The growth and regeneration of axons: an *in vitro* model for studying interactions in 3D

Gerardo-Nava J[1], Hodde D[1], Katona I[1], Bozkurt A[2], Grehl T[3], Steinbusch HWM[4], Weis J[1], Brook GA[1]

[1] Institute of Neuropathology, Uniklinik RWTH Aachen, Aachen 52074, Germany
[2] Department of Plastic, Hand and Burns Surgery, Uniklinik RWTH Aachen University, Aachen, Germany
[3] Department of Neurology, Ruhr-University Bochum, BG-Kliniken Bergmannsheil GmbH, Bochum 44789, Germany
[4] Department of Translational Neuroscience, Maastricht University Medical Center, Maastricht, The Netherlands

Investigations of the factors influencing mammalian axonal growth and regeneration are currently addressed using a range of *in vitro* and *in vivo* models. The *in vitro* models often employ relatively simple 2D culture systems (or the more recent development of axon diodes in micro-fluidics [5,6]) and present the advantage of ready access, manipulation and visualization of the axons and the environment in which they are growing. The *in vivo* models employ relatively expensive laboratory animals and present the advantage of studying axon growth in a more natural 3D environment of supporting cells and extracellular matrix. Our earlier *in vitro* attempts to demonstrate the support of axon regeneration by 3D bioengineered scaffolds involved the use of explanted dorsal root ganglia (DRG) obtained from adult rats [2,3]. This approach has been useful for demonstrating the influence of the scaffold on the properties of glial cell adhesion, proliferation, migration and process extension as well as on axon regeneration and orientation of growth. However, such studies were limited sensory neurons which are easy to dissect and can be maintained in culture for extended periods of time. However, patterns of DRG axonal growth do not necessarily reflect those that may be demonstrated would be expected by motor axons: differences in axonal growth between the motor and sensory neurons (in both *in vivo* and *in vitro* models) have been demonstrated by others in response to specific cellular and molecular cues, including differences when presented with environments containing a range of growth factors [1]. Such differences highlight the value of assays capable of evaluating neuron subtype-specific responses to their environment. Here, we describe the use of the *in vitro* organotypic spinal cord slice preparation for the investigation of motor axon regeneration from early post natal (P7-P9) rat pups and demonstrate the influence of scaffold microstructure on the trajectories the axons (for a complete description see Gerardo-Nava and colleagues [4]). To date, *in vitro* investigations of such motor axon-3D scaffold interactions remain poorly characterized, largely due to the difficulties of culturing motor neurons (MN) for extended periods *in vitro*. The organotypic spinal cord slice preparation has the advantage of studying motoneurons in a relatively normal cellular niche of the ventral horn gray matter in which their survival and axonal regeneration can be maintained for many weeks or even months.

The rat pup spinal cords were rapidly isolated in ice-cold Gey's balanced salt solution and the dura mater and superficial blood vessels carefully removed. The lumbar spinal cord was sectioned transversely (350 µm thick) with a McIlwain tissue chopper and the slices transferred to Millicell membrane inserts where previously dissected ventral roots or biomaterials (fibrin hydrogel or orientated collagen scaffold, Matricel GmbH) could be

carefully apposed to the ventral surface of the slices (as described by Vyas and colleagues [7]). The preparations were then maintained in an incubator at 37°C with 5% CO_2.

Myelin clearance within the explanted ventral roots was rapid with the events following a similar pattern to that already described for Wallerian degeneration *in vivo.* By 7-14 days in culture, axons regenerated for substantial distances along the explanted nerve root, with most axons following a trajectory within the root itself. The interactions between regenerating motor axons and Schwann cells revealed different stages of maturity from simple, initial axon-Schwann cell contacts, to the ensheathment and the formation of compact myelin surrounded by basal lamina. A similar sequence of events could also be seen within the longitudinally orientated porous framework of the collagen scaffold, however, these interactions took place between migrating Schwann cells (from the residual root stumps remaining at the ventral surface of the slice) and the regenerating motor axons which formed longitudinally orientated fascicles. Although similarly extensive Schwann cell migration and axon growth was also supported by the fibrin hydrogel, the trajectory of both cells and axons followed the surface of the hydrogel rather through it.

Although the organotypic spinal cord preparation is capable of reproducing many of the events taking place in PNS Wallerian degeneration with subsequent axonal regeneration, the model has clear limitations, including the lack of any functioning vasculature, an incomplete immune system and the absence of appropriate end organ targets for the regenerating motor axons. However, despite these limitations, the model affords a number of significant advantages over assays that attempt to employ cultures of freshly isolated embryonic motor neurons (or cells lines), or studies involving laboratory animals. These include the simplicity and ease of preparation, the length of time such preparations can be maintained and the financial and ethical benefits of reducing the number of animals required to perform such studies. The model thus represents, in our opinion, an excellent platform for investigating how regenerating motor axons interact with any 3D growth-promoting environment and can be used as a cost-effective means of screening or assaying bioengineered scaffolds or the mechanism of action of drugs and bioactive agents that modify axon growth and regeneration.

References:

1. Allodi I, Guzmán-Lenis MS, Hernàndez J, Navarro X, Udina E. In vitro comparison of motor and sensory neuron outgrowth in a 3D collagen matrix. J Neurosci Methods. 2011; 198: 53-61
2. Bozkurt A, Brook GA, Moellers S., Lassner F, Sellhaus B, Weis J, Woeltje M, Tank J, Beckmann T, Fuchs P, Olde Damink L, Schügner F, Heschel I, Pallua N. In-vitro assessment of axonal growth using dorsal root ganglia (DRG) explants in a novel 3D collagen matrix. Tissue Engineering. 2007: 13: 2971-2979
3. Bozkurt A, Deumens R, Beckmann C, Olde Damink L, Schügner F, Heschel I, Selhaus B, Weis J, Jahnen-Dechent W**,** Brook GA**,** Pallua N. In vitro cell alignment obtained with a Schwann cell enriched microstructured nerve guide with longitudinal guidance channels. Biomaterials. 2009; 30: 169-179
4. Gerardo-Nava J, Hodde D, Katona I, Bozkurt A, Grehl T, Steinbusch HW, Weis J, Brook GA. Spinal cord organotypic slice cultures for the study of regenerating motor axon interactions with 3D scaffolds. Biomaterials. 2014; 35:4288-96

5. Kim Y, Karthikeyan K, Chirvi S, Dave DP. Neuro-optical microfluidic platform to study injury and regeneration of single axons. Lab Chip. 2009; 9: 2576-2581
6. Peyrin JM, Deleglise B, Saias L, Vignes M, Gougis P, Magnifico S, Betuing S, Pietri M, Caboche J, Vanhoutte P, Viovy JL, Brugg B. Axon diodes for the reconstruction of oriented neuronal networks in microfluidic chambers. Lab Chip. 2011; 11:3663-73
7. Vyas A, Li Z, Aspalter M, Feiner J, Hoke A, Zhou C, O'Daly A, Abdullah M, Rohde C, Brushart TM. An in vitro model of adult mammalian nerve repair. Exp Neurol. 2010; 223:112-8