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Intranasal erythropoietin therapy in nervous system disorders

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Importance of the field: Erythropoietin (EPO) is a growth hormone and cytokine that plays an important role in erythropoiesis and neuroprotection. However, EPO treatment for neurological diseases requires repeated injections or high-dose systemic administration, which may cause systemic side effects. The lack of any effective treatment of acute and chronic neurodegenerative diseases and the promising outcome by EPO in animal models *in vivo* demand a critical evaluation of intranasal EPO delivery to the brain as an alternative administration method.
Areas covered in this review: The current use and intranasal administration of EPO and its derivatives in preclinical studies and recent clinical trials with EPO in neurological diseases.
What the reader will gain: This paper gives an overview of the therapeutic considerations of intranasal EPO and EPO derivatives for neuroprotection.
Take home message: Intranasal delivery (ID) of neuroprotective drugs is an area of great interest. Among the administration strategies used at present, ID of EPO is the most promising. Further preclinical and clinical studies are needed to evaluate the potential significance of this alternative route for increasing EPO bioavailability and decreasing side effects.

Keywords: blood–brain barrier, erythropoietin, nasal drug delivery, nervous system, neuroprotection, stroke

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1. Introduction

Erythropoietin (EPO) is a hematopoietic growth factor and cytokine that stimulates erythropoiesis by inhibiting apoptosis of erythroid progenitors in bone marrow. Human EPO is a 30.4 kDa glycoprotein consisting of 165 amino acids and is produced mainly in the adult kidney. Human EPO was isolated in 1977 [1] and its gene cloned in 1985 [2,3] (for a historical review of earlier EPO studies, see [4]). For the past 20 years, recombinant human EPO (rhEPO) has been used for the treatment of anemia related to chronic renal failure and prematurity [5]. A growing body of evidence indicates that the therapeutic efficiency of EPO could extend far beyond the stimulation of erythropoiesis, such as tissue-protective effects and prevention of tissue damage during ischemia and inflammation in the nervous system, suggesting that this cytokine may be an attractive drug candidate for the treatment of neurological diseases [6].

In the central nervous system (CNS), EPO exerts its cellular effects by binding to the EPO receptor (EPOR) expressed by neurons, glial cells and cerebrovascular endothelium [6-8]. EPO induces its effects by dimerizing EPOR molecules, which leads to the activation of the EPOR-associated Janus tyrosine kinase 2 and secondary signaling molecules such as Stat5. However, EPO neuroprotection also involves PI3K and protein kinase B (Akt) signaling [6].

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Article highlights.

- Erythropoietin is neuroprotective in many animal models of neurodenerative disorders.
- Erythropoietin showed systemic side effects in clinical trial.
- New, reliable and effective methods of administration should be developed for EPO delivery into the brain.
- Single or combined intranasal administration of EPO is neuroprotective in animal studies. Intranasal delivery of EPO should be considered for clinical trial in neurodegenerative disorders.

This box summarizes key points contained in the article.

Neuronal expression of EPO and EPOR peaks during brain development, suggesting that they are crucial during early embryonic development of the neural system [9]. The weak constitutive expression in the adult brain is stress-responsive and can be rapidly increased by hypoxia and acute metabolic stress in chronic brain diseases such as schizophrenia and Alzheimer's disease (AD) [10,11]. As relatively low levels of endogenous EPO are produced in the CNS, administration of exogenous rhEPO can support endogenously the neurorestoration process. Indeed, numerous studies have shown that EPO ameliorates or prevents neuronal injury by different mechanisms in cell culture and animal models of neurological diseases, including stroke, neonatal hypoxic-ischemic encephalopathy, multiple sclerosis (MS), subarachnoid hemorrhage, traumatic brain injury, epileptic seizures, Parkinson's disease (PD) and spinal cord injury (**Figure 1**) [12-15]. The beneficial effects of rhEPO have also been demonstrated in clinical studies (**Table 1**) [16-24].

As circulating EPO, a large and highly glycosylated negatively charged molecule, was thought not to cross the blood–brain barrier (BBB), the early studies used a direct intraventricular or intracerebral route of administration of EPO to demonstrate its potent tissue protective activity in animal models of neurological diseases [25-27]. However, these invasive delivery routes, which are not suitable for clinical use, require surgical expertise and can cause brain damage and infections. More recent studies, in several species including humans, confirmed the ability for a high dose of systemically administered EPO to cross the BBB in pharmacologically sufficient amounts [28-31]. During the last decade, an enormous number of experimental studies in animals used a high dose of systemically administered EPO to show its potential as a neuroprotective drug. Although EPO is accepted as a safe therapeutic in the treatment of anemia, the clinical use of EPO as a neuroprotective drug at high doses raises concerns about its possible adverse side effects, such as thromboembolism, hypertension and tumor progression in patients with cancer. Various strategies may be used to increase EPO bioavailability and decrease side effects in clinical use in the treatment of neurological diseases: i) targeting delivery of EPO to effective CNS sites; ii) the use of non-hematopoietic derivatives of EPO; and iii) using alternate administration routes such as intranasal delivery (ID) of EPO across biological barriers to obtain neuroprotection. Formulation considerations for EPO relating the first strategy is now an area of great interest and has been comprehensively reviewed [32]. Here, the last strategy is focused on in light of the current literature. Recently developed non-hematopoietic derivatives of EPO are also discussed briefly.

2. Intranasal drug delivery as an alternative route to treat the central nervous system diseases

Intranasal delivery of neuroprotective drugs targeting the CNS is an area of great interest at present, as reviewed elsewhere [33-38]. Here, the pathways and mechanisms of the transport of drugs from nose to brain and experimental and practical considerations with intranasal drug delivery of CNS therapeutics are briefly reviewed. The anatomy, physiology and brain delivery characteristics of the nasal cavity regarding intranasal drug transport have been comprehensively reviewed elsewhere [34,36]. For their different functions and tissue structures, each of the nasal cavities can be basically subdivided into three regions; namely, the nasal vestibule, the olfactory region and the respiratory region. The nasal vestibule has almost no absorption function. Following intranasal administration, drugs come into contact with the nasal mucosa, which is comprised of the nasal epithelium and contains various cell types, and the underlying lamina propria, a thin layer of loose connective tissue that contains blood vessels, lymphatic vessels, axons and glands.

2.1 Pathways and mechanisms

Intranasally administered therapeutics may reach the CNS through several pathways after nasal instillation: olfactory, trigeminal and systemic pathways involving the olfactory and trigeminal nerves, respiratory and olfactory mucosa, the nasal vasculature, cerebrospinal fluid (CSF), and the lymphatic system (**Figure 2**). Possible mechanisms of transport may involve bulk flow and diffusion within perineural channels, perivascular spaces, or lymphatic channels directly connected to brain tissue or CSF. It is likely that a combination of transport pathways into the brain on absorption by means of nasal mucosa with different transport rates is responsible, although one pathway may predominate, depending on the properties of the therapeutic, the characteristics of the formulation, and the delivery device used [34].

Both the olfactory and trigeminal nerves innervate the nasal cavity, providing a direct connection with the CNS. In the olfactory region, olfactory receptor neurons are interspersed among supporting cells and basal cells to form the olfactory epithelium [34]. These cells are bipolar sensory neurons that mediate the sense of smell by conveying sensory information

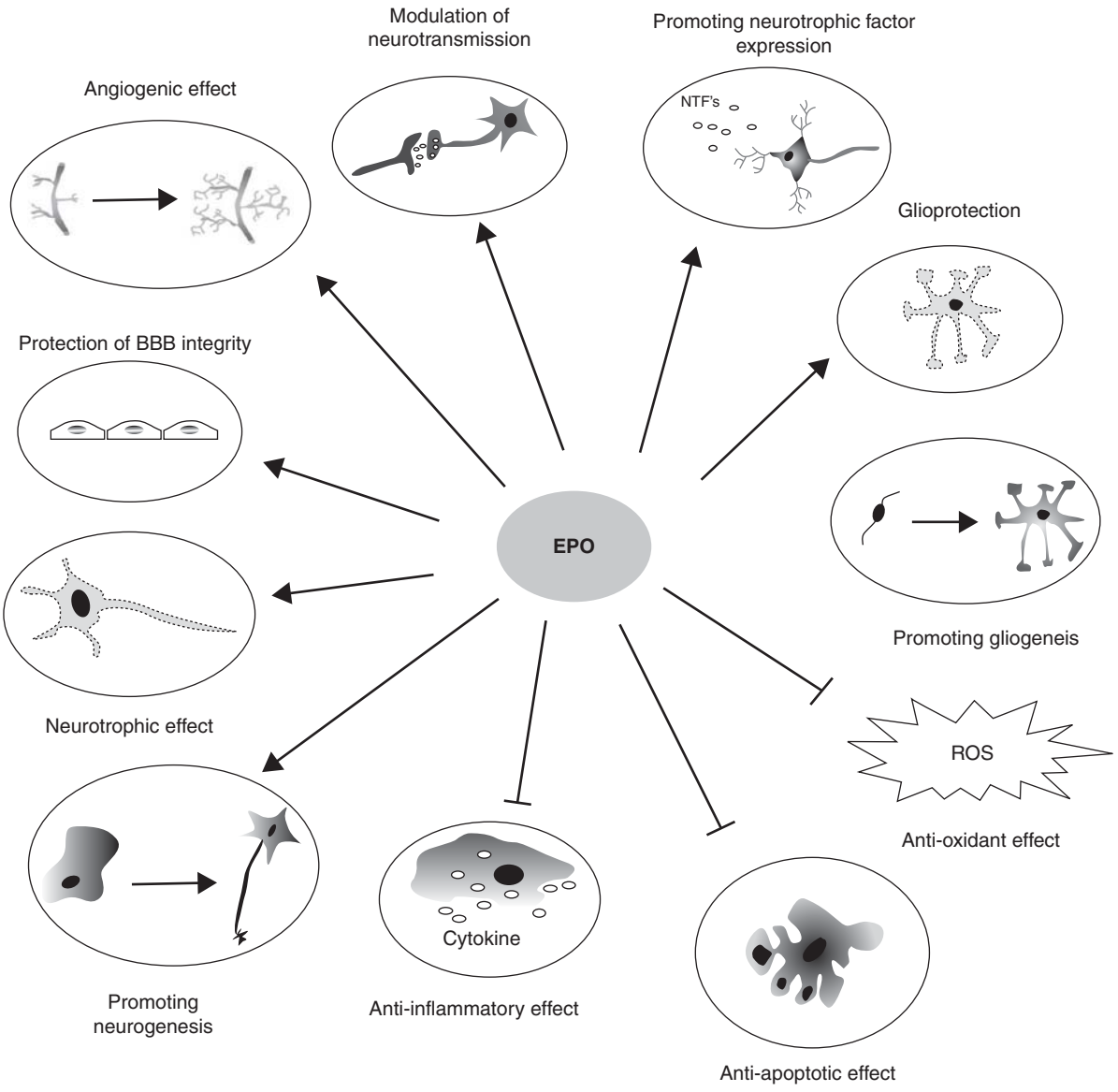

Figure 1. Protective effect mechanisms of EPO.
BBB: Blood–brain barrier; EPO: Erythropoietin; ROS: Reactive oxygen species.

from the peripheral environment to the CNS, and their dendrites extend into the mucous layer of the olfactory epithelium. Drugs that reach the olfactory mucosa can enter the olfactory dendrites by pinocytosis, simple diffusion or receptor-mediated endocytosis and are transported by slower axonal transport through the olfactory nerve axons [39]. These axons terminate on mitral cells in the olfactory bulbs. From there, neural projections extend to multiple rostral brain regions, including the olfactory tract, anterior olfactory nucleus, piriform cortex, amygdala and hypothalamus. Solute transport takes several hours to days for a drug to appear in the olfactory bulbs and these brain areas [34,40]. The trigeminal nerve innervates the respiratory epithelium of the nasal cavity and conveys sensory information from the nasal cavity to the CNS via its ophthalmic and maxillary divisions. The trigeminal nerve enters the brain at the level of the pons, terminating in the spinal trigeminal nuclei in the brainstem [34]. The trigeminal nerve pathway to intranasal drug delivery to the CNS, especially to caudal brain regions, the brainstem and the spinal cord, has recently been recognized [40,41].

In the olfactory epithelial pathway, drugs may enter the supporting cells by means of pinocytosis or diffusion as part of a transcellular transport mechanism or they may enter paracellularly through the tight junctions between the supporting cells and clefts between the supporting and olfactory receptor cells into the intercellular fluid [36,42]. Thus, the nasal barrier to the CNS could be considered highly permiable from the constant turnover of the olfactory receptor neurons.

Table 1. Completed clinical trials with erythropoietin in nervous system diseases except stroke.

Disease	Phase	Number of patients	Treatment protocol	Result	Ref.
Neonatal hypoxic- ischemic encephalopathy	I/II	167	Started within 48 h of birth 300 or 500 U/kg on alternate days (2 weeks)	improved long-term outcomes only for infants with moderate HIE	[24]
Preterm infant with IVH, PVL, ROP, septicemia cerebral palsy, NEC bronchopulmonary dysplasia	IIa/IIb	45	3000 U/kg, i.v., at 3, 12 – 18, 36 – 42 h after birth	No differences on the short-term outcome No significant adverse effects	[20]
Extremely low birth weight infants	I/II	60	500/1000/2500 U/kg, i.v. 3 times at 0, 24, 48 h	EPO is well tolerated No excess morbidity or mortality	[21]
Schizophrenia	IIb	39	40,000 U i.v. weekly (12 weeks)	improvement in schizophrenia- related cognitive performance	[18]
Multiple sclerosis	IIa	8	Combined with Mpred 8000/48,000 U weekly (12 weeks) bi-weekly (12 weeks)	No adverse events, improvement of motor function and cognition	[19]
Subarachnoid hemorrhage	IIb	80	Started within 72 h of SAH, 30,000 U i.v./d (3 day)	Reduce delayed ischemic deficits following SAH Poor clinical condition with EPO	[23]
	IIb	73	500 U/(kg d) (3 day)		[22]
Friedreich's ataxia	IIa	8	5000 U s.c. 3 times weekly for 8 weeks 2000 U s.c. 3 times weekly for 6 months	Increase in frataxin levels Reduction of oxidative stress markers	[17]
	IIa	12			[16]

EPO: Erythropoietin; HIE: Hypoxic ischemic encephalopathy; i.v.: Intravenous; IVH: Intraventricular hemorrhage; Mpred: Methylprednisolone; NEC: Necrotizing enterocolitis; PVL: Periventricular leukomalacia; ROP: Retinopathy of prematurity; SAH: Subarachnoid hemorrhage; s.c.: Subcutaneous.

Extracellular transport mechanisms involve the rapid movement of molecules between cells in the nasal epithelium, requiring only a few minutes to 30 min for a drug to reach the olfactory bulbs and other areas of the CNS after intranasal administration. Special Schwann cell-like cells called olfactory ensheathing cells envelope the axons of olfactory receptor neurons and create continuous, fluid-filled perineural channels [43]. After reaching the lamina propria, drugs can enter perineurial channels, where they can access the CSF and olfactory bulbs. Extracellular drug transport probably involves bulk flow mechanisms within the perineural channels. From the CSF, drugs can be distributed by means of bulk flow mechanisms and mix with brain interstitial fluid throughout the brain.

Intranasally applied drugs may enter the systemic circulation by passing through the nasal epithelium and entering the blood capillaries that lie in the submucosal tissue. The resulting perivascular pump can account for the rapid distribution of therapeutic molecules throughout the brain [34,40,44].

Although there are no conventional lymphatics in the brain, physiological studies have revealed a substantial and immunologically significant lymphatic drainage from brain to cervical lymph nodes [45]. If the drug remains in the lymph flow it may eventually appear in the systemic blood circulation and contribute to the nasal-systemic pathway [37]. The pathways between the nasal passages and the CSF are important and functional, evidenced by the fact that therapeutics pass directly to the CSF following intranasal delivery, without entering the blood to an appreciable extent [34,46].

2.2 Experimental and practical considerations

It is important to consider the different methodologies used in intranasal drug delivery studies, as factors such as administration position of animals, administration technique, the influence of anesthetics, and delivery volume, can all influence drug deposition in the nasal cavity and the pathway a drug follows to the CNS. In addition, interspecies differences, age of the animals and the methods used for the assessment of drug distribution in the CNS should be considered as interfering experimental factors [34,38].

Optimal delivery to the CNS along neural pathways requires targeting of the drug to the upper third of the nasal cavity. Most experimental studies have been carried out in anesthetized mice and rats, with animals positioned in the supine position [34,39,40,47]. In non-human primates and clinical studies with human subjects, different head positions can also influence the deposition of nasal drops in the nasal cavity. The supine position and the praying-to-Mecca position, with the head down and forward, may prove to be better, as drugs can be accumulated in the olfactory region, which is likely to increase further the uptake of drugs [34,38].

In correlation with one another, drug concentration, dosage and delivery volume are three major factors that affect nasal absorption of drugs [38]. The nasal absorption of most drugs increases with the increase of concentration, especially those

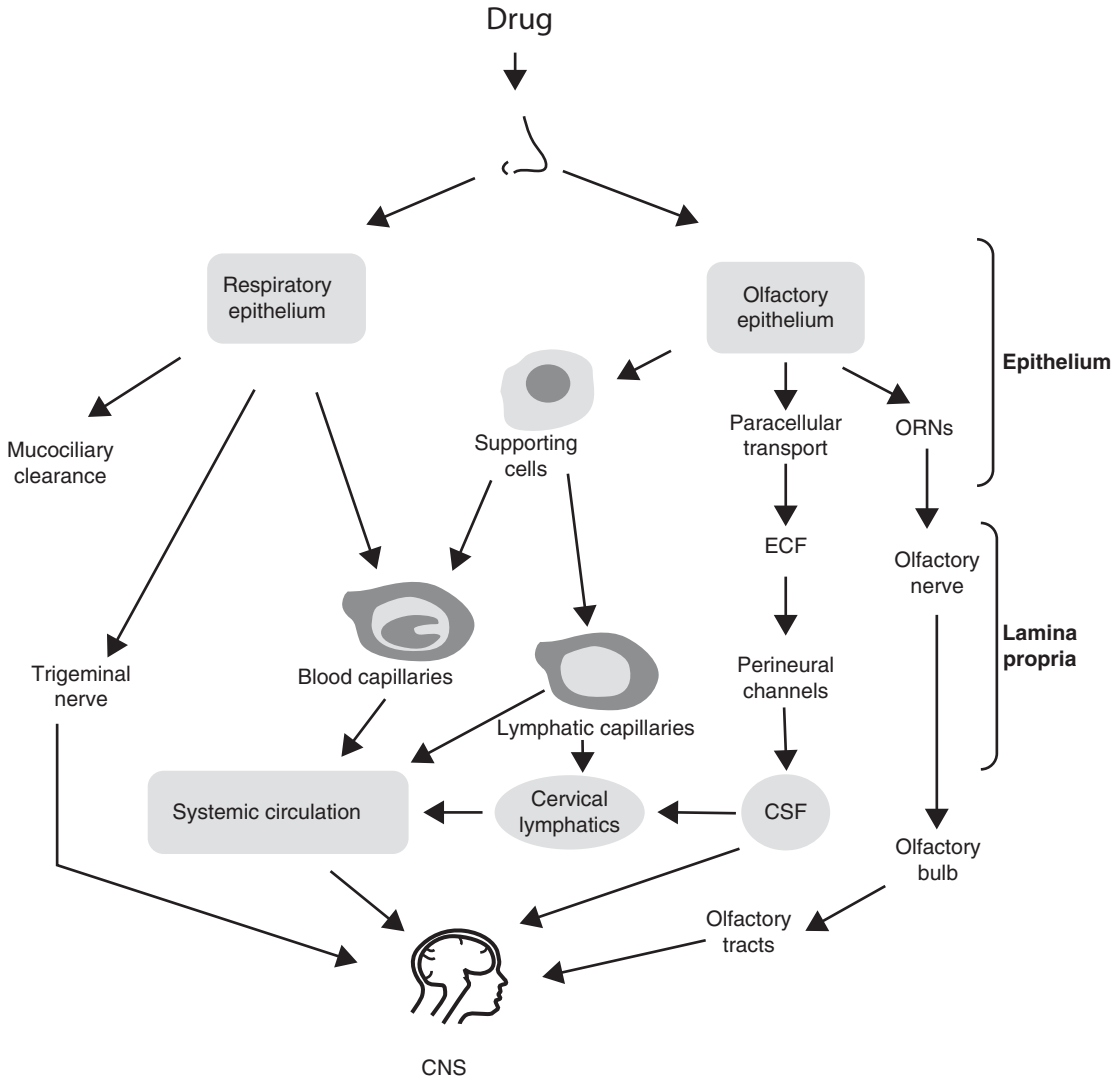

Figure 2. The possible pathways involved in the direct and indirect delivery of therapeutics from the nasal cavity to the CNS.
CNS: Central nervous system; CSF: Cerebrospinal fluid; ECF: Extracellular fluid; ORN: Olfactory receptor neuron.

with an absorptive mechanism of passive diffusion. However, the volume of the nasal cavity is limited and the dosage for nasal administration is relativly low at 25 – 100 µl, thus constricting the amount of drug transport from nose to brain [38].

Interspecies differences in nasal and brain anatomy and physiology must be considered in intranasal drug delivery studies [48-50]. The CSF production rate, volume and its spreading rate also significantly affect the brain uptake of drugs following intranasal administration, which varies largely in different species [38]. The differences in CSF volume in rodents and humans and the turnover time for CSF in these species can also lead to reduced efficiency of the intranasal drug delivery to brain in humans when compared with rodents. The CSF volume (~ 160 ml in humans versus 35 ml in mice) is replaced every 2 h in mice compared with every 5 h in humans, which may affect the interpretation of nose-to-brain drug delivery studies, especially those in experimental protocols that utilize CSF concentrations as an indication of brain uptake [51]. Thus, interspecies differences in nasal and brain anatomy and physiology may confound the extrapolation of results to humans. These factors must be considered before any assessment can be made regarding utility of this method for drug delivery in humans [36,51].

Despite anatomical differences between rodents and humans, similar pathways are involved in ID to the CNS. Translation into the clinic is now underway, with clinical trials of intranasal treatments for AD demonstrating success [50,52]. Some experiments on the nose-to-brain transport process have been reported in non-human primates [41,53]. Clearly, the primate model can be considered to be more realistic for providing information on the human transport process than the rodent models [36].

2.3 Experimental methods for the assessment of central nervous system distribution

In intranasal drug delivery studies, a variety of techniques can be used, depending on the subject of the research. The exact transport pathway(s) of a specific drug can be investigated by radiotracing and immunochemical techniques [37]. Assessing concentrations and distribution to different brain regions provides insight into pathways involved in the transport to the CNS. For example, greater distribution in the olfactory bulbs and frontal cortex compared with the cerebellum and brainstem would be consistent with pathways involving the olfactory nerves following intranasal administration [34]. Chromatographic as well as spectroscopic techniques and enzyme-linked immunosorbent assay (ELISA) can be used for the measurement of drug present in collected samples.

Interestingly, brain injury may change the distribution properties of the drugs in the CNS. In brain injury, independent from the administration route, drugs are directed to the damaged area [54-56]. The mechanism(s) of this process remains to be clarified.

CSF drug concentration is also measured by microdialysis. This method permits continuous monitoring of the drug concentration in the CSF under controlled experimental conditions without removing any body fluids. It can also be used for monitoring drug concentration in other brain regions of freely moving animals [36], but according to current protocols animals are restrained for microdialysis to get more reliable results.

2.4 Formulation considerations

The rate and capacity of drug transport from the nasal cavity to the brain depend primarily on the drug's physicochemical properties and protective barriers in the nasal mucosa. Research efforts have focused on the development of various approaches to overcome the barriers present in the nasal mucosa in order to improve the efficiency of drug transport from the nose to the brain for the treatment of CNS disorders [34,38]. Generally, drugs with a molecular mass > 1000 Da show poor capability in penetrating the physiological barrier. Recent findings indicate that the nasal mucosa pathway is more advantageous for hydrophilic and polar high-molecular-mass drugs such as EPO. Many large water-soluble drugs also reach the brain following intranasal administration by traveling along the olfactory and trigeminal neural pathways [35,36].

Protective barriers in the nasal mucosa contribute to the low efficiency of delivery observed following intranasal administration, with typically < 1% of the administered dose reaching the brain [34,36]. Drugs can be encapsulated in carriers, such as cyclodextrins, microemulsions, liposomes and nanoparticles, to overcome these issues for ID to the CNS [42]. Various strategies for systematic EPO delivery have been investigated for increasing EPO bioavailability and decreasing side effects, including nano/microparticles, PEGylation of EPO and transport-mediated delivery systems [32]. However, these formulations still need to be tested intranasally.

2.5 Intranasal delivery of neuroprotective agents

Early studies showed tracers such as wheat-germ agglutinin conjugated to horseradish peroxidase were transported within olfactory nerve axons to reach the olfactory bulbs in the CNS [57]. Direct ID of therapeutics to the brain was first proposed and investigated in 1989 [34]. In animals, detailed pharmacokinetic and pharmacodynamic studies showed that small, lipophilic molecules, such as cocaine, morphine and testosterone, are able to reach the brain after intranasal administration and exert effects on CNS-mediated behaviors within a short time frame [34]. Intranasal administration of larger therapeutics, such as proteins and peptides, also results in direct delivery to the CNS [39]. Numerous experimental studies have shown that a wide variety of peptide and protein therapeutics given by the intranasal route have the potential to treat nervous system disorders [34,35]. Intranasal nerve growth factor, insulin-like growth factor-1 (IGF-1) and EPO have been shown to protect the brain against stroke in animal models [35,47,54,56]. Intranasal activity-dependent neurotrophic factor and its active peptide fragment NAP have been shown to reduce neurodegeneration, tau pathology, amyloid accumulation and memory loss in mouse models of AD [58].

Recent studies have also provided evidence for the transport of drugs from the nose to the CNS in humans. Using the ID method to target insulin to the CNS, Born *et al.* [46] demonstrated that cerebrospinal fluid insulin levels significantly increased after treatment of normal adults with insulin, without altering blood insulin and glucose, and improved memory and mood [49,52].

In addition to insulin, other peptides and proteins applied intranasally exert beneficial effects in humans. The effects of intranasally delivered peptide hormone, oxytocin, on human social behavior have been studied extensively [59].

3. Safety concerns with erythropoietin in systemic delivery

Several safety concerns of systemic administration of EPO require necessary alternative routes of administration, such as intranasal administration. Here, the safety concerns arsing from systemic administration of EPO are summarized.

As the brain tissue is separated from the circulating blood by the BBB that significantly influences the direct exchange of molecules between the nervous system and the vascular component, it becomes ever more imperative to determine the best delivery method of neurotherapeutic drugs to the CNS [12]. In 2000, Brines *et al.* showed biotinylated EPO crosses BBB in mice [29]. ELISA and nuclear imaging studies supported the transport of EPO via BBB of both healthy individuals and patients with schizophrenia and stroke [30,60,61]. Radiolabeled EPO and albumin cross the BBB and enter the brain parenchyma in similar kinetics, suggesting that the transport of

EPO across the BBB is rather mediated by the extracellular pathways [31,62]. Increased permeabilization of the BBB in acute or chronic pathological conditions such as stroke, traumatic brain injury, AD or MS probably facitilates the entry of EPO to the brain parenchyma [62]. However, the pharmacologically neuroprotective doses of systemically administered EPO are still much higher than those needed for stimulation of hematopoiesis [6]. The pilot safety study in patients with ischemic stroke suggests systemic administration of EPO is safe and well tolerated, but the dose (100,000 U/patient, human mean weight is 70 kg) is lower than used in most preclinical studies encouraging larger clinical trials [30]. Unfortunately, a Phase II/III EPO Stroke Trial has failed to show any protective effects of EPO [60]. There was a significant increase in the death rate when patients with stroke received EPO within 6 h of stroke onset compared with the death rate in the placebo group. Actually, Philip and co-workers recently showed that the quality of preclinical EPO studies as measured by a Stroke Therapy Academic Industry Roundtable (STAIR)-derived quality score was relatively low [63,64]. Meta-analysis studies showed that STAIR recommendations were only incompletely followed by preclinical EPO stroke studies, because the combination therapy and co-morbidity studies such as hypertension and diabetes are still missing [13,65]. Indeed, the combination of EPO with thrombolytic therapy caused serious side effects, such as intracerebral hemorrhage, brain edema and thromboembolic events in the clinical trial [60]. This combination of treatments was not investigated in either preclinical models or in the clinical pilot trial. A preceding investigation of EPO–rtPA (recombinant tissue plasminogen activator) interactions could have prevented side effects of a combination therapy in the clinical trial [65]. Indeed, a recent mouse stroke study by Zechariah *et al.* showed that a combination of rhEPO and rtPA induces BBB permeability and extracellular matrix disaggregation and EPO promotes matrix metalloproteinase-9 (MMP-9) activity that was markedly elevated by rtPA [66]. When administered 6 h after the insult in a rat model of middle cerebral artery occlusion, EPO exacerbates rtPA-induced brain hemorrhage without reduction of ischemic brain damage, whereas the combination therapy started 2 h after stroke significantly reduced ischemic damage without increasing the hemorrhagic transformation [67]. Although the dose of rtPA used was 10 times higher than the dose used in humans, these results are consistent with observations made from the clinical trial [60]. Thus, the timing of the use of EPO (i.e., in different phases of the stroke) and long-term outcomes should also be evaluated in future preclinical studies. Actually, safety concerns such as the risk of cardiovascular events were raised for systemically administered EPO even when administered in the dose range determined to be safe [12,68]. In adults with diabetes, chronic kidney disease and moderate anemia, darbepoetin-alpha, a hyperglycosylated analogue of EPO, did not reduce the risk of death or cardiovascular events and was even associated with a higher rate of stroke [69].

As EPO also has a tissue repair capacity promoting neuroregeneration, repeated chronic administration of the drug might also be beneficial both in adult and developing brain injury. A potential problem in the chronic use of EPO for neurodegenerative disorders is the undesirable erythropoietic side effects. For example, in a clinical pilot trial in patients with Friedreich's ataxia, increases in hematocrit requiring phlebotomies occurred in 50% of the patients [17]. Another safety concern with the repeated administration of rhEPO or its analogues and derivatives is immunogenicity of recombinant protein therapeutics [70]. Furthermore, modified derivatives of recombinant proteins, especially in the form of prolonged therapy, may elicit epitope spreading and increase the immune responses, which could trigger thrombotic complications [71].

In this context, the strategy to develop derivatives of rhEPO lacking erythropoietic activity but retaining neuroprotective potential may avoid the side effects reported in the acute stroke trial and allow for multiple and chronic use of EPO in CNS disorders (**Table 2**) [6,11]. Several strategies have been used to identify neuroprotective non-hematopoietic EPO derivates. One of these derivates results from the total enzymatic removal of sialic acid from EPO. AsialoEPO displays neuroprotective activities without stimulation of hematopoiesis [72]. In contrast to EPO, asialoEPO has an extremely short plasma half-life and is so rapidly degraded in the plasma that EPO–EPOR interactions do not reach the level necessary to stimulate hematopoietic precursor cells, and thus erythropoiesis is not activated. However, asialoEPO can cross the BBB and its degradation is much slower, allowing binding and activation of EPOR in the brain. Neuro-EPO (rhEPO with low sialic acid content), another structural variant of EPO, is very similar to the one that occurs in the mammalian brain [53]. When brain EPO is compared with serum EPO, brain EPO is smaller in size and more active *in vitro* at low ligand concentrations owing to the lower extent of sialylation [73]. Owing to its low sialic acid content, Neuro-EPO is rapidly degraded by the liver. Thus, this molecule could be administered by a non-systemic route, such as the intranasal route, to prevent its degradation in the liver. Neuro-EPO reaches the brain rapidly, does not stimulate erythropoiesis, and shows efficacy in some rodent models of brain ischemia and in non-human primates [53].

Another approach is carbamoylation of EPO, which strongly reduces the binding affinity of EPO for EPOR. Carbamoylated EPO (CEPO) is neuroprotective and does not stimulate hematopoiesis [74]. Intravenous CEPO crosses the BBB in rodents [74,75]. The results of a continuing clinical trial with CEPO in acute stroke are still being awaited. More recently, two peptides modeled after EPO helix B, which is not involved in EPOR binding, were shown to have neuroprotective but not erythropoietic effects [76]. A recent study has shown that a peptide encompassing the C alpha-helix region of EPO (amino-acid residues 92 – 111), termed Epotris, binds to EPOR and induces neurite outgrowth and neuronal survival [77]. Systemically injected Epotris does not induce erythropoiesis. Epotris crosses the BBB

Table 2. Protein and peptide derivates of erythropoietin.

Name	Molecular mass (kDa)	Neuroprotective effect	Erythropoietic effect	Ref.
rhEPO	30 – 34	Cerebral ischemia, TBI, SAH, HIE, epilepsy, Parkinsonism, EAE, spinal cord injury	+	[6,26]
CEPO	40	Cerebral ischemia, spinal cord injury, diabetic neuropathy, EAE	-	[72]
Asialo-EPO	30 – 34	Cerebral ischemia, spinal cord injury, sciatic nerve crush	-	[70]
Neuro-EPO	30 – 34	Cerebral ischemia	-	[54]
Helix B Peptide	20 aa aa residues of hEPO (58 – 82)	Cerebral ischemia, sciatic nerve crush	-	[74]
Pyroglutamate 11-mer helix B surface peptide	11 aa	Cerebral ischemia, sciatic nerve crush	-	[74]
EpoTris	19 aa aa residues of hEPO (92 – 111)	Seizure, kainic acid toxicity	-	[75]

aa: Amino acids; CEPO: Carbamoylated erythropoietin; EAE: Experimental autoimmune encephalomyelitis; EPO: Erythropoietin; hEPO: Human erythropoietin; HIE: Hypoxic ischemic encephalopathy; rhEpo: Recombinant human erythropoietin; SAH: Subarachnoid hemorrhage; TBI: Traumatic brain injury.

and has anticonvulsive and neuroprotective effects in a mouse model of kainic acid-induced neurotoxicity. In spite of these advances, some safety problems remain. Marketed pharmaceutical products of rhEPO and its derivatives or peptide mimetics are used as injections. Patients may be intravenously or subcutaneously injected many times, which causes a high risk for infective dermatitis at the injection site, reducing the patient's acceptance. Such unsolved safety concerns with EPO still indicate the reqirement of the use of the intranasal route as the most promising alternative drug delivery route [12,53].

4. Intranasal delivery of erythropoietin to the central nervous system

It has been shown that rhEPO is absorbed through the nasal mucosa of rats without enhancers after a single intranasal administration and stimulates erythropoiesis in a dose-dependent manner as evaluated by the percentage of circulating reticulocytes [78]. These results indicate that intranasal EPO at high doses does not prevent its systemic effects. In 2005, Yu *et al.* showed that intranasal administration of rhEPO at smaller doses than those in systemic administration protected rats from acute brain injury after transient focal cerebral ischemia, although the dose dependence of intranasal rhEPO was not compared with intraperitoneal rhEPO [47]. The range of effective doses of rhEPO is ~ 16 – 80 U/kg in intranasal administration, which is similar to the recommended clinical doses (75 – 150 U/(kg week)) in subcutaneous or intravenous administration, but much smaller than those in animal experiments with intraperitoneal injections (mostly 1000 – 5000 U/kg). Importantly, to exclude the non-specific effect, the inactivated rhEPO (by heating at 56°C for 30 min) was administered intranasally as a control in that study [47]. These results suggest that intranasal EPO may be a more effective administration route for treatments of ischemic stroke.

More recent studies using radiolabeled rhEPO further supported the transport of EPO from nose to the CNS in normal or injured rodent brain [54,79]. A short of period time (25 min) following intranasal delivery, rat olfactory bulbs and trigeminal nerve had extremely elevated levels of EPO (160.3 ± 53.1 and 152.4 ± 21.6 ng/ml, respectively) compared with other nervous system structures, suggesting that EPO may penetrate to the CNS via multiple pathways [79]. By 60 min these levels had decreased significantly, whereas cortical EPO levels increased slightly. Systemic doses in animal experiments range up to 5000 U/kg, which translates into an approximate blood level of 40 ng/ml and CSF EPO levels of 1000 mU/ml (which is < 8.4 ng/ml) [29]. Minimal EPO level has been detected in the liver, indicating that intranasally administered EPO at low doses does not significantly pass to the systemic circulation [79]. The penetration of radioiodinated EPO and/or IGF-1 from nose to the brain was significantly higher compared with intravenous, subcutaneous and intraperitoneal injections both in normal and ischemic mouse brain [54]. In that study, EPO doses were 5000 U/kg for systemic administration and 100 U for intranasal delivery. Intranasal delivery showed the highest concentrations and earliest peak concentrations in the brain compared with other delivery routes. The accumulation of intranasally administered EPO in ischemic brain tissue has been questioned recently [80]. Although whole-brain lysates were used for liquid scintillation measures and the levels of the agents have not been quantitatively evaluated in different brain structures, autoradiography studies by Fletcher *et al.* showed the accumulation of radiolabeled cytokines in stroke core and penumbra regions [54]. In contrast to this, there was not a statistically significant difference in rhEPO or CEPO levels in

the damaged hemisphere versus the contralateral non-ischemic hemisphere following peripheral administration of rhEPO and CEPO in a rat model of embolic stroke [75]. The differences between the studies may be due to administration route, animals, stroke model and measurement techniques. Increased endogenous EPO level resulting from ischemic insult could have interfered with the measurements by ELISA in the study of Wang Y *et al.* [75]. However, as the antibodies used in the ELISA assay to quantitate rhEPO in that study are specific for human EPO, endogenous rat EPO does not contribute to the observed drug levels and therefore neither rhEPO nor CEPO was detected in plasma or brain of rats treated with saline as control. Fletcher *et al.* showed that ID achieved peak brain tissue concentrations 7 – 31 times higher for EPO and 12 – 20 times higher for IGF-1, and at an earlier time interval than the other delivery methods. The peak concentrations for all delivery methods were higher in the post-stroke brain than in the normal brain, most probably owing to increased BBB permeability from ischemia-induced vascular dysfunction. As revealed by stroke volume measurements 24 h after reperfusion and neurobehavioral outcome assessment up to 90 days following stroke, intranasal coadministration of these neuroprotective agents provided more efficient neuroprotection than the treatment with either EPO or IGF-1 alone in a mouse model of transient focal cerebral ischemia, suggesting that the intranasal route could potentially provide a fast and efficient treatment to prevent acute and chronic effects of stroke.

Neuro-EPO is the only derivative of EPO tested intranasally. Pharmacokinetic studies with Neuro-EPO in mice, gerbils and non-human primates have recently been reviewed [53]. Radioiodinated Neuro-EPO can rapidly cross to the brain in gerbils and Neuro-EPO improves histopathological and clinical signs of cerebral ischemia [81]. Although the distribution of the drug has not been evaluated in cortical structures or ischemic region, radioiodinated Neuro-EPO shows time-dependent transport from olfactory bulbs to the cerebellum in a rostral to caudal direction. Neuro-EPO has been detected in CSF of primates following ID [53]. Furthermore, the amounts of intranasally administered Neuro-EPO are significantly lower than the amounts used in intravenous administration. Single or multiple dose regimens with intranasal Neuro-EPO decrease ischemic region and brain edema and improve neurobehavioral outcomes in rodent models of cerebral ischemia [53]. It has been concluded that, in contrast to other EPO derivatives, the structural similarity of Neuro-EPO to the endogenous EPO constitutes a potential advantage over the other variants of EPO for the chronic treatment of neurodegenerative diseases.

5. Limitations, challenges and solutions for intranasal use of erythropoietin

Increasing experimental evidence suggests that intranasal EPO delivery may be more practical, safe and efficient than systemic administration for protection against brain injury in humans. In spite of the significant merit of bypassing the BBB, direct nose-to-brain delivery still bears limitations of low efficiency and volume for capacity owing to the limited volume of the nasal cavity, the small area ratio of olfactory mucosa to nasal mucosa and the limitations of low dose and short retention time of drug absorption [34,38]. Despite ethical considerations that exist, non-human primate studies would be more informative for the translation of experimental results to human patients [41,53].

There are numerous studies with systemically administered rhEPO in healthy volunteers. Several recent human studies performed in either healthy volunteers or depressed patients have evaluated the effect of EPO on brain functions using non-invasive neuroimaging, neurophysiological or neuropsyhcological techniques [82,83]. EPO positively influences human cognition and exerts an antidepressant effect in these studies. In a more recent study, EPO injections for 3 days at high doses increased CSF EPO concentration and cerebral glucose and lactate metabolism, but had no effect on cognition [61]. These studies may encourage intranasal use of EPO in human subjects. The possible sleep modulatory effect of EPO evidenced by animal experiments could also be tested in human beings using intranasal EPO [84].

Several therapeutic considerations such as optimal dosing, nasal inflammation, the patience and compliance of intended subjects and variability of intranasal dosing should be kept in mind in human studies with intranasal EPO. As ID is believed to occur from the nasal mucosa to the CNS and does not require absorption into the general circulation with subsequent delivery across the BBB, it should not be assumed that the effective dose range is a direct function of body weight [33]. Although a contradictory finding, most studies suggested that intranasal drug pharmacokinetics and/or pharmacodynamics are not affected by the presence of rhinitis and nasal inflammation [33]. Inter- and intra-subject variability in pharmacokinetics and/or pharmacodynamics can be affected by numerous factors, including those arising from the subject, delivery device, formulation, and the drug itself [33]. For high-molecular-mass drugs such as peptides and proteins including EPO, intranasal pharmacokinetics show relatively low bioavailability and relatively high variability compared with injections. This can be ameliorated by the use of permeation enhancers, which can enhance bioavailability and reduce variability [33,38].

A recent study has reported no significant local nasal irritation or hematological side effects in acute and subacute nasal dosing of EPO with a low content of sialic acid in adult rats [85]. However, enhanced immune response in EPO-treated mice and rats has been reported with either systemic or intranasal administration [85]. As EPO has also been implicated in the pathogenesis of CNS tumors, similar safety concerns coud still be valid with ID of EPO [86,87]. An interesting opinion is that structural modifications in the EPO molecule abolishing its hematopoietic effects or delivery of the molecule to the CNS by means of a route other than systemic

administration, such as the intranasal route, may exclude beneficial non-neuronal effects of EPO, including BBB protection, stimulation of angiogenesis, or regulation of vascular tone [14,80]. These advantages of systemically administered rhEPO are more important in infants, who do not show serious side effects even after a long-term systemic EPO treatment, in contrast to adults. Following a single EPO injection, the circulating concentrations of EPO in rats given 5000 U/kg EPO is comparable to that for infants given 500 or 1000 U/kg, suggesting that 500 – 1000 U/kg is a suitable dosing range [21]. Plasma concentrations fall more rapidly after intravenous dosing, so more frequent dosing might be required [14]. A recent clinical trial has shown that repeated doses of systemic rhEPO reduce the risk of disability in newborns with hypoxic-ischemic encephalopathy, without apparent side effects [24].

6. Conclusions

Intranasal drug delivery, administered as nose drops or nasal spray, has great clinical potential owing to simplicity of administration, non-invasive drug delivery, rapid drug absorption and relatively rapid CNS delivery for a given drug when compared with systemic administration, ability to repeat dosing easily, no requirement for drug modification, and minimal systemic exposure. Intranasally administered neurotherapeutic agents bypass the BBB and enter the CNS via several pathways, such as the olfactory, trigeminal and systemic pathways. Intranasal formulations are now more widely used because of improved tolerability compared with injections. Intranasal dosing may be particularly suited for chronic dosing of patients who are unqualified to administer therapies with needles, as well as when oral dosing is problematic. At present, EPO is administered either as an intravenous or a subcutaneous injection two to three times a week, depending on the patient requirement. Thus, ID of EPO seems to be a patient-friendly route. The results of recent preclinical stroke studies suggest that the ID of EPO provides a non-invasive method that enhances further the efficiency of drug by lowering the dosage needed and providing a faster delivery route to the brain. The therapeutic effect of intranasal EPO delivery in preclinical models of other nervous system disorders, including traumatic brain injury, MS, schizophrenia and chronic neurodegenerative diseases such as AD and PD, remains to be tested.

7. Expert opinion

The blood–brain barrier restricts the use of numerous neuroprotective agents that were developed to treat acute and chronic CNS disease neurodegeneration, because it limits CNS penetration, depending on drug size or charge. The use of intranasal delivery as a practical and non-invasive method of bypassing the BBB allows drugs that do not cross the BBB to be delivered to the brain and spinal cord rapidly, reducing systemic exposure and its potential side effects. This new method has already been used successfully in delivering rhEPO or rhEPO with a low content of sialic acid (Neuro-EPO) to the brain in the preclinical models of cerebral ischemic stroke. Although EPO is accepted as a safe therapeutic in the treatment of anemia, the clinical use of EPO as a neuroprotective drug at high doses raises concerns about its possible adverse side effects. Various strategies such as targeted delivery of EPO to diseased brain regions, the use of non-hematopoietic neuroprotective derivatives of EPO and EPO mimetic peptides, and ID of EPO can be used for increasing EPO bioavailability and decreasing side effects in clinical use in the treatment of neurological diseases. Although ID does not require any modification to therapeutic agents, further studies are needed to compare the pharmacokinetics and neuroprotective effects of rhEPO and its structural variants in intranasal use. The probability of the entry of low doses of intranasal rhEPO to the systemic circulation and exerting systemic effects seems to be very low. However, intranasal use of a non-hematopoietic variant may be more advantegous in this situation. CEPO and EPO mimetic peptides have not been tested intranasally. In particular, the low molecular mass of peptide mimetics may pose a potential advantage for transport to the CNS.

Although the requirement of multiple dosing may favor use of the intranasal route in infants, intranasal EPO delivery may not provide further advantage over systemic administration because of the important contribution of systemic effects of rhEPO to neuroprotection and much fewer systemic side effects in this age group. The immaturity of BBB in infants may also facilitate the crossing of systemically administered EPO to the brain. It has been proposed that systemic effects such as enhanced erythropoiesis, which increases iron utilization thereby decreasing free iron and reducing oxidative brain injury, complement the direct neuroprotective effects of EPO and may explain why lower dosing strategies also improve outcome in infants [14]. Systemic EPO may provide long-term neurorestoration after an insult by providing increased oxygen-carrying capacity through increased erythropoiesis and angiogenesis. Thus, it is important to proceed cautiously with clinical trials because risks may vary among specific populations, ages and disease states.

Although the exact mechanisms are still unclear, multiple transport pathways are probably involved in the transport of EPO from nose to brain. Whether related structures including nasal cavity, nasal vasculature, trigeminal nerve, olfactory mucosa and nerve express EPOR or its other putative receptors is not known. Conversely, the presence of a specific neurotrophic factor receptor in olfactory mucosa may be a limiting factor for the transport of this neurotrophic factor, as demonstrated for brain-derived neurotrophic factor and its receptor trkB, which is highly expressed in the olfactory epithelium [79]. The exact mechanisms of intranasal transport of EPO to the injured brain regions are also unclear. In general, the identification of the endogenous mechanisms of

the drug targeting to the injured brain areas may help in the development of drug targeting strategies that can be used exogenously. This may be especially useful in chronic use of the intranasal route for the therapy of chronic CNS diseases such as AD and PD that predominantly influence the specific brain regions.

At present, there are no studies available profiling a dose response in rodent and human brain. Therefore, the minimum dosage of intranasal EPO required to exert a protective effect in the rodent or human brain is not known. The absorption and distribution of EPO must be critically evaluated to determine the amount of drug delivered to the CNS. Further preclinical testing is necessary to identify the characteristics of the temporospatial accumulation of EPO and its derivatives in the brain tissue, the synergistic beneficial effects of EPO with other drugs, and the efficacy of EPO in animal models of CNS disorders other than stroke. Recently, the synergistic effects of EPO with β-human chorionic gonadotropin (β-hCG) were evaluated in a Phase IIa study [88]. The results of this study provide data supporting the safety of combined treatment of EPO with β-hCG when initiated 24 – 48 h after stroke onset. Combined treatment of EPO with other drugs could be used in intranasal administration. Preclinical studies have shown that combined use of EPO with other drugs in intranasal administration will be a good therapeutic approach against neurodegenerative disorders [80,81]. It has been found that intranasal delivery of EPO with insulin-like growth factor (IGF-1) is effective for the treatment of an animal model of stroke and HIV-associated neurocognitive disorder [89]. These results suggest that intranasal delivery of EPO with other drugs should be considered for clinical trial in neurodegenerative disorders.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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