Maturation and physiological aging of the BBB and the neurovascular unit

BBB during embryogenic development and postnatal period

For better understanding of its function and the complex composition of BBB, it is important to overview the processes of development, maturation and maintenance of its structural elements. The genetic program of embryogenesis coordinates the development of CNS including the development of BBB. This main stream of the process is characterized by a strong building of the brain with neuronal cells surrounding with vasculature. The developing vasculature is critical for vessel remodeling and maturation events. Nutrition is a part of fundamental basis of embryogenesis and neonatal development of CNS with BBB. Here we emphasize the role of growth factors and some other factors in the development of BBB. The permeability of the BBB is high in the early developmental phases, but later on it becomes a more compact structure providing an almost complete protection against xenobiotics and toxic agents. During the aging process BBB partially loses again its general protective, barrier function. The timeline of BBB development during embryonic phases and postnatal days in mice is shown in **Figure S1** and will be summarized in general in the next section.

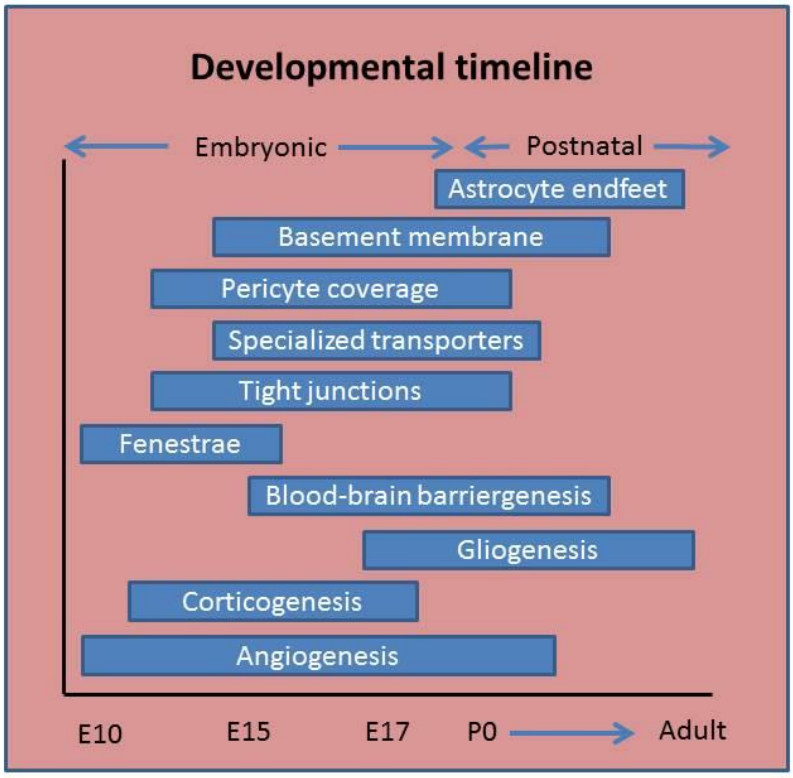

Figure S1. The timeline of the development of BBB during the embryonic and postnatal days in mice. (Modified from Zhao et al, 2015) [1]

ECs and pericytes both contribute to the basement membrane development and induction the expression of integrin. The EC-derived factors, platelet derived growth factor-BB (PDGF-BB) and heparin binding-epidermal growth factor (HB-EGF) support growth, and they are critical in tube maturation. The new vessels built up from pre-existing vessels with sprout into the embryonic neuroectoderm. The early vessels have TJs, transporters, transcytotic vesicles and leukocyte adhesion molecules. The mature BBB vessels come into close contact with cells of NVU (pericytes, astroglia, neurons), transcytosis is decreased and the regulated efflux transport increased [2]. Maturation is completed by sealing of TJs.

Vascular endothelial growth factor (VEGF) has a fundamental role in embryonic angiogenesis including VEGF receptor 2 (Vegfr-2); fetal liver kinase 1 (Flk-1); and kinase insert domain receptor, (Kdr) [3]. Activation of PI3K-AKT/PKB and similar the p38/MAPK-HSP27 pathways support EC survival and promotes EC migration [4].

During embryogenesis the Wnt/beta-catenin pathway is activated in CNS ECs and therefore it drives angiogenesis specifically in the CNS [5]. Wnt ligands inhibit the degradation of beta-catenin in the proteasome. Downstream signaling element of beta-catenin in ECs results in failed vascularization of CNS [5]. Wnt induces the expression of BBB genes, including nutrient transporters. The same signal that drives EC migration into the CNS also induces BBB functions. Endothelial beta-catenin also regulates the formation of TJs so it has a key role in embryonic and postnatal BBB maturation [6].

GPR124, a member of the G protein-coupled receptor family (also known as tumor endothelial marker 5, TEM5), has a pivotal role in the brain-specific angiogenesis [7-9]. The phenotype is characterized by impaired EC survival, growth and migration, which result in an eligible vascular sprout to embryonic neuroectoderm. GPR124 seems to act independently from VEGF in vessel sprouting. Expression of Vegfr was unaffected in the absence of GPR124 [7, 8].

BBB formation in embryonic stage is regulated together with other factors by an important protein, Sonic Hedgehog (SHH). Shh knockout mice exhibit embryonic lethality between E11 and E13.5. Their phenotype is associated with abnormalities in BBB formation with decreased expression of TJ proteins (occludin, claudin-5). Moreover, when Smoothened (Smo), a downstream signaling protein was selectively deleted from ECs resulting a lower TJ protein expression associated with vessel leakage. These data suggest that Shh is required for the maturation of the BBB vessels. Shh upregulates TJ protein expression in human BBB ECs and decrease the permeability, indicating that SHH could also have a role in maintaining BBB functions [10]. Shh can induce Vegf and angiopoietin (Ang) expression, which are both strong angiogenic factors [11].

The infant brain is vulnerable to neurotoxic substances connecting partly to the immature BBB. The neonatal BBB cells have lower barrier and p-glycoprotein (P-gp) functions than the adult BBB cells and well associated with lower expressions of the barrier-related proteins (P-gp) and the age-dependent BBB permeability of drugs [12].

Two astrocyte markers, the glial water channel aquaporin-4 (AQP4) and the glial fibrillary acidic protein (GFAP), have been implicated in several physiological and pathological conditions in the CNS as well as in BBB breakdown. AQP4 and GFAP increase expression in the cerebellum of neonate (14-day-old) and adult (8-week-old) rats regulating the age- and time-related water/electrolyte balance [13].

In the postnatal period the permeability of water and low/high molecular-mass tracers is connected to the activity of pericytes and regulated by the endothelial and astrocyte collaborations [14]. The main activity of postnatal period remains the development and reproduction.

Astroglial perivascular connectivity occurs and develops during postnatal BBB maturation (days 2-20). The absence of astroglial connexins (Cx30 and Cx43) weakens the BBB, which opens upon increased hydrostatic vascular pressure and shear stress demonstrating that astroglial connexins are necessary to maintain BBB integrity [15, 16].

Below, first, the general characteristic of a selection of neurodegenerative diseases is presented. Then, main neurodegenerative processes and disorders are generally described, followed by more specific information on BBB dysfunction in these neurodegenerative diseases.

Neurodegenerative diseases

Alzheimer's disease. Alzheimer's disease is characterized by brain processes that include formation of amyloid (senile) plaques; neurofibrillary tangles in the intracellular space of neurons, with a high content of the hyperphosphorylated protein "tau" , as well as brain atrophy and shrinkage [17]. In this disease the neurons of typically the temporal lobe and parietal lobe, and parts of the frontal cortex and cingulate gyrus degenerate, leading to gross atrophy of these brain areas [18]. Amyloid-β (Aβ) is produced from the amyloid-β precursor protein (APP), both in the brain and in peripheral tissues. Clearance of amyloid-β from the brain normally maintains its low levels in the brain. Aβ accumulation in the Alzheimer's affected brain is likely due to its faulty clearance from the brain [19-22].

Multiple sclerosis. Multiple sclerosis is a typical inflammatory disease, but axonal loss and neurodegeneration have been observed even in its earliest stages [23-26]. Endothelial cell stress and apoptosis are characteristic hallmarks of multiple sclerosis. The inflammatory demyelinating disease processes in early multiple sclerosis triggers a cascade of events that lead to neurodegeneration, which are amplified by pathogenic mechanisms related to brain ageing and accumulated disease burden [27].

Parkinson's disease. Parkinson's disease is a chronic, progressive neurological disorder. The roles of oxidative stress, apoptosis, mitochondrial dysfunction, inflammation, and impairment of the protein degradation pathways have been highlighted by work with animal models [28, 29]. The mechanism by which the brain cells in Parkinson's disease are lost may consist of an abnormal accumulation of the protein alpha-synuclein bound to ubiquitin in the damaged cells. The alpha-synuclein-ubiquitin complex cannot be directed to the proteosome. This protein accumulation forms proteinaceous cytoplasmic inclusions called Lewy bodies, which are one of the hallmarks of Parkinson's disease [30].

Pharmacoresistant epilepsy. In very broad and general terms, pharmacoresistance is the failure of seizures to come under complete control or acceptable control in response to antiepileptic drugs. About 30-40% of all people with epilepsy do not become fully seizure free with present medication, even when treated at the maximal tolerated dose. This pharmacoresistance is particularly prominent in partial epilepsies and some severe syndromes in infants, but essentially it can occur in nearly all

types of epilepsies and epileptic syndromes. In addition, unresponsiveness in these patients is not limited to a specific drug or drug class, but occurs with the complete range of antiepileptic drugs [31, 32].

Neurodegenerative processes

Neurodegenerative diseases are characterized by a continuous progress of neuronal death and progressive nervous system dysfunction. The neurodegenerative diseases have many processes in common, though these processes may be qualitatively, quantitatively, temporally and spatially distinct. The main processes include gene defects and variants, oxidative stress, protein misfolding and accumulation, which will affect different cellular and intercellular signaling pathways to cause neuronal cell death by necrosis or apoptosis. Here we shortly describe these main processes with references for further reading, as this review focus is on changes at the level of the BBB, which will be discussed afterwards in more detail for Alzheimer's disease, Multiple sclerosis, Parkinson's disease and pharmacoresistant epilepsy.

Gene defects and variants

Over the years, many genetic defects related to human neurodegeneration have been identified, which in the presence of environmental factors determine the course of progressive neurodegenerative processes [33, 34]. In **Table S1** the genes that have been found to be associated with Alzheimer's disease, Multiple sclerosis, Parkinson's disease, and pharmacoresistant epilepsies are displayed.

It should be noted that genome-wide association studies have successfully revealed numerous susceptibility genes for neurodegenerative diseases, but that it has been found that odds ratios (a statistical number, based on the link between absence/presence of property A and absence/presence of property B in a given population) associated with risk alleles are generally low. This indicates that it is mere the "common disease- multiple rare gene variants" than the "common disease-common gene variants" determine the susceptibility for a given neurodegenerative disease [35].

TableS1. Disease condition and associated changes in genes.

Disease condition	Genes	References
Alzheimer's disease	APOE, APP, PSEN1, PSEN2	[36]
Multiple sclerosis	CYP27B1, HLA-DRB1, IL2RA, IL7R TNFRSF1A	[36]
Parykinson's disease	ATP13A2, GBA, LRRK2, PARK2, PARK7, PINK1, SNCA, UCHL1, VPS35	[36]
Pharmacoresistant epilepsies	ABCB1, CSTB, EMPA, GABRG2, NHRC1, SCN1a, SCN9A, SLC2A1, TSC1, TSC2	[37]

Oxidative stress

Disturbances in the normal redox state of cells can cause toxic effects via the production of peroxides and free radicals (reactive oxygen species (ROS) and reactive nitrogen species (RNS)) which may damage all components of the cell, such as proteins, lipids, and DNA. Such oxidative stress can cause disruptions in normal cellular signaling and seems to have a ubiquitous role in neurodegeneration via the induction of cell death [38-41]. In response to oxidative stress, cells increase and activate their cellular antioxidant mechanisms. Glutathione (GSH) is the major antioxidant in the brain, and as such plays a pivotal role in the detoxification of reactive oxidants.

For Alzheimer's disease oxidation/dysfunction of a number of enzymes specifically involved in energy metabolism have been found, that support the view that reduced glucose metabolism and loss of ATP are crucial events triggering neurodegeneration and progression of Alzheimer's disease [42].

For multiple sclerosis, oxidative stress has been strongly implicated in both the inflammatory and neurodegenerative pathological mechanisms. The onset of multiple sclerosis is characterized by inflammation-mediated demyelination due to lymphocyte infiltration from peripheral blood and microglial cell activation. In different disease stages accumulation of ROS and RNS have been observed. Also, it has been shown that GSH homeostasis is altered in multiple sclerosis [43-45].

For Parkinson's disease, oxidative stress is generally recognized as one of the main causes of Parkinson's disease, and excessive ROS can lead to dopamine neuron vulnerability and eventual death [46, 47].

Finally, no information is available on the role of oxidative stress in specifically pharmacoresistant epilepsies.

Protein misfolding and accumulation

Protein misfolding is a naturally occurring process in living cells. Under healthy conditions the so-called Protein Quality Control Systems dispose misfolded proteins. When the capacity of these systems is limiting, misfolded proteins may accumulate and aggregate. It seems that proteins that have repetitive amino acid motifs are mostly prone to changing into a misfolded state. Such state is often toxic and can be viewed as "infective" when the misfolded protein is able to induce the conversion of other, normally folded proteins into the toxic configuration, which may lead to an amplification loop. Accumulation and aggregation of misfolded proteins further lead to impairment of proper cellular functioning and ultimate cell killing (**FigureS 2**). The prion proteins are important and well known example of this catastrophal cascade [30, 48-50] . For Alzheimer's, the accumulation proteins are amyloid-β and hyperphosphorylated tau ([19-22]), and for Parkinson's disease the accumulating protein is α-synuclein [51, 52].

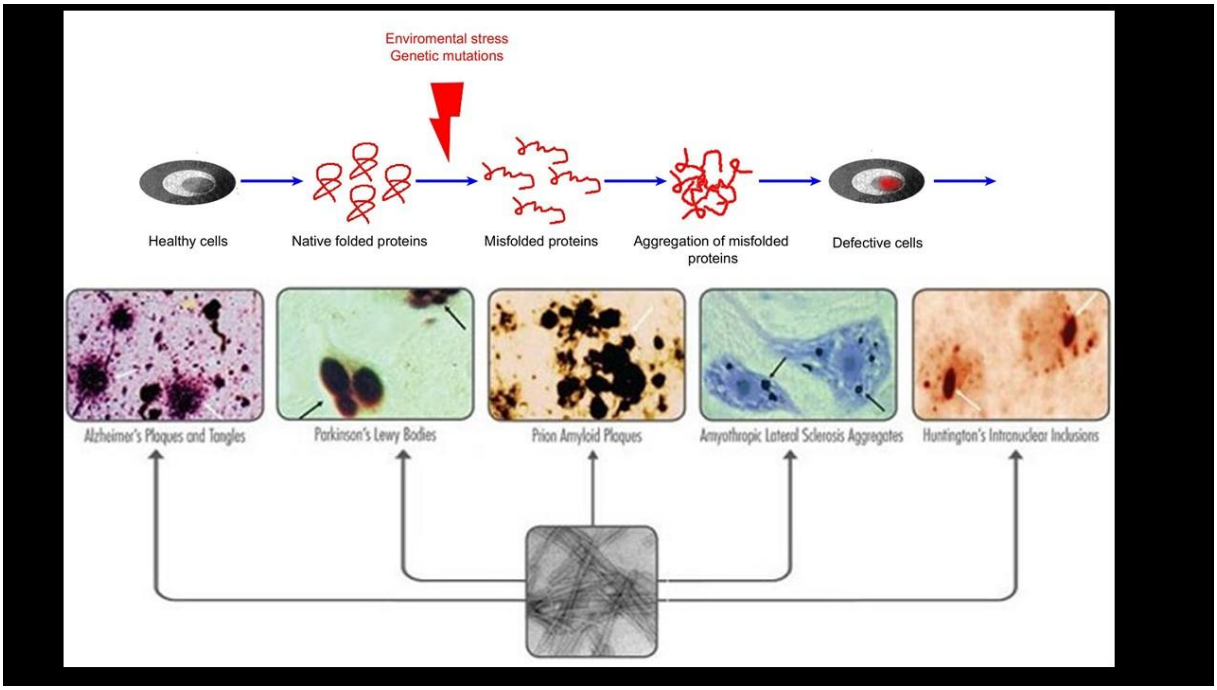

Figure S2. Upper panel: The cascade from normal cells to defective cells due to aggregation of misfolded proteins. Lower panel:. Many neurodegenerative disorders involve the accumulation of misfolded proteins, although the exact protein involved and the location where it tends to accumulate is specific to each disease [53].

Cell death

Inappropriate death of cells in the nervous system is the cause of multiple neurodegenerative disorders. Pathological neuronal death can occur by apoptosis, by necrosis or by a combination of

both. Elevated intracellular calcium is the most ubiquitous feature of neuronal death with the concomitant activation of cysteine calcium-dependent proteases, calpains. Calpains and lysosomal, catabolic aspartyl proteases, play key roles in the necrotic death of neurons [54].

Neuronal loss is almost invariably accompanied by abnormal insoluble protein aggregates, either intra- or extracellular. Ambegaokar et al (2010) have reviewed methods by which Drosophila melanogaster has been used to model aspects of Parkinson's disease and Alzheimer's disease [55].

In the pathogenesis of neurodegeneration, an aberrant regulation of apoptosis is known to play a role. For example, ROS are known to be able to initiate apoptosis via the mitochondrial and death receptor pathways [56]. Abnormality in mitochondrial Ca(2+) handling has been detected in a range of neurodegenerative diseases, and emerging evidence from disease models suggests that mitochondrial Ca(2+) may play a role in disease pathogenesis [57].

Excess cells are eliminated through programmed cell death or apoptosis. The initiation of neuronal apoptosis in response to numerous extracellular agents has been widely reported and lethal functions for the lipid effectors ceramide and sphingosine have been identified in both normal and pathophysiological conditions. Inappropriate initiation of apoptosis by deregulated ceramide and sphingosine has been proposed to underlie the progressive neuronal attrition associated with, amongst other neurodegenerative diseases, Alzheimer's disease and Parkinson's disease [58].

Bains et al [59] (1997) proposed a GSH-depletion model of neurodegenerative disorders. Oxidative stress-mediated neuronal loss may be initiated by a decline in GSH. GSH plays multiple roles in the nervous system including free radical scavenger, redox modulator of ionotropic receptor activity, and possible neurotransmitter. GSH depletion can enhance oxidative stress and may also increase the levels of excitotoxic molecules; both types of action can initiate cell death in distinct neuronal populations. Evidence for a role of oxidative stress and diminished GSH status is present, among other neurodegenerative diseases, for Parkinson's disease, and Alzheimer's disease [59].

Autophagy is a highly conserved intracellular pathway involved in the elimination of proteins and organelles by lysosomes. Known originally as an adaptive response to nutrient deprivation in mitotic cells, autophagy is now recognized as an arbiter of neuronal survival and death decisions in neurodegenerative diseases. Studies using postmortem human tissue, genetic and toxin-induced animal and cellular models indicate that many of the etiological factors associated with neurodegenerative disorders can perturb the autophagy process. Emerging data support the view that dysregulation of autophagy might play a critical role in the pathogenesis of neurodegenerative disorders, such as Alzheimer's and Parkinson's disease [60].

The DNA damage response is a key factor in the maintenance of genome stability. As such, it is a central axis in sustaining cellular homeostasis in a variety of contexts: development, growth, differentiation, and maintenance of the normal life cycle of the cell. The DNA damage response defects in neurons may result in neurodegeneration. Barzilai et al (2010) have provided an overview on the potential role of the DNA damage response in the etiology and pathogenesis of neurodegenerative diseases [61].

Macroautophagy is a cellular process by which cytosolic components and organelles are degraded in double-membrane bound structures upon fusion with lysosomes. A pathway for selective

degradation of mitochondria by autophagy, known as mitophagy, has been described, and is of particular importance to neurons. It appears that the regulation of mitophagy shares key steps with the macroautophagy pathway, while exhibiting distinct regulatory steps specific for mitochondrial autophagic turnover. Mitophagy has been linked to the pathogenesis of Parkinson's disease, through the study of recessively inherited forms of this disorder, involving PINK1 and Parkin. Recent work indicates that PINK1 and Parkin together maintain mitochondrial quality control by regulating mitophagy. In the Purkinje cell degeneration (pcd) mouse, altered mitophagy may contribute to the dramatic neuron cell death observed in the cerebellum, suggesting that over-active mitophagy or insufficient mitophagy can both be deleterious [62].

Exposure of hippocampal neuronal/glial co-cultures to β-amyloid peptides activates the glial nicotinamide adenine dinucleotide phosphate oxidase, followed by predominantly neuronal cell death, which is mediated by poly(ADP-ribose) polymerase in response to oxidative stress generated by the astrocytic nicotinamide adenine dinucleotide phosphate oxidase [63].

Aneuploidy is an abnormal number of copies of a genomic region. Genomic instability has been associated with aneuploidy, however, can also lead to developmental abnormalities and decreased cellular fitness. Arendt et al (2010) have shown that neurons with a more-than-diploid content of DNA are increased in preclinical stages of Alzheimer's disease and are selectively affected by cell death during progression of the disease. Present findings show that neuronal hyperploidy in Alzheimer's disease is associated with a decreased viability. Hyperploidy of neurons thus represents a direct molecular signature of cells prone to death in Alzheimer's disease and indicates that a failure of neuronal differentiation is a critical pathogenetic event in AD [64].

The metabolic turnover of sphingolipids produces several signaling molecules that profoundly affect the proliferation, differentiation and death of cells. It is well-known that specifically ceramide and sphingosine-1-phosphate play an important role in the so-called cell death pathways. Wide body of evidence indicates that ceramide and amyloid beta protein plays a key role in attacking mitochondria to set in the pathways of cell death in Alzheimer's disease [65]:

Mechanisms by which dopaminergic neurons die in Parkinson's disease seems to be related to, (i) defects in ubiquitin-proteasome pathway and protein misfolding and aggregation caused by α-synuclein and Parkin gene defects; (ii) defects in mitochondrial morphology and function in PINK1/Parkin and DJ-1 mutations; (iii) increased susceptibility to cellular oxidative stress which appear to underlie defects in α-synuclein, Parkin and DJ-1 genes [66].

It is known that Parkinson's disease is characterized by the progressive loss of select neuronal populations, but prodeath genes mediating the neurodegenerative processes is a novel proposed concept by Aimé et al (2015). They have proposed a pathway involving Trib3 (tribbles pseudokinase 3), which is a stress-induced gene with proapoptotic activity, to have a role in neuronal death associated with Parkinson's disease [67].

Then, for Parkinson's disease, Michel and colleagues (2016) have provided an overview of cell-autonomous mechanisms that are likely to participate in DA cell death in both sporadic and inherited forms of Parkinson's disease. Damage to vulnerable DA neurons may arise from cellular disturbances produced by protein misfolding and aggregation, disruption of autophagic catabolism (a conserved catabolic process that degrades cytoplasmic constituents and organelles in the lysosome),

endoplasmic reticulum stress, mitochondrial dysfunction, or loss of calcium homeostasis. Where pertinent, they show how these mechanisms may mutually cooperate to promote neuronal death [68].

For multiple sclerosis it has been postulated that glutamate excitotoxicity, as a result of an excessive amount of glutamate that over-activates its cellular receptors and induces cell death, could be a missing link between inflammatory and neurodegenerative processes evident in multiple sclerosis [69].

Glutamate-mediated excitotoxicity is the principal mechanism driving neuronal death after status epilepticus, whereby excessive glutamate release leads to intracellular calcium overload, oxidative stress, organelle swelling and rupture of intracellular membranes, activation of proteases and necrosis. Based on the work of Meldrum and others, it has generally been accepted that status epilepticus, even in the absence of systemic complications, causes neuronal death in vulnerable brain regions, often but not necessarily including the hippocampus [70-72].

Finally, it can be said that in the different neurodegenerative diseases a selective vulnerability of particular neuronal groups or brain structures exists. What makes such specific vulnerability to be associated with a particular neurodegenerative disease remains to be further elucidated.

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