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REVIEW PAPER

The Promises and Challenges of Erythropoietin for Treatment of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder in the world, and intracellular neurofibrillary tangles and extracellular amyloid-beta protein deposits represent the major pathological hallmarks of the disease. Currently available treatments provide some symptomatic relief but fail to modify primary pathological processes that underlie the disease. Erythropoietin (EPO), a hematopoietic growth factor, acts primarily to stimulate erythroid cell production, and is clinically used to treat anemia. EPO has evolved as a therapeutic agent for neurodegeneration and has improved neurological outcomes and AD pathology in rodents. However, penetration of the blood–brain barrier (BBB) and negative hematopoietic effects are the two major challenges for the therapeutic development of EPO for chronic neurodegenerative diseases like AD. The transferrin receptors at the BBB, which are responsible for transporting transferrin-bound iron from the blood into the brain parenchyma, can be used to shuttle therapeutic molecules across the BBB. In this review, we discuss the role of EPO as a potential neurotherapeutic for AD, challenges associated with EPO development for AD, and targeting the BBB transferrin receptor for EPO brain delivery.

Keywords Erythropoietin · Alzheimer's disease · Transferrin receptor · Blood–brain barrier · Molecular Trojan horse

Pathological Mechanisms and Recent Insights of Alzheimer's Disease

Alzheimer's disease (AD), a neurodegenerative disorder accounting for more than 60% of the 36.5 million dementia cases worldwide, is characterized by progressive memory loss, cognitive decline, and an inability to complete daily tasks such as driving, housekeeping, and keeping track of finances (Sosa-Ortiz et al. 2012). In 2017, the average cost of health care and long-term care for a patient with AD was $48,000/year. Furthermore, care provided by family members and unpaid caregivers was estimated at $232 billion (2018 Alzheimer's disease facts and figures. 2018). Since AD incidence in individuals ≥85 years old is expected to increase from 40% in 2001 to 62% in 2050 with the aging of the baby boomer population, the disease burden of AD in coming years will be immense (Hebert et al. 2001).

During early stages of AD, patients may exhibit either anosognosia, an inability to perceive their illness, or simply ignore their symptoms, attributing their memory loss to normal aging (Barrett et al. 2005). These factors greatly contribute to the delay in diagnosis and the underestimation of disease incidence and prevalence.

As the disease progresses, memory impairment is followed by motor and visuospatial deficits, with patients experiencing extrapyramidal symptoms, apraxia, or seizures (Portet et al. 2009; Giannakopoulos et al. 1998; Vossel et al. 2017). In late-stage AD, patients may become disinhibited, withdrawn, or mute, therefore requiring daily supervision while eating, dressing, and bathing (Sarazin et al. 2005). These patients begin to rely heavily on caregivers, leading to an increased incidence of caregiver burnout (Dauphinot et al. 2015). This burnout often persists for years, as the median time from diagnosis to death in AD patients is 3.8 years,

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with many dying of malnutrition or infection (James et al. 2014; Mitchell et al. 2009). Some patients may live with the disease much longer, causing a strain on financial and societal resources for decades.

Pathogenesis

Many hypotheses have been developed to elucidate AD pathogenesis; however, the amyloid cascade, cholinergic, and inflammatory hypotheses remain among the most heavily studied (Hardy 2009; De Felice et al. 2008; Eikelenboom et al. 2006; Bartus et al. 1982). An infectious origin of the disease has also gained traction in recent years (Mawanda and Wallace 2013).

The Amyloid Cascade Hypothesis

The leading model for AD progression is the amyloid cascade hypothesis, which posits that the deposition of β-amyloid (Aβ) is the initial pathological event in AD leading to the formation of senile plaques followed by neurofibrillary tangles (NFT). This leads to downstream neuronal cell death and ultimately dementia due to central nervous system (CNS) dysfunction (Hardy and Higgins 1992). Aβ is a protein consisting of 38–43 amino acids; it is formed proteolytically when amyloid precursor protein (APP) is cleaved by β- and γ-secretases via the amyloidogenic pathway (Haass and De Strooper 1999; Chow et al. 2010; Gremer et al. 2017). The role of APP in synaptic transmission helps explain its endogenous abundance within the brain (Priller et al. 2006). Two forms of Aβ that are abundantly found in the AD brain are $A\beta_{40}$ and $A\beta_{42}$, the latter being more abundant and exhibiting greater CNS toxicity (Potter et al. 2013; Wildburger et al. 2017). As Aβ accumulates within the brain, it forms insoluble neuritic plaques, which contain a central amyloid core surrounded by activated microglia (Hong et al. 2016). Furthermore, Aβ plaque accumulation induces hyperphosphorylation of tau protein resulting in further toxicity to the CNS (De Felice et al. 2008). Hyperphosphorylated tau is unable to bind and stabilize microtubules, and instead aggregates to form NFT, leading to downstream synaptic loss and impaired neuroregeneration (Zhang et al. 2014). In concurrence with their involvement in AD pathology, Aβ and tau are often measured in the cerebrospinal fluid of patients for disease staging and prognostication (Braak et al. 2006; Thal et al. 2002), and a lot of research efforts are focused on reducing the burden of Aβ and NFT aggregation in the brain to stop AD progression (Panza et al. 2012).

Neurotransmitter Dysfunction and Synaptic Loss

Aβ and NFT disrupt normal synaptic transmission to neighboring cerebral areas (DeKosky and Scheff 1990). Neurotransmitters are often seen in abnormal amounts in AD patients (Lombardo and Maskos 2015; Wenk 2003; Kandimalla and Reddy 2017). For example, choline acetyltransferase, which is involved in the synthesis of acetylcholine, one of the most studied neurotransmitters in AD pathogenesis, is decreased in post-mortem AD brains (Slotkin et al. 1990). The excitatory neurotransmitter, glutamate, accumulates in AD brains, leading to increased *N*-methyl-D-aspartate (NMDA) receptor activation (Revett et al. 2013) and Aβ plaque formation (Dinamarca et al. 2012). Furthermore, Aβ plaque formation weakens synaptic inhibition via downregulation of *gamma*-Aminobutyric acid receptors, causing increased excitotoxicity in the CNS (Ulrich 2015). Serotonin receptors, which are involved in cognitive processing, are also downregulated in the cortical and hippocampal areas, contributing to cognitive decline in AD brains (Reynolds et al. 1995). Histamine, another neurotransmitter important in cognitive functioning, also plays a crucial role in the pathogenesis of AD (Kandimalla and Reddy 2017). Overall, improper synaptic communication has been linked to an imbalance in neurotransmitter levels, causing downstream synaptic loss and cognitive decline. Thus, drugs that work to replenish normal neurotransmitter levels have been developed for AD (Reisberg et al. 2003; Bartus et al. 1982).

Mitochondrial Dysfunction and Oxidative Stress

Mitochondrial dysfunction, a naturally occurring part of the aging process, is an early finding in AD pathogenesis. Aβ and tau aggregates impair mitochondrial function, causing oxidative stress within the cell that results in free radical damage (Eckert et al. 2012). This oxidative stress induces further Aβ accumulation and tau phosphorylation, causing a self-perpetuating cycle of mitochondrial dysfunction, oxidative damage, and plaque formation (Leuner et al. 2012; Melov et al. 2007).

Microglial Activation and Inflammation

The role of neuroinflammation in AD pathogenesis is now widely recognized. In the CNS, microglia are involved in synaptic remodeling and tissue surveillance. However, pathologic stimuli, including Aβ plaque accumulation, can result in sustained microglial activation, and in the AD brain, Aβ plaques and NFT are often found surrounded by activated microglia (Wang et al. 2015). Activated microglia are involved in Aβ plaque clearance; however, sustained microglial activation can perturb this clearance process. Activated microglia secrete pro-inflammatory cytokines like tumor necrosis factor α (TNF-α), and chemokines that further propagate an inflammatory response and neuronal degeneration in AD (Fan et al. 2015; Rogers et al. 1996; Heneka et al. 2015). Astrocytes, T-cells, complement proteins, and other

acute phase reactants are also recruited to the sites of inflammation, causing an inflammatory storm that results in tissue damage. Moreover, widespread inflammation encourages free radical formation, inciting further damage (McGeer and McGeer 2003). Besides accelerating the progression of AD, growing evidence shows that inflammatory processes may drive AD pathology, thus providing a long window of opportunity for therapeutic intervention (Wyss-Coray 2006). Therapeutic strategies to mitigate neuroinflammation in AD, including the development of non-steroidal anti-inflammatory drugs, peroxisome proliferator-activated receptor (PPAR)-γ agonists, and cytokine inhibitors, have been/or are under investigation (Ardura-Fabregat et al. 2017).

Genetic Risk Factors

Many genes play a role in the development of AD; however, the most heavily associated is the gene encoding apolipoprotein-E (Apo-E), a protein involved in cholesterol transport (Castellano et al. 2011). The Apo-E ε4 allele contributes to poor Aβ clearance from the brain and is a strong genetic risk factor for late-onset, sporadic AD. On the other hand, the Apo-E ε2 allele confers AD protective effects and is associated with increased age at onset (Castellano et al. 2011). Apart from Apo-E, increased APP expression greatly contributes to disease progression. Encoded by a gene on chromosome 21q21.3-22.05, higher levels of APP cause familial early-onset AD in patients with Down syndrome, owing to their extra copy of chromosome 21 (Schupf et al. 1998). Lastly, mutations in the genes encoding presenilins, neuronal proteins widely expressed in the CNS, are also associated with familial early-onset AD. Presenilin 1 and presenilin 2 found on chromosome 14q24.3 and 1q31-42, respectively, are critical components of the γ-secretase complex and are involved in the amyloidogenic cleavage of APP (Brunkan and Goate 2005; Rogaev et al. 1995). Other emerging genes associated with AD risk include genes involved in synapse turnover, clathrin-mediated endocytosis, amyloid clearance, and inflammation cascades (Karch and Goate 2015).

Current Treatment Options

Currently, there is no pharmacologic cure for AD, and drugs are prescribed to enhance cognition and treat AD symptoms. FDA-approved drugs for AD belong to two classes: acetylcholinesterase inhibitors and NMDA receptor antagonists. Acetylcholinesterase inhibitors currently approved for AD are donepezil, rivastigmine, and galantamine. These drugs act by increasing the amount of acetylcholine in the brain to reestablish proper synaptic communication (Bartus et al. 1982). However, these drugs only perform with modest efficacy and are approved for mild-to-moderate AD. The NMDA receptor antagonist memantine, on the other hand, works by blocking NMDA receptors to regulate glutamatergic neurotransmission and prevent synaptic loss, which is thought to improve cognition in AD (Danysz et al. 2000). AD patients taking memantine show improved functioning and decreased rate of decline upon cognition testing (van Marum 2009).

Apart from symptomatic treatment for AD, there are a number of anti-Aβ and anti-tau therapies in different stages of clinical development (Bachurin et al. 2017). AN1792 was the first active immunotherapy against $A\beta_{42}$, however was discontinued due to reports of meningoencephalitis (Nicoll et al. 2003). Passive forms of immunotherapy against Aβ, including bapineuzumab and solanezumab, which act on soluble Aβ, performed poorly in Phase III clinical trials (Panza et al. 2014). Other anti-Aβ agents under development include antibodies that target aggregated Aβ, including aducanumab and gantenerumab. Aducanumab slowed cognitive decline and reduced Aβ plaques in patients with early-stage AD, and is currently being tested in Phase III trials of early-onset AD. Gantenerumab is currently in phase III trials for prodromal AD.

Additionally, β- and γ-secretase inhibitors that prevent the cleavage of APP to pathogenic Aβ, like verubecestat and semagacestat, have been investigated to reduce the burden of Aβ plaques in the brain; however, clinical trials were largely unsuccessful (Wan et al. 2009; Doody et al. 2013). Meanwhile, tau-based immunotherapies are currently in early stages of clinical development. RG7345, which is a humanized monoclonal antibody targeting the tau phosphoepitope pS422, caused an improvement in a transgenic mouse AD model but was discontinued after Phase I clinical trials (Collin et al. 2014; Sigurdsson 2018). Besides these approaches, drug candidates that target different neuronal receptors (e.g., serotonin receptor ligands), enzyme inhibitors (e.g., lipoprotein-associated phospholipase A2 inhibitor), neurotrophic drugs, and anti-inflammatory (e.g., etanercept) are currently under development (Bachurin et al. 2017).

Erythropoietin and AD

Erythropoietin (EPO), a 30.4 kDa hematopoietic growth factor produced in the kidney, acts primarily to stimulate erythroid cell production by supporting the survival, proliferation, and differentiation of erythroid progenitor cells (Jelkmann 2013). Recombinant human EPO is FDA approved and clinically used to treat anemia (Ng et al. 2003). In the past decade, over 60 studies have demonstrated the neuroprotective role of EPO in neurodegenerative conditions (Jelkmann 2005). As shown in Fig. 1, EPO improves neurological outcome in experimental AD by reducing Aβ load, inflammation, oxidative stress, apoptosis, and neuronal loss (Sargin et al. 2010). Overall, EPO has evolved as a therapeutic agent

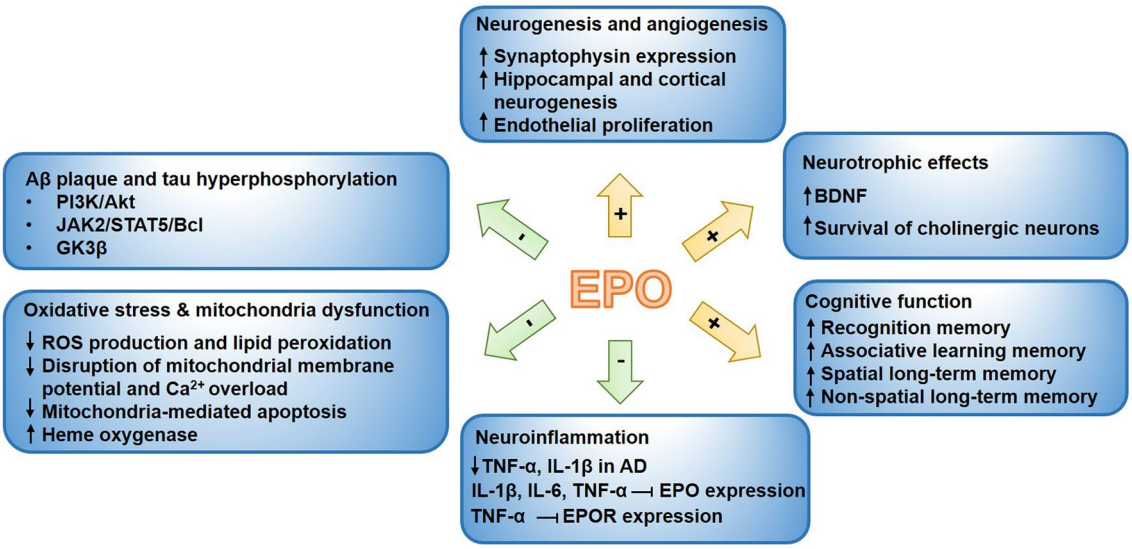

Fig. 1 The neuroprotective effects of EPO in AD. In experimental AD, EPO reduces Aβ toxicity, neuroinflammation, and oxidative stress, and enhances neurogenesis and neurotrophic factor expression

for CNS disorders given its robust non-hematopoietic neuroprotective effects.

Expression of EPO and EPO Receptor in the CNS

The EPO receptor (EPOR) is a highly conserved member of the cytokine receptor superfamily. EPOR expression in the brain is observed during development and adulthood in humans and other mammals (Marti et al. 1996). EPO expression is detected in neurons, astrocytes, and pericytes, while EPOR is expressed in neurons, astrocytes, and microglia (Ji 2016; Nagai et al. 2001). EPOR is prominently expressed in astrocytes, oligodendrocytes, and certain neuronal populations in fetal brain during gestation (Jelkmann 2005), and brain EPOR expression is significantly reduced with development, indicating the vital role of EPO in brain maturation (Juul et al. 1998).

The expression of EPO and EPOR is controlled by hypoxia inducible factor-1 (HIF-1). This hypoxia-dependent transcription of EPO is regulated via the hypoxia response element in the 3′-flanking region of EPO gene that specifically binds to HIF-1 under hypoxic conditions (Haase 2013). Neuronal production of EPO is related to the oxygen tension of the medium in vitro, which is enhanced by hypoxia (Masuda et al. 1994). Further, astrocytic EPO expression is increased by more than 100-fold under hypoxic conditions (Marti et al. 1996). Radio-iodinated EPO studies in rats indicate that EPOR expression is localized to the hippocampal and cortical areas, which are most vulnerable to AD pathology and ischemic insult (Digicaylioglu et al. 1995). EPOR expression is also observed on the endothelial cells forming the blood–brain barrier (BBB), and EPOR is upregulated in the temporal cortex and hippocampus of patients with mild cognitive impairment and AD (Cornford and Hyman 2005; Assaraf et al. 2007).

Effect of EPO on AD pathology

Amyloid Toxicity

AD is pathologically characterized by the accumulation of Aβ and tau hyperphosphorylation in the hippocampus and cortex. Therefore, it is important for therapeutic AD agents to act on toxic Aβ buildup, association of newly cleaved Aβ fragments into oligomers and plaques, and tau phosphorylation. Intraperitoneal injections of EPO induce a 20% reduction of total Aβ load and a 59% reduction of $Aβ_{40}$ protein level in mice; however, no significant decrease in $Aβ_{42}$ load is observed after EPO treatment (Armand-Ugon et al. 2015). Tg2576 mice also exhibit a reduced amount of amyloid plaque after daily intraperitoneal injection of EPO for 5 days (Lee et al. 2012). Similarly, EPO reduces hippocampal Aβ accumulation in an intracerebroventricular streptozotocin-induced AD rat model (Samy et al. 2016). Intranasal administration of EPO also decreases Aβ deposits in an APP/PS1 transgenic AD mouse model (Rodriguez Cruz et al. 2017). In vitro, EPO is reported to attenuate $Aβ_{25–35}$-induced rat pheochromocytoma cell death by regulating anti-apoptotic protein expression (Ma et al. 2009). Further, EPO protects microglia-mediated neuronal regeneration and Aβ clearance, in vitro (Shang et al. 2012). A recent study reported that EPO alleviates tau hyperphosphorylation via regulation of glycosynthase kinase-3β in a rodent AD model (Li et al. 2015).

Mitochondrial Dysfunction and Oxidative Stress

Oxidative stress is linked to mitochondrial dysfunction in AD (Eckert et al. 2012), and EPO relieves oxidative stress by preventing the accumulation of lipid peroxidation in the hippocampus of AD mice (Maurice et al. 2013). In vitro, EPO treatment prior to $A\beta_{25–35}$ exposure significantly elevates cell viability, decreases ROS production, and stabilizes mitochondrial membrane potential (Li et al. 2008). EPO further attenuates nitric oxide and staurosporine-induced oxidative toxicity in neonatal rat astrocytes by promoting the expression of heme oxygenase-1 (Diaz et al. 2005), and EPO-mediated anti-oxidant effects reduce neuronal damage by activated microglia (Wenker et al. 2013).

A massive release of synaptic glutamate in brain regions susceptible to Aβ-toxicity leads to excitotoxicity and oxidative stress, resulting in neurotoxicity. EPO prevents glutamate-induced excitotoxicity and cell death by inhibiting mitochondrial calcium overload in cultured rat neurons (Morishita et al. 1997). EPO activates several kinase cascades to confer resistance against oxidative stress-induced apoptosis (Li et al. 2008). It has also been demonstrated that EPO inhibits Bax/Bcl complex-mediated apoptosis induced by Aβ (Rodriguez Cruz et al. 2017; Li et al. 2015).

Neurotrophic Effects

The role of EPO as a neurotrophin and in neurogenesis has been well documented. Intracerebroventricular administration of EPO significantly increases brain-derived neurotrophic factor (BDNF) expression in the brain. EPO also induces BDNF production and long-term activation of its specific receptor, tyrosine receptor kinase B, resulting in EPO-mediated neuroprotection against neurotoxin trimethyltin (Viviani et al. 2005). Notably, BDNF levels are reduced in AD, and exogenous BDNF offers protection against Aβ- and tau-related neurodegeneration (Jiao et al. 2016). The role of EPO-derived BDNF in AD, however, needs further investigation.

EPO augments choline acetyltransferase activity in mouse embryonic primary septal neurons and a cholinergic hybridoma cell line. Moreover, EPO promotes survival of septal cholinergic neurons in adult rats which have undergone fimbria-fornix transections (Konishi et al. 1993). Intraperitoneal administration of EPO spurs significant neurogenesis in the dentate gyrus in the streptozotocin-induced AD rat model; however, EPO does not change neurogenesis in the dentate gyrus of intact animals (Arabpoor et al. 2012). Tg2576 mice treated with EPO show increased hippocampal and cortical neurogenesis identified by 5-bromo-2-deoxyuridine fluorescent labeling, and increased synaptophysin expression. Further, EPO enhances endothelial proliferation in the brains of Tg2576 mice, indicating the positive role of EPO in angiogenesis (Lee et al. 2012). Overall, these studies indicate that EPO acts as a potent neurotrophic factor to influence differentiation, maintenance, and regeneration in the CNS.

Neuroinflammation

Increasing evidence suggests that neuroinflammation in AD is implicated as a significant contributor to disease pathogenesis and progression. With respect to neuroinflammation, EPO prevents the $A\beta_{25–35}$-induced increase in TNF-α and interleukin (IL) production in a rodent AD model (Maurice et al. 2013). Besides its effect in AD rodent models, EPO has been reported to decrease production of TNF-α, IL-6, and monocyte chemoattractant protein-1 in an ischemic stroke model (Villa et al. 2003). Moreover, EPO also diminishes inflammation and glial activation in a model of brain inflammation (Shang et al. 2011). These data collectively suggest that EPO act as a protective cytokine in inflammatory pathologies of neurodegeneration, including AD.

The effect of immunomodulatory peptides on EPO expression in cultured human neurons, microglia, astrocytes, and oligodendrocytes has also been investigated. EPO expression in human astrocytes was reduced by pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α, whereas EPOR expression was markedly increased in human neuronal cells by TNF-α (Nagai et al. 2001). EPOR and EPO expression may thus be regulated by pro-inflammatory cytokines in the CNS.

Cognitive Function

AD is the most common cause of senile dementia and is characterized by a progressive decline in cognitive function. Behavioral tests commonly used in animal AD studies attempt to mimic deficits in memory and cognition of AD patients. In a contextual fear-conditioning test, EPO improves associative learning memory in aged transgenic AD mice (Lee et al. 2012). In a step-down inhibitory avoidance test, which measures short-term retention of an aversive stimulus, intraperitoneal EPO treatment restores $A\beta_{42}$-induced loss of step-down latency (Li et al. 2015). Intranasal EPO alleviates place learning and spontaneous alteration deficits in the Morris Water Maze, which is used to measure spatial and long-term memory by recording escape latency, distance, and velocity (Rodriguez Cruz et al. 2017). EPO administration also prevents deficits in alternation behavior in Y-maze and novel object recognition procedures in $A\beta_{25–35}$-injected mice (Rodriguez Cruz et al. 2017; Maurice et al. 2013). Non-spatial long-term memory function deficits, tested by the passive avoidance procedure, are observed in AD and stroke models, and these are improved by EPO treatment (Esmaeili Tazangi et al. 2015; Sakanaka et al.

1998). Using place learning in water maze, hippocampus-dependent spatial memory is improved by EPO (Maurice et al. 2013). Similarly, bilateral injection of Aβ into the rat cortex leads to memory loss and synaptic plasticity defect, which are recovered by EPO (Esmaeili Tazangi et al. 2015).

Limitation of EPO as a CNS-Penetrating Drug

Though EPO and EPOR have a role as an "endogenous neuroprotective system" in the brain, BBB penetration and negative hematopoietic effects are the two major challenges that have to be addressed before EPO can be developed as a neurotherapeutic. EPO is a 30.4 kDa polar macromolecule and is larger than the molecular weight cut-off for lipid-mediated free diffusion across the BBB (Pardridge 2005; Konofagou et al. 2012). The finding that the EPOR is expressed at the brain capillaries suggested that peripheral EPO may cross the BBB to produce therapeutic effects in the brain (Brines et al. 2000). Further, systemically administered biotinylated-EPO was detected surrounding the brain capillaries, and this transport of biotinylated-EPO was eliminated by co-administration of unlabeled EPO (Brines et al. 2000). Based on these findings, it was suggested that EPO is transported across the BBB via a specific receptor-mediated transport system; however, the origin of the biotin label detected in these studies (biotin conjugated to EPO or free biotin) was not confirmed. The presumption that EPO enters the CNS is also largely based on measurement of systemically administered EPO in the cerebrospinal fluid (CSF) (Brettschneider et al. 2006). It should be noted that movement from blood into the CSF is controlled by the blood–CSF barrier, and all molecules present in the blood enter the CSF at a rate that is inversely related to their molecular weight (Pardridge 2016). Follow-up brain uptake studies using radiolabeled EPO in rodents and primates showed that the rate of EPO entry into the brain was very slow and that EPO entry into the brain was via a non-specific mechanism (Boado et al. 2010; Banks et al. 2004). Using the capillary depletion technique, which separates the brain homogenate into vascular pellet and post-vascular supernatant, the rate of EPO entry into the brain was found to be comparable to that of a marker of brain blood volume (human IgG1) showing the limited transport of EPO across the BBB (Boado et al. 2010).

EPO is administered systemically or intranasally in experimental AD (Esmaeili Tazangi et al. 2015; Maurice et al. 2013; Lee et al. 2012; Li et al. 2015; Armand-Ugon et al. 2015; Samy et al. 2016; Arabpoor et al. 2012). Systemic routes, though less invasive than the intracranial or intracerebroventricular routes used to bypass the BBB, require high dosage to infiltrate the brain and therefore result in heightened peripheral toxicity risk. As a red blood cell growth factor, high systemic EPO doses induce negative hematopoietic side effects including increased hematocrit, arterial hypertension, cerebral convulsion, thromboembolism, iron deficiency, and influenza-like syndrome (Lapchak 2010; Lundby et al. 2007). Such hematopoietic side effects associated with high systemic doses limit the applicability of the systemic route of administration for EPO delivery for chronic diseases like AD that require long-term treatment.

EPO analogs with reduced erythropoietic activity and associated vascular complications are currently under investigation (King et al. 2007). Asialo-EPO, which is produced by the removal of sialic acid residues from EPO to yield a molecule with enhanced clearance, has no effect on erythropoiesis but exerts neuroprotective effects in animal models of neural diseases (Erbayraktar et al. 2003; Mennini et al. 2006). Another non-erythropoietic EPO derivative, carbamylated-EPO (CEPO), which is produced by the modification of lysine residues to homocitrulline, produces its protective effects through the EPOR/β common receptor, and not the classical EPOR. In an AD mouse model, CEPO treatment improved memory and modulated the expression of molecules involved in neurotransmission. However, compared to EPO, CEPO did not decrease Aβ plaque burden and soluble $A\beta_{40}$ (Armand-Ugon et al. 2015). The CEPO-Fc variant, which is engineered to prolong the half-life of CEPO, was also found to be protective in a rodent AD model (Hooshmandi et al. 2018). Notably, these non-erythropoietic variants of EPO are also macromolecules with limited BBB penetrability across the BBB, comparable to that of EPO (Torup and Leist 2006).

Another variant of EPO, neuro-EPO, which contains low sialic acid, has been designed. Neuro-EPO is devoid of hematopoietic effects and is administered intranasally. Neuro-EPO prevents oxidative damage, neuroinflammation, apoptosis, and cognitive deficit in an AD animal model (Rodriguez Cruz et al. 2017; Maurice et al. 2013). Even though intranasal administration is advantageous over systemic administration with respect to systemic side effects, the efficacy of intranasal drug delivery decreases as molecular weight and hydrophilicity of the drug increase (Lu et al. 2014). Moreover, intranasal delivery relies on widespread drug diffusion from the olfactory CSF to brain entry sites, and drug diffusion decreases exponentially with distance (Lochhead and Thorne 2012; Pardridge 2012). Large diffusion distances in human brains compared with smaller rodent brains may limit the efficacy of the intranasal route of administration in humans. This is in contrast to the transvascular route across the BBB that results in widespread drug delivery throughout the brain because of the density and ubiquity of the cerebral vasculature (Pardridge 2005). Hence, modifying EPO for direct and specific penetration of the BBB via the transvascular route and minimal hematopoietic effects is needed for AD.

BBB Receptor-Mediated Transport of EPO

Molecular Trojan horse (MTH) technology holds promise for efficiently transporting neurotherapeutics across the BBB (Pardridge 2006, 2017). MTH technology harnesses endogenous receptor-mediated transcytosis (RMT) systems to ferry macromolecules into the brain. For this, a fusion protein of the neurotherapeutic and a MTH, which is an antibody against an endogenous receptor expressed on the luminal side of brain endothelial cells, is engineered. Systemic injection of the neurotherapeutic-MTH complex triggers its binding to the endogenous RMT receptor on the luminal side of the BBB, followed by subsequent endocytosis and release of the complex on the abluminal side (Pardridge 2012, 2009). This approach can therefore be used to shuttle therapeutic molecules across the BBB non-invasively to treat CNS pathology (Preston et al. 2014). The two widely studied MTH vehicles are antibodies against the human insulin receptor and transferrin receptor (TfR) (Jones and Shusta 2007; Pardridge 2006).

The TfR system is responsible for transporting transferrin-bound iron from blood across the brain endothelium into the brain parenchyma (Roberts et al. 1993; Jefferies et al. 1984; Fishman et al. 1987). TfR is a type II transmembrane protein and is expressed at both the luminal and abluminal surface of brain endothelial cells (Huwyler and Pardridge 1998). There are two TfR isoforms, TfR1 and TfR2, and the primary TfR isoform expressed at the BBB is TfR1 (Li et al. 2001). Upon binding of the iron–transferrin complex on the luminal surface, TfR enters the brain endothelial cells via endocytosis. Once inside the brain endothelial cells, the remaining mechanism for iron entry into the brain is debatable. One proposed mechanism is transport of the iron–transferrin–TfR complex from the luminal to the abluminal membrane of brain endothelial cells followed by release of iron and transferrin into the brain interstitium with subsequent reverse transcytosis of iron-free transferrin into the blood, while another is dissociation of the iron from the transferrin–TfR complex within the acidified endosomes in the brain endothelial cells and transcytosis of the iron across the abluminal membrane to enter the brain (Roberts et al. 1993; Lee et al. 2000; Dautry-Varsat et al. 1983; Moos et al. 2007; Zhang and Pardridge 2001).

The common mechanism of transcytosis of varying MTHs across the abluminal membrane is also debated, and it is clear that antibodies against the TfR may differ based on their affinity for the TfR. Both high- and low-affinity, as well as monovalent and bivalent TfR antibodies have been engineered, and these different antibodies for the same TfR may warrant different success rates of entry into the brain parenchyma (Niewoehner et al. 2014; Yu et al. 2011; Weber et al. 2018; Hultqvist et al. 2017; Chang et al. 2018). Some studies suggest that TfR antibodies with high affinity can be trapped within the brain vasculature due to their high affinity, which prevents their transportation across the abluminal side of brain capillaries (Yu et al. 2011). Reducing the affinity of the TfR antibody to enhance RMT across the BBB has been suggested as an alternative. It should, however, be noted that high injection doses are needed to produce a therapeutic effect while using low-affinity TfR antibodies, and such high doses result in effector function-associated toxicity (acute-clinical signs and reticulocyte suppression) (Couch et al. 2013). Low therapeutic doses of high-affinity TfR antibodies on the other hand do not produce an immune-response or reticulocyte suppression (Pardridge et al. 2018). Overall, as the second most abundant protein on the brain endothelial cells compared to other RMT systems, TfR is considered an ideal candidate for MTH drug delivery of therapeutics across the BBB (Bien-Ly et al. 2014; Pardridge 2015).

To facilitate non-invasive transvascular EPO delivery into the brain, human EPO was re-engineered as a TfR antibody–EPO fusion protein, designated as cTfRMAb–EPO (Fig. 2). The cTfRMAb domain of the fusion protein that enables BBB penetration is a rat/mouse chimeric antibody that is specific to the mouse TfR and binds to the mouse TfR with high affinity (K_D of 2.6 ± 0.3 nM) in a bivalent configuration (Boado et al. 2009). The EPO domain of the fusion protein binds to the mouse EPOR with an $ED_{50} = 0.33 \pm 0.04$ nM to produce neuroprotection in brain behind the intact BBB (Zhou et al. 2010). Intravenous injection of radiolabeled cTfRMAb–EPO in mice results in a brain uptake of $2.0 \pm 0.1\%$ injected dose/g of brain and ~70% of the fusion protein is transcytosed across the BBB into the brain parenchyma measured using the capillary depletion technique (Zhou et al. 2010). The plasma clearance of the cTfRMAb–EPO fusion protein is > 10-fold higher than that of EPO due to the rapid peripheral TfR-mediated clearance of the fusion protein (Kato et al. 1998). Such a pharmacokinetic profile of the cTfRMAb–EPO is desirable given that increased plasma clearance lowers the potential for peripheral hematopoietic side effects associated with the EPO domain of the fusion protein. In fact, desialylated variants of EPO have a reduced half-life and increased clearance, and offer the possibility to uncouple stimulation of erythropoiesis from tissue-protective effects of EPO (Erbayraktar et al. 2003; Torup and Leist 2006).

Chronic 3 days a week intravenous treatment with the cTfRMAb–EPO fusion protein for 3 weeks offered neuroprotection in a mouse model of neural disease and resulted only in a 10% increase in hematocrit after 2 weeks of treatment with no further hematocrit increase (Zhou et al. 2011).

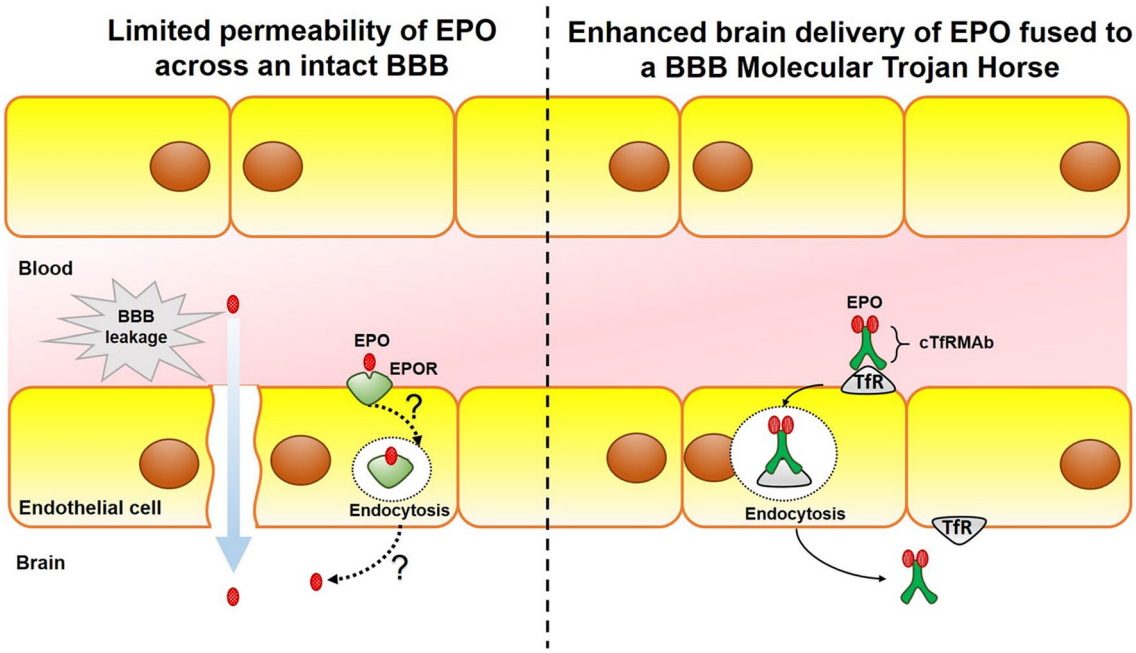

Fig. 2 Schematic of routes of EPO brain delivery. Penetration of the BBB is a major challenge of EPO administration for CNS diseases including AD. One hypothesis is the existence of EPOR on the luminal side of the brain capillaries that is presumed to provide a route for circulating EPO to enter the brain. This route of EPO entry remains debatable. In the absence of such a receptor, brain delivery of EPO requires compromised BBB permeability. Alternatively, endogenous transferrin receptors (TfR), which are receptor-mediated transcytosis systems, and are highly expressed at the BBB can be harnessed to ferry EPO into the brain. A fusion protein of EPO and a chimeric monoclonal antibody against TfR has been engineered and designated, cTfRMAb–EPO. Upon binding to the BBB TfR, the cTfRMAb–EPO fusion protein is ferried into the brain to enable EPO delivery into the brain across the BBB

The dose of the cTfRMAb–EPO fusion protein in the aforementioned study was 1 mg/kg which is equivalent to an EPO dose of 200 µg/kg, since the cTfRMAb–EPO fusion protein is 20% EPO based on the amino acid sequence of the fusion protein. Since the clearance of the cTfRMAb–EPO is > 10-fold faster than EPO, the equivalent EPO dose is 20 µg/kg per dose or 60 µg/kg per week. This is lower than the doses of EPO that are therapeutic in experimental models of AD (Lee et al. 2012; Armand-Ugon et al. 2015). In a recent study, chronic treatment with the cTfRMAb–EPO fusion protein for 8 weeks reduced Aβ pathology, microglial activation, and synaptic loss in a transgenic mouse model of AD. cTfRMAb–EPO further improved performance in the modified Y-maze, showing that treatment with the BBB-penetrable EPO improved spatial memory in experimental AD (Chang et al. 2018). High-affinity humanized TfR antibodies targeting the human TfR have been engineered and their ability to act as efficient MTHs has been confirmed in primates (Pardridge et al. 2018; Yu et al. 2014). Species-specific TfR antibody–EPO fusion proteins can thus be engineered to translate these effects of the BBB-penetrable-EPO into other species. Overall, engineering EPO variants that target the BBB TfR to enable BBB penetration may provide a promising approach to further the development of EPO for chronic neurodegenerative conditions including AD.

Summary

EPO has evolved as a therapeutic agent for neurodegenerative conditions given its robust non-hematopoietic neuroprotective effects. However, penetration of the BBB and negative hematopoietic effects are the two major challenges for the development of EPO for chronic neurodegenerative diseases like AD. Brain drug delivery strategies, such as the MTH technology that harnesses endogenous RMT systems like the BBB TfR, hold promise for efficiently transporting EPO across the BBB for AD, while increasing systemic clearance to minimize hematopoietic side effects.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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