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Review

Involvement of hemoglobins in the pathophysiology of Alzheimer's disease

Meric A. Altinoz[a,*], Sinan Guloksuz[a,b], Rainald Schmidt-Kastner[c], Gunter Kenis[a], Bahri Ince[d], Bart P.F. Rutten[a]

[a] Department of Psychiatry and Psychology, School for Mental Health and Neuroscience, Maastricht University Medical Centre, Maastricht, the Netherlands
[b] Department of Psychiatry, Yale School of Medicine, New Haven, CT, USA
[c] Integrated Medical Science Department, Charles E. Schmidt College of Medicine, Florida Atlantic University (FAU), Boca Raton, FL, USA
[d] Department of Psychiatry, Mazhar Osman Bakirkoy Mental Diseases Research and Education Hospital, Istanbul, Turkey

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ABSTRACT

Hemoglobins (Hbs) are heme-containing proteins binding oxygen, carbon monoxide, and nitric oxide. While erythrocytes are the most well-known location of Hbs, Hbs also exist in neurons, glia and oligodendroglia and they are primarily localized in the inner mitochondrial membrane of neurons with likely roles in cellular respiration and buffering protons. Recently, studies have suggested links between hypoxia and neurodegenerative disorders such as Alzheimer Disease (AD) and furthermore suggested involvement of Hbs in the pathogenesis of AD. While cellular immunohistochemical studies on AD brains have observed reduced levels of Hb in the cytoplasm of pre-tangle and tangle-bearing neurons, other studies on homogenates of AD brain samples observed increased Hb levels. This potential discrepancy may result from differential presence and function of intracellular versus extracellular Hbs. Intracellular Hbs may protect neurons against hypoxia and hyperoxia. On the other hand, extracellular free Hb and its degradation products may trigger inflammatory immune and oxidative reactions against neural macromolecules and/or damage the blood-brain barrier. Therefore, biological processes leading to reduction of Hb transcription (including clinically silent Hb mutations) may influence intraerythrocytic and neural Hbs, and reduce the transport of oxygen, carbon monoxide and nitric oxide which may be involved in the (patho)physiology of neurodegenerative disorders such as AD. Agents such as erythropoietin, which stimulate both erythropoiesis, reduce eryptosis and induce intracellular neural Hbs may exert multiple beneficial effects on the onset and course of AD. Thus, evidence accumulates for a role of Hbs in the central nervous system while Hbs deserve more attention as possible candidate molecules involved in AD.

1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder comprising a complex etiopathogenesis, which ultimately leads to progressive loss of memory and other cognitive functions (Burns and Iliffe, 2009). AD has a devastating impact on global human health and economy and has an increasing prevalence due to an aging society (Burns and Iliffe, 2009; Dementia Fact Sheet, 2016). As no efficient treatment exists, there is a great need for improving our understanding of the etiopathogenesis and pathophysiology, which will hopely inform innovative strategies for intervention. Until now, a plethora of scientific experiments were conducted to discover novel treatments of AD and many clinical trials were conducted with several candidate molecules, yet few drugs with limited activity exist in the armamentarium against AD. Hence, illuminating novel pathways of pathogenesis and discovery of new therapeutic molecules in AD is an urgent and essential need. The current manuscript reviews the available evidence on hemoglobins (Hbs) and their links with AD. We will illustrate that i) the presence of hemoglobin chains was recently discovered in neurons (Biagioli et al., 2009), ii) Hbs play key roles in the pathophysiologies of a range of disorders, iii) modulation of Hbs is known to have significant impact on cellular functions (e.g. in cellular respiration and buffering oxygen radicals). Based on published findings on Hbs, we propose that i) neuronal intracytoplasmic and mitochondrial Hbs may exert protective functions in AD, ii) aberrations in structure (e.g. undetected minor thalassemias and hemoglobinopathies), translation, or amount of neuronal Hbs may play a role in the etiology of AD, iii) structural abnormalities of Hbs may trigger supraphysiological accumulation of Hbs in mitochondria and cause pathological interactions with β-amyloid and α-synuclein, and iv) extracellular Hb released from degraded red blood cells (RBC) may induce toxicity, disruption and/or degeneration of neurons as well as the blood brain barrier (BBB) in AD. Three tables

* Corresponding author.
E-mail address: maltinoz@gmail.com (M.A. Altinoz).

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Table 1
Clues suggesting that hemoglobin chains may be regulators of neuronal physiology in healthy conditions and physiological aging[a].

Anatomical and cellular localization of hemoglobins in neural cells and tissues	References
Hbs are expressed in cortical and hippocampal astrocytes and in oligodendroglia localizing in all brain regions including striatum, corpus callosum, and medulla oblongata	Biagioli et al., 2009
Hb α-and β-globin exist in mesencephalic dopaminergic neurons in the ventral tegmental area and substantia nigra	Biagioli et al., 2009
Hbα-positive cells juxtaposing Hbα-negative cells display higher oxygenation than their neighbours and can be prominently distinguished from those	Schelshorn et al., 2009
Neurons expressing Hb β-chain are more frequent in the internal layers of the cortex (layers IV–VI) compared to the external layers (layers I–III).	Brown et al., 2016
The majority of Hb β-chain expressing neurons exerted pyramidal morphology with large size, apical dendrites and long axons	Brown et al., 2016
In neurons overexpressing Hb chains, 46% of the stimulated-genes encode subunits of mitochondrial complex I–V	Biagioli et al., 2009
In neurons, five of the fourteen Hb β-chain interacting proteins were mitochondrial, including ATP5A1 and ATP5B, MDH2, SLC25A31, and SLC25A3	Brown et al., 2016
Neuronal Hb expression is highly reduced in rats treated with the mitochondrial toxin rotenone	Richter et al., 2009
Both Hba and Hbb co-localize within mitochondria in the mammalian brain	Shephard et al., 2014
Binding partners of Hb β-chain in neurons also include histone H3 (HIST2H3A) and lysine-specific histone demethylase 8 (KDM8)	Brown et al., 2016
Aβ-binding proteins were purified from rat brain and three new Aβ-binding proteins were detected including Hb α-chain	Oyama et al., 2000
Hb chain levels are higher in rat hippocampal CA1 region during normal aging process	Blalock et al., 2003
In mice, the top 5 genes with a lower expression in aged compared with young astrocytes comprised Hba-a2 (10.6-fold decrease), Hbb-b1 (10.2-fold decrease), Hba-a1 (8.2-fold decrease)	Orre et al., 2014
α-Synuclein and Hb can form a complex in brain tissue and RBCs in aging cynomolgus monkeys. Free Hb and Hb-α-syn complex levels in mitochondria of the striatum decreased with age; which was negatively correlated with the cytoplasmic Hb levels. Hb overexpression enhanced free Hb levels in mitochondria, stabilized mitochondrial membrane potential, and lowered α-syn-induced apoptosis	Yang et al., 2016

[a] The discrepancy between the opposite results is explained within the manuscript.

Table 2
Clues suggesting that hemoglobin chains may involve in neurodegeneration with special emphasis to Alzheimer's disease (AD)[a].

	References
Relative mitochondrial to cytoplasmic ratios of Hba and Hbb exerted differential distributions in the brain in the Purkinje Cell Degeneration (pcd^{5J}) mouse model. A significant decrease in mitochondrial Hba and Hbb content occurs when dynamic neuronal loss reaches to peak levels in the pcd^{5J} mouse	Shephard et al., 2014
Mitochondrial Hba and Hbb levels decline in aging brains	Shephard et al., 2014
Substantia nigra accumulated mitochondrial Hb in PD brains, but Western blotting of mitochondrial fractions from PD and control brains indicated significantly less Hb in PD brain mitochondria. A specific loss of neurons accumulating mitochondrial Hbs?	Shephard et al., 2014
Hb-Aβ-complexes exist in a soluble fraction of the AD cerebral cortex	Oyama et al., 2000
Hb is present in amyloid plaques and brain cells, mainly in neurons and oligodendrocytes	Chuang et al., 2012
Hb colocalized with senile plaques and cerebral amyloid angiopathy in AD brains. Hb levels are elevated in amyloid pathology-associated brain regions (inferior temporal gyrus, cerebral parietal gray matter and parietal white matter)	Wu et al., 2004
Lower β-globin protein levels in hippocampus and entorhinal cortex of people with AD in comparison to normal controls	Schonberger et al., 2001
α-Globin levels prominently declined in brain synaptosomes in a rat model of AD	Yang et al., 2011
Decreases of α- and β-globin chains, in almost all neurons with hyperphosphorylated tau deposits in the frontal cortex and hippocampus in AD and in pre-tangles in argyrophilic grain disease (AGD). Loss of Hb in all swollen neurons in the amygdala in AD and AGD	Ferrer et al., 2011
Higher Hba and Hbb levels in the cerebrospinal fluid (CSF) could differentiate AD-converters from non-converters within the mild cognitive impairment (MCI) subjects	Spellman et al., 2015
Almost absent Hb in about 80% of dopaminergic neurons of the substantia nigra, and in other altered neurons of the brain stem that contain abnormal α-synuclein small inclusions or Lewy bodies	Ferrer et al., 2011
Aβ binding to Hb and other hemoproteins via the iron-containing heme moiety, may be an intrinsic protective mechanism to prevent Hb/heme/iron-induced cytotoxicity. Binding of Hb to Aβ required iron-containing heme	Chuang et al., 2012
Hbs increased in aging and APP/PS1 transgenic mice and microinjection of human Hb into the dorsal hippocampi of the APP/PS1 transgenic mice developed envelope-like structures composed of Aβ surrounding Hb	Chuang et al., 2012
In astrocyte cultures, exposure to $Aβ_{1–42}$ strongly induced production of IL-1β, GM-CSF and RANTES, which were all reduced by cotreatment with heme or Hb	Sankar et al., 2018
Hb chains are significantly lowered in AD brains which were also confirmed at the protein level	Vanni et al., 2018

[a] The discrepancy between the opposite results is explained within the manuscript.

Table 3
Clues suggesting that free hemoglobin may act neurotoxic.

	References
RBCs in AD patients contain more surface bound IgG and display increased proteolysis and oxidative stress which may trigger their lysis and subsequent increase of free Hb fragments	Slemmon et al., 1994
Free Hb increased astrocytic activity and IgG extravasation through the blood-brain barrier (BBB). Significant increases of oxidative end-products in BBB-endothelia were found following free Hb infusion	Butt et al., 2011
Hb- and heme enhanced endothelial adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), which increase leukocyte adhesion and transmigration in BBB endothelia	Butt et al., 2011
Both free-Hb and heme exacerbate inflammatory responses and activate the innate immunity through Toll-like receptor 4 (TLR4) on microglia and other innate immune cells	Bamm and Harauz, 2014

were created to summarize the involvement of Hbs in neuronal (patho) physiology with special emphasis to AD. The first table lists the clues suggesting that Hb chains may be regulators of neuronal physiology in healthy conditions and physiological aging (Table 1). The second table lists the clues suggesting that Hb chains may involve in neurodegeneration with special emphasis to AD (Table 2). Lastly, the third table lists the clues suggesting that free Hbs may act neurotoxic (Table 3).

2. Expression and function of hemoglobins in neural tissues

2.1. A short biochemistry of Hb chains

The human α-globin gene cluster resides at chromosomal region 16p13.3 and contains seven loci (Song et al., 2012): 5′ - zeta(ζ) – pseudozeta (Ψζ) – mu(μ) – pseudoalpha1 (Ψα1) – α2–α1–theta(θ) – 3′. The human β-globin gene cluster resides at chromosomal region 11p15.5 and contains five loci (Song et al., 2012): 5′ – epsilon(ε) – gammaG (Gγ) – gammaA(Aγ) – delta(δ) – beta(β) – 3′. Three Hbs are produced in adults: the major adult HbA (α2β2, ~97%) and the minor Hbs, HbA2 (α2δ2, < 3%) and HbF (< 1.2%) (Rochette et al., 1994). HbA consists of two α- and two β-globin chains assembling into a α2β2 heterotetramer, where each globin binds a heme group (Hardison, 2008). Intracellular Hb also exerts antioxidant and superoxide and hydrogen peroxide scavenger properties (Masuoka et al., 2003).

2.2. Expression of Hbs in neural cells and tissues

Hb acts likely as an oxygen-storage molecule, which is particularly relevant to counteract effects of hypoxia on neurons, which have an obligate demand to sustained energy (Biagioli et al., 2009). Hbs were recently found to be expressed in cortical and hippocampal astrocytes and in oligodendroglia localizing in all brain regions including striatum, corpus callosum, and medulla oblongata (Biagioli et al., 2009). Hb α-and β-globin were found in mesencephalic dopaminergic neurons in the ventral tegmental area and substantia nigra (Biagioli et al., 2009), where Hb is expressed in the majority of A9 cells, but only in 5% of A10 neurons (Biagioli et al., 2009). To reveal the physiological roles of Hbs in neural cell lineages, knock-out strategies in cell lines and in living animals would provide more precise results. Currently, there is a lack of such studies but some overexpression studies may provide partial hints about their likely physiological functioning. For instance, dopaminergic neurons transfected with α- and β-chains of Hb showed changes in genes modifying oxygen homeostasis and oxidative phosphorylation (Biagioli et al., 2009). In neurons overexpressing Hb chains, 46% of the stimulated-genes encode subunits of mitochondrial complex I–V (Biagioli et al., 2009). Both α- and β-chains of Hb can be observed in the soma of neuronal cells, while mainly β-chains exist in dendrites (Richter et al., 2009). Hitherto, there is a lack of information in regard to their intracellular existence in other neural cell lineages.

Neurons expressing Hb β-chain are more frequent in the internal layers of the cortex (layers IV–VI) as compared to the external layers (layers I–III) (Brown et al., 2016). The majority of Hb β-chain expressing neurons exerted pyramidal morphology with large size, apical dendrites and long axons (Brown et al., 2016). Coimmunoprecipitation and mass spectrometry were used to determine the proteins binding to the Hb β-chain in neurons (Brown et al., 2016). Five of the fourteen Hb β-chain interacting proteins were mitochondrial, including ATP synthase subunits α and β (ATP5A1 and ATP5B), mitochondrial malate dehydrogenase (MDH2), ADP/ATP translocase 4 (SLC25A31), and a mitochondrial phosphate carrier (SLC25A3) (Brown et al., 2016). The analyses revealed unexpected binding partners of Hb β-chain, including histone H3 (HIST2H3A) and lysine-specific histone demethylase 8 (KDM8) (Brown et al., 2016). Hence, it was postulated that Hbs may transmit signals between the neuronal mitochondria and nuclei. A role of Hbs in mitochondrial respiration and redox system is supported by the observations that neuronal Hb expression is highly reduced in rats treated with rotenone (Richter et al., 2009). Rotenone (a toxin used to induce Parkinson's Disease (PD) in animal models) blocks the mitochondrial complex I and inhibition of this complex under physiological NO-levels inhibits the transcription factor HIF1α, which subsequently lowers Hb mRNA (Richter et al., 2009). Another mechanism explaining the decrease of Hb may be the reduction of free heme, which is a strong inducer of globin chain transcription (Richter et al., 2009). Heme is synthesized in the mitochondria via ferrochelatase, which inserts the ferrous iron into a tetrapyrrole (Richter et al., 2009).

3. Possible pitfalls of detecting Hb mutations and cognitive deficits in thalassemia carriers

About 1720 different Hb variants have thus far been identified among globin chains (Giardine et al., 2014), and only a minority of these cause clinical expression in signs or symptoms (Modell and Darlison, 2015). It has been estimated that 5.2% of the world population (i.e. about 400 million) suffers from clinical symptoms linked to Hb variants. This percentage is higher globally when non-expressing carriers of those variants are also considered (Cousens et al., 2010). Thalassemias are in principle caused by mutations affecting the level of Hb-chain synthesis rather than mutations impacting on the qualitative function of Hbs. β-thalassemia is caused by β-globin mutations in Hbs and comprises the most frequent single-gene inherited condition in the world; each year aproximately 70,000 infants are born with β-thalassemia major in the world (Cousens et al., 2010). Many cases of β-thalassemia trait are not being identified and misdiagnosed as iron deficiency (without further diagnostic work-ups) because both conditions present with microcytic anemia (Ntaios et al., 2007; Usman et al., 2011).

α-Thalassemia is caused by α-globin mutations (Bernini and Harteveld, 1998). When only a single allele is mutated, this is called “silent α-thalassemia” with lack of clinical signs and absence of any laboratory abnormality. When two alleles are mutant, this is called the “α-thalassemia trait” which is characterised by mild microcytic hypochromic anemia, that is als often overlooked as iron deficiency anemia (Bernini and Harteveld, 1998). Only when three α-globin alleles are mutant, this is called “Hemoglobin H (HbH) Disease”, characterised by manifest microcytic hypochromic anemia and hepatosplenomegaly (Bernini and Harteveld, 1998). Given that Hb function is still quantitatively compromised in people with silent or trait α-thalassemia, one could speculate that their neuronal cells (having very high metabolic rates and relying on mitochondrial respiration) might be functionally impacted. In support, the functional read-out parameters of P300 event related potentials in β-thalassemia carriers was compromised as compared to subjects not harbouring Hb variants (Nevruz et al., 2007). Based on evidence of overt and latent genetic variations in Hbs in relation to a range of other medical disorders (reviewed in Altinoz et al., 2016a, 2016b and Altinoz and Ince, 2017), it is tempting to speculate that hematologically silent or manifest mutations of Hbs and/or transcriptional deregulation in neurons may also be linked with aberrant function of neurons, and may be involved in pathogenesis of – at least a fraction – of age-related neurodegenerative diseases such as AD.

4. Protective roles of intraneuronal Hb's in AD and aging?

4.1. Mitochondrial localization of Hbs

Both Hba and Hbb co-localize within mitochondria in the mammalian brain (Shephard et al., 2014). It was proposed that the Hbs provide adequate levels of oxygen in close proximity to the oxidative phosphorylation machinery to prevent local hypoxia. Hbs reside within the inner membrane space, which is compatible with the observation that heme is synthesized within the mitochondrion; hence, it is plausible that heme containing proteins may also localize in the organelle where they are constructed (Shephard et al., 2014). Relative mitochondrial to cytoplasmic ratios of Hba and Hbb showed differential distributions in the brain in the Purkinje Cell Degeneration (pcd^{5j}) mouse model (Shephard et al., 2014) which is a model of ataxia, caused by a fast loss of Purkinje cells in the cerebellum. A significant decrease in mitochondrial Hba and Hbb content occurs at 31 days after birth, when dynamic neuronal loss reaches to peak levels in the pcd^{5j} mouse (Shephard et al., 2014). This study also reported changes in mitochondrial Hba and Hbb levels in aging mouse brain and demonstrated

a decline of Hba levels in aged brains. During analyses of PD brains, it was observed that cell bodies in the substantia nigra accumulated mitochondrial Hb (Shephard et al., 2014). However; Western blot analysis of mitochondrial fractions from PD and control brains indicated significantly less Hb in PD brain mitochondria (Shephard et al., 2014). One explanation could be a specific loss of cells (nigral neurons) containing mitochondria loaded with Hb proteins.

4.2. Levels of brain Hbs in patients with dementia and animal models

Lower β-globin protein levels were observed in the hippocampus and entorhinal cortex of people with AD in comparison to normal controls by a proteomics approach (Schonberger et al., 2001). In 2011, Yang et al. demonstrated that α-globin levels prominently declined in brain synaptosomes in a rat model of AD (Yang et al., 2011). Ferrer et al. investigated Hb expression in the brains of patients diagnosed AD, argyrophilic grain disease (AGD), Parkinson's disease (PD) and Dementia with Lewy bodies (DLB) (Ferrer et al., 2011). They showed decreases of α- and β-globin chains, in almost all neurons with hyperphosphorylated tau deposits in the frontal cortex and hippocampus in AD and in pre-tangles in AGD (Ferrer et al., 2011). Loss of Hb was also detected in swollen neurons in the amygdala in AD and AGD (Ferrer et al., 2011). Decreased Hb was also observed in about 80% of dopaminergic neurons of the substantia nigra, and in other altered neurons of the brain stem that contain abnormal α-synuclein small inclusions or Lewy bodies; on the other hand, Hb was preserved in neurons without intracytoplasmic inclusions (Ferrer et al., 2011). A transcriptome study of cortical astrocytes and microglia from young and aged mice showed changes of Hbs (Orre et al., 2014) Strikingly, the top genes with decreased expression in aged astrocytes included Hba-a2 (10.6-fold decrease), Hbb-b1 (10.2-fold decrease), and Hba-a1 (8.2-fold decrease) (Orre et al., 2014).

Analyses of samples from peripheral tissues obtained from aged subjects or patients with neurodegenerative disorders showed that Hbs levels change at different stages of the disorder. For example, higher Hba and Hbb levels in the cerebrospinal fluid (CSF) could be used to differentiate between AD-converters from non-converters in subjects with mild cognitive impairment (MCI), while Hbs did not indicate significant differences between AD or MCI and controls (Spellman et al., 2015). Hbs may first increase in response to certain toxic insults inside the neurons (specifically in mitochondria); but if the injury persists, Hbs may leak from dying neurons into the CSF explaining their statistically higher levels when MCI converts into manifest AD. A stringent protocol was employed to remove plasma proteins from CSF samples to avoid false results (Spellman et al., 2015). There is evidence that post-translational modifications of Hbs may also play important roles in AD pathogenesis. In samples of the inferior parietal lobule obtained from AD subjects, analysis of S-glutathionylated proteins revealed that oxyhemoglobin was among the main proteins that underwent glutathionylation in comparison to normal samples (Newman et al., 2007). Under normal conditions, glutathione (GSH) is a powerful antioxidant defense molecule and exists in neural cells at high levels (0.5–10 mM). The thiol group on the GSH's cysteine maintains the cellular redox state under oxidative conditions, but ongoing oxidative events may induce formation of disulfide bonds between GSH and protein cysteine side chains, which can be reversed by glutaredoxin (Newman et al., 2007). One could speculate that sustained and high levels of oxidative events may exhaust the potential of glutaredoxin and increase protein glutathionylation to supraphysiological extents. The attachment of glutathione to Hb results in an enhancement for oxygen affinity and a decline in the Bohr effect, which consequently reduces the release of oxygen and causes a decline in ATP production and mechanistically contributes to the pathogenesis of AD (Newman et al., 2007).

4.3. Affinity between Hb chains and amyloid-β. Compensatory or detrimental?

When Aβ-binding proteins were purified from rat brain, Hb α-chain was identified (Oyama et al., 2000). Aβ-complexes with Hb α-chain were found in the soluble fraction cerebral cortex in AD (Oyama et al., 2000). Hb was also identified as a major Aβ-binding protein in brain in a second study (Wu et al., 2004), and higher Hb levels were detected in AD brain (Wu et al., 2004). Further, Hb was colocalized with senile plaques and cerebral amyloid angiopathy in AD brains (Wu et al., 2004). Importantly, these studies also demonstrated that the Hb levels were elevated in amyloid pathology-associated brain regions (inferior temporal gyrus, cerebral parietal gray matter and parietal white matter) (Wu et al., 2004). In addition, Hb chain levels were reported to be increased in rat hippocampal CA1 region during normal aging by a microarray approach (Blalock et al., 2003).

Detailed mechanistic studies were used to characterize the binding of Aβ with different components of Hb: 1) apohemoglobin (apoHb) or heme-free Hb, 2) heme and 3) protoporphyrin IX (PpIX) ring or iron-free heme (Chuang et al., 2012). Neurotoxicity of heme and Aβ or their mixture was also investigated in vitro (Chuang et al., 2012). In vivo interaction between Hb and Aβ was tested by injection of Hb into the hippocampi of APP/PS1 tg (APP expressed with prion promoter-transgenic) mice. These studies showed that 1) the binding of Hb to Aβ required iron-containing heme; 2) other heme-containing proteins, such as myoglobin and cytochrome C also bound to Aβ; 3) heme cytotoxicity was lowered in neuroblastoma cells by low levels of Aβ; 4) Hb existed in neurons and glia of brains and was increased in aging and APP/PS1 transgenic mice; 5) after microinjection into the dorsal hippocampi of the APP/PS1 transgenic mice, envelope-like structures composed of Aβ surrounding Hb developed (Chuang et al., 2012). It was also observed that Hb was present in amyloid plaques, in neurons and oligodendrocytes (Chuang et al., 2012).It was postulated that elevated Hb in cells of the aging brain serves as a compensatory component against hypoxia (Chuang et al., 2012). Aβ binding to Hb and other hemoproteins via the iron-containing heme moiety may be an intrinsic protective mechanism to prevent Hb/heme/iron-induced cytotoxicity (Chuang et al., 2012). Heme-induced cytotoxicity was reduced by Aβ, presumably by the Aβ-iron interaction. Hb and other hemoproteins are potentially harmful (especially when existing in the extracellular milieu) because their heme iron can trigger a Fenton reaction to generate a hydroxyl-radical (Chuang et al., 2012). These studies also indicated that by binding to Aβ, the hemorrhage-associated Hb/heme toxicity can be limited (Chuang et al., 2012). When microhemorrhages occur during aging, traumatic brain injury or cerebrovascular disease, Aβ can act as a vascular sealant preventing further leakage of free Hb and heme iron (Chuang et al., 2012). A vessel-sealing function of Aβ is suggested by the observation that Aβ is up-regulated after traumatic brain injury, and removal of deposited Aβ from the vessels by anti-Aβ antibodies enhanced the frequency of intracerebral hemorrhage in APP tg mice (Chuang et al., 2012). It is also possible that continous extravasation of Hb and release of iron may augment Aβ-toxicity during chronic phases of AD, since iron aggravates the neurotoxicity of high levels of Aβ, lipid peroxidation and neurotoxicity (Chuang et al., 2012). On the other hand, high-molecular weight oligomeric soluble Aβ species caused proinflammatory activation of astrocytes which was reduced when Aβ was associated with Hb or heme (Sankar et al., 2018). In astrocyte cultures, exposure to $A\beta_{1-42}$ strongly induced production of IL-1β, GM-CSF and RANTES, all of which were reduced by cotreatment with heme or Hb (Sankar et al., 2018). Thus, Hbs shall be in the right place at the right time to alleviate neuronal injury.

5. Any role of blood contamination in studies of Hb's in brain?

Since samples of brain may contain variable amounts of blood, it is unclear to what extent Hbs found in brain specimens is located in

neurons or blood cells. However, Hb expression in neurons was shown at the cellular level using immunohistochemistry (IHC) e.g. in the cytosol of hippocampal pyramidal neurons (Wu et al., 2004) and cortical, hippocampal and cerebellar neurons (Schelshorn et al., 2009). Hbα-positive cells were shown to display higher oxygenation than their unstained neighbours (Schelshorn et al., 2009). In transcriptomics studies of laser-capture microdissected rat nigral dopaminergic neurons and mouse striatal neuron, Hbα, adult chain 2 (Hba-a2) and Hbβ (Hbb) transcripts were found, whereas erythroid markers were absent (Richter et al., 2009). qRT-PCR analyses proved the expression of Hba-a2 and Hbb in rat nigral dopaminergic neurons, striatal GABA-ergic neurons, and cortical pyramidal neurons. Combined IHC with the neuronal marker (NeuN) and in situ hybridization proved the existence of Hb mRNAs in neurons in rat brain (Richter et al., 2009). Taken together, IHC identified Hbα- and β-chains in both human and rat brains, and Hb proteins were found by Western blotting in mesencephalic neuron cultures, further excluding blood contamination (Richter et al., 2009). The Hb presence of Hbs in MS cortex was also shown by IHC (Brown et al., 2016).

Hb mRNA levels were analyzed in cerebral tissue of patients afflicted by Creutzfeldt-Jakob disease (CJD), in different genetic types of prion diseases (gPrD), in AD patients and in age-matched controls (Vanni et al., 2018). RT-qPCR was employed to determine Hb transcripts HBB and HBA1/2, and four different reference genes were used for normalization. Importantly, expression analysis of the specific red blood cell marker ALAS2 was performed to exclude the possibility of blood contamination (Vanni et al., 2018). The expression of Hbα1/2 and Hbb proteins was also determined with confocal and immunofluorescence microscopy (Vanni et al., 2018). A significant increase of HBA1/2 in variant CJD brains and a significant decrease of HBB in iatrogenic CJD was revealed. Transcription of Hb chains did not change in sporadic and genetic types of prion diseases but Hb chains were significantly decreased in AD brains which was confirmed at the protein level (Vanni et al., 2018). The findings for Hbs in regard to AD are in the opposite direction as in previous studies (Blalock et al., 2003; Wu et al., 2004). This discrepancy may be associated with the stage of disease. While Hbs may increase as compensatory mechanisms against neurodegenerative actions of Aβ, sustained injury may lower Hb levels.

6. Hb chains and α-synuclein

The role of Hbs in aging associated neural damage can also be discussed in relation to studies on Multiple System Atrophy (MSA) and Parkinson's Disease (PD). MSA is defined as an α-synucleinopathy, an umbrella definition which covers neurodegenerative disorders in which aggregation of α-synuclein (α-syn) is a key step in pathophysiology (Mills et al., 2015). Major α-synucleinopathies include PD, dementia with Lewy bodies (DLB) and a variety of other neuroaxonal dystrophies (Galvin et al., 2001; Mills et al., 2015). By employing a transcriptomics approach, an enhanced expression of the α- and β-globins was shown in the cerebral white matter of MSA patients (Mills et al., 2015). Abnormal accumulation of the monomeric protein and formation of small α-syn aggregates is harmful to neurons (Yang et al., 2016). Importantly, α-syn and Hb can form complexes in brain tissue and RBCs in aging cynomolgus monkeys (Yang et al., 2016). Free Hb and Hb-α-syn complex levels in mitochondria of the striatum decreased with age which was negatively correlated with cytoplasmic Hb levels (Yang et al., 2016). Cytoplasmic α-syn aggregates may sequester Hb-α-syn complex and hinders Hb translocation from the cytoplasm to mitochondria (Yang et al., 2016). Mitochondrial α-syn accumulation would increase Hb-α-syn complex formation and reduce free mitochondrial neuronal Hb. Hb overexpression enhanced free Hb levels in mitochondria, stabilized mitochondrial membrane potential, and lowered α-syn-induced apoptosis (Yang et al., 2016). Interestingly, RBCs contain 1000-fold higher levels of α-synuclein than the cerebrospinal fluid, and RBC-derived microvesicles penetrate the CNS and carry this protein to the microglia in inflammatory conditions (Matsumoto et al., 2017). In this study it was proposed that misfolded Hb proteins may cause RBC injury and propagate microvesicle formation which would enter the brain and activate microglia; and that Hb may increase aggregate formation of α-synuclein and then can cause RBCs to release more α-synuclein and leading to a vicious circle (Matsumoto et al., 2017). The degree of accumulation and complexation with other proteins may determine the net outcome of Hb effects on mitochondrial injury and cell death. While appropriate levels of Hbs may be neuroprotective, supraphysiologically high levels of Hbs may propagate dopaminergic cell injury. A surprising finding is that Hbs produced aggregates and lead to impairment of motor learning when adeno-associated viruses carrying α- and β-chains were stereotaxically injected into mouse substantia nigra (Codrich et al., 2017). An increase of HBA1, HBA2 and HBB was found in amygdala of schizophrenic patients which remains to be explained (Chang et al., 2017). Chronic unpredictable mild stress induced depression-like behaviors in mice which was accompanied by increases of Hb mRNAs in nucleus accumbens (Ma et al., 2019).

7. High levels of extracellular Hb in circulation may facilitate further Hb entry into neuropil via damaging the BBB

Vascular risk factors including high homocystein and cholesterol levels and type II diabetes are harmful to the cerebrovascular system by inducing silent strokes and perturbing of β-amyloid clearance at the blood-brain barrier resulting in enhanced cerebral levels of β-amyloid (Humpel, 2011; Foidl and Humpel, 2019). In this context, it should be emphasized that free Hb may contribute to blood-brain barrier (BBB) damage. The effects of free Hb on the endothelial tight junction proteins (zonula occludens 1 (ZO-1), claudin-5), astrocyte activation, IgG extravasation, heme oxygenase (HO), iron deposition and oxidative end-products were determined in guinea pigs (Butt et al., 2011). ZO-1 expression was reduced after free Hb transfusion, and the distribution of Claudin-5, but not the total expression, was shifted from small- to medium-sized vessels (Butt et al., 2011). Transfusion of free Hb increased astrocytic activity and IgG extravasation, and significant increases of oxidative end-products in BBB-endothelia were found (Butt et al., 2011). CD163, the macrophage receptor for Hb-haptoglobin complexes, was expressed in perivascular/meningeal macrophages and pericytes (Butt et al., 2011). Hb- and heme-triggered inflammatory pathways also enhanced endothelial adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), which enhance leukocyte adhesion and transmigration. Inflammatory leukocytes release oxidant molecules, which will further damage BBB and trigger a vicious cycle. These studies lead to the concept that an intact BBB plays a key role in blocking the entry of neurotoxic free Hb into the CNS (Butt et al., 2011). However, prolonged hemolysis may saturate the buffering mechanisms of free Hb (such as plasma haptoglobin), which may facilitate Hb entry to the CNS via damaging BBB. Furthermore, both free-Hb and heme exacerbate inflammatory responses and activate the innate immunity through Toll-like receptor 4 (TLR4) on microglia and other innate immune cells (Bamm and Harauz, 2014). Indeed, neurologic complications are frequent in conditions such as chronic or intermittent intravascular hemolysis, genetic and drug-induced hemolytic anemias and microbial infections (Butt et al., 2011).

8. Neurotoxicity of Free Hb diffused into the brain in AD

Studies on the relationship between the entry of Hb's into the brain tissue and AD date back to 1994 when the cerebellar peptides in AD were studied by reverse-phase HPLC (Slemmon et al., 1994). Cerebellum was chosen in initial studies because it does not show significant neuronal loss but does show plaque formation in AD. Several fragments of Hb were found and immunolocalization of Hb was preferentially around blood vessel walls and granule cells (Slemmon et al., 1994).

Elevated Hb peptides suggested either disruption of the BBB for Hb or selective extravasation of smaller peptides (Slemmon et al., 1994). The presence of Hb cleavage products in AD cerebellum was proposed to be neurotoxic. The hypothesis was proposed that RBCs in AD patients contain more surface bound IgG and display increased proteolysis and oxidative stress which may trigger their lysis and subsequent increase of Hb fragments at the endothelial wall (Slemmon et al., 1994).

Cerebrovascular diseases common in AD, including cerebral amyloid angiopathy, microvascular degeneration (capillary tortuosity, string vessels, vascular fibrohyalinosis and lipohyalinosis), microinfarcts, subcortial lacunes and micro-hemorrhages, alter cerebral vessel integrity and hinder brain perfusion (Chuang et al., 2012). It remains unclear whether leakage from microvasculature is critical to plaque formation (Stone, 2008). The colocalisation of heme and plaques suggested that an elevation in brain Hb via leakage or perturbations of Hb gene regulation may participate in AD pathogenesis (Stone, 2008). A vicious positive-feedback mechanism between microbleeds and amyloid plaque formation in AD was proposed (Stone, 2008) in which plaque formation begins with a microhemorrhage due to increased fragility in aging, that releases blood into the local neuropil and causes local ischaemia via endothelial retraction. Ischaemia increases neural secretion of soluble monomeric β-amyloid, and then components of blood (including Hbs) interact with β-amyloid, causing oligomers to self-assemble into insoluble amyloid plaques that are neurotoxic. Additionally, excess β-amyloid perturbs the BBB and the blood-related proteins enter to create a nidus for plaque formation (Stone, 2008). In this model, interaction between Hb entering the brain tissue and β-amyloid is one of several possible mechanisms; indeed, Hb was found to co-precipitate with β-amyloid in homogenized brain tissue from AD patients (Stone, 2008).

Furthermore, the γ(A)-globin subunit of fetal Hb (HbF) was determined as an Aβ-binding partner (Perry et al., 2008). Surface plasmon resonance studies confirmed that both HbA and HbF specifically bind to Aβ1-42, and metHbA binds to Aβ with significantly higher affinity than metHbF (Perry et al., 2008). Further experiments showed that Aβ may preferably enhance the injury and lysis of RBCs containing HbA versus those RBCs containing HbF which is related to peroxidase activity of the Aβ-heme complex (Perry et al., 2008). The role of HbF is of interest, because 89% percent of adult HbF and F cell (RBCs carrying HbF) variance is inherited and the *Xmn*I polymorphism (C → T) at position −158 of the *HBG2* promoter accounts for the greatest share of this genetic variability (Perry et al., 2008). The *Xmn*I polymorphism is associated with higher F cell/HbF levels (Perry et al., 2008). A reduced frequency of the T allele was found in the AD patients relative to the healthy subjects which associates with the higher HbF:HbA ratio (Perry et al., 2008). Because metHbF does not bind Aβ1-42 as avidly as metHbA, metHbF-Aβ complex may be less toxic resulting in lesser RBC lysis and lesser release of neurotoxic free Hb (Perry et al., 2008).

9. Abnormal levels of Hb are detrimental for brain health and cognition

Low Hb levels (anemia) are a major risk factors for increased frailty, decreased executive functioning, impaired motor performance and increased mortality (Peters et al., 2008). The association between AD and anemia has been studied the elderly (Beard et al., 1997). In a case-control study, an almost twofold increase in occurrence of AD (OR, 1.88) was associated with anemia (Hb level < 12 g/dl for women and < 13 g/dl for men) Association of dementia with Hb levels was studied in a rural elderly sample in the Indian population (Pandav et al., 2004). Hb was inversely associated with AD after adjustment for age, sex, and literacy with every increase in Hb concentration by unit 1.0 g/dl decreasing the probability of AD by 30% (Pandav et al., 2004). Using baseline data from a community-based longitudinal study, 1435 non demented subjects aged 75–95 years were followed for 3 years to detect incident dementia cases (DSM-III-R criteria) (Atti et al., 2006). Subjects with anemia (baseline Hb < 13 g/dl (for men) and < 12 g/dl (for women)), had higher hazard ratios (1.6) of developing dementia 3 years later (Atti et al., 2006). In persons with good baseline cognition (MMSE ≥ 26), the association was stronger and remained statistically significant after adjustments for chronic diseases, inflammatory markers, and indicators of nutrition (Atti et al., 2006).

A meta-analysis examined the relationship between cognitive decline or dementia in the elderly and anemia, i.e. Hb level (Peters et al., 2008). Inclusion criteria were longitudinal studies of subjects aged ≥65, with primary outcomes of incident dementia or cognitive decline. The pooled hazard ratio was determined as 1.94 (CI 1.32–2.87) showing a significantly increased risk of incident dementia with anemia (Peters et al., 2008). In the Rush Memory and Aging Project, global cognitive functioning, semantic, episodic and working memory, perceptual speed and visuospatial abilities were measured (Shah et al., 2009). Using regression models adjusted for several variables, low Hb levels were associated with lower global cognitive function ($p = 0.019$), and Hb was related to semantic memory and perceptual speed (Shah et al., 2009). In an second study, annual cognitive assessments for AD were performed on 881 community-dwelling older persons participating in the Rush Memory and Aging Project without dementia (Shah et al., 2011); during an average of 3.3 years of follow-up, 113 persons developed AD. When compared to participants with clinically normal Hb ($n = 717$), participants with anemia ($n = 154$) had a 60% increased risk for developing AD (Shah et al., 2011). The same group analyzed Hb and neuropathological changes in the Rush Memory and Aging Project (Shah et al., 2012). Using logistic regression models adjusted for age at death, gender, and education, each g/dl lower Hb level enhanced the odds for having a chronic macroscopic infarction by 37% (Shah et al., 2012). In the Australian Imaging Biomarker and Lifestyle (AIBL) participants with mild cognitive impairment (MCI), with AD and healthy controls were studied for the role of anemia (Faux et al., 2014). Individuals with AD had significantly lower Hb, mean cell Hb concentrations, packed cell volume and higher erythrocyte sedimentation rates (adjusted for age, gender, APOE-ε4 and site) (Faux et al., 2014). There was a strong association between anemia and AD (OR = 2.43), and anemia emerged as a stronger potential risk for AD than aging 5 years (Faux et al., 2014). The significantly greater prevalence of abnormally high serum ferritin in AD together with lower plasma iron levels and transferrin saturation in AD, argue that tissue iron stores are not adequately mobilized in AD which is consistent with iron accumulation in the AD neocortex (Faux et al., 2014). It was postulated that Aβ enriched in RBC-membranes in AD oxidizes RBC Hb and potentially contributes to anemia in AD (Faux et al., 2014).

Two independent cohorts were assessed as to whether anemia associates with cognitive dysfunction and AD: AddNeuroMed includes a longitudinal analysis of 738 subjects including AD and age-matched controls with hemogram values and cognitive evaluations; and UK Biobank, a study including 502,649 healthy subjects, aged 40–69 years with cognitive test assessments and hemogram indices at baseline (Winchester et al., 2018). Analysis of the AddNeuroMed revealed a significantly greater decline in RBC indices for AD patients in comparison to controls (p values between 0.05 and 10–6). Lower Hb levels were found in subjects with reduced cognitive function in the UK Biobank cohort (Winchester et al., 2018). A significant association existed for MCH and RBC distribution width (a measure of cell volume variability) compared to four different cognitive function assessments which include reaction time and reasoning ($p < 0.0001$). By employing Mendelian randomisation, a significant influence of MCH on the verbal–numeric and numeric traits was revealed, indicating that anemia has a causative effect on cognitive dysfunction (Winchester et al., 2018). The relationship between Hb levels, cerebral small-vessel disease (CSVD), and cortical atrophy was investigated using neuroimaging in cognitively healthy persons (Park et al., 2016). Decreased Hb levels were associated with decreased cortical thickness in the frontal, temporal, parietal and occipital regions (Park et al., 2016). Taken together,

reduced oxygen transport to the brain due to anemia influences cognitive functions and increases the risk of dementia.

10. Hemorphin's and Alzheimer's disease

Another interesting connection between Hb and brain function arises from the study of the peptide LVV-hemorphin 7 (LVV-H7) which is a degradation product of the β-globin chain (Albiston et al., 2004). The angiotensin AT4 receptor was originally described as the specific, high affinity binding site for the hexapeptide angiotensin IV (Ang IV) but binding of LVV-H7 was also demonstrated at the AT4 receptor (Albiston et al., 2004). High affinity AT4 receptor-binding sites are found in brain areas associated with memory, i.e. basal forebrain cholinergic nuclei, the medial septal complex and the basal nucleus of Meynert, and their terminal fields, such as the hippocampus and neocortex (Albiston et al., 2004). Importantly, both Ang IV and LVV-H7 increased memory function in normal and scopolamine treated rats (Albiston et al., 2004). Future studies should examine whether minor Hb mutations (without clinical manifestations) can influence the Hb cleavage to LVV-H7, and whether altered LVV-H7 levels subsequently play a role in AD.

11. Erythropoietin/Hb axis with particular relevance to treatment of AD

Erythropoietin (EPO) is responsible for regulating red cell production, including stimulation of Hb synthesis (Teramo et al., 2018). EPO is expressed in adult human brain (Chin et al., 2000) and EPO has been reported to exert neuroprotective, antiapoptotic, antiinflammatory and antioxidant effects (Teramo et al., 2018). It appears that EPO may be also involved in regulating brain levels of Hb, as treatment of mice with EPO (5000 IU/kg i.p.) increased expression of brain Hbβ, mitochondrial complex III, complex V, enhanced mitochondrial respiration and levels of H3K4me3 (Singhal et al., 2018). In the cuprizone mouse model of multiple sclerosis (MS), EPO prevented the mitochondrial injury and the reductions of complex III, NAA and Hbβ (Singhal et al., 2018). EPO (1000 IU/kg) treatment of mice reduced Aβ42-induced cognitive decline and tau hyperphosphorylation via glycogen synthase kinase-3β (GSK-3β) and reduced the Aβ42-induced cerebral mitochondrial injury and apoptosis (Li et al., 2015). In a transgenic mice model of AD (APPSwe mice), an intranasal formulation of EPO (Neuro-EPO (125, 250 μg/kg)) reduced cognitive deficits and amyloid toxicity as well as amyloid load (Rodríguez Cruz et al., 2017). Neuro-EPO also reduced oxidative injury, apoptosis and neuroinflammation (Rodríguez Cruz et al., 2017). In a rat model of AD induced by intracerebroventricular injection of streptozotocin, the increased latency time on the passive avoidance learning tests, enhanced cerebral TNF-α levels, reduced choline acetyltransferase activity, and the loss of hippocampal CA1 and CA3 neurons were observed, which were all rescued by EPO treatment (Cevik et al., 2017). In a rat model of AD, induced by intrahippocampal administration of $A\beta_{25\text{-}35}$, an increase of MAPKs (Mitogen Activated Protein Kinases) and MMP-2 (Matrix Metalloprotease-2) activity and a dysregulation in Akt/GSK-3β pathway was observed along with cognitive deficits (Hooshmandi et al., 2018). In this model, a carbamylated EPO-Fc analog (CEPO-Fc) reduced memory deficits and blocked the increase of hippocampal of ERK, P38, MMP-2 and also Akt/GSK-3β pathway perturbations induced by $A\beta_{25\text{-}35}$ (Hooshmandi et al., 2018). Importantly, very recent studies demonstrated that an EPO variant without hematopoiesis-stimulating efficacy called EpoL protected against Aβ-induced neurotoxicity via EpoR (Castillo et al., 2019). Erythropoietin is also shown to reduce oxidative stress-induced death (eryptosis) of red blood cells (Sun et al., 2018). In future studies, the effect of EpoL on Hb synthesis in the brain should be explored.

12. Conclusions and future prospects

In this review, we aimed to integrate findings linking Hbs with the pathogenesis of AD. Thus, Hbs are present in neurons, with both α- and β-globin chains being localized in soma and β-chains in dendrites. Hbs also localize in the inner membrane of mitochondria in neurons. In AD, reduced levels of Hb chains have been observed in cells with pretangles and tangles while other studies have found increased expression of Hb chains in brain homogenates; this discrepancy may reflect different roles of intracellular versus extracellular Hbs. While intracellular Hbs may protect neurons against hypoxia, extracellular free Hb (and its degradation products heme and free iron) may damage the blood-brain barrier and facilitate further Hb entry from the blood into the brain. Chronic hemolysis may saturate free-Hb scavenging systems and lead to accumulation of free-Hb around cerebral microvessels. Indeed, amyloid-β (Aβ) also exists in RBCs and Aβ-heme complex acts as a prooxidant peroxidase. Further, RBCs contain more surface bound IgG and display higher proteolysis and oxidative stress in AD patients. Avidity between Aβ and Hb may be beneficial in mutual ways that Aβ reduces toxicity of free Hb and vice versa, Hb may reduce detrimental effects of Aβ against BBB (Zhao et al., 2013). However, at advanced stages of AD, this avidity may facilitate aggregation of Aβ fibrils. There is also a strong epidemiological link between anemia and decline of cognitive function, including AD. One could speculate that a pathogenic mechanism associated with AD may reduce intra-erythrocytic Hbs as well as neural Hbs, which reduces oxygen delivery to the brain and oxygen-storage within the neurons. Stimulating erythropoiesis, and inducing intracellular neural Hbs may exert multiple benefits against chronic course of AD. EPO is a candidate to exert these multiple benefits including improvement of cognitive functioning.

In future, experimental models and clinical studies should focus on specific issues to explore the roles of Hbs in AD: i) Different neural cell lineages can be created to overexpress certain Hb chains and their vulnerability to β-amyloid toxicity can be tested. ii) In transgenic mouse models, cell type (neuron, astrocyte, oligodendroglia)-specific deletions and overexpression of selective Hb chains can be performed to ascertain whether these changes could ameliorate or increase the disease burden in AD-mice. iii) Laser capture-microdissection and in situ proteomic analyses on AD-brains can be performed to illuminate the discrepant results which showed increased and decreased levels of Hbs in AD brains and to reveal whether these differences occur due the differential extent of injury. iv) Signaling mechanisms which direct Hb chains into mitochondria should be studied. v) It should be tested whtther genetic mechanisms reducing the level Hb transcription or occult minor Hb mutations may be involved in pathogenesis of AD whereby anemia may be used for screening. vi) Levels of protective hemorphins shall be measured in AD patients with proteomic methods. Overall, studies focusing on the role of Hbs in AD may provide precious novel data for illuminating disease pathogenesis and discovery of novel treatment strategies.

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