Title: The Benefit of Extracellular Vesicles at the Blood-Brain Barrier

An editorial on: Manuscript 315031: Neural Progenitor Cell-Derived Extracellular Vesicles Enhance Blood-Brain Barrier Integrity by NF-κB (Nuclear Factor-κB) Dependent Regulation of ABCB1 (ATP-Binding Cassette Transporter B1) in Stroke Mice, By Zhang et al ATVB

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Editorial

Stroke is an extremely debilitating disease and the second most common cause of death and disability worldwide [1]. In addition to causing neuronal cell death, the cerebral ischaemia that occurs during stroke causes disruption of the blood-brain barrier (BBB), resulting in direct exposure of the brain to intravascular blood proteins, blood cells, pathogens and toxins. Thus, if a treatment can be found to preserve the integrity of the BBB in stroke patients, it should also greatly improve their outcome.

Several studies have shown that stem and progenitor cell transplantation following ischaemic stroke protects the BBB, thereby limiting brain injury and neuroinflammation, and improving post stroke recovery. For example, Doeppner et al showed in 2014 that transplantation of neural progenitor cells (NPCs) induced acute post ischaemic neuroprotection by stabilizing the BBB in mice that had been subject to middle cerebral artery occlusion (MCAO) [2]. Importantly, the cells did not need to be injected intracranially, but can be administered intravenously, and still provided the same benefit despite their low levels of intracerebral engraftment. This can be explained by small, lipid-bilayer nanoparticles called exosomes, which allow cells to communicate in a paracrine manner [3].

Various techniques can be used to isolate exosomes from cells in tissue culture, however, most techniques don't result in completely pure exosomes, but exosome-enriched fractions that also contain some contaminating non-exosomal vesicles and protein [4]. For this reason, current guidelines advise that the isolates be referred to as extracellular vesicles (EVs) rather than exosomes [5]. EVs isolated from NPCs using a precipitation-based method have been shown to enhance post-stroke neurological recovery and neuro-regeneration for as long as 3 months following systemic administration in mice [6]. Surprisingly, however, the majority of the EVs are detected in extracranial

organs such as the liver and the lung [6], leaving the mechanism by which they provided neuroprotection unclear.

Now, Zhang et al.[7] have conducted a detailed investigation of NPC-EVs and their effect on the BBB, to establish whether this contributes to their neuroprotective activity. The BBB consists of capillary endothelial cells and basement membrane, neuroglial membrane, glial podocytes (end-feet) and perivascular microglia. Therefore, for their initial, *in vitro* experiments Zhang et al. made use of a tissue-culture model of the BBB consisting of a mixture of endothelial cells and astrocytes, subjecting them to a model of simulated ischaemia using oxygen and glucose deprivation [7]. The observations were then validated *in vivo* using a murine stroke model. In both experimental models, ischaemia activated the stress-responsive transcription factor NF-κB (nuclear factor-κB). In astrocytes, NF-κB activation caused an increase in the levels and activity of MMP9 (matrix metalloproteinase 9), which breaks down the extracellular matrix, reducing BBB integrity (**Figure 1**). In endothelial cells, NF-κB increased the levels of ABCB1 (ATP-binding cassette transporter B1). ABCB1 (also known as P-gp or P-glycoprotein), is an important functional component of the BBB, effluxing potentially damaging molecules and drugs from the brain parenchyma back into the blood to protect the brain tissue. However, ABCB1 also exports inflammatory cytokines, fuelling the post stroke inflammatory response.

Zhang et al. found that systemically administered NPC EVs suppressed NF-κB activation in both astrocytes and endothelial cells, thereby preventing the increases in both MMP2 and ABCB1 [7]. Importantly, using Evans blue-albumen to examine the intactness of the BBB, the authors found that NPC EVs maintained BBB integrity following stroke. Further, indirect support for the increase in BBB integrity following EV administration was provided by the decrease in inflammatory leukocyte infiltration following stroke. The authors suggest that this could be because, with less ABCB1, there is less release of cytokines – although it might also be a secondary effect of there being less tissue injury.

A vital step was to confirm that the systemically administered EVs reached their hypothesized destination *in vivo* - the astrocytes and endothelial cells. To this end, the NPC EVs were labelled with a fluorescent dye to allow their uptake to be detected. Lipophilic dyes are commonly used as a means to visualize EV uptake into cells and organs, but their use requires careful control experiments due to the dyes' promiscuous binding [8]. Zhang et al. took pains to separate free dye by density gradient centrifugation, and also confirmed that the fraction *lacking* EVs did not result in any fluorescent uptake [7]. This provides strong evidence that the labelling is EV-specific and not tissue autofluorescence or artefact.

While the current results demonstrate an important ability of NPC EVs to preserve BBB integrity following ischaemic stroke, future investigations will be required to understand exactly how they suppress NF-κB activation. One possibility is that the EVs transfer miRNAs known to suppress the NF-κB pathway [9]. It is also possible that EVs reduce the production of pro-inflammatory cytokines (e.g.: TNF-α and IL-6) by other cell types such as microglia, thereby reducing the stimulus for NF-κB activation. By their nature, *in vitro* studies are reductionist models, bypassing the intractable complexity of the *in vivo* scenario. An unavoidable limitation of a reductionist model is that you cannot see what isn't there! The *in vitro* culture model of the BBB used here included endothelial cells and astrocytes, but omits the wider context of the neurovascular unit, including neurons, pericytes, astrocytes, and microglia and the blood vessels, which might also be target of the EVs. This simply illustrates the complexity of relating *in vitro* studies to the *in vivo* scenario. Nevertheless, the experiments by Zhang et al. are an important step along the pathway to understanding the mechanism of stem cells and their tiny progeny in benefiting stroke.

Figure 1

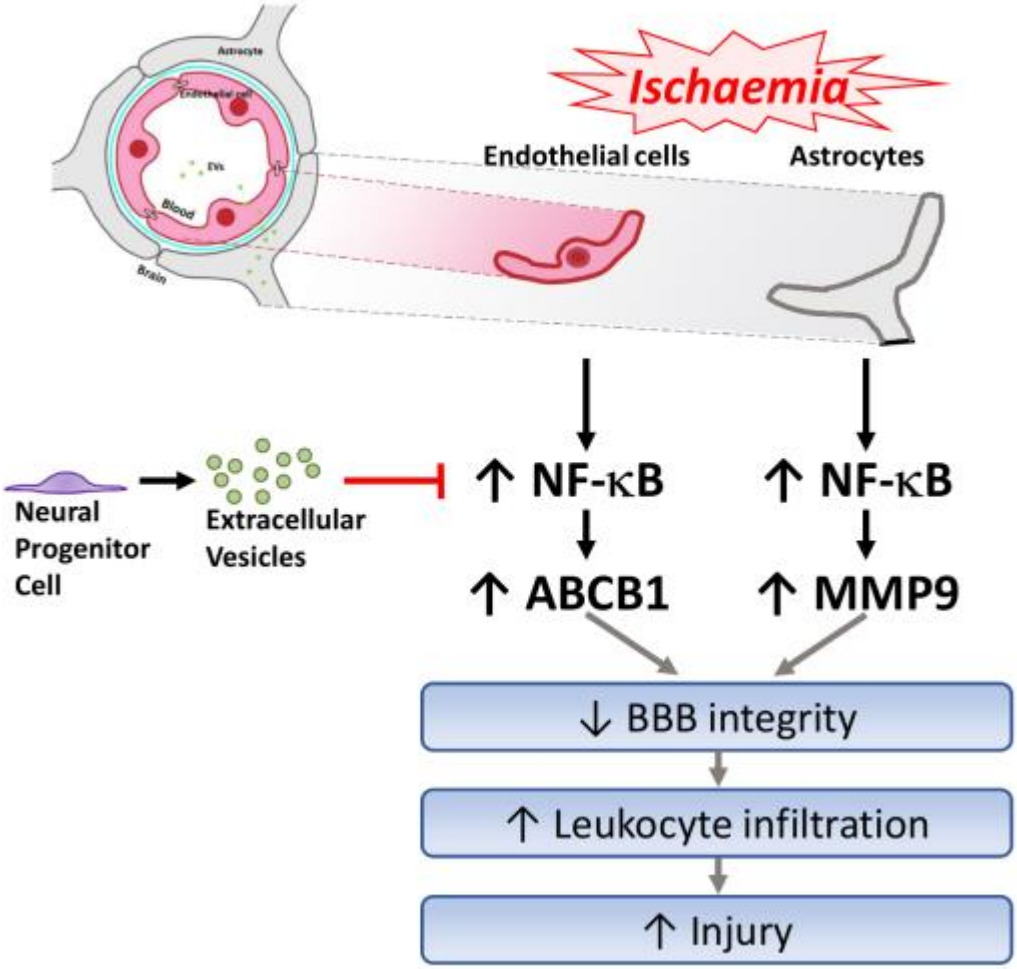

Figure legend

Figure 1. The mechanism proposed by Zhang et al.[7] for how ischaemic stroke damages the blood brain barrier (BBB), leading to inflammation and brain lesions.

Ischaemic stroke results in an increase in NF-κB in endothelial cells and astrocytes. In astrocytes, NF-κB increases MMP9 expression which degrades the basal membrane. In endothelial cells, it increases expression of the ABCB1 drug efflux transporter, which also releases cytokines and chemokines that recruit inflammatory leukocytes and Ly6C^{high} monocytes. Zhang et al. show that neural progenitor cells release extracellular vesicles that inhibit the increase in NF-κB, thereby maintaining BBB integrity, suppressing inflammation and decreasing injury.

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