholesterol trafficking and changed intracellular cholesterol distribution harbored trisomy 21 cells; (4) young wild-type mice fed high and low cholesterol diets developed aneuploidy in spleen but not in brain cells within 12 weeks; (5) like with the studies on Aβ-induced aneuploidy, calcium (Ca^{2+}) chelation reduced OX-LDL and LDL-mediated chromosomal instability; and (6) altering plasma membrane fluidity with ethanol attenuated OX-LDL and LDL-induced aneuploidy.

These results suggest a novel biological mechanism by which disrupted cholesterol homeostasis may promote both atherosclerosis and AD by inducing chromosome mis-segregation and development of aneuploid cells. Understanding the cause and consequence of chromosomal instability as a common pathological trait in AD and CVD may be beneficial to designing therapies relevant for both diseases.

CHAPTER ONE

INTRODUCTION[1]

The accurate transmission of genetic material from a mother cell to the daughters requires not only a faithful replication of the genome, but also a precise segregation of chromosomes during the cell division. To accomplish this remarkable process, the cell has to disentangle replicated DNA molecules and pull them accurately to the opposite poles of the mitotic spindle before completing cytokinesis (Figure 1). In eukaryotic, including mammalian cells, the cohesion between sister chromatids, a duplicated set of chromosomes, is sustained long after DNA replication, which allows for successful completion of mitosis (M). During the M phase of the cell cycle, microtubules (MT), highly dynamic protein polymers of the mitotic spindle, attach the kinetochores of the sisters chromatids to opposite spindle poles and drive chromosome movement and traction (reviewed in Morgan, 2007). A tightly controlled interaction between activating and inhibiting factors of the cell cycle assures chromosomal stability and integrity (Draviam et al., 2004), whereas dysregulation of the genes that

[1] Portions of the results in this chapter have been published (Granic et al., 2010) and are utilized with the permission from the publisher. The contributions of the authors are: A. Granic, experimental designs and implementation, data collection and analysis, and manuscript preparation; J. Padmanabhan, important scientific insights for the manuscript, M. Norden, mice genotyping and assistance with the surgical procedures, and H. Potter experimental designs and manuscript preparation.

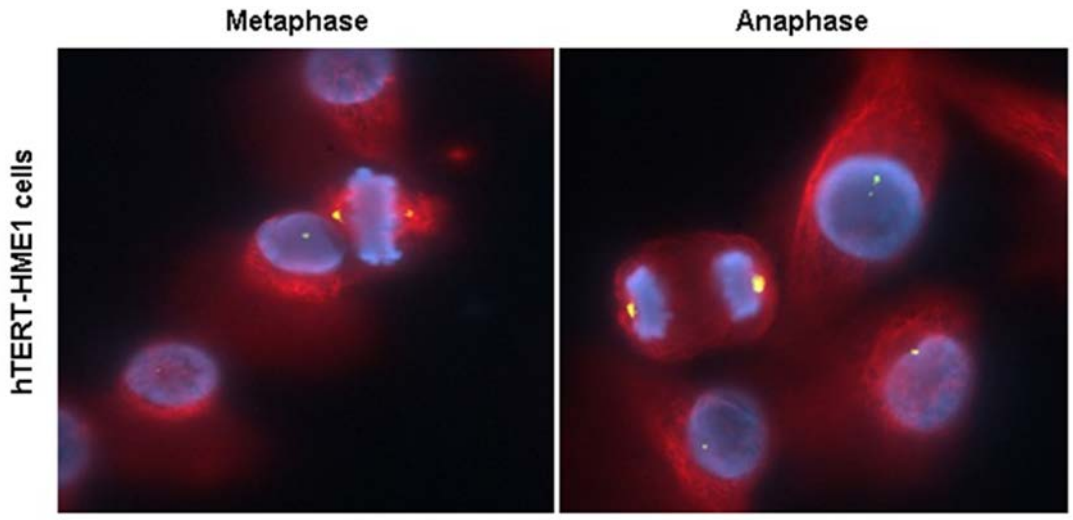


Figure 1. Metaphase to Anaphase Transition in hTERT-HME1 Cells
Immortalized human epithelial cells were stained for mitotic spindle (α-tubulin, red), centrosomes/spindle poles (γ-tubulin; green) and nuclear DNA (DAPI, blue) to visualize the transition through the M phase of the cell cycle. Granic and Potter, unpublished data.

govern the spindle checkpoints (Bharadwaj and Yu, 2004; Kinzler and Voglstein, 1997) and DNA repair result in various genomic alternations, including mutations, chromosomal rearrangements, deletions, inversions and aneuploidy (Draviam et al., 2004; Rao et al., 2009). Despite numerous cytogenetic pathways that ought to preserve this fundamental principle of life, spontaneous and induced chromosome mis-segregation happens frequently in humans during normal and pathological conditions (Cimini et al., 1999; Rasnik, 2000; Peterson et al., 2008; Rao et al., 2009; Rehen et al., 2005; Shi et al., 2000; Wojda et al., 2007).

Aneuploidy, a numerical abnormality of chromosomes compared to the normal genome (King and Stansfiled, 1990) arises as a consequence of mitotic errors (Kops et al., 2005; Rao et al., 2009) and has been linked to (1)

tumorigenesis of solid tumors (Boveri 1929; reviewed in Duesberg and Rasnik, 2000; Jefford and Irminger-Finger, 2006; Ried 2009); (2) spontaneous abortions and embryonic lethality (Lebedev et al., 2004; Ljunger et al., 2011); (3) etiology of genetic diseases with abnormal phenotype such as Down syndrome (DS) (reviewed in Hassold and Sherman, 2000; Nižetić, 2008); (4) aging (Aviv et al., 2001; Carere et al., 1999; Faggioli et al., 2011; Ly et al., 2000; Vijg and Dolle, 2007); (5) atherogenesis (Matturri et al., 1997; 2001); (6) neurogenesis (Peterson et al., 2008; Rehen et al., 2005; Yang et al., 2003); and (7) neurodegeneration and dementia (Arendt et al., 2010; Potter, 2004). Mitotic division of an aneuploid cell is believed to be an evolutionary experiment resulting in cellular diversity and constant genomic redistribution (Rasnick, 2000), but also a phenomenon characterizing or contributing to the disease process.

This chapter will focus on genomic instability and chromosomal aneuploidy in normal and diseased human brain, the functional consequence and relevance thereof, the theoretical foundation for the role of altered gene dosage and abnormal cell cycle in dementia of the Alzheimer's type, cancer, and cardiovascular disease (CVD).

GENOMIC INSTABILITY IN NORMAL HUMAN BRAIN

Ever since the numbers of human chromosomes have been determined (2n = 46; Tjio and Levan, 1956), numerous molecular cytogenetic studies reported genomic imbalance and the presence of euploid and aneuploid cells (i.e.,

mosaicism) in various pathogenic and nonpathogenic human conditions. The cells from normal tissues have been assumed to contain identical genomes in which aging, environmental and endogenously-induced insults may compromise the accuracy of mitotic machinery, initiating or causing the diseased state (Aviv et al., 2001; Beetstra et al., 2005; Benassi-Evans and Fenech, 2011; Mukherjee and Thomas, 1997; Ried, 2009). In addition, higher cellular proliferation rates of renewable tissues in a complex human organism during embryogenesis (Biancotti et al., 2010; Mantzouratou and Delhanty, 2011), neurogenesis (Yurov et al., 2005; 2007; Westra et al., 2008), tissue development and cell differentiation put at risk tightly regulated mitotic checkpoints, often resulting in chromosome mis-segregation and aneuploidy (i.e., aneugenesis) (Draviam et al., 2004). Primary neurons in the normal human brain have been traditionally viewed as post-mitotic and in G0 (Obrenovich et al., 2003), and thus genetically stable. However, with advancements in cytogenetic techniques (Figure 2) from the classic karyotype analysis of colcemid-arrested cells at metaphase (Barnicot and Huxley, 1961) to more sophisticated examination of dividing and post-mitotic cells via fluorescent DNA probes to target a part of a chromosome in interphase cells (i.e., fluorescent in situ hybridization, FISH; Pinkel et al., 1986) or different chromosome paints to visualize one or all chromosomes in dividing cells (i.e., spectral karyotyping, SKY; Schröck et al., 1996; Speicher et al., 1996), the mosaic nature of developing and adult normal mammalian, including human brain has been identified (Rehen 2001; 2005; Peterson et al., 2008; Yurov et al., 2005; 2007).

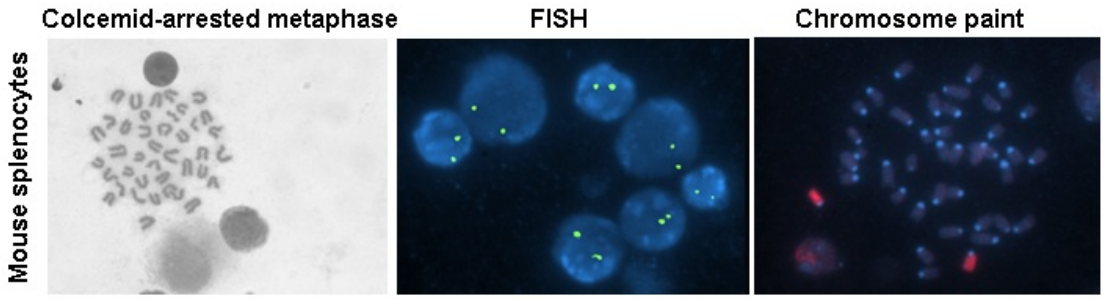


Figure 2. Examples of Cytogenetic Methods for Analysis of Chromosome Complements in Metaphase and Interphase Cells
Chromosomes in dividing cells can be visualized and analyzed via colcemid-arrested metaphase spread (left panel), FISH with fluorescent DNA probe (middle panel), and chromosome paint (right panel). Granic and Potter, unpublished data.

Mosaic Nature of Neurogenesis

For almost a century, a central dogma in neuroscience (Ramon y Cajal, 1913; Rakic, 1985) viewed neurogenesis as a process confined to the embryonic stage of mammalian development, and only recently a series of studies in various animal models and human brain samples confirmed that new neurons are indeed being born in distinct regions of the adult brain throughout life (reviewed in Christie and Cameron, 2006; Gross, 2000; Kempermann and Gage, 2000; Ming and Song, 2005; Zhao et al., 2008). Adult neurogenesis, a continuous generation of functional neurons in the dentate gyrus, olfactory bulb and neocortex from the neural precursor cells (NPCs) occurs via mitosis (Eriksson et al., 1998; Christi and Cameron, 2006) adding thousands of new neurons to the brain per day (Cameron and McKey, 2001; Hastings et al., 2000), which become an integral part of existing neuronal circuits (Carleton et al., 2003; Kingsbury et al., 2005; Van Praag et al., 2002).

Methodological limitations of the techniques used to identify neurogenesis (e.g. incorporation of the synthetic thymidine into mitotically active cells indicates DNA synthesis and not necessarily cell division, and neuronal-specific markers cross-react with other brain cells or do not detect all types of neurons) (Cristie and Cameron, 2006; Ming and Song, 2005; Rakic, 2002) suggest that the renewal of the brain tissue may be happening at higher rates and in other brain regions than has been reported. In addition, several modulators of adult neurogenesis have been recognized (Ming and Song, 2005). Unfavorable conditions such as exposure to acute and chronic stress and aging may decrease (Kuhn et al., 1996; Gould and Tanapat, 1999; Jin et al., 2003), whereas environmental complexity (Brown et al., 2003) and learning of hippocampal-dependent tasks, may increase the proliferation of neuronal cells in the dentate gyrus (Gould et al., 1999; Leuner et al., 2004). Accumulated evidence has shown that chronic neurodegenerative diseases (e.g., Huntington's and Alzheimer's disease; Curtis et al., 2003; Jin et al., 2004) and brain injury (Magavi et al., 2000) trigger a significant increase in neuronal marker-positive cells, suggesting an induction of brain's self-repair mechanism under pathologic stimuli.

Advanced cytogenetic analyses by FISH and SKY have provided new insights into the nature of the developing and adult mammalian brain. Numerous chromosomal aberrations in the embryonic cerebral cortex, postnatal ventricular zone (VZ) and other adult cortical regions of normal mouse and human brains were observed among neuronal stem cells, NPCs and mature neurons (e.g., Kaushal et al., 2003; Kingsbury et al., 2006; Peterson et al., 2008; Rehen et al.,

2001; 2005; Yang et al., 2003; Yurov et al., 2005; 2007; Westra et al., 2008), which were functionally active and had been incorporated into the present neuronal circuitry (Kingsbury et al., 2005). Up to 33% metaphases cultured from mouse embryonic neural cells exhibited both loss and gain of autosomal and sex chromosomes (Rehen et al., 2001), and about one-third of postnatal NPCs that give rise to neurons and glia were aneuploid for more than one chromosome (Kaushal., et al., 2003). Analyzing mitotic defects in prenatal cortical hemispheres by co-immunolabeling for histone H3 to detect chromosomes and for vimentin, a cytoplasmic marker of NPC, Yang et al. (2003) identified 4.6% of NPCs with lagging chromosomes, 3.2% with multipolar cell division events, and various aneuploidies by SKY. The rate of aneuploidy per mouse chromosome in developing brain may be estimated as high as 1.65% assuming that every chromosome has an equal chance to be gained or lost (Faggioli et al., 2011). Similarly, application of multicolor interphase FISH with DNA probes for the chromosomes exhibiting a frequent mosaic autosomal aneuploidy in humans (i.e., chromosome 13, 18, and 21; Iourov et al., 2006) on isolated fetal brain nuclei revealed between 30-35% of aneuploidy or estimated aneuploidy rate of 1.25-1.45% per chromosome (Yurov et al., 2005; 2007). Taken together, the data indicates that genetic mosaicism or co-existence of euploid and aneuploid cells is a universal feature of the developing mammalian brain.

Several hypotheses have been put forth to explain the susceptibility of the central nervous system (CNS) to genomic instability, aneuploidy generation (i.e., aneugenesis) and the phenotypic benefits thereof (Dierssen et al., 2009).

Extreme brain complexity manifested by cellular diversity, lateral asymmetry, regional interconnection of specialized microdomains, functional and structural plasticity, and enhanced responsiveness to environmental stimuli and cues make the brain more sensitive to genomic variations (Gericke, 2008) compared to other organs. Chromosome mis-segregation in neural precursors and consequent genetic mosaicism could be a mechanism for biological variability and diversity in neurons contributing to the functional complexity of the brain and differences in intellectual abilities, personality, and psychiatric diseases in humans (Muotri and Gage, 2006). Neuronal genomic heterogeneity as a universal organizational and functional property of developing and adult brain may play a role in the late-onset neurodegenerative diseases if the level of aneuploidy exceeds a physiologically sustainable threshold and the mechanisms of aneuploidy clearance fail (Arendt et al., 2009; Potter, 2004). Increased apoptosis of aneuploid neuroblasts during cortical development (Rehen et al., 2001) suggests an inherent susceptibility to the cell death and/or decreased proliferative potential (Blaschke et al., 1996; Kai et al., 2009; Williams et al., 2008).

Advancements in neuroscience in the past couple of decades challenged two scientific dogmas: (1) the adult brain is not capable of renewal, and (2) normal neural cells possess identical genomes. However, complete understanding of the mechanism, extent, regional distribution and the role of both neurogenesis and aneugenesis under normal and pathological conditions in developing and mature human brain remains to be explored.

Mechanism of Aneuploidy Generation

Numerous tightly regulated processes and checkpoints throughout mitosis should ensure equal chromosome segregation and accurate separation of synthetized genetic material into two daughter cells (reviewed in Morgan, 2007). Molecular biology and cytogenetic studies using mostly human cancer cells revealed

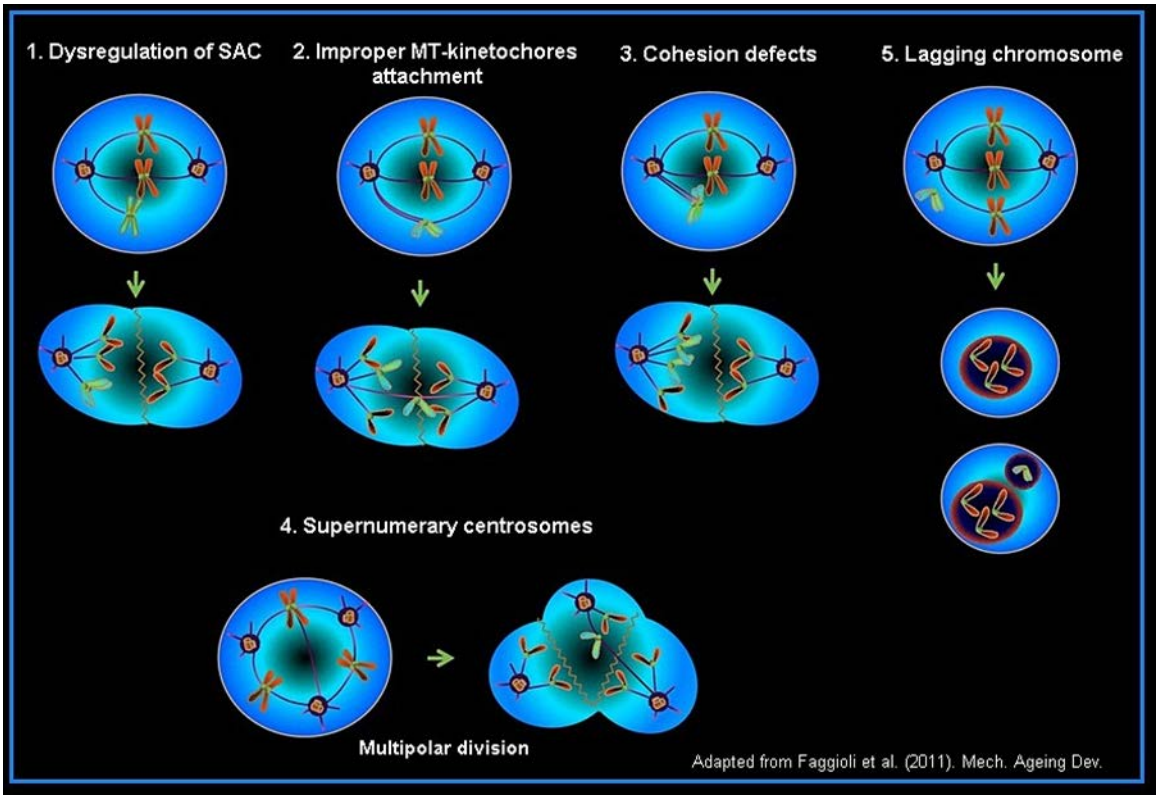


Figure 3. Mechanisms of Aneuploidy Generation
During the separation of sister chromatids, several pathways may lead to gain or loss of chromosomes such as: (1) dysregulation in the spindle assembly checkpoints (SAC) genes in which one daughter cell gets both chromosome copies after cell division; (2) improper attachment of microtubules and kinetochores to spindle poles (e.g., one kinetochore attached to both spindle poles); (3) incorrect cleavage of cohesin; (4) multiple spindle poles leading to asymmetric cell segregation; and (5) lagging chromosomes left after anaphase may result in one or both daughter cells to be hypoploid.

several pathways that lead to aneugenesis: (1) dysregulation of spindle assembly checkpoint (SAC) genes; (2) improper cross-linking of microtubules and kinetochores; (3) sister chromatid cohesion defects; (4) supernumerary centrosomes; and (5) incomplete cytokinesis (Chi and Jeang, 2007; Gisselsson, 2011; King, 2008; Yang et al., 2003). These mechanisms of neoplastic aneuploidy generation and persistence of chromosomal instability in tumors may also explain chromosomal aberrations in the normal CNS (schematically presented in Figure 3).

The SAC is a surveillance system responsible for delaying the separation of the sister chromatids and progression into the anaphase until all chromosomes are properly oriented across the mitotic plate and attached on the mitotic spindle via kinetochores (Ito and Matsumoto, 2010; Straight, 1997). Dysfunction and epigenetic modification of the signaling within the SAC (e.g., in the Mad and Bub protein family) can result in a mother cell with mis-aligned or unattached chromosomes proceeding with chromosome segregation towards opposite spindle poles, producing a daughter cell with both chromosome copies. Furthermore, inhibition of mitotic spindle motors such as kinesins, a group of proteins that aid connecting antiparallel MT and segregation of spindle poles resulted in massive aneuploidy in cultured cells (Borysov et al., 2011).

Proper cleavage of cohesin, a complex of four proteins that holds duplicated DNA molecules together from the S phase through the metaphase is the central event in chromosome segregation (Nasmyth, 2001; Uhlmann, 2003). Experimental evidence in cellular and animal models has indicated that both

defects in expression of cohesin (Barbero, 2011) and cohesin protease separase leads to mitotic errors, aneuploidy and tumor formation (Meyer et al., 2009; Mukherjee et al., 2011; Zhang et al., 2008).

Additionally, erroneous number of spindle poles due to centrosome replication defect (Godinho et al., 2009) may separate chromosomes to several nuclei or give rise to asymmetric segregation and generation of laggards (Falck et al., 2002; Norppa and Falck, 2003), which are chromosomes left at the mitotic plate after anaphase and frequently captured into the micronuclei (Figure 3).

Studies on human neoplastic tumors have shown that despite bipolar mitosis and accurate metaphase-anaphase transition, cytokinesis failure in combination with spindle multipolarity may generate clones with high levels of aneuploidy evident as trisomies and tetrasomies of multiple chromosomes, which continue to propagate in similar fashion (Gisselsson et al., 2010), allowing for long-term clonogenic survival of aneuploid cells (Gisselsson, 2011).

Chromosome gain and chromosome loss observed in human tissues under normal and pathologic conditions may also arise through bipolar mitotic nondisjunction in which chromosomes fail to separate, and both sister chromatids move to one of the daughter cells (Kirsch-Volders et al., 2002; Torosantucci et al., 2002; Weaver et al., 2006), resulting in trisomic and monosomic progeny.

Lastly, correct disassembly of both membrane layers, the nuclear pore complexes and the nuclear lamina of the nuclear envelope (NE) at the onset of mitosis requires the concerted action of duplicated centrosomes, microtubules and mitotic motors (i.e., dynein complexes) to generate tension and

fragmentation of the NE (Burke and Ellenberg, 2002; Foisner, 2002; Gönczy,. 2002, Salina et al., 2003). Changes in the composition and dynamics of the nuclear envelope prior to the M phase may affect the dismantling process of the NE and its dynein-mediated attachment to MT and centrosomes, consequently influencing the release and arrangement of the chromosomes.

In summary, several mechanisms may lead to the formation of trisomic and monosomic cells during mitosis such as anaphase lagging, multipolar centrosomes, sister chromatid nondisjunction (Yang et al., 2003), as well as other pathways commonly observed in cancer cells, which allow for stable propagation and persistence of aneuploid karyotype (Gisselsson, 2011).

Aneuploidy in Developing and Aging Brain

As mentioned previously, mitotic division of neuroblasts in embryonic human brain is characterized by chromosomal instability in which nondisjunction is the essential mechanisms of aneuploidy formation (Peterson et al., 2008; Yang et al., 2003; Yurov et al., 2005). The invention of interphase FISH with DNA probes (i.e., centromeric, locus-specific, and translocation) or chromosome paints combined with immunocytochemistry allowed for analysis of low-level mosaicism in non-dividing cells, and refuted the idea that mature neurons must possess identical genomes to be functionally active. Studies utilizing various FISH approaches on isolated nuclei from normal adult brains revealed aneuploidy for chromosome 1, 13, 21, 18, X and Y at a total aneuploidy rate of 2.3% (Yurov et

al, 2005), and 4% of monosomy and trisomy 21 (nondisjunction) in neurons and glia which varied within the cell types and age of an individual (Rehen et al., 2005).

In a more comprehensive study of aneuploidy incidence in normal brain tissue, Iourov et al (2009) used interphase chromosome-specific multicolor banding (Iourov et al., 2007) and multicolor FISH (mFISH) for chromosomes 1, 7, 11, 13, 14, 17, 18, 21, X and Y, and determined the average rate of aneuploidy per chromosome as 0.5% and total aneuploidy as 10%, but noted wide variations between the chromosomes and individuals. Constitutive or basal aneuploidy, including trisomy 21 in normal human brains increased with advancing age (Rehen et al., 2005; Yurov et al., 2007; Iourov et al., 2009), suggesting that chromosomal instability may account not only for intrinsic genomic diversity but also for susceptibility to aging and disease.

FISH analysis of the neurons prepared from several brain regions (i.e., cerebral cortex, hippocampus, and cerebellum) of normal human brains (aged 40-75) using the probes for chromosomes 1, 3, 6, 7, 8, 9 and 11 identified about 40% of total aneuploidy and 20% of loss of heterozygosity (LOH) and allelic instability events in isolated subgroups of neurons (Pack et al., 2005). The authors speculated that constitutive neuronal aneuploidy may be necessary for some neurons to preserve differentiated state and interconnectivity under changing environmental conditions (Pack et al., 2005).

Furthermore, FISH analysis of non-telencephalic brain cells such as cerebellar neurons and glia of both adult mice and humans with DNA probes for

mouse chromosome X and 16, and human chromosome 6 and 21 revealed chromosome gains and losses at the level of 1% per chromosome in both cell populations (Westra et al. 2008). This study reported that mosaic aneuploidy in both developing and adult cerebellum of mice and humans arises at a lower rate than in, for example in cerebral cortex (~20% verses ~33%), suggesting that aneuploid NPCs not only survive into adulthood as an integral part of brain circuits, but that the level of genomic variation may be region specific and contribute to overall CNS diversity.

Combining retrograde immunocytochemistry with the neuronal tracer FluoroGold in vivo with immunolabeling to identify mature neurons in tissue sections and FISH probes for chromosomes X and Y, Kingsbury et al. (2005) showed that aneuploid neurons in the adult mouse brain possess axonal connections, demonstrate functional activity, and are likely to be present in all brain regions, and probably contain mis-segregated autosomal chromosomes.

Another study compared a slide-based cytometry (SBC) and PCR amplification of alu-repeats with chromogenic in situ hybridization (CISH) for chromosome 17 to quantify the DNA content of a single neuron on a fixed brain slice (Mosch et al., 2007). About 12% of adult neurons isolated from the brains without any known neurological or psychiatric disease (mean age ~72 years) had hyperploid DNA content (here defined as more than diploid [2n] and less than tetraploid [4n] karyotype), and included a subpopulation of cells expressing cyclin B1, an early M phase marker (Darzynkiewicz, 1996). In addition, between 6-7% brain cells showed three hybridization spots for chromosome 17 and about 0.4-

0.7% cyclin B1-negative neurons were tetraploid, representing constitutional tetraploidy.

Taken together, the results from all the studies suggest that: (1) the brain genome is not constant—mosaic aneuploidy is a constitutive complement of the developing and mature mammalian brain; (2) aneuploid neurons are functionally active and incorporate into brain circuits; (3) aneuploidy levels may vary between brain regions and change through the developmental stages; (4) basal aneuploidy may be needed for brain diversification, cellular heterogeneity and complexity of gene expression; (5) aberrant mitosis of neuroblasts in the ventricular zone may not be the sole source of aneuploid neurons; and (6) overall aneuploidy prevalence and incidence in human brain remains to be determined. To date, comprehensive cytogenetic studies of chromosomal variations in normal human brain compared to other somatic tissues throughout life are lacking, but it is generally postulated that even a low level of aneuploidy may significantly contribute to neuronal diversity, brain function and structure, and susceptibility to aging, psychopathological and neurodegenerative conditions (Arendt. et al., 2009; 2010; Muotri and Gage, 2006; Potter, 2004; Rehen et al., 2001).

Consequence of Genomic Instability in CNS

Gain or loss of at least one chromosome is associated with increased or decreased expression of many genes in cancer cells (e.g., Sellmann et al., 2010) and yeast strains (e.g., Pavelka et al., 2010), and exert a profound change at the

transcriptome and proteome level, altering cell physiology and function (Torres et al., 2007). Similarly, analysis of gene expression of aneuploid NPCs born in the postnatal subventricular zone and olfactory bulb revealed loss of heterozygosity and altered gene expression in cells missing one copy of chromosome 15 (Kaushal et al., 2003). Some aneuploid neurons survive into adulthood (Rehen et al., 2001), escape 'apoptotic pruning' (Blaschke et al., 1996; Kai et al., 2009; Harrison et al., 2000), and function aside mostly euploid cells (Kingsbury et al., 2005). This suggests that a mosaic brain network may produce a unique variation in gene dosage and consequent gene expression profile with both beneficial and detrimental consequences as observed in cancer cells (Shelzer and Amon, 2011; Weaver et al., 2007;) and genetic diseases (Dowjat et al., 2007; Gardiner et al., 2010). For example, Down syndrome (DS), a full trisomy of chromosome 21 (HSA 21) is a genetic condition that provides some insights into how constitutive aneuploidy (a change in one chromosome copy number) may lead to altered gene expression, brain dysfunction and degeneration (discussed below).

It is unclear how much aneuploidy a single brain cell can sustain under normal developmental conditions (Kingsbury et al., 2006; Arendt at al., 2009), and what overall 'aneuploidy burden' would prompt the susceptibility to disease and consequent neurodegeneration. It has been suggested that the neural aneugenesis beyond the basal levels may represent an early or contributing event in the brain disease process (Arendt et al., 2009; Potter, 2004), triggered either by unfavorable genetic polymorphism and/or environmental stimulus.

GENOMIC INSTABILITY IN DISEASED BRAIN

Recent cytogenetic studies have revealed an increase in genomic instability in several human brain diseases compared to normal aging and development, including Alzheimer's disease (AD) (Arendt et al., 2010; Mosch et al., 2007; Iourov et al., 2009; reviewed in Kingsbury et al., 2006; Thomas and Fenech, 2007), mosaic variegated aneuploidy (MVA) (Matsuura et al., 2000), schizophrenia (Yurov et al., 2008), autism (Yurov et al., 2007b), ataxia-telangiectesia (AT) (Iourov et al., 2009; Iourov et al., 2009b), and frontotemporal dementia (FTD) (Granic and Potter, unpublished data; Rossi et al., 2008). All aforementioned diseases share chromosome mis-segregation and cell cycle defects as an underlying pathological trait that may be associated with a gene mutation, environmental insults or gene-phenotype interaction leading to brain dysfunction and/or neuronal death.

Aneuploidy in Alzheimer's Disease (AD)

Hypotheses of AD Neuropathology

Alzheimer's disease is a multifactorial neurodegenerative disorder characterized by the aberrant accumulation of two misfolded proteins—extracellular amyloid beta (Aβ), a main constituent of senile plaques, and intracellular hyperphosphorylated tau protein (p-tau) assembled into neurofibrillary tangles (NFTs) (reviewed in Selkoe 2004; Querfurth and LaFerla 2011). Both early diagnosis and successful treatment of AD will be greatly aided by a complete

understanding of initiators of pathological processes that lead to formation of neurotoxic proteins, inflammation, oxidative damage, impaired calcium homeostasis, and neurodegeneration.

Over the past decades several hypotheses have been developed to explain complex and heterogeneous nature of AD pathogenesis, which may not be mutually exclusive. For example, a central hypothesis of AD, the Amyloid Cascade Hypothesis, postulates that the imbalance between Aβ production, aggregation and clearance from the brain is the major driver of the disease process leading to synaptic loss, formation of senile plaques, inflammation, oxidative stress, neurodegeneration, and dementia (Blennow et al., 2006; Hardy 2006; Potter et al, 2001). A highly neurotoxic isoform of the Aβ protein, Aβ42, is produced by consecutive action of two cleaving enzymes, β- (BACE-1) and γ-secretase, from a larger transmembrane protein, the amyloid precursor protein (APP) (Haass and Selkoe, 1993; Wolfe, 2003) within cholesterol-rich membrane domains (Wahrle et al., 2002). A crucial step in AD pathogenesis is oligomerization or polymerization of Aβ peptide driven by inflammatory proteins (Potter et al., 2001; Hardy, 2006), coupled with Aβ-mediated phosphorylation of tau that causes destabilization of microtubules (Small and Duff, 2008). In addition, intracellular Aβ accumulates in diseased neurons and contributes to disease progression by affecting the function of mitochondria, calcium ion channels and synapses (La Ferla et al., 2007). NFTs clustering within the neurons cause disruption in neuronal signaling, synaptic failure, and impaired nutrient trafficking through the cell body and axons, and ultimately lead to

neuronal death (Alonso et al., 2006). Because of their branched cell structure and large surface, neurons are especially sensitive to depletion of oxygen and nutrients, and impaired transport of key molecules and organelles essential for the cell survival and communication (Mattson and Magnus, 2006; Stokin and Goldstein, 2006).

The Vascular Hypothesis of AD (de la Torre and Mussivand, 1993) posits that cardiovascular risk factors (CRF) play a substantial role in AD pathogenesis, especially in the pre-clinical phase, by lowering cerebral blood flow (CBF) to a critical point where the high metabolic demand of the brain for oxygen, glucose and other nutrients cannot be sustained, leading to chronic cerebral brain hypoperfusion (CBH) and a neuronal energy crisis (de la Torre, 2004). Reduced ATP synthesis and depletion in affected neurons may promote oxidative stress, dysfunction in ion pumps, neurotransmitter failure, abnormal APP metabolism and increased production of Aβ, and microtubule damage (reviewed in de la Torre, 2010). While CBH can be present in cognitively intact older adults decades before the appearance of first clinical symptoms and classic AD markers (Vermeer et al., 2003), a combination of unfavorable vascular factors (e.g., hypertension, hypercholesterolemia and atherosclerosis) and vascular-related susceptibility genes (e.g., apolipoprotein (*APOE*) ε4 allele) can exacerbate the disturbance in the cerebrovascular environment and weaken brain hemodynamic function (Bell and Zlokovic, 2009; de la Torre, 2004).

The mechanisms that could lead to impaired cerebral perfusion in AD include: (1) atherosclerotic changes in small and large vessel walls of both

central and peripheral vascular systems (e.g., Hofman et al., 1997; Beach, et al., 2007); (2) anatomical defects and atrophy of cerebral arterioles and capillaries resulting in reduced microvascular density and injury to brain endothelium (Farkas and Luiten, 2001; Grammas et al., 2002); (3) microvascular lesions; (4) loss of cholinergic innervations in brain blood vessels (Farks and Luiten, 2001); and (5) development of cerebral amyloid angiopathy (CAA) and Aβ deposits in the smooth muscle cell (SMC) layers of cerebral microartheries (Olichney et al., 1995), causing damage to the neurovascular unit (NVU) and decreased Aβ clearance across the blood brain barrier (BBB) (Bell and Zlokovic, 2009). The NVU at the BBB includes neurons and glia, vascular and perivascular brain cells (e.g. vascular SMC, endothelial cells and perivascular macrophages), which are responsible for cerebral homeostasis by regulating blood flow and molecular exchange across the BBB, and by providing immune surveillance and trophic support to the brain (Iadecola, 2010).

Both hypotheses try to answer several important questions regarding AD initiation and treatment: (1) whether vascular or cerebral (i.e., Aβ-related) pre-clinical pathologies are the early markers of the disease process; (2) which markers should be targeted therapeutically before progressive clinical symptoms (i.e., cognitive decline) emerge, and (3) which preventive strategies should be implemented so that neurovascular integrity and brain health remain at the optimal level in old age.

The Down Syndrome Model/Chromosome Mis-segregation Hypothesis of AD (Potter, 1991; 2008) suggests that genomic instability and a slow

accumulation of aneuploid, including trisomy 21 cells through mitotic defects and aberrant cell cycle events in peripheral organs and in the brain of an individual over a lifetime could initiate or promote both familial (FAD; caused by mutations in presenilin 1 and 2 [*PS1* and *PS2*] and *APP* genes) and sporadic form of Alzheimer's. The universal presence of AD-like pathology and neurodegeneration in Down syndrome (Epstein, 1990; Glenner and Wong, 1984), the development of FAD in patients with a duplicated APP region on one chromosome 21 (McNaughton et al., 2010; Rovelet-Lecrux et al., 2006; Sleegers et al., 2006), and occurrence of ectopic cell cycle events and chromosome mis-segregation in the cells and mice expressing FAD genes (e.g., Boeras et al., 2008; Granic et al., 2010; Nagy et al., 1997; Potter, 2004; Vincent et al., 1996; Yang et al., 2006), and in fibroblasts, lymphocytes, buccal cells, and neurons of AD patients (e.g., Arendt et al., 2010; Geller and Potter, 1999; Iourov et al., 2009; Kingsbury et al., 2006; Migliore et al., 1999; Mosch et al., 2007; Thomas and Fenech, 2008; Trippi et al., 2001) reinforced the hypothesis that chromosomal instability and mosaic aneuploidy could be a 'cytogenetic hallmark' of AD neurodegeneration, initiated by either genetic mutations, APP overexpression, Aβ overproduction or by environmental insults (reviewed in Granic and Potter, 2011). Thus several molecules and processes associated with AD pathology may act as aneugens and contribute to chromosome mis-segregation and generation of aneuploid, apoptosis-prone neurons (Busciglio and Yankner, 1997) and neural progenitors. Increase in surviving hyperploid and hypoploid brain cells might contribute to other commonly observed features of AD, such as

overexpression of inflammatory proteins, amyloidogenic processing of APP and defective microtubule function and protein trafficking between and within the cells (Potter, 2004).

The Level of Central Aneuploidy in AD

The advancements in DNA technologies and visualization of chromosomal aberrations in mitotically active and dormant cells allowed for the establishment of aneuploidy mosaicism in healthy and diseased tissues, including neuronal. However, the hypothesis of unscheduled cell cycle re-activation in quiescent neurons under pathological conditions of AD is not novel. Analyzing with the less refined technique the brain tissue from Alzheimer's patients, Ramon y Cajal and Bouman suggested in the early twentieth century that the senile plaques may arise from hyperproliferation and subsequent degeneration of diseased neurons that re-enter a de novo cell cycle trying to self-repair (Bouman, 1934; Ramon y Cajal, 1913). It would take another three-quarter of the century for the evidence of the ectopic cell cycle events in fully differentiated neurons in AD brains to emerge (e.g., McShea et al., 1997; Nagy et al., 1997; Vincent et al., 1996; Smith and Lippa, 1995), which challenged the dogma of their post-mitotic nature. Several studies have shown that mature neurons are capable of re-entering the cell cycle in vitro and in vivo, and that, depending on the external cues, they are either committed to finishing the M phase and divide, or abort the cell cycle via apoptosis (Howard et al., 1993; reviewed McShea et al., 1999; Yang and Herrup, 2007).

As discussed above, genomic instability in the normal adult and developing fetal human brain in the form of mosaic aneuploidy (~10% or 0.5% per chromosome, and 30-35% or 1.25-1.45% per chromosome, respectively) with the gains and losses of whole chromosomes is part of neuronal complexity and diversity (e.g., Iourov et al., 2009; Yurov et al., 2005; 2007; Arendt et al., 2010), but could also play a pathogenic role if the level of aneuploidy exceeds the sustainable physiological threshold, and the mechanisms responsible to correct erroneous cells fail. To date, the evidence drawn from animal models of AD and autopsy studies of human brains indicates that (1) reactivation of the cell cycle and DNA duplication (Yang et al., 2001; Zhu et al., 2008) in the vulnerable population of AD neurons may present a fundamental initiator of AD pathogenesis (e.g., Heintz, 1993; McShea et al., 1999; Nagy, 2005; Obrenovic et al., 2003; Varvel et al., 2008; Vincent et al., 1996; Yang et al., 2006) and occur before fibrilar Aβ deposition and cell death; and (2) AD brains harbor up to 35% of hyperploid, including trisomy 21 neurons, which are detectable at mild stages of AD before neuronal loss (Arendt et al., 2010; Iourov et al., 2009). These cycling or aneuploid mature neurons may arise during prenatal and/or adult neurogenesis throughout an individual's lifetime (Eriksson et al., 1998) and may either die or be functionally active.

The above-mentioned study by Mosch et al. (2007) that used three independent methods to compare single-cell DNA content in normal and AD entorhinal cortices immunolabeled with the cell cycle markers, revealed about 20% cyclin B1-positive neurons with hyperploid DNA content (i.e., >2n and <4n),

and about 12% neurons from early and 13% from advanced AD brains with trisomy 17 compared to 6% in the age-matched control group. In addition, ~2% of cyclin B1-positive neurons had tetraploid DNA content (4n karyotype) compared to ~0.3-0.5% in the controls. The authors concluded that hyperploid AD neurons undergo an incomplete DNA replication compared to a complete replication observed in cyclinB1-positve tetraploid cells, and that these cells, although committed to the cell cycle (i.e., progressing beyond the G1 restriction checkpoint; Bybee and Thomas, 1991), would rather die than finish the cell division. The majority of neurons with a more than diploid content which were elevated in the early-stage of AD by 2-3 folds remained stable through the mild-stage, but died off as the patients progressed to more severe dementia, accounting for about 90% of total cell loss (Arendt et al., 2010). A shift from physiologically sustainable levels of ~10% of hyperploidy to pathological ~30-35% in the preclinical AD might trigger selective clearance of vulnerable, genetically unstable neurons during AD development and progression. Thus, mature neurons residing in the brain regions susceptible to AD pathology entered a lethal cell cycle, but could survive in a hyperploid or diseased state for years or decades (Morsch et al., 1999; Yang et al., 2001).

A recent study by Iourov et al. (2009) assessed aneuploidy in AD brains using eight different FISH DNA probes and paints, and observed a 10-fold increased level of trisomy and monosomy 21 (10.7% versus 1.7% in controls, 95% CI 6.5-14.7%) as the most predominant chromosome mis-segregation event, and less than 0.04% tetrasomic (i.e., a chromosomal duplication of a

single chromosome pair) and polyploid (4n) neuronal and glial cells. To date, this is the latest and the most comprehensive analysis of neural genomic instability in AD utilizing the FISH assay. Similarly, a study by Westra et al. (2009) that used a combination of DNA fluorescence-activated cell sorting (FACS), FISH for chromosomes 4, 6 and 21, and immunolabeling to analyze the level of tetraploidy (4n karyotype) verses tetrasomy (2n+2) for a specific chromosome in AD and normal brains determined that tetraploid cell are mostly non-neuronal, do not occur explicitly in AD, and are present at extremely low level (0.003%).

The discrepancies among the described studies in the level and type of aneugenesis and genomic instability, and the emphasis on either tetraploidization (Arendt et al., 2010; Frade and López-Sánchez, 2010; Mosch et al., 2007; Yang et al., 2001; Yang and Herrup, 2007) or aneuploidization of the brain cells (Granic and Potter, 2011; Iourov et al., 2009; Iourov et al., 2011; Potter, 2004; 2008) as an important hallmark of AD pathology could be explained by the differences in working hypotheses, methodology employed, and definition of hyperploid state. Nonetheless, the accumulated evidence suggests that elevated chromosome mis-segregation and aneuploidy in cycling neurons in AD may represent a critical molecular event contributing to neurodegeneration (Potter, 2004; Arendt et al., 2009; Iourov et al., 2011; Yang and Herrup, 2007; McShea et al., 1999).

It is generally accepted that (1) cell cycle dysregulation and aberrant cell cycle re-entry in a subpopulation of susceptible neurons are early events in AD pathogenesis that can ultimately lead to cell death (cycle-related neuronal death

[CRND]; reviewed in Fiorelli and Padmanabhan, 2011); (2) how far into the cell cycle postmitotic AD neurons progress and whether they complete the cell division after DNA replication is unknown—an authentic mitotic event has yet to be recorded; and (3) neurons with abnormal DNA content (>2n) are present at an elevated level in AD brain regions prone to neuropathology and are capable of surviving throughout early to mid-stage of AD and contributing to the brain physiology.

The Level of Peripheral Aneuploidy in AD

Early cytogenetic studies have shown an increase in chromosomal abnormalities in human lymphocytes with advancing age in which sex chromosomes and chromosomes belonging to E, F and G group (i.e., short submetacentric and very short acrocentric chromosomes; Robinson, 1960) were more readily lost or gained than other autosomes (Jacobs et al., 1963; Galloway and Buckton, 1978). The aging process has been recognized as the strongest risk factor for dementia and AD; the risk of AD increases exponentially and doubles every 5-6 years after about age of 60 (Jorm et al., 1987; Kawas et al., 2000; Ziegler-Graham et al., 2008). The discovery that Down's syndrome patients who live beyond the 4th decade of life invariably develop AD-like neuropathology (Glenner and Wong, 1984; Olson and Shaw, 1969; Wisniewski et al., 1988), and the fact that APP gene resides on chromosome 21 (HSA21) (Goldgaber et al., 1987; Neve et al., 1988; Tanzi et al., 1987), a small acrocentric chromosome, instigated the

research on nondisjunction in AD and on the common biological mechanisms between AD and DS (Potter, 1991; 2008; Geller and Potter, 1999).

Initial cytogenetic studies investigating the rate and type of chromosomal aberrations in patients with dementia used a classic karyotype analysis and G-banding on blood cultures, and yielded inconclusive (e.g., Nordensen et al., 1980) or negative results (e.g., Buckton et al., 1987; Kormann-Bortolotto et al., 1993; Mark and Brun, 1973; Moorhead and Heyman, 1983) due to a small sample size and technical limitations of the assay. The advent of FISH (Ried, 1998) allowed for analysis of chromosome mis-segregation in poorly dividing or non-dividing cells or cells with a low-level aneuploidy such as human lymphocytes, which in a mosaic living person are subjected to negative selection, and may be 'masked' by diploid cells in the tissue culture and thus may not represent the accurate level of chromosomal abnormality of an individual (discussed in Potter, 2004).

Utilizing the advantages of FISH, Geller and Potter (1999) analyzed primary fibroblasts from AD patients carrying familial mutations in the *PS1*, *PS2* or *APP* gene and those of sporadic origin, and revealed twice the frequency of trisomy 21 (~5%) compared to age-matched controls (~2.5%), which was not associated with the age of the affected individual. A parallel assessment of trisomy 18 nondisjunction revealed an increase in aneuploidy, indicating that the mitotic defects in AD likely affected other chromosomes. Both trisomy 21 and 18 are the most common trisomies in newborns linked to erroneous maternal meiosis I or II, respectively and to 5-15% of postzygotic (mitotic) nondisjunction

(reviewed in Nicolaidis and Petersen, 1998). In both trisomies, errors in maternal meiosis I is associated with reduced genetic recombination between non-disjoined chromosomes and an increased recombination in meiosis II errors (Lamb et al., 1996). The susceptibility of chiasmata, the points of crossing over and genetic material exchange between to homologous chromosomes observed in meiosis, and the fact that HSA21 is the smallest, gene-poor autosome in humans (Gardiner and Davisson, 2000) might also explain a preferential occurrence of HSA21 nondisjunction in genetic disorders and in AD.

Thomas and Fenech (2008) used FISH analysis and DNA probes for chromosome 21 and 17 to compare aneuploidy levels in buccal cells (BC) collected from young and older healthy adults, DS individuals, and clinically diagnosed AD patients. Young controls harbored 5% of trisomy 17 and 21, whereas older adults had 13.8% trisomy 17 and 9.6% trisomy 21, confirming the previous results that aneuploidy levels increase with advancing age. There was a 1.5-fold increase in trisomy 21 and a 1.2-fold increase in trisomy 17 in AD subjects compared to age-matched cognitively intact older adults. DS individuals had 16% aneuploidy of chromosome 17 compared to 5% of young controls. The use of BC in this study has several advantages: they are easy to collect and share the same origin as a neural tube epithelium from which nervous system is developed, and thus they may exhibit chromosomal defects similar to the brain tissues.

Along with the studies described above, separate lines of investigation provided evidence that other mitotic defects and mitosis-specific proteins may be

present in the cells of AD patients (reviewed in Potter, 2004). For example, the mitotic spindles in dividing AD cells exhibited abnormalities and susceptibility to premature centromere division (PCD) and micronucleation (MN) upon chemically induced (e.g., colchicines, griseofulvin) microtubule damage (e.g., Fitzgerald et al., 1975; Spremo-Potparević et al., 2004; Trippi et al., 2001; Potter et al., 1995). The PCD in which individual sister chromatids are separated by a clear gap and not connected at the centromeres, has been observed in lymphocytes and fibroblasts of AD patients and individuals prone to chromosome mis-segregation, and recently confirmed in neurons of individuals with sporadic AD (Spremo-Potparević et al., 2008).

In summary, the universal presence of genomic instability and increased aneuploidy, including trisomy 21 in peripheral tissues (i.e., blood, skin, and mucosa) from patients with both familial and sporadic AD indicates that mitotic defects may not only be associated with FAD mutations but also with exogenous risk factors, and possibly other susceptibility AD genes. A larger scale cytogenetic autopsy and biopsy study employing various FISH techniques at the single-cell level is needed to establish the type and level of genomic instability in AD and other neurodegenerative disorders.

The Evidence for the Down Syndrome/Chromosome Mis-segregation Hypothesis

Down Syndrome: Consequence of Altered Gene Dosage

As indicated previously, chromosomal instability and the resulting chromosomal aneuploidy in somatic and germ-line cells have been implicated in the etiology of genetic diseases and cancers in which the presence or absence of an entire chromosome(s) alters the expression of the genes and gene products resulting in a specific, abnormal phenotype (reviewed in Iarmarcovai et al., 2008; Ye et al., 2007). Down syndrome, a phenotypic result of trisomy 21, is the most common chromosomal instability in surviving humans. Triplication of HSA21 is responsible for about 95% of DS cases (reviewed in Sherman et al., 2005), and arises as a direct result of meiotic error mostly of maternal origin (Ghosh et al., 2008; Nicolaidis and Petersen, 1998; Warburton, 2005). The remaining cases are due to mosaicism or translocations of parts of chromosome 21 with a hetero- or homologous acrocentric chromosome (i.e., 13, 14, 15, 22) (Kim and Sheffer, 2002), and are usually associated with a less prominent DS phenotype (Devlin and Morrison, 2004a). Overexpression of the critical genes in DS within the Down syndrome critical region (DSCR; 21q22; Korenberg et al., 1990) has been associated with several DS phenotypes, and instituted the research on genotype-phenotype relationship. Besides intellectual disability, DS individuals share over 80 clinical traits including congenital heart disease, muscle hypotonia, leukemia, deficits in vision and hearing, seizures, immune system deficiency, accelerated aging and neurodegeneration of the Alzheimer's type (Epstein, 1990;

Antonarakis and Epstein, 2004; Patterson, 2009). There are two hypotheses that explain the relationship between the DS specific phenotype and triplication of HSA21. Certain gene combinations might predispose DS individuals to a disease (e.g., Alzheimer's) while other gene groupings might be protective (e.g., lower incidence of atherosclerosis and solid tumors; Draheim et al., 2010; Hasle et al., 2000) because of (1) gene dosage imbalance (Korenberg et al., 1990) or (2) increased gene expression and altered genetic homeostasis (Shapiro, 1983). For example, overexpression of *APP* gene may reflect the increased gene dosage of 50% leading to overproduction of Aβ, increased ratio of more amyloidogenic Aβ42 over Aβ40 (Teller et al., 1996) and neurodegeneration.

Trisomy 21: Links between DS and AD

Epidemiological evidence suggesting that chromosome mis-segregation and trisomy 21 mosaicism might be associated with AD pathogenesis originated from studies showing a significantly higher frequency of DS children in some families with familial AD (Heston et al., 1981; Heyman et al., 1983). Another important result that provided support for the Trisomy 21 Model of AD came from a retrospective study of young mothers (aged <35) of Down syndrome children who showed a 5-fold increased risk of developing AD later in life compared to either older DS mothers or the general population (Schupf et al., 1994; 2001). In the vein of the Trisomy 21 Model of AD, it could be postulated that the young DS mothers most likely had HSA21 mosaicism and were predisposed for genomic instability, which resulted in DS offspring and their own increased risk of AD later

in life. Recently, Migliore et al. (2006; 2009) confirmed the susceptibility to aneuploidy and trisomy 21 nondisjunction in young mothers of DS children. Furthermore, case studies of patients with HSA21 mosaicism and no mental impairments of the DS type who developed AD by the age of 40, strongly suggest that a small percentage of chromosomal instability sufficed to result in early-onset AD (e.g., Ringman et al. 2008; Schapiro et al., 1989).

Therefore, a slow accumulation of a low number of trisomy 21 and other aneuploid cells over the course of life may initiate and/or contribute to the pathogenesis of both genetic and sporadic form of AD. Furthermore, a dose response effect could be suggested in which full trisomy 21 in DS individuals produces AD-like pathology by age 20 and by middle age in familial AD and even later in sporadic Alzheimer's patients, who also belonged to the trisomy 21 mosaic group with an increased frequency of DS children before the age of 35. The recent discovery that an extra copy of *APP* gene on one HSA21 is sufficient to cause early-onset AD (McNaughton et al., 2010; Rovelet-Lecrux et al., 2006; Sleegers et al., 2006) suggests that *APP* overexpression and consequent overproduction of Aβ peptide is the likely cause of AD in both DS and trisomy 21 mosaic individuals.

Experimental Evidence for DS Model/Chromosome Mis-segregation Hypothesis

Several predictions under the Down Syndrome Model/Chromosome Mis-segregation Hypothesis have been tested so far in the laboratory of Dr.

Huntington Potter and others: (1) AD patient should harbor mis-segregated, including trisomy 21 cells which could contribute to dementia onset and neurodegeneration but at slower pace than in DS due to modulating effect of normal cells in the body; (2) FAD mutations should occur in genes directly or indirectly involved mitosis; and (3) AD cells should exhibit defect in microtubules, mitotic spindle and mitosis-related proteins that could lead to aneuploidy and trisomy 21 mosaicism.

As mentioned previously, the cytogenetic studies confirmed the first prediction: the cells and tissues obtained from both familial and sporadic AD patients are mosaic for several chromosomes, including HSA21.

Over the past two decades the lab of H. Potter investigated the role of mutant *PS1*, *PS2* and *APP* genes and their proteolytic product, the Aβ peptide in the cell cycle, chromosome mis-segregation and induction of HSA21 aneuploidy. All assays, tissues and cells from FAD transgenic mice and cells harboring FAD genes or treated with Aβ peptide yielded comparable results: overexpression of presenilin or *APP* in vivo and in vitro or exposure to Aβ peptide induced genomic instability and trisomy 21 mosaicism through several defects in mitotic spindle, dysfunction of microtubules and inhibition of mitotic motors (Boeras et al., 2008; Borysov et al., 2011; Granic et al., 2010; Potter et al., 2008).

Additional experimental and epidemiological evidence has also suggested the involvement of presenilins and APP in mitosis: (1) PS1/2 proteins localized to the centromeres and nuclear envelope in dividing cells, and the kinetochores during interphase (Li et al., 1997; Honda et al.,2000); (2) the *PS1* gene inhibited

the cell cycle (Janicki and Montero, 1999) and induced apoptosis in the cultured cells (Wolozin et al., 1996); (3) APP was found to get increasingly phosphorylated (Judge et al., 2011) and to localize to the centrosomes and nuclear membrane during mitosis (Nizzari et al., 2007; Zitnik et al., 2006); and (4) polymorphisms in the *PS1* gene have been associated with an increased risk of AD (e.g., Wragg et al., 1996; Higuchi et al., 1996) and with an increase of DS offspring via a meiosis II defect (Lucarelli et al., 2004; Petersen et al., 2000) .

The possible mechanisms by which PS and APP mutations may induce chromosome mis-segregation involves their inability to properly link the chromosomes to the nuclear envelope and to release them at the appropriate time during mitosis, which may lead to chromosome mis-segregation and other cell cycle defects. Another possibility links *PS1/PS2* and *APP* to the increased production of neurotoxic Aβ42 as a likely effector molecule responsible for the cell cycle dysfunction, including mitotic spindle abnormalities and chromosome mis-segregation (Boeras et al., 2008; Borysov et al., 2011, Granic et al., 2010).

To determine whether genomic instability observed in fibroblasts and lymphocytes from FAD and sporadic individuals could be mimicked in peripheral and brain tissues of AD mice, we investigated the level of total and chromosome-specific aneuploidy in animals carrying *PS1* and *APP* mutations. For instance, quantitative FISH analysis with a mouse chromosome 16 BAC probe (Kulnane et al., 2002) of neurons isolated from APP-V177F mutant mice showed about 6.5% of trisomy 16 compared to 1.5% in nontransgenic littermates (Figure 4; Granic et al., 2010). Similar levels of aneuploidy were observed in lymphocytes and brains

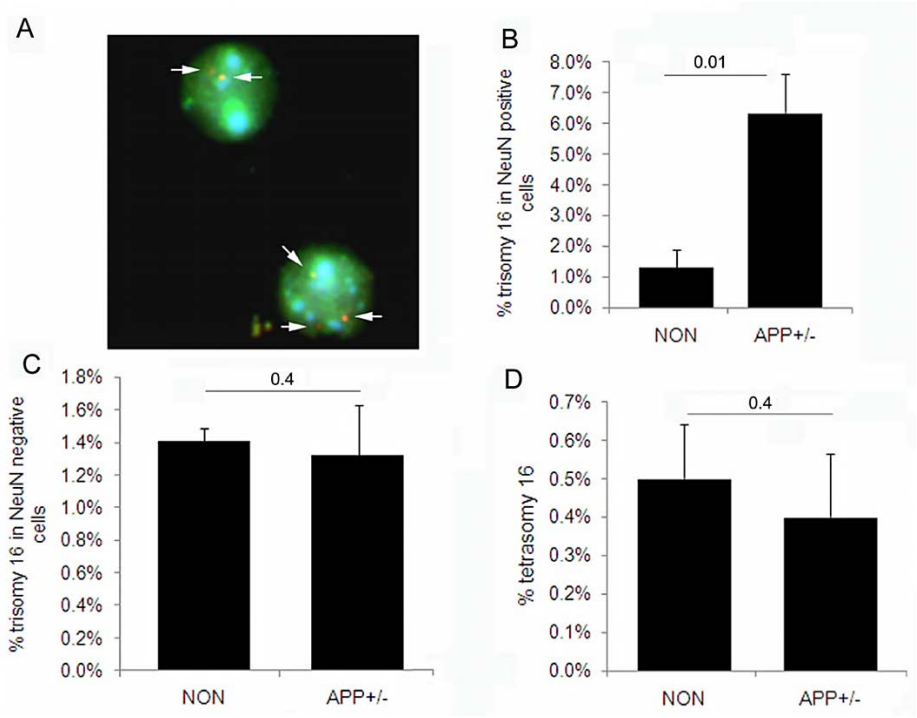


Figure 4. Induction of Trisomy 16 in Neurons of APP-V177F Mice
(A-B) Quantitative FISH analysis with a mouse chromosome 16 BAC probe (orange) revealed significantly higher levels of trisomy 16 NeuN-positive (green, neurons) cells in transgenic mice compared to controls but no induction of tetrasomy in whole brain single-cell suspension (D).
(C) No induction of trisomy 16 was observed in NeuN-negative cell.
"Reprinted from Antoneta Granic, Jaya Padmanabhan, Michelle Norden, and Huntington Potter, "Alzheimer Aβ Peptide Induces Chromosome Mis-Segregation and Aneuploidy, Including Trisomy 21: Requirement for Tau and APP", Molecular Biology of the Cell, Vol. 21, 511-520, February 15, 2010 with the permission of The American Society for Cell Biology."

of animals carrying either a mutant *PS1* or *APP* human gene or in mutant PS1 knock in mice (Boeras et al., 2008; Granic et al., 2010).

To investigate whether FAD mutations or some other factors were responsible for the chromosomal instability observed in mouse models of AD, parallel cultures of human cells with a stable karyotype were transiently transfected with plasmids carrying either *PS1* or *APP* mutant genes (e.g., M146L or Swedish mutation [K595N/M596L], respectively). Overexpression of these genes induced about 2-3% of trisomy 21 and/or trisomy 12, and up to 30% of

total aneuploidy in metaphase cells (Granic et al., 2010; Boeras et al., 2008), indicating that an aneugenic effect of FAD mutations likely affected all chromosomes, and that chromosome mis-segregation was not restricted only to transfected cells. We hypothesized that Aβ peptide itself found at increased levels in both familial and sporadic AD might be the effector molecule interfering with the cell cycle and chromosome segregation (Potter, 2008). Immunocytochemistry of PS1-expressing cells revealed several abnormalities in the mitotic spindles, with disarrayed microtubules, multiple centrosomes and lagging chromosomes as the most frequent spindle errors (Boeras et al., 2008). Following the initial observation that more cells became aneuploid than transfected, we examined the aneugenic effect of Aβ42 peptide in vitro. Human epithelial cells treated with 1μM Aβ40 and Aβ42 for 48 hr develop more than 20% total aneuploidy and 2% of trisomy 21 and 12 compared to 6% total and less than 1% chromosome 21 aneuploidy, respectively in the cells treated with various control peptides (e.g., Aβ42-1) (Figure 5, Granic et al., 2010).

Further in vitro studies investigated the mechanism(s) by which Aβ exerts its aneugenic activity on dividing cells, especially those related to microtubule function, mitotic spindle integrity and tau. It has been shown that Aβ requires downstream changes in tau protein and in microtubules to exert its toxic effect on neurons—Aβ induces tau phosphorylation (Small and Duff, 2008) and Aβ neurotoxicity depends on the presence of tau (Rappaport et al., 2002). Our data indicated that (1) mouse splenocytes lacking one or both *Tau* genes developed up to 5% aneuploidy; (2) Aβ aneugenic activity depended on tau, suggesting that

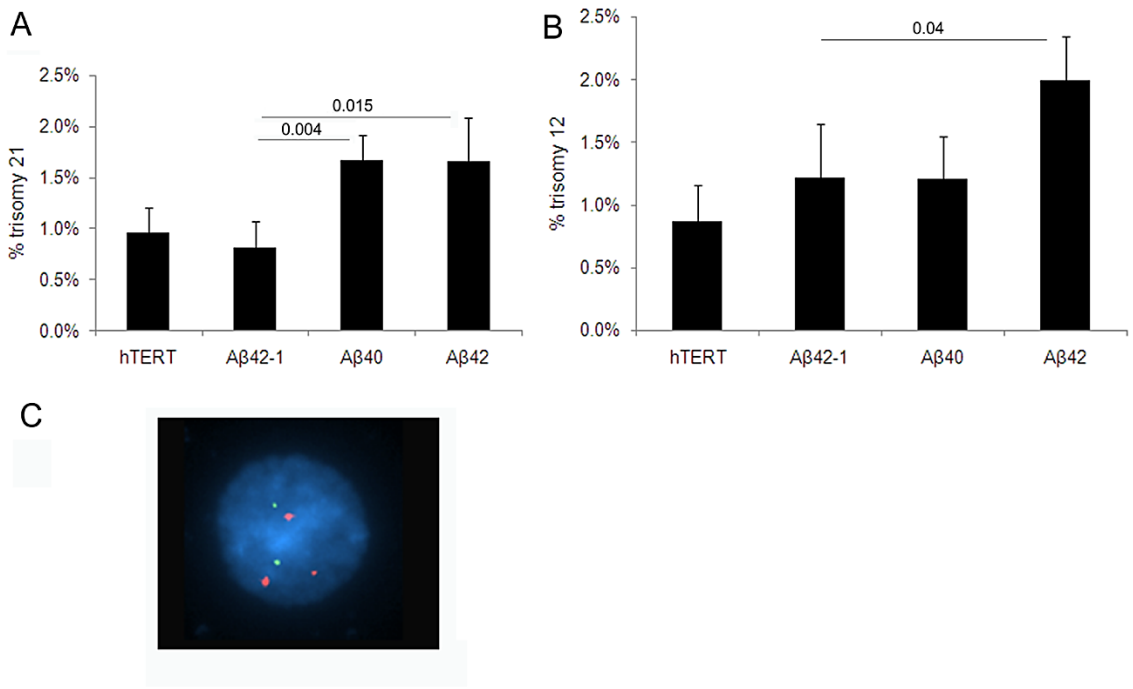


Figure 5. Aβ Peptides-induced Trisomy 21 and 12 In Vitro
(A-C) Quantitative FISH analysis with a dual color probe (Abbott, Vysis) showed an increase in trisomy 21 (SpectrumOrange) and trisomy 12 (SpectrumGreen) in Aβ-treated cells. Adapted and used with permission from Granic et al., 2010, Molecular Biology of the Cell, The American Society for Cell Biology.

the peptide interfered with the tau's normal MT-stabilizing function; (3) Aβ induced similar mitotic spindle defects observed in the cell expressing *PS1* mutations, and inhibited three motor kinesins, the Eg5, KIF4A, and MCAK, required for proper spindle formation and chromosome mis-segregation (Borysov et al., 2011), and (4) a functional *APP* gene was necessary for aneugenic effect of Aβ. Additionally, we determined that chelation of extracellular Ca^{2+} with BAPTA and thus inhibition of, for example, calpain, and inactivation of GSK-3β by LiCl, reduced Aβ-induced aneuploidy. Previous studies have shown that both BAPTA and LiCl inhibit Aβ toxicity (Sofola et al., 2010; Kuwako et al., 2002).

Future studies will determine if the interference with microtubule function

by Aβ contributes to defective neuronal plasticity, neurotoxicity, as well as impaired neurogenesis and generation of aneuploid neurons prone to Aβ overproduction and neurodegeneration. Chromosomal instability, including trisomy 21 may be a cause and an effect of Aβ-induced microtubule dysfunction creating a feed-forward loop that stimulates AD progression.

Aneuploidy in Other Neurodegenerative Diseases

Ataxia-telangiectasia (AT) and frontotemporal dementia (FTD) are examples of neurodegenerative diseases in which mutations in genes involved in mitosis and the cell cycle regulation may be associated with chromosomal instability and neuronal death.

AT is an autosomal recessive genetic disorder caused by mutation in a single gene, the 'ataxia telangiectasia mutant' (*ATM*) which encodes the ATM protein, a kinase responsible for the G1 cell cycle checkpoint, maintenance of genomic integrity and elimination of cells with damaged DNA (Abraham, 2001; McKinnon, 2004; Shiloh, 2003). This single point mutation leads to a wide range of defects including neurodegeneration, cognitive impairment, immune dysfunction and high susceptibility to malignant tumors (McKinnon, 2004). Because of the loss of function in ATM, one of the main pathological hallmark of the disease is a significant increase in aneuploid cells, including neuronal cells early in disease progression both in murine models of AT (~38%; McConnell et al., 2004) and in humans (2-3 fold increase over normal human brain; Iourov et

al., 2009). The data suggest that absence of *ATM* results in failed clearance of mis-segregated cells, which in turn instigates increased incident of lymphatic tumors, immunodeficiency because of accumulated oncogenic T and B cells, and defective neurogenesis and progressive neurodegeneration (Iourov et al., 2009b).

A recent study by Rossi et al. (2008) proposed another function for the protein tau—protection of genomic stability and chromosomal integrity. Cytogenetic analysis of lymphocytes and skin fibroblasts from individuals harboring a P301L mutation in *Tau* gene showed several chromosomal instabilities compared to non-demented controls (e.g., aneuploidy, chromosome and chromatid breaks, translocations and de-condensed chromosomes). In the Potter laboratory, we have been investigating the role of microtubule defects in AD and FTD by determining the level of MT-based transport deficiency in mouse models of FTD and human brains evidenced by aneuploidy and reduced expression of neurotransmitters and neurotrophin receptors. Preliminary data indicate an increased induction of trisomy 21 in human brains with FTD, and elevated levels of trisomy 16 in spleens and brains of mice harboring P301S mutation compared to non-transgenic controls.

GENOMIC INSTABILITY IN CARDIOVASCULAR DISEASE

Atherosclerosis is a leading cause of death in developed countries and a major component of cardiovascular disease (CVD). The pathogenesis of

atherosclerosis is complex and involves multiple factors. It has been generally accepted that three dominant pathways are implicated in atherogenesis: disturbed cholesterol homeostasis, inflammatory response, and oxidative damage. Therefore, atherosclerosis has been described as an inflammatory disorder that is induced and progresses within a high cholesterol environment (Steinberg, 2002). As with AD, several hypotheses have been proposed to elucidate the initiating agent or process responsible for atherogenesis.

The Response to Injury Hypothesis postulates a tear in the endothelial lining of an artery caused by e.g., mechanical injury or oxidized low density lipoprotein (OX-LDL) (Ross, 1993; Carmena et al., 2004) as a first atherogenic step followed by overexpression of adhesion molecules, infiltration of inflammatory cells, an excessive migration and proliferation of smooth muscle cells, accumulation of cholesterol, fats, and cellular debris, and consequent plaque formation and calcification (Ross, 1993; Libby et al., 2002; Steinberg, 2002).

The Monoclonal Proliferation Theory posits a monoclonal expansion of SMC and chromosomal instability as an initiating mechanism for the development of atherosclerosis, similar to the development of aneuploid, benign tumors (Benditt and Benditt, 1975; Casalone et al., 1991; Fernandez et al., 2000; Lavezzi et al., 2005; Matturi et al. 1997; 2001; Murry et al., 1997; Vanni and Licheri, 1991). Cytogenetic analysis of unstable atherosclerotic plaques revealed clonal proliferation of SMC in artery intima and the presence of several chromosomal instabilities, including trisomy and tetrasomy 7, trisomy 10, 18, and

20, monosomy 11, and loss of Y, suggesting the involvement of genomic alternations in atherosclerotic process.

THEORETICAL FRAMEWORK

Discovering the genetic origin of Aβ (Glenner and Wong, 1984) and the mapping of the APP on chromosome 21 (e.g., Goldgaber et al., 1987), allowed for new insights into pathogenic pathways of AD, and for the establishment of a unique link between genomic instability in DS and Alzheimer's (Potter, 1991). Constitutive trisomy 21 has been associated with intellectual impairment, and development of Alzheimer's like neuropathology by the 4th decade of life (Epstein, 1990). Overexpression of *APP* due to altered gene dosage, may lead to overproduction of Aβ and neurodegeneration (Head and Lott, 2004). Similarly, accumulated trisomy 21 and other aneuploid cells in AD patients both in the brain (e.g., Arendt et al., 2010; Mosch et al., 2007; Iourov et al., 2009) and peripheral tissues (Geller and Potter, 1999; Migliore et al., 1999; Thomas and Fenech, 2008) over the course of life may present an initiating event in AD pathogenesis and/or promote neurodegeneration (Potter, 2008). Likewise, the evidence from animal models of AD and autopsy studies of human brains suggests that reactivation of the cell cycle and DNA duplication (e.g., Yang et al., 2001) in the vulnerable population of AD neurons may present an important initiator of AD (Nagy, 2005; Vincent et al., 1996; Varvel et al., 2008; Yang and Herrup, 2007;

Yang et al., 2006) present before Aβ deposition and neuronal death, and could also lead to chromosome mis-segregation and aneuploidy.

A unifying hypothesis for Alzheimer's and Down syndrome neurodegeneration has been proposed (Potter, 1991)—development of a mosaic population of aneuploid, including trisomy 21, cells and alternation in genomic homeostasis may lead to classic AD neuropathology observed in both diseases. The Trisomy 21 Hypothesis of familial and sporadic AD posits that slow development and accumulation of a small number of trisomy 21 cells over a lifetime could cause or help promote neurodegeneration (Potter, 1991; 2004; 2008). Trisomy 21 mosaicism could be the first step in AD pathogenesis, and might lead to: (1) neurodegeneration and apoptosis; (2) higher expression of a neurotoxic version of Aβ peptide; (3) induction of an inflammatory cascade; and (4) defects in microtubule function and protein trafficking between and within the neurons (Potter, 2008).

Genomic imbalance has been observed in other diseases of the brain such as ataxia telangiectasia (Iourov et al., 2009; 2009b), schizophrenia (Yurov et al., 2008), autism (Yurov et al., 2007b), and it has been recently implicated as an early event in frontal temporal dementia (FTD) (Rossi et al., 2008). Taken together, chromosome mis-segregation and aneuploidy in human brain may not only represent neural complexity, cellular diversity, and a universal genetic state of the human mind, but a mechanism for neurodegeneration if the level of imbalance reaches a pathogenic threshold.

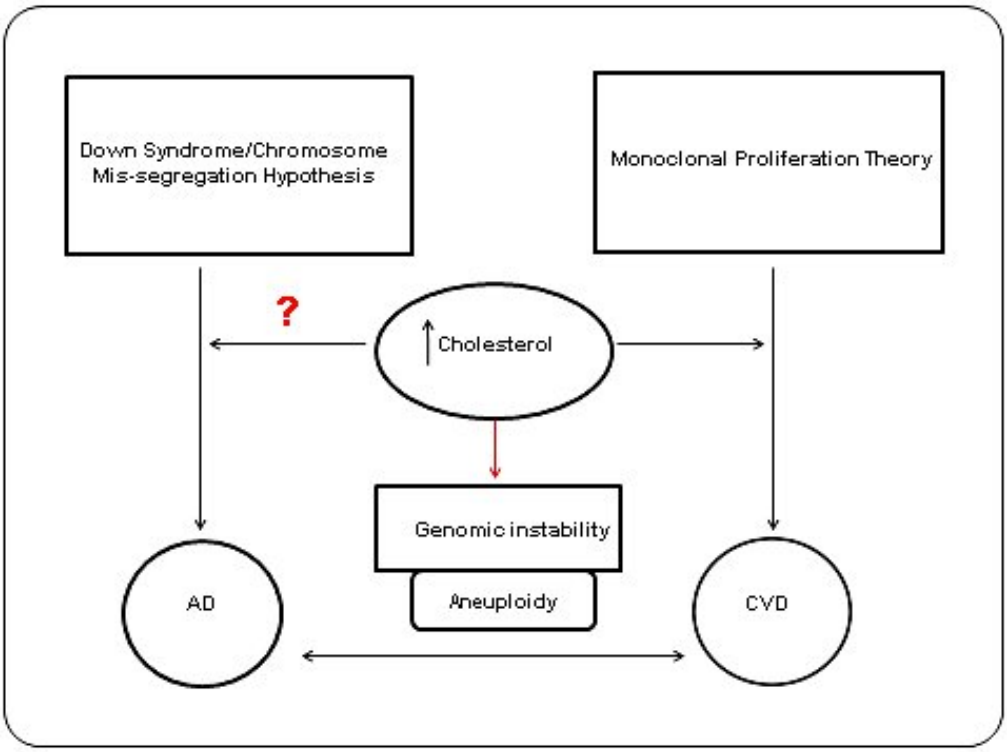


Figure 6. Genomic Instability in AD and CVD as a Common Pathogenic Trait
High cholesterol as a common risk factor for AD and CVD (atherosclerosis) may initiate aneugenesis and contribute to development of both diseases.

Cytogenetic analysis by FISH of the chromosome complement in atherosclerotic plaques showed clonal expansion of aneuploid smooth muscle cells in artery intima, with trisomy 7 and monosomy 11 as the most predominant chromosomal instability (Matturri et al., 1997; 2001). Thus, the development of atherosclerotic lesions may be initiated or promoted by clonal expansion of aneuploid smooth muscle cells.

In line with the Down Syndrome/ Chromosome Mis-segregation Hypothesis of AD and the Monoclonal Proliferation Theory of Atherosclerosis, it could be inferred that elevated blood cholesterol lipoproteins, common risk factors for AD and atherosclerosis, may act as atherogenic and aneugenic

agents, and that AD and CVD (atherosclerosis) may share chromosomal instability and development of aneuploidy as a unique cytogenetic and pathogenic trait (Figure 6).

Therefore, the purpose of this dissertation was to investigate whether disrupted cholesterol homeostasis (e.g., elevated lipoproteins/cholesterol and modified intracellular cholesterol distribution) may contribute to genomic instability observed in AD and CVD using in vitro and in vivo models.

CHAPTER TWO

BIOLOGICAL LINKS BETWEEN AD AND CVD

Brain aging is characterized by the gradual and progressive biochemical and physiological changes in the structure and function of brain cells, its subcellular components, molecules and brain vasculature responsible for its optimal functioning and integrity (Shankar, 2010). For example, alternation in dynamic communication between the cells of the neurovascular unit at the BBB may lead to decreased cerebral blood flow, impaired clearance of neurotoxic proteins, and increased brain vulnerability to the changes and insults from peripheral systems (Zlokovic, 2008; Iadecola, 2010). Furthermore, abnormal metabolism of the key brain molecules like cholesterol and lipoproteins may promote several pathophysiological processes observed in age-associated brain disorders such as Alzheimer's (e.g., reviewed in Canevari and Clark, 2007; Liu et al., 2010; Panza et al., 2009; Pfrieger, 2003; Poirier et al., 2003; Wolozin, 2004).

Accumulated evidence from epidemiology, genetics, pharmacology, and molecular, cell, and animal biology indicates that disturbance in central and peripheral cholesterol homeostasis may play a role in AD initiation and progression (Hartmann et al., 2007; Puglielli et al., 2003; Shobab et al., 2005; Wellington et al., 2004). For instance, in line with the Vascular Hypothesis of AD

(de la Torre, 2004), epidemiological studies propose a link between AD, cardiovascular risk factors (e.g., high plasma cholesterol) and cardiovascular disease (CVD) (e.g., atherosclerosis and coronary heart disease) (e.g., Alonso et al. 2009; Anstey et al., 2008; Casserley and Topol, 2004; Dolan et al. 2010; Hofman et al., 1997; Laitinen et al., 2006; Li et al., 2005; Kivipelto et al., 2001; Solfrizzi et al., 2004; Solomon et al., 2007; 2008; Sparks et al., 1990; van Oijen et al., 2007). Cerebral hypoperfusion and compromised neurovascular unit early in pathology of AD may lead to decreased clearance of Aβ and cerebral amyloid angiopathy (CAA), impaired cholesterol efflux across the BBB and atherosclerotic buildup in cerebral blood vessels (Bell and Zlokovic, 2009; Iadecola, 2010). The presence and severity of atherosclerotic lesions in systemic and intracranial vasculature correlates with the extent of AD pathology (Beach et al., 2007; Casserley and Topol, 2004; Honig et al., 2005; Li et al., 2003; Sparks et al., 1997). Additionally, genetic and molecular studies identified ApoE as a major cholesterol transporter in the brain (Mahley, 1988) that serves as a ligand for the low density lipoprotein receptor (LDLR) family in neurons and glia, coordinating cholesterol intake and distribution (Andersen and Willow, 2006; Brown and Goldstein, 1976; 1986; May et al., 2007; Poirier, 2003). Likewise, the inheritance of the *ApoE* ε4 allele has been recognized as the strongest genetic risk factor for sporadic AD (Roses, 1996; Strittmatter et al., 1993a). ApoE, particularly apoE4 directly interacts with Aβ and catalyzes its polymerization into neurotoxic assemblies (Ma et al., 1994; Wisniewski et al., 1994; Potter et al., 2001). Results from several observational studies have also shown a reduced incidence of AD in

older adults taking both lipophilic or lipophobic statins (e.g., Fassbender et al., 2001; Jick et al., 2000; Wolozin et al., 2000; reviewed in Kandiah and Feldman, 2009; Wolozin, 2004b).

In line with the Amyloid Cascade Hypothesis (reviewed in Hardy, 2006), a number of cellular and animal studies have indicated a reciprocal relationship between cholesterol levels and APP processing and Aβ production. The evidence includes the following: (1) cholesterol depletion or inhibition reduces cellular amyloid load and aggregation in vitro and in vivo (Fassbender et al., 2001; Refolo et al., 2001; Schneider et al., 2006; Simons et al., 1998); (2) plasma membrane cholesterol levels correlate with, and presumably influence the activity and expression of APP secretases involved in Aβ generation (Bodovitz and Klein, 1996; Ehehalt et al., 2003; Kojro et al., 2001; Xiong et al., 2009); (3) APP processing, γ-secretase and Aβ regulate cholesterol and other lipids (reviewed in Grösgen et al., 2010); (4) Aβ aggregates preferentially bind to cholesterol (Adulov et al., 1997; reviewed in Beel et al., 2010) and cause accumulation of cholesterol in senile plaques and neuronal terminals in cortex and hippocampus of human and mouse AD brains (Gylys et al., 2007; Mori et al., 2001; Panchal et al., 2010); and (5) animals, especially FAD transgenic mice, fed a high cholesterol diet increase Aβ production and develop greater amyloid burden, possibly by increasing the expression of ApoE to accommodate the extra cholesterol, and thus further catalyzing Aβ polymerization (Hooijmans et al., 2007; Levin-Allerhand et al., 2002; Oksman et al., 2006; Refolo et al., 2001; Shie et al., 2002; Sparks et al., 1994). Also, modification of membrane cholesterol

content and accumulation of lipids in cytosolic compartments, and deregulation of brain cholesterol metabolism has been implicated in other neurodegenerative disorders such as Niemann-Pick, Huntington's and Parkinson's disease (reviewed in Liu et al., 2009; Pfrieger, 2003).

Despite the plethora of aforementioned evidence that points to an intrinsic role of cholesterol and CVD (atherosclerosis) in the etiology of AD, the nature of the mechanistic connection between them is not completely explained, and warrants further investigation. This dissertation sought to explore a novel pathological trait that may underlie both AD and CVD—chromosome mis-segregation and development of aneuploidy in which high cholesterol may act as an environmental aneugen. To our knowledge, this would be the first study to investigate the effect of elevated lipoproteins/cholesterol on chromosomal integrity and the relevance thereof for AD and CVD.

The first part of this chapter will define the general hypothesis and three specific research questions under the study, and the second part will focus on underlying scientific background relevant for each by delineating some important aspects of peripheral and central cholesterol homeostasis, and its role in AD pathology, neurodegeneration and atherosclerosis.

GENERAL HYPOTHESIS AND RESEARCH QUESTIONS

This dissertation sought to investigate the **general hypothesis that disturbed cholesterol homeostasis may lead to chromosome mis-segregation in vitro and in vivo and therefore contribute to genomic instability observed in AD and CVD.** Under this hypothesis, three specific research questions were examined (Figure 7):

(1) Do elevated lipoproteins (i.e., oxidized low density lipoprotein [OX-LDL], low density [LDL], and high density lipoprotein [HDL]) and cholesterol induce chromosome mis-segregation and aneuploidy through LDL receptor or APP-mediated pathways, and whether there is an association between altered intracellular cholesterol distribution and mitotic defects in vitro?

(2) Does peripheral hypercholesterolemia in nontransgenic mice fed a high cholesterol diet affect chromosomal integrity in visceral and brain cells?

(3) Is there a mechanistic link between calcium homeostasis (Mechanism I) and membrane dynamics (i.e., fluidity) (Mechanism II) and lipoproteins/cholesterol-induced cell cycle defects in vitro?

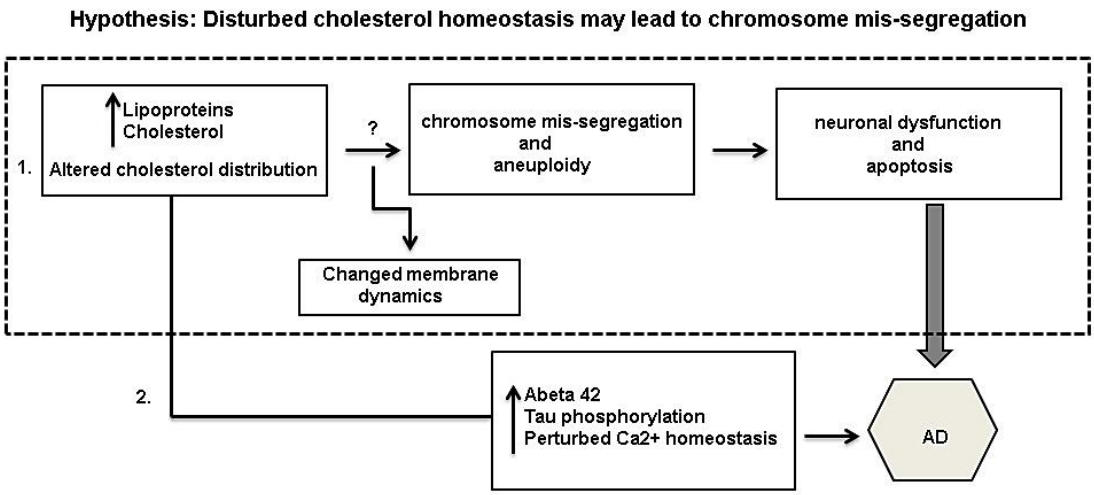


Figure 7. Research Questions under the General Hypothesis
Disturbed cholesterol homeostasis may lead to chromosome mis-segregation and aneuploidy in vitro and in vivo, and this biological effect may initiate or contribute to AD pathogenesis similar to the development of cell cycle defects observed in cellular, mouse models of AD and patients with dementia (path 1). Under the general hypothesis, this dissertation investigated following research questions (path 1 and 2): Do elevated lipids (in vitro and in vivo) and modifications in intracellular cholesterol lead to aneugenesis, and is this biological process LDLR-mediated or independent of cholesterol-APP/Aβ interaction (referenced above); Is there a mechanistic link between calcium homeostasis or plasma membrane dynamics and lipids-induced mitotic defects?

CHOLESTEROL HOMEOSTASIS AND AD

Since its isolation from gallstones in late 18th century, cholesterol's biosynthesis, molecular structure and metabolism as well as its role in pathogenesis of cardiovascular, brain and several genetic diseases has been extensively investigated. Cholesterol is a molecule essential for life, yet excess or deficiency and erroneous compartmentalization of cholesterol within the cells and body organs can be detrimental. For instance, cholesterol is a main component of eukaryotic cell membranes (Bloch, 1991) where it regulates membrane fluidity, permeability and integrity, affects functioning of membrane-bound or membrane-associated proteins, and facilitates transmembrane signal transduction (reviewed

in Maxfield and Tabas, 2005). In the brain, among other functions, cholesterol is required for neurite formation, synaptogenesis, microtubule stability, and myelination of axons (Björkhem and Meaney, 2004; Koudinov and Koudinova, 2001; Pfrieger, 2003). Excess of cholesterol and otherwise disturbed cholesterol homeostasis has been implicated in the pathogenesis of several vascular, metabolic, hepatic and brain disorders.

A renewed interest in the role of high blood cholesterol and CVD in etiology of AD was spurred by several important discoveries, the main being the identification of ApoE as key cholesterol transporter in the brain (e.g., Brown and Goldstein, 1986), and the *APOE* ε4 allele as the strongest risk factor for sporadic AD (e.g., Strittmatter et al., 1993a). Additionally, two groundbreaking studies by Sparks and colleagues reported abundant amyloid plaque deposits in 75% of the brains of cognitively intact older adults who died from coronary artery disease (CAD) compared to 12% of control brains of individuals with a healthy heart (Sparks et al., 1990), and an increase in Aβ immunoreactivity in the brains of rabbits fed a high cholesterol diet (Sparks et al., 1994). A decreased prevalence of AD was also shown in older adults using statins (Wolozin et al., 2000), and reduced Aβ42 and Aβ40 production in neuronal culture and in a guinea pig model treated with lovastatin and simvastatin (Fassbender et al., 2001). Despite the sound criticisms of the Amyloid Cascade Hypothesis which emphasized the inability to demonstrate causality (Neve and Robakis, 1998; Joseph et al., 2001), and provide effective treatments (de la Torre, 2004), a body of research that followed concentrated on the relationship between peripheral and central

cholesterol homeostasis and APP processing/Aβ production, and the involvement of the genes associated with cholesterol metabolism in susceptibility to AD (reviewed in Sagin and Sozmen, 2008). In parallel, increasing evidence supporting a vascular etiology in AD (de la Torre, 2004) suggested the involvement of other APP-independent pathways in disease initiation and progression. However, the complete understanding of biological mechanism in which combined influence of plasma cholesterol, related lipoproteins, protein carriers (e.g., ApoE), brain hypoperfusion, cerebrovascular Aβ and the brain cholesterol homeostasis contribute to neuropathology and cognitive dysfunction is yet to be attained.

Cholesterol Metabolism, Function and Organization

Cholesterol homeostasis is a tightly regulated process that involves cholesterol synthesis, transport, storage and removal. In periphery, cholesterol originates from two sources: de novo synthesis in the liver and intestine and from diet. The liver releases cholesterol into the circulation mainly in esterified form (i.e., cholesteryl esters) bound to ApoB, within LDL which is then taken up by peripheral cells via receptor-mediated (e.g., LDL receptor family) endocytosis. In endosomal and lysosomal compartments, internalized cholesterol is rapidly freed from the lipoprotein particles and recycled back to the plasma membrane through either the endoplasmic reticulum (ER) or Golgi network (reviewed in Maxfield and van Meer, 2010; Soccio and Breslow, 2004). The studies of Niemann-Pick

disease type C (NPC), which is characterized by abnormal accumulation of cholesterol and other lipids in late endosomes and lysosomes have shown that two proteins, the NPC1 and NPC2 are responsible for cholesterol egress from these organelles to the ER (Karten et al., 2009; Mukherjee and Maxfield, 1999; 2004). Deletion or mutations in either of these two non-homologous genes leads to altered trafficking of endogenous or LDL-derived cholesterol and its sequestration into cytoplasmic organelles. Because of the delayed movement of internalized LDL-cholesterol to the ER and back to plasma membrane, the production of the endogenous cholesterol and LDL receptor is increased (Wojtanik and Liscum, 2003).

The ER is the major regulatory site of cellular cholesterol homeostasis. Cholesterol biosynthesis requires more than 20 steps for which the key enzymes are localized in the ER, including the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, a rate-limiting enzyme and target of statins. An elaborate sterol-sensing mechanism (e.g., SREBP, the sterol regulated element binding protein and SCRAP, SREBP-cleavage activating protein; and INSIG) controls the cholesterol synthesis and expression of lipoprotein receptors (e.g., LDLR) based on the change in intracellular cholesterol levels. The surplus of cholesterol is esterified in the ER by the Acyl-CoA acyltransferase (ACAT) and deposited in the cytoplasm as lipid droplets or transported via nonvesicular pathways from the ER to the plasma membrane through the ATP-binding cassette transporter family (ABC) located at the membrane surface. Cholesterol efflux from the intra- to extracellular pools is achieved via HDL that serves as extracellular cholesterol

acceptor (with ApoA-I as a major apoprotein) taking cholesterol back to liver, where it is excreted in the form of bile salts. Furthermore, unesterified cholesterol can be converted into oxysterols by cholesterol-metabolizing enzymes localized in the ER such as cholesterol 7α-hydroxylase (*CYP7A*) and cholesterol 24-hydroxylase (*CYP46*) and excreted from the cell via the ABC-transporters. Oxysterols also serve as ligands for the liver X receptor (LXR) which in turn regulates the genes for APOE and ABC (reviewed in Huttunen and Kovacs, 2007; summarized in Figure 8).

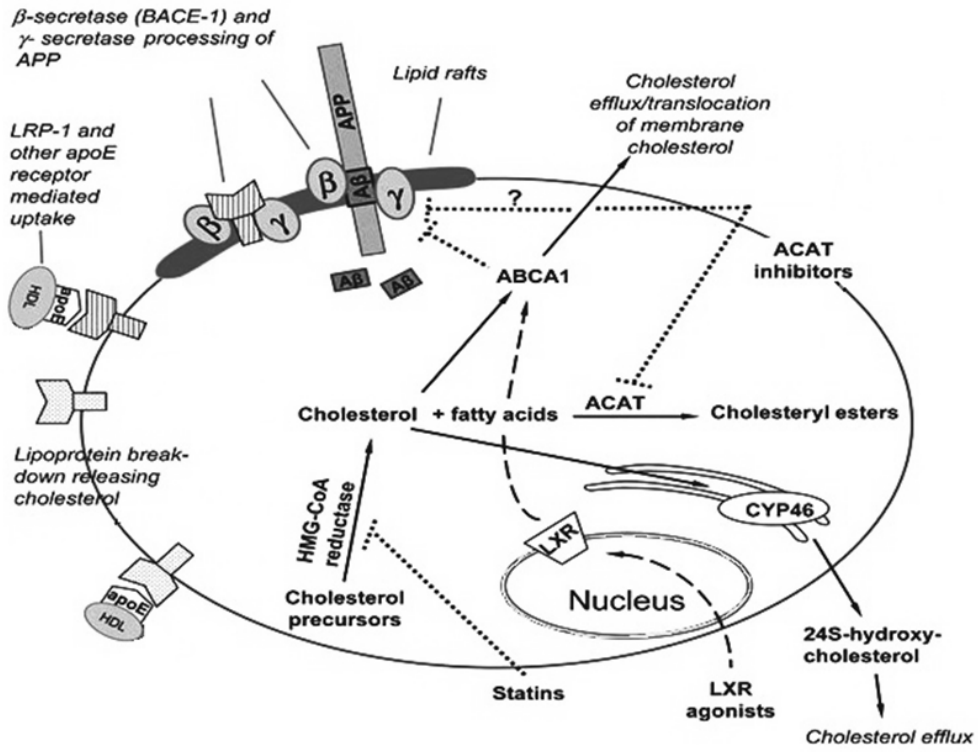


Figure 8. Elements of Cholesterol Homeostasis
Cholesterol homeostasis which includes cholesterol influx from the periphery, synthesis in the ER, storage (esterification), trafficking to the plasma membrane and efflux (hydroxylation and export to extracellular acceptors) is a tightly regulated process not fully explained. Several key proteins associated with the endoplasmic reticulum regulate intracellular cholesterol levels. Plasma membrane is enriched with cholesterol compartmentalized into lipid rafts, where APP and amyloidogenic APP-processing enzymes, β- and γ-secretases reside. Schematic diagram was adapted from Martins, et al. (2006), Mol. Psychiatry.

Thus, the ER as the central cholesterol sensing site regulates cholesterol synthesis, esterification into lipid bodies, trafficking to intracellular and plasma membranes and catabolism into oxysterols for excretion.

Cholesterol distribution between the organelles and intracellular membranes is heterogeneous, the highest being in the plasma membrane (~40% of lipid molecules; Maxfield and Wustner, 2002)—mostly packed into lipid raft microdomains (Brown and London, 2000; Crane and Tamm, 2004; Simons and Toomre, 2000), and the lowest in the ER (~1% of total cell cholesterol; Lange, 1991). Endocytic organelles and Golgi complex are also enriched in cholesterol (Hao et al., 2002), but how this asymmetric compartmentalization is achieved, and the mechanisms of cholesterol transport and movement within the cells is not completely revealed (reviewed in Ikonen, 2008; Prinz, 2007). It is widely accepted that cholesterol content and organization within the membrane affects many cellular functions such as signal transduction and membrane trafficking (Maxfield and Tabas, 2005). Cholesterol interacts with other membrane lipids and proteins, and because of its biophysical properties (e.g., rigid ring structure), it preferentially associates with hydrocarbon chains of saturated lipids causing increased lateral ordering and decreased membrane fluidity (Demel and De Kruyff, 1976; Mukherjee and Maxfield, 2004; Simons and Vaz, 2004). According to the 'fluid mosaic model' of the plasma membrane, membrane lipids and proteins are organized into bilayer leaflets (Ikonen, 2008) in either highly-ordered (gel-like), liquid-ordered, or liquid-disordered (the most fluid) phases, which often coexist with different types of microdomains. For example, the disordered lipid

phases are characterized by poorly-organized and loosely-packed acyl chains, which allow for easy diffusion of water and other molecules into the bilayer structure (Mukherjee and Maxfield, 2004; Simons and Vaz, 2004), and is usually cholesterol poor. Cholesterol-rich plasma membrane, on the other hand, is consistent with the ordered lipid phase—because of its rigid ring structure, cholesterol has the ability to increase the order of more fluid membranes. The lipid microdomains (e.g., lipid rafts) are involved in signal transduction and segregation of the membrane-anchored proteins based on their preference for different types of lipid ordering.

Cholesterol, as an important regulator of plasma membrane organization, can be manipulated in culture, and the effect of changed biophysical membrane properties on proteins and other cellular functions can be readily studied. In mammals, dietary intake of cholesterol and saturated/unsaturated fats are significant factors affecting membrane organization and therefore cell functioning (e.g., Aoun et al., 2011; McMurray et al., 2011; Raza, 2010; Wang and Schreurs, 2010; Wassall and Stillwell, 2009).

In summary, intracellular cholesterol distribution and organization between different organelles and membranes in eukaryotic cells is heterogeneous in nature and characterized by differential lipid ordering which, if changed, may affect the functioning of various proteins and lead to a diseased state. The mechanism by which cells maintain these differences, and keep in check the sterol sensing response over a narrow range of cholesterol concentration, and readily transport and distribute intracellular cholesterol is only partly understood.

Brain Cholesterol Homeostasis and AD Pathology

Cholesterol is essential for normal brain development and function, including formation of synapses and synaptic plasticity, neurotransmission, signaling transduction, membrane biosynthesis and repair (Göritz et al., 2002; Koudinov and Koudinova, 2001; Pfrieger, 2003). Brain cholesterol homeostasis is a complex, strictly regulated process not fully explained (Pfrieger and Ungerer, 2011). As in the periphery, this process includes cholesterol synthesis, shuttling, storage and turnover, which are mostly independent of hepatic cholesterol pools, and protected by the BBB (Björkhem and Meaney, 2004). The tight junctions between cerebral endothelial cells that form the barrier prevent the brain to compete for cholesterol from circulation (Ballabh et al., 2004). Alternations in cholesterol/lipoprotein metabolism and brain cholesterol homeostasis have been linked to several pathologic processes that, for example, lead to production of neurotoxic proteins and changes in plasma membrane integrity (reviewed in Liu et al., 2010). To understand the complexity of the role that cholesterol may play in neurodegenerative diseases, one has to appreciate the unique aspects of brain cholesterol homeostasis, the proteins and transporters involved in its maintenance, and intermediary steps and enzymes interacting between peripheral and central cholesterol pools.

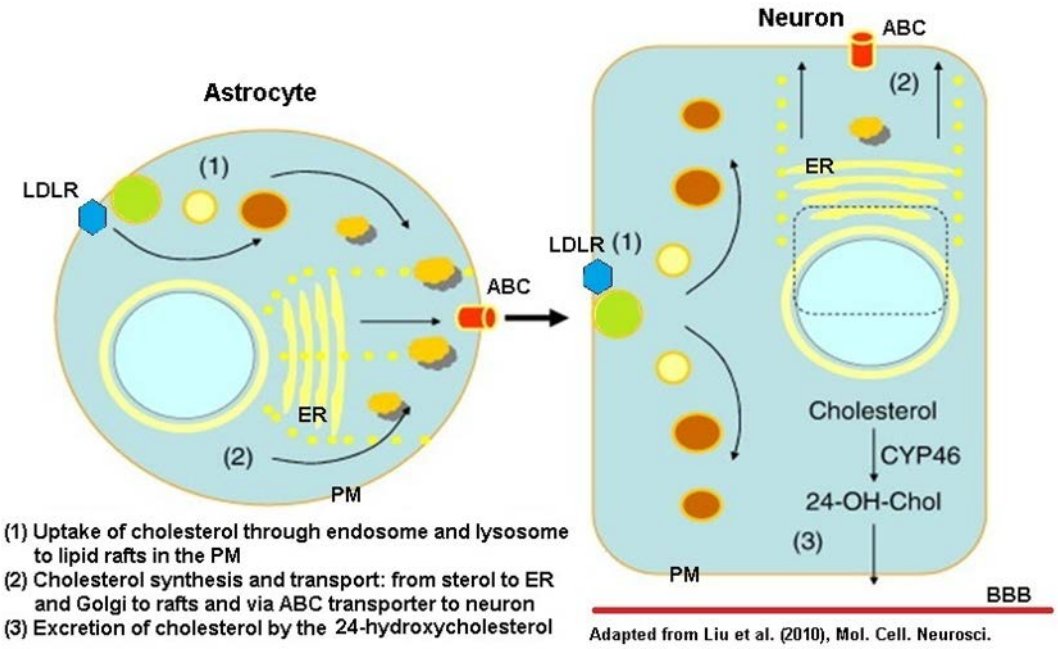


Figure 9. Brain Cholesterol Homeostasis
Brain cholesterol homeostasis is accomplished by a tight cooperation between the two main cell types of CNS, astrocytes (left) and neurons (right). Astrocytes provide mature neurons with cholesterol, which is synthetized in the ER from sterols and transported to Golgi apparatus and lipid rafts in the plasma membrane (PM). Cholesterol with ApoE is exported to neurons via the ABC transporter family, internalized via receptor-mediated endocytosis (e.g., LDLR), and further shuttled through the endosomes, lysosomes and ER to the PM. The excess cholesterol is converted by 24-hydrocylase (CYP46) to 24-hydroxycholesterol (24-OH-Chol) which crosses the BBB.

The brain is the most cholesterol-rich organ in the human body: it contains 25% of the total body cholesterol, but accounts only for 2% of total body weight (Dietschy and Turley, 2004). Intracellular cholesterol is present mainly in the myelin sheaths and cell membranes (e.g., lipid rafts), where it is essential for membrane fluidity and elasticity, synaptogenesis, signal transmission, and functioning of membrane-associated proteins (Pfrieger, 2003). Brain cholesterol is synthesized de novo by neurons, astrocytes, and oligodendrocytes, independently of peripheral production. While oligodendrocytes supply cholesterol for myelin formation, it is believed that astrocytes, the major neuronal

supporting cells, produce cholesterol required to sustain the integrity of neuronal membranes and synapse (Björkhem and Meaney, 2004; Pfrieger and Ungerer, 2011). As the neurons mature, they reduce production of endogenous cholesterol and become dependent on effective cholesterol synthesis and transport from adjacent cells, primarily astrocytes (Figure 9; Liu et al., 2010). Slowed rate of cholesterol biosynthesis in adult neurons may reflect the overall reduced requirement for surplus cholesterol in the brain after maturation, and the need for optimal energy efficiency. Furthermore, astrocytes not only synthesize, but also recycle extracellular cholesterol released from dying neurons (Jurevics and Morell, 1995).

Cholesterol exchange and uptake between the brain cells is a multifaceted process not fully elucidated (reviewed in Liu et al., 2010; Pfrieger and Ungerer, 2011). As in peripheral cells, several key acceptors, receptors, transporters and enzymes involved in regulation of the brain cholesterol homeostasis have been recognized. For example, both ApoE (the main carrier protein) and lipoprotein receptors are essential for cholesterol transport and clearance within the brain. The ATP-binding cassette transporter (ABCA1) is responsible for cholesterol flux from neurons to glia, preventing accumulation of excess cholesterol inside the cells (Dietschy and Turley, 2004). The neurons also internalize cholesterol in several intercellular compartments (e.g., endosomes, lysosomes, and Golgi apparatus), where it is released from its lipoprotein complex in the form of free cholesterol, or transformed into cholesterol esters for storage. Free intracellular cholesterol serves as a negative feedback loop to HMGR to reduce cholesterol

synthesis in the ER, while cholesterol esters have a role in dendritic and synaptic remodeling (Pfrieger, 2003; Poirier, 2003). Because the brain has no mechanism for cholesterol degradation, excess cholesterol is transformed into 24S-hydroxycholesterol (24OH) by oxidation (encoded by *CYP46* gene), a brain-specific lipophilic cholesterol metabolite that passes through the plasma membranes and crosses the BBB (Björkhem et al, 1998). Another oxysterol, 27-hydroxycholesterol (27OH) is produced in the periphery, and enters the brain at an estimated rate of 1% a day. The exchange between the two oxysterols across the intact BBB has been proposed as a missing link between cerebral and plasma cholesterol pools, suggesting that the brain cholesterol homeostasis is not entirely independent of the periphery (Dietschy and Turley, 2004). Moreover, vascular insults compromise the integrity of the BBB causing an increase in exchange of oxysterols between the brain and circulation (Xie et al., 2003).

Any disturbance in the brain cholesterol homeostasis due to, for example, presence of interfering molecules such as Aβ; and diminished efficiency of transporting proteins (e.g., ApoE); or compromised BBB and injury to the neurovascular unit could contribute to neurodegeneration.

Differential distribution of cholesterol in the cell membranes and intracellular cell compartments makes the analysis of total cholesterol in aged and diseased brain technically challenging. Studies involving aged mouse and human brains have shown an increase of cholesterol in the membranes (Igbavboa et al, 1996; Schroeder, 1984) and decreased synthesis in hippocampus but stable levels of total cholesterol (Thelen et al., 2006).

However, there is a little consensus about the change in bulk brain cholesterol in AD (Panza et al., 2006). Some studies reported reduced levels of cholesterol in the brain areas with extensive Aβ and tau pathology, especially in the patients with APOE ε4 allele (Wood et al., 2002), but increased cholesterol flux in early stages of AD (Dietschy and Turley, 2004). Distribution of the membrane cholesterol changes with the progression of disease severity—the levels of cholesterol within the cell membrane increase, altering membrane fluidity. Higher membrane cholesterol content is associated with the increased Aβ-induced toxicity both in neurons and astrocytes (Abramov et al, 2011). Also, excess cholesterol accumulates in the form of ester droplets within senile plaques (Gómez-Ramos and Asunción Morán, 2007; Mori et al., 2001; Panchal et al, 2010) and in Aβ-positive neuronal terminals (Gylys et al., 2007), whereas tangle-bearing neurons in AD brains contain more free cholesterol compared to phospho-tau free cells (Distl et al., 2001). These findings fit with the hypothesis that change in intracellular cholesterol and not in total cholesterol may be the major force behind AD progression that alters APP processing and Aβ dynamics (Eckert et al., 2000).

Another measure of cholesterol turnover and neurodegeneration in AD may be the disruption in oxysterol homeostasis. Several studies reported a change in oxysterol levels during disease progression. At early stages of the disease, the amount of 24OH cholesterol increases in the brain and periphery, suggesting increased cholesterol turnover from destroyed synapses and dying neurons (e.g., Papassotiropoulos et al., 2002; Lütjohann et al., 2000). In the later

stages, concentrations of 24OH fall, indicating lower brain cholesterol metabolism and the loss of neurons expressing *CYP46* gene proportionally to the degree of brain atrophy. The ratio between brain and circulatory oxysterols has been suggested as a biochemical biomarker for AD progression (Leoni et al., 2004; Leoni and Caccia, 2011).

Supposedly a bidirectional relationship between cholesterol and Aβ has been observed in cellular and animal studies that manipulated the levels of cholesterol by depletion or synthesis inhibition, or by dietary interventions. At least a part of cell's APP and APP-cleaving enzymes that generate amyloidogenic Aβ peptide, the β- and γ-secretases are localized in lipid rafts (Chen et al., 2006; Wahrle et al., 2002), whereas the non-amyloidogenic α-secretase activity favors more fluid, non-lipid raft environment (Kojro et al., 2001; reviewed in Wolozin, 2004). Alternations of the membrane lipid content may therefore shift the activity of the key enzymes involved in APP processing from harmful (Ehehalt et al., 2003; Xiong et al., 2008) to neuroprotective (Bodovitz and Klein, 1996; Kojro et al., 2001). For example, cholesterol depletion/inhibition with different drugs (e.g., statins, cyclodextrins) reduced Aβ production and cellular amyloid load in cultured neurons and in brains of treated animals (e.g., Burns et al., 2006; Fassbender, et al., 2001; Refolo et al., 2001; Schneider et al., 2006; Simons et al., 1998), whereas increased membrane cholesterol in mature neurons led to Aβ-induced calpain (a calcium-dependent cysteine protease) activation and generation of neurotoxic tau fragments (Nicholson and Ferreira, 2009; reviewed in Wray and Noble, 2009). Small rodents, especially FAD

transgenic mice fed a high cholesterol diet increased production Aβ and develop greater amyloid burden (e.g., Hooijmans et al., 2007; Levin-Allerhand et al., 2002; Oksman et al., 2006; Refolo et al., 2001; Shie et al., 2002; Sparks et al., 1994) compared to animals on regular diet.

It could be postulated that an altered ratio between the more amyloidogenic Aβ42 and less harmful Aβ40 affects membrane lipid content, and, in turn, membrane fluidity could affect activity of the enzymes involved in the amyloidogenic pathway, causing a vicious circle. The data suggest that intracellular and not total cholesterol regulates APP processing and Aβ production (Burns et al, 2006), but the interconnection between all proteins involved in APP proteolysis and cholesterol homeostasis is not fully elucidated. Similarly, the mechanism linking the increase in extracerebral cholesterol content and intracerebral Aβ load is not completely clear, but it points to disrupted oxysterol homeostasis, vascular insults and compromised BBB (reviewed in Martins et al., 2006).

In summary, disturbance in cholesterol homeostasis and specific changes in cholesterol distribution in plasma membrane may be involved in AD pathogenesis by: (1) overproduction of toxic Aβ that affects membrane properties; (2) altered plasma membrane fluidity and elasticity due to improper cholesterol trafficking or/and receptor mediated uptake; (3) increased calcium influx and calpain activity leading to generation of neurotoxic tau species; (4) change in cholesterol flux and recycling; and (5) inability to maintain cholesterol steady-state levels for synaptogenesis and repair. The research on cholesterol

involvement in neurodegenerative diseases and the role it plays in health of central nervous system is fairly new with a plethora of ‘brain secrets’ to be discovered.

Hypercholesterolemia and AD Risk

A positive association between high blood cholesterol and dementia has been reported in some but not all epidemiological studies (Panza et al., 2009). The studies differed depending on: (1) the ‘time period at exposure’ (late vs. mid-life blood cholesterol); (2) the length of follow up; (3) number of cholesterol measurements; (4) dementia ascertainment method; (5) availability of ApoE ε4 genotype; (6) choice of covariates; and (7) population under study. All of the above may bias the results and account for inconsistencies. Dementia has a long latency period and the concepts of time at exposure and follow up period may be of essence to detect a true relationship between exposure and disease. Proponents of a ‘life-course perspective’ seek to understand at which stage of life an exposure (total cholesterol) might have the most effect on the outcome (cognitive impairment), and if the effect of exposure is additive or synergistic (Fratiglioni et al., 2010).

For example, some of the recent prospective studies reported positive, negative or no association between blood cholesterol and AD and/or vascular dementia (VaD). The Seven Countries Study of 10,211 men and 40 years follow-up reported a positive association between total cholesterol (TC) and dementia

mortality (HR = 1.73, [95% CI 1.05-2.84]) in individuals with >250 mg/dl of blood cholesterol compared to men with <200 mg/dl (Alonso et al., 2009). In the Honolulu Asia Aging Study of 1027 men, the serum TC declined significantly 15 years before AD diagnosis, the strongest in those with *ApoE ε4* allele (Stewart et al., 2007). A recent meta-analysis of 12 prospective studies of AD and VaD (Anstey et al., 2008) that included about 23,800 subjects over, on average, 13 years of follow-up revealed no association between late-life TC, LDL or HDL and any type of dementia, but positive association between mid-life TC and AD and VaD which was not dependent on ApoE genotype. Similarly, in the Kaiser Permanente Study with 21 years of follow-up, Solomon et al. (2009) reported a positive association between high mid-life TC (>240 mg/dl) and AD and VaD (HR = 1.57, [95% CI 1.23-2.01; and HR = 1.26, [95% CI 0.82-1.9], respectively).

Current research suggests that cholesterol and the risk of dementia may have a nonlinear association with age; at mid-life, high blood cholesterol may pose a risk for cognitive decline 30-40 years later, while higher cholesterol levels may be protective later in life (Duron and Hanon, 2008; Panza et al., 2006). Protective effects of higher cholesterol on cognition may be due to cholesterol involvement in neuronal plasticity (Koudinov and Koudinova, 2001), while the decrease in cholesterol from mid to late life may result from pathological process associated with neurodegeneration (Solomon et al., 2007).

The Role of Atherosclerosis in AD

As mentioned previously, a number of epidemiological and pathological studies provided evidence for a pronounced vascular pathology in AD, and the importance of vascular risk factors and CVD in AD development (reviewed in de la Torre, 2004; 2010). Autopsy studies revealed that white matter lesions, ischemic infarcts, lacunes, microvessel hemorrhages, CAA and atherosclerotic plaques as the most frequent types of vascular pathology in AD (Honig et al., 2005; Jellinger, 2008), that contribute to steeper cognitive decline especially in the early stages of the disease (Esiri et al., 1999). For instance, a comparative pathological study showed a higher incidence of vascular lesions in 48% of AD patients compared to 32.8% age-matched controls (Jellinger and Mitter-Ferstl, 2003), whereas in the autopsy study of elderly Japanese-American males, these lesions were as common as classic AD markers (White et al., 2002).

A number of epidemiological and autopsy studies provided important, yet inconsistent evidence regarding the etiologic role of systemic and central atherosclerosis in AD onset and development. In a cross-sectional study, Hofman et al. (1997) showed that indicators of atherosclerosis in carotid arteries and large vessels of the legs were associated with an increased risk of AD and VaD especially in ApoE ε4 carriers (OR = 3.9 [95% CI 1.6-9.6], and OR = 19.8 [95% CI 4.1-95.0], respectively). In a recent autopsy study, atherosclerosis of coronary arteries associated strongly with the density of neuropathological lesions of AD in older adults with at least one ApoE ε4 allele (Beeri et al., 2006).

No association between coronary atherosclerosis and VaD was found. In a large prospective study of 6,647 participants, two noninvasive measures of carotid atherosclerosis (i.e., the intima media thickness and carotid plaques) were associated with AD regardless of ApoE genotype (HR = 2.4 [95% CI 1.25-4.60], and HR = 2.53 [95% CI 1.21-5.32], respectively) after controlling for vascular covariates (van Oijen et al., 2007). The Baltimore Longitudinal Study of Aging (BLSA) utilized a series of 200 brain and 175 full body autopsies from prospectively followed participants to examine the relationship between systemic and central atherosclerosis, AD pathology and the risk of dementia (Dolan et al., 2010). The results indicated that the presence of intracranial but not cardiac or aortic atherosclerosis significantly increased the odds for dementia (OR = 2.0 [95% CI 1.4-2.9] per unit increase in atherosclerotic grade), independently of AD pathology and cerebral infarcts. Quantitative analysis of atherosclerosis in systemic and central vessels showed no association between the degree of atherosclerotic burden and AD pathology, suggesting that there is an additional association between intracranial atherosclerosis and dementia that is not related to cerebral infarcts and AD lesions. Furthermore, recent retrospective histopathological studies confirmed the association between the intracranial atherosclerosis in the circle of Willis of AD subjects compared to age-matched controls, and a strong correlation between the density of senile plaques and NFTs and the degree of the atherosclerotic narrowing independently of possession of ε4 allele (Beach et al., 2007; Honig et al., 2005; Roher et al., 2003). For example, in a study of 397 older adults with confirmed

neuropathological diagnoses, Beach et al. (2007) showed that the circle of Willis atherosclerosis score was a significant predictor of AD (OR = 1.31 [95% CI: 1.04-1.69]) and VaD (OR = 2.50 [95% CI: 1.52-4.10]) but not for non-AD dementia for each unit of increase in atherosclerotic grade, independently of the ApoE ε4 genotype.

In line with the aforementioned evidence, several hypotheses have been proposed to explain the relationship between AD and CVD: (1) atherosclerosis may exacerbate AD pathogenesis and act synergistically with neurodegeneration; (2) atherosclerosis may present a parallel pathology that adds to cognitive burden independently of classic AD markers and susceptibility genes (e.g., ApoE ε4); and (3) both diseases may share a common biological mechanism linking the two pathologies (e.g., cell-cycle defects). While larger scale prospective and autopsy studies are needed to address the validity of association between systemic and central atherosclerosis and AD risk, the proponents of the Vascular Hypothesis argue that scientific evidence supporting vascular origins of AD outweighs that supporting the Amyloid Hypothesis (de la Torre, 2004; Casserly and Topol, 2004).

Thus, atherosclerosis and AD may be independent but converging diseases that share several common genetic and environmental risk factors (i.e., ApoE ε4 polymorphism, hypercholesterolemia, hypertension, diabetes, systemic inflammation, obesity, smoking, and high fat diet) [Casserly and Toppol, 2004]. A better understanding of one disease could aid in providing better therapies and prevention strategies for the other. Because most of the vascular risk factors are

preventable, skillful and timely prevention and/or treatment of vascular disease preferably at middle age could help preserve both cardiovascular and cognitive health (de la Torre, 2004; 2010). For instance, several non-invasive techniques have been developed to detect systemic vascular pathology such as the narrowing of carotid artery (e.g., carotid artery ultrasound [CAUS]) caused by intima-media thickness [C-IMT]; the velocity and direction of the blood flow from the heart to brain [e.g., pulse-wave Doppler scanning]; changes in the structure and function of the heart muscle [e.g., echocardiography]; and the extent of peripheral arterial disease (e.g., ankle-brachial index [ABI]). All the above may be valuable screening tools that could aid in designing strategies to prevent ongoing or future cognitive decline (de la Torre, 2010). Similarly, CVD-related clinical trials could routinely include measures of cognitive function to investigate the therapeutic potentials of anti-inflammatory, anti-angiogenic, and anti-hypercholesterolemic agents on dementia development. In sum, additional epidemiological and clinical studies looking at the benefits of either lifestyle changes or therapies for CVD (e.g., atherosclerosis) at middle age would provide valuable insights for prevention strategies aimed to stall cognitive decline later in life.

CONCLUSIONS: DISRUPTED CHOLESTEROL HOMEOSTASIS AND AD

Taken together, the cellular, animal and epidemiological studies referenced above implicate cholesterol as an important component of neurodegenerative

process in AD. While hypercholesterolemia may contribute to AD pathology via systemic and cerebral atherosclerosis by causing diminished cerebral perfusion and neuronal energy crisis, other mechanisms may also take place. First, altered cholesterol homeostasis within the brain and plasma membrane may promote Aβ production and increase both amyloid and tau-related pathologies. Second, similar to atherogenesis in peripheral vessels, high cholesterol may disrupt communication between neurons and vascular cells within the neurovascular unit resulting in increased inflammation, impaired cerebral Aβ clearance and consequent CAA within vessel walls, endothelial injury and compromised BBB (e.g., Farks and Luiten, 2001; Bell and Zlokovic, 2009). Third, a unique cytogenetic mechanism may underlie both AD and atherosclerosis contributing to pathogenesis—disrupted cholesterol homeostasis may interfere with the cytogenetic properties of mitotic cells (peripheral and central) by affecting the process of proper chromosome segregation.

CHAPTER THREE

EXPERIMENTAL PROCEDURES

To assess the effect of lipoproteins and cholesterol on chromosome segregation in vitro and in vivo, this study utilized various human cell lines and primary cells derived from human and mouse peripheral tissues and brains, dietary intervention on wild-type mice, and karyotype and fluorescence in situ hybridization (FISH) analyses as the main methods for the quantification of chromosomal aberrations in a single cell. This chapter describes in detail the cell lines, cell sources, reagents, experimental procedures, and data acquisition and analysis used to address the research questions under the general hypothesis (Figure 7).

CELL LINES

hTERT-HME1 Cell Line

The hTERT-HME1 cell line (Clontech) is a primary human mammary epithelial cell line that permanently expresses the telomerase reverse transcriptase

(Clontech). Stable expression of this enzyme preserves telomere length and allows the cells to divide indefinitely while retaining normal function, phenotype, and karyotype (Jiang et al., 1999; Morales et al., 1999). The cell line was cultured in the mammary epithelium basal medium (MEBM, Lonza) supplemented with 52 µg/ml bovine pituitary extract (BPE), 0.5 µg/ml hydrocortisone, 10 ng/ml hEGF, 5 µg/ml insulin, 50 µg/ml gentamicine, and 50 µg/ml amphotericine-B, and subcultured every 2-3 days. The cells were maintained for no more than 6 population doublings (PD) from the $PD_{initial}$.

HASM Cell Line

The Human Primary Aortic Smooth Muscle Cells (HASMC) (ScienCell Research Laboratories) were isolated from the human aorta and cryopreserved at secondary culture at the density > 5 x 10^4 cells per vial. The cells were maintained in smooth muscle cell medium (SMCM; ScienCell Research Laboratories) consisting of 500 ml basal media, 2% fetal bovine serum (FBS), 5 ml smooth muscle cell supplement, and 5 ml 1x penicillin/streptomycin (PS; 5,000 I.U/ml each; Cellgro) solution according to manufacturer's specifications. The cells were propagated when they were 90% confluent in 2 µg/ml poly-L-lysine (Sigma)-coated cell culture dishes, and the growth media was changed every other day.

Human Fibroblasts

LDL receptor-deficient human skin fibroblasts harboring two mutations, *C240-F* and *Y160-ter*, that cause a severe form of familial hypercholesterolemia (FH), and human fibroblasts with a functional LDL receptor from an unaffected age and race matched donor were purchased from Coriell Cell Repositories. Both cell lines were cultured following company's protocol in Minimum Essential Medium (MEM; with Eagle-Earle salts and non-essential AA) (Gibco/Invitogen) supplemented with 2 mM L-glutamine (Gibco), 5 ml MEM essential AA 50x (Gibco), 2.5 ml MEM Vitamins 100x (Gibco) and 15% heat inactivated FBS (Cellgro).

Four different human fibroblasts harboring a Niemann-Pick C1 (*NPC1)* mutation (Coriell, NIGMS Human Genetic Cell Repository) and four age-matched controls (Coriell, NIA Aging Cell Culture Repository) (Table 1) were maintained in Dulbecco's modified MEM (with 2 mM L-glutamine; Gibco/Invitrogen), supplemented with 15% FBS (Atlanta Biological), and 5 ml of 1x PS solution (5,000 I.U/ml each; Cellgro). All fibroblasts and HASMC were grown in 37˚C, 5% CO2 incubator and subcultured every 3-5 days.

Table 1. Characteristics of the Niemann-Pick Type C (NPC) and Control Samples

Repository #	Age (years)	Gender	Clinical Dx	Mutation or NPC assay
Control fibroblasts				
AG02101	27	female	None (healthy non-fetal tissue)	N/A
AG02603	35	female	None (healthy non-fetal tissue)	N/A
AG09319	24	female	None (healthy non-fetal tissue)	N/A
AG09429	25	female	None (healthy non-fetal tissue)	N/A
NPC1 fibroblasts				
GM18422	NK	female	NPC1	813_815delCAT & ASP874VAL
GM18390	NK	female	NPC1	ASP242HIS & SER940LEU
GM22871	4	female	NPC1	1920delG & IVS9-1009G>A
GM17912	19	female	NPC1	PRO1007ALA & THR1036MET
Control brains				
UMB754	11.5	female	asthma	N/A
UMB1864	2.5	female	streptococcus infection	N/A
UMB4725	32.2	female	hypertension	N/A
UMB55	19.6	female	auto accident injuries	N/A
UMB4590	20.5	female	dilated cardiomyopathy	N/A
NPC brains				
UMB5372	11.3	female	NPC1	filipin & cholesterol esterification
UMB4214	32.3	female	NPC	confirmed neuropathology of NPC
UMB4237	19.8	female	NPC	confirmed neuropathology of NPC
UMBM4992M	20.1	female	NPC	clinical diagnosis of NPC

NK = not known. Human fibroblasts were purchased from Corielle and brains from NICHD Brain and Tissue Bank for Developmental Disorders. Independent t-test revealed no age difference between NPC and control brain donors (data not shown).

PRIMARY CELLS

Generation and Maintenance of mNPC

Neurosphere cultures containing mouse neuronal precursor cells, mNPC) were established from non-transgenic prenatal brains (E17-E18) following a modified protocol by Pacey et al. (2006) and maintained according to the procedure

developed by Marchenko and Flanagan (2007). Specifically, timed-pregnant C57BL/6J mice (Jackson Labs) were anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal (i.p.) injection and the fetuses retrieved through the transabdominal incision. Six to eight pups were removed from the amniotic sack, decapitated with a sterile blade and their brains cleaned of skulls, meningeal tissue and cerebella and pooled into a 60 mm culture dish in cold 1x PBS (Cellgro). The remaining cortical tissues were enzymatically and mechanically processed to obtain a single-cell suspension and grown in the presence of mitogens (i.e., growth factors). First, the cortices were minced and incubated in 5 ml of pre-warmed 0.25% trypsin (Gibco/Invitrogen) for 4 min at room temperature followed by a gentle trituration with a fire polished Pasteur pipette. After a centrifugation at 1500 rpm for 5 min, the majority of the trypsin was removed with the supernatant and the rest neutralized in 5 ml of Trypsin Neutralization Buffer (Lonza) for 4 min at 37˚C. The tissue aggregates were triturated with a fire-polished pipette for at least 10 times. Following the second centrifugation (1500 rpm for 5 min), the cell pellet was further resuspended and triturated in 1 ml of mouse neural precursor cell media (mNPC media, described below) (Schwartz et al., 2003; Marchenko and Flanagan, 2007) with fire polished pipettes of two different pore sizes (each 60-70 times) to yield a single-cell suspension. Lastly, after the viability and the cell concentration was established by Trypan Blue (Invitrogen) dye exclusion, the cells were plated in 24 multi-well tissue culture plates (Falcon) at a density of 50-100 cells/µl (Pacey et al., 2006) in 0.5 ml of mNPC media and grown in 37˚C, 5% CO_2 incubator. The mNPC

media consisted of DMEM:F12 growth media (Gibco) supplemented with 20% BIT-9500 (5x) serum substrate (Stem Cell Technologies), 1x PS (Cellgro), and the following growth factors at the concentration of 40 ng/ml: human epithelial growth factor (EGF), fibroblast growth factor (FGF)-basic, and platelet-derived growth factor (PDGF-AB) (PeproTech).

After 5-7 days, developed neurosphere clones were dissociated in 2 ml of Cell Dissociation Buffer (Invitrogen) for 5 min at 37˚C, centrifuged, resuspended and triturated in 0.5 ml of NPC media, and plated in 6-well plates (Fisher

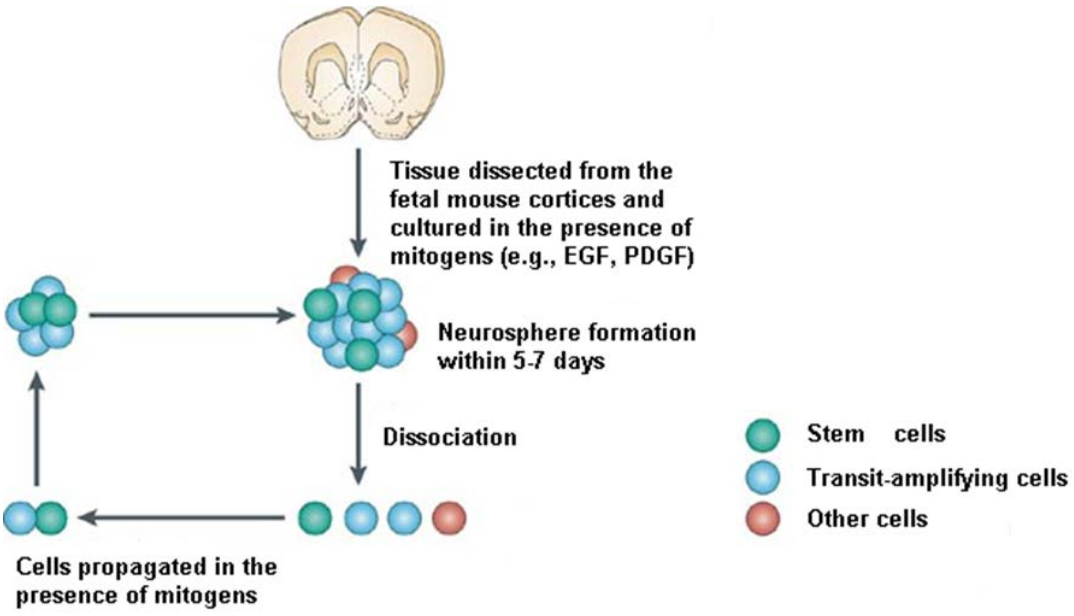


Figure 10. Development of Mouse Neural Precursor Cells
The cells were isolated from the mouse fetal cortices (E17-E18) and were mechanically and enzymatically processed to yield a single-cell suspension. The development of neurospheres clones was achieved by culturing dissociated clusters in growth factors-containing media, which resulted in newly formed spheres. Adapted and re-drawn from Chojnacki et al. (2009).

Scientific). Mature neurospheres (~100 µm in diameter, Pacey et al., 2006) were passaged once a week and grown in 25 cm2 tissue culture flasks (Falcon) in conditioned mNPC media (½ supernatant of old and ½ fresh growth media) to assure optimal cell growth. Similarly, only half of the media was changed every other day in between the passaging (Figure 10).

Mouse Splenocytes and Brain Cells

Mouse spleens and brains were harvested and processed to yield a single-cell suspension and prepared for karyotype and FISH analysis according to procedures described elsewhere (Boeras et al., 2008; Granic et al., 2010). Briefly, mouse spleens were removed, washed in sterile 1x PBS and triturated in RPMI 1640 medium (Gibco/Invitrogen) using the ends of two sterile Superfrost Plus slides (Fisher) to dissociate single cells. The cell suspension was grown for 44 hr in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-Glutamine, 5% PSA, 50 µM β-mercaptoethanol, and 5 µg/ml Concanavalin-A (Sigma) which was used as a mitogen.

Mouse brains were cleaned of meninges and mechanically dissociated in ice-cold 1x PBS using 5 ml and 2 ml pipettes and fire polished Pasteur pipettes of three different pore sizes (10-15 times each). The brain cells prepared as a single-cell suspension were centrifuged at 1500 rpm for 5 min, and then immediately resuspended and fixed in cold 3:1 anhydrous methanol (Fisher

Scientific):acetic acid (Fisher Scientific) (fixative) and incubated on ice for at least 30 min prior to any downstream assays being performed.

Human Brain Cells

Frontal cortices from the patients with the Niemann-Pick type C disease (aged 17.26 ± 11.07) and age, gender and race matched control brain tissues (age 20.88 ± 8.64) were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD) (Table 1). To generate a single-cell suspension, we used a technique established previously in the laboratory of H. Potter with fresh and frozen mouse brains (Granic et al., 2010). Briefly, 2 g of frozen brain tissue was triturated with fire polished Pasteur pipettes of different pore sizes in ice-cold 1x PBS. The cells and cell aggregates were centrifuged at 1500 rpm, the supernatant removed, and the cell pellet resuspended and fixed in either ice-cold fixative (3:1 anhydrous methanol and acetic acid) and incubated on ice for at least half an hour, or in 4% paraformaldehyde (PFA, Electron Microscopy Science) in 1x PBS and incubated in 37˚C water bath for 30 min.

MICE

APPKO Mice

Six transgenic mice lacking an *APP* gene and their non-transgenic littermates were 3-4 months of age (Jackson Labs) and had C57BL/6J background strain. The genotype was confirmed using QPCR Bio-Rad iCycler (Bio-Rad). Mice spleens were used for primary cell culture and lipoprotein/cholesterol treatments.

Wild-type Mice

Twelve one month old non-transgenic C57BL/6J mice (6 males and 6 females) (Jackson Lab) were caged in standard housing in pairs, and were fed ad libitum for 12 weeks with either regular rodent chow, low cholesterol or high cholesterol diet (Harlan Laboratories) with free access to fresh water. Food intake and weight gain were monitored and recorded weekly for each mouse. After 12 weeks, the mice were anesthetized with i.p. injection of sodium pentobarbital (50 mg/kg weight), and transcardially perfused with 0.9% saline before any internal organs of interest were removed. Livers, spleens and brains were visually inspected for pathological changes. All animals, including the two time-pregnant C57BL/6J mice were housed at the animal housing facility at the University of South Florida (USF) in a 12-hours light and dark cycle room at 22˚C. All animal

studies were approved and conducted in accordance with the guidelines stated by the USF Institutional Animal Care and Use Committee.

DIETS

Regular mouse chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan Laboratories) consisted of 5% crude plant-based oil (fat), 0% cholesterol, and supplied 3.3 Kcal/g of Digestible Energy (DE). Low cholesterol diet (7001 Teklad 4% Mouse/Rat Diet, Harlan) contained 4.2% crude oil, 0.02% cholesterol, and 3.23 Kcal/g of DE. The custom made high cholesterol diet (TD.95286, Harlan) consisted of 21% milk fat (fat), 1% of cholesterol, and provides 4.5 Kcal/g of DE.

REAGENTS

Lipoproteins

Human oxidized LDL (protein concentration 2 mg/ml), human LDL (protein concentration 5 mg/ml) and human HDL (protein concentration 10 mg/ml) were purchased from Biomedical Technologies Inc. The content of protein in each lipoprotein complex was assayed by the supplier using the Lowry method (Lowry et al., 1951), and the lipid concentration calculated according to chemical composition established by Fellin et al. (1974) and Barclay et al. (1972). For

example, chemical composition of human LDL is 22% protein and 78% lipid which brought the lipid content of the sample to 17.7 mg/ml. Water-soluble cholesterol (WsCh) was obtained from Sigma (31.8 mg of pure cholesterol in 0.6 g total weight of solid, balanced in methyl-β-cyclodextrin) and resuspended in sterile water.

Salts

For the calcium ions chelation, two different Ca^{2+} blockers were used. Chelator BAPTA (Calbiochem) served as an intracellular Ca^{2+} buffering reagent at the concentration of 1 mM. Four carboxylic functioning groups in BAPTA molecule bind to two calcium ions. EGTA chelator was added to the cells at the concentration of 1.5 mM. The reagent is selective for Ca^{2+}, and binds one cation per molecule (soluble in 1 M NaOH). hTERT-HME1 cells are cultured in the medium that contains 294 mg/L of calcium (or 2.65 mM).

OTHER REAGENTS, KITS, AND DNA PROBES

Absolute (without benzene) ethyl alcohol (Sigma-Aldrich) was prepared as a sterile aqueous 1 M solution and aliquoted. Filipin complex (Sigma) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at -20˚C in small aliquots protected from light. Giemsa stain (Gibco/Invitrogen) was diluted freshly

in the 1x GURR buffer (GibcoBRL Life Technologies) to stain metaphase spreads according to standardized cytogenetic protocol. Trypan blue (0.4%, BD Biosciences) exclusion assay was used to determine the viability of the hTERT cells grown in the closed chamber system with and without ethanol. Mouse anti-neuronal nuclei (NeuN) Alexa Fluor® antibody (Millipore) and VectaShield with 4',6-diamino-2-phenylindole (DAPI) (Vector Laboratories) was used to label neurons and nuclei, respectively. Nick Translation Kit (Abbott Molecular Inc.) and SpectrumGreen™ or SpectrumOrange™ dUTPs (Abbot, Vysis) were used to generate BAC probe following an established protocol (Kulnane et al., 2002; Boeras et al., 2008). Ethanol Assay Kit (BioVision) and BioTek Synergy HT micro-plate reader (BioTek Instruments) for colorimetric assay (optical density 570 nm; OD_{570}) and Gen5™ (BioTek Instruments) data analysis software were used to measure ethanol concentration in the media at the baseline, after 24 and 48 hr of exposure following manufacturer's instructions. Oil-Red-O staining kit (American MasterTech) was used to detect fatty deposits in hepatocytes of mice fed a high cholesterol diet. LSI® ETV6(TEL)/RUNX1(AML1) ES Dual Color Translocation Probe, LSI® IGH (14q32)/MALT1(18q21) Dual Color Dual Fusion Probe, and LSI® D7S522 (7q31) SpectrumOrange™/CEP® 7 SpectrumGreen™ were purchased from Abbott, Vysis.

IN VITRO INCUBATION WITH LIPOPROTEINS/CHOLESTEROL

Two days prior to treatment/incubation, freshly passaged hTERT cells (1-3 x 10^5 cells/2 ml) were plated in 100 mm tissue culture dishes (56 cm^2/dish) with supplemented Mammary Epithelium Basal Medium (MEBM, Lonza) to assure 60%-70% confluency on the day of the treatment. The cells were incubated for 48 hr at the concentration of 20 µg/ml of lipoproteins and 2 and 4 µg/ml of WsCh. Higher concentrations of WsCh were cytotoxic (data not shown).

First, 7 parallel experiments were conducted to assess the effect of lipoproteins on chromosome segregation (i.e., karyotype analysis and chromosome specific aneuploidy) and 6 separate experiments to test the aneugenic effect of cholesterol. Second, the same cell passages were used for the set of parallel experiments involving Ca^{2+} chelating reagents, BAPTA or EGTA. The cells were pretreated with the 1 mM and 1.5 mM of chelators, respectively for 3 min before lipoproteins were added. Prior to the harvest, the cells were treated with 33 ng/ml colcemid for 10 hr, fixed according to the standardized cytogenetic procedure, and prepared for metaphase chromosome and FISH analysis. Third, two sets of triplicate experiments involving lipoproteins and ethanol co-incubations were conducted separately with the same low cell passage (described below).

Similarly, two days before the treatment with lipids, freshly passaged HASMC were seeded onto pre-coated (2 µg/ml of poly-L-lysine, Sigma) single well glass chamber slides (8.6cm^2 growth surface/well) (BD Bioscience) at the

density of 10^5 cells per slide. After 48 hr of lipids/cholesterol incubation, the cells were rinsed twice with 1x PBS without Ca and Mg (Cellgro) and immediately fixed with cold 3:1 anhydrous methanol and acetic acid fixative and put at -20°C for 30 min. Fresh fix was added to the cells before storage at -20°C.

Human fibroblasts with and without functional LDL receptor were seeded at a density of ~2 x 10^5 cells per single chamber slide pre-coated with 2µg/ml of poly-L-lysine. The same procedure involving incubation with lipids and fixation was followed as with the HASMC.

Mouse non-transgenic and APPKO spleen cells were incubated with the lipoproteins at the same concentration mentioned above for 48 hr, harvested and fixed for FISH analysis using a BAC probe for mouse chromosome 16 according to established protocol (Kulnane et al., 2002; Boeras et al., 2008).

CLOSED CHAMBER FOR ETHANOL AND LIPIDS INCUBATION

To assure an exact and consistent ethanol (EtOH) concentration in treated cells, a modified closed chamber system protocol by Adickes et al. (1988) was used (described below). Prior to ethanol and lipoproteins co-treatments, several pilot experiments were conducted to examine the viability of hTERT-HME cells in the closed chamber, and to establish cytotoxicity of the two EtOH concentrations using the Trypan Blue dye exclusion assay and hemocytometry. First, the effect of 25 and 50 mM of EtOH for 6, 12 and 24 hr on cell morphology, proliferation

and survival was assessed (data not shown). Second, based on these results, triplicate experiments were conducted with 25 mM EtOH for 24 hours followed by lipoproteins/cholesterol and EtOH co-incubation for 48 hr.

Specifically, a day before pretreatment with EtOH in the closed chamber (a 27 x 16.5 x 11 cm tightly sealed polystyrene container), hTERT cells were seeded in 6-well plates at the density of 6 x 10^4/well and grown in 37˚C, 5% CO_2 incubator. Next, established cultures were incubated in 25 mM EtOH containing media for 24 hr and cultured in the sealed chamber alongside with an open dish (10 x 7 x 6 cm) filled with 300 ml 25 mM EtOH aqueous solution, which was changed daily to counteract alcohol evaporation. The next day, lipoproteins (20 µg/ml) and water soluble cholesterol (4 µg/ml) were added to the fresh culture media with EtOH, and the cells were grown for 48 hr. A small aliquot of EtOH-containing media from two samples was collected at the baseline, 24 and 48 hr later to measure alcohol concentration using a colorimetric assay (Ethanol Assay Kit, BioVision). Lastly, the cells were harvested and fixed for FISH analysis.

METAPHASE CHROMOSME ANALYSIS

After colcemid treatment, hTERT-HME1 cells were harvested according to the standard cytogenetic procedure described previously (Boeras et al., 2008). Briefly, the cells were hypotonically treated in 75 mM KCl for 15 min in a 37°C water bath, fixed in 3:1 anhydrous methanol: acetic acid fixative for a minimum of

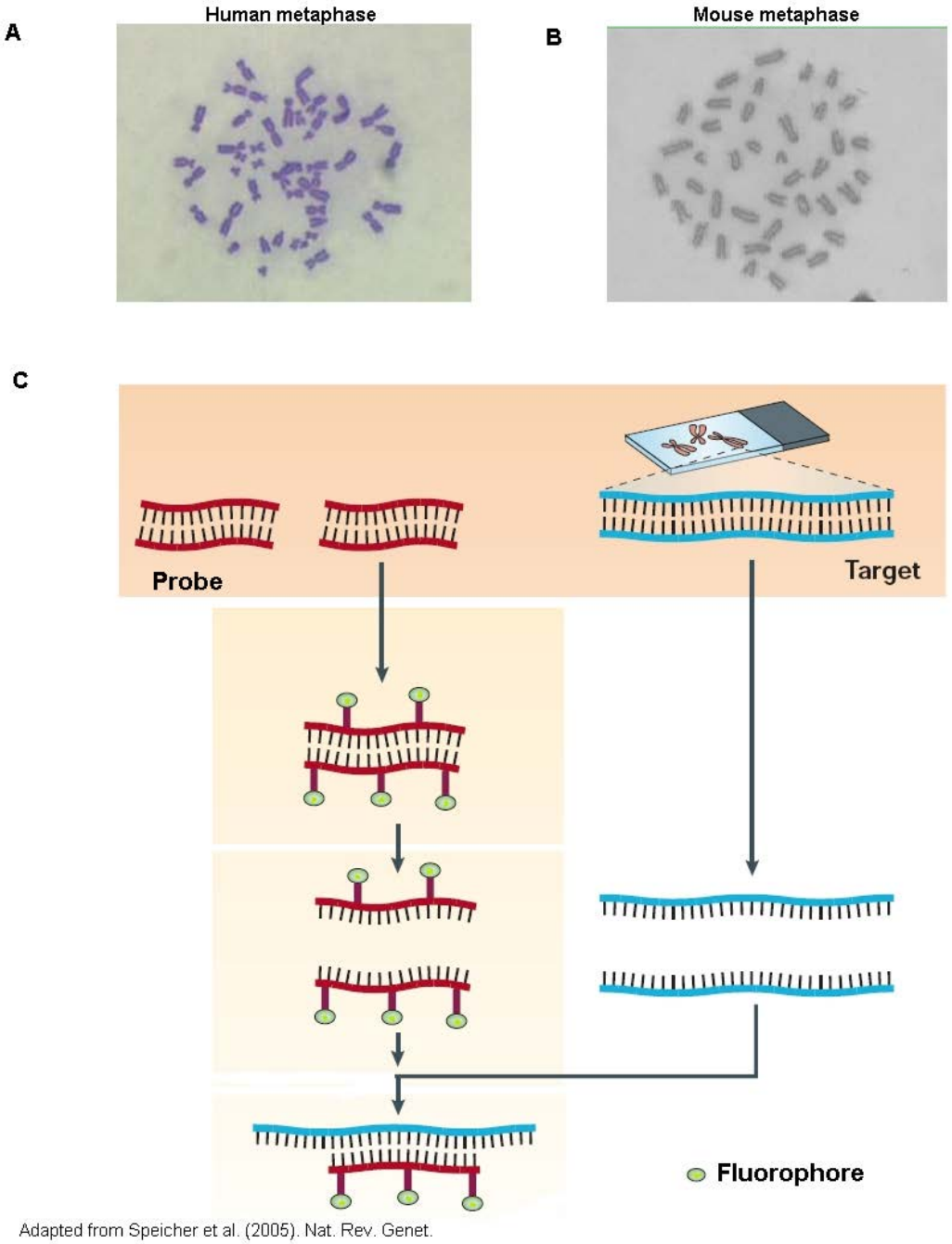
30 min on ice, followed by two fixative washes and resuspension in fresh fix. Metaphase spreads were stained with Giemsa stain (Gibco/Invitrogen Corporation) diluted in the 1x GURR buffer (GibcoBRL Life Technologies) and the chromosomes counted (Figure 11A, B). Genus 2.81® software was used for chromosome analysis (Applied Imaging). At least 40-45 metaphases were analyzed per each sample.

FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Fluorescence in situ hybridization (FISH) is a cytogenetic technique that allows one to detect the number of copies of a particular chromosome in both metaphase and interphase cells at the single-cell level (Ried, 1998; Tibiletti et al., 1999; Speicher and Carter, 2005) with great sensitivity and specificity (Tibiletti, 2004). This method is especially suitable for poorly and non-dividing cells, or for cells with a very low level of aneuploidy (e.g., lymphocytes). The basic principle of FISH consists in hybridization of fluorophore containing DNA probe with a unique genomic sequence to its complimentary DNA in cytological samples prepared on slides (Figure 11C). Annealed probe and target-DNA is then visualized in situ using fluorescent microscope filters that should match the wavelength of the selected fluorophore for proper detection and analysis (reviewed in Jiang and Katz, 2002; Tibiletti, 2007). In the present studies, for human samples commercially available single or dual-labeled locus specific

chromosome probes (Abbott, Vysis) were used, and for mouse samples, a ~300 kb unique mouse DNA sequence cloned into the bacterial artificial chromosome (BAC) cloning vector and labeled by Nick translation (Zhu et al., 1994) was utilized.

Prior to hybridization, all slides were aged at room temperature followed by hybridization procedures recommended by Abbott, Vysis and by protocols described elsewhere (Boeras et al., 2008; Granic et al., 2010; Kulnane et al., 2002). Briefly, aged slides were placed in 37˚C 2x SSC buffer for 45 min and dehydrated in serial ethanol washes (70%, 80%, and 90% for 2 minutes each), and targeted with a DNA probe mixture (7 µl hybridization buffer, 1 µl fluoroprobe, and 2 µl DNase-free water). Interphase FISH of all lipoprotein/cholesterol and reagents-treated human cell lines and primary cells was performed with either the LSI® ETV6(TEL)/RUNX1/(AML1) ES Dual Color Translocation Probe, LSI® D7S522 (7q31) SpectrumOrange ™/CEP® 7 SpectrumGreen™ or LSI IGH/MALT1 Dual Color Dual Fusion probe (Abbott, Vysis) in a HyBrite hybridization chamber (Vysis) according to manufacturer's specifications for a selected probe for at least 16 hr at 37˚C. A dual color detection protocol allows one to simultaneously assess two chromosomes under appropriate dual-band pass fluorescence microscope filters. The next day, slides were washed with 0.4x SSC and 2x SSC containing NP-40 detergent to remove nonspecifically-bound probe, and mounted with DAPI II (Abbott, Vysis) or Vectashield with DAPI (Vector Laboratories) counterstain.



Adapted from Speicher et al. (2005). Nat. Rev. Genet.

Figure 11. Metaphase Chromosome Spreads and FISH Procedure

(A-B) Human aneuploid metaphase (A) (>46 chromosomes) and mouse (B) euploid metaphases (40 chromosomes) were prepared following standardized cytogenetic protocol (Barnicot and Huxley, 1961) and stained with Giemsa.

(C) In the FISH procedure, DNA probe is labeled with fluorophore-containing nucleotides followed by the co-denaturing of the target and probe DNA and annealing of the complimentary DNA sequences.

A bacterial artificial chromosome (BAC) containing a mouse chromosome 16-specific sequence (a gift from Dr. Bruce Lamb at Case Western Reserve of University) was labeled by Nick translation and fluorophore-containing dUTP as described previously (Kulnane et al., 2002; Boeras et al., 2008; Granic et al., 2010). Briefly, 1 mM of either SpectrumGreen™ dUTP (2'-Deoxyuridine, 5'-Triphosphate; Abbott, Vysis) or SpectrumOrange™ dUTP (Enzio Life Sciences) was used to label 1 µg of BAC DNA in Nick translation enzymatic reaction/incubation (Nick Translation kit, Abbott) at 15˚C for 8-16 hr. Precipitated and pre-incubated (37˚C overnight) BAC probe was co-denatured with mouse spleen or brain cells at 74˚C for 4 min and hybridized at 37˚C in HyBrite chamber for either 22 or 48 hours, respectively, followed by two or three consecutive washes in SSC detergent-containing buffers of various stringencies (0.4x SSC, 2x SSC, and 4x SSC) and counterstained with DAPI II (Abbott, Vysis) or Vectashield (Vector) nuclear DNA stain.

FISH FOLLOWED BY IMMUNOCYTOCHEMISTRY

Studying the effect of PS1 mutation and overexpression on the cell cycle and chromosome mis-segregation in mouse models of FAD, this laboratory previously used a modification of the protocol by Liesi et al (2001) to develop primary mouse neuronal cultures followed by FISH analysis, therefore depending on availability of live animals and successful isolation of neuronal cells. Other

researchers that examined the levels and distribution of aneuploidy in normal and AD brains in humans and mice utilized either brain slices and immunofluorescence staining and cytometry to measure the DNA content in single neurons (e.g., Arendt et al., 2010), and mechanically isolated nuclei from different brain regions and FISH (e.g., Iourov et al., 2009), or FACS analysis of PFA-fixed brain cells labeled with a neuronal specific marker followed by FISH assay (e.g., Westra et al., 2008).

To circumvent the technical complexity of each method (e.g., a single-cell DNA quantification on the brain slices requires specialized instruments and multistep immunocytochemistry protocol), the lab developed a relatively simple technique which combines FISH and immunostaining performed in two consecutive steps on a single slide (Granic et al., 2010). To assess neuronal versus glial cell aneuploidy in the cells derived from either fresh or frozen brain tissue utilizing only fluorescence microscopy, mechanically dissociated single cells (nuclei) were first subjected to FISH assay with a single or dual labeled DNA probe followed by immunocytochemistry using a conjugated primary antibody against the neuronal nuclear antigen, NeuN labeled with AlexaFluor 488 or with other fluorophore. With this method, both neurons and glial cells from any brain region could be assessed for aneuploidy for any chromosome of interest.

Thus, a single-cell suspension of NPC and control frontal cortices (Table 1) were hybridized with a DNA probe for chromosome 21, and immediately after FISH, incubated in 1x PBS for 10 min, blocked in 10% goat serum/0.1% Triton X-100 PBS solution for 1 hr followed by overnight incubation in conjugated Ms X

Neuronal Nuclei AlexaFluor 488 (Millipore) 1:100 antibody prepared in 1% BSA/0.1% TritonX-100 1x PBS. After final washes in 1x PBS, the cells were stained with DAPI II counterstain and coverslipped.

IMMUNOCYTOCHEMISTRY, FILIPIN AND OIL-RED-O STAINING

Filipin is a cholesterol-specific fluorescent dye (Norman et al., 1972) used to quantify plasma membrane and intracellular free cholesterol and to demonstrate a correlation between decreases in membrane cholesterol and increased membrane fluidity (Coxey et al. 1993). To co-stain NPC brain cells with neuronal marker and filipin, a modification of the protocol described by Millard et al. (2000) was used. A single-cell suspension of 4 NPC and 5 control frontal cortices (Table 1) were fixed in 4% PFA in 1x PBS for 30 min at 37˚C. The cells were dropped onto pre-wetted slides (Fisher) that were marked around the edges with the ImmEdge pen (Vector Laboratories) to create a single chamber, and allowed to age overnight. After the three consecutive washes with 1x PBS for 10 min, the slides were stained/permeabilized with 50 µg/ml filipin in PBS/10% normal goat serum overnight at 4˚C, and then incubated in conjugated Ms X Neuronal Nuclei AlexaFluor 488 (Millipore) 1:100 antibody prepared in filipin/1x PBS/10%goat serum for 60 min in 37˚C hybridization oven on a shaker. Following final washes in 1x PBS, the cells were mounted with Fluoro-gel with Tris Buffer (Electron Microscopy Sciences) and coverslipped.

Oil-Red-O stain is used to detect intracellular neutral lipids in hepatocytes and adipogenic cultures (e.g., De Gottardi et al., 2007), and for detection of hepatic steatosis in liver cryosections (e.g., Zhao et al., 2011). Visual accumulation of cytosolic lipids in more than 5% of hepatocytes in human liver is considered as diagnostic of steatosis (Klein et al., 2005) as a consequence of an increased efflux of free fatty acids and triglycerides formation (Bradbury, 2006). A single-cell suspension of 4% PFA/1x PBS-fixed livers were dropped onto slides and stained with the Oil-Red-O staining kit following manufacturer's procedures (American MasterTech). Hematoxylin stained single cells (blue) and cell aggregates were evaluated under phase microscopy for the presence of lipid droplets (red).

IMAGE AQUISITION AND ANALYSIS

FISH Visualization and Scoring

Hybridization signals were visualized using a Nikon Eclipse E1000 fluorescence microscope with a 4912 CCIR high performance COHU CCD Camera and Genus 2.81® software (Applied Imaging) or a Zeiss Imager.M1 microscope with a CV-M4+CL high resolution camera and Axiovision 4.6 software (Zeiss). The corresponding optical filters and their spectral bandwidths were: DAPI (peak excitation/emission (ex/em) 350 and 470 nm), Fluorescein Isothiocyanate (FITC)

(ex/em 490/525 nm), and Tetramethyl Rhodamine Isothiocyanate (TRITC) (ex/em 557/576 nm) for the Nikon Eclipse scope, and Zeiss filter sets 49 DAPI/Hoechst 33258 (ex/em 365/445[450] nm), 38 HE eGFP/FITC/Alexa488 (ex/em 470[40]/525[550] nm) and 43 HE Cy3/DsRed/Rhodamine (ex/em 550[525]/605[670] nm) from Chroma.

Only intact nuclei (DAPI) and compact and bright signals were scored for aneuploidy separately for each probe/fluorophore following the Abbott/Vysis (http://www.abbottmolecular.com/contactus/fishtechsupport/techtipsandinfo/fish-lab-quality-control.html) guidelines. For example, overlapping cells were not analyzed and closely adjacent spots or spots connected with a fluorescent strand were evaluated as a split signal and counted as one chromosome.

During the course of our studies, this laboratory experienced high hybridization efficiency (> 98%) with both commercially available (Vysis) and homemade BAC probes (data not shown) on cell lines and primary cells as a source of target-DNA. The evaluation of chromosomal abnormalities in the samples lacking more than 10% of fluorescent spots is not recommended because it leads to underestimation of trisomies and overestimation of monosomies and miscalculation of aneuploidy (Tibiletti, 2007). A similar problem arises when the nuclei are processed from paraffin sections (slices) or isolated from paraffin-embedded tissue blocks if the architecture of analyzed cells is not correctly assessed (Tibiletti, 2007). To avoid any biases in scoring of euploid and aneuploid cells in the study samples, only non-overlapping single nuclei derived from a single-cell suspension were evaluated. A portion of the

hybridization area was routinely recounted for reliability. On average, between 700 and 1,000 interphases per each sample/treatment were scored for aneuploidy, and about 400 NeuN-positive and NeuN-negative NPC brain cells were analyzed for DNA hybridization spots.

Filipin Semi-quantitative Analysis

First 50 NeuN-positive NPC and control brain cells were semi-quantitatively assessed for the most intense filipin stain and cholesterol localization and categorized into three following groups: membrane staining, intracellular staining and both. For filipin visualization and image acquisition, a Zeiss Imager.M1 microscope with a CV-M4+CL high-resolution camera and 49 DAPI/Hoechst 33258 filter set (Chroma) was used, along with the Axiovision 4.6 software (Zeiss). Also, a Zeiss Imager.M1 and phase contrast was used to assess the presence of Oil-Red-O staining in mouse hepatocytes.

STATISTICAL ANALYSIS

To establish whether lipoprotein/cholesterol induce chromosome mis-segregation in various human and mouse cells (i.e., hTERT-HME, HASMC, human fibroblasts, mouse splenocytes, and mouse neural precursors), a Paired Student's t-test was used to compare the aneuploidy levels (mean ± SEM) between treated cells and controls. Similarly, to answer the question if a

functional *LDLR* or *APP* gene is required to observe aneugenic effect of lipids in vitro, a Paired t-test was employed to contrast the levels of aneuploidy in lipoproteins-treated fibroblast harboring malfunctional *LDLR* compared to controls, and in lipid-treated mouse splenocytes lacking *APP* gene compared to untreated cells (Research Question 1).

To investigate the role of intracellular cholesterol homeostasis in chromosome mis-segregation in a neurodegenerative disease model, an Independent t-test was used to compare the aneuploidy levels between the NPC1 fibroblasts and the NPC brain cells and corresponding age, gender and race matched controls. The same statistical analysis was utilized to contrast filipin staining between the NeuN-positive NPC cells and normal neurons (Research Question 1).

To establish the effect of a dietary-induced hypercholesterolemia on chromosome segregation in peripheral tissues in a mouse model, a Paired t-test was used to compare the levels of aneuploidy in spleens of the mice (littermates) fed a high cholesterol to mice consuming regular diet, and the mice fed a low cholesterol diet to mice on regular chow (Research Question 2).

To explore the mechanism of aneuploidy induction in vitro, and to establish whether Ca^{2+} chelation and plasma membrane fluidization by ethanol may attenuate lipoproteins/cholesterol-induced aneuploidy, a Paired Student t-test was used to compare aneuploidy levels between the lipids-treated cells with and without chelator, and the cells co-incubated with lipids and ethanol compared to appropriate controls (Research Question 3).

Three to 7 treatments with lipids for each cell line, and triplicate experiments with the chelator and ethanol were conducted and scored for aneuploidy. Four to 5 primary NPC cells sources and controls (Table 1) and at least 6-12 mice were analyzed for each graph. All data were presented as mean ± SEM of treatments and controls with $p < 0.05$ considered as statistically significant.

SUMMARY

To summarize, the following materials and methods were used to answer the research questions. For the Research Question 1 (Do lipids induce aneugenesis in vitro and is the induction dependent on a functional LDL receptor and APP gene?), hTERT-HME1, HASM cells, human fibroblasts with and without functional LDLR, mouse APPKO splenocytes and neural precursors, human NPC1 fibroblasts and brain cells where treated either with lipoproteins/cholesterol and/or assessed for aneuploidy utilizing the FISH assay, and for the intracellular cholesterol distribution using filipin staining. For the Research Question 2 (Does diet-induced hypercholesterolemia affect chromosome mis-segregation in visceral and brain tissues of wild-type mice?), young nontransgenic animals were fed three different diets, and the spleen and brain cells analyzed for chromosome 16 aneuploidy using a BAC probe and FISH. For the Research Question 3 (Is there a mechanistic link between calcium homeostasis (Mechanism I) and plasma membrane dynamics (Mechanism II) and lipid-induced chromosome mis-

segregation in vitro?), hTERT cells were treated with lipoproteins in the presence or absence of calcium chelators (BAPTA and EGTA) and EtOH, respectively, and analyzed for aneuploidy using FISH.

CHAPTER FOUR

RESULTS

To investigate the role of lipoproteins and cholesterol in chromosome segregation in vitro and in vivo, and to establish the effect of disrupted cholesterol homeostasis on the cell cycle, we used: (1) human and mouse primary cells with a functional LDL receptor; (2) mouse primary cells lacking *APP* gene; (3) human primary cells with defective *LDLR* and Niemann-Pick Type C1 (*NPC1*) genes responsible for lipoprotein uptake and intracellular cholesterol trafficking, respectively; (4) young wild-type mice fed high (1.05%) and low (0.02%) cholesterol diet for 12 weeks to induce hypercholesterolemia; and (5) metaphase chromosome analysis and FISH to examine the levels of total and chromosome specific aneuploidy, respectively. Specifically, we assessed the level and distribution of trisomy and tetrasomy of human chromosomes 7, 12, 14, 18, 21, and mouse chromosome 16 in various peripheral and brain cells. Mis-segregation of these chromosomes has been implicated in AD (e.g., Boeras et al., 2008; Geller and Potter, 1999; Granic et al., 2010; Migliore et al., 1999; Thomas and Fenech, 2008) and in development of atherosclerotic lesions (e.g., Casalone et al., 1991; Fernandez et al., 2000; Matturri et al., 1997; 2001). Lastly, (6) we examined the mechanisms by which lipoproteins and cholesterol

may cause chromosome mis-segregation, and asked whether (a) alternation in extracellular Ca^{2+} homeostasis by either EGTA or BAPTA, and (b) a change in membrane fluidity and elasticity by ethanol, may decrease lipoproteins/cholesterol-induced chromosome mis-segregation in the cell culture.

This chapter describes individual research questions, scientific rationale and study results in three sections: (1) lipoproteins/cholesterol-induced chromosome mis-segregation in vitro (Research Question 1); (2) diet-induced peripheral genomic instability in vivo (Research Question 2), (3) mechanistic link between calcium homeostasis and plasma membrane fluidity and lipids-induced aneuploidy in vitro (Research Question 3).

LIPOPROTEINS/CHOLESTEROL-INDUCED ANEUPLOIDY IN VITRO

hTERT-HME1 Cells Treated with Lipoproteins Developed Aneuploidy within 48 Hours

Recently, this laboratory has shown that overexpression or mutation in the *PS1* or *APP* genes causes chromosome aneuploidy, including trisomy 21 in cellular and animal models of AD (Boeras et al., 2008; Granic et al., 2010) partly via Aβ peptide by disrupting the microtubule integrity and the function of mitotic spindle motors (Borysov et al., 2011). To broaden the knowledge that cell cycle defects and accumulation of aneuploid/hyperploid cells, including neurons may contribute

to neurodegeneration and dementia (e.g., Arendt et al., 2010; Iourov et al., 2009; Obrenovich et al., 2003; Potter, 2004), this dissertation sought to investigate the aneugenic effect of other effector molecules implicated in pathology of AD and AD-related risk factors (i.e., atherosclerosis), such as LDL and OX-LDL. To answer the question whether lipoproteins at physiologically elevated levels induce mitotic defects and chromosome mis-segregation in dividing cells with low aneuploidy background and a functional LDL receptor, human primary epithelial cells with a stable karyotype (i.e., permanently expressing a telomerase reverse transcriptase gene; Jiang et al., 1999; Morales et al., 1999), the hTERT-HME1 cells were exposed to OX-LDL, LDL and HDL for 48 hr at the concentration of 20 µg/ml and assessed for aneuploidy by metaphase chromosome analysis and FISH. First, seven parallel cultures of hTERT-HME1 cells were incubated with lipoproteins, then arrested at metaphase by colcemid and assessed for aneuploidy (metaphase spreads both of < and > of 46 chromosomes). The results showed a significant increase in chromosome mis-segregation, especially in OX-LDL and LDL-treated cells compared to controls ($p = 0.0001$, and $p = 0.005$, respectively; Paired t-test; Figure 12A). LDL-cholesterol induced significantly more mis-segregated cells compared to less aneugenic HDL ($p = 0.005$) and OX-LDL ($p = 0.01$). Second, the induction of chromosome specific aneuploidy was analyzed by quantitative FISH analysis (Figure 12B-G), and revealed the induction of: (1) trisomy 21 ($p = 0.02$) and 12 by OX-LDL ($p = 0.02$; Paired t-test) and a borderline significant induction of trisomy 21 and 12 by LDL

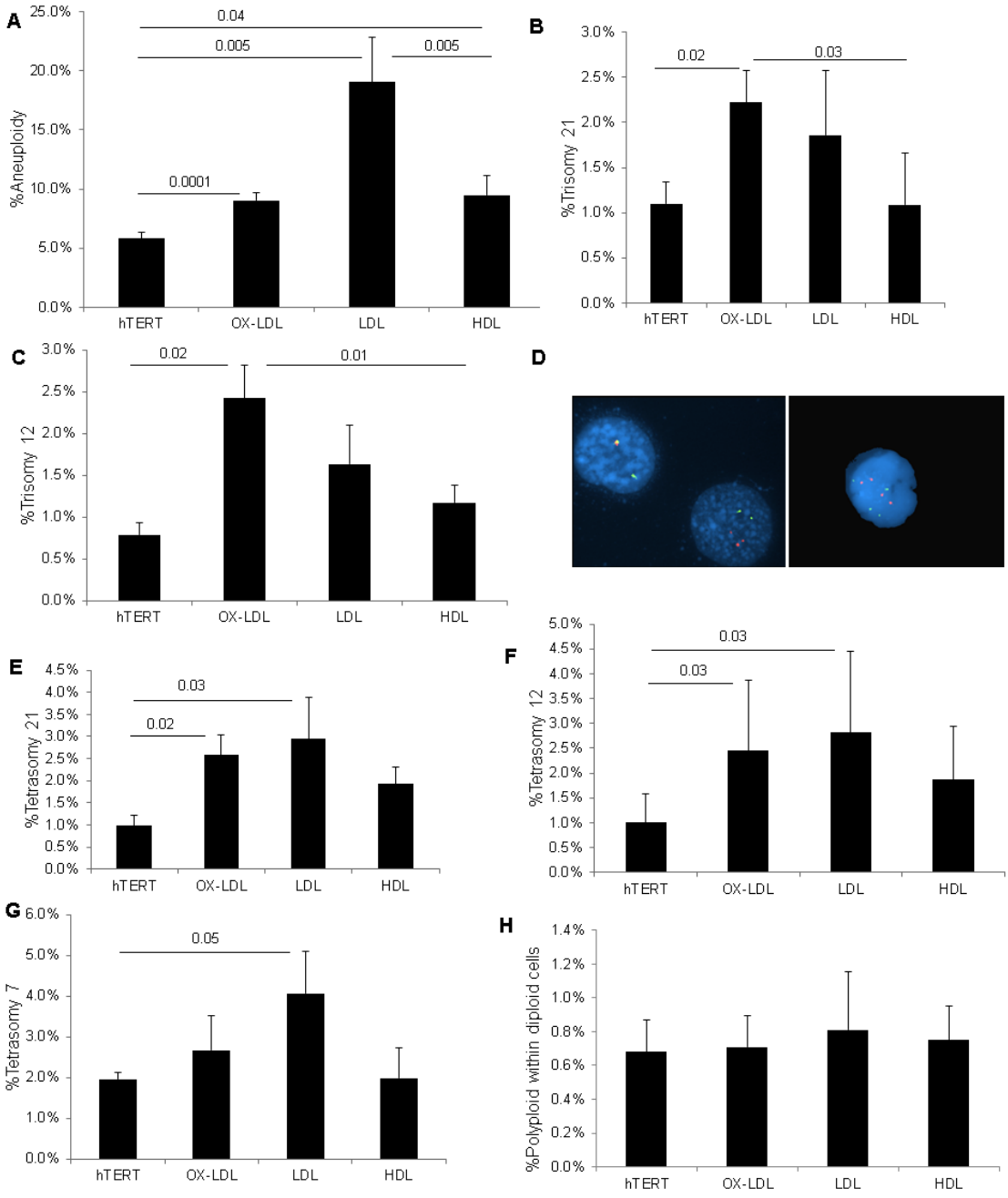


Figure 12. Chromosome Aneuploidy, Including Trisomy and Tetrasomy 21, 12 and 7 Induced in Lipoproteins-treated hTERT-HME1 Cells

(A) hTERT-HME1 cells were treated with 20 µg/ml of OX-LDL, LDL and HDL for 48 hr and arrested in metaphase by incubation in 33 ng/ml of colcemid for 10-12 hr. Hypotonic treatment in 0.075 M KCl was followed by a cell fixation in 3:1 methanol:acetic acid. Fixed cells were Giemsa stained for karyotype analysis. All lipid-treated cells harbored significantly higher levels of aneuploidy compared to controls, especially those treated with LDL-cholesterol.

(B-G) FISH analysis of the same lipoproteins-treated cells revealed OX-LDL-induced trisomy 21 (B) and trisomy 12 (C), and OX-LDL and LDL-induced tetrasomy 21 (E), tetrasomy 12 (F), and tetrasomy 7 (G).

continued>

LSI® ETV6(TEL)/RUNX1(AML1) ES Dual Color Translocation Probe (SpectrumOrange™ 21/SpectrumGreen™ 12) was used to detect complimentary DNA sequence on chromosome 21 and 12 in interphase cells (D), whereas LSI® D7S522 (7q31) SpectrumOrange™/CEP 7 SpectrumGreen™ probe was used to detect LDL-induced tetrasomy 7 (G). Atherogenic OX-LDL induced significantly higher levels of trisomy 21 and 12 compared to HDL-cholesterol. No induction of trisomy 7 in any lipid-treated sample was observed (data not shown).
(H) Karyotype analysis of an aliquot of the cells from the same treatments showed low number of polyploidy cells, suggesting that the tetrasomies observed were due to chromosome mis-segregation of chromosomes 21, 12 and 7, and not a result of chromosome duplication without cell division.
A Paired t-test was used to compare aneuploidy levels (percentages) between lipids-treated and control cells with $p < 0.05$ considered to be statistically significant.

(not significant; Figure 12B-D); and (2) tetrasomy 21, 12, and 7 induced by OX-LDL and/or LDL ($p = 0.03$, Paired t-test; Figure 12D-G). Compared to HDL, OX-LDL-cholesterol induced significantly more trisomic 21 and 12 cells within 48 hr ($p = 0.03$ and $p = 0.01$, respectively; paired t-test).

Taken together, the data suggest that OX-LDL and LDL-induced tetrasomy in interphase cells happed possibly due to either cell reduplication with incomplete cell division or due to chromosome mis-segregation for a particular chromosome. The presence of tetraploid neurons has been observed in AD brains of humans and mice, and explained by some researchers as due to aberrant cell cycle activation and DNA duplication without the cell cycle completion (e.g., Arendt et al., 2009; Obrenovich et al., 2003; Yang et al., 2001; 2006; Yang and Herrup, 2007; Zhu et al., 2008). In order to determine why there is more than diploid content of DNA in interphases of lipid-treated hTERT cells, a total number of metaphase hyperploid/polyploid and diploid cells were scored in the portion of the slide initially analyzed for karyotypes. There was no elevation of polyploid (>4n) within diploid (2n) cells in OX-LDL and LDL-treated samples indicating that the increases in interphase tetrasomies were most likely due to

chromosome mis-segregation for the particular chromosome of interest (p = 0.4 and p = 0.3, respectively; Figure 12H).

LDL Induced Trisomy 7 in HASM Cells

Cytogenetic analyses of atherosclerotic plaques from diseased human arteries and isolated primary smooth muscle cells (SMC) from these plaques have shown a variety of chromosomal instabilities, including trisomy and tetrasomy 7, monosomy 11, trisomy 10, 18, 20, and loss of the Y chromosome (Casalone et al., 1991; Fernandez et al., 2000; Lavezzi et al., 2005; Matturri et al., 1997; 2001). According to the Monoclonal Proliferation Theory of atherogenesis, individual SMCs occasionally undergo a somatic mutation and chromosomal alternations resulting in increased proliferative activity and consequent clonal expansion to yield atherosclerotic lesions (Benditt and Benditt, 1975; Casalone et al., 1991; Murry et al., 1997; Vanni and Licheri, 1991) similar to development of aneuploid benign tumors. Therefore, the effect of lipoproteins (OX-LDL, LDL and HDL) on chromosome segregation in primary human aortic smooth muscle cells (HASMC) was assessed, and the cells treated with lipoproteins (20 μg/ml) for 48 hr, fixed, and analyzed by FISH with fluorescent DNA probe for chromosome 7. Quantitative FISH analysis showed a statistically significant 3-fold increase in trisomy 7 (p = 0.0007, Paired t-test; Figure 13A, B) in LDL-treated cells, and a borderline significant increase in tetrasomy 7 over the background (6.2% over 3.4%, respectively; data not shown). Compared to HDL, atherogenic and

aneugenic LDL-cholesterol induced significantly more trisomy 7 (p = 0.01, Paired t-test) within 48 hr.

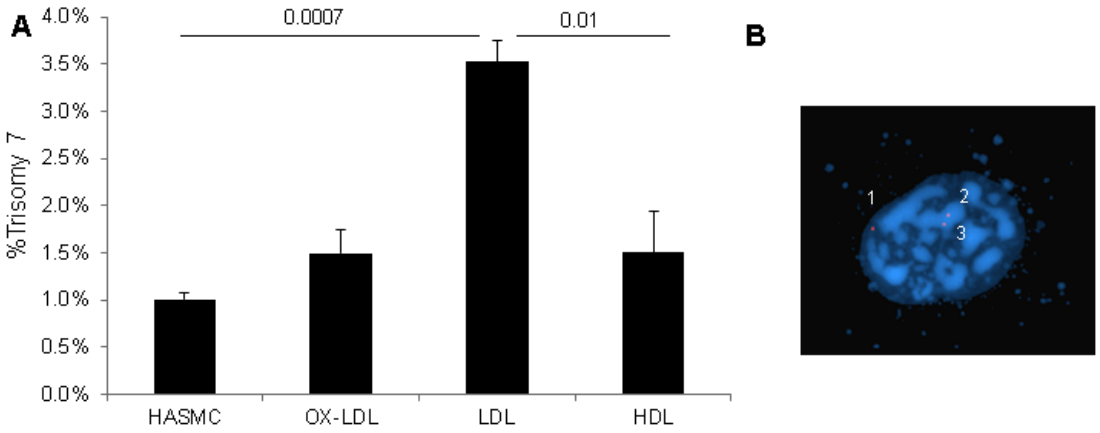


Figure 13. LDL-induced Trisomy 7 in HASM Cells
(A) Quantitative FISH analysis of HASM cells showed an increase in trisomy 7 when incubated with 20 µg/ml of LDL for 48 hours. Atherogenic LDL-cholesterol induced significantly more trisomy 7 compared to HDL-treated cells. A Paired t-test was used to compare aneuploidy induction between treated and untreated cells, and between LDL and HDL-treated cells.
(B) LSI® D7S522 SpectrumOrange™ probe was used to detect a region on chromosome 7 in interphase cells.

LDL-induced Trisomy 7 may be Dependent on a Functional LDL Receptor

To establish whether lipoprotein-induced chromosome mis-segregation may be mediated through the LDLR and thus dependent on intracellular signaling, human primary cells from the patients diagnosed with familial hypercholesterolemia (FH) were obtained, and the aneuploidy levels were compared to control cells with a functional LDLR after exposing them to various lipids under the same conditions.

LDL receptor belongs to a large family of structurally and functionally homologous cell-surface receptors, the LDLR receptor family (Brown and Goldstein, 1976; 1986; Herz and Bock, 2002; Hussain et al., 1999) which are involved in diverse cellular functions implicated in AD pathogenesis (Jaeger and Pietrzik, 2008; Andersen and Willow, 2006), from cholesterol metabolism and Apolipoprotein E (ApoE) binding (Herz and Beffert, 2000) to APP trafficking and processing (Abisambra et al., 2010; Marzolo and Bu, 2009). The LDLR is a ubiquitously expressed transmembrane protein that mediates clearance of cholesterol rich LDL particles circulating in the blood and facilitates LDL internalization into cells (Brown and Goldstein, 1976; 1986; Goldstein and Brown, 1976; 1987), and is, therefore, essential for cholesterol homeostasis. To date, there are more than 1066 unique LDLR genetic alternations that lead to FH (Leigh et al., 2008) causing three to four times higher levels of LDL cholesterol than normal (Khachadurian, 1964) and premature CVD events, including severe atherosclerosis (reviewed in Fahed and Nemer, 2011).

To investigate whether lipoprotein-induced chromosome mis-segregation requires a functional LDLR, FH primary human fibroblasts (Coriell repository number GM01915) harboring two mutations in the *LDLR* gene and normal human fibroblasts (NHF, repository number GM01661) were treated with 20 µg/ml of lipoproteins for 48 hr and assessed for aneuploidy. Quantitative FISH analysis revealed that LDL treated NHF develop significantly higher levels of trisomy 7 compared to FH fibroblasts exposed to the same concentration of LDL ($p = 0.03$, Paired t-test; Figure 14A, B). Compared to HDL-cholesterol,

atherogenic LDL induced more aneuploidy cells which reached a borderline significance (p = 0.07). The data indicates that a functional LDLR is necessary to observe LDL-induced chromosome mis-segregation and aneuploidy generation in vitro.

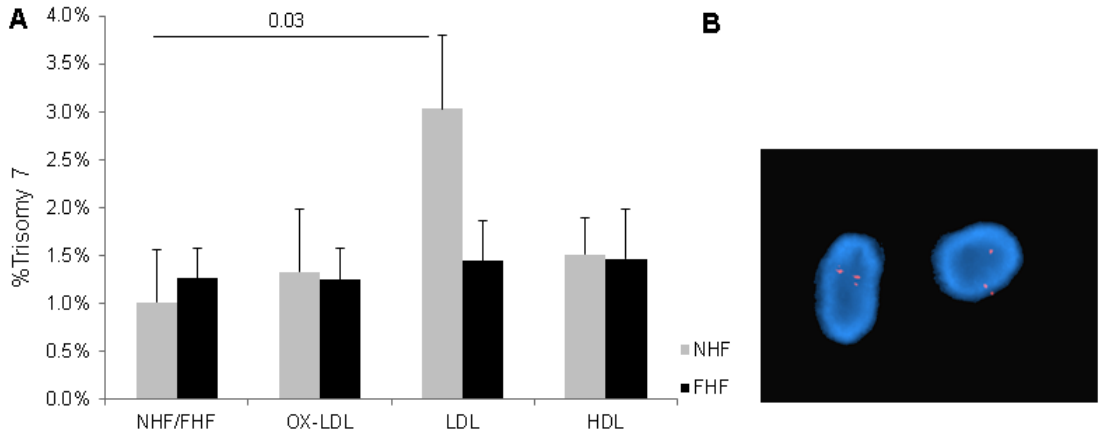


Figure 14. LDL-induced Trisomy 7 in Primary Human Fibroblasts may be LDLR Dependent
(A) Normal human fibroblasts (NHF, light gray bars) developed significantly higher levels of trisomy 7 when treated with 20 µg/ml of LDL within 48 hr compared to LDL receptor deficient human fibroblasts obtained from the patient diagnosed with familial hypercholesterolemia (FH, black bars). Compared to HDL-treated NHF, LDL-cholesterol induced a 2-fold increase in trisomy 7 that reached a borderline significance (p = 0.07, Paired t-test). The data suggest that a functional LDLR may be required for aneugenic effect of LDL in vitro.
(B) FISH analysis and LSI® D7S522 SpectrumOrange™ probe was used to detect chromosome 7 in interphase cells.

Lipoprotein-induced Aneuploidy is Independent of APP

As discussed previously, accumulated evidence from experimental, animal and human studies points to the role of cholesterol in APP processing and Aβ generation and clearance (e.g., Eckert et al., 2000; Panza et al., 2009; Pfrieger,

2003; Schneider et al., 2006; Solomon et al., 2008). High lipoprotein/cholesterol within the plasma membrane promotes (Ehehalt et al., 2003; Hooijmans et al., 2007; Refolo et al., 2001; Sparks et al., 1994; Xiong et al., 2008), and low cholesterol reduces, the production of Aβ (Fassbender, et al., 2001; Refolo et al., 2001; Simons et al., 1998), partly by changing the activity of the key membrane-bound secretases involved in APP proteolysis (e.g., Bodovitz and Klein, 1996; Kojro et al., 2001; Schneider et al., 2006; Wahrle et al., 2002) within lipid rafts microdomains (Lee et al., 1998; Parkin et al., 1999; Refolo et al., 1991; Simons and Toomre, 2000). Furthermore, Aβ and presenilin (PS1/2) affect lipid/cholesterol metabolism (Cutler et al., 2004; Grimm et al., 2005) and cholesterol content of the plasma membranes, influencing in turn their fluidity and function (Grimm et al., 2006).

As mentioned previously, this lab reported that familial AD-causing (FAD) mutations in the *PS1* and *APP* gene and their pathogenic product, the Aβ peptide induced aneuploidy in transgenic mice and transfected cells in culture (Boeras et al., 2008; Granic et al., 2010) by disrupting the formation and stability of mitotic spindle and by interfering with normal microtubule function (Borysov et al., 2011). Moreover, Aβ-induced aneuploidy is dependent on a functional *Tau* gene and tau-stabilized microtubules, and on interaction with endogenous APP (Granic et al., 2010). Similarly, Aβ-induced cytotoxicity requires a full-length APP possibly as a cell-surface receptor to aid endocytosis and generation of intracellular or for the uptake of extracellular Aβ (Lorenzo et al., 2000; Shaked et al., 2006).

To test the hypothesis that observed lipoprotein/cholesterol induced chromosome mis-segregation in vitro may be independent of APP, APP processing and consequent Aβ production, primary splenocytes from APPKO mice and nontransgenic littermates were isolated and exposed to lipoproteins for 48 hr. The cells were fixed and hybridized with the mouse chromosome 16 BAC

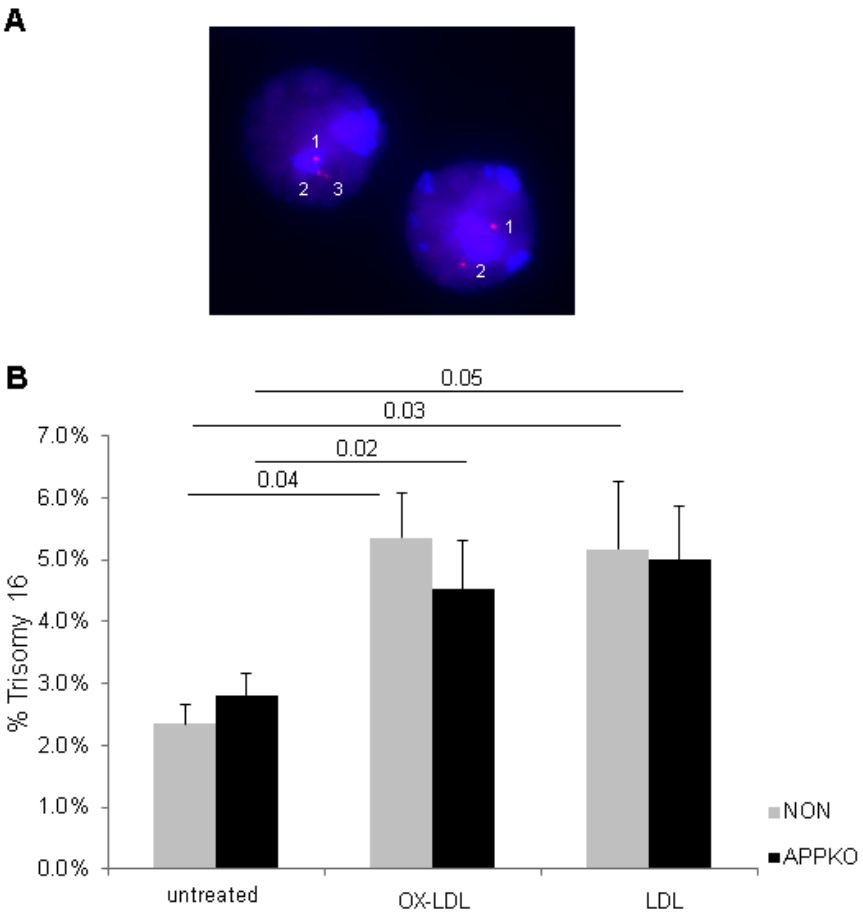


Figure 15. Primary Mouse Splenocytes Lacking the APP Gene Expression Develop Aneuploidy Upon Lipoproteins Treatment

(A) In situ DNA FISH with a BAC plasmid containing mouse chromosome 16 labeled with the SpectrumOrange™ dUTP was used to detect the levels of aneuploidy in lipoprotein-treated primary splenocytes derived from non-transgenic (NON) and APP gene deficient mice (APPKO).

(B) Quantitative FISH analysis revealed comparable levels of trisomy 16 in both cell types upon incubation with 20 µg/ml of OX-LDL and LDL within 48 hours. The data suggest that aneugenic activity of lipoproteins may be independent of a functional *APP* gene and thus probably independent of Aβ. Paired t-test was used to compare the levels of aneuploidy in lipid-treated and untreated cells.

probe (Kulnane et al., 2002) labeled with SpectrumOrange™ dUTPs by Nick translation (Enzio Life Sciences) and scored for aneuploidy (Figure 15A). OX-LDL and LDL induced significant increases in trisomy 16 in both cells types compared to controls ($p = 0.04$ and $p = 0.02$ in OX-LDL-treated normal and APPKO splenocytes, and $p = 0.03$ and $p = 0.05$ in LDL-treated cells, respectively, Paired t-test; Figure 4B), suggesting that aneugenic effect of lipoproteins may be independent of APP expression and Aβ production. Thus, endogenous APP may not be required for lipoproteins-induced aneuploidy, though it is needed for aneugenic activity of Aβ (Granic et al., 2010), Aβ-induced tau phosphorylation (Pigno et al., 2001; Small and Duff, 2008) and Aβ cytotoxicity (Rapoport et al., 2002; Roberson et al., 2007).

Water-soluble Cholesterol Induced Aneuploidy In Vitro

Cholesterol is the most abundant lipid in eukaryotic cell membranes (Bloch, 1991) and is compartmentalized into microdomains (e.g., lipid rafts; Brown and London, 2000; Crane and Tamm, 2004; Pike, 2003), where it is required for essential cellular functions and membrane structure, such as signaling transduction and plasma membrane dynamics (Mukherjee and Maxfield, 2004; Simons and Toomre, 2000; Weber et al., 2006). It is well established that membrane cholesterol content affects membrane stiffness and fluidity (Demel and De Kruyff, 1976), which can be changed by either cholesterol enrichment or depletion, respectively (e.g., Abramov et al., 2011; Mukherjee and

Chattopadhyay, 2005; Weber et al., 2006). Also, cyclodextrins as cholesterol acceptors (e.g., methyl-β-cyclodextrin, MβCD) have been effectively used to manipulate intracellular cholesterol levels (Christian et al., 1997; Yancey, et al., 1996) and to aid the delivery of hydrophobic cholesterol into the plasma membrane and cytosolic compartments (Härtel et al., 1998; Ohvo et al., 1997).

To examine the effect of MβCD-delivered exogenous cholesterol on chromosome segregation in vitro, water-soluble cholesterol (WsCh), a MβCD:cholesterol complex, and karyotype and FISH analysis was used to assess the levels of overall and chromosome specific aneuploidy. First, hTERT-HME cells were incubated with either 4 μg/ml or 2 μg/ml of WsCh for 48 hr, and the metaphases analyzed for chromosome mis-segregation. The cells treated with 4 μg/ml of cholesterol showed a 2-fold increase in total aneuploidy compared to untreated cells ($p = 0.01$, Paired t-test) but no induction of chromosome mis-segregation at the concentration of 2 μg/ml of WsCh was observed (not significant; Figure 16A). Second, FISH analysis for a specific chromosome revealed a significant increase in trisomy 21 and trisomy 12 in 4 μg/ml WsCh treated cells ($p = 0.01$ and $p = 0.03$, respectively, Paired t-test; Figure 16B) and no induction of tetrasomy 21 or 12 (data not shown). Taken together, the data suggest that aneugenic effect of cholesterol is likely to affect all chromosomes, and that this biological effect may be related to the changed membrane fluidity in which cholesterol-enriched, more rigid membranes of the nuclear envelope fail to properly attach chromosomes during mitosis, leading to generation of aneuploid cells.

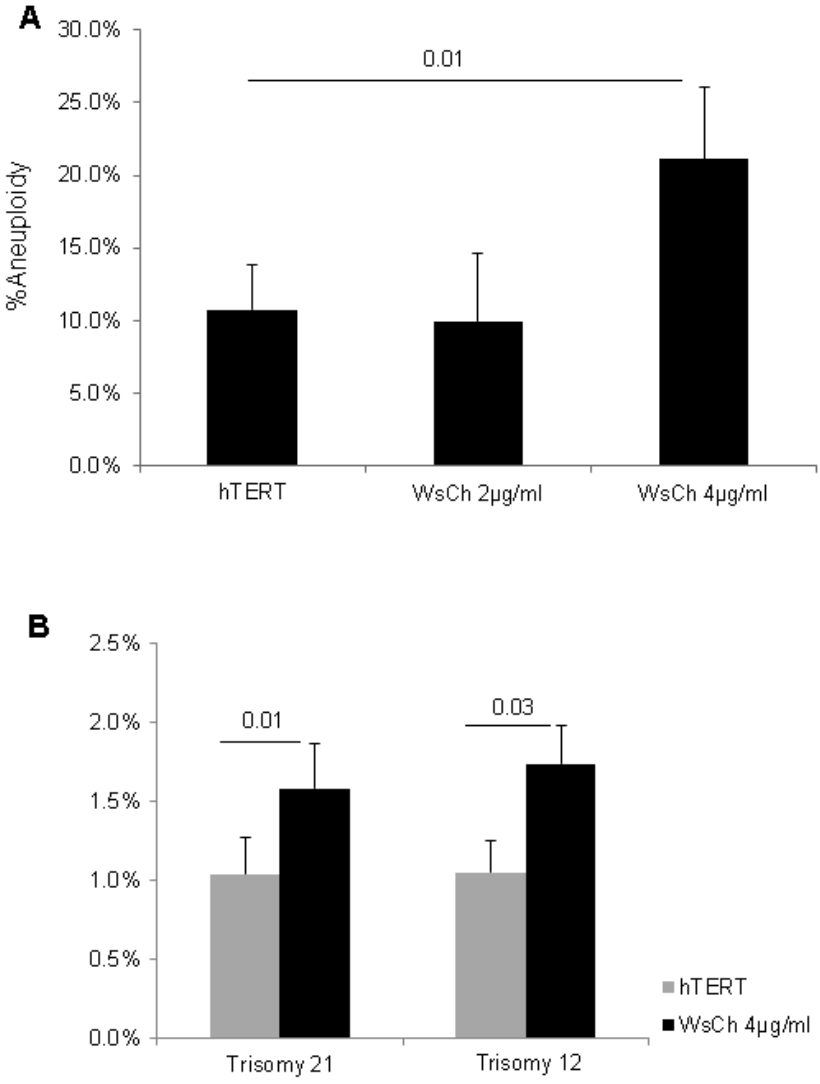


Figure 16. Cholesterol: MβCD Complex-induced Aneuploidy in hTERT Cells

(A) Karyotype analysis of hTERT cells treated with 4 µg/ml water-soluble cholesterol (WsCh) showed the induction of aneuploidy within 48 hr. Cell death was observed at the concentration of 10 and 20 µg/ml (data not shown). A Paired t-test was used to evaluate the difference in aneuploidy between treated and untreated cells.

(B) Quantitative FISH analysis with dual-labeled DNA probe revealed an increase in trisomy 21 and trisomy 12 but no induction of tetrasomy 21 or 12 (data not shown). The data suggests that exogenously derived cholesterol balanced in MβCD is disturbing an important aspect of mitotic machinery probably by changing plasma membrane fluidity, which in turn may affect chromosome segregation in general.

Water-soluble Cholesterol Induced Trisomy 16 in mNPCs

Recently, this lab reported that Aβ-induced cell cycle abnormalities and chromosome mis-segregation (Granic et al., 2010) may involve selective inhibition of mitotic spindle motors and microtubule defects (Borysov et al., 2011), which would especially be detrimental during neurogenesis and regeneration upon neuronal injury (Ming and Song, 2005; Zhao et al., 2008), resulting in malfunctional, apoptosis-prone neural progenitors and neurons. Trisomy 21 cells that accumulate in the brains of AD patients (e.g., Iourov et al., 2009) and in FAD transgenic mice (Boeras et al., 2008; Granic et al., 2010), over-express APP and Aβ the imbalance of which may contribute to disease onset and progression, as evident in individuals harboring trisomy 21 mosaicism (Migliore et al., 2006; Schupf et al., 1994) or an *APP* gene duplication (McNaughton et al., 2010; Rovelet-Lecrux et al., 2006; Sleegers et al., 2006). Like Aβ, cholesterol-induced mitotic defects in neural progenitors may lead to the development of aneuploid, genetically-imbalanced neuronal and glial cells susceptible to apoptosis and degeneration in vivo.

To test this hypothesis and to investigate the effect of cholesterol on the development of aneuploidy, including trisomy 21 in mouse neural precursor cells (mNPCs), neurospheres derived and cultured from prenatal brains of nontransgenic mice (described in Chapter Three) were utilized, and subjected to FISH with a fluorescently labeled BAC probe for chromosome 16 (Kulnane et al., 2002), a mouse analog for human chromosome 21. Parallel mNPCs cultures

were incubated with 4 µg/ml of WsCh for 7 days (equivalent to two cell divisions), and fresh cholesterol-containing media replenished every other day to allow for expansion of the spheres, a sign of mNPCs proliferation (Pacey et al., 2006). More than 4% trisomy 16 was detected in untreated cells (Figure 17A), which represents a constitutional genomic imbalance that has been previously reported

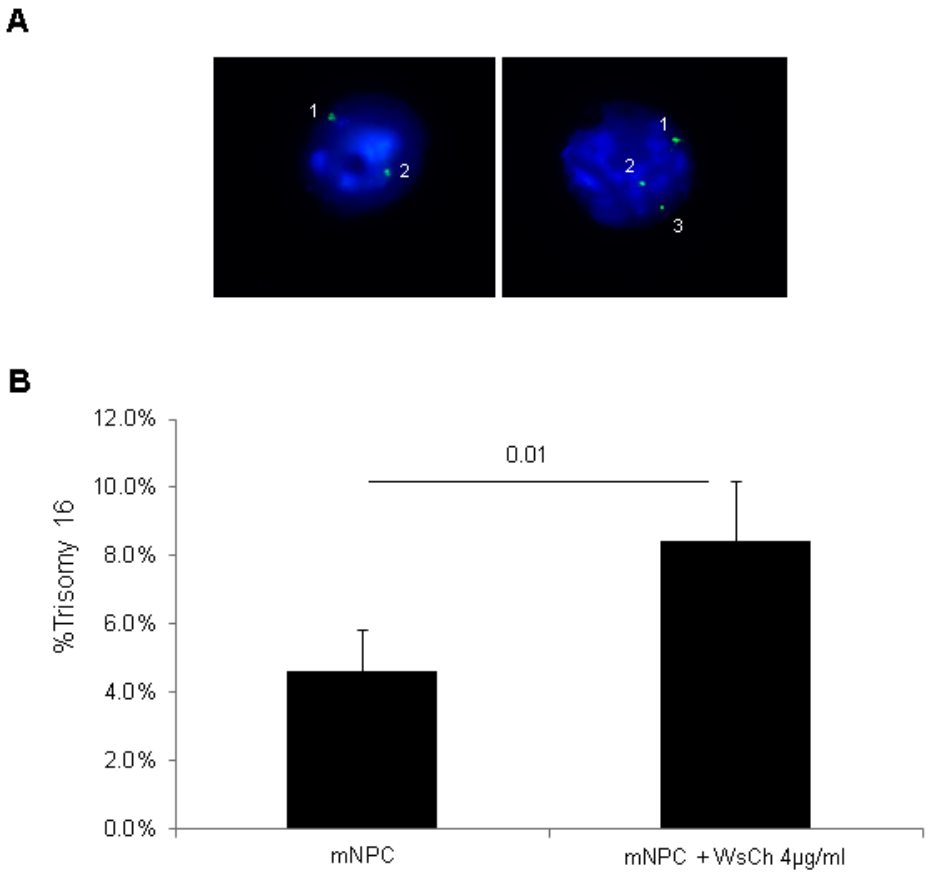


Figure 17. Cholesterol-induced Trisomy 16 in Mouse Neural Precursor Cells (mNPC)

(A) Fluorescently labeled BAC DNA probe containing a sequence of mouse chromosome 16 was used to detect aneuploidy levels in mouse neural precursor cells (mNPC) derived from prenatal brains of wild-type mice (E17-18). Established neurospheres were treated with 4 µg/ml of WsCh for 7 days.

(B) Quantitative FISH analysis showed a 2-fold increase in trisomy 16 in WsCh-treated cells compared to controls (Paired t-test). mNPC harbor up to 4.6% endogenous aneuploidy reported previously in developing mouse and human brains (Peterson et al., 2008; Rehen et al., 2001; 2005).

in developing and adult healthy human and mouse brains (Peterson et al., 2008; Rehen et al., 2001; 2005; Westra et al., 2008; Yang et al., 2003; Yurov et al., 2007). Therefore, to detect measurable levels of cholesterol-induced aneuploidy over the background, mouse NPCs were incubated with the aneugenic concentration of cholesterol for longer time, and revealed 2-fold increases in trisomy 16 compared to untreated cells ($p = 0.01$, Paired t-test; Figure 17B).

Differential Distribution of Cholesterol and Trisomy 21 in NPC1 Neurons, Glia, and Fibroblasts

To investigate the role of disrupted central and peripheral cholesterol homeostasis in chromosome segregation in a human neurodegenerative disease model, primary cells derived from the patients diagnosed with Niemann-Pick Type C (NPC) disease (Table 1 in Chapter Three) were utilized. All patients donors were clinically diagnosed with NPC, and the presence of disorder assessed with either filipin staining or the cholesterol esterification assay (Park et al., 2003), and by DNA analysis to confirm the mutation in *NPC1* gene. Mutations and deletions in the *NPC1* and *NPC2* genes cause impaired trafficking of unesterified cholesterol (both LDLR-internalized and endogenously synthesized) and other lipids (e.g., glycosphingolipids) that accumulate in late endosomes and lysosomes and fail to travel to the plasma membrane and ER (reviewed in Karten et al., 2009; Mukherjee and Maxfield, 2004; Peake and Vance, 2010). Like with exogenously delivered cholesterol that changes plasma

membrane stiffness, the ‘lipid traffic jam’ (Mukherjee and Maxfield, 1999) and differential redistribution of intracellular cholesterol in NPC1/2 cells, including neurons and glia away from plasma membrane (Karten et al., 2002; 2003; Mukherjee and Maxfield, 2002) may affect membrane fluidity and elasticity (Mukherjee and Maxfield, 1999), and proper chromosome segregation during mitosis, which may lead to the development of aneuploid cells susceptible to other cellular insults (e.g., oxidative stress) and apoptosis (Brusciglio and Yankner, 1997). Thus, intracellular cholesterol imbalance as a pathological process that causes neurodegeneration in NPC disease may affect plasma membrane structure of dividing cells and neural precursors, making them prone to mitotic errors.

To test the association between cholesterol redistribution and genomic instability in NPC disease, following assays were used: (1) immunofluorescence labeling and fluorescence microscopy to compare the localization of unesterified cholesterol between the neurons in NPC and age-matched normal brains; and (2) FISH analysis to detect the level of chromosome mis-segregation in NPC neurons, glia, and fibroblasts. First, to visualize cholesterol distribution in neurons from the sample (Table 1), a single-cell suspension derived from frontal cortices of NPC cases and controls was fixed in PFA and co-stained with neuronal marker (Neu-N Alexa Fluor® 488 antibody) and filipin (Figure 18A) as described (Millard et al., 2000), and quantified into three distinct categories based on filipin stain intensity (i.e., plasma membrane, intracellular and both). Pronounced filipin-positive staining in endocytic organelles as indicative of

intracellular cholesterol sequestration has been reported previously in cellular (Jin et al., 2004; Millard et al., 2005), mouse models (Hawes et al., 2010; Kodam et al., 2010; Treiber-Held et al., 2003) of NPC, and in fibroblasts and neurons from NPC patients (Karten et al., 2002; reviewed in Morris and Carstea, 1998; Vanier, 2010). Semi-quantitative analysis of cholesterol localization of the first 50 NeuN/filipin-positive cells revealed significantly more cholesterol staining in intracellular compartments of NPC neurons (18%) compared to controls (2%, $p = 0.02$, Independent t-test) in which cholesterol was associated mostly with the plasma membrane (85% verses 37%) ($p = 0.02$, Independent t-test; Figure 18B).

Second, a separate single-cell suspension of NPC and control brain cells was fixed in methanol:acetic acid fixative and hybridized with the chromosome 21 probe or dual-color DNA probes for chromosome 21 and 12 (Figure 18C), and chromosome 18 and 14 followed by immunocytochemistry with the NeuN antibody to distinguish between neuronal and glial cell. Quantitative FISH analysis of Neu-N-negative and Neu-N-positive cells showed 4 to 6-fold increases in trisomy 21 in both neurons and glia with the NPC mutation, respectively compared to the cells from control brains ($p = 0.02$ and $p = 0.0003$, Independent t-test; Figure 7C middle and right panel and Figure 18D). However, there was no increase in aneuploidy of chromosomes 12, 14 and 18 in the mixed brain cells population in NPC cases compared to controls (0.55% vs. 0.24%, $p = 0.11$; 1.61% vs. 1.46%, $p = 0.41$; and 0.75% vs. 0.78%, $p = 0.45$, respectively).

Third, primary fibroblasts from the patients harboring NPC1 mutations and fibroblasts from controls were subjected to FISH analysis with DNA probes for

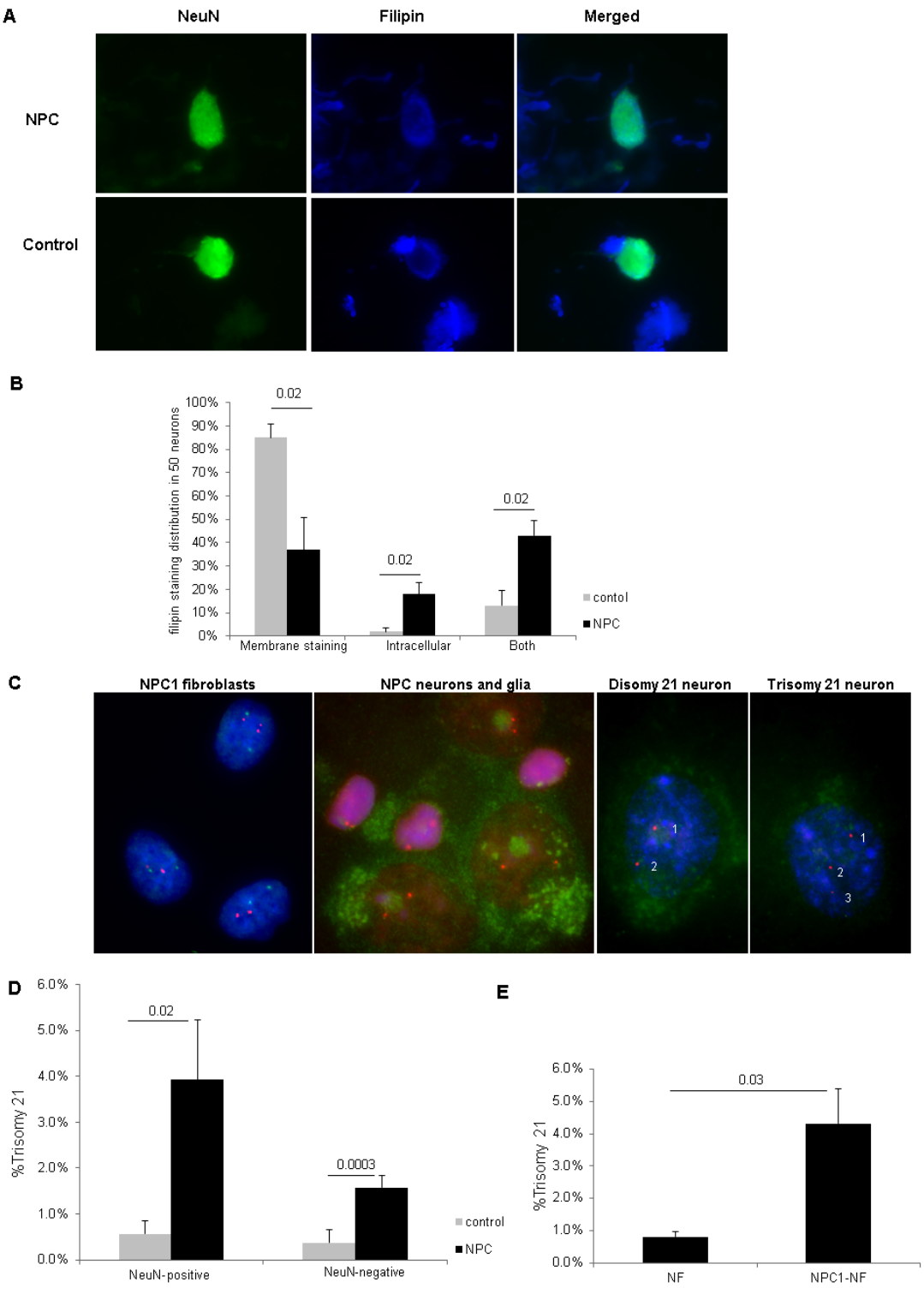


Figure 18. Neurons, Glia, and Fibroblasts Derived from Niemann-Pick Type C1 (NPC1) Patients Harbored Aneuploidy and Altered Cholesterol Distribution

(A) NPC and control brain cells were co-stained with the NeuN antibody (green) and filipin (blue) to detect

continued>

the localization of the intracellular cholesterol in neurons.
(B) Semi-quantitative analysis and comparison of cholesterol distribution between NPC and control neurons revealed mostly perinuclear/cytoplasmic filipin staining in NPC and membrane staining in control cells.
(C) NPC1 fibroblasts were analyzed by FISH with the DNA probe for chromosome 21 (red) and 12 (green), and counterstained with DAPI (blue) (first left panel). NPC brain cells from frontal cortices were subjected to FISH analysis with DNA probe for chromosome 21 (red) followed by co-staining with NeuN antibody (green) and DAPI (blue) nuclear stain, which revealed mostly disomic NeuN-positive (neurons) and NeuN-negative (glia) cells (middle panels) but significantly higher levels of trisomy 21 in NPC neurons (right panel) and glia compared to controls.
(D) FISH analysis of NeuN-positive and NeuN-negative cells derived from patients harboring NPC mutations showed an increase in trisomy 21 cells compared to age-matched normal brain cells.
(E) Likewise, human fibroblasts from NPC1 individuals showed an increase in trisomy 21 compared to age-matched controls. Independent t-test was used to compare cholesterol localization and the levels of trisomy 21 between cases and controls.

chromosome 21 and 12 and compared for chromosome mis-segregation. We observed a 4-fold increase in trisomy 21 in NPC1 cells ($p = 0.03$, Independent t-test; Figure 18C left panel and Figure 18E) and an induction of aneuploidy 12 that did not reach statistical significance compared to normal fibroblasts (2.1% vs. 0.4%, $p = 0.12$).

LIPOPROTEINS/CHOLESTEROL-INDUCED ANEUPLOIDY IN VIVO

Low and High Cholesterol Diets Induced Trisomy 16 in Splenocytes

An increasing body of evidence from observational and experimental studies suggests that the disruption in systemic cholesterol homeostasis plays a role in AD onset and progression (reviewed in Canevari and Clark, 2007; Panza et al., 2009; Puglielli et al., 2003; Wolozin, 2004). For example, recent epidemiological studies suggested a positive association between hypercholesterolemia at midlife and the risk of AD decades later (Anstey et al., 2008; Kivipelto et al., 2001;

2002a; Solomon et al., 2007; 2009), and an increased risk of dementia/AD among individuals consuming high cholesterol and/or saturated fats diet (Engelhart et al., 2002; Kalmijn et al., 1997; Laitinen et al., 2006; Luchsinger and Mayeux, 2004; Morris et al., 2003; Parrot and Greenwood, 2007). Likewise, murine and other animal models, including FAD transgenic mice fed a high cholesterol diet (1-1.3% w/w) developed pronounced amyloid burden and increased Aβ production (both central and peripheral), cerebrovascular disturbance, inflammation and steeper cognitive decline and learning impairment compared to animals on regular diets (Galloway et al., 2007; 2008; Hooijmans et al., 2007; 2009; Li et al., 2003; Levin-Allerhand et al., 2002; Oksman et al, 2006; Refolo et al., 2000; Shie et al., 2002; Sparks et al., 1994; Thirumangalakudi et al., 2008; Tibola et al., 2010) within 5-12 weeks. Wild-type mice on long-term cholesterol enriched regimens (e.g., Typical Western, Atherogenic, Thomas-Hartroft Diet) developed dyslipidemia (i.e., high total plasma cholesterol), altered VLDL, LDL and HDL metabolism, cerebrovascular disturbances, neuroinflammation, increased hepatic steatosis, atherosclerosis, and aerobic and/or cognitive dysfunction (Li et al., 2003; Maxwell et al., 2009; Paigen et al., 1990; Sullivan et al., 1997; Thirumangalakudi et al., 2008). Furthermore, inhibition of endogenous cholesterol synthesis with statins has been associated with a reduced risk of AD and decreased amyloid deposits in several human (Dufouil et al., 2005; Haag et al., 2009; Jick et al., 2000; Li et al., 2007; Sparks et al., 2005; Wolozin et al., 2000) and animal studies (Fassbender et al., 2001; Refolo et al., 2000). The mechanistic link between increased extracerebral

cholesterol content and intracerebral Aβ load is not fully understood (Puglielli et al., 2003), but it points to disrupted oxysterol homeostasis (Papassotiropoulos et al., 2002; Xie et al., 2003), vascular insults (reviewed in Bell and Zlokovic, 2009; Casserly and Topol, 2004; de la Torre, 2010; Iadecola, 2010) and compromised blood-brain-barrier (Takeshi et al., 2010; Zlokovic et al., 2008).

In the following in vivo experiment, it was investigated whether diet-induced hypercholesterolemia in atherosclerosis-susceptible wild-type mice, the C57BL/6J strain (Li et al., 2003; Paigen et al., 1990) has an effect on chromosome-segregation in peripheral (splenocytes) and brain cells. Twelve one month old nontransgenic mice (6 males and 6 females), the offspring from the same breeding pair were randomly assigned to three dietary regiments (two mouse pairs per group) for 12 weeks: (1) a regular rodent diet (18% protein, 5% crude fat from oil and 0% cholesterol w/w; Harlan Laboratories); (2) a low (25% protein, 4.2% crude fat and 0.02% cholesterol w/w, Harlan Laboratories); and (3) a high cholesterol diet (17% protein, 21% milk fat, and 1% cholesterol w/w, total cholesterol of 1.05%, Harlan Laboratories). The characteristics of the mice throughout the study are summarized in Table 2.

Table 2. Characteristics of the Mice Fed Three Different Diets for 12 Weeks

Dietary group	n/gender	Food intake g/mouse pair/week	Caloric intake DE/mouse pair/week	Weight gain/g/mouse after 60 days	Oil-Red O staining of hepatocytes
Regular diet	2/female 2/male	52.02±7.21 49.23±9.53 Total: 50.62±8.34	176.86±24.52Kcal/g 167.37±32.41Kcal/g 172.11±28.52Kcal/g	3.55±1.34 7.75±0.78 5.65±2.59	sparse or none
Low cholesterol diet	2/female 2/male	57.14±11.44 60.16±11.14 Total: 58.65±11.15***	184.57±36.97Kcal/g 194.31±35.97Kcal/g 189.44±36.01Kcal/g***	4.6±2.12 5.55±2.05 5.08±1.79	sporadic
High cholesterol diet	2/female 2/male	57.04±18.49 44.61±7.28 Total: 50.83±11.14	256.46±83.33Kcal/g 200.63±32.82Kcal/g 228.54±68.19Kcal/g***	13.8±2.69 13.45±0.92 13.63±1.65*	pronounced

Compared to other diets, mice fed a low cholesterol diet consumed more food per mouse pair/week ($p < 0.001$, Paired t-test). Also, in each dietary group except for a low cholesterol diet, female pairs consumed more food/g/mouse pair/week ($p < 0.05$). Correspondingly, mice fed a low and high cholesterol diet digested significantly more DE compared to mice fed a regular diet ($p < 0.001$; Paired t-test). All female pairs except in a low cholesterol diet group consumed more DE than male pairs ($p < 0.05$). Thus, mice fed high cholesterol diet gained significantly more weight within 60 days compared to mice on a regular and low cholesterol diet ($p < 0.05$, Paired t-test).
Digestible Energy (DE) is the total amount of energy in the diet minus the energy in indigestible portion of the food.
All values are presented as M ± SD. Paired Student t-test was used to compare the differences in food and caloric intake and weight gain between dietary groups. *p<0.05, **p<0.01, ***p<0.001

Compared to other diets, the mice fed a low cholesterol diet consumed more food per mouse pair/week ($p < 0.001$, Paired t-test), and the mice on either a low and high cholesterol diet digested significantly more Digestible Energy per week compared to the mice fed a regular chow ($p < 0.001$, Paired t-test). Because of the difference in caloric value between the diets, the mice on a high cholesterol diet gained on average 2 ½ times more weight than the mice fed either a regular chow or low cholesterol diet within 60 days of intervention ($p < 0.05$; Paired t-test). All four mice in the high cholesterol group developed hepatic steatosis, a sign of dyslipidemia while mice in the two other dietary groups showed no indication of fatty deposits in the livers (observational data). Oil-Red-O staining

of isolated hepatocytes revealed pronounced Oil-Red-O staining in mice fed a high cholesterol diet compared to mice consuming regular chow (Figure 19A)

Primary splenocytes cultures were grown according to established protocol (Boeras et al., 2008) for 44 hours in Con A containing media, fixed and prepared for FISH analysis with fluorescently labeled BAC probe for chromosome 16. Brains were processed to yield a single-cell suspension as described elsewhere and analyzed for aneuploidy (Granic et al., 2010). FISH analysis of spleen cells from mice fed a low and high cholesterol diet showed a significant increase in trisomy 16 compared to splenocytes of mice fed a regular diet ($p = 0.005$ and $p = 0.02$, respectively, Paired t-test; Figure 19B), and no induction of aneuploidy in the brain cells (data not shown). While there are no animal studies to date demonstrating that diet-induced hypercholesterolemia in nontransgenic mice affects brain cholesterol homeostasis, the data suggest that development of aneuploidy cells in peripheral (visceral) tissues of animals fed high cholesterol may be an initiating event that precedes the induction and accumulation of aneuploidy in the brain resulting from, for example, compromised BBB (Zlokovic, 2008). Lipoproteins and Aβ complexes from the periphery may pass across weakened BBB as demonstrated by co-localization of plasma ApoB and Aβ close to the cerebral vasculature (Takechi et al., 2010) and thus contribute to genomic instability in the brain.

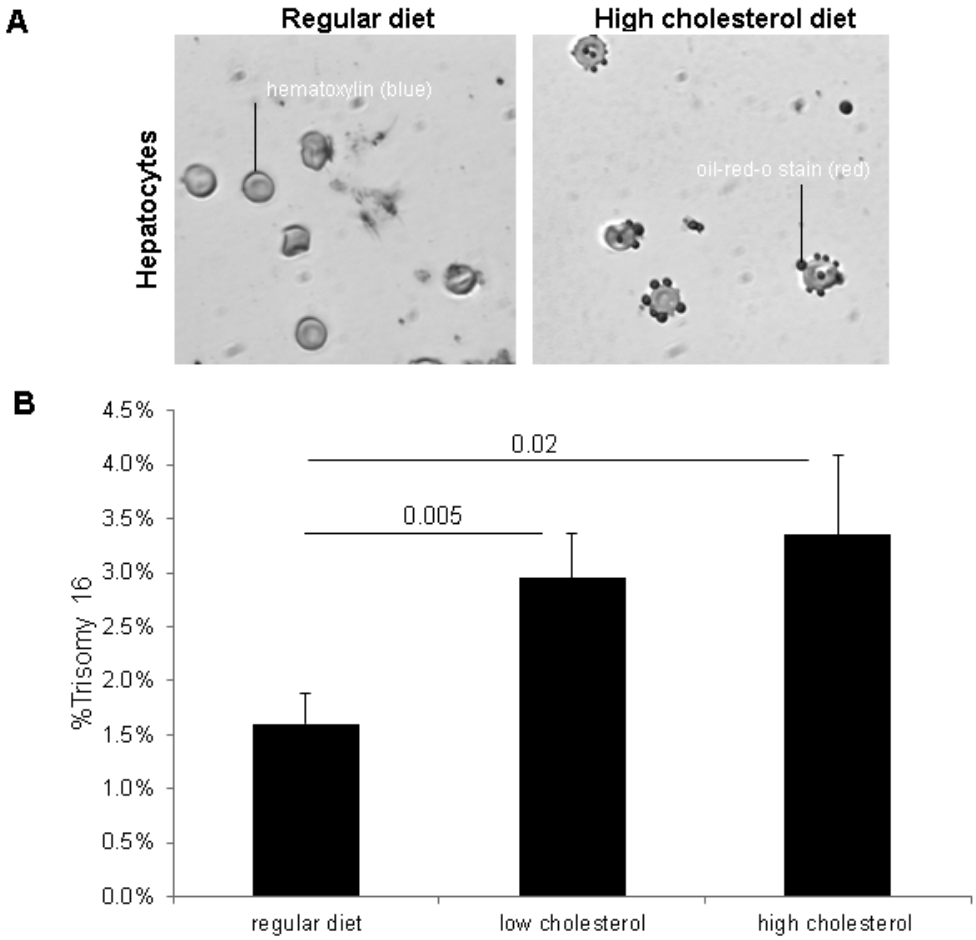


Figure 19. Young Wild-type Mice Fed Low (0.02%) and High (1.05%) Cholesterol Diets for 12 Weeks Developed Higher Levels of Trisomy 16 in Spleen Cells Compared to Mice Fed Regular Diet

(A) Mouse hepatocytes (hematoxylin; light gray in micrograph) were stained with Oil-Red-O stain (dark gray in micrograph) to detect accumulation of lipid droplets, a sign of liver steatosis and consequence of dyslipidemia.

(B) Quantitative FISH analysis of primary splenocytes revealed a 2 to 3-fold induction of trisomy 16 in a low and high cholesterol diet fed mice compared to the animals on a regular diet (Paired t-test). No induction of aneuploidy in the brain cells was observed in either low or high cholesterol diet fed mice (data not shown).

MECHANISM OF LIPIDS-INDUCED CHROMOSOME MIS-SEGREGATION

Alterations in Extracellular Ca^{2+} may Attenuate OX-LDL and LDL-induced Aneuploidy In Vitro

It has been postulated that perturbed calcium homeostasis plays an important role during aging and in AD pathogenesis (Eckert et al., 1994; Khachaturian, 1994; LaFerla, 2002). Aβ peptide neurotoxicity is partly explained by Ca^{2+} dysregulation in synapses early in AD (Green and LaFerla, 2008). Increased production of a longer, more toxic version of Aβ peptide, Aβ42, by mutations in *PS1* and *PS2* gene leads to overload of intracellular calcium stores and deficits in Ca^{2+} refilling mechanism, and reductions in γ-secretase/PS1-dependent Ca^{2+} levels in the ER (Mattson and Chan, 2003). Recently, it has been shown that oligomeric Aβ incorporates into neuronal membranes forming 'amyloid channels' that contribute to Ca^{2+} disturbances prior to the accumulation of Aβ deposits and classic senile plaque burden (Kawahara et al., 2011). Furthermore, calcium augments Aβ formation and tau phosphorylation (reviewed in LaFerla, 2002; Yu et al., 2009), and it is required for Aβ-induced inactivation and cleavage of tau (Park and Ferreira, 2005), as well as for the proper function of kinesin-like MT motors important for spindle formation and mitosis (Vinogradova et al., 2009; Wang and Schwarz, 2009). In the study of Aβ-induced aneuploidy, this laboratory has shown that chelation of extracellular Ca^{2+} with BAPTA prevented chromosome mis-segregation in vitro (Granic et al., 2010), possibly by

inactivation of calpain-mediated tau cleavage and consequent destabilization of microtubules.

To elucidate the role of Ca^{2+} in lipoproteins/cholesterol-induced aneuploidy and to investigate whether the blockage of Ca^{2+} influx may normalize chromosome mis-segregation, several experiments with hTERT-HME cells using two different chelating reagents, EGTA and BAPTA were conducted. First, the cells were pre-treated with 1.5 mM of EGTA for 3 minutes before the incubation with 20 µg/ml of lipoproteins for 48 hr, then fixed by standard cytogenetic procedures and analyzed by FISH. Karyotype and FISH analysis revealed a slight increase in total aneuploidy and lower mitotic index in EGTA-treated cells, but similar levels of trisomy 21 and 12 compared to controls (data not shown). Contrasting the aneuploidy levels of the cells treated with EGTA and lipoproteins to cells incubated only with lipids, we observed a 3-fold decrease in OX-LDL induced trisomy 21 ($p = 0.04$, Paired t-test; Figure 20A), tetrasomy 21 ($p = 0.01$, Paired t-test), and tetrasomy 12 ($p = 0.03$, Paired t-test; Figure 20B). Second, to confirm these results, the hTERT-HME1 cells were pre-incubated with 1 mM of extracellular Ca^{2+} chelator BAPTA for 3 minutes, and treated with lipids for 48 hr. The results showed a statistically significant BAPTA-dependent reduction in OX-LDL-induced trisomy 21 ($p = 0.02$; Paired t-test), trisomy 12 ($p = 0.01$; Paired t-test; Figure 20C) and a borderline decrease in trisomy 21 in LDL-treated samples ($p = 0.065$; Paired t-test; Figure 20C).

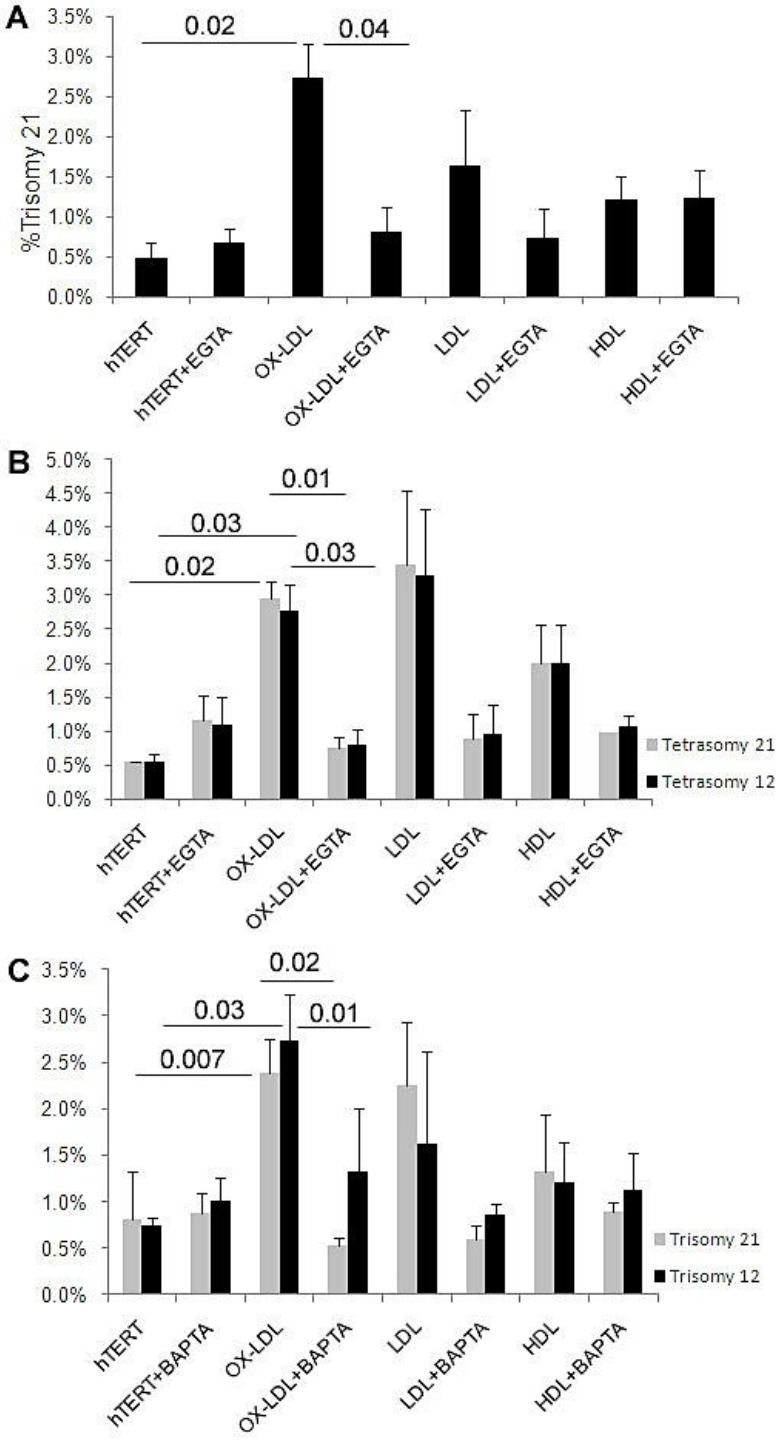


Figure 20. Alternations in Extracellular Ca^{2+} with Chelators may Attenuate OX-LDL-induced Aneuploidy In Vitro

(A-C) Quantitative FISH analysis with dual-labeled DNA probe for chromosomes 21 and 12 showed a significant reduction in trisomy 21 (A), tetrasomy 21 (light gray bars, B) and tetrasomy 12 (black bars, B) in hTERT cells pretreated with 1.5 mM of Ca^{2+} chelator EGTA compared to the cells treated only with OX-LDL.

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(C) Also, cells pretreated with 1 mM BAPTA had significantly lower levels of OX-LDL-induced trisomy 21 (light gray bars, C) and trisomy 12 (black bars, C) compared to the cells grown without chelator. Paired t-test was use to compare aneuploidy levels between the lipids-treated cells with and without chelators.

Changing Membrane Fluidity by Ethanol Decreased Lipids-induced Chromosome Mis-segregation In Vitro

The membrane fluidizing property of ethanol (EtOH) and its effect on the biophysiology of different mammalian cell including neuronal membranes, has been recognized (Alling, 1983; Goldstein, 1983; Rubin and Rottenberg, 1982) and actively investigated (e.g., Bae et al. 2005; Marques et al., 2011; Serget et al., 2004). The membrane disordering effect of acute and chronic ethanol exposure has been attributed to several processes, including (1) perturbation of acyl chains' ordering within the membrane (Holte and Gawrisch, 1997; Marques et al., 2011); (2) altered binding to the lipid-water surface (Barry and Garwrisch, 1994); (3) changed membrane cholesterol content compared to other lipids (Yamada and Lieber, 1984), and (4) oxidative stress induced by ethanol metabolism (Sergent et al., 2004). As mentioned before, both lipoprotein- and MβCD-delivered cholesterol increase the membrane cholesterol content in vitro (Abramov et al. 2011), decreasing membrane fluidity and elasticity (Demel and De Kruyff, 1976). It could be hypothesized that the cells with lipoprotein/cholesterol-enriched membranes may be prone to mitotic errors, and that this biological effect may be counteracted by membrane-fluidizing actions of ethanol.

To determine whether enhancing membrane fluidity with ethanol prior to lipoproteins/cholesterol treatment may reduce aneugenic effect of lipids, the hTERT cells were pretreated with 25 mM of EtOH for 24 hr and further co-incubated with lipids in EtOH-containing media. The rationales for using this concentration of EtOH were: (1) 25 mM or 0.115% correspond to the blood-alcohol levels of moderate drinkers (1-3drinks a day, 15-45 g), which has been associated with a decrease in CVD (Rimm et al., 1999) and AD (Luchsinger et al., 2004) risk; 2) increased cholesterol efflux and cholesterol transport but unchanged cholesterol synthesis have been observed in human astrocytes in vitro at the concentration of 25 mM of EtOH in the presence and absence of cholesterol acceptors (Guizzetti et al., 2007); (3) preincubation of hepatic cells with 0.5% EtOH did not have an effect on LDLR or LDL (Dashti et al., 1996); (4) 25mM EtOH fluidized the exofacial leaflet of the synaptic membranes (Schroeder et al., 1988; Wood et al., 1989); and (5) pilot experiments showed low cytotoxicity and up to ~95% of cell viability within 48 hr of EtOH exposure and non-significant induction of aneuploidy (data not shown). To avoid evaporation and assure consistent concentration of EtOH in the culture (Eysseric et al., 1997), a modified closed chamber system was used (i.e. 'compensating system', described below; Adickes et al., 1988; Rodriguez et al., 1992) with EtOH replenished every 24 hr. A colorimetric assay (OD_{570}; Ethanol Assay Kit, BioVision) was used to measure the ethanol concentrations in two random EtOH-containing samples at the baseline, 24 and 48 hr later and calculated according to manufacturer's specifications (mean ± standard deviation of three dilutions; M ± SD; 22.32 ±

0.36, 20.39 ± 0.58, and 18.29 ± 0.59 mM, respectively). A slight decrease in EtOH levels within 24 hr of each time point was not statistically significant (Paired t-test, data not shown).

Quantitative FISH analysis with dual-labeled translocation DNA probe of lipoproteins/EtOH-treated cells showed a significant decrease in OX-LDL and LDL-induced trisomy 21 ($p = 0.015$, Paired t-test; Figure 21A), and OX-LDL and LDL-induced trisomy 12 ($p = 0.012$, Paired t-test; Figure 21B), suggesting that fluidization of the membranes by EtOH may stabilize chromosome segregation upon lipid treatment. In this experiment, a statistically significant LDL-induced aneuploidy for chromosomes 21 and 12 was observed ($p = 0.02$ and $p = 0.03$, respectively, Paired t-test), which separately reached a borderline significance in previous experiments (Figure 12B, C), a variation that may be contributed to the clonal change in hTERT aneuploidy background (Granic and Potter, unpublished data) and to a difference in LDL-cholesterol composition between different lipoprotein batches.

It has been reported previously that ethanol can affect cell proliferation (Guizzetti et al., 1997; Luo and Miller, 1998) by affecting the length of G1 and S phase (Hicks et al., 2010), and by delaying the progression though M phase of the cell cycle (Mashimo et al., 1999). Also, recent in vitro studies showed ethanol-induced chromosomal instability and chromosomal damage in human cell lines, including neurons (Benassi-Evans and Fenech, 2011, Kayani and Parry, 2010; Lamarche et al., 2004). Here, a non-significant increase in trisomy 21 (Figure 21A), tetraploidy 21 and 12 in 25 mM EtOH-treated cells was

observed within 72 hours, and no reduction in OX-LDL and LDL-induced tetraploidy in EtOH pre-incubated samples (data not shown). An opposing effect of lipoproteins and ethanol on chromosomal stability in vitro could be postulated in which EtOH-induced fluidization of the membrane may attenuate or normalize its rigidity caused by exogenous lipoproteins/cholesterol delivery and increased membrane lipid content.

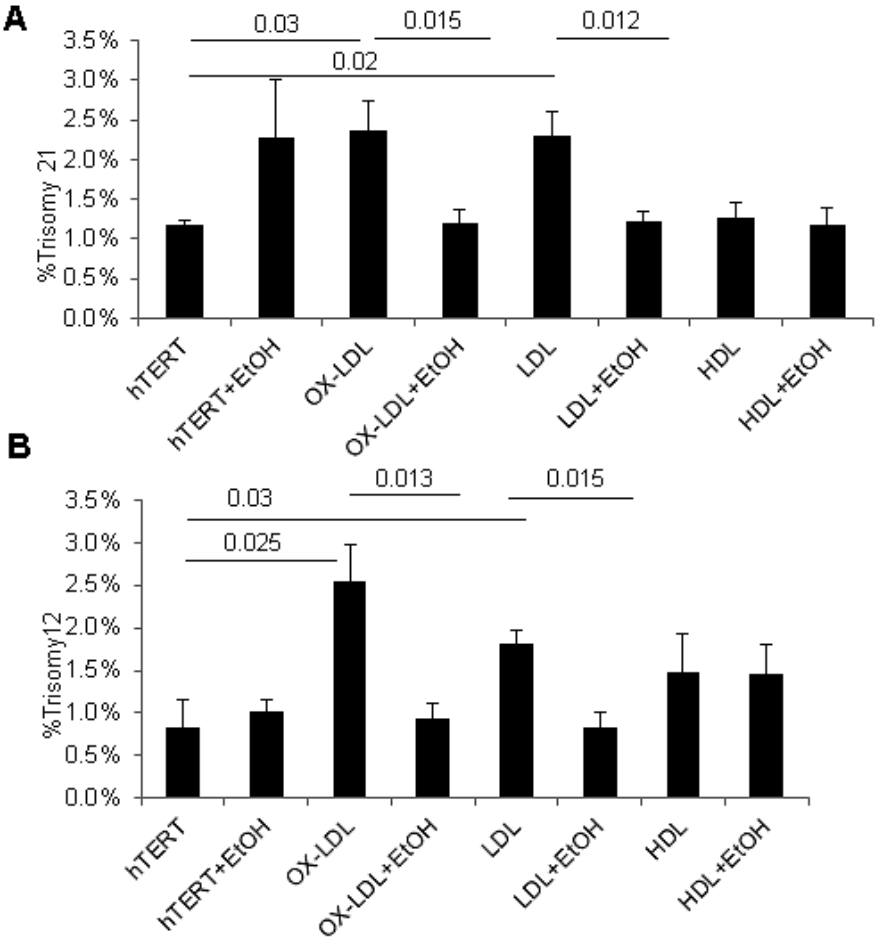

Figure 21. Altering Membrane Fluidity with Ethanol Decreased Lipoproteins-induced Aneuploidy In Vitro

(A-B) Quantitative FISH analysis of hTERT cells pre-treated with 25 mM of ethanol (EtOH) for 24 hr and co-incubated with lipoproteins and EtOH for next 48 hr revealed a decrease in OX-LDL and LDL-induced

continued>

trisomy 21 (A) and trisomy 12 (B) (Paired t-test). No change in tetraploidy 21 or 12 was observed (data not shown). This experiment was conducted in the closed chamber system to counteract EtOH evaporation and to assure consistent EtOH concentration. Colorimetric assay (OD_{570}) was used to measure EtOH levels in two random samples at the baseline, 24, and 48 hr later. A slight decrease in EtOH concentration within 48 hr was observed, but stayed at 82% of the baseline.

To summarize, this study reported several novel findings investigating the cytogenic link between CVD and AD: (1) atherogenic lipoproteins (i.e., LDL, OX-LDL) and cholesterol induced chromosome mis-segregation, including trisomy and tetrasomy 12 and 21, and trisomy 7 in primary human and mouse cells in vitro, and apparently require a functional LDL receptor but not the presence of APP gene to exert their aneugenic effect; (2) disturbance in intracellular cholesterol homeostasis and obstructed cholesterol trafficking to the plasma membrane may be associated with an increase in trisomy 21 in NPC1 fibroblasts, neurons and glial cells; (3) long-term exposure to dietary cholesterol may induce chromosome mis-segregation in peripheral tissues of young wild-type mice; and (4) alternation in either extracellular Ca2+ homeostasis or plasma membrane fluidity may decrease lipoproteins/cholesterol-induced aneuploidy in vitro.

CHAPTER FIVE

DISCUSSION

The main premise of this dissertation was that atherogenic lipoproteins/cholesterol disrupt genomic stability in vitro and in vivo and thereby contribute to the etiology of Alzheimer's disease. Several important ideas and discoveries prompted the research described here, including that (1) the cell cycle defects and chromosome mis-segregation as a pathological trait of Alzheimer's disease are associated with overexpression or mutations in FAD genes, *PS1/2* and *APP*, and its proteolytic product, the amyloid-beta peptide, which acts as a mitotic spindle toxin leading to accumulation of aneuploid and hyperploid, apoptosis prone neurons and neural progenitors (e.g., Arendt et al., 2010; Boeras et al., 2008; Borysov et al., 2010; Granic et al., 2010; Iourov et al., 2009; Obrenovic et al., 2003; Potter, 1991, 2008); (2) atherosclerotic plaques are characterized by a monoclonal expansion of smooth muscle cells in arterial walls under high LDL (OX-LDL)-cholesterol stimuli (e.g., Matturri et al., 1997; 2001), which trigger chromosomal aberrations; (3) disturbed cholesterol homeostasis as a risk factor for AD is associated with enhanced amyloidogenesis and atherogenesis in the brain and blood vessels, changed plasma membrane dynamics, and compromised integrity of the BBB (e.g., Casserly and Topol,

2004; Dolan et al., 2010; Solomon et al., 2007; Xiong et al., 2009; Zlokovic, 2008), leading to decreased clearance of neurotoxins, inflammation and neuronal death. Despite a body of molecular, cellular and epidemiological evidence pointing to the central role of lipoproteins/cholesterol in AD initiation and progression, the mechanistic connections between cardiovascular and brain disease risk factors (e.g., hypercholesterolemia) are only partly understood. One challenge has been that there is a separation and limited exchange between peripheral and central cholesterol pools, suggestive of independent pathological pathways.

Cholesterol is a molecule essential for life, involved for example in brain development and function—yet excess, shortage or erroneous distribution of extracellular or intracellular cholesterol could be detrimental and lead to neurodegeneration. Investigating further the event of chromosomal instability as a 'cytogenetic hallmark' of AD and the involvement of other dementia-associated molecules in aneugenesis, this study revealed that the cell lines with a functional LDL receptor developed mitotic defects and aneuploidy of several chromosomes, including HSA21 upon treatment with elevated levels of lipoproteins (OX-LDL and LDL) and cholesterol, the common risk factors for AD and CVD (atherosclerosis), independently of Aβ-mediated genomic instability. The cells exposed to cardioprotective HDL-cholesterol (Assmann and Gotto, 2004) were less prone to genomic errors. Furthermore, disrupted intracellular cholesterol homeostasis in Niemann-Pick skin and brain cells was associated with HSA21 mosaicism, possibly due to the changed distribution of the cellular cholesterol

between the cytosolic compartments and plasma membrane. In the animal model of diet-induced hypercholesterolemia, atherosclerosis susceptible wild-type mice fed a high cholesterol diet soon developed aneuploidy in peripheral cells. Attenuation of the extracellular calcium levels and therefore the inactivation of, for example calpain-mediated tau cleavage, decreased the lipid-induced chromosome mis-segregation in cultured cells. Also, changing the plasma membrane property with ethanol prior to and during lipoproteins and cyclodextrin-derived cholesterol incubation reduced aneugenesis by 2-3-fold in vitro. Taken together, the data suggest a common pathological trait and a unique cytogenetic mechanistic link between AD and atherosclerosis in which elevated cholesterol acts as environmental aneugen.

This chapter will explore (1) the relevance of lipid-mediated aneuploidy in AD with regards to the recent findings involving membrane cholesterol-Aβ-tau and calcium homeostasis connections (2) the possible role of plasma membrane fluidity in cholesterol-induced genomic instability; (3) the limitations of employed experimental designs; (4) future experiments to further address the research questions; and (5) diagnostic and therapeutic implications of the chromosome mis-segregation in AD and CVD.

LIPOPROTEINS/CHOLESTEROL-INDUCED ANEUPLOIDY IN VITRO

Several human cell lines (mostly derived from primary cultures) used in this study developed a rapid chromosome mis-segregation and aneuploidy of

chromosomes 7, 12 and 21 upon treatment with atherogenic lipoproteins, OX-LDL and LDL, compared to HDL-treated cells or untreated controls (Figures 12B-G; 13A; 14A). The studies of atherogenesis have shown that smaller and denser LDL particles are prone to oxidation and easily penetrate into subendothelial arterial space, initiating the atherosclerotic process (Berliner and Haberland, 1991; reviewed in Ross, 1993; Tsimikas and Miller, 2011). The data presented here suggest another biological role of atherogenic lipoproteins—induction of cell cycle defects and aneuploidy that is likely to affect other chromosomes besides 7,12, and 21.

Mis-segregation of chromosomes 7, 12 and 21 has been reported in previous studies in the brains and peripheral tissues of AD patients (e.g., Geller and Potter, 1999; Iourov et al., 2009), as well as in the mouse and cellular models of AD (e.g., Boeras et al., 2008), and in atherosclerotic plaques (e.g., Matturri et al., 1997; 2001). To date, it is not known whether or how the relationship between the AD candidate genes and mis-segregation of carrier chromosomes, and thus altered gene (gene product) expression, bears on the etiology of AD. Whereas aneuploidy of HSA21 fits well with the Down Syndrome Model of AD (Potter, 1991), altered genomic homeostasis (i.e., *APP* overexpression) and the Amyloid Cascade Hypothesis (e.g., overproduction of Aβ) (Hardy, 2006), other chromosomes susceptible to mitotic errors may carry the genes involved in other pathways significant to AD, such as cholesterol metabolism and de novo lipogenesis. For example, recent genome scan and association studies reported at least three late-onset AD candidate genes located

on chromosome 12 (e.g., Scott et al., 2000); of which oxidized LDL (lectine-like) receptor 1(OLR1), a gene implicated in lipid metabolism, has been proposed as a risk factor for AD in some French and American families (Lambert et al., 2003), and has been associated with increased Aβ40 levels and pronounced CAA in frontal cortices of AD patients (Shi et al., 2006). Moreover, OLR1 has been linked to tumorigenesis (Hirsch et al., 2010), upregulation of NF-κB and its target oncogenes responsible for inhibition of apoptosis (Khaidakov et al., 2011) and to development of atherosclerotic lesions (Chen et al., 2000), suggesting a mechanistic overlap between atherogenic and oncogenic pathways.

A future study utilizing SKY or mFISH techniques could determine, for example, the frequency and type of chromosomal aberrations for each chromosome in mitotically active cells upon lipoprotein treatments, and establish whether, under certain aneugenic stimuli, some chromosomes are lost or gained more than others.

It is well established that ApoE and LDL receptors are key players in the etiology of AD (e.g., reviewed in Gopalraj et al., 2005; Jaeger and Pietrzik, 2008; Kim et al., 2009) and are involved in 'lipid' and 'amyloid cascade' pathways. Both candidate genes reside on chromosome 19 (19q13.2 and 19p13.2, respectively) (Francke et al., 1984; Lin-Lee et al., 1985; Smit et al., 1988) within the A-T rich and gene poor chromosome bands as revealed by G-banding (Rønne, 1990; Ushiki et al., 2002), but the loss or gain of this chromosome has not been reported in relation to AD. Early molecular studies in the laboratory of H. Potter that investigated the role of ApoE allelic variants in Aβ polymerization (Ma et al.,

1994; 1996) and their possible involvement is the cell cycle defects in AD, determined that apoprotein alone had no aneugenic properties in vitro, but rather the lipid cholesterol bound to it could be the candidate effector molecule that triggered erroneous chromosome segregation (Lee and Potter, 1995; unpublished data). This dissertation confirmed the initial hypothesis. However, it is not known whether the absence of ApoE or presence of its different allelic variants (i.e., ε2, ε3, and ε4; Strittmater et al., 1993a) would result in a more or less pronounced aneugenic karyotype upon lipoprotein treatment. Similarly, whether AD transgenic mouse models harboring mutant *APP* or *PS1* genes in combination with different *APOE* alleles develop variable aneuploidy profiles may be worthwhile to investigate.

As mentioned above, ApoE, a major cholesterol transporting molecule in the brain and a ligand for LDLR in neurons and glia, is produced de novo by astrocytes (reviewed in de Chaves and Narayanaswami, 2008), and performs several functions that are isoform-specific. ApoE coordinates cholesterol intake and redistribution during development or after neuronal injury (May et al., 2007; Poirier, 2003); it associates with Aβ, promoting its aggregation in an isoform-specific manner (e.g.,Ma et al., 1994; Wisniewski et al., 1994), and is involved in inflammation, tau aggregation, signaling transduction, and brain cells survival (Gee and Keller, 2005; Kim et al., 2008). To date, *APOE* ε4 gene is the most investigated and best established risk factor for late-onset of AD. There is no consensus among the researchers about the relationship between cholesterol homeostasis, ApoE and AD (Kivipelto et al., 2002a). One of the hypotheses

reviewed by de Chaves and Narayanaswami (2008) emphasizes the isoform-specific effect of ApoE in AD pathogenesis. Animal and cellular studies revealed several pathology-promoting features of *APOE* ε4 gene, which include: (1) less efficient cholesterol delivery to neurons for regeneration and repair; (2) impaired antioxidant mechanism; (3) promotion of tau and Aβ aggregation; (4) reduced Aβ clearance; (5) dysregulation of neuronal signaling; (6) altered LDL receptor binding; (7) greater susceptibility to proteolytic degradation and aggregation; (8) and lesser cellular cholesterol release (reviewed in de Caves and Narayanaswami, 2008). It is believed that compared to ε4, other isoforms of the *APOE* gene are neuroprotective (Ma et al., 1994; Wisniewski et al 1994; Rebeck et al., 2002), and are less involved in AD-promoting pathways. Taken together, it could be hypothesized that the carriers of APOE ε4 genotype in combination with unfavorable AD genes and environmental risk factors may be more at risk for AD and neurodegeneration than non-carriers, but whether they are more susceptible to chromosome instability is currently not determined.

The role of APP and LDLR in Lipid-induced Aneuploidy

This study provided in vitro evidence that aneugenic effect of lipoproteins may be independent of APP expression and Aβ production, and that both aneugens may act separately, at least in their early steps. On the other hand, a functional LDL receptor was required for aneugenic activity of LDL.

Accumulated evidence from molecular and animal studies indicates that cholesterol is involved in APP processing and Aβ generation (e.g., Eckert et al., 2000; Refolo et al., 2001; Solomon et al., 2009), partly by affecting the activity of membrane-associated APP-secretases (e.g., Ehehalt et al., 2003; Kojro et al., 2001). Aβ toxicity includes the interference with a proper mitotic spindle function (Borysov et al., 2011) and consequent aneuploidy that is dependent on a functional *Tau* gene and on the presence of endogenous *APP* (Granic et al., 2010). To determine whether lipids and Aβ-induced chromosome mis-segregation are mechanically related, mouse spleen cells lacking the *APP* gene were exposed to lipoproteins, and the aneuploidy levels compared to lipid-treated cells with a functional gene (Figure 15B) under the same experimental conditions. Comparable levels of OX-LDL and LDL-induced trisomy 16 (up to ~5.5%) were observed in both cell lines, suggesting that neither endogenous APP or Aβ, nor Aβ-induced Tau phosphorylation was required for lipoprotein-induced aneuploidy.

To confirm these results in other cell models, the following experiments could be conducted. To determine the role of APP in lipid-induced aneugenesis in the brain cells, neural precursor cells (Pacey et al., 2006) derived from young APPKO mice with the established genotype could be treated with isolated lipids prepared with ApoE as a protein carrier. Human monocyte-derived macrophages exposed to OX-LDL exhibit increased production of lipid-free ApoE (Cader et al., 1997), which could be immunoprecipitated from the culture supernatant, and, coupled with purified lipids, extracted and isolated by the

method described by Bligh and Dayer (1959) to generate ApoE containing LDL particles. In addition, siRNA gene silencing technology (Elbashir et al., 2001) could be employed to transiently block the expression of APP in a human cell line followed by the treatment with lipoproteins.

As discussed previously, the LDL receptor family has been implicated in several processes relevant to AD pathogenesis, which include isoform-specific ApoE binding (Hertz and Beffert, 2000), APP trafficking and processing (Abisambra et al., 2010; Marzolo and Bu, 2010), decreased amyloid deposition and increased Aβ clearance upon overexpression (Kim et al., 2009), and synaptic plasticity (Herz, 2009). In the periphery, the main function of LDLR is to mediate the removal of cholesterol from the bloodstream (Brown and Goldstein, 1986), and to reduce the risk of atherogenesis and coronary heart disease (CHD). The number of LDL receptors in liver, and thus the efficiency of LDL removal are regulated by the cholesterol content in hepatocytes (Goldstein and Brown, 1987). The data reported here suggest another potential role of LDLR—mediation of LDL-induced aneuploidy in vitro (Figure 14A). Future studies could determine the relationship between downregulation and upregulation of LDL receptor and lipid-induced aneuploidy in vitro by manipulating the expression of the receptor prior to LDL-cholesterol treatment.

Genetic defects in the *LDLR* gene result in impaired clearance of lipoproteins from the circulation and severe atherosclerosis and the risk of CHD. Mutations in *LDLR* are classified into several classes based on the characteristics of the mutant receptor (e.g., class 1 mutations lead to absence of

the protein; class 2 (50% of the mutations) affect the intracellular transport of the receptor; in class 3, mutant receptors can reach the cell membrane but are defective in binding to lipoprotein; class 4 mutations prevent the ligand-receptor internalization) (Gent and Braakman, 2004). The class of the mutant FH cell line used in this study is not known, but it could be hypothesized that similar results would be obtained with cell lines harboring a different class of LDLR mutation. Alternatively, gene silencing technology could be used to completely block the receptor in hTERT-HME1 cells prior to lipid treatment to confirm the results obtained with the primary fibroblasts.

In summary, it could be suggested that the presence of LDLR and absence of APP play a complex, interdependent role in lipoprotein-induced chromosome mis-segregation in vitro, which could be further explored by utilizing different cell models and methods.

HIGH CHOLESTEROL DIET-INDUCED ANEUPLOIDY

Epidemiological evidence suggests an association between high consumption of cholesterol and saturated fats and dementia (e.g., Engelhart et al., 2002; Kalmij et al., 1997; Luchsinger and Mayeux, 2004; Morris et al., 2003; Solfrizzi et al., 2005). Similarly, familial AD (FAD) and nontransgenic animals on a long-term high cholesterol regimen develop dyslipidemia, neuroinflammation, increased amyloid burden, atherosclerosis, hepatic steatosis, and cognitive dysfunction (e.g., Levin-Allerhand et al., 2002; Li et al., 2003; Maxwell et al., 2008; Oksman

et al., 2006; Refolo et al., 2000) compared to animals on regular diets. Compromised BBB and vascular insults (Iadecola, 2010; Zlokovic, 2008) due to altered peripheral cholesterol homeostasis may affect the integrity of the central cholesterol metabolism and exacerbate AD pathology.

In this study, diet-induced hypercholesterolemia in nontransgenic young mice (1 month) was associated with mitotic defects and the accumulation of aneuploid cell in the periphery (splenocytes) (Figure 19) at a higher levels than in mice fed a low cholesterol diet or regular (non-animal fat containing) chow, suggesting that change in systemic cholesterol homeostasis may compromise genomic stability in dividing cells. As discussed previously, patients diagnosed with sporadic AD harbor aneuploidy in various peripheral cells (lymphocytes, skin fibroblasts, buccal cells; Geller and Potter, 1999; Migliore et al., 1999; Thomas and Fenech, 2008), which could be contributed to several environmental aneugens and not related to Aβ-mediated chromosome mis-segregation.

Analysis of brain extracts of the mice fed different cholesterol diets in this study with ELISA (enzyme-linked immunosorbent assay) and immunoblotting for Aβ40 and Aβ42 levels, and for the expression of APP, phospho-tau and ApoE, respectively yielded inconclusive results (data not shown). However, no difference in Aβ42 levels between the dietary groups was detected (ranged from 22.3 to 23.4 pg/ml, $p > 0.2$), and similar performance on the task of spatial memory and learning utilizing a modified version of the Morris water maze (Nunez, 2008) over 12 consecutive trials (data not shown). The diets in this study were not energy adjusted, and the caloric intake and weight gain between

the groups was statistically significant (Table 2), which influenced animals' behavior and performance during cognitive testing (e.g., mice with more adipose tissue floated better and were less stressed; observational data), but may also have affected the aneuploidy result. Future study with the middle aged animals (5-6 months) should be conducted with cholesterol and regular diets of similar digestible energy and controlled intake, and mice cognitive performance assessed with other hippocampus-based memory test such as object recognition (Clark et al., 2000).

POSSIBLE MECHANISMS OF ANEUPLOIDY INDUCTION

The Role of Cholesterol in Biological Membrane

Lipids are essential for membrane integrity, structure and function. The plasma membrane is organized into two main domains, the exofacial (outer) and cytofacial (inner) leaflet, which differ in cholesterol and phospholipids content, ordering (cohesion and packing of e.g., acyl chains), fluidity, electric charge, location of microdomains (e.g., lipid rafts and calveolae), and the functioning of associated enzymes, receptors and proteins (Ikonen, 2008; Wood et al., 2002). Lateral and transbilayer distribution of the cholesterol within the plasma membrane is believed to be essential for several membrane functions such as: (1) permeability of ions and maintenance of solute concentrations on each side of the membrane; (2) anchoring and diffusion of the proteins; (3) vesicle formation;

and (4) organization of signaling transduction (Maxfield and Tabas, 2005). Membrane lipid domains and asymmetry in lipid distribution are not stationary and could be affected by aging (Schroeder, 1988), chemical treatments (e.g., ethanol, MβCD; Christian et al., 1997; Rubin and Rottenberg, 1982), and neuropathological conditions such as Alzheimer's, Niemann-Pick, and Tangier disease (Eckert et al., 2000; Lee et al., 1998; Koike et al., 1998; Mori et al., 2001; Mukherjee and Maxfield, 1999; Orsó et al., 2000; Wood et al., 2002). Manipulation of the cholesterol content in the leaflets results in changed membrane fluidity which, for example, in the nervous system may affect the functioning of the membrane-bound neuronal receptors and modulate the binding of ligand molecules. Several techniques have been developed to detect the difference and change in leaflet fluidity, including cationic and anionic fluorescently labeled probes (Schroeder, 1985) such as Laurdan fluorescence (Parasassi et al., 1990) or fluorescence quenching (Schroeder, 1985). For example, a spectrofluorometer measures the intensity of the fluorescence polarization of Laurdan as a function of the change in the dipole moment of the fluorophore after excitation (Mukherjee and Chattopadhyay, 2005).

The experiments of this dissertation have shown that human cells treated with the MβCD:cholesterol complex, which serves as an acceptor of hydrophobic cholesterol and aids its delivery into the plasma membrane by inserting it into a central nonpolar cavity (Härtel et al., 1998), develop aneuploidy within 48 hours (Figures 16A, B; 17B). This biological effect could be a consequence of the rigidifying effect of cholesterol in which cholesterol-enriched, more ordered

membranes fail to properly align and attach MT-bound chromosomes prior to anaphase, resulting in aneuploid progeny. To determine whether changed membrane fluidity could be a mechanistic link between cholesterol exposure and chromosome mis-segregation, the change in membrane ordering prior and after cholesterol treatment could be measured by generalized polarization (GE) of Laurdan fluorescent probe (Parasassi et al., 1990).

Early studies by Schroeder et al. (1988) and Wood et al. (1989) utilizing fluorescent quenching to measure the synaptic membrane fluidity upon treatment with 25mM of ethanol determined that the disordering (fluidizing) effect of this molecule affected primarily the exofacial leaflet. The mechanism by which ethanol changes membrane fluidity has been ascribed to several processes, one being the change in the order and packing of acyl chains, which in rigid membranes are elongated and tight. Upon ethanol exposure, the chains become disordered and loose, which allows for water and other small molecule to easily enter the bilayer space (Marques et al., 2011; Mukherjee and Maxfield, 2004). It could be hypothesized that membrane rigidifying effect of cholesterol could be counteracted by membrane fluidizing action of ethanol, which should result in attenuation of lipid-induced aneuploidy (Figure 21A, B). Whether lower concentrations and different pre-incubation times also have membrane-fluidizing effects, and whether lipoprotein-induced aneuploidy could be counteracted by ethanol in the dose-response manner over different time points is worthwhile to investigate, especially in cells directly involved in the atherosclerotic process. A moderate red-wine consumption has been associated with the reduced incidence

of CVD in consumers of a typical French diet high in saturated fats and cholesterol ('French Paradox') (reviewed Lippi et al., 2010). Alcohol and polyphenols (e.g., resveratrol) in red wine may act synergistically and increase the cholesterol efflux from blood vessel walls, decrease oxidative stress and lipid peroxidation, and prevent downstream atherosclerotic events such as foam-cell formation, but may also change membrane fluidity and reduce the aneugenic effect of dietary cholesterol as another mechanism of atherogenesis.

The Role of Calcium in Aβ and LDL-Induced Aneuploidy

Findings from this and a recent study (Granic et al., 2010) suggest that both LDL-cholesterol and Aβ-induced chromosome mis-segregation could be attenuated by chelation of extracellular calcium (Figure 20A-E). Whereas Aβ-mediated aneuploidy depends on functional *APP* and *Tau* genes, lipid-induced aneugenesis is independent of APP, but requires a functional LDL receptor. Tau is essential for Aβ-induced neurotoxicity (Rapoport et al., 2002), and the calcium-dependent protease calpain is activated by Aβ and generates neurotoxic tau fragments leading to neurodegeneration (Park and Ferreira, 2005). Increased membrane cholesterol regulates the calcium influx (Bialecki et al., 1991), and increases the susceptibility of mature neurons to Aβ-induced calpain activation and production of 17kDa neurotoxic tau fragment. A recent study by Abramov et al. (2011) showed that a higher cholesterol content in the plasma membrane increased Aβ-induced calcium signaling and apoptosis in both astrocytes and

neurons. Whereas Nicolson and Ferreira (2009) were the first to propose a role for plasma cholesterol in a tau-Aβ-calcium mediated neurotoxicity pathway, the data presented here indicate that restoring calcium homeostasis and therefore, de-activating calpain-mediated tau cleavage and destabilization of microtubules may attenuate LDL-cholesterol induced chromosome mis-segregation in vitro (Figure 22).

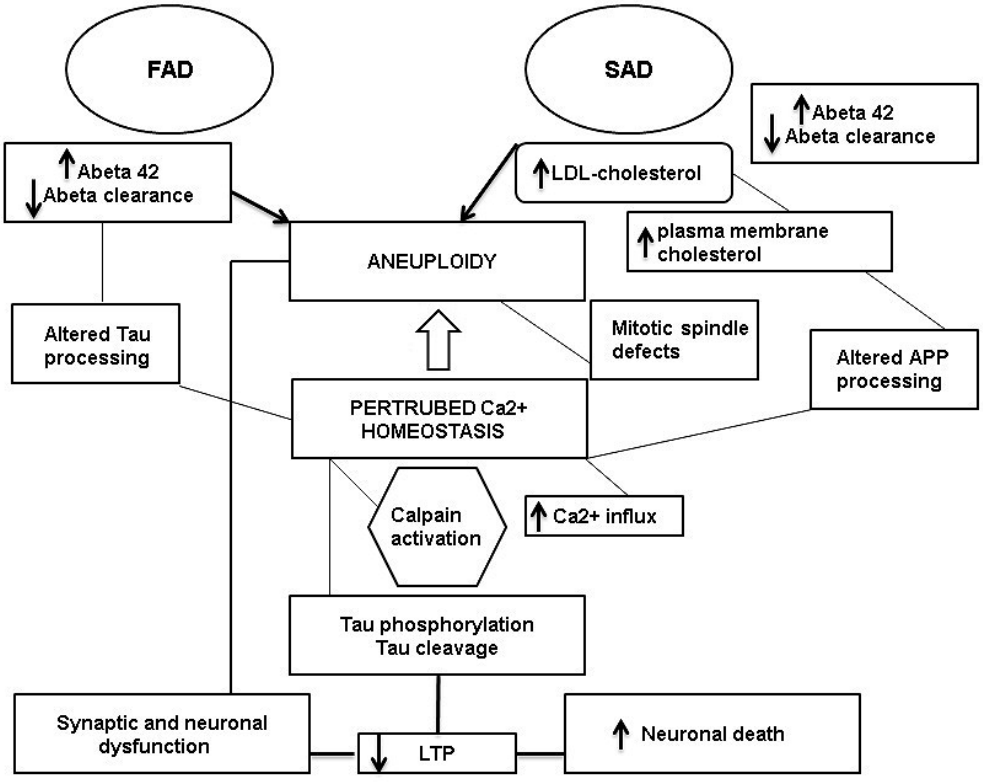

Figure 22. Possible Pathways Involving Calcium Homeostasis, Aβ and LDL-cholesterol-induced Aneuploidy in Pathogenesis of AD

Multiple interrelated pathways may be involved in the complex etiology of familial (FAD) and sporadic AD (SAD). Both Aβ and LDL induce chromosome mis-segregation in vitro within 48 hr. Removal of extracellular calcium decreased both Aβ and LDL-mediated aneuploidy. Elevated cholesterol changes plasma membrane fluidity (Weber et al., 2006) and increases Aβ-induced calcium signaling (Abramov et al., 2011), which in turn may increase the susceptibility of neurons to Aβ-dependent calpain activation, tau cleavage into neurotoxic fragments and apoptosis (Nicholson and Ferreira, 2009). Calcium chelation may attenuate both Aβ and LDL-induced mitotic defects by restoring calcium homeostasis and de-activate calpain, and, consequently, preventing tau cleavage and destabilization of microtubules.

IMPLICATIONS OF CHROMOSOME MIS-SEGREGATION IN AD AND CVD FOR DIAGNOSIS AND THERAPY

The mechanistic implication of the results presented in this dissertation is that disruption in cholesterol homeostasis may contribute to Alzheimer's disease pathogenesis by inducing genomic instability and chromosome mis-segregation, and thus contributing to disease progression. AD as the most common type of dementia in old age, currently affects 35.6 million people in either genetic (familial) or sporadic form, and that prevalence is expected to quadruple worldwide by the mid of the 21st century (Ferri et al., 2005). Whereas genetic or early-onset AD accounts for only 5% of all cases (Cummings, 2004), 95% of sporadic or late-onset AD is attributed to the interaction of a number of factors such as advancing age, environmental insults, and to several risk-enhancing genetic polymorphisms (Bertram and Tanzi, 2004). Likewise, CVD (atherosclerosis) is the most prevalent disorder and the number one killer in developed countries (Daviglus et al., 2006).

The search for more effective diagnoses and therapy for AD and CVD should take into account mitotic defects in peripheral and central tissues of individuals at risk, especially those whose genetic predispositions and lifestyle factors (e.g., high-fat diet) increases the odds of developing both diseases. Chromosome analysis and detection of low levels of aneuploid cells utilizing FISH or spectral karyotyping in skin fibroblasts or buccal cells in patients at risk for cardiovascular events during the pre-clinical phase of dementia could be a

potential diagnostic tool. Another implication of the data presented above that cholesterol-induced genomic instability in AD may be the initiating event in disease pathogenesis also suggests new approaches to prevention and treatments (Potter, 2004). For example, elevated total and LDL-cholesterol as risk factors for AD and atherosclerosis in midlife should be treated early both as an atherogenic and aneugenic agent. Molecules that would restore the mitotic defects and strengthen the fidelity of mitotic machinery could be searched for and used as a prophylactic measure. Furthermore, other aneugenic environmental agents aside from high cholesterol that cause chromosome instability could be identified and countered with drugs that restore genomic homeostasis. Alternatively, a more difficult but still effective approach to therapy would be to identify and remove mis-segregated cells from the body by utilizing their unique cell biology and/or gene profile.

To summarize, the results from this dissertation and several other laboratories over the past twenty years have shown that AD patients are susceptible to genomic instability, accumulating elevated levels of aneuploid including trisomy 21 cells throughout the body compared to age matched healthy controls. Whether the patients suffering from CVD (atherosclerosis) are at higher risk for AD because of disturbed genomic homeostasis may be important to investigate. The precise mechanism by which abnormal cells arise in the periphery (e.g., skin and arterial walls) and especially in the brain during the life of an individual and how or whether they contribute to disease initiation and progression warrant future investigation. The better understanding of these

novel findings has the potential to contribute to the development of future diagnoses and therapies for AD and CVD.

CONCLUDING REMARKS

This dissertation presented several novel findings, the main being that Alzheimer's disease and CVD (atherosclerosis) may be mechanistically related through a cytogenetic pathway. While dysfunction of cholesterol homeostasis may contribute to AD pathology via systemic and cerebral atherosclerosis causing cerebral hypoperfusion, disrupted communication between neurons and vascular cells at the BBB, inflammation, impaired cerebral Aβ clearance and consequent CAA, and compromised BBB (Vascular Hypothesis), other mechanisms may also be involved. Altered cholesterol homeostasis within the brain and plasma membrane may promote or inhibit amyloid production and clearance (Amyloid Cascade Hypothesis) leading to neurodegeneration. Finally, this dissertation presents evidence that a unique common cytogenetic mechanism may underlie both AD and CVD (atherosclerosis). Specifically, disturbed cholesterol homeostasis may interfere with the cytogenetic property of mitotic cells in the periphery and brain by changing their ability to properly segregate their chromosomes, similar to development of mis-segregated, including HSA21 aneuploid cells in blood, skin, mucosa and brain of AD patients (Chromosome Mis-segregation Hypothesis), further perpetuating the pathology.

Another novel finding of this dissertation suggests an association between disturbed intracellular cholesterol homeostasis and differential distribution of lipids between cell compartments and aneuploidy, as observed in the NPC1 cells. The mechanism by which impaired cholesterol trafficking and storage leads to accumulation of neurofibrillary tangles and neurodegeneration in the brains of NPC1 patients is obscure (Karten et al., 2009), but understanding the correlation between the aneugenesis and dysfunction in cholesterol homeostasis may provide new insights into the pathogenic pathways of both AD and NPC.

This study had several limitations including: (1) mis-segregation of only a few of chromosomes was tested and the extent of chromosome-specific aneuploidy is not known; (2) the role of the more or less neuroprotective APOE variants in genomic instability and aneugenesis in conjunction with lipids is undetermined; (3) the extent of lipoprotein-induced aneuploidy in the brain cells has not been investigated; (4) mice on a high cholesterol diet gained significantly more weight compared to the mice in other dietary groups, which may have biased the results; and (5) the known fluidizing effect of ethanol in vitro has been hypothesized as a possible mechanism for the reduction in aneuploidy in the cells co-incubated with lipids and alcohol—however, the membrane fluidity has not been measured.

In summary, the fact that several age-related diseases (e.g., Alzheimer's, cancer, atherosclerosis, and FTD) share a common cytogenic trait—genomic instability and chromosome mis-segregation—opens new avenues for the research and therapeutic approaches.

GLOSSARY

acrocentric chromosome: a chromosome with a very short p (small) arm and centromere (a region of the chromosome where sister chromatids are in close contact) positioned towards its end. Observed in human chromosomes 13, 14, 15, 21, and 22.

anaphase: the stage of mitosis when sister chromatids are separated by mitotic spindle.

aneugen: process, agent or molecule that induces improper segregation of the chromosomes during the cell division.

aneuploidy: a numerical abnormality of chromosomes compared to normal genome (e.g., normal human somatic cells contains 46 chromosomes or 22 pair of autosomes and one pair of sex chromosomes).

apoptosis: programmed cell death.

BAC (bacterial artificial chromosome): an artificially constructed cloning plasmid in which a DNA sequence of interest could be inserted and transformed into bacteria to generate identical clones resistant to a specific antibiotic.

calpain: a calcium-dependent protein expressed ubiquitously in mammalian cells.

cerebral amyloid angiopathy (CAA): accumulation of amyloid-β protein in blood vessels of the central nervous system.

cerebral hypoperfusion: decrease blood flow in cerebral vasculature especially in microvessels.

chelator: an organic compound that serves as a ligand to other molecules via formation of a single central atom bond.

chiasma (pl. **chiasmata):** a X-shaped chromosomal structure and the site of cross-over (a reciprocal exchange of DNA) between homologous chromosomes during meiosis.

chromogenic in situ hybridization (CISH): an alternative cytogenetic method to FISH that employs enzymatic reactions and the brightfield microscopy to

assess gene alternations, aneuploidy and other chromosomal aberrations in formalin fixed or paraffin-embedded tissue slices.

clonal proliferation: division of identical cells derived from the same original cell.

cholesteryl esters: highly hydrophobic esterified form of cholesterol.

circle of Willis: a circle of arteries that supply the blood to the brain.

colcemid: microtubule toxin, a drug that inactivates spindle formation and arrests the cells in metaphase.

cyclodextrines: a family of organic compounds composed of several sugar molecules in a ring structure able to form stable aqueous complexes with other molecules.

cytokinesis: a process of division of the mother cell into two daughters.

cytosol: intracellular fluid-filled space of the cell divided into compartments by membranes.

diploid: containing two copies or homologs of each chromosome (one inherited from one parent and one from the other). Most somatic cells are diploid.

dynein: a microtubule (MT) motor protein complex that transports various cellular cargo along MT toward its minus-end and centrosomes.

efflux: any exit of ions or molecules from the cell into extracellular space.

endocytosis: a process of ingestion of molecules in which plasma membrane folds inwards to form an intracellular vesicle.

endoplasmic reticulum (ER): an organelle in eukaryotic cell composed of interconnected network of membranes responsible for transport, production, metabolism and regulation of different proteins, lipids, ions and other molecules.

endosome: membrane-associated vesicle inside the cell that transports molecules for further processing to other organelles or back to the plasma membrane.

euploid: containing normal chromosome compliments for the species (opposite of aneuploid).

fluorescence-activated cell sorting (FACS): a technique that uses modified flow cytometer (an instrument that counts and differentiates different types of cells based on intensity of fluorescent dye) to sort cell into different containers.

fluorescence in situ hybridization (FISH): a cytogenetic technique in which a specific DNA sequence labeled with a fluorescent dye binds to complimentary DNA of a target cell of specimen.

fluorophore (chromophore): a compound that bound to other molecule causes fluorescence (e.g., DAPI, fluorescein isothiocyanate, SpectrumOrange).

G-banding: a classic cytogenetic technique in which metaphase chromosomes are enzymatically treated (trypsin) and stained with the Giemsa stain to obtain unique chromosomal bands (light and dark).

Golgi network (apparatus): a membrane structure in the cell responsible for processing and packing of the proteins before secretion.

hepatic steatosis: fatty liver, accumulation of neutral lipids in hepatocytes.

hyperploid: containing more than diploid chromosome compliment.

hypoploid: containing less than diploid chromosome compliment.

influx: flux of ions and molecules into the cell or subcellular organelles.

in vitro: (Latin ‘within glass’) studies in cellular biology done in the cells isolated from tissues and organs.

in vivo: studies done in an intact living organisms.

karyotype: a set of condensed chromosomes in metaphase stained or otherwise prepared in order to detect the unique features of each chromosome pair.

kinase: proteins that transfer phospho groups onto other molecules and thus regulate different cellular processes.

kinetochore: a protein complex located at the centromere (a region of the chromosome), where the microtubules are attached during mitosis.

ligand: (Latin ‘binding’) a biomolecule that forms a bond (e.g., ionic bonds) with other substance or molecule to perform certain biological function.

loss of heterozygosity: loss of function of one allele of the gene in which the other one is already non-active.

lysosome: (Greek lysis [to separate] and soma [body]) an organelle in the cell filled with digestive enzymes that break down proteins, sugars, nucleic acids and cellular debris.

meiosis: a specialized nuclear division in formation of the gametes in which each cell contains haploid nuclei with a single homolog of each chromosome (e.g., 23 chromosomes in humans).

microtubule (MT): a straw-like hollow structure composed of tubulin subunits that supports cell cytoskeleton in interphase and forms mitotic spindle during mitosis.

mitochondria: cell energy plant that converts complex sugars into energy packets, the adenosine triphosphate (ATP) via glycolysis or Kreb's cycle.

mitosis: a process of division in which the duplicated chromosomes are separated via mitotic spindle.

mitogen: an agent that stimulates the proliferation of the cells.

mosaicism: the presence of the euploid and aneuploid cell population in one organ or organism.

nuclear envelope (NE): the inner cellular membrane that surrounds and protects nucleus from various insults and damage.

PCR (polymerase chain reaction): a molecular biology technique used to amplify a specific DNA sequences and fragments.

retrograde immunocytochemistry: a combination of protein staining and tracing technique used to identify, for example, neuronal projections.

separase: a protein that initiates the separation of sister chromatids.

sister chromatids: a duplicated set of chromosomes.

spectral karyotyping (SKY): a cytogenetic technique that allows for visualization of all chromosomes in metaphase by using different fluorescent probe for each.

telomerase: a specialized enzyme that synthetizes the repetitive sequence of DNA at the end of the chromosomes, the telomeres that maintain their integrity and regulate the number of cell divisions.

tetraploid: containing four copies (or homologs) of each chromosome as a result of failed cytokinesis.

Xenopus cell extract: cells prepared from frog oocytes.

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