Accepted Manuscript

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Please cite this article as: Guardia Clausi M, Paez PM, Pasquini LA, Pasquini J.M.Inhalation of growth factors and apo-transferrin to protect and repair the hypoxic-ischemic brain.*Pharmacological Research* http://dx.doi.org/10.1016/j.phrs.2016.01.010

Inhalation of growth factors and apo-transferrin to protect and repair the hypoxic-ischemic brain

Running title: Inhalation of drugs and brain injury.

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Graphical abstract
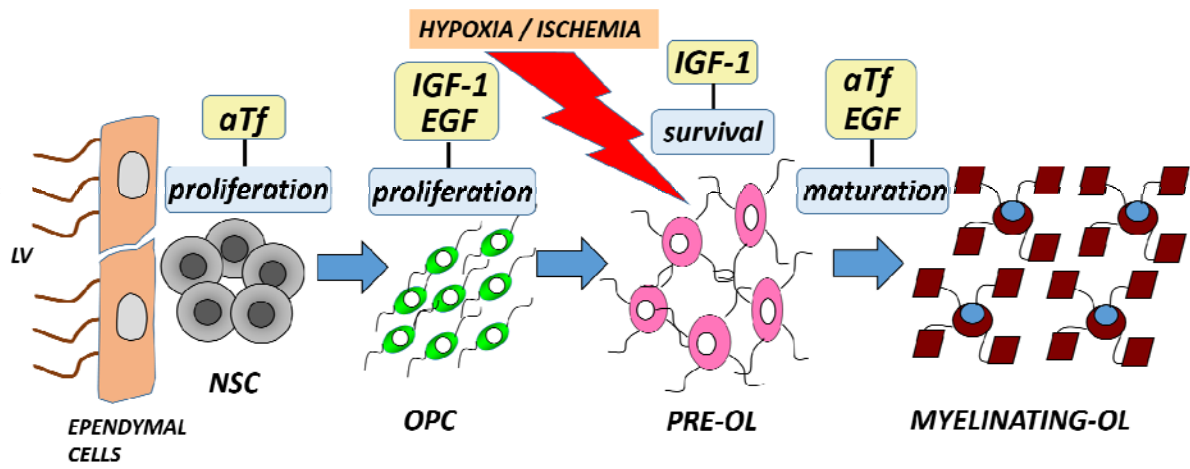
ABSTRACT

Hypoxic-ischemic brain damage is a major contributor to chronic neurological dysfunction and acute mortality in infants as well as in adults. In this review, we summarize recent publications demonstrating that the intranasal administration (INA) of apo-transferrin (aTf) and different growth factors provides neuroprotection to the mouse and rat brain after a hypoxic-ischemic event. The intranasal delivery of growth factors such as insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) has been found to improve neurological function and reduce infarct size in adult rats after a hypoxic-ischemic event. On the other hand, INA of aTf and epidermal growth factor (EGF) were effective in reducing white matter damage and inflammation and in promoting the proliferation and survival of oligodendroglial progenitor cells (OPCs) in a model of hypoxic-ischemic encephalopathy. Therefore, data summarized in this review suggest that INA of growth factors and aTf can be used in combination in clinical treatment in order to protect and repair the hypoxic-ischemic brain.

Drug instillation

A promising way to deliver drugs to the brain is the intranasal route. The olfactory region is the only site where the central nervous system (CNS) is in contact with the external environment due to the presence of the olfactory receptor neurons, whose axons end in the olfactory bulb. Illum and coworkers (2000; 2004) [1,2] determined the existence of a direct pathway connecting nose to brain and provided details of the possible routes of entry of substances introduced into the nose of different animals and humans. The authors postulated that, depending on the size, charge and hidro or lipophilicity of the molecule, different substances could be transported into the brain. Essentially, two routes have been proposed for the direct passage of peptides and proteins from the nose to the brain: an intraneuronal and an extra-neuronal pathway [2]. Intraneuronal transport includes the internalization of the peptide into olfactory neurons, followed by axonal transport. However, this route poses a great risk of proteolysis, resulting from lysosomal degradation, and requires several hours for substances to reach the olfactory bulb [2]. It therefore seems more probable that peptide molecules travel by the extracellular route, passing through patent intercellular gaps in the olfactory epithelium to diffuse into the subarachnoid space [2].

Intranasal administration (INA) has many advantages from a clinical point of view: it is noninvasive and easily carried out given the capacity of bypassing the blood-brain barrier [3,4]. The potential utility of INA derives from the fact that biologically effective concentrations of neuropeptides and proteins can reach the human brain without serious systemic side effects. Such effects limit the systemic administration of peptides to quantities too small to exert significant effects in the brain. A wide range of studies has explored the transport of various drugs from the nasal cavity to the brain and, although most of them have been conducted in rat models, studies in mice, rabbits and monkeys have also been reported. INA has not only been used in the basic research field [5-8], but has also found applications in human health [9,10]. Several reports confirm the positive outcome of nose-to-brain delivery not only for drug

molecules with various molecular weights [11,12] but also for living cells [13,14]. Hanson et al (2009) [11,12] reported that INA targets deferoxamine to the brain and reduces systemic exposure, and that intranasal deferoxamine prevents and treats stroke damage after middle cerebral artery occlusion in rats. On the other hand, Danielyan and collaborators (2001) [13,14] have revealed noninvasive intranasal delivery of stem cells to the rat brain for the first time, showing that the intranasal application of mesenchymal stem cells resulted in the appearance of cells in the olfactory bulb, cortex, hippocampus, striatum, cerebellum, brainstem and spinal cord. Therefore, INA represents a highly promising alternative to target and deliver stem cells or neurotrophic factors to the brain with the option of chronic application.

Intranasal administration of growth factors

The INA of neurotrophic factors and other substances, including certain hormones, has received increasing attention in recent years [15]. Different reports on successful INA of insulin-like growth factor-1 (IGF-1) in the treatment of various brain injuries have been recently published. Thorne and colleagues (2004) [8] demonstrated that IGF-1 administered intranasally in rats can reach distant areas such as the cerebral cortex, the hypothalamus, the cerebellum, the brain stem and the medulla in concentrations considered to be of therapeutic value **(Figure 1)**. The intranasal delivery of nerve growth factor (NGF) has been reported to ameliorate or prevent neurodegeneration and memory deficits in the AD11 mouse model of Alzheimer's disease [16,17]. In addition, NAP (an 8-amino acid peptide derived from activity-dependent neuroprotective protein ADNP) has been observed to improve the performance of normal and cognitively impaired rats in the Morris water maze test. Moreover, NAP has been shown to alleviate anxiety and enhance cognition after chronic intranasal treatment [18]. Additionally, the intranasal delivery of activity-dependent neurotrophic factor (ADNF) to the brain has been reported to play a neuroprotective role [19]. Most importantly, intranasal neurotrophins such as

fibroblast growth factor-2 and heparin-binding epidermal growth factor-like growth factor have been shown to enhance neurogenesis in the subventricular zone of the adult mouse brain [20].

Growth factor inhalation and hypoxic-ischemic brain injury

There are no clinically relevant treatments for people suffering hypoxic-ischemic brain injury and the accessibility of different therapeutic molecules to the specific brain damage areas remains problematic. In recent years, growth factor inhalation has been studied as a therapy for hypoxic-ischemic brain injury or brain stroke. IGF-I has been shown to exert protection against stroke when administered intracerebro-ventricularly in rats, although this invasive method of administration is not practical for the large number of individuals who require treatment. However, intranasal delivery of IGF-1 has been found to improve neurological function in adult rats after hypoxic-ischemic brain damage [21,22]. In a work by Liu et al. (2001), INA of IGF-1 was shown to significantly reduce infarct volume and improve neurological function following focal cerebral ischemia in rats, solving deficit in motor, sensory, reflex and vestibulomotor functions [21]. Similarly, intranasal delivery of IGF-1 has been found to recuperate neurological function in adult rats after middle cerebral artery occlusion [21,22]. Intranasal IGF-1 significantly reduced infarct volumes and hemispheric swelling and improved neurologic function, assessed by the postural reflex, flexor response and adhesive tape tests. In the same line, Lin et al. (2009) confirmed that INA of IGF-1 is an effective way to target this growth factor to the neonatal rat brain following cerebral hypoxia-ischemia [23]. Intranasal delivery of IGF-1 not only attenuated pathological changes induced by hypoxia-ischemia in the neonatal brain, but also enhanced neurological functions [23] **(Figure1)**. It has been also demonstrated that IGF-1 treatment activates the pAkt pathway and inhibits the activation of caspase-3 after cerebral hypoxia ischemia [23]. Moreover, it has been shown to promote the proliferation of neural progenitor cells during the tissue repair stage in a neonatal hypoxic-ischemic model [23]. Similarly, Yang and colleges (2009) [12] have evaluated dose effectiveness in the intranasal

delivery of vascular endothelial growth factor (VEGF) in the treatment of experimental stroke, reporting that INA of VEGF was effective in reducing infarct volume, improving behavioral recovery and enhancing angiogenesis in the stroke brain [12].

The epidermal growth factor (EGF) is an important player in the development of oligodendrocytes [24]. Using an established model of preterm brain injury, Scafidi et al. (2014) have demonstrated that INA of heparin-binding EGF immediately after hypoxic injury decreases oligodendroglia cell death, increases the production of new oligodendroglial cells and promotes brain recovery [25]. Furthermore, these interventions diminish ultrastructural abnormalities and alleviate behavioral deficits in white-matter-specific paradigms [25]. Thus, these results provide direct evidence that INA of EGF at a specific time after the hypoxic damage is clinically feasible and potentially applicable to the treatment of premature children with white matter injury.

In summary, these studies indicate that INA of growth factors holds significant promise as a noninvasive and efficacious method for the treatment of hypoxic-ischemic brain damage **(Figure 1).**

Intranasal administration of apo-transferrin

Previous studies have shown that the intracerebral injection of apo-transferrin (**aTf**) alleviates white matter damage and accelerates remyelination in neonatal rat models of neurodegeneration [26-28]. Nevertheless, the intracerebral injection of aTf might not be adequate for clinical treatments. Therefore, the development of less invasive techniques for the delivery of aTf to the CNS has been investigated in order to use this protein in clinical studies and, in particular, our group has explored the possibility of delivering aTf into the brain using INA of radioactive iodine-labeled aTf. ^{125}I-aTf of high specific activity was prepared and delivered through the nostrils of anesthetized young rats. Two hours later, the animals were perfused and the brains excised. The cerebral hemispheres were divided into three areas (anterior, middle and posterior, including the brain stem and cerebellum) and the distribution of

radioactivity present in the tissue was analyzed by autoradiography of coronal brain slices. Our results show that, although in small amounts, the radiolabeled aTf introduced through the nostrils reached distant areas of the brain (**Figure2**), which suggests that INA is a feasible procedure to deliver aTf into the brain.

Similar experiments were performed in a model of hypoxic-ischemic encephalopathy [29]. We have found that aTf reaches the brain parenchyma and increases its presence in the different areas of the CNS. We have also shown that aTf was present in the right olfactory bulb and in the frontal and posterior brain in both the control and hypoxic-ischemic animals after INA. The mechanisms of protein transport from the nasal cavity to the brain are not entirely known, although several possible pathways have been proposed [2]. Our results indicate that anterograde axonal transport is the pathway for aTf delivery into the perinatal mouse brain. In support of this conclusion, a very low concentration of aTf was detected in the olfactory bulb when cytochalasine B or colchicine was administered before the INA of aTf. Colchicine inhibits microtubule assembly and reduces axonal transport [30], while, in the optic nerve, cytochalasin B is an inhibitor of neurofilament axonal transport [31]. However, further studies are necessary to describe the mechanism of transport of aTf from the olfactory area to the brain.

Apo-transferrin inhalation and hypoxic-ischemic brain damage

In the CNS, transferrin (Tf) is produced by oligodendrocytes and is vital for normal brain development. We have found that aTf is essential for oligodendrocyte maturation and myelination in vitro as well as in vivo. Since 1994, our laboratory has published a number of paper describing the effects of aTf on oligodendroglial cell differentiation and myelination. We have reported that a single intracranial injection of aTf upregulates the expression of diverse myelin constituents and significantly increases myelin deposition, especially in areas close to the lateral ventricles in rats [32,33]. This promyelinating effect was also seen in primary cultures of oligodendrocytes [34], as well as in oligodendroglial cell lines treated with aTf [35,36]. We

have demonstrated that aTf modulates the expression of myelin basic protein (MBP) through different signaling pathways and, furthermore, we have shown that aTf overexpression promotes oligodendrocyte differentiation and myelination of cortical neurons [36,37].These data have been confirmed by other authors who showed that aTf regulates MBP expression [38] and that transgenic mice overexpressing the human Tf gene in the CNS evidence increased myelination [39].

In a rat model of neonatal hypoxia-ischemia encephalopathy (**HIE**) [29], we have demonstrated that the INA of aTf can remyelinate areas of demyelination. Intranasal delivery of aTf decreased astrogliosis and neuronal loss in HIE animals and increased oligodendrocyte survival in different areas of the brain [29]. We also found that the INA of aTf enhanced the proliferation of OPCs in the corpus callosum and the subventricular zone and protected these cells against apoptotic death after the hypoxic-ischemic incident. For instance, the number of PDGFRα-positive OPCs was higher in mice treated with aTf than in untreated brains two days after the hypoxic-ischemic event [29]. Additionally, the number of OPCs positive for caspase-3 in the corpus callosum, cortex and striatum was lower in aTf-treated hypoxic-ischemic mice. A summary of aTf effects on the neonatal hypoxic-ischemic brain is shown in **Figure 1**. These results seem to indicate that aTf is an inducer of myelinating oligodendrocytes in the neonatal mouse brain in acute demyelination caused by HIE. Additionally, this study shows that the intranasal delivery of aTf promotes the survival and maturation of OPCs after demyelination and suggests that the INA of aTf can be used for clinical treatment to induce remyelination in demyelinating hypoxic-ischemic events.

The authors declare no competing interests.

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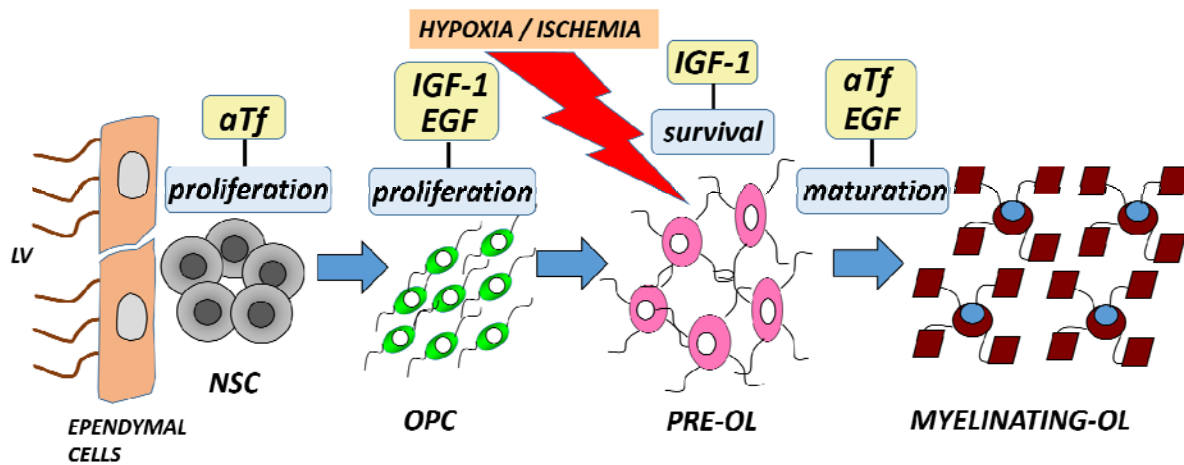
Figure Captions

Figure 1: Promyelinating effects of intranasal apotransferrin (aTf), EGF and IGF-1 after neonatal hypoxia ischemia. Intranasal aTf, EGF and IGF-1 restore white matter development disrupted by a hypoxic ischemic insult (H/I). aTf induces proliferation of neural stem cells (NSC) located in the wall of the lateral ventricle (LV). IGF-1 and EGF promote the proliferation of oligodendrocyte precursor cells (OPCs). IGF-1 prevents apoptosis of pre-oligodendrocytes (pre-OL), which are particularly vulnerable to H-I. aTf and EGF accelerate the maturation of pre-oligodendrocytes to become myelinating oligodendrocytes.
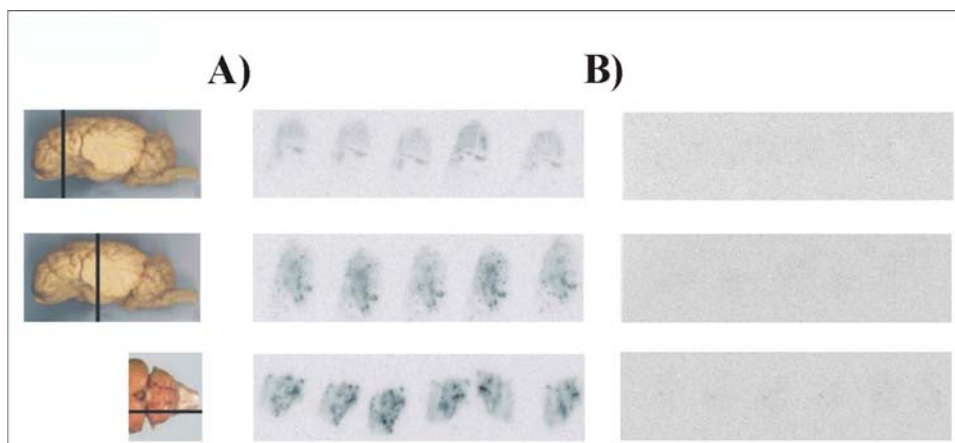
Figure 2: No radioactivity was found in the controls treated with unlabeled aTf. Tissue radioactivity in brains treated with iodine-labeled aTf (^{125}I-aTf) was evidenced by the presence of the intact molecule of aTf, which was checked by gel electrophoresis followed by radioautography, and the identification of the radioactive band by its molecular weight. No radioactive byproducts were detected. This indicates quite clearly that aTf can be safely delivered into the brain by intravascular infusion. Experiments were done following the method described in Hill et al.,1985 with slight modifications