1 *Review*

2 # Type 2 Diabetes Mellitus Increases The Risk of Late-
3 # Onset Alzheimer's Disease: Ultrastructural
4 # Remodeling of the Neurovascular Unit and Diabetic
5 # Gliopathy

6 **Melvin R Hayden** [1,2] *

7 [1] Diabetes and Cardiovascular Center, University of Missouri School of Medicine, Columbia, MO 65212,
8   USA.
9 [2] Division of Endocrinology and Metabolism, Department of Medicine, University of Missouri, Columbia,
10   MO 65212, USA
11 * Correspondence: mrh29pete@gmail.com; Tel.: +1-573-346-3019

12 **Abstract:**

13 Type 2 diabetes mellitus (T2DM) and late-onset Alzheimer's disease-dementia (LOAD) are
14 increasing in global prevalence and current predictions indicate they will only increase over the
15 coming decades.   These increases may be a result of the concurrent increases of obesity and aging.
16 T2DM is associated with cognitive impairments associated with metabolic factors and increases the
17 cellular vulnerability to develop the age-related increased risk of LOAD. This review addresses
18 possible mechanisms due to obesity, aging, multiple intersections between T2DM and LOAD and
19 mechanisms for the continuum of progression. Multiple ultrastructural images in female diabetic
20 *db/db* models are utilized to demonstrate marked cellular remodeling changes of mural and glia cells
21 and provide for the discussion of functional changes in T2DM.   Throughout this review multiple
22 endeavors to demonstrate how T2DM increases the vulnerability of the brain's neurovascular unit
23 (NVU), neuroglia and neurons are presented. Five major intersecting links are considered: *i*. aging
24 (chronic age-related diseases); *ii*. metabolic (hyperglycemia - advanced glycation end-products and
25 its receptor (AGE/RAGE) interactions and hyperinsulinemia – insulin resistance (a linking linchpin);
26 *iii*. oxidative stress (reactive oxygen-nitrogen species); *iv*. inflammation (peripheral macrophage and
27 central brain microglia); *v*. vascular (macrovascular accelerated atherosclerosis - vascular stiffening
28 and microvascular NVU/neuroglial remodeling) with resulting impaired cerebral blood flow.

29 **Keywords:** Aging; Alzheimer's disease; brain insulin resistance; *db/db* diabetic mouse model;
30 diabetic cognopathy; insulin resistance; metabolic syndrome; mixed dementia; obesity; type 2
31 diabetes mellitus

32

1. Introduction

34   The human central nervous system culminates in the brain that contains a virtual galaxy of cells
35 within a universe.   Estimates of brain cell numbers includes: 100 billion neurons [1], 85-100 billion
36 glia (astrocytes, microglia and oligodendrocytes) [2] and 0.15 quadrillion synapses [3], which   makes
37 our brain cell numbers somewhat comparable to that of our own Milky Way galaxy.   Additionally,
38 there are mural vascular cells coursing throughout the brain consisting of endothelial cell(s) (EC),
39 pericyte(s) (Pcs) and vascular smooth muscles cells.   Each of these cells have multiple unique
40 identifying ultrastructural forms and functions [4-9].   This review focuses on type 2 diabetes mellitus
41 (T2DM) and the increased risk of late-onset Alzheimer's disease (LOAD) and the capillary
42 neurovascular unit(s) (NVU) and neuroglia.   m mesh-like networks of microvessels that insure
43 proper nutrient delivery (specifically oxygen and glucose) and nutrient exchange of metabolic
44 byproducts from glia and neuronal cells to the systemic circulation.   Importantly, the NVUs provide

45 the necessary cerebral blood flow to allow for functional hyperemia of regional active neurons.   It
46 has been estimated that there are approximately 100 million capillary NVUs that course through the
47 brain for approximately 400 miles, which also allow for a vast surface area of approximately 20 square
48 meters in order to provide molecular transport to and from the systemic circulation [10].
49 Understanding how these two disparate disease mechanisms may intersect as a continuum of
50 progression with similar risk factors along with genetic, epigenetic and environmental conditions are
51 essential, since T2DM and LOAD are occurring in parallel with increasing global prevalence. A better
52 understanding of how these two diseases with very similar risk factors intersect with one another is
53 especially important since they are both age-related diseases.   Thus, the brain parenchyma and its
54 vascular supply are the structural and functional substrate of the mind.

55 *1.1. T2DM, Late -onset Alzheimer's Disease-Dementia (LOAD) and Societal Aging*

56 T2DM is a heterogeneous, multifactorial, polygenic, endocrine, metabolic, chronic and age-
57 related disease characterized by obesity, insulin resistance (IR) and hyperglycemia.   Further, T2DM
58 is a result of the relative or complete impairment of insulin actions, signaling, and associated with a
59 progressive decline in pancreatic beta cell function [4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16].
60 We are now living in a period of time where there exists one of oldest-living global populations
61 [16] and currently there is evidence that suggests the number of older people in our global population
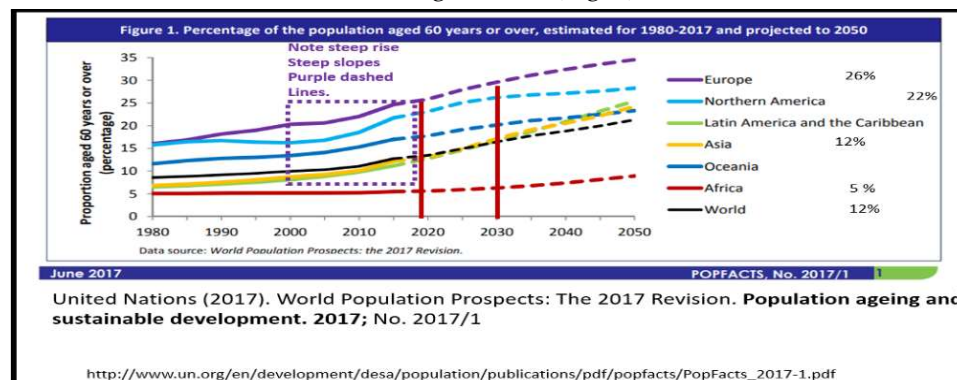62 is going to increase even further in the coming decades (Fig. 1) [17].

64 **Figure 1.** Graphical representation of the percent of the global population age 60 or greater.   This
65 figure is divided into global regions and age groups from United Nations (2017) population aging
66 and sustainable development – the United Nations.
67 https://www.un.org/en/development/desa/population/publications/pdf/popfacts/PopFacts_2017-
68 1.pdf accessed July 10, 2019.

69 This aging population is largely due to the aging baby boom generation that benefited from the
70 advancement of modern medicine including immunizations, antibiotics and cerebrovascular -
71 cardiovascular care including hypertension and cholesterol control, coronary artery stenting; bypass
72 grafting and stroke care. Additionally, this global aging generation resulted from the high birth rates
73 following World War II and is partially responsible for driving this global increase.   As this aging
74 generation continues to age, they also undergo an increase in age-related loss of skeletal muscle mass
75 (extreme cases known as sarcopenia, moreso in males) with increasing skeletal muscle insulin
76 resistance.   Also, there is an associated decrease in physical activity and increased consumption of
77 compact calorie-dense Western diets, which contribute to the parallel global increase of obesity,
78 T2DM and LOAD.   Globally, there are 425 million patients estimated to have T2DM, and by 2040, it
79 is expected that this figure will rise to 642 million [18, 19, 20].   Importantly, it has been recently
80 estimated that as this generation ages, 13.5 million individuals in the United States may manifest
81 LOAD by the year 2050 (https://www.alz.org/help-support/resources/publications/trajectory_report
82 *accessed July 10, 2019*) and the current lifetime risk of a 65-year-old individual for LOAD is now
83 estimated at 10.5% [21].

84   It is important to note before proceeding that the multicellular maladaptive ultrastructural
85   remodeling figures shared in this review may represent a unique phenotype in Leptin receptor
86   deficient (monogenic (*Lepr^{db}*) female diabetic db/db (BKS.Cg*Dock7^m*+/+*Lepr^{db}*/J) db/db female models
87   with elevations in leptin and may not be a general phenotype in multiple other diabetic models. This
88   caution was provided in order to encourage other indepent investigator to study some of the other
89   multiple diabetic models.

90   *1.2. Aging, Obesity, Insulin Resistance, Metabolic Syndrome and the H and E Phenomenon*

91   The current societal aging phenomenon as presented in section 1.1 is associated with excesses
92   and these excesses have been incorporated into a syndrome that the late Gerald M Reaven initially
93   termed Syndrome X (Fig. 2) [22], which was later termed the now familiar metabolic syndrome (MetS)
94   or the "H" phenomenon (H = hyper) and the "E" phenomenon (E = excesses) (Fig. 3).   MetS and the
95   H & E phenomenon may be defined as a clustering or constellation of metabolic factors and clinical
96   syndromes, which are intimately linked together by insulin resistance and known to increase the risk
97   of not only cerebro-cardiovascular (CVD) disease and T2DM but also may link age-related LOAD.
98   Importantly, visceral obesity is felt to be the initiating driver and insulin resistance is felt to be the
99   linking factor of each of the four arms of the insulin resistance syndrome X and the H and E
100   phenomenon (Fig. 2, 3). The ATP III guidelines provide the proper clinical parameters to identify
101   those individuals with the MetS as follows: three of following five factors need to be confirmed:
102   central obesity (≥ 94 cm (for males), 80 cm (females)), triglycerides concentration ≥ 150 mg/dl, HDL
103   cholesterol concentration < 40 mg/dl (males), < 50 mg/dl (females), values of blood pressure ≥ 130/85
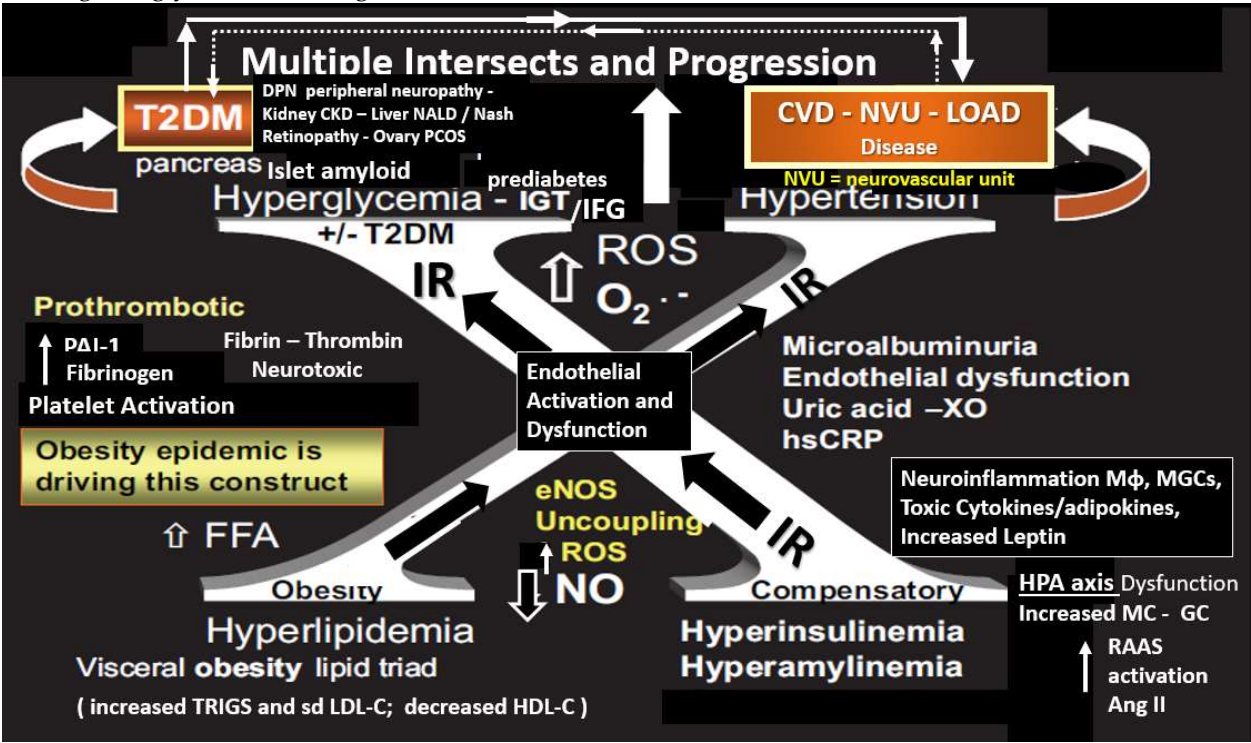104   mm Hg and glycemia ≥ 100 mg/dl [23].

105

106   **Figure 2.** Metabolic syndrome with multiple intersects and progression. This image illustrates the
107   Metabolic syndrome utilizing the letter "X" in order to honor Reaven's original term Syndrome X
108   who championed the concept that resistance to insulin-mediated glucose disposal was a characteristic
109   of patients with T2DM, which was later termed the Metabolic syndrome (MetS). Note the four
110   different hyper-conditions associated with the MetS on each arm of the X. This figure depicts
111   peripheral IR; however, there may be a contribution to central brain insulin resistance (BIR). *CKD =*
112   *chronic kidney disease; CVD = cardio-cerebrovascular disease; DPN = diabetic peripheral neuropathy; eNOS =*
113   *endothelial nitric oxide synthase;ET-1= endothelin 1; GC = glucocorticoid; IR = insulin resistance; FFA = free*
114   *fatty acids; hs CRP = highly sensitive C-reactive protein; IGT = impaired glucose tolerance; IFG = impaired*

115   *fasting glucose; LOAD = late onset Alzheimer's disease; MGC = Microglial cell; Mϕ = macrophage; MC =*
116   *mineralocorticoid;   NO = nitric oxide; NALD = non-alcoholic liver disease; NASH = non-alcoholic*
117   *steatohepatitis; O2- = superoxide; PCOS = polycystic ovary syndrome; T2DM = type 2 diabetes mellitus;*
118   *RAAS = renin angiotensin aldosterone system; ROS = reactive oxygen species; TNFα = tumor necrosis alpha;*
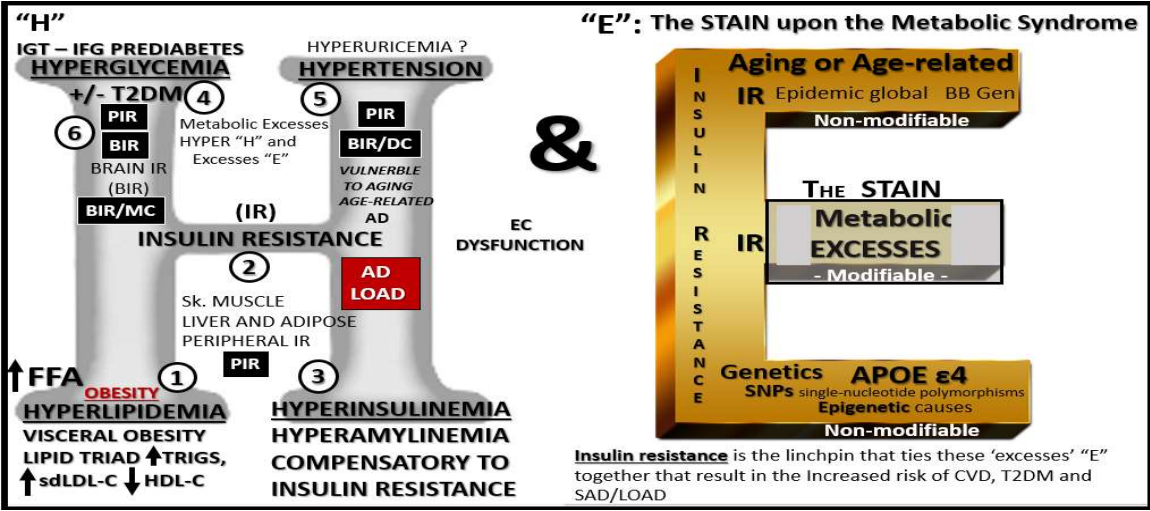119   *XO = xanthine oxidase.*

120

121   **Figure 3.** "H" and "E" hyper and excess phenomenon stain upon the metabolic syndrome. The "H"
122   phenomenon represents the numerous HYPER-metabolic/clinical abnormalities associated with the
123   metabolic syndrome in Fig. 2 and represents hyperlipidemia, hyperinsulinemia – hyeramylinemia,
124   hyperglycemia, hypertension and the emerging hyperuricemia hypothesis.   Furthermore, it
125   illustrates at least six steps in the progression of this process that may be responsible for T2DM
126   progression and increased vulnerability to the development of late onset Alzheimer's disease
127   (LOAD).   Note how insulin resistance (IR), step two, provides a linchpin to link the arms of the H
128   and the metabolic stain upon T2DM and LOAD.   Additionally, the "E" represents the excesses,
129   which also may be associated with the metabolic syndrome. *APOEε4 = apolipoprotein E epsilon 4; BIR*
130   *= brain insulin resistance; BB Gen = baby boomer generation; EC= endothelial cell; HDL-C = high density*
131   *lipoprotein cholesterol;   IFG = impaired fasting glucose; IGT = impaired glucose tolerance; PIR = peripheral*
132   *insulin resistance; Sk. = skeletal; SNPs= single nucleotide polypeptides Trigs = triglycerides; sdLDL-C = small*
133   *dense low-density lipoprotein cholesterol.*

134   1.2.1. Insulin Resistance: Peripheral and Central-Brain Insulin Resistance

135   It is difficult to sort out the differences of peripheral insulin resistance (PIR) and hyperglycemia
136   - glucotoxicity, since they are both present in T2DM and in brain insulin resistance (BIR) in LOAD
137   (Box 1).

138

139   **Box 1.** Difficulty in sorting out the contributions of insulin resistance and hyperglycemia in T2DM
140   and LOAD.   Insulin resistance and hyperglycemia are both important in the development of the end-
141   organ complications associated with T2DM including the brain.   It is very difficult to sort out the
142   individual roles of hyperinsulinemia due to peripheral insulin resistance (PIR) in type 2 diabetes and
143   central brain insulin resistance (BIR) in LOAD as well as hyperglycemia in T2DM and LOAD.   What
144   makes this so difficult is that T2DM has both a singular early insulin resistance state that compensates
145   and initially protects individuals from the development of hyperglycemia but not without a price to
146   pay (due to the damaging effects of hyperinsulinemia) not only in peripheral tissues but also in the
147   brain followed over time and aging with ongoing insulin resistance.   Hyperglycemia eventually
148   evolves due to the inability of the pancreatic islet beta cells to compensate (islet beta cell exhaustion-
149   dysfunction and/or loss due to apoptosis) with ensuing hyperglycemia. This box attempts to outline
150   what is currently thought to be known regarding insulin resistance and hyperinsulinemia and
151   compare these effects to hyperglycemia in the brain that may contribute to the development of LOAD.
152   Aβ = amyloid beta; AGE = advanced glycation end-products; BIR = brain insulin resistance; EC =
153   endothelial cells; IDE = insulin degrading enzyme; KATP = Adenosine triphosphate-sensitive
154   potassium channel; LOAD = late-onset Alzheimer's disease; MetS = metabolic syndrome; NVU =
155   neurovascular unit; O2- = superoxide; PHF = paired helical fragments; PIR = peripheral insulin
156   resistance; NFT = neurofibrillary tangles; RAGE = receptor for advanced glycation end-products; RNS
157   = reactive nitrogen species; ROS = reactive oxygen species; T2DM = type 2 diabetes mellitus.

158   Further, the effect PIR and glucotoxicity may have important bearings on the development of
159   brain insulin resistance (BIR) in LOAD.   For example, Ferreira LSS et al. [24] utilize the concept that
160   visceral omental obesity induces excessive toxic free fatty acids and hyperglycemia induces advanced
161   glycation end-products (AGE) to result in blood-brain barrier (BBB) permeabilization. This was
162   followed by brain neuroglia (astrocyte and microglia) inflammation with the production of a host of
163   toxic cytokines to induce neuronal BIR and endoplasmic reticulum stress with synaptic dysfunction
164   and/or loss and its association with neuronal dysfunction and neurodegeneration in age-related
165   LOAD.   However, it is currently becoming more and more evident that there exists a primary BIR
166   in LOAD [25-29].

167   *1.3. Hypothalamic-Pituitary-Adrenal (HPA) Axis Dysfunction in the Metabolic Syndrome H and E*
168   *Phenomenon*

169   The HPA axis in homeostatic conditions is a tightly regulated system that represents one of the
170 body's response mechanisms to acute and chronic physiological or psychological stress.   HPA axis
171 dysfunction is manifest in obesity, insulin resistance, MetS and T2DM as excesses "E" of
172 mineralocorticoids (aldosterone), glucocorticoids (corticosterone in rodents and cortisol in humans)
173 and may be associated with increased sympathetic tone [8, 30].   However, in diet induced obesity
174 (DIO) Western models and preclinical diabetic db/db models and humans it is observed to become
175 dysregulated – HPA axis dysfunction.   Elevated levels of aldosterone and corticosterone contribute
176 to insulin resistance and cerebrocardiovascular disease and may even implicate the cerebral arteriole
177 and capillary NVUs.

178   Previously, in DIO Western model cohorts [8] the adrenal gland underwent considerable
179 ultrastructural remodeling (unreported data). This included adrenal cortical hyperplasia and
180 hypertrophy in zona glomerulosa and zona fasciculata, which may result in increased aldosterone
181 and corticosterone with markedly dilated capillaries within both cortical and medullary regions
182 suggesting increased adrenal blood flow (Fig. 4) and also may be associated with adrenal medullary
183 catecholamine output into the peripheral systemic circulation with increased sympathetic vascular
184 tone (however in these models we did not demonstrate an increase in blood pressure at 8, 12, 16 or
185 20-weeks of observation).   Importantly, the above abnormalities in the Western models strongly
186 suggested the presence of inappropriate and impaired HPA feedback control with dysregulation
187 between these neuroendocrine and systemic endocrine organs. These observations and findings in
188 the Western model agreed with another study of the adrenal gland [31].
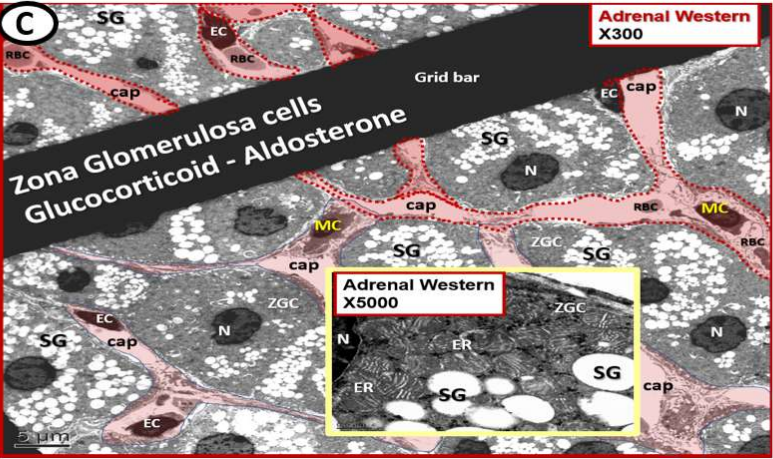189
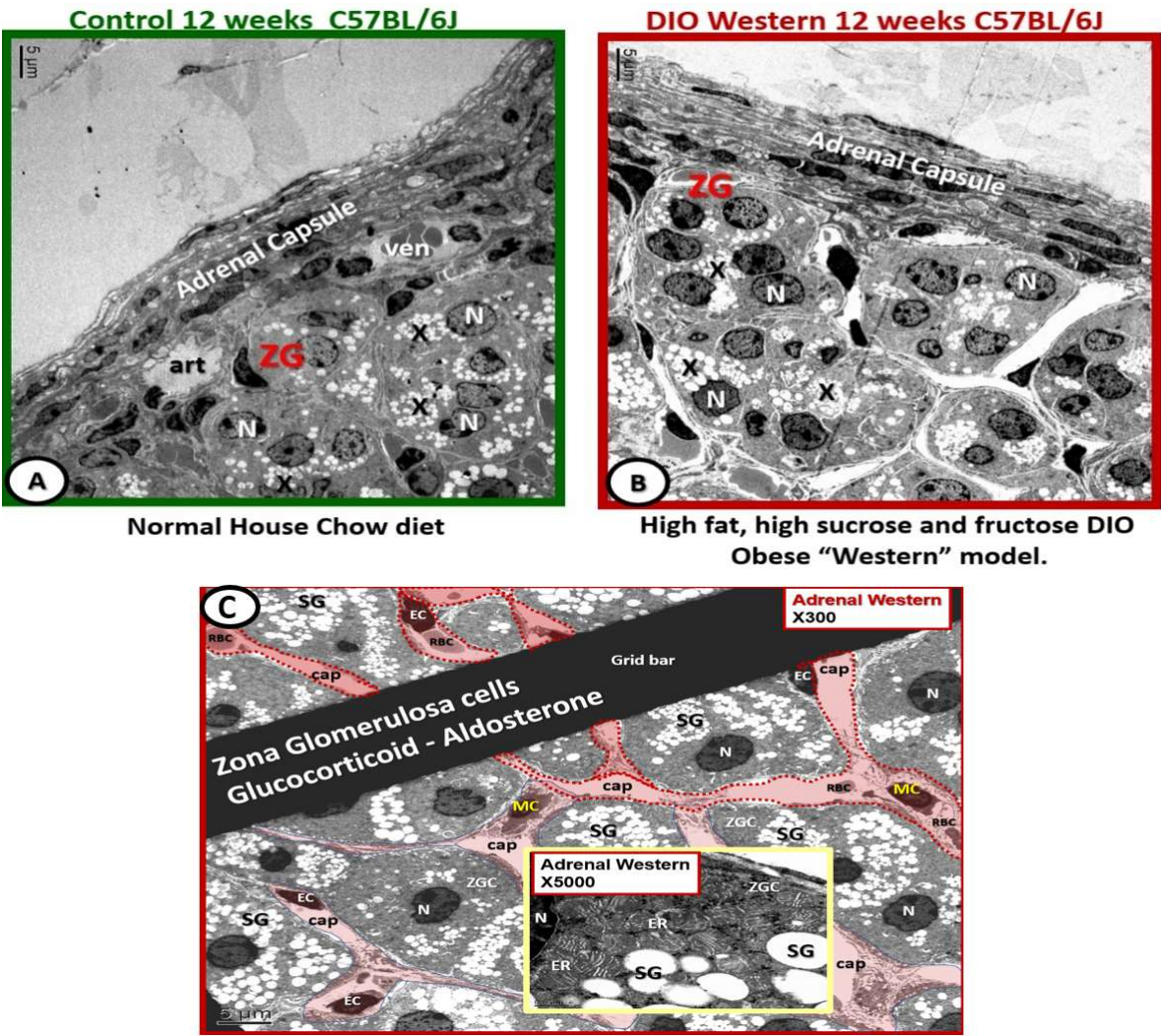
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Figure 4. Adrenal gland zona glomerulosa hypertrophy and hyperplasia with capillary dilation in diet induced obesity Western models. Panel A illustrates the normal control zona glomerulosa (ZG). Panel B depicts the abnormal remodeling changes found in the ZG cells containing spherical secretory granules (electron lucent) that contain aldosterone (X). Note the hyperplasia and hypertrophy and dilated capillaries as compared to the control models in panel A. Panel C depicts dilated capillaries bathing the zona glomerulosa cells in Western models. This image illustrates in greater detail the dilated capillaries (cap; pseudo-colored red) that appear to be continuously bathing the zona glomerulosa cells (ZGCs) and nuclei (N). Insert emphasizes the zona glomerulosa secretory granules (SG) and the extensive endoplasmic reticulum (ER). Magnification x300; scale bar = 5 μm. Insert magnification X5000; bar = 0.5 μm. EC = capillary endothelial cell(s); MC = mononuclear inflammatory cells; RBC = red blood cells Magnification X300; scale bar = 5 μm.

In addition to the HPA axis dysfunction it is thought that the visceral-omental adipose tissue excess is associated with an increased compressive effects on the kidney that may result

in an increase in the renin-angiotensin-aldosterone system (RAAS). Also, the excess aldosterone

may also be associated with a visceral adipose derived aldosterone releasing factor [32, 33, 34].

2. Multiple Intersections Between Type 2 Diabetes Mellitus and Late-Onset Alzheimer's Disease

LOAD, like T2DM, is a chronic age-related disease with a long preclinical or prodromal phase (up to 10 years which is somewhat similar to prediabetes - impaired glucose tolerance and impaired fasting glucose). Once diagnosed, LOAD may have a clinical duration of 8-10 years. Furthermore, in age groups over the age of 65 years of age LOAD has a prevalence (10-30%) and incidence (1-3%) with a current lifetime risk of LOAD of approximately 10.5%. [35].

The MetS and "H" and "E" phenomenon coupled with aging allow for multiple intersects between T2DM and LOAD with IR as the possible linking linchpin between these two age-related diseases (Box 2).

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Box 2. Multiple intersections between T2DM and late-onset Alzheimer's disease. This figure illustrates multiple (at least 18) intersects between T2DM and LOAD. These multiple intersects contribute to the increase of LOAD in those individuals with T2DM. Importantly, note that insulin degrading enzyme (IDE) is related to #7 Insulin Resistance and hyperinsulinemia in that elevated insulin of IR could compete with amyloid beta for degradation by the IDE and that advanced glycation end-products (AGE) and its receptor RAGE are important to # 8 hyperglycemia in generating reactive oxygen species and AGE could also compete with RAGE for amyloid beta degradation. *AD = Alzheimer's disease; BBB = blood-brain barrier; ER = endoplasmic reticulum; LOAD = late-onset AD; NVU = neurovascular unit; T2DM = type 2 diabetes mellitus; UPR/UPS = unfolded protein response.*

Both T2DM and LOAD are known to have multifactorial risk profiles and therefore, it is likely that the relationship between these two diseases have multiple intersects and multifactorial risks that promote cognitive abnormalities and interact during the development of age-related LOAD with underlying T2DM.

The above converging global situations in 1.1. and 1.2. gives rise to great concern, in that, T2DM has recently been demonstrated to increase the risk of developing other chronic age-related diseases such as LOAD [36-47]. LOAD is responsible for 90 - 95% of all dementias. This is in contrast to early onset familial Alzheimer's disease (EOFAD), which develops prior to age 65 and may be related to specific genetic defects such as those in the amyloid precursor protein or presenilin genes and contributes to only approximately 4-5% of dementias [48]. Furthermore, there may be a continuum of progression from obesity, metabolic syndrome, T2DM to vascular dementia (VaD), LOAD and mixed dementia (MD). Importantly, there has been a recent trend to soften the once hard-fixed clinical and histopathologic boundary-lines drawn between VaD and LOAD. Additionally, LOAD may now be considered to be under the umbrella of mixed dementia's (MD) (Fig. 5).
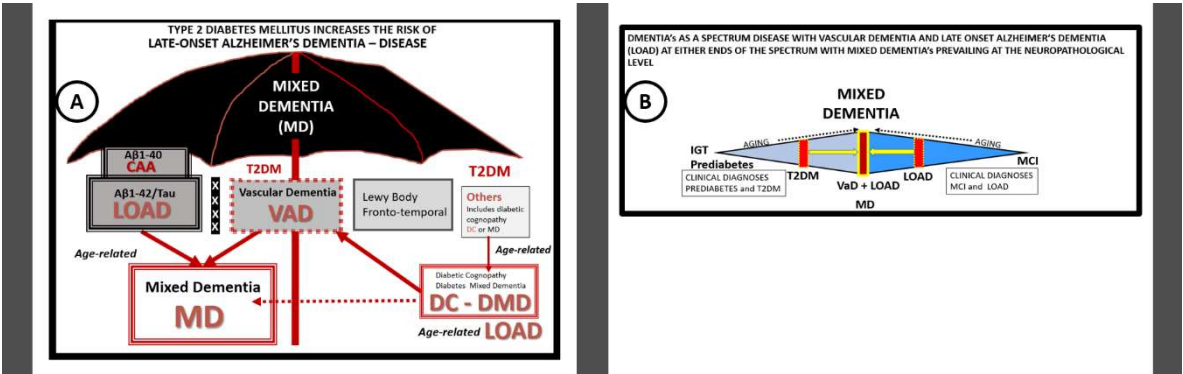
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244   **Figure 5.** The overarching umbrella and the spectrum of   mixed dementias in late-onset Alzheimer's disease
245   (LOAD).   Panel A depicts the overarching umbrella of mixed dementia and diabetic mixed dementia in LOAD.
246   LOAD is by far the most common of all dementias – 90 -95% with early-onset Alzheimer's disease contributing
247   approximately 4-5%.   What has been previously thought of as distinct dementias with rigid separating lines
248   may now be thought to belong under the overarching umbrella of mixed dementia(s) (MD).   Individuals who
249   are clinically diagnosed with LOAD, to best of the clinician's ability, during life, are frequently found have a
250   mixed dementia at the time of autopsy and neuropathologic examination .   The histopathology consists of not
251   only amyloid beta extracellular neuritic plaques, microtubule associated tau paired helical fragments and
252   neurofibrillary tangles but also cerebral amyloid angiopathy (CAA) and evidence of microvascular capillary
253   neurovascular unit (NVU) and arteriolar abnormalities in structure and function.   Interestingly it is now being
254   found that NVU dysfunction may precede the deposition of amyloid beta and tau in LOAD.   Note the X's within
255   the thickened bar (representing a softening) that divides LOAD from vascular dementia (VaD) and the other
256   dementias under this overarching umbrella. The others box in the figure (far right) may include the following:
257   Metabolic e.g. diabetic cognopathy or diabetic mixed dementia (DMD), trauma, infection, Parkinson's disease,
258   alcohol, rare genetic causes, drug-induced and depression-related causes of dementia. Panel B illustrates the
259   spectrum of mixed dementias (MD) found in LOAD. LOAD and vascular dementia (VaD) are the two most
260   common dementia's in older individuals with LOAD being the most common. However, when brains are
261   examined in those patients clinically diagnosed as LOAD it is found by autopsy and neuropathological
262   examination that most have a mixed dementia (MD) of LOAD, cerebral amyloid angiopathy (CAA) and VaD
263   making them mixed (co-occurrence) dementia's (MD).   Therefore, MDs may now be considered one of the most
264   common forms of dementia in older adults age 65 and older.

265

266   This fading phenomenon of the demarcation between VaD and LOAD is largely due to the
267   findings of mixed dementia at the time of autopsy and the early finding of neurovascular -
268   microvascular disease in LOAD (Fig. 5) [49-51].   The findings of mixed dementia consisting of LOAD
269   (neuritic Aβ plaques and tau neurofibrillary paired helical fragments (PHF) and neurofibrillary
270   tangles (NFT), cerebral amyloid angiopathy (CAA)   and VaD are being found to co-exist more and
271   more when post-mortem tissues are examined by neuropathologists providing their mixed
272   histopathologic findings, which suggests strongly that LOAD may be a spectrum disorder or disease
273   (Fig. 5).
274   Also, recently recognized community-based neuropathologic studies have shown that there may
275   be many different complex constellations of underlying pathologies, which may lead to cognitive
276   decline in LOAD [52].   Mixed dementia's equals co-occurrence dementia's for example LOAD + VaD
277   + CAA = mixed dementia when we include neuropathologic findings of post-mortem autopsy.
278   Furthermore, it has been recently shared that neurovascular disease, particularly microvascular -
279   NVU disease (vascular contributions of impaired cognition and dementia (VCID – microvascular,
280   Micro-VCID), may be one of the earliest findings in LOAD [53, 54].   Also, the 2-hit vascular
281   hypothesis has been placed into acceptance (in addition to other multiple LOAD hypotheses) [55, 56]
282   and recently the paper with 69 authors, which discussed in detail the importance of the NVU and its
283   constituent cells as well as other related risk factors [57].   Microvascular NVU dysfunction, cellular
284   remodeling and NVU uncoupling may result in hypoperfusion and decreased CBF (discussed in

285 greater detail in section 5.1.), which appear to be early markers of neurodegeneration.   These
286 remodeling changes may set in motion a serious aberrant cascade of remodeling and functional
287 events in the brain that may be concurrent or even precede the synaptic dysfunction and deposition
288 of neuritic plaques or neurofibrillary tangles and result in the cognitive decline of age-related LOAD
289 neurodegeneration.   It is commonly known that T2DM increases the risk for VaD and a recent study
290 of 28 prospective observational studies in 2012, which included 89,708 diabetic patients demonstrated
291 a 73% increased risk of all type dementias (which included LOAD), a 56% increase of LOAD and a
292 127% increase of VaD in diabetic human patients [58]. However, it might be very interesting if one
293 would consider LOAD as a mixed dementia (inclusive of VaD, CAA and LOAD) in regards to how
294 these percentages might change.

295 **3. Continuum of Progression: From Obesity, Insulin Resistance to T2DM to LOAD**

296 There appears to be a continuum of progression in the natural history of T2DM inclusive of
297 obesity; MetS to LOAD.   From obesity (specifically visceral or omental) to MetS to T2DM and the
298 multiple intersects and increasing cellular vulnerability to the development of LOAD and mixed
299 dementia(s) (MD) (Fig. 5).

300 3.1. Obesity

301 As mentioned earlier in section 1.2, obesity seems to be the driver of both the Hyper "H" and
302 "E" phenomenon and the Met S. (Fig.2, 3) with associated mild cognitive impairment (MCI) to T2DM
303 to NVU remodeling, neuroinflammation, neurodegeneration and LOAD.   Peripheral insulin
304 resistance (PIR) and central brain insulin resistance (BIR) and impaired insulin signaling seem to be
305 the link between T2DM and LOAD.   So it seems that T2DM may increase the vulnerability and the
306 risk of LOAD via a progressive continuum: from visceral-omental mid-life overweight and obesity
307 that is known to independently increase LOAD as well as VaD [59].   This progressive continuum to
308 LOAD via the MetS and   "H" and "E" phenomenon to T2DM seems to increase the risk of
309 neurodegeneration via a 2-hit vascular hypothesis: VCID to LOAD (Fig. 6).
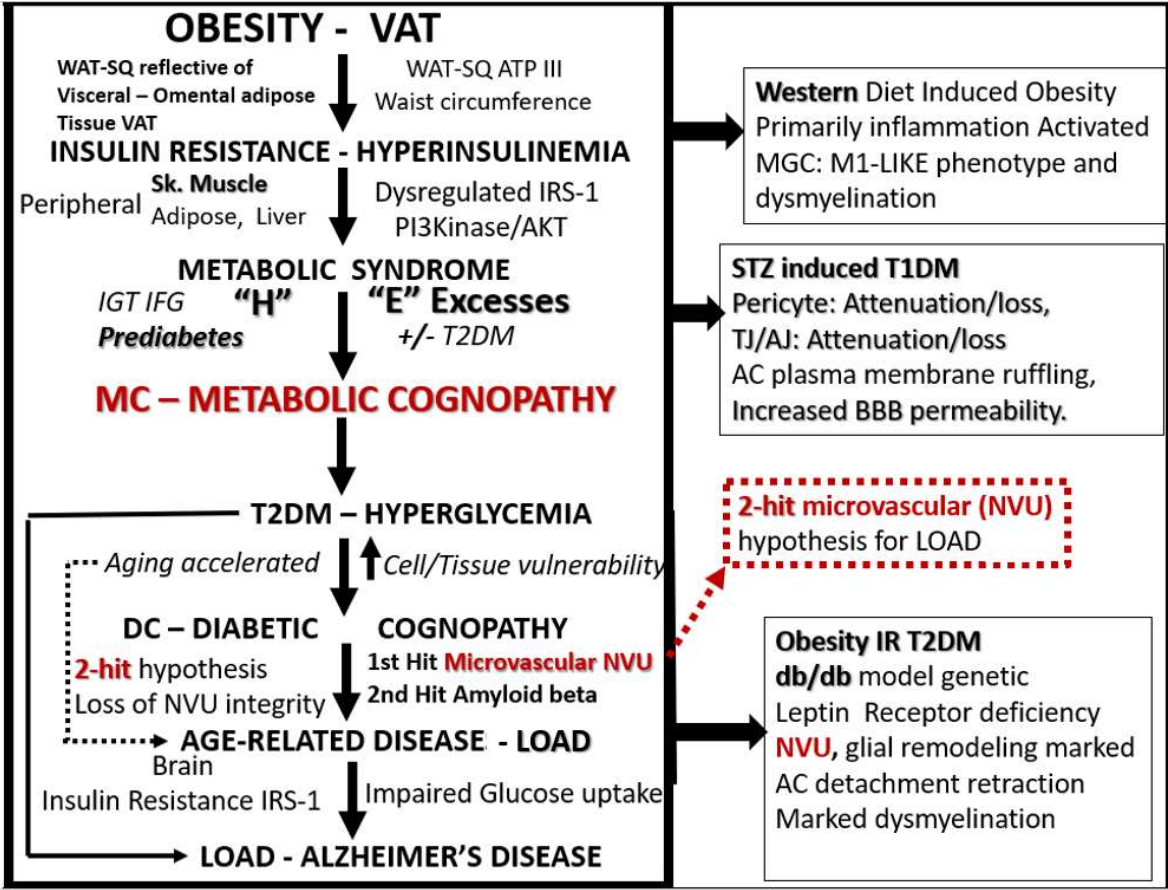

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Figure 6. The progressive continuum from visceral obesity to type 2 diabetes mellitus to neurodegeneration and late-onset Alzheimer's disease (LOAD). This continuum of progression (downward arrows) may occur independently as independent risk factors or interact synergistically and concurrently over time (aging). This figure also represents the continuum of progressive metabolic excesses and stresses involved to advance to LOAD via obesity, insulin resistance, metabolic syndrome and the "H" and "E" phenomenon and a metabolic cognopathy to prediabetes and/or overt T2DM with glucotoxicity to accelerated aging and increased neurovascular unit, vascular mural cells, neuroglia, neuronal cellular vulnerability and remodeling to metabolic cognopathy, diabetic cognopathy and aging with the increased risk of neurodegeneration and LOAD. In this figure we propose that these above precursors are more of a continuum of progression from various stages, one to the next; however, some have posited that they could each represent separate pathologies that may be differentially expressed over time and thus, remain age-related .   The three boxes on the right side of this figure refer to our independent findings in the brains of the diet induced obesity, insulin resistant model with impaired glucose tolerance or prediabetes Western mouse model [8], the streptozotocin induced type 1 diabetic mouse model [9] and the db/db mouse model of obesity, insulin resistance and T2DM [4-7].   Note the intersects between #10 in Box 2 to the red dashed outline of the 2-hit microvascular hypothesis and vascular contributions to cognitive impairment and dementia (VCID). *ATP III = adult treatment panel III of the National Cholesterol Education program; cognopathy = disease of or impaired cognition; DC = diabetic cognopathy; IFG = impaired fasting glucose; IGT = impaired glucose tolerance; IRS-1 = insulin receptor substrate-1; MC = metabolic cognopathy; NVU = neurovascular unit; PI3kinase/AKT = phosphatidylinositol 3/protein kinase B; Sk = skeletal;   SQ = subcutaneous; VAT = visceral adipose tissue; WAT = white adipose.*

Previous studies in obese-prediabetic models with impaired glucose tolerance have demonstrated that the diet-induced obesity Western model (with high fat, high sucrose and fructose in C57B6 models) were observed to have lipofuscin-like bodies within the interstitial spaces that were being avidly consumed by activated MGCs with some dysmyelination (markedly less electron density of myelin) of axons in layer III of the mid cortical grey matter. Overall, this model was primarily a neuroinflammatory model with activation and senescence of microglia without any NVU remodeling [8].   The next model was the lean streptozotocin induced type 1 diabetes model, which demonstrated marked NVU remodeling with blood-brain barrier (BBB) disruption and neurovascular unit dysfunction with increased permeability and loss of NVU BBB integrity.   In this model we deduced that glucotoxicity resulting in excessive oxidative stress was the primary cause of NVU remodeling, since treatment with a specific mitochondria carbonic anhydrase inhibitor protected these NVU remodeling changes as well as increased permeability [9]. The most recent model studied was the obese, insulin resistant, T2DM female *db/db* mouse model [4-7].   This model demonstrated marked NVU, microglia cell(s) (MGC) and oligodendrocyte(s) OL and myelin remodeling that was protected utilizing a 10-week treatment period with the glucose lowering anti-diabetic sodium glucose transporter 2 (SGLT2) inhibitor from 10 – 20 weeks of age [7].   In this diabetic obese *db/db* model we were also able to demonstrate a marked expansion in aortic visceral perivascular adipose tissue that contained hypertrophic unilocular adipocytes that were rupturing with the spillage of neurotoxic proinflammatory free fatty acids (unpublished data).   These remodeling changes were also associated with chronic inflammation due to peripheral systemic macrophage infiltration and thus provided for excessive neurotoxic cytokines and adipokines to be available to the aortic wall and systemic circulation, which was associated with vascular stiffening. This vascular stiffening could increase abnormal pulsatile mechanical forces associated with NVU -microvascular structural damage and remodeling to the NVU of brain due to increased pulse wave velocity and increase the expansion via increased pulse pressure in addition to also being a source of chronic inflammation due to systemic cytokines-adipokines from the perivascular adipose tissue. Thus, the *db/db* model [4-7] was able to 'fill-in' many of our gaps in knowledge between the diet-induced obesity Western model [8] and the type 1 streptozotocin induced diabetic models [9].

3.2. Capillary Neurovascular Unit (NVU)

363   The capillary NVU is a readily identifiable ultrastructural constant when viewing brain tissue
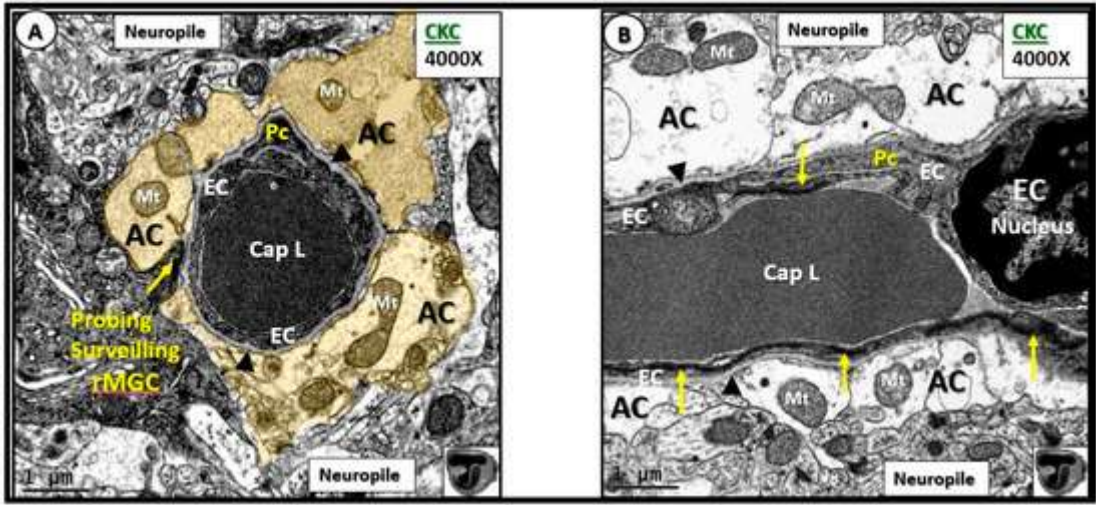364   with transmission electron microscopy (TEM) (Fig. 7) [4-7].

365

366   **Figure 7.** Normal neurovascular unit (NVU) morphology in control wild-type non-diabetic models
367   (C57BL/KsJ; CKC control). Panels (**A**) (cross-section) and (**B**) (longitudinal section) illustrate the
368   normal cellular ultrastructure of the NVU. Panel (**A**) depicts an electron-dense ramified microglia cell
369   (rMGC) surveilling the NVU (arrow). The NVU capillary consists of an endothelial cell (EC) encircling
370   a capillary lumen (Cap L) whose basement membrane (BM) splits (arrowheads) to encompass the
371   pericyte (Pc) foot process. Note how the pseudo-colored golden astrocyte (AC) end-feet encompass
372   and tightly abut the capillary EC and Pc BMs. Note that the AC clear zone in panel (**A**) was pseudo-
373   colored golden to emphasize its importance in the NVU, while it exists as a clear-zone with a reduced
374   electron-dense cytoplasm as compared to other cells within the brain, and represents not only a
375   golden halo, but also a clear zone or corona of AC foot processes surrounding the EC and Pc cells of
376   the NVU (panel (**B**)). Panel (**B**) illustrates the electron-lucency of the AC clear zone halo or corona that
377   tightly abuts and encircles the NVU EC and Pc BMs. Note the EC nucleus (far right side) and the
378   highly electron-dense tight junctions/adherens junctions (TJ/AJ) complex (yellow arrows) that are not
379   readily visible in panel (**A**) (arrows). Also, note that the mitochondria (Mt) have an electron-dense Mt
380   matrix and that cristae may be noted even at this magnification. Note that the NVU is encompassed
381   by the outermost abluminal neuropil (neuropil). In the bottom right-hand corner, note the logo of red
382   blood cells within a capillary NVU that are in the shape of the letter T overlying the letter J, which are
383   used to abbreviate tight junction(s). Magnification ×4000; scale bar = 1 μm. Image available via
384   license CC BY 4.0. [4].

385   This multicellular structural and functional unit in the brain consists of the following cells: ECs,
386   Pcs, ACs, MGCs, OLs and neurons.   Within the NVU is contained the EC paracellular BBB
387   synthesized primarily by ECs and supported structurally and functionally by Pcs and additionally
388   supported and maintained by a corona of surrounding-encircling AC foot processes (ACfp).   The
389   two primary mural cells of the capillary NVU are the luminal continuous monolayer of ECs and
390   supportive Pcs, which are embedded within the EC basement membrane (BM) creating an inner and
391   outer BM.   This is in contrast to the arterial NVU, in which vascular smooth muscle cells provide for
392   mural arteriole support with similar abutting of ACfp.   The third contiguous cells belong to the
393   multiple ACfps that tightly abut the EC and Pc outer BMs.   Probing ramified microglia cells,
394   oligodendrocytes and oligodendrocyte linage precursor cell(s) (OPC) (especially in subcortical and
395   white matter regions) along with neuronal axons (unmyelinated and myelinated) may also be noted
396   to be adjacent to the outer basement membrane of the NVU [4-7].
397   There are basically three to four barriers of the NVU from luminal to abluminal regions: *i.*
398   endothelial glycocalyx (ecGCx); *ii.* endothelium (ECs) inclusive of BBB tight and adherens junctions
399   (TJ/AJ) and the EC cytoplasm itself, which also utilizes transcytosis as a method of EC cellular
400   transport; *iii.* EC and pericyte (Pc) and/or its pericyte foot processes (Pcfp) basement membrane (BM),

401 which shares the common inner and outer basement membrane (BM); *iv.* astrocytes (ACfp) and
402 their outer basal lamina.   Of these three to four barriers, the BBB TJ/AJ that reside between EC cell-
403 cell junctions of the NVU and between the blood and brain interstitial fluid provides the greatest
404 trans-endothelial electrical resistance (TEER) control over the immediate microenvironment of
405 brain cells.   Additionally, there are other interfaces-barriers that are also important such as the
406 choroid plexus epithelium between the blood and ventricular cerebrospinal fluid (CSF), and the
407 arachnoid epithelium between the blood and subarachnoid CSF.

408 *3.2.1. Endothelial Cell(s) (EC) – Endothelium*

409   The endothelium is the continuous, epithelial, highly polarized, monolayer cell that lines the
410 luminal surface of the NVU and serves as the interface between the peripheral circulating blood and
411 its cellular, molecular proteins, ion constituents and the brain parenchyma (Fig. 7).   The NVU ECs
412 are responsible for creating and maintaining the NVU permeability and lumen formation [60].   The
413 EC is responsible for 2 initial barriers, which create the BBB consisting of TJ/AJ, junctional adherens
414 molecules and cytoskeletal zona-occludin-1 and beta catenin's that are formed between adjacent
415 endothelial cells [4-7] and the EC surface layer the endothelial glycocalyx (ecGCx).   The apical
416 luminal and basolateral abluminal polarity of the brain EC is of utmost importance in creating the
417 normal requisite physiologic functions of the NVU in that many of its influx - efflux and specific
418 transporters proteins require a specific location to be physiologically functional [4, 60].   It is also
419 important to note that while the integrity of the paracellular TJ/AJ proteins in ECs are of critical
420 importance in EC permeability, there also exists an intracellular transcytotic route for increased EC
421 transcellular transport and permeability mechanisms.   In fact, our diabetic *db/db* models have
422 demonstrated that there exists not only an ultrastructural abnormality in TJ/AJ with attenuation
423 and/or loss but also an increase in the EC transcytotic-pinocytotic vesicles plus other important
424 remodeling change such as inflammation and basement membrane thickening [4, 7] (Fig. 10).

425
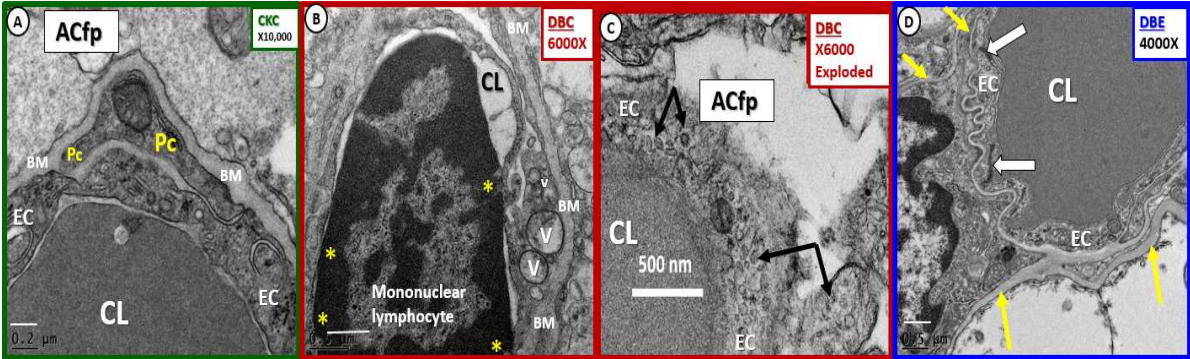
426 **Figure 8.** Multiple endothelial cell remodeling changes in diabetic *db/db* models.   Panel A Illustrates
427   the normal endothelial cell (EC), pericyte (Pc) and astrocyte foot process (ACfp).   Note that the
428   basement membrane appears almost as thick as in the known BM thickening in panel B; however,
429   note that the magnification is higher (x10,000) and the scale bar is not as wide in panel A (200nm) as
430   in panel B (500nm).   Panel B depicts adherent mononuclear lymphocyte and also note EC vacuoles
431   (V), vesicles (v) and pinocytosis arrows in panel C.   Panel D demonstrates the protective quality of
432   empagliflozin.   Magnification X×10,000 panel A; ×6000 panel B. ×6000; bar = 500nm panel C; ×4000
433   panel D with scale bars 0.2μm, 0.5 μm, 0.5 μm in panels   A, B, D respectively. Image available via
434   license CC by 4.0 [4].

435   Also, the EC in the diabetic *db/db* model demonstrated the following remodeling changes: *i.*
436 endothelial thinning and loss of electron density; *ii.* increased vesicles-pinocytosis-vacuoles; *iii.*
437 aberrant mitochondria; *iv.* hyperplasia with some reduplication; *v.* endothelial cell activation with
438 adherent erythrocytes and leukocytes (lymphocytes and monocytes).   Importantly, the EC is capable
439 of synthesizing at least 2 main autocrine paracrine vasodilating molecules nitric oxide (NO) and
440 prostaglandin E2 (PGE2) or prostacyclin and the autocrine paracrine potent vasoconstrictor

441 endothelin-1 (ET-1).   Recently, normal aging in Wistar rats increases EC permeability in 14-16-
442 month-old cohorts as compared to younger cohorts at 2-3 month of age [61].   Bors L. et al were able
443 to demonstrate that there was increased thickness of EC BM, TJ/AJ were attenuated, ACfp were
444 extended with increased GFAP staining and a decrease in the efflux permeability glycoprotein (P-gp)
445 or the ABCB-1 efflux EC protein [61].   These findings correlate with previous sections 1.1 and 1.2.
446 The endothelium is responsible for synthesizing the luminal ecGCx and also the inner BM that are
447 continuous with the Pc assisted synthesis and maintenance of the inner and outer BMs of the NVU.

3.2.2. Endothelial Glycocalyx (ecGCx)

449 The ecGCx is a decorative 'coat of many colors' and consists of a sugar-protein endothelial
450 screen-like mesh; gel-like slime surface coating that covers and decorates the luminal side of the
451 highly polarized endothelium and is synthesized primarily by the EC with some plasma
452 contributions of albumin, fibrinogen and soluble plasma proteoglycans and glycolipids (Fig. 9).
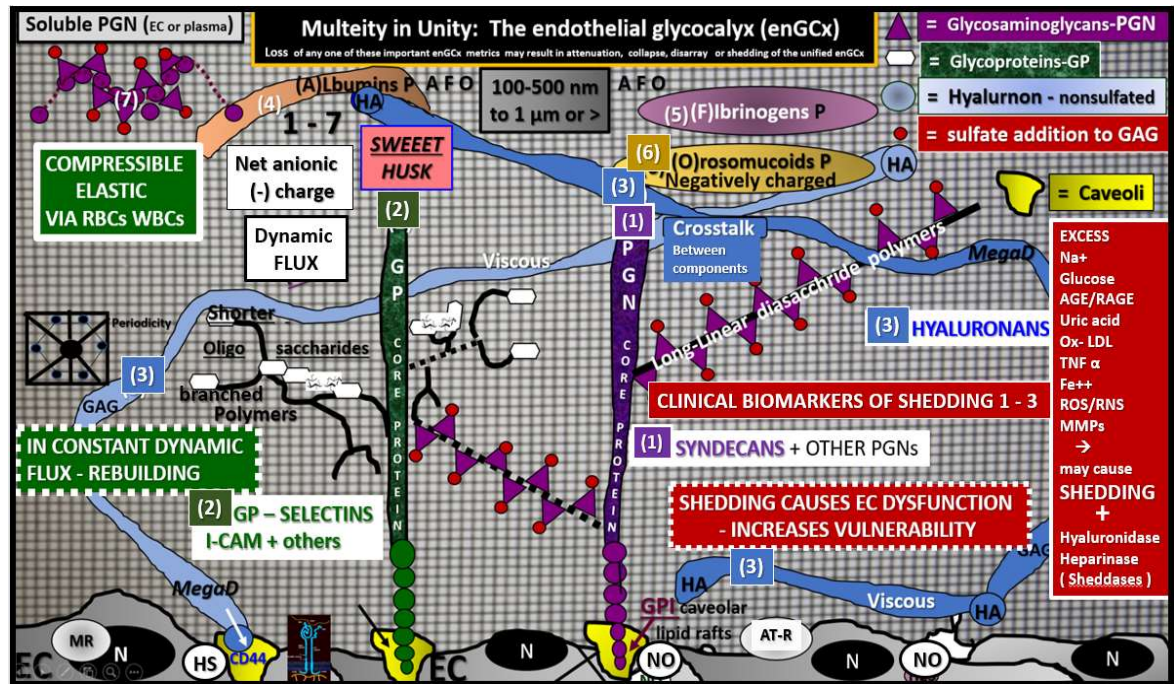

Figure 9. The endothelial cell glycocalyx (ecGCx) of the capillary neurovascular unit.   This cartoon
depicts the three major anchoring proteins of the ecGCx: proteoglycan(s) (PGN) (1) (purple),
glycoproteins (GP) (2) (green) and Hyaluronan (HA) (3) (blue).   This ecGCx sweet husk of the
endothelial cell (EC) is in a constant state of flux being constantly resynthesized by the EC and is
highly compressible by the blood's cellular constituents (red and white blood cells RBC, WBC). The
proteoglycans (PGN) are noted for their syndecans (SYN) (plus others) and the glycoproteins (GP)
are known for their selectin's such as I-CAM, P and E-Selectin plus others.   One will note the
glycosaminoglycans (GAGs) long-linear disaccharides of the PGNs and shorter highly branched
GAGs oligosaccharides of the glycoproteins.   This surface layer has a net negative anionic charge
(due to orosomucoids protein and opposes negatively charged molecules in the blood utilizing Van
der Wall forces) and size selective semipermeable surface barrier (hindering access of 70 kiloDalton-
kDa or larger proteins) and additionally serves as a glucose and sodium sink initially in health that
can be rapidly overcome with attenuation and/or shedding in diabetes (glucotoxicity-oxidative stress)
and clinical excesses of sodium chloride salinity.   The intact ecGCx contains the protective
extracellular superoxide dismutase (*ec-SOD*) and antithrombin III (*AT III*) while serving as a
mechanotransducer.   The thickness of the ecGCx varies from 100-500nm (300nm mean), from 1- 2
micrometer in length and the percentage of EC covered by the ecGCx was approximately 40% in the
NVU capillaries in the transmission electron microscopic studies and was greater in the brain as
compared to the heart or lung continuous capillaries without a BBB (Fig. 9) [67].   Glucotoxicity;
oxidative stress and neuroinflammation (as in the diabetic *db/db* model); traumatic shock injuries;

474   ischemia/reperfusion and sepsis are known to be capable of ecGCx shedding and/or reduction in
475   volume and *plasma levels of* syndecans, I-CAMs, selectins and hyaluronans can be measured clinically
476   with elevated levels reflecting the reduced ecGCx due to thinning, loss or shedding. *AT-1R =*
477   *angiotensin 1 receptor; BBB = blood-brain barrier; CD44 = the hyaluronan receptor of the ecGCx and cell-*
478   *surface glycoprotein involved in cell–cell interactions and binds HA via the amino-terminal domain; EC =*
479   *endothelial cell; GP = glycoprotein(s); H2S = hydrogen sulfide; HA = hyaluronan; I-CAM = intercellular*
480   *adhesion molecule 1; MR = mineralocorticoid receptor; N = nucleus; P = protein; PGN = proteoglycans; RBC =*
481   *red blood cell; WBC = white blood cell.*

482   The intact ecGCx is important for vascular wall arteries, arterioles and capillary integrity and it
483   does not decorate veins or venules [62].   The ecGCx is anchored to the EC via proteoglycans
484   (syndecans plus others) and glycoproteins (selectins plus others including I-CAM), while the third
485   major component protein consists of hyaluronan.   Hyaluronans may be free or anchored to the
486   endothelium via CD44.   Interestingly, this protective coating is very similar to the brain's interstitial
487   extracellular matrix in molecular contents, which is difficult to examine by ultrastructure imaging
488   due to its extreme thinness even at very high magnification due to the tightly packed glia and neurons
489   within a confined space created by the exoskeleton the skull.   The elusive ecGCx is in a constant state
490   of flux and also in a constant state of being regenerated and repaired by the endothelium and plasma
491   constituents [63, 64, 65, 66].   The systemic cells and molecules of the blood seldom see or come into
492   direct contact with the plasma membrane-plasmalemma of the EC, because it is constantly and nearly
493   continuously covered by the protective ecGCx surface coating, which prevents direct exposure of
494   systemic blood components to the EC plasma membrane in health.
495   Recently, the brain's NVU ecGCx has been beautifully studied, visualized and documented by
496   lanthanum nitrate staining by both transmission electron microcopy (TEM) and scanning electron
497   microscopy (SEM) making this EC surface coating layer visible even though TEM preparations
498   undergo numerous dehydration steps [67].   The perfusion fixation with lanthanum nitrate pre-
499   sacrifice allows the ecGCx to be beautifully stained and visualized with TEM and SEM studies from
500   cortical brain regions of the C57B6 mice (Fig. 10).
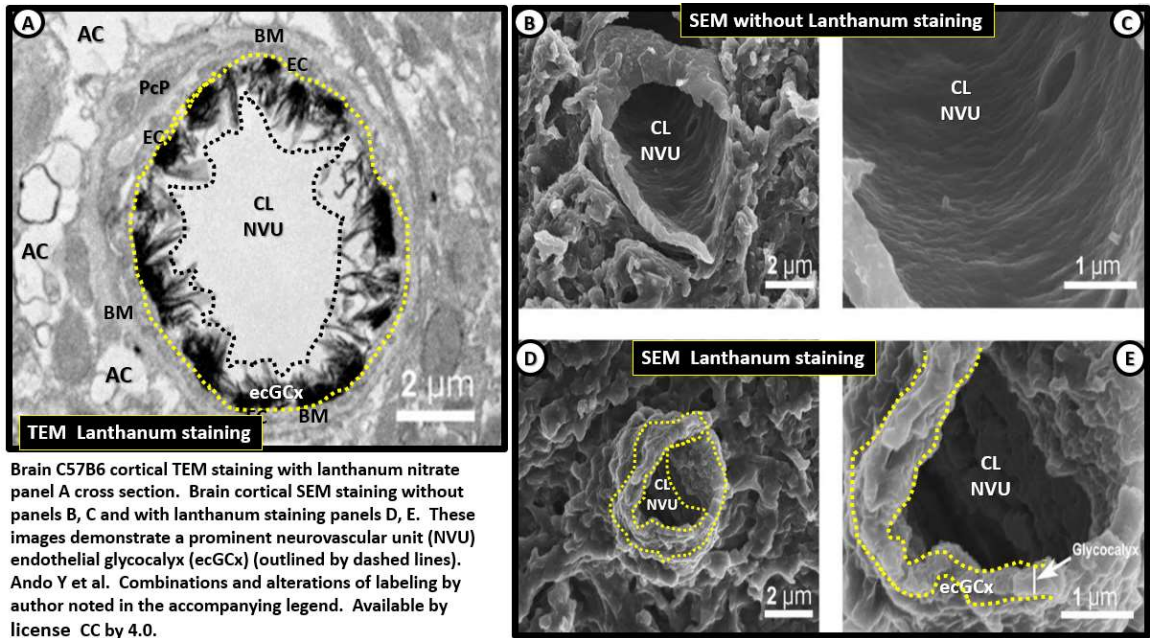
Brain C57B6 cortical TEM staining with lanthanum nitrate panel A cross section. Brain cortical SEM staining without panels B, C and with lanthanum staining panels D, E. These images demonstrate a prominent neurovascular unit (NVU) endothelial glycocalyx (ecGCx) (outlined by dashed lines). Ando Y et al. Combinations and alterations of labeling by author noted in the accompanying legend. Available by license CC by 4.0.

502   **Figure 10.** TEM and scanning electron micrographs of the endothelial cell glycocalyx. With And
503   Without Lanthanum Staining.   Panel A depicts a transmission electron microscopic (TEM) cross
504   sectional image of the endothelial cell glycocalyx (ecGCx) of the neurovascular unit, outlined by
505   yellow dashed lines abluminally and black dashed line luminally. Note how this electron dense
506   stained ecGCx by lanthanum nitrate with perfusion fixation covers nearly the entire luminal surface
507   of the endothelial cell (EC) with intermittent varying electron dense cores and extend outwardly into
508   the lumen in control non-diseased models of the C57B6 mice from the cerebral cortical brain. Scale

509   bar = 2 μm. Panels B and C illustrate scanning electron microscopy (SEM) micrographs the NVU
510   without lanthanum nitrate staining and panels D and E depict lanthanum nitrate staining and now,
511   note the visible elusive ecGCx outlined by yellow dashed lines from the EC on the luminal side. This
512   image actually combines figures 1 and figures 2 from   Ando Y [67]. Scale bar = 2 μm Panels A, B and
513   D.   Scale bar = 1 μm panels C, E.   Image available via license CC by 4.0 [67].

514   Recently, our transmission electron microscopy core was surprisingly able to identify a highly
515   electron dense endothelial surface coat utilizing a secondary staining technique that is utilized for
516   focused ion beam/scanning electron microscopy (FIB/SEM) with the use of traditional electron
517   microscopy (osmium tetroxide, potassium ferrocyanide, en bloc uranyl acetate and Walton's Lead
518   Nitrate solution staining) in control C57B6 mouse models (Fig. 11).

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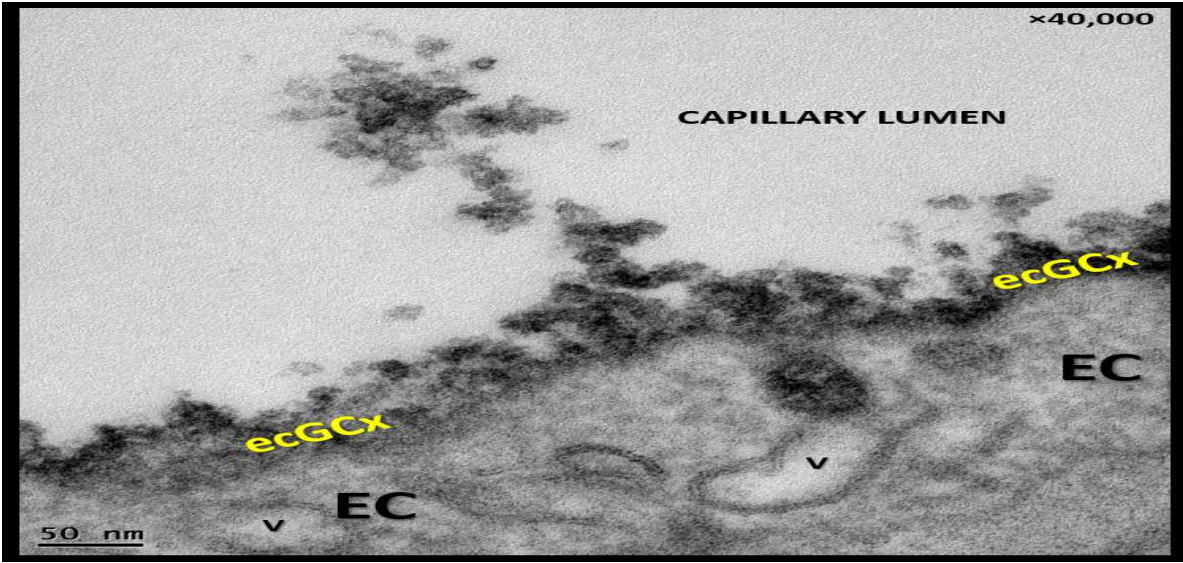
520   **Figure 11.** Transmission electron microscopy identification of endothelial cell glycocalyx.   Our TEM
521   core was recently able to identify the extremely electron dense endothelial glycocalyx (ecGCx).   Note
522   base regions with approximately 50nm thickness with periodic spikes in thickness of 200-300nm on
523   the surface capillary luminal side of the endothelial cell (EC).   Also note within EC cytoplasm the
524   presence of large 50-100nm vacuoles (V). Magnification ×40,000; scale bar = 50nm. This is an original
525   unpublished image.

526   Also, our core has more recently been able to identify the elusive ecGCX with perfusion fixation
527   by lanthanum nitrate in the cerebral NVU capillaries of control C57B6 mouse models and it will be
528   interesting to observe for attenuation/thinning, shedding, degradation of this endothelial surface
529   coating in age-matched diabetic models in the coming future (Fig. 12).

Figure 12. Lanthanum nitrate perfusion fixation staining of control C57B6 mouse models in the cortical layer III capillary neurovascular unit endothelial cell glycocalyx (ecGCx). Note the extreme electron dense staining of lanthanum nitrate covering the endothelial surface of the ecGCx (arrows) similar to figures 12 and 13 that notably does not penetrate the endothelium or any other aspects of the neurovascular unit with lanthanum nitrate staining. Its thickness appears to vary fro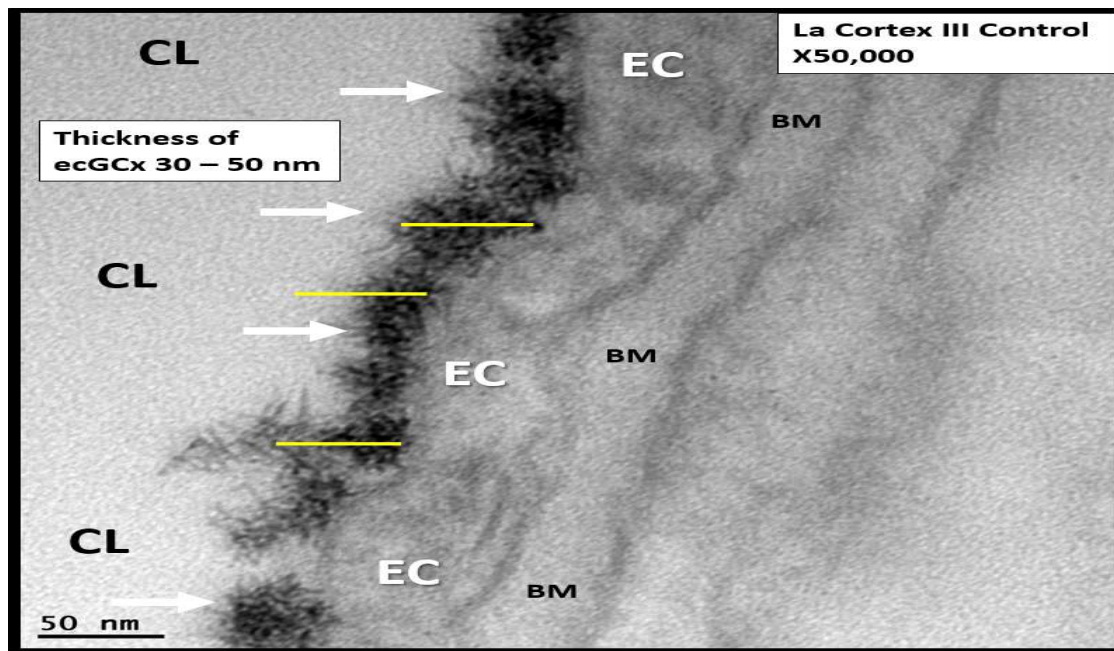m 30 to 50 nanometers (nm). Yellow scale bars of 50nm are placed overlying the electron dense lanthanum nitrate staining ecGCx. Magnification x50,000; scale bar = 50 nm. *BM = endothelial cell basement membrane; CL = capillary lumen; EC = endothelial cell cytoplasm.*

Aging is known to be the major risk factor for the development of LOAD, and it has recently been demonstrated that aging results in a thinning of the ecGCx by 50% when comparing young (6 months) to old (24 months) C57B6 mice (mesenteric and skeletal microvessels) and 30% thinning of the ecGCx when comparing young (23 years) and old (60 ± 2 years) humans (sublingual microvessels) [68]. Now that we may have a reliable and reproducible method for determining ecGCx disruption, thinning, degradation and/or shedding we may be able to demonstrate by TEM with perfusion fixation staining of lanthanum nitrate that indeed there is disruption of the endothelial surface layer - ecGCx and in some cases be able to determine if the increased permeability that was once thought to be non-disruptive may now be considered to be disruptive due to alterations of the ecGCx [69].

3.2.3. Endothelial cell Basement Membrane (BM)

The BM consists of type IV collagen, fibronectin, hyaluronan, laminin, nidogen, and heparin sulfate proteoglycans (agrin and perlecan) and envelops the basilar portion of the EC and splits to encompass the Pc and forms its base to which the EC and Pc BM forms the adhesion anchoring structure for the corona of ACfps [4, 70].   The BM is synthesized primarily by the EC; however, the Pc and AC also contributes to the synthesis and maintenance of the NVU BM.   Interestingly, as one studies the ultrastructure of the NVU, one notes that the polarized ECs create a barrier at the luminal surface layer (ecGCx) and also the paracellular and basilar abluminal side of the EC's monolayer.   In diabetic *db/db* models there is BM thickening and even though it is thickened, its structure is remodeled with rearrangement and thickening, which allows for increased permeability (Fig. 13).
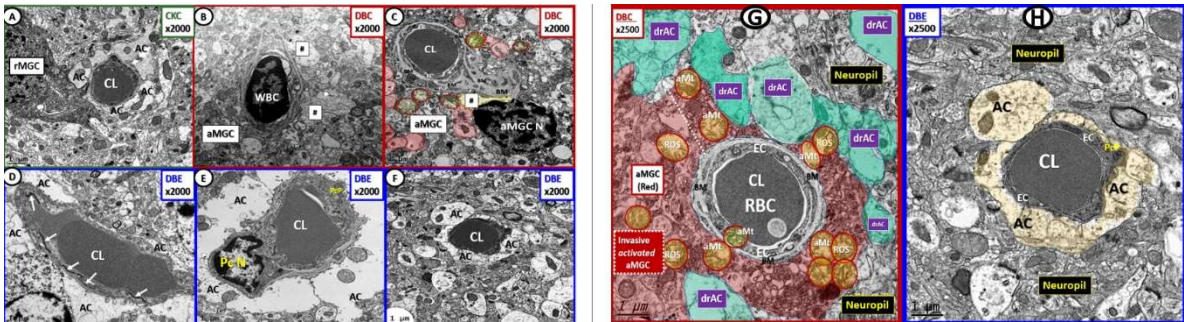
Figure 13. Empagliflozin protects the neurovascular unit from basement membrane thickening: Loss Of blood-brain barrier endothelial tight and adherens junctions and pericytes. Panel A and D depict the normal ultrastructure of the basement membrane (BM) and BBB in control non-diabetic CKC models. Panels B and C depict the Pc ghost (panel B) and complete loss of Pcs in panels B and C as well as the detachment of astrocyte foot processes in panel A (hashtags) with invading activated microglia cell (aMGC) in the *db/db* DBC diabetic models outlined in red. Panels D, E and F demonstrate that empagliflozin protects from EC activation, loss of EC tight and adherent junctions (arrows), Pc loss and AC detachment in the DBC treated models with empagliflozin (DBE) outlined in blue. Magnification X2000;scale bar = 1μm. AC = Astrocyte end feet processes; aMGC = activated microglial cell; CL = capillary lumen; Pc N = pericyte nucleus; PcP = pericyte process; rMGC = ramified microglia cell. Image available via license CC by 4.0 [4, 7]. Panels G and H depict the thickening of the BM In db/db models and the normal appearing BM in empagliflozin treated models respectively. Note the invasive aMGC and the detached retracted ACfp in addition to the basement membrane thickening in panel G as compared to the empagliflozin treated models in panel H. Magnification. x2500; scale bar = 1 μm. *aMGC = activated microglia cell; aMt = aberrant mitochondria; drAC = detached retracted AC EC = endothelial cell; CL = NVU capillary lumen; Pc = pericyte foot process; RBC = red blood cell; ROS = reactive oxygen species.* Image available via license CC by 4.0. [4, 5, 7]

3.2.4. Neurovascular Unit (NVU) Pericyte(s) (Pc)

The pericyte (Pc) is a ubiquitous - systemic (found both in peripheral and brain microvascular capillaries), requisite, mesenchymal derived, pluripotent and postnatally undifferentiated vascular mural cell important for mediating physiological and pathological repair processes. The Pc serves other microcirculation functions including post-natal vascular development (angiogenesis), important for maturation and remodeling of the NVU. Additionally, pericytes provide microvascular structural stabilization as well as provide a supportive-protective role to capillary endothelial cells and is known to be an innate immune and antigen presenting cell of the NVU and may be capable of differentiating into a microvascular niche mesenchymal stem cell (Fig.14) [71, 72, 73, 74, 75, 76, 77, 78, 79, 80].

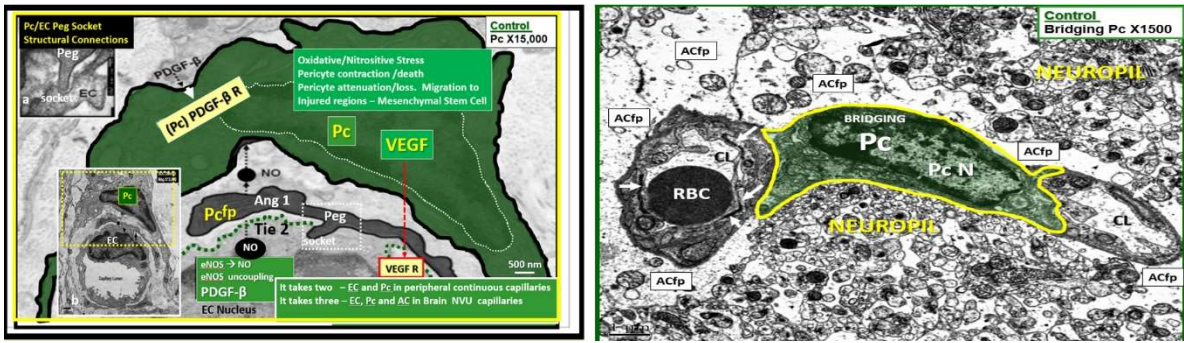Figure 14 Normal pericyte - endothelial morphology and crosstalk in control CKC models. Left panel depicts a peripheral continuous capillaries "*It takes two*". Indeed for both peripheral continuous capillaries and brain continuous capillaries of the neurovascular unit (NVU with blood-brain barrier (BBB) tight and adherens junction (TJ/AJ)) it takes these two highly integrated vascular mural cells

(endothelial cells (EC) and pericyte (Pc) interaction and crosstalk. However, in the brain " it takes three " since ECs, PCs and astrocytes (ACs) must properly   interact in order for the proper development and maintenance of the BBB TJ/AJ – junctional adherens and zona-occludin-1 (ZO-1) as well as the glial astrocyte (AC) to allow for this microvascular system to develop proper cerebral blood flow, prevent increased permeability, and assure proper NVU coupling with regional neurons to assure proper functional hyperemia. This image focuses on peripheral continuous capillaries in order to demonstrate normal EC-Pc morphology. The Pericyte soma is pseudo-colored green and its nucleus is enveloped with white dashed line. Importantly the Pc stains positive for alpha smooth muscle actin and allows for contraction and importantly relaxation to allow for NVU coupling and vasodilation. Insert (a) depicts the peg socket interaction while insert (b) allows the entire capillary to be viewed in its environment without colorization.   Magnification ×15,000; bar = 500nm in all images. Also, note that the EC provides nitric oxide (NO) and platelet derived growth factor (PDGFβ) and that the Pc provides vascular endothelial growth factor (VEGF) and makes possible the contraction and relaxation of the capillary NVU via Pc actin which is essential for these two cells survival and the Ang1/Ti2 system via crosstalk for proper function and protection of the endothelial capillary NVU of control CKC models. *eNOS = endothelial nitric oxide; NVU = neurovascular coupling; PDGF-β = platelet derived growth factor beta; PDGF-R its receptor; VEGF = vascular derived growth factor. and VEGF* receptors - VEGF-2 (Flk-1/KDR = type III receptor tyrosine kinase). *Right panel Illustrates the* Pericyte connectivity between two capillary neurovascular units in cerebral cortex of the C57B6 mouse control model layer III. *ACfp = astrocyte foot processes; arrows = tight and adherens junctions; CL = capillary lumen; Pc = pericyte; PcN = pericyte nucleus.*

Importantly, pericytes are contractile cells and contribute to the regulation of capillary cerebral blood flow, hydrostatic balance and maintenance of proper intracapillary pressure and permeability between the microvascular NVU and interstitial tissue   [73, 74, 75, 76, 77,78, 79].   The EC provides the Pc with essential NO and platelet derived growth factor beta (PDGFβ) to allow for prevention of contraction and allows for dilation of the capillary NVU – capillary neurovascular unit maintenance. Also, the EC relies on the Pc to provide VEGF production (essential cellular crosstalk).   While each of these above interactions between pericytes and endothelial cells are extremely important it is beyond the scope of this review to discuss them in their entirety, especially in wound healing and angiogenesis.

Previously, some have considered the Pc to be the "*guardian angel*" or sentinel of the EC microcirculation within the peripheral continuous capillaries (Fig. 14) [61, 80, 81, 82, 83].   This same "guardian angel" analogy may also be applied to the EC of capillary NVU as a functional and structural unit in the brain, since platelet derived growth factor beta receptor (PDGFR-*β*) knockout models in Pcs have demonstrated the abnormal formation of the NVU in adult models with increased permeability due to impaired BBB TJ/AJ development and function and impaired clearance of Aβ [73, 74, 75, 76, 77, 78, 79].   Additionally, it's very important role within the NVU has undergone considerable expansion regarding its role in the effect of the regional neuronal activity of the neurons via the connecting astrocyte to result effectively in dilation in order for NVU coupling and functional hyperemia to support the necessary vasodilating properties of the capillary NVU and the smallest of arterioles [4, 7, 73, 74, 75, 76, 77, 78, 79].

Recently, it has been shared that pericytes (Pc) in the female diabetic *db/db* model undergoes marked remodeling changes including attenuation, retraction and/or complete loss from the cortical layer III NVU. These remodeling changes have also been demonstrated to be protected with empagliflozin treatment for 10 weeks duration from 10 to 20 weeks of age (Fig. 15) [4, 7].
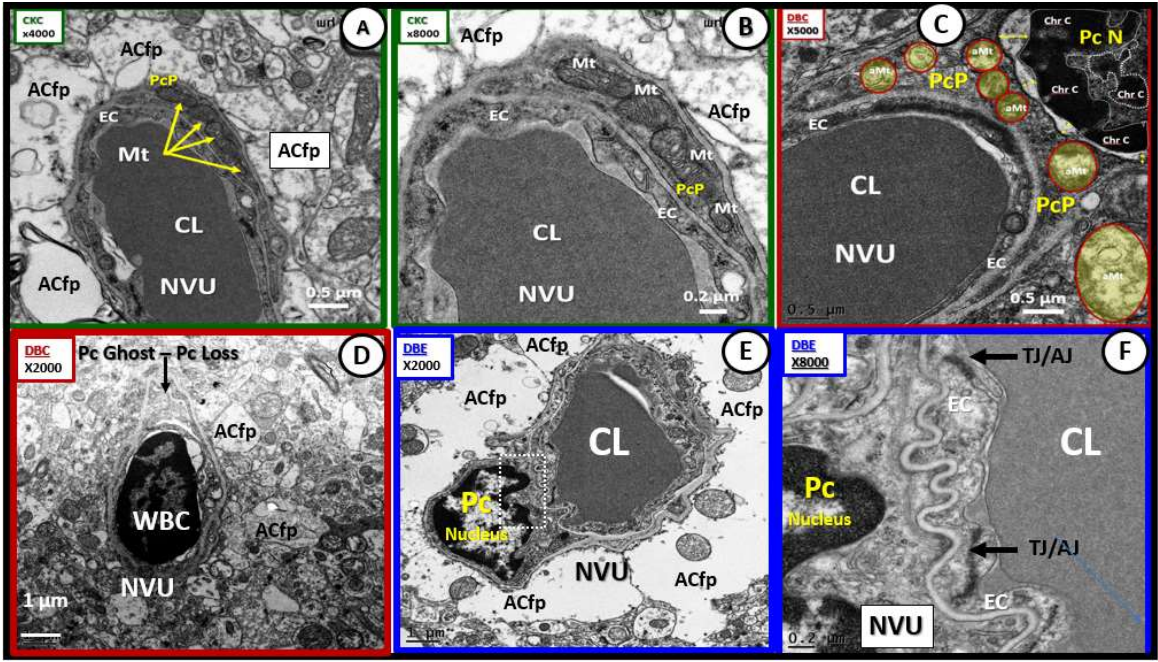
Figure 15. Pericyte injury and loss in diabetic female *db/db* with protection by empagliflozin. Panels A and B demonstrate the normal appearance of pericyte (Pc) and foot processes (Pcfp) in relation to the neurovascular unit (NVU) endothelial cell (EC) in control CKC models. Magnification ×4000; bar = 0.5 μm in A and ×8000; bar = 0.2 μm in B. Panel C illustrates injury – abnormalities (aberrant mitochondria and Pc Nuclear retraction and increased chromatin condensation) in diabetic DBC outlined in red. Magnification x3000; scale bar = 0.5μm. Panel D illustrates an activated endothelial cell with white blood cell adherence (lymphocyte) with loss of pericyte and foot processes in diabetic DBC outlined in red. Magnification ×1000; bar = 0.1 μm ghost cell. Panels E and F depict the protection of the Pc and Pcfp in the empagliflozin treated modes (DBE). Magnification ×2000; bar = 1 μm (panel E) and magnification ×8000; bar = 0.2 μm (panel F). Image available via license CC by 4.0 [4].

3.2.5. Astrocyte (AC) – Diabetic Gliopathy

In the type 2 diabetic db/db model, I have put forth the term "diabetic gliopathy" – impaired glia function and ultrastructure in order to describe the dysfunctional and maladaptive ultrastructural remodeling response due to the injuries imposed upon glia cells (astrocyte 3.4.5., microglia 3.2.6. and oligodendrocyte 3.2.7.) as a result of obesity, insulin resistance-hyperinsulinemia and diabetic hyperglycemia-glucotoxicity of the db/db models [4, 5, 6] along with the multiple intersections between T2DM and LOAD – especially oxidative/nitrosative stress - in previous section 2; Box 2.

ACs are unique cells localized to only the brain and spinal cord tissues and they are the major strategic connecting cell within the brain. The astrocytic end-feet – foot processes (ACfp) of the capillary NVU are tightly adherent to the BM of the EC and Pc cells and play a specialized role for water, amino acid and ionic homeostasis; provide a reservoir for glucose storage via glycogen and are the primary source within the brain for localized antioxidant production of glutathione in addition to communicating with adjacent AC and bidirectionally with regional neurons for functional hyperemia - NVU coupling and cerebral blood flow (Fig. 7, 12, 15, 16**)** [4, 84, 85, 86, 87, 88, 89, 90]. Importantly, AC foot processes (ACfp) connect regional neurons to the mural cells of the NVU and are responsible for signaling the mural cells via glutamate signals, increases in calcium transients (Ca++ flux) from the neurons to the mural cell pericyte to dilate in the presence of increased neuronal activity and create the capillary NVU coupling which results in functional hyperemia and CBF. ACs are also connective in that they form a syncytium amongst other AC, which are in a constant state of sending or receiving information from other like AC cells. While it is a well-known and

672 accepted theory that active neurons increase their nutrient-energy supply and oxygen by dilating
673 nearby arterioles and capillary NVUs to provide for neurovascular coupling or functional hyperemia
674 there does remain somewhat of controversy regarding whether it is the arteriole or capillary that play
675 the major role. It is the opinion of the author that even though the capillary NVU may be the first to
676 dilate that both capillary and arterioles of the brains vascular supply are affected. To this end , Mishra
677 A *et.al.* have recently demonstrated that AC's signal pericytes but not the vascular smooth muscle
678 cells of arterioles [91].
679   It is important to note that in *db/db* models that ACfp of the capillary NVU were detached,
680 separated and retracted (Fig. 8B, 13, 15B) [92]. Importantly, this would result in the loss of the
681 connecting ACs from regional neurons to the capillary NVU and impair functional hyperemia (Fig.
682 20, 21, 22) [4, 7, 92] and CBF. These ultrastructural changes in the preclinical rodent *db/db* models if
683 found to be present in human brain tissues could impair functional hyperemia and result in decreased
684 CBF and contribute to a loss of energy substrate sources and decrease in oxygen resulting in ischemia
685 and premature neuronal dysfunction in synapses and eventually dysfunction with known cognitive
686 impairment and neurodegeneration. This detachment may be due to the increased endothelial,
687 pericyte and AC oxidative stress generating excessive ROS/RNS due to glucotoxicity as previously
688 proposed [4, 7, 91, 92, 93], which was protected with a SGLT2 inhibitor (empagliflozin) (Fig. 16) [7].
689
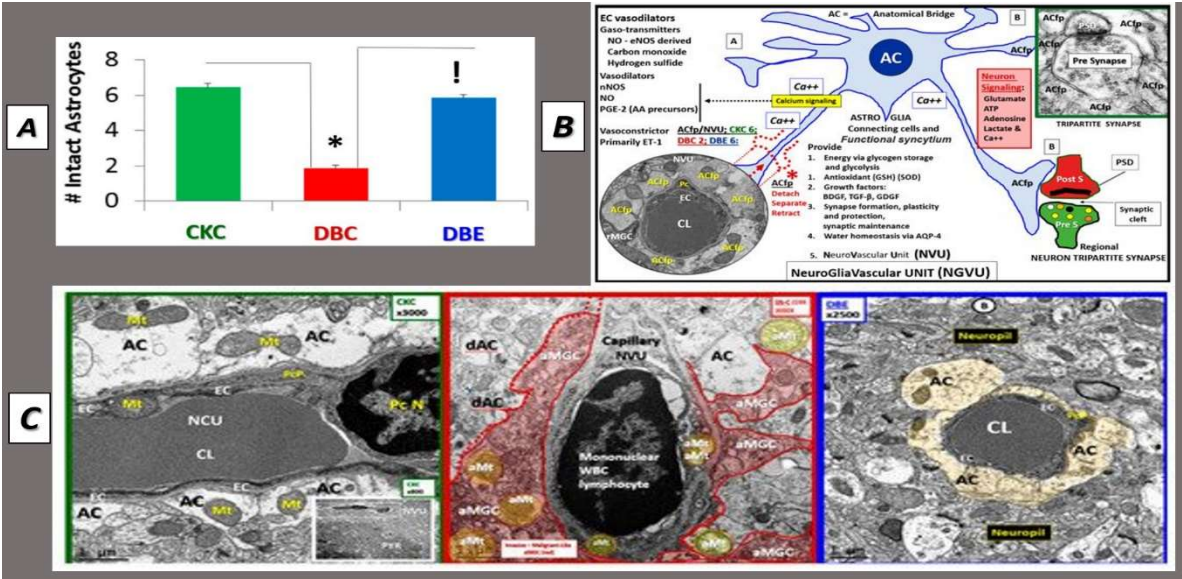
690
691
692 Figure 16. Detached Retracted Astrocytes in db/db Models. Panel *A* illustrates the normal relationship between
693 the NVU astrocytes and neuronal synapses. Panel *B* depicts the reduction in the numbers of intact-attached
694 astrocyte foot processes in the diabetic db/db models. Panel *C* demonstrates the detachment and retraction of
695 astrocyte foot processes (ACfp) (middle panel) and the protection of ACfp loss or detachment in empagliflozin
696 treated models (right-hand panel). CC by 4.0 [92].

697 *3.2.6. Microglial Cell(s) (MGC) - Diabetic Gliopathy*

698   MGCs are currently thought to be yolk sac-derived (mesoderm/mesenchymal), colonize the
699 brain prenatally (embryonic day 10-14 in the mouse and week 4-24 in human brain), the innate first
700 line of resident immune cell defense and thus, the immune guardians or gatekeepers of the brain [94,
701 95]. MGCs may be considered the resident immunocompentent and phagocytic cell of the CNS and
702 are critical to its normal functioning in health and homeostasis. Interestingly, they precede the
703 appearance of the NVU, astrogliogenesis, oligodendrogenesis, neurogenesis, migration, and
704 myelination and are known to be regionally distributed. Ramified MGC being) are constantly at
705 work as a consummate gardener to provide a cleaning – housekeeping function and provide
706 surveillance for waste clean-up from normal wear and tear of the brain's cellular milieu. Their
707 ramified processes are constantly moving about the brain ready to identify and phagocytose any

708 unwanted accumulation of structural byproducts of metabolism in homeostasis or in response to
709 injury mechanism [5, 96].   In focused ion beam/scanning electron microscopic (focused ion beam/
710 scanning electron microscope - FIB/SEM) videos one can even observe the rMGC cytoplasmic
711 extensions as they appear moving about between neurons and the NVUs providing these important
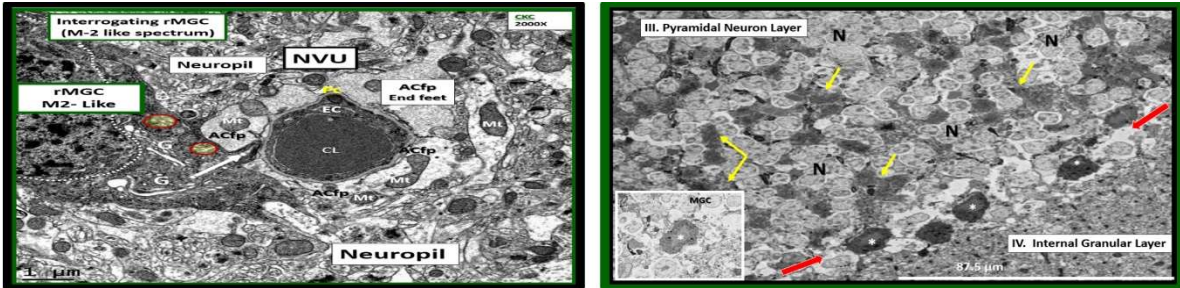712 homeostatic functions (Fig. 17) [5].
713

714
715

716 **Figure 17.** Ramified Microglia Interrogating Neurovascular Unit and Pyramidal Neurons Layer III.
717 Left panel illustrates a ramified microglial cell (rMGC) interrogating the neurovascular unit (NVU) in
718 C57B6 control models (CKC). Note the rMGC protruding its cytoplasmic process and inserting
719 between the astrocyte foot processes (ACfp) (arrow) or end-feet (~ 50-100nm space) that encompass
720 the capillary endothelium (EC) surrounding the capillary lumen (CL). Also note the prominent
721 Golgi system within the rMGC cytoplasm (G).   Even in the rMGC there will be occasional aberrant
722 mitochondria (pseudo-colored yellow with red outline) (Mt).   Magnification ×2000; bar = 1µm. Image
723 available via license CC BY 4.0. [4] CC by 4.0 [4].   Right-hand image depicts rMGCs with electron
724 dense cytoplasmic extensions (yellow arrows) intermixed with pyramidal layer III neurons (N) of the
725 cortical grey matter in control CKC 16-week-old mice.   Note what we term, a train of rMGCs. with
726 remodeled lipid bodies to form lipid cores (asterisks)   in the interstitium between layers III and IV
727 (red arrows).   Inset is an exploded - highlighted image of the MGC with lipid core (asterisk).   Scale
728 bar ~ 87.5 micrometer.   Image information from focused ion beam/scanning electron microscopy
729 (FIB/SEM technology): Voltage: 3 kV; Pixel Size: 350 nm; Field of View: 175 x 175 µm^2 ; Image Size 491
730 x 497;Vacuum: 0.25 Torr [5].

731   MGCs have a large number of membranous/intracellular microglial markers and a large number
732 of signaling molecules, which include numerous microglia, cytokines and chemokines [97].
733 Additionally, they contribute to the regulation of brain development, shaping synaptic connectivity
734 within neuronal networks and are of major importance in brain defense injury [5, 97, 98, 98].
735   MGCs are readily capable of producing large amounts of free radicals (superoxide, reduced
736 nicotinamide adenine dinucleotide phosphate oxidase (NADPH Ox), inducible nitric oxide synthase
737 (iNOS) and mitochondrial-derived ROS and are the major killing - phagocytic cell for infectious
738 processes in the brain. Damage/danger-associated molecular patterns (DAMPs) or pathogen-
739 associated molecular patterns (PAMPs) and MGC remain in an activated state until the
740 DAMPS/PAMPs signal subside. Importantly, microglia are able to return to their surveilling-ramified
741 phenotypes once the danger–damage signals or infectious invaders have been eradicated   and
742 assume their normal cellular debris housekeeping role of rMGCs [5, 94, 95, 96, 97, 98].   While MGCs
743 are essential to homeostasis they may also become injurious to the CNS cells as in the invasive
744 damaging role they play in NVU BBB loss of integrity in diabetic models as a result of their
745 invasiveness that is associated with AC detachment and retraction previously discussed in section
746 3.2.5. [4].
747   rMGCs are genetically programed to constantly be prepared to undergo a rapid diverse
748 phenotypic remodeling functional change to what may be termed activated-amoeboid microglial cell
749 phenotype (aMGC).   These changes may be due to morphological remodeling and/or the
750 expressions of their cell surface receptors in response to danger or damage signals such as PAMPs or
751 DAMPs due to oxidized/glycated proteins/polypeptides, lipids, and nucleic acids from their diabetic
752 hyperglycemic microenvironment.

753   aMGC have been classified by some to be similar to peripheral macrophages, i.e., M1-like
754   (classically activated macrophages) and M2-like (alternatively activated macrophages) cells [99,
755   100].   However, the possibly more preferred method of identification of MGCs relies on individual
756   cell surface markers or their response to inducible cytokines and/or neurotoxins such as LPS
757   (lipopolysaccharide) [101, 102].   Author has chosen to utilize only the terms ramified (rMGC) or
758   activated (aMGC) when referring to the morphofunctional–pathomorphologic phenotypic
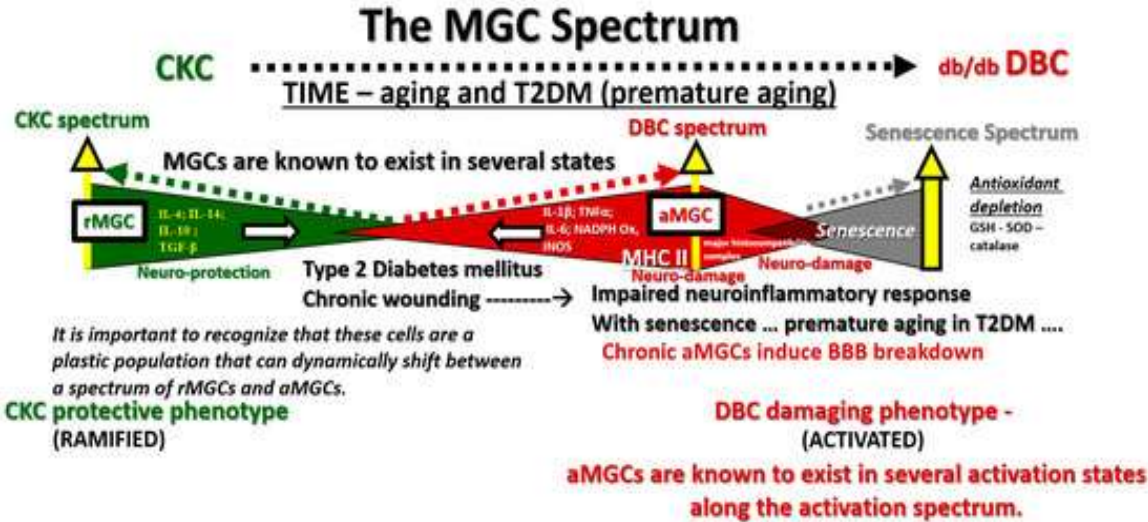759   polarization in TEM observations (Fig. 18) [6].

760

761   **Figure 18.** Microglia spectrum phenotypes in type 2 diabetes mellitus (T2DM). The microglia cell(s)
762   (MGC) are very unique and capable of undergoing a marked diversity of morphological and
763   functional phenotypic remodeling change as they pass along a spectrum of phenotypes from the
764   ramified MGC (rMGC) (green colorization) on the far left-hand side of this cartoon to the chronically
765   activated-amoeboid microglia cells (aMGC) (red colorization) on the right hand in addition to some
766   microglia progressing to a senescent type of MGC on the far right (grey colorization), which may
767   implicate advanced aging. Note the various cytokines profiles associated with each of the rMGC and
768   the aMGC phenotypes. In our age-matched nondiabetic control (CKC) models, the predominate MGC
769   would be rMGC and in the diabetic DBC models the predominant microglia would be aMGC.   Note
770   that in health or homeostasis we demonstrate that the MGC may be in a flux or spectral change
771   between rMGCs and more activated/amoeboid MGCs. The regions between open arrows may even
772   represent a range of homeostasis between rMGCs and versatile aMGCs phenotypes, while the dashed
773   lines suggest multiple spectral morphologic phenotypes. *IL-1β = interleukin 1 beta; IL-14 = interleukin*
774   *14; IL-10 = interleukin 10; TGF-β = Transforming growth factor beta; TNFα= tumor necrosis factor alpha; IL-*
775   *6= interleukin 6; NADPH Ox = reduced nicotinamide adenine dinucleotide phosphate; iNOS = inducible nitric*
776   *oxide synthase; GSH = glutathione; SOD= superoxide dismutase.*   Image available via license CC BY 4.0.
777   [5].

778   Recently, female diabetic *db/db* models were observed to primarily harbor aMGC in cortical layer
779   III in the grey matter in contrast to rMGCs in controls.   Additionally, these aMGCs had a marked
780   increase in aberrant mitochondria (aMt) and their nuclei contained a definite increase in chromatin
781   condensation (Fig. 19) [5].
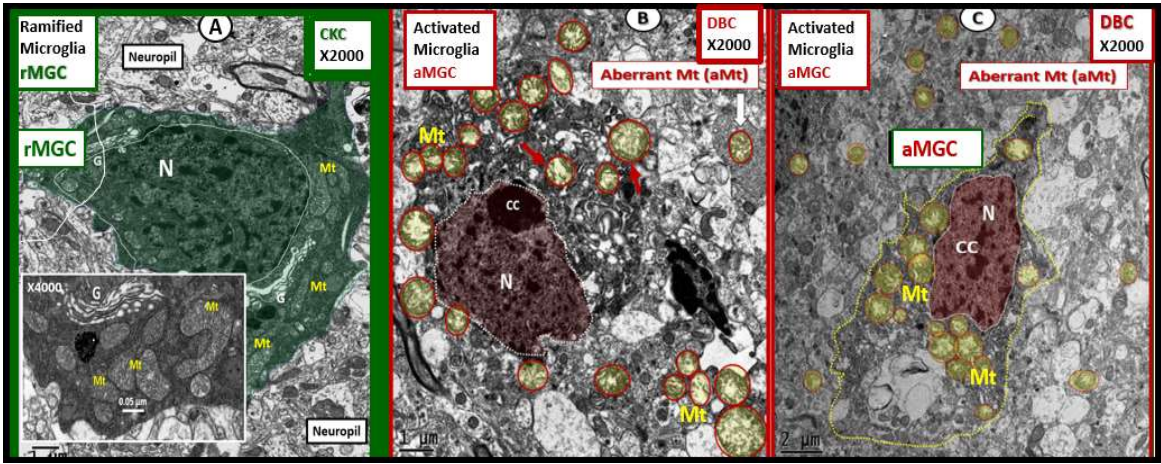
Figure 19. Activated microglia with aberrant mitochondria in diabetic db/db compared to ramified microglia in control non-diabetic models. Panel A illustrates the normal appearing ramified microglia (rMGC) in control CKC. Insert demonstrates the normal electron dense mitochondrial matrix with prominent crista.   In contrast panels B and C depict the activated-amoeboid microglia cell (aMGC) a marked increase in aberrant mitochondria (Mt) and also not the nucleus (N) chromatin condensation (CC) in the diabetic DBC models. *G = Golgi; Mt = mitochondria; N = nucleus. Magnification ×2000; bar = 1 μm. Exploded insert magnification ×4000; bar = 0.05 μm.* Image available via license CC BY 4.0 [5].

In addition, aMGC were   demonstrated to be invasive of the NVU in the diabetic *db/db* models (Fig. 20).
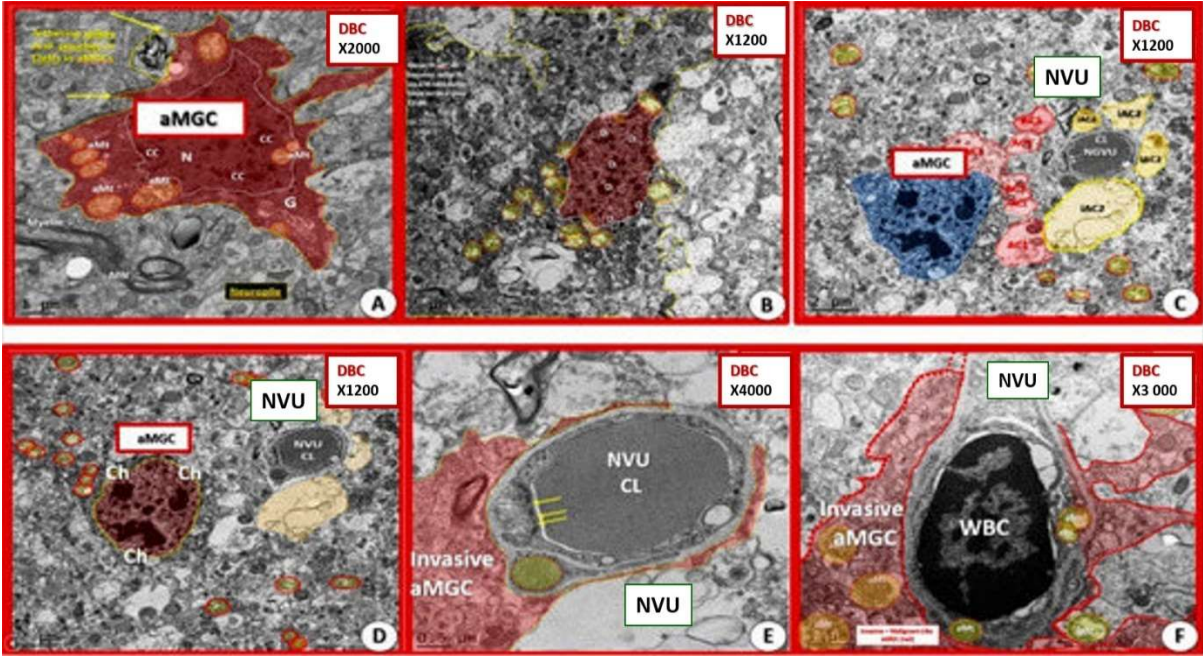
Figure 20. Activated microglia become invasive of   the neurovascular unit in diabetic *db/db* models. Panels A and B portray activated microglia cells (aMGC) in diabetic DBC. Panels C,D,E and F depict the invasion of the neurovascular unit (NVU) by aMGCs in DBC. Note the frown-faced chromatin condensation in Panels C and D. Magnification ×2000 Panel A; 1200 Panel B, C, D; ×4000 panel E; ×3000 panel F with varying scale bars lower left. Image available via license CC BY 4.0 [5].

Importantly, empagliflozin (SGLT2 inhibitor) a glucose lowering treatment for 10 weeks ameliorated these abnormal remodeling changes in MGCs [5].

While the study and knowledge of MGCs is expanding exponentially in regards to neuroinflammation and its relation to T2DM and LOAD, the author would like to direct those with

802   beginning or ongoing interests in MGCs to read the following physiology review by Kettenmann H
803   et al. [103]. Herein, the following nine postulates from the book chapter "Microglia" by Pio del Rio-
804   Hortega in 1932 still hold true to this very time and will be entered in this section (Table 1) [103, 104].

805   **Table 1.** Pio del Rio-Hortega's nine postulates regarding the microglia from 1932.   Please note that
806   number eight is, in compliance with, the recapitulation of embryonic genetic memory upon injury or
807   receiving injury signals such as damage-danger-associated molecular patterns (DAMPs). It is amazing
808   that these nine postulates stand-up to rigorous scrutiny 87 years later.

---

1) Microglia enter the brain during early development.

2) These invading cells have amoeboid morphology and are of mesodermal origin.

3) They use vessels and white matter tracts as guiding structures for migration and enter all brain regions.

4) They transform into a branched, ramified morphological phenotype in the more mature brain

5) In the mature brain, they are found almost evenly dispersed throughout the central nervous system and display little variation.

6) Each cell seems to occupy a defined territory.

7) After a pathological event, these cells undergo a transformation.

8) Transformed cells acquire amoeboid morphology similar to the one observed early in development.

9) These cells have the capacity to migrate, proliferate and phagocytose

---

809

810   *3.2.7. Oligodendrocyte and Myelin - Diabetic Gliopathy*

811   Oligodendrocytes (OLs), oligodendrocyte precursor cells (OPC), and oligodendrocyte lineage
812   cells are specialized glial cells responsible for the synthesis, wrapping ensheathment, and compacting
813   of myelin in myelinated axons [105, 106, 107].   OL derived myelin additionally serves as a protective
814   sheath in myelinated axons in order to provide for long-term axon integrity, maintenance and
815   survival as well as increasing the speed of information transmission from neuron to neuron or
816   regional bundles to distant bundles via white matter tracts as in optic nerves and corpus callosum.
817   These white matter tracts are important for carrying - transferring large amounts of information from
818   one region of the brain to distant regions and must rapidly transmit this information.   This rapid
819   transmission is primarily due to its compacted electron dense myelin sheaths.   As a result, if there is
820   any abnormal remodeling change there may be delays in the arrival of information to the more distant
821   regional neurons with resulting cognitive impairment [108].   Of note, the newer technology of
822   diffusion tensor imaging may lead the way into the future in regards to our better understanding of
823   white matter tract abnormalities in T2DM and LOAD [108].   Certainly, it is already known that
824   prediabetes and T2DM are associated with structural brain abnormalities including lacunar infarcts,
825   white matter hyperintensities (WMHs), cerebral microbleeds (CMBs) and brain atrophy [109].
826   Previous information demonstrating prominent remodeling changes of oligodendrocytes with
827   increased nuclear chromatin condensation and volume and increased numbers of active myelination
828   sites of the cytoplasm in subcortical transition zones beneath the cortical layers I-VI in the diabetic
829   *db/db* models with known impaired cognition [6. 82].   Marked dysmyelination has also been
830   observed in outer myelin lamellae sheath with splitting, separation, and ballooning with aberrant
831   mitochondria in grey matter and similar myelin remodeling changes with marked myelin disarray
832   and additional axonal collapse in transitional zones in DBC as compared to control CKC models [6],
833   which were protected with the glucose lowering effects of empagliflozin (Fig. 21) [6].
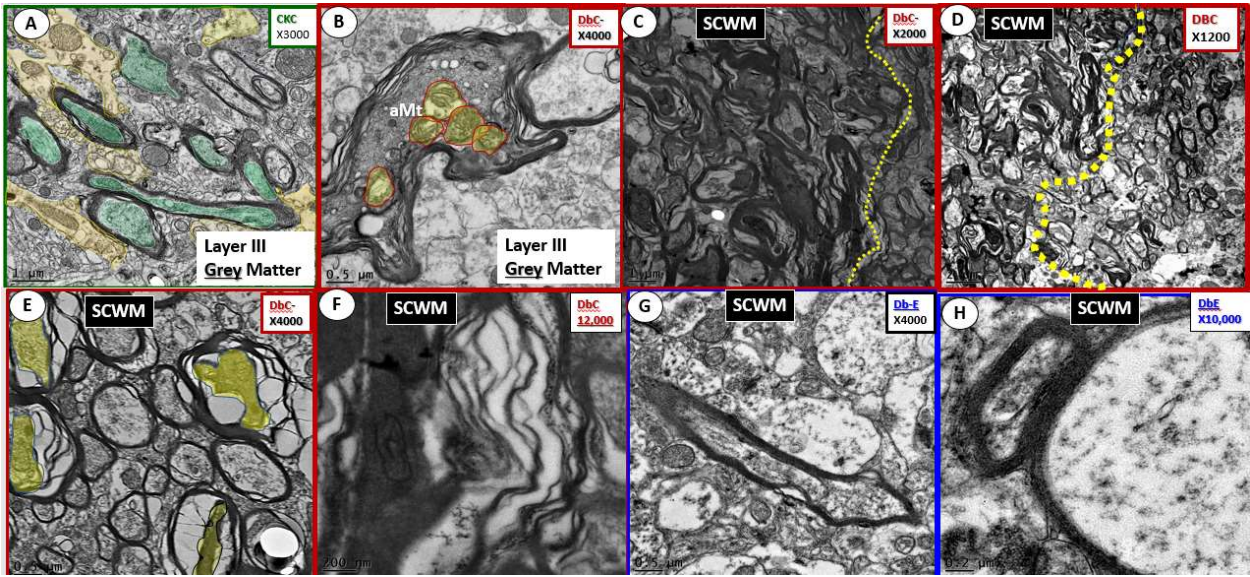
834

Figure 21. Myelin grey and subcortical white matter comparisons. Panel A illustrates normal electron dense myelin ensheathing axons in Layer III of the grey matter in control CKC models. Magnification X3000;bar = 1μm. Panels B-F depict the abnormal myelin remodeling in the diabetic *db/db* (DBC) models with panel B in grey and panels D, E, F, and G in subcortical white matter transitional zone just beneath Layers I-VI in the subcortical white matter at various magnifications with scale bar of 0.5 μm (B);1 μm (C); 2 μm (D); 0.05 μm E; 200 nm (F); 0.5 μm (G); 0.2 μm (H). *aMt =aberrant mitochondria; SCWM = subcortical white matter*. Image available via license CC by 4.0 [6].

Additionally, Desai MK et al. have demonstrated that OL/myelin remodeling changes are an early manifestation of triple transgenic AD mice (3×Tg-AD) as they age and this could possibly also be pertinent to development of   LOAD in humans [110].   This possibility may be even more relevant since Met S, obesity, insulin resistance and T2DM are associated with earlier OL/Myelin remodeling in *db/db* diabetic models [6].   Importantly, Ramos-Rodriguez JJ et al., demonstrated gross observable cortical brain atrophy and decreased brain weights in 26-week-old diabetic db/db mice [111].   Also, previous MRI studies in T2DM humans have been reported wherein, smaller total brain volumes and cortical grey matter and hippocampal atrophy was evident [112, 113].   The ultrastructural images in this section and others throughout this review have been from a 20-week-old diabetic *db/db* models. Even though aging remains the strongest risk factor to develop LOAD and VAD, T2DM remains a risk factor for each of these dementias that indeed may be synergistic via microvascular (NVU) and white matter OL/myelin remodeling with impaired barrier function.

As can be observed in the previous images each of the glia cells (AC, MGC and OL) may be associated with a diabetic gliopathy.

3.2.8. Neurovascular Unit and Barrier functions

Earlier in the section *3.2.* it was mentioned that there may be three or four barriers provided by the capillary NVU depending if you count the EC BM and the PC inner and outer membrane separately or as continuous BM encasing both the EC and Pc and Pc foot processes (Pcfp).   Recently, there has been a newer understanding of brain endothelial cells, NVU and BBB, in that, Kutuzov N et al. have examined the penetration of large (40 and 150kDA dextran) and smaller molecular weight sized (376Da sodium fluorescein and 463Da Alexa flor) hydrophobic tracers from carotid artery into the brain to study passive transport from blood to brain [114].   They were able to elegantly demonstrate that the ecGCx is a significant 1st barrier by utilizing fast scanning two-photon microscopy via a cranial window and further, that the EC cytoplasm and its transport systems and adjacent tight and adherence junctions are the 2nd barrier and that the endothelial cell, Pc and Pcfp

869   BMs and ACs create yet a 3rd barrier due to differences in diffusion partition coefficients barrier
870   properties on the brain side of the NVU.   Furthermore, they suggested that these three sequences of
871   diffusional constraints or barriers (ecGCx, endothelium and extravascular compartment) be termed
872   the "Tripartite BBB" [114]
873   These functional diffusional barrier compartments coincide with the ultrastructural findings in
874   this review with the exception that this review initially considered the endothelial BM as its own
875   separate diffusion barrier because it was remodeled and thus four barriers vs. the tripartite barriers
876   by Kutuzov N et al.   Certainly any remodeling changes or alterations to the enGCx, EC and Pc BMs
877   and ACs as a result of T2DM in previous images would have deleterious consequences to the integrity
878   of the NVU due to a decrease in barrier functions with increased permeability and create an increased
879   cellular vulnerability due to disruptions and loss of integrity to increase the risk and development of
880   LOAD over time and aging.

881   **4. Oxidative Stress: Reactive oxygen/nitrogen species (ROS/RNS) redox stress and aberrant**
882   **mitochondria (aMt)**

883   aMt were found to be markedly increased in aMGC in the diabetic *db/db* models in section *3.2.6.*
884   and were also found to be present in other vascular mural cells (EC and Pc) of the NVU, AC, OL and
885   neurons (myelinated and unmyelinated) in the grey matter cortical regions layer III and the
886   transitional subcortical white matter regions of the diabetic *db/db* brain (Fig. 22) [4, 5, 6, 7].

887
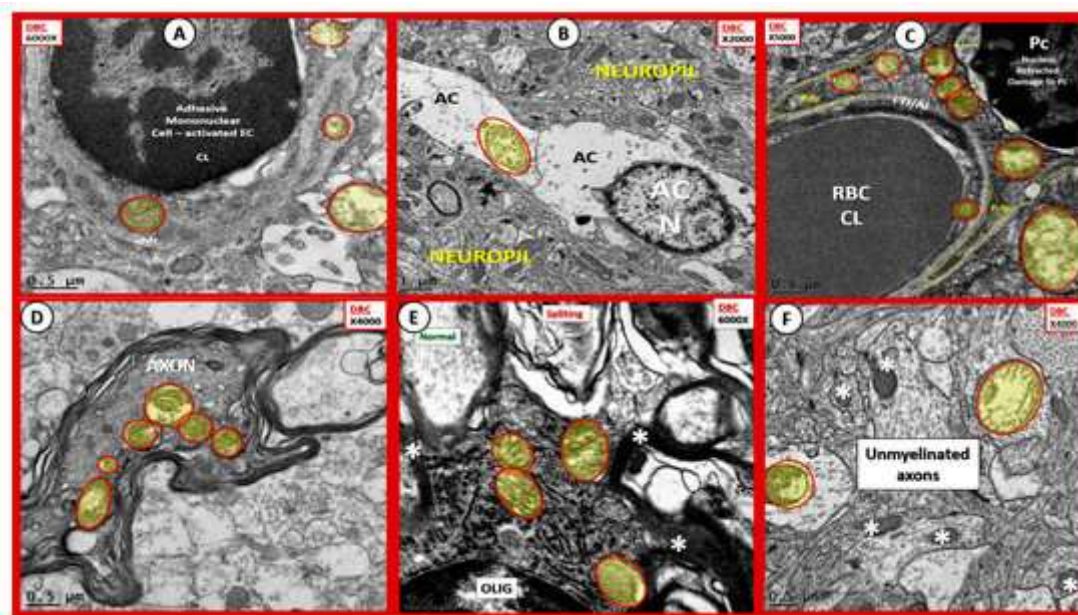
888   **Figure 22.** Aberrant mitochondria in endothelial cells, pericytes and foot processes, astrocytes,
889   oligodendrocytes, myelinated and unmyelinated neurons in the diabetic DBC models. Panels (A–F)
890   demonstrate that aberrant mitochondria (aMt) are found to be present in multiple cells in addition to
891   activated microglia cells (aMGCs). The aMt are pseudo-colored in each of these panels (yellow
892   outlined in red lines) in order to allow rapid recognition. Panels (B,F) are especially important since
893   they demonstrate the aMt characterized by swollen mitochondria (Mt), loss of electron-dense Mt
894   matrix and crista. Panel (A) illustrates the aMt within the endothelial cells and surrounding aMGC.
895   Panel (B) depicts aMt in ACs. Panel (C) demonstrates aMt in pericytes and foot processes (Pc and
896   Pcfp). Panel (D) depicts aMt in a dysmyelinated neuronal axon. Panel (E) depicts aMt in an
897   oligodendrocyte and Panel (F) illustrates aMt in an AC to the left and an unmyelinated axon on the
898   right within the neuropil. Magnifications are noted in the upper part of each panel and scale bars are
899   located at the lower left-hand side of all panels. Scale bars = 0.5 μm in all images except for panel (B)
900   with scale bar = 1μm. Image available via license CC by 4.0 [4, 5, 6].

901   Oxidative Stress (ROS/RNS) seems to be of extreme importance at every turn of events or
902 downward arrow from obesity to T2DM to LOAD in the continuum of progression section 3.1. (Fig.
903 6). ROS beget ROS independently and via previously presented aMGC section 3.2.6. (Fig. 19, 20).
904 The neuroinflammatory aMGCs enzymatic   iNOS, NADPH Ox and Mt-derived excessive ROS due
905 to aberrant mitochondria may interact and fuel other sources of ROS/NOS such as: metabolic excess,
906 hormonal excess, renin angiotensin aldosterone system activation, inflammation systemic or
907 neuroinflammatory, hypoxia-ischemia, ischemia-reperfusion, eNOS uncoupling associated with EC
908 dysfunction as in the MetS section1.2. (Fig. 2).   These accumulating ROS/NOS of oxidative stress
909 may be synergistic in promoting brain injury with subsequent response to injury remodeling changes
910 in structure and functional abnormalities (Box 3).

911
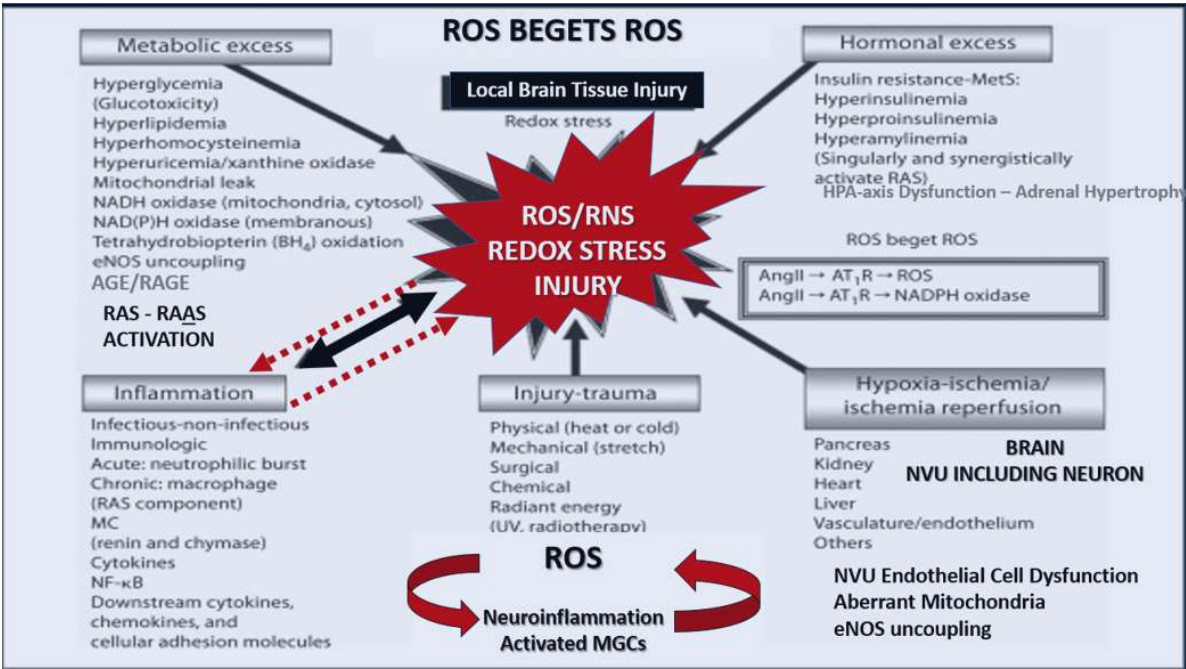
912   **Box 3.** ROS beget ROS. This figure illustrates how the metabolic excesses, hormonal excesses of the H
913   & E phenomenon interact to produce reactive oxygen/nitrogen species (ROS/RNS) and redox stress.
914   Also note how neuroinflammation, brain injury and hypoxia-ischemia and reperfusion may all work
915   individually or synergistically to produce redox stress damage to the brain resulting in accelerated
916   aging and neurodegeneration.   At every downward arrow in the continuum of progression in figure
917   8, ROS/NOS and redox stress are involved in the progression of age-related LOAD and support the
918   oxidative stress hypothesis of LOAD.   From obesity and insulin resistance of the Met S to T2DM to
919   NVU remodeling to LOAD. *AGE/RAGE = advanced glycation end-products/ receptor for advanced glycation*
920   *end-products; Ang II = angiotensin II; AT1R =angiotensin type 1 receptor; eNOS = endothelial nitric oxide*
921   *synthase; HPA = hypothalamic pituitary adrenal; MC = mast cell; MGCs = microglia cells; NADPH –*
922   *NADPH Ox = reduced nicotinamide adenine dinucleotide phosphate oxidase; NVU = neurovascular unit; NF-*
923   *kB = nuclear factor- kappa B; RAS = renin angiotensin system; RAAS = renin angiotensin aldosterone system;*
924   *ROS/RNS = reactive oxygen species/reactive nitrogen species.*

925 *4.1. Oxidative Stress (ROS/NOS): Aberrant Mitochondrial-Derived ROS/NOS Leakage In T2DM End-*
926 *Organ Complications*

927   Previous studies have demonstrated an important role of mitochondria in diabetic end-organ
928 complications (Box 4).

Type 2 DIABETIC COMPLICATIONS – END-ORGAN DISEASE

I. Central and Peripheral Nervous System
- a). Diabetic Cognopathy – Encephalopathy
- b). VAD, MD, LOAD
  cerebral vascular disease,
  stroke (occlusive / hemorrhagic) WMH –microbleeds
  OL/myelin abnormalities
- c). Peripheral Neuropathy

II. Eye
- a). Retinopathy   Blindness
- b). Cataracts

III. Cardiovascular
- a). Endothelial Cell dysfunction – activation
- b). Peripheral Arterial Disease (PAD)
- b). Diabetic Cardiomyopathy
- c). Coronary Artery Disease – myocardial infarction
- d). Accelerated atheroscleropathy – atherosclerosis
- e). Vascular arterial stiffness
- f). Hypertension

IV. Kidney
- a). Nephropathy
- b). Glucosuria
- c). Proteinuria (microalbuminuria)
- d). Renal Failure – end stage renal disease - dialysis

V. Genitourinary
- a). Importance
- b). Decreased libido - hypogonadism
- c). Genitourinary infections (monilial / bacterial)

VI. Skin – Diabetic Dermopathy
- a). Infection
- b). Impaired wound healing

VII.) Oral disease
- a). Caries, gingivitis, periodontal disease, infections

VII. Gastrointestinal
- a). Delayed gastric emptying - gastroparesis
- b). Diarrhea/constipation/dyspepsia
- c). Exocrine insufficiency

VIII. Bone
- a) osteopenia –osteoporosis: falls and fractures
  bone mineral quality bone matrix quality
  even with Bone mineral density is normal
  females > males

VIII. Foot
- a) Diabetic foot
- b) Ulceration – amputation

Box 4. Type 2 diabetes mellitus and end-organ remodeling complications. Aberrant mitochondria (aMt) are a central figure in most, if not all, of the end-organ complications. Diabetic end-organs may all have problems with decreased energy production (ATP) and excessive Mt-derived oxidative stress (ROS) production due to abnormalities in structure as well as function. LOAD = late-onset Alzheimer's disease; MD = mixed dementia; OL = oligodendrocyte; VAD= vascular dementia; WMH =white matter hyperintensities.

In general mitochondria lie at the intersection of many critical cellular pathways involving energy substrate metabolism (ATP generation), calcium homeostasis (acting as a calcium sink), reactive oxygen species (ROS) generation and apoptosis signaling. Furthermore, in healthy cells mitochondria exist as a dynamic reticulum network (in constant fusion and fission) that move around within the cytoplasm assisted primarily by a microtubule actin motor network [115]. Mitochondria are primarily formed within the soma and move down the axon to high energy requiring synapses and if they undergo aberrant mitochondrial (aMt) remodeling changes, they are sent antegrade for repair via (fission and fusion mechanisms) or undergo mitophagy. Thus, if there is impaired autophagy or mitophagy or impairment in microtubule transport due to paired helical fragments and or neurofibrillary tangles even in the early stage of tau formation there may be impaired mitochondrial movement and repair i.e. hyperphosphorylated and aggregated tau appears to damage the axonal transport, leading to abnormal mitochondrial structural defects in synapses [116].

In many of the end-organs that have been previously studied by the author with obesity- genetic or diet induced, insulin resistance, MetS, impaired glucose tolerance or overt T2DM the mitochondria have demonstrated either functional and/or an ultrastructural abnormalities suggesting that oxidative stress may be at the very core of diabetic end-organ complication damage similar to those found in the brain [117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130]. Thus, ultrastructural aMt in peripheral or central CNS tissues may allow one to strongly suspect decreased ATP generation – hypometabolism, excessive aMt-derived ROS production leakiness and oxidative stress. Of interest, is that in patients and genetic models with LOAD and AD respectively experience mitochondrial dysfunction (aMt) that are associated with bioenergetic deficits and oxidative stress that occur early and may promote Aβ and Tau pathologies [131].

Nunomura A et al. have demonstrated that oxidative damage is one of the earliest events and furthermore as Aβ deposition increases and LOAD progresses there is an associated decrease in oxidative damage. They utilized measurements in both neuronal oxidized RNA 8-hydroxyguanosine (8OHG) and the oxidized amino acid nitrotyrosine (3-NT) in LOAD patients. They also demonstrated that the 8OHG immunogold studies labeled the cytoplasmic ribosomal

963 endoplasmic reticulum and not the nucleus regions of the neurons. Authors suggested these
964 findings may be due to an increased antioxidant response to Aβ and tau formation [132].
965 Importantly, if there were chronic ongoing oxidative stress associated with concurrent and/or
966 preexisting ongoing chronic MetS, insulin resistance and T2DM then antioxidant depletion would be
967 more plausible.   Swerdlow RH et al. during a decade (2004-2014) have progressed the concept of the
968 mitochondrial hypothesis such that it has now been included in the evolving hypotheses (Box 5) [133,
969 134, 135, 136].

5. Major Existing And Emerging Hypotheses for LOAD

971 LOAD is an age-dependent, heterogeneous, multifactorial and neurodegenerative disorder
972 functionally characterized early on at its onset by mild cognitive impairment with a progressive
973 cognitive decline progressing to the sixth leading cause of death. Pathologically, LOAD may be
974 characterized by progressive deposition of extracellular amyloid-β (1-42) (Aβ) neuritic plaques
975 derived from amyloid precursor protein (APP), vascular deposition of amyloid-β (1-40) in the
976 arteriole vascular media and paired helical fragments (PHF) and neurofibrillary tangles (NFT)
977 derived from abnormal hyperphosphorylation of tau proteins primarily within the neuron.
978 Aβ is formed after sequential cleavage of APP by the proteolytic enzymes β- and γ-secretases.
979 The γ-secretase cleavage at the C-terminal end of the transmembrane region of APP generates a
980 number of isoforms of 36–43 amino acid residues. The most abundant isoforms are Aβ(1-40), which
981 is found primarily in cerebral amyloid angiopathy and Aβ(1-42) in neuritic plaques.   Also varying
982 lengths of oligomers of Aβ have been found to be the toxic form of Aβ rather than the Aβ neuritic
983 plaques [137].
984 Tau is a neuron microtubule associated protein that is essential for proper function.   The
985 microtubules are associated with the cytoskeletal actin motor unit elements of neurons and are
986 essential for the mobility of neurotransmitter vesicles and mitochondria cellular movement along
987 with other organelles within neurons during their long passage from the soma to the dendritic
988 synapses to provide synaptic transmission from one neuron to another. In LOAD tau becomes
989 hyperphosphorylated and is known to disrupt normal microtubule functions with impaired
990 transport of neuronal cellular organelles to dendritic synapses and if necessary, to transport damaged
991 mitochondria antegrade to the soma regions to undergo macro-autophagy or mitophagy. Tau is
992 thought to become hyperphosphorylated and in this hyperphosphorylated state it becomes
993 polymerized into paired helical filaments (PHF) admixed with straight filaments, which ultimately
994 form neurofibrillary tangles (NFTs) and the second hallmark lesion of misfolded proteins along with
995 Aβ associated with LOAD. [138].   Currently it is thought that Aβ formation and deposition occurs
996 earlier than the PHF and NFT of tau.   Additionally, PHF and NFT of misfolded tau correlate more
997 closely with the clinical progression of the disease in LOAD [137, 138].
998 Over the last century, since Alois Alzheimer made his initial discovery in a younger early onset
999 50-year-old female patient suffering from dementia in 1906, there have emerged numerous
1000 hypotheses regarding the development of LOAD [139].   These numerous major and emerging
1001 hypotheses (Box 5) have developed from many different laboratories and multiple researchers and
1002 will undoubtedly continue to grow and/or change as new information comes forward but for now
1003 these are currently felt to be the best candidate hypotheses, which we have attempted to illustrate
1004 and discuss in the important intersects between T2DM and LOAD in section 2.

Major Existing And Emerging Hypotheses for LOAD

I. **AGING Hypothesis:** Considered the greatest risk factor for the development of LOAD

II. **Cholinergic Hypothesis:** The **earliest hypothesis** and generated our first clinical treatment with anticholinesterase medication.

III. **Aβ cascade Hypothesis:** has been the mainstay hypothesis for at least 2 decades. **A Trigger that may be necessary but not sufficient** (*see IX. Vascular 2 hit hypothesis*).

IV. **Tau Hypothesis:** neuronal loss atrophy. Insulin resistance of importance in relation of dysfunctional glycogen synthase kinase (GSK3β) signaling, which allows for hyperphosphorylation of Tau and misfolding aggregation of tau proteins with malfunctioning of microtubules with disturbance of neurotransmitter carrier and Mt energy source carrying functions.

*** HUGH intersect with obesity, insulin resistance and T2DM – LOAD and

Traumatic brain injury (TBI) via GSK3β intersect

V. **Inflammation Hypothesis:** Reactive gliosis and neuroinflammation are hallmarks of AD. Microglia activation. In response to multiple stressors i.e. oxidative stress – ROS begets inflammation and inflammation begets ROS. Toxic cytokine release ROS. MGC activation thus far in all models examined: **Western Brain**, T1DM streptozotocin models, and especially T2DM i.e. obesity, Insulin resistance and **T2DM db/db** and **BTBR ob/ob** models thus far studied.

VI. **Oxidative Redox Stress Hypothesis**: *HUGE intersect with LOAD and may be the greatest and one of the earliest intersects b/t T2DM and LOAD.

ROS beget ROS aMt beget aMt via Neuroinflammation aMGCs and toxic cytokines.

VII. **Hypometabolism Hypothesis:** Glucose Metabolism **Hypoglycemia** and **Hyperglycemia**. For example, glucose metabolism brain imaging such as ¹⁸FDG-PET (Positron emission tomography with 2-deoxy-2-fluorine-18-fluoro-D-glucose) has become valuable indicator for diagnosis of neurodegenerative diseases that cause dementia, including AD. Dysfunctional Mt are a central and important finding in diabetic brains. Of importance is that BOLD PET scans and studies contribute data that are in addition to measuring the amount of glucose delivered as it also measures the CBF since the vasculature delivers the glucose that is delivered and measured.

VIII. **Genetic Hypothesis:** ApoE-ε4 and various genetic single nucleotide polymorphisms. SNPs and possibly includes maternal mitochondria inheritance by the genetic hypothesis.

IX. **Vascular Hypothesis - 2 Hit Hypothesis** and the **(micro)-vascular cognitive impairment and dementia (VCID) Hypothesis:** These paired hypotheses have merged into accepted hypotheses in the present time not only for vascular dementia (VAD) but also late-onset Alzheimer's Disease (LOAD).

X. *Evolving Hypotheses:*

1. *Mitochondrial Cascade Hypothesis: Rapidly emerging as a sustaining hypothesis.*

2. *Microbiota-gut-brain-immune axis Hypothesis:* Most certainly this hypothesis will be equally accepted (as I-IX) over time and is definitely an exciting story to observe. *Rapidly emerging as a sustaining hypothesis.*

1005

1006 **Box 5.** Major existing and emerging hypotheses for late-onset Alzheimer's disease (LOAD). *AD =*
1007 *Alzheimer's disease; APOE-ε4 = apolipoprotein E epsilon 4; BOLD = blood-oxygen-level-dependent imaging;*
1008 *CBF = cerebral blood flow; LOAD = late-onset Alzheimer's disease; MGC = microglia cell; PET = positron*
1009 *emission tomography; ROS = reactive oxygen species; T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes*
1010 *mellitus; VAD - VaD = vascular dementia.*

1011 *5.1. Endothelial Cell Activation – Dysfunction And Impaired Cerebral Blood Flow (CBF) In Diabetic db/db*
1012 *Models: Hypometabolism (VII) and Vascular (IX) Hypotheses Elaborated*

1013   The brain receives 20% of the cardiac output and also 20% of the body's oxygen and glucose in
1014 order to maintain proper homeostasis. CBF is responsible for the delivery of essential oxygen and
1015 glucose and therefore healthy blood vessels are essential and if interrupted, damage may occur to
1016 cerebral neurons in minutes. This estimated 400-mile trek of capillary NVUs allows the cellular

1017 elements to be perfused and when there is increased neuronal activity this neurovascular coupling
1018 allows for increased CBF to provide the necessary nutritional supply of glucose and oxygen, which
1019 is dependent on intact NVU coupling and functional hyperemia [140].   Thus, if there is any
1020 dysfunction or interruption of CBF to supply the increased neuronal activity the neurons suffer
1021 initially with dysfunction and later neurodegeneration due to oligemia - ischemia.

1022 EC function plays a huge role in maintaining the competence of NVU coupling and CBF in the
1023 brain via functional hyperemia.   EC dysfunction or activation is associated with impaired NO
1024 production via eNOS enzyme uncoupling due to oxidative stress, decreased   tetrahydrobiopterin
1025 (BH4), increased vasoconstrictors such a endothelin-1 (ET-1), angiotensin II (Ang II) and disturbed
1026 regulation of inflammation and thrombosis.   Since NO is known to be an inhibitor of platelet
1027 aggregation and leukocyte adhesion, EC activation has detrimental effects on cerebrovascular - NVU
1028 functions and CBF [141].   EC dysfunction – activation (Fig. 2) was presented in section 2. and is
1029 known to be present as an early abnormality in MetS and T2DM.   EC activation in the NVU due to
1030 impaired nitric oxide generation and eNOS enzyme uncoupling with decreased NO bioavailability
1031 could contribute to NVU uncoupling and decreased CBF [141].

1032 Altered platelet, red and white blood cell rheology is known to be present in T2DM [142].
1033 Adherence of activated platelets, erythrocyte and leukocytes are known to be associated with EC
1034 activation in preclinical models and humans with T2DM [143, 144].   Previous studies in the diabetic
1035 female T2DM *db/db* models have demonstrated major remodeling changes to the EC as a result of EC
1036 activation in the NVU, which consisted of platelets, red blood cells (RBCs), leukocyte adherence in
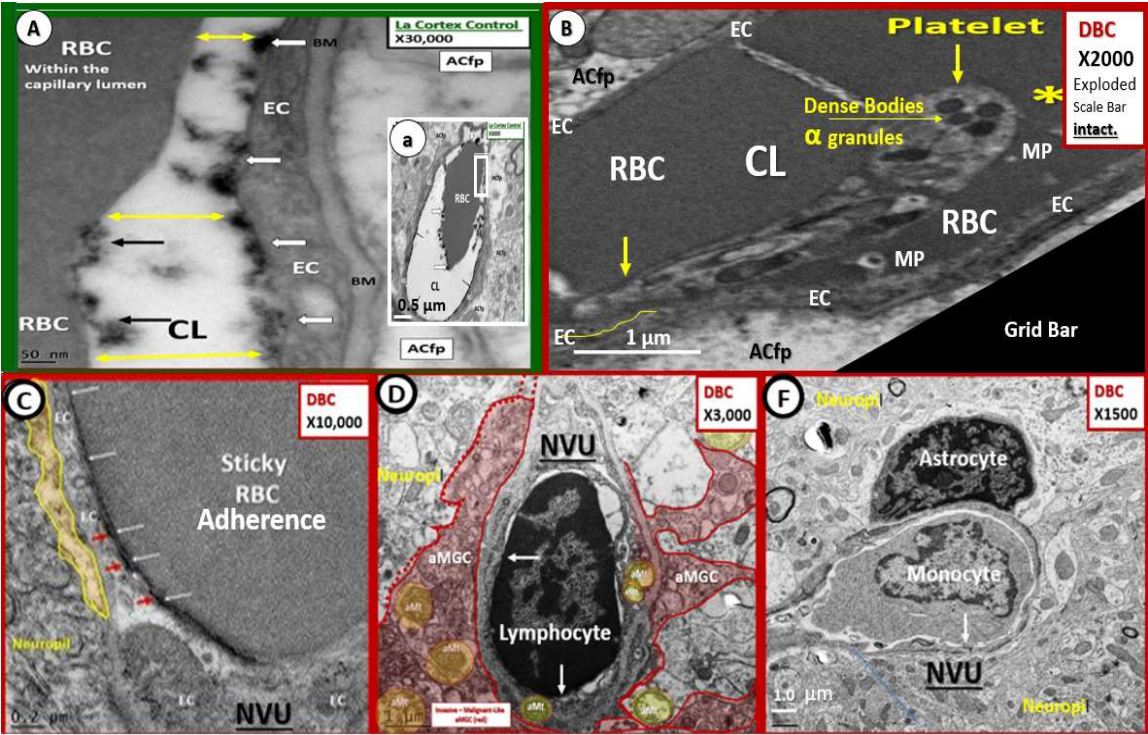1037 addition to the well-known fibrin network dysfunctional abnormalities (Fig. 23).

1038

1039 **Figure 23.** Adherent red blood cells, leukocytes and activated platelets to activated endothelial cells
1040 in *db/db* Models.   Panel A depicts an intact endothelial glycocalyx (ecGCx) on both the intraluminal
1041 red blood cell (RBC) and the luminal surface of the endothelial cell (EC) with lanthanum nitrate
1042 staining (arrows) with magnification x30,000; scale bar = 50nm in a non-diabetic control C57B6 mouse
1043 model. Insert (a) demonstrates this same capillary neurovascular unit (NVU) with intraluminal RBC
1044 with lower magnification x3000; scale bar = 0.5 μm to depict the entire capillary NVU. Note that the
1045 EC and RBC are each decorated by an extremely electron dense protective surface coating of an ecGCx
1046 (arrows) that are separated and not adherent in control models with pre-sacrifice lanthanum nitrate
1047 infusion that has been found to be lost or shed in diabetic models (such as in the db/db and ob/ob
1048 models - not shown). Panel B depicts an adherent activated platelet (arrows and yellow line lower
1049 left) to the EC and note the elongation of this platelet suggesting activation in addition to its adhesion

1050   to the capillary NVU endothelial cell. Magnification x2000 enlarged in paint program with intact scale
1051   bar = 1μm. Panel C illustrates adhesion of a RBC to the EC in the *db/db* diabetic DBC model with an
1052   electron dense adhesion plaque (electron dense (arrows) of the NVU. Magnification x10,000; scale bar
1053   = 0.2 μm. Panels D and F depict the tight adherence of leukocytes to the endothelium (adherence
1054   points depicted with arrows) in the diabetic *db/db* DBC models.   A lymphocyte in panel D and a
1055   monocyte in panel F. This tight adherence of RBC and leukocytes and activated platelet are
1056   representative of an activated endothelium in the diabetic DBC models. Various magnifications
1057   utilized to demonstrate the adherence. Magnification ×3000; bar = 1 μm (panel D); magnification
1058   ×1500; bar = 1.0 μm (panel F).   Panels C and D available via CC by 4.0. [4].

1059   The adhesion of RBCs, leukocytes and activated platelet to the NVU activated endothelium
1060   observed in the obese insulin resistant diabetic *db/db* DBC models as observed in figure 33 would
1061   definitely result in increased inflammation and oxidative stress but equally important would be their
1062   physical obstruction to CBF and decreased distal perfusion and resulting ischemia to neurons due to
1063   a stalling of CBF.
1064   Just as there were adherent and stalled neutrophils in the study by Cruz Hernández JC et al. the
1065   *db/db* DBC diabetic mice have demonstrated adherent stalled RBCs, leukocytes and platelets.   So in
1066   addition to the impaired CBF that may be associated with AC retraction and loss of neurovascular
1067   coupling and impaired vascular hyperemia there may also be stalled RBCs and leukocytes due to
1068   adhesion to an activated and dysfunctional endothelium with adherent activated platelets.   As a
1069   result of our findings regarding the AC detachment and adhesion of RBCs and leukocytes in the
1070   diabetic *db/db* models we can now demonstrate at least two morphological ultrastructural changes in
1071   the *db/db* that could interfere with CBF that appear to be protected with empagliflozin [7].   This type
1072   of oligemia and decreased CBF in the *db/db* diabetic mouse model would certainly accelerate the
1073   remodeling changes and contribute to the increased risk that would be associated with a pre-existing
1074   T2DM on the age-related development of LOAD.   Additionally, impaired CBF as measured by
1075   cerebral glucose metabolic rate (CMRglu) by fludeoxyglucose F 18-positron emission tomography
1076   (FDG-PET) are known to be associated with LOAD risk and even observed prior to the onset of
1077   dementia [145, 146,147, 148].   Thus, brain hypoperfusion and impaired CBF may participate in the
1078   pathogenesis/pathophysiology of neurodegenerative diseases such as in LOAD with underlying
1079   T2DM.
1080   A brief synopsis of some of our findings certainly tie into the preceding paragraphs in that, the
1081   five major conditions – categories as listed in the abstract consisted of *i*. aging; *ii*. metabolic
1082   (hyperglycemia and advanced glycation end-product and receptor AGE/RAGE interaction resulting
1083   in oxidative stress and inflammation in addition to insulin resistance as a linchpin between T2DM
1084   and LOAD; *iii*. oxidative stress; *iv*. inflammation; *v*. vascular (extra and intracranial macrovascular,
1085   vascular stiffening and NVU microvascular disease)   all come into play in discussing the increased
1086   risk of LOAD with underlying T2DM and the phenomenon of impaired CBF.   Additionally, in the
1087   *db/db* (DBC) diabetic model we have learned that there is microvascular disease of the NVU with EC
1088   activation with RBC and WBC adherence resulting in   decreased CBF due to cellular adhesion
1089   resulting in stalling of CBF.   Additionally, attenuation and/or loss of the BBB TJ/AJ, thickening of the
1090   BM, detachment of the AC with impairment of functional hyperemia and neurovascular uncoupling
1091   between active neurons and dilation of the NVU in T2DM could markedly increase the vulnerability
1092   of these cells as age-related LOAD begins to develop and   would contribute to the brain atrophy
1093   noted by Ando Y [67].

6. Neuropathological changes in LOAD

1095   The neuropathologic substrate of LOAD consists of extracellular neuritic plaques (Aβ 1-42) and
1096   CAA (Aβ1-40) and intracellular-intraneuronal hyperphosphorylated misfolded microtubule-
1097   associated tau proteins [149].   Despite all of the newer sophisticated diagnostic procedures and tests,
1098   autopsy and neuropathological examination still remains the 'gold standard' for the definitive
1099   diagnosis of LOAD.   The only visible gross finding in LOAD is cortical atrophy and enlargement of
1100   the lateral ventricles; however, this is not specific for LOAD and could include many other

neurodegenerative diseases, but this finding certainly raises a red flag to the neuropathologist. Numerous regions within the brain are affected by the neuritic plaques and neurofibrillary tau tangles; however the entorhinal cortex, the CA1 and subicular regions of the hippocampal regions are in particular heavily involved along with other regions such as the amygdala, and the deeper layers (layers III, V, and superficial VI) of the neocortical regions of the brain.   The extent and distribution of neurofibrillary tangles in cases of LOAD correlate with both the degree of dementia and the duration of illness as well as staging [150] and have been thought of as one of the cardinal histopathological lesions along with neuritic plaques of LOAD.   There has also been demonstrated to be a synaptic loss of neurons in LOAD (up to 45%) and this finding is important to the neuron to neuron communication and synaptic loss is felt to contribute to the cognitive impairment found in these individuals along with cerebral atrophy.   Even though LOAD is the sixth leading cause of death, it is often not the direct cause of death and many of these patients may die from other associated diseases such as sepsis, pneumonia, traumatic falls with injury and cardiovascular diseases including stroke due to the older age of these patients with multiple comorbidities and frailties.   The most commonly associated condition with LOAD is stroke and ischemic infarction. Additionally, another co-morbid condition, Parkinson's disease with or without Lewy bodies, is often associated with clinical diagnosis of LOAD at the time of neuropathologic study.   While the above may be somewhat of a superficial   sketch of the neuropathological findings, it does add to the overall knowledge and background of this important topic [149].   These previous observations speak out strongly for the importance of mixed dementias as discussed in section 2. (Fig. 5).

7. Discussion and Concluding Remarks

T2DM is known to be associated with macro and microvascular complications in the peripheral vasculature such as in accelerated atherosclerosis, peripheral neuropathies, retinopathies, nephropathies and cardiomyopathies for many years.   In contrast the central brain has been studied as an end-organ for a much briefer time period.   We are now gaining knowledge rapidly and still learning on a regular basis regarding the various mechanisms of functional and structural change that occur in the brain end-organ of T2DM.   Although, the mechanisms implicated have not been completely elucidated it is now known   that cognitive impairment, VaD, stroke, anxiety/depression and LOAD have been related to T2DM.   This knowledge, plus the role of the macro and microvessels being the provider of essential oxygen and nutrients and to dispose of or efflux metabolic byproducts of metabolism are all important.   Additionally, astrocytes and their very unique ultrastructure and functions of being connected and signaled by high activity of regional neurons providing neurovascular coupling and functional hyperemia to increase cerebral blood flow in times of need are essential for proper brain perfusion.   These various cells and functional substructures such as the NVU with their readily identifiable ultrastructure has directed this review to heavily focus on both its structure and its function in order to autoregulate its vascular supply.   Prior to this time the BBB along with its necessary TJ/AJ held the role of protecting the neurons but now we understand that there is more to this highly dynamic structure as we study the NVU with the many cells it employs in order to undergo a great deal of crosstalk between one another.   Once this important NVU structure is injured and remodeled by T2DM, the neurons may be deficient of proper function and allow dysfunctional remodeling change with synaptic dysfunction, loss and eventually neuronal dysfunction and loss with neurodegeneration and atrophy.

LOAD and VaD are the most frequent contributors to cognitive dysfunction and dementia. Further, they frequently and commonly co-occur as a mixed dementia (MD) in autopsy studies.   The risk for stroke or dementia is great for both sexes, i.e. at age 65, one out of four men and one out of three women will have an increased risk of developing stroke, dementia or both [151]. Additionally, when the first baby boom generation began turning 65 years of age at a rate of approximately 10,000 per day in 2011, they were estimated to be just under 77 million strong in the United States population [https://www.census.gov/prod/2014pubs/p25-1141.pdf] *accessed July 10, 2019.*

1151   T2DM and LOAD are two independent chronic age-related diseases that are each associated
1152   with high morbidity and mortality in our current aging global societies.   Since both are age-related
1153   and chronic diseases, it is not unusual for them to co-occur along with cerebro-cardiovascular
1154   disease and LOAD.   Both have multifactorial causations and risk, in which, there are at least 18
1155   intersects (Box 2) with insulin resistance, deficiency and/or insensitivity emerging as a linchpin
1156   between the two disparate diseases.   In fact, as these two diseases merge in our aging society they
1157   may form a troublesome excess or a "bottleneck" of senior citizen's over the next two decades due to
1158   the world-wide aging societies.   This conundrum will undoubtedly create   a strain on our health
1159   care system, a financial burden to our society and much stress to individual caregivers and families.
1160   A great deal still remains unknown; however, a wonderful discussion regarding what is and what is
1161   not known regarding brain insulin resistance can be found and is strongly suggested [152, 153. 154].

1162   As previously discussed in section 1.2.1 peripheral insulin resistance is a definite core feature of
1163   T2DM that is rapidly emerging as a core feature of LOAD as brain insulin resistance [155].   BIR may
1164   be defined as the failure of brain cells to respond to insulin [154, 155].

1165   In the near future we will come to better understand BIR, since intranasal insulin is to be studied
1166   in human clinical trials [156].   Already, it has been determined that intranasal insulin has been able
1167   to not only improve multiple systems that are known to be involved with LOAD like impaired
1168   glucose uptake but also improve memory, focus and activities of daily living in multiple studies
1169   without causing hypoinsulinemia [157].   Six intranasal insulin treatment trials have been rigorously
1170   reviewed in regards to MCI and LOAD by Avgerinos KI et al. [158, 159, 160, 161,162, 163, 164].

1171   The evidence continues to grow stronger that there is impaired insulin signaling (brain insulin
1172   resistance) in the brains of those human individuals with LOAD.   Additionally the combination of
1173   T2DM and LOAD suggest that the two disparate diseases may be synergistic when they co-occur and
1174   demonstrated the greatest decrease in insulin signaling proteins [164].   Recently, the brain insulin-
1175   PI3K-AKT signaling pathway from autopsied frontal cortices (nine with LOAD, 10 with T2DM, eight
1176   with T2DM and LOAD were compared to seven control human autopsies) and found that the greatest
1177   PI3K–AKT signaling impairment was more severe in individuals with both T2DM and LOAD [164].
1178   Further, this group found in T2DM and LOAD patients there was a decrease in insulin receptors, IRS-
1179   1 total, PI3K(85) total - phosphorylated (p)PI3K(p85), PDK1total - (p)PDK1, AKT total - (p)AKT,
1180   GSK-3βtotal - (p)GSK-3β (allowing hyperphosphorylation of   tau and PHF and NFT) and increased
1181   Calpain-1, which could allow for increased degradation of these decreased insulin signaling proteins
1182   [164].   Also, this group found that there were decreases in glucose transporters, namely GLUT1
1183   (endothelial GLUT) and GLUT3 (neuronal GLUT) as well as decreased HIF-1 and O-GlcNAcylation,
1184   which correlated with increased hyperphosphorylation of tau in short time post-mortem AD brains
1185   (<3-hour average postmortem delay).   These findings strongly support the notion that GLUT1 and
1186   GLUT3 deficiencies could cause impaired brain glucose uptake and/or metabolism, which could
1187   contribute to neurodegeneration in correlation to a decrease in O-glcNAcylation and
1188   hyperphosphorylation of tau in AD [165].

1189   The following two additional emerging hypotheses may be 'game changers' in regard to both
1190   the novel treatments of LOAD by repurposing insulin and insulin sensitizing medication and aiding
1191   in the understanding of the development and/or the continuum of progression in LOAD.

7.1. Metabolic Hypothesis: Hyperglycemia and Hyperinsulinemia

1193   Hyperglycemia - glucotoxicity and hyperinsulinemia of T2DM and MetS with peripheral insulin
1194   resistance (PIR) and central – brain insulin resistance (BIR) may be extremely injurious   to brain
1195   metabolic functioning and brain end-organ remodeling associated with the increased risk in the
1196   development of LOAD (Box 1), section 1.2.1 [166].

1197   The importance of this evolving hypothesis allows one to consider the possible impact of
1198   excessive amounts of amyloidogenic amylin (islet amyloid polypeptide – IAPP) – hyperamylinemia
1199   to be deposited in the brain's perivascular and extracellular matrix.   Amylin is co-synthesized, co-
1200   packaged and co-secreted from the islet pancreatic beta cell's insulin secretory granule due to
1201   peripheral insulin resistance (PIR) [167, 168].   Amyloidogenic amylin may be deposited as amyloid

1202   amylin (Aα) and eventually function as a possible niche region in the brain for cross-seeding with Aβ
1203   and allow Aβ and Aα to undergo mixed perivascular and extracellular co-deposition in addition to
1204   Aα independent deposition in the perivascular regions and extracellular matrix, which may possibly
1205   interfere with Aβ efflux or clearance [169, 170, 171, 172, 173, 174].   The amylin hypothesis involving
1206   the amyloidogenic Aα might also lead to the use of an amylin analog (pramlintide) for clinical study
1207   that has already been approved and utilized to treat type 1 and T2DM and known to improve glucose
1208   levels [175].   Incidentally, pramlintide has been reported to improve the feeling of "well-being" by
1209   those having used it in the original treatment trials (authors personal communication in 2004) [176].
1210   Additionally, fibrinogen is one of the biomarkers of inflammation and an important risk factor
1211   for many cardiovascular and cerebrovascular diseases including T2DM and LOAD [177].   Misfolded
1212   proteins and their deposition are also important to the development of LOAD.   Additionally, this
1213   concept of misfolded proteins allows for the fifth misfolded protein in LOAD that being amyloid
1214   fibrinogen-fibrin (AFib) due to activated platelets and its membrane amyloid precursor protein,
1215   which contribute to Aβ deposition and known to be associated with MetS and T2DM [178].
1216   Currently, there are at least five known and distinct misfolded proteins consisting of Aβ(1-40), Aβ(1-
1217   42), tau (NFT), amyloid amylin (Aα) and now AFib that contributes to Aβ(1-42) via the activated
1218   platelets, which increase the amyloid precursor protein and AFib.   In the future, there may be even
1219   more misfolded proteins found to be involved in the development of LOAD.

1220   *7.2. The Antimicrobial Protection Hypothesis: Antimicrobial peptide(s) (AMP)*

1221   Aβ is known to be an antimicrobial peptide [179].   Previous infections such as *Human*
1222   *alphaherpesvirus 1* (HSV-1 - viral), *Chlamydia pneumoniae* (bacterial), *Borrelia burgdorferi or recurrentis*
1223   *(spirochete)* plus other pathogens or the ongoing chronic sterile inflammatory process (activated
1224   MGCs) have all been implicated in activating AMPs [178 179].   Interestingly, this infectious
1225   hypothesis or antimicrobial protection hypothesis is somewhat reminiscent of the *Helicobacter pylori*
1226   (gastritis and duodenal ulcer story) and certainly an emerging hypothesis to follow closely, while
1227   keeping in mind the potential of antiviral and antibacterial drug   repurposing [180, 181].   One of
1228   the antibiotics that the author is interested in following is minocycline, because it is known to pass
1229   the BBB and is also known to be a matrix metalloproteinase inhibitor [182].
1230   The ongoing chronic sterile inflammatory process (activated MGCs) as occurs in LOAD and
1231   T2DM may result in excessive AMP-APP production of Aβ(1-42) due to activation of excessive APPs.
1232   The chronic sterile inflammatory process may be perceived as a pathogenic-like threat, which results
1233   in increased APP synthesis and cleavage to oligomeric forms of Aβ(1-42) to form amyloid fibrils in
1234   order to entrap pathogens and serve as an innate immune pathway as a response to a perceived active
1235   infection or a sterile inflammatory response.   This process could act in a feed-forward viscous cycle
1236   to result in ongoing Aβ(1-42) deposition of extracellular neuritic plaques.   This emerging
1237   antimicrobial protection hypothesis does not interfere with the existing Aβ cascade hypothesis in Box
1238   5 (III), Section V; however, it does add a novel dimension to this exiting hypothesis and may suggest
1239   that an innate immune-mediated inflammation could propagate LOAD Aβ(1-42) deposition and
1240   ongoing neurodegeneration [183, 184, 185].   Additionally, this could allow for the consideration of
1241   antibacterial, anti-spirochetal and antiviral treatments if an underlying source of infective agent could
1242   be identified via past medical history of previous infections or current diagnostic findings.
1243   Historically, the NVU concept emerged and was formalized at the 2001 Stroke Progress Review
1244   Group meeting of the National Institute of Neurological Disorders and Stroke and currently the NVU
1245   has definitely come of age [186]. It is the author's current hypothesis that the overarching concept
1246   and beauty of the intact NVU is the dynamic interplay or coupling between neural activity and
1247   cerebral blood flow and functional hyperemia from which, functional magnetic resonance
1248   imaging measures **t**o blood oxygenation level dependent (BOLD) images evaluate human
1249   individuals clinically and rodent preclinical models.   These images rely on regional differences in
1250   cerebral blood flow to delineate regional activity. NVU function may now be considered a
1251   multidimensional process involving signals from multiple cells that engage in distinct highly
1252   orchestrated signaling pathways and effector systems across the cerebrovascular capillary mesh-like

1253   network of NVUs in a symphonic-like process.   In addition, the NVU with its BBB – blood-brain
1254   interface performs as both an endocrine target and an endocrine secretory tissue and therefore,
1255   should also be viewed as an endocrine-like organ between the CNS and peripheral tissues, since it is
1256   capable of autocrine, paracrine and endocrine-like functions [187].   Furthermore, the NVUs tripartite
1257   BBB functions along with its interface functions, which incorporates signaling to its own supporting
1258   cellular network via crosstalk with Pcs, ACs, MGCs, OLs and even neurons provide a vital link to
1259   CNS homeostasis.
1260   The multiple weights of multiple cellular injuries in T2DM and their intersects along with the
1261   emerging hypotheses of the *metabolic hypothesis and antimicrobial protection hypothesis* may increase the
1262   cellular vulnerability and tip the balance for the brain to develop an increased risk of LOAD (Fig. 24).
1263   Further, there may also be novel findings, as in the preclinical *db/db* models, waiting to be gleaned
1264   from studying the ultrastructure of short timed post-mortem or biopsies of human brain specimens
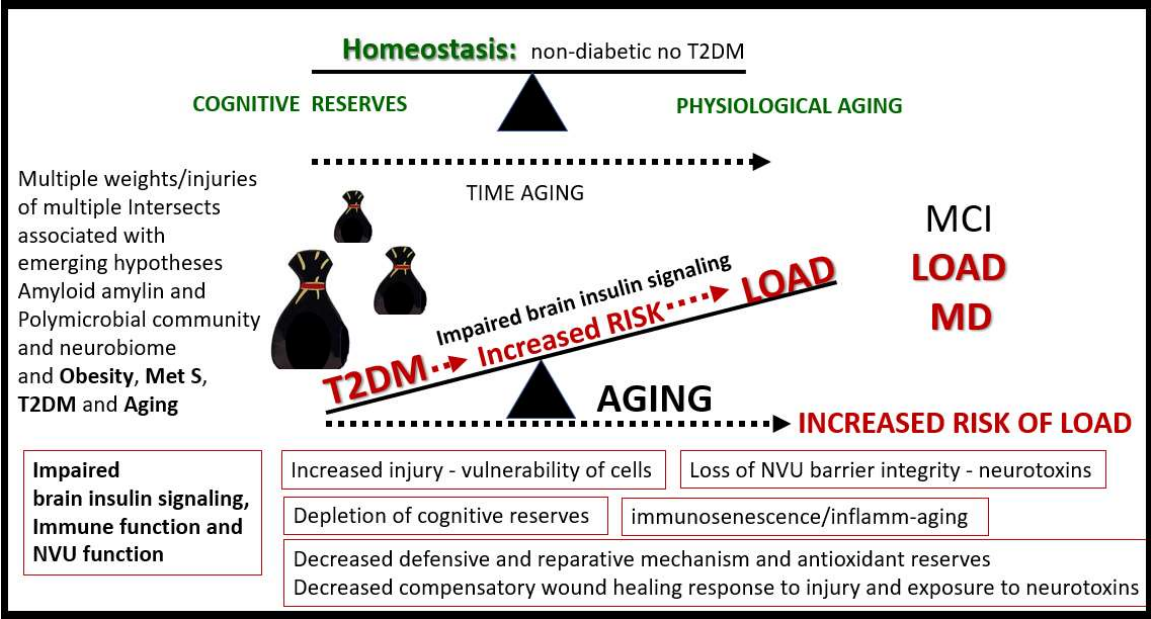1265   [188] from T2DM, LOAD and LOAD +T2DM when compared to one another.

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Figure 24. T2DM tips the balance for the development of LOAD.   The multiple weights of injuries,
1268   metabolic insults, impaired brain insulin signaling, immune function, NVU dysfunction, intersects,
1269   ongoing chronic wounding of obesity, MetS, T2DM, aging and the increased burdensome weight of
1270   wounding injuries (multifactorial insults and injuries) set in motion the multiple wound healing
1271   responses to injury mechanisms with resulting increases in cellular vulnerability along with impaired
1272   signaling mechanisms for the development and increased risk of age-related LOAD. *LOAD = late-
1273   onset Alzheimer's disease; MCI = mild cognitive impairment; MD = mixed dementia; MetS = metabolic
1274   syndrome; NVU = neurovascular unit; T2DM = type 2 diabetes mellitus.*

1276   Throughout this review multiple efforts have been made to comprehensively utilize supportive
1277   ultrastructural TEM images from the cortical grey matter capillary NVUs of layer III and its
1278   constitutive cells from the obese, insulin resistant, diabetic *db/db* preclinical mouse model.
1279   For most of the adult life, the brain neurons do not experience exposure to peripheral
1280   neurotoxins in health; however, the aged brain and especially those individuals who develop T2DM
1281   in mid-life and older age groups experience exposure to peripheral neurotoxins as a result of the
1282   NVUs barrier loss of integrity.   Importantly, brain neurons are not properly equipped to respond to
1283   this increase in exposure to peripherally derived neurotoxins and therefore remain dependent on an
1284   intact NVU with barrier integrity for proper homeostasis and function.   Therefore, the
1285   ultrastructural cellular remodeling changes as shared in this *db/db* diabetic model along with the
1286   associated functional changes demonstrate the important role of how structure and function are

1287 tightly related to the importance of obesity, aging, intersects and the continuum of progression in
1288 regards to the presence of T2DM and the increased risk of LOAD.

1296 **Conflicts of Interest:** The author declares no conflict of interest.

1297 **Abbreviations**

1298 Aα = Amyloid alpha

1299 AC = astrocyte

1300 Aβ = amyloid beta

1301 ACfp = astrocyte foot processes

1302 AD = Alzheimer's disease

1303 AFib = amyloid fibrinogen/fibrin

1304 AGE = advanced glycation end-products

1305 AMP = antimicrobial peptide(s)

1306 aMt = aberrant mitochondria

1307 APP = amyloid precursor protein

1308 aMGC = activated microglia cell

1309 BB = baby boom generation following WWII

1310 Boomer = baby boom generation following WWII

1311 BBB = blood-brain barrier

1312 BIR = brain insulin resistance

1313 BM = basement membrane

1314 CAA = cerebral amyloid angiopathy

1315 CBF = cerebral blood flow

1316 CKC = C57B6-J control models

1317 CSF = cerebral spinal fluid

1318 CVD = cerebro-cardiovascular disease

1319 DBC = diabetic *db/db* models

1320 DC = diabetic cognopathy

1321 DIO = diet induced obesity

1322 EC = endothelial cell

1323 ecGCx = endothelial cell glycocalyx

1324 eNOS = endothelial nitric oxide synthase

1325 FFA = free fatty acids

1326 HPA = Hypothalamic-Pituitary-Adrenal (HPA) axis

1327 H and E = hyper and excess phenomenon

1328 HSV-1 – herpes simplex virus type 1

1329   IAPP = islet amyloid polypeptide

1330   IR =insulin resistance

1331   LOAD = late onset Alzheimer's disease

1332   MC = metabolic cognopathy

1333   MD = mixed dementia

1334   MetS = metabolic syndrome

1335   MGC = microglia cell

1336   MT = microtubule

1337   NO = nitric oxide

1338   NFT = neurofibrillary tangles

1339   NVU = neurovascular unit

1340   OL = oligodendrocyte

1341   Pc = pericyte

1342   Pcfp = pericyte foot process

1343   PHF = paired helical fragments

1344   PIR = peripheral insulin resistance

1345   RAAS = renin-angiotensin-aldosterone system

1346   RAGE = receptor for advanced glycation end-products

1347   RBC = red blood cell

1348   rMGC = ramified microglia cell

1349   ROS/RNS = reactive oxygen species/ reactive nitrogen species

1350   T2DM = type 2 diabetes mellitus

1351   Tau = microtubule associated protein

1352   TJ/AJ = tight junctions/adherens junctions

1353   VaD: VAD = vascular dementia

1354   VCID = vascular contributions of impaired cognition and dementia

1355   VSMC = vascular smooth muscle cell
1356   WBC = white blood cell

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