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Marie Curie Intra-European Fellowship Project Summary

Application of Mechanical Forces on Axon Growth Cones via Magnetic Nanoparticles to Enhance Axon Regeneration in Central Nervous System (MagForce4AxonGrowth 273420)

The overall goal of the project is to apply mechanical tension on central nervous system (CNS) axon growth cones, by means of novel magnetic nanoparticles functionalized with neuronal cell adhesion molecule (NCAM), in order to modulate axon outgrowth in a chemically- and mechanically-defined environment and to investigate the mechanotransduction-related signalling pathways that govern this modulation. The complex axon guidance process in CNS neurons is controlled by the growth cone cytoskeleton (CSK) that integrates chemotactic factors and mechanical cues received from the environment. It is hypothesized that this process may be modulated by applying external mechanical tension on axon growth cones. Enhanced axon outgrowth could be a repair strategy for spinal cord injury (SCI), where regenerating axons are unable to penetrate the stiff glial scar that forms following the injury.

To test this hypothesis, a series of research objectives have been formulated: (i) To develop a controlled force transduction system based on magnetic tweezers method in order to apply mechanical tension on growth cones via magnetic particles functionalized with NCAM; (ii) To develop a microfluidic neuron culture device in order to grant exclusive access to multiple growth cones, in which concentration gradients of soluble factors can be imposed; (iii) To examine the effect of mechanical tension on axon outgrowth in response to chemotactic factors; (iv) To determine the role of RhoGTPases in the NCAM-mediated mechanotransduction at axon growth cones. To this end, Semaphorin 3A (Sema3A) was identified as the negative guidance cue and several pathways related to Sema3A signalling, i.e., Rho and Calpain pathways, were identified as the likely candidates for pharmaceutical intervention.

Primary cortical neurons isolated from embryonic mouse are cultured in three-compartmental microfluidic devices. The devices are designed in such a way that axons are able to invade the central (or axonal) compartment while cell bodies are not. The third compartment is used as a source of a diffusing guidance molecule being studied. If connected with driven flows in the two side compartments, the device offers a stable linear concentration gradient in the axonal compartment; if no flow is induced, a temporary unstable gradient forms.

The magnetic tweezers technique is based on applying forces to magnetic particles and the biological entity the particles are attached to. Two different types of particles, spherical superparamagnetic particles 1.4 µm in diameter, and FeAu cylindrical particles 250 nm in diameter and 2µm in length were synthesized in the course of this project. The spherical particles were synthesized in higher quantities and therefore used for axon pulling experiments. The advances made in cylindrical particle surface chemistry for the purposes of axon pulling were utilized in a collaborative project involving cancer cell lines. However, the functionalization of cylindrical particles did not match the timeline of the current project. To target the spherical particles to axonal growth cones, particles were first coated with a self-assembling layer of polyethylene glycol. A fraction of this layer contained protected amine groups which were then conjugated to an antibody that recognizes the extracellular portion of NCAM. Using this approach, growth cones in the microfluidic devices were successfully targeted with a single or a few magnetic particles, suitable for pulling experiments. The particles were commonly observed in the neck regions of the growth cones indicating that the particle had engaged with the actin cytoskeleton and transported retrograde.

Two magnetic tweezers apparatus were created for this project and both systems were integrated with an inverted epi-fluorescence microscope. A permanent magnet assembly consisting of two pairs of NdFeB magnets with their same poles facing each other over a 1 mm air gap provides approximately 10 pN force per bead at a distance < 3mm, which was easily achieved with the microfluidic circuit design. Since the maximum achievable force with the permanent magnet assembly was miniscule compared to the intrinsic forces generated by neuronal growth cones, an electromagnet was designed and implemented. The design parameters were optimized to achieve the highest magnetic field gradient (approximately 70 T/m for 6 A current) in the axonal compartment.

The force was calibrated at different lateral and vertical particle – electromagnet tip distances. At 0.5 mm lateral and 0.5 mm vertical tip distances, up to 74 pN pulling force can be applied per bead. To enable these lateral and vertical distances, the microfluidic device was further improved.

Low force magnitudes (< 10 pN) proved to be insufficient to modulate axon elongation under control conditions, whereas high forces (< 100 pN) does increase the elongation rate ~ 7×. When combined with axonal inhibition of actin polymerization, approximately 5 pN force results in membrane tethers. When combined with axonal inhibition of ROCK, pulling at similar force magnitudes result in 2× faster growth in a small fraction of axons. Growth cone force application paradigm established in control conditions was suitable for combination experiments were axons were pulled towards an uncontrolled gradient of Sema3A by simply adding this compound in the distal compartment. However, the effect of Sema3A on axon elongation had to be established before it could be combined with force application experiments.

The uncontrolled Sema3A concentration experiments were conducted in combination with drugs that inhibit Rho, ROCK, Myosin, Calpain, and local protein synthesis. Both uniform and diffusing Sema3A resulted in a slight decrease in the scalar velocity and significant decrease in vector velocity. Interestingly, diffusing but not uniform Sema3A caused axons to deviate from the source as quantified by the change in the elongation direction from 30-60 min to 90-120 min. The reduction in the scalar axon velocity was blocked by inhibiting axonal protein synthesis, Calpain activity and ROCK signalling, whereas the reduction in the vector velocity was blocked by inhibiting axonal Calpain activity and Rho and ROCK signalling. Most interestingly, the deviation in the axon path was blocked by inhibiting Calpain activity and ROCK signalling. These results identified Calpain and ROCK pathways as the most plausible targets to combine with axonal pulling experiments.

By using the permanent magnet assembly, constant pulling forces were applied to axonal growth cones. Two different responses were observed when beads on growth cones were pulled on. In the first category, the bead did not engage with the growth cone and remained on the axon shaft (80% of cases). In the second category the bead engaged with the growth cone and towed the growth cone at high velocities and towards the pulling direction (20% of the cases). Axonal vector velocity was quantified for the combination of Sema3A gradient and pulling forces with a magnitude of ~10pN. The results suggest that for axonal growth cones to be towed against a gradient of negative guidance cues, the contractile mechanisms in axons need to be partially blocked and the particle needs to engage with the growth cone. The mechanism of engagement has previously been shown to involve a coupling with the actin cytoskeleton. Therefore the blocking of the contractile mechanism cannot involve an intervention to the actin cytoskeleton. Such intervention, indeed, proved to cause membrane tethers to form and no successful axon pulling.

In summary, the project has achieved to establish the proposed experimental model system that (i) physically isolates axons of central nervous system neurons; (ii) exposes axons to linear concentration gradients of Sema3A and Netrin 1; (iii) targets NCAM-conjugated magnetic microspheres to developing growth cones; (iv) applies forces in the range of 30-75pN with a precisely controlled time profile; and (v) enables continuous monitoring of developing neurons under these conditions. Using some aspects of this model system we show that

1. Embryonic mouse cortical neurons deviate from a distal source of Sema3A. This deviation is controlled by Rho/ROCK and Calpain pathways.
2. Forces lower than 10pN acting on growth cones through native NCAM receptors can slightly accelerate and direct axon elongation if the inherent Rho/ROCK/Myosin-based force generation is impaired.
3. Forces at these magnitudes acting on growth cones can accelerate and direct axon elongation towards a source of negative guidance cues, such as, Sema3A, if the bead engages with the growth cone and if the ROCK pathway is inhibited.

The results of this project demonstrate that it is possible to tow central nervous system axons towards a source of negative signalling, such as the glial scar environment, by applying mechanical tension at low magnitudes. This suggests that when synchronized with local pharmaceutical intervention(s), magnetic tweezers-based mechanical stimulation may lead to novel therapies for SCI where the miniscule forces utilized in this project are likely to be achieved by simple magnetic field sources further optimized for clinical practice.

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