PERSPECTIVE

Magnetic nanotechnology to study and promote axon growth

After injury of the central and peripheral nervous systems, functional recovery is impaired by axon regeneration failure. Various approaches for promoting axon growth have been attempted but their low efficacy has prevented them from being clinically applicable. It is possible that in spite of all the research that has been performed regarding axon growth, we are still missing key aspects of axon growth biology that are essential in order to design effective treatments for axon regeneration. Most of what we know about how an axon grows has been discovered by using tools that delete, add, block, or activate macromolecules in the whole cell and by looking at its corresponding effect on axon growth. Less is known about the spatiotemporal actions of macromolecules and organelles at the growth cone and its relation with axon growth. Macromolecules and organelles are heterogeneously distributed in the cell and their resulting function depends heavily on when and where they exert their actions. We need tools that allow us to manipulate these macromolecules and organelles in a temporal window during which axon growth events are taking place. The development of such tools will provide enhanced knowledge of how an axon grows and lead to design more effective therapies to promote axon growth.

Superparamagnetic iron oxide nanoparticles (SPIONs) have ideal physical properties for manipulating macromolecules and organelles in seconds and studying the resulting biological effects. SPIONs have a size (1–100 nm), within the size range of macromolecules and organelles. SPIONs can be coupled to moieties that bind specific macromolecules or organelles (*e.g.*, antibodies), or to functional proteins (Hoffmann et al., 2013). Macromolecules or organelles bound to SPIONs can be then manipulated in space and time using magnetic fields and the response of the cell to the perturbation can be studied.

Several authors have been able to manipulate macromolecules in cells using SPIONs. Hoffman et al. (2013) were able to induce microtubule assembly at specific locations of an artificial cell by using SPIONs coupled to Ran-GTP or to the Ran guanine exchange factor, RCC1. The authors encapsulated *Xenopus* cytoplasm extracts and functionalized SPIONs within spherical droplets and applied a magnetic field. Both RAN-GTP SPIONs and RCC1 SPIONs clustered in response to the magnetic field and triggered an enzymatic cascade involving importins and MAPs that ended with the formation of asymmetric arrays of microtubule fibers. The positions of these arrays were modified after its formation just by rotating the magnetic field. As RAN-GTP plays a central role in axon growth (Hall and Lalli, 2010), it would be interesting to adapt a similar approach to neurons to assess whether RAN-GTP SPIONs can induce axon formation or growth in response to an applied magnetic field. Other authors have used SPIONs to cluster protein receptors at the cellular membrane and induce intracellular signaling responses (Mannix et al., 2008; Lee et al., 2010). In those studies, receptor specific antibodies were coupled to SPIONs and added to cell cultures to freely bind their corresponding receptor. Application of magnetic fields induced SPION magnetization and receptor clustering at the cell membrane. Due to the proximity of these receptors, an intracellular signaling cascade was triggered and a cellular response was induced (cell proliferation in one case and secretion of inflammatory cytokines in the other). These studies demonstrated that ligand-receptor clustering produces more responses than simple ligand-receptor binding. Similarly, SPIONs may also be used to cluster growth cone receptors such as integrins or other growth cone proteins to generate intracellular signaling cascades and study their effect on axon growth (**Figure 1A**).

In addition to local protein concentration and receptor clustering, manipulation of whole organelles using magnetic nanotechnology can also be achieved as we have recently demonstrated (Steketee et al., 2011; Pita-Thomas et al., 2015). Our lab has manipulated neuronal signaling vesicles and filopodia in order to study the effect on axon growth. In our first study (Steketee et al., 2011), we functionalized SPIONs with an agonist anti-TrkB antibody inducing the rapid endocytosis of SPIONs into retinal ganglion cells (RGCs). As the antibody was also coupled with a fluorescent dye, we were able to track TrkB vesicle transport along the axon and at the growth cone in real time. We observed that disrupting the transport of the TrkB vesicles by applying a magnetic field induced growth cone halt. After the magnetic field was turned off, the TrkB vesicle trafficking was reestablished and the growth cone advanced. These experiments highlighted the importance of TrkB signal vesicle trafficking in axon growth. In our second study (Pita-Thomas et al., 2015), we were able to manipulate filopodia using SPIONs and magnetic fields. We induced filopodia growth by applying mechanical tension through membrane targeted SPIONs (**Figure 1B**). These elongated filopodia behaved as *bona fide* filopodia as they were filled by actin cytoskeleton and capable of generating retrograde forces, demonstrating that mechanical tension alone can induce filopodia growth. However, when the elongated filopodia were linked to the substrate through their membrane integrin receptors, the filopodial retrograde forces were unable to induce growth cone advance, suggesting that other signals or structural components are required for axon growth and undermining the importance of filopodia as the main force for inducing growth cone advance. Overall, these studies demonstrate that magnetic nanotechnology has been successfully implemented recently to study cell biology and axon growth. These techniques are now ready to be used more routinely in labs and answer questions related to spatiotemporal actions of macromolecules or organelles.

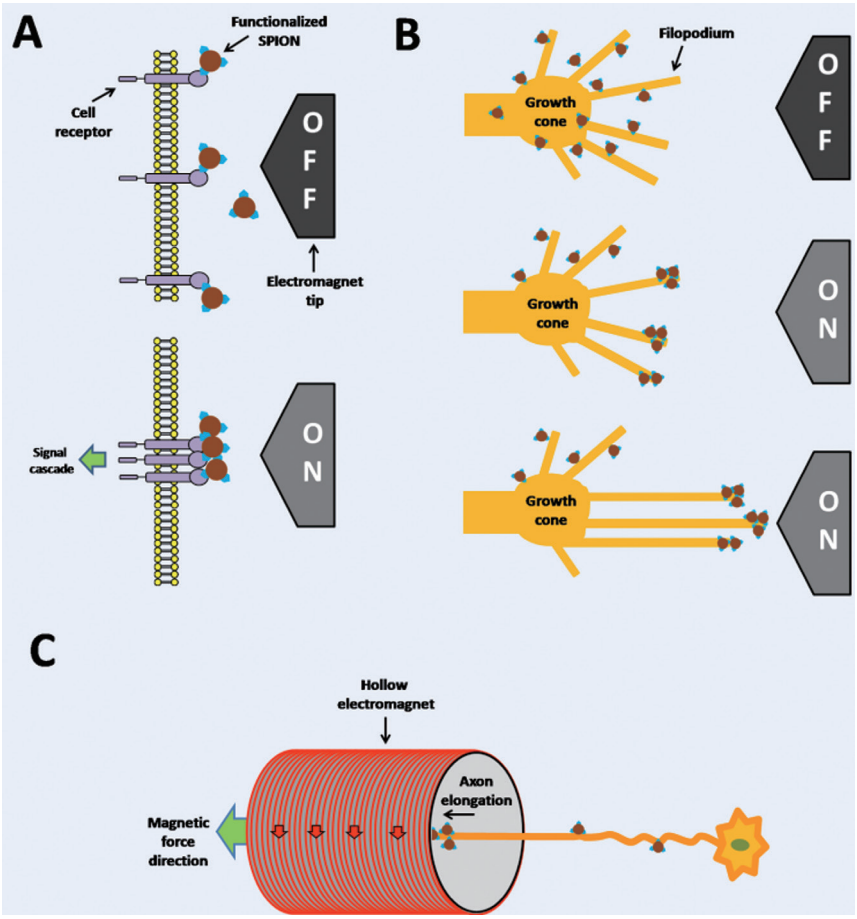

Figure 1 Superparamagnetic oxide nanoparticles (SPIONs) as a tool to study and promote axon growth.
(A) Functionalized SPIONs specifically bind cell receptor proteins present in the axon membrane. SPIONs can be magnetized by close apposition of electromagnet to induce clustering of receptors and a resulting intracellular signaling cascade. (B) Functionalized SPIONs attach to growth cone membrane and then accumulate at the end of filopodia when an electromagnet is turned on. The magnetic force exerted by these SPIONs induces rapid filopodia elongation (Pita-Thomas et al., 2015). (C) A hollow electromagnet made by tightly wrapping copper wire around a hollow metallic cylinder will generate a magnetic force (green arrow) inside the cylinder that is perpendicular to the electric current flowing through the copper wire (orange arrows). SPIONs located at the axon cell membrane may generate magnetic forces and induce axon elongation when axons are located inside the hollow electromagnet.

In addition, magnetic nanotechnology may also offer a new therapy to promote axon growth.

Axon elongation by mechanical forces (*e.g.*, by attaching a pipette tip to the growth cone membrane and pulling) is a well-known phenomenon in cultured neurons (Bray, 1984). However, applying mechanical tension *in vivo* is limited by the inaccessibility of the axons inside the neural tissue. SPIONs have been used extensively as a contrast agent in clinical magnetic resonance imaging due to their low toxicity. SPIONs surface can be modified with moieties that bind specific molecules and, due to SPIONs small size, they can diffuse through tissues and bind specific targets to allow magnetic resonance imaging in, for example, sites of inflammation or tumors (Pita-Thomas and Goldberg, 2013; Sharifi et al., 2015). Grafted cells bound to SPIONs can also be monitored inside the organism by magnetic resonance (Sharifi et al., 2015). The paramagnetic nature of SPIONs also allows the manipulation of these grafted cells inside the organism using external magnets. For example, grafted mesenchymal stem cells bound to SPIONs can be enriched in the spinal cord injury site by applying an external magnet system (Tukmachev et al., 2015). Similarly, drug loaded SPIONs can be directed to specific organs by applying an external magnet; increasing the local drug concentration at the target and reducing side effects (Laurent et al., 2014). SPIONs can also bind to and be endocytosed into neurons *in vivo*. Harrison et al. (2012). showed that after injection of SPIONs at the injured optic nerve site, SPIONs can be found at RGC axons and bodies, demonstrating that SPIONs were attached to the axon membrane and then endocytosed and transported all the way back to the retina. In our recent publication, we were able to target SPIONs to cultured neuron membranes and induce filopodia elongation at high speeds (Pita-Thomas et al., 2015). The sharp needle electromagnet used in our study has a small range and must be placed very close to the neuronal membrane in order to magnetize SPIONs (**Figure 1B**). As a consequence, this type of magnet can only induce SPION accumulation at the filopodial tip and promote filopodia but not axon growth. From our study, we hypothesize that magnetic tension from larger extensions of growth cone membrane is needed to induce axon growth. Could a different type of magnet improve SPION mechanical tension at the end of the axon and induce axon growth *in vitro* and *in vivo*? If axon tracts with membrane localized SPIONs are placed inside of a hollow electromagnet and oriented parallel to the magnetic field, SPIONs along the axon membrane would exert a magnetic force parallel to the magnetic field and tend to accumulate at the end of the axon (**Figure 1C**). The provided SPION mechanical tension may then induce axon

elongation. Larger versions of these hollow electromagnets may be designed for *in vivo* use in order to apply non-contact magnetic force.

For the *in vivo* context, it would be also very important to find the right functionalization to target SPIONs to axons. Moieties that bind specifically axons and no other cell types are desirable as would minimize off target effects. Functionalization with an antibody against Thy1 may limit binding to axons as it is a specific marker of postnatal neurons. We have previously shown that functionalized anti-Thy1 SPIONs bind neuronal membranes and induce magnetic mechanical tension. It would be interesting to test whether injection of these anti-Thy1 SPIONs at the injury site may specifically target axons *in vivo*. The presence of phagocytic cells in the injury site engulfing SPIONs may limit the availability of these particles at the axon membrane. SPIONs with a coating that reduces immune system response would be certainly desirable. Another possibility is that SPIONs are injected far away from the injury, in regions with less presence of phagocytic cells and transported by the endosomal neuronal system. Cholera toxin B functionalization may be an interesting choice to achieve this goal. Cholera Toxin B binds to the ganglioside GM1 present in neurons and then it is transported throughout the whole axon. In our publications, we demonstrated that SPIONs can be attached to Cholera Toxin B, bind the neuronal membrane, and generate magnetic mechanical forces at the neuronal membrane in a similar fashion than anti-Thy1 SPIONs. As we demonstrated previously, anti-trkB SPIONs can be endocytosed by specific signaling endosomes and sorted through the neurite (Steketee et al., 2011). It would be interesting to test whether CtxB SPIONs can also be sorted in their corresponding vesicles and distributed throughout the axon. Once SPIONs are located at the membrane of axons *in vivo*, a magnetic field parallel to axon tracts may be applied to test whether it induces SPION accumulation at the end of the axon and in turn, axon elongation.

In summary, SPION magnetic manipulation can be used to study the spatiotemporal role of macromolecules and organelles in axon growth. Functionalized SPIONs can target axon membranes and generate magnetic mechanical forces to induce filopodia growth and potentially axon growth. New magnet designs may be useful to induce axon growth *in vitro* and *in vivo* using the magnetic mechanical forces of SPIONs. Thus, magnetic nanotechnology offers new ways to study axon biology and may open the door to new therapies for neurological diseases that course with axon regeneration failure, such as traumatic brain or spinal cord injury.

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