Directed Axonal Outgrowth Using a Propagating Gradient of IGF-1

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The human nervous system is a highly organized ensemble of neurons wired with the intricate interplay of attractive and repulsive guidance cues during development.[1] Many neurological disorders arise due to the deficiency of axons and dendrites in recognizing and locating their appropriate synaptic partners.[2] Although some guidance molecules persist beyond the embryonic period, adult neural networks fail to regenerate injured or misguided axonal connectivity in the central nervous system (CNS).[3] This is due in part to the presence of inhibitory extracellular factors and the absence of cues that guide axonal re-growth for regeneration.[4,5] Various approaches have been proposed to understand how neurons integrate guidance information to make growth decisions for axon extension.[6] Several classes of guidance cues have been documented, including immobilized and soluble protein gradients, topographical features, electric fields, and modulation of neuronal activity.[7–12] Much of our understanding of guidance cues in development is based on the identification of soluble guidance proteins and their receptor-mediated downstream signaling pathways.[13] However, because current experimental approaches are largely dependent on isotropical and unrestricted diffusion of these proteins in free solution, there is the inherent diffusion-based limitation to assess and/or modulate the temporospatial extent of axonal outgrowth for the purpose of reliable preclinical evaluations. Here, we report the successful construction of a biocompatible axonal guidance device in which the linear propagation of insulin-like growth factor 1 (IGF-1) gradients sequentially directs axon outgrowth of up to 5 mm in 5 days.

As a model system, we have utilized murine cortical spinal motor neurons (CSMN), one of CNS neurons, appropriately patterned from embryonic stem cells,[14] and examined axonal outgrowth in response to IGF-1. IGF-1 has been previously reported to enhance the rate and length of murine CSMN axon outgrowth.[15] In the present study, IGF-1 was encapsulated in biodegradable poly-lactic-co-glycolic acid (PLGA) particles that can be formulated to control the release rate of IGF-1. The sequential IGF-1 releases were obtained based on a previous work[16] where targeting specific drug release profiles was achieved by admixing different types of drug-encapsulated microspheres and integrating an array of the individual release profiles.

PLGA particles were prepared by the water-in-oil-in-water (w/o/w) method (Figure 1a), known to be efficient for carrying easily-denaturing peptide or protein-based drugs.[17] Due to the immiscibility between drug-containing aqueous droplets and non-polar polymer solution, the incorporated drugs could be isolated during the manufacturing process and stably encapsulated within the polymer matrices. Figure 1b shows the images of PLGA particles prepared by the w/o/w method. Particles with different release kinetics were prepared by modulating design parameters such as particle concentration and composition (Figure 1c). Drug encapsulation efficiencies were calculated as the actual amounts of the encapsulated IGF-1 in the particles compared to the theoretical amount of the incorporated IGF-1 during the particle preparation[16] and each particle type had about 40% to 50% efficiency (Figure 1c). The distinct drug release profiles of each particle type were empirically determined (Figure 1d). These profiles were used for designing three targeted release profiles (‘early’, ‘intermediate’, and ‘late’) with temporally sequential peaks of IGF-1 release, using the method developed by Lee et al.[16] It is based on the observation that the resulting drug release profile of a particle admixture can be predicted as the linear summation of the individually measured drug release profiles of each particle type in an admixture. This strategy allowed us to formulate a matrix equation from Figure 1d and determine how much of each particle type should be included in an admixture in order to have a specific drug release profile (additional details are described in Supporting Information). Because the temporal span of the peak release levels from each particle type limit the controllable range of the release kinetics of the admixtures, we targeted the span of the propagation of IGF-1 to be within 10 days. However, the gradient or the time of peak drug release can be easily extended by modulating other well-known design parameters, such as molecular weight and physical configuration, or by providing an outer layer that surrounds the core unit containing the drug.[16]

The three final admixtures were empirically tested for IGF-1 release kinetics and the three targeted IGF-1 releasing profiles were successfully obtained (Figure 1e). The ‘early’ release profile had the peak level of released IGF-1 for the first 2 days, the ‘intermediate’ release profile peaked at 3–4 days and the ‘late’ release profile generated a delayed IGF-1 release that decayed after 10 days. The raw data of the optical density (O.D.) are shown in Figure S1. We also verified that the overall process did not deteriorate the bioactivity of the incorporated IGF-1 measured by proliferation of C6 rat glioma cell cultures (Figure 1f).
Plain PLGA particles prepared with PBS instead of IGF-1 solution showed no biological effect (Figure 1f).

Once the targeted sequential release profile of an axon attractant was established, a biocompatible axonal guidance device was developed using a synthetic poly(ethylene glycol) (PEG) hydrogel (Figure 2a). PEG is one of the most extensively utilized hydrogels for biomedical applications because, besides its biocompatibility, the network structure can be easily modified to mimic critical physicochemical aspects of the human extracellular matrices. In order to promote neurite growth, we conjugated the well-known immobilized guidance proteins, fibronectin and laminin, to the surface of the hydrogel matrix. We then determined the optimal spacing between the CSMN and the IGF-1 encapsulating particle admixtures that the incorporated CSMN could recognize the released IGF-1 gradient and extend their axons enough to be clearly observed through a microscopic view. The axonal densities were assessed by scoring microscopic images. When the admixture with the early release was placed at 3 mm away from the CSMN, the density of the extended axons was significantly reduced compared to when the admixture was placed at 2 mm from the CSMN (Figure 2b and Figure S2). Based on this observation, we limited the spacing between the cells and the admixtures to less than 2 mm and targeted the total length of axonal outgrowth to be about 5 mm. The three particle admixtures were placed at even intervals within this 5 mm range: the ‘early’, ‘intermediate’, and ‘late’ release admixtures were loaded at approximately 1.7 mm, 3.3 mm, and 5 mm away from the cell loading zone, respectively.

Green fluorescent protein (GFP) expressing CSMNs were then placed into the cell loading zone and the entire device was incubated for 10 days. It was important to place the cells right next to the entrance of the axon guiding conduit where the generated IGF-1 gradient would be well preserved. Green fluorescent protein positive (GFP+) axonal growth along the guidance conduit was monitored over time and the growth rates were estimated by measuring the length of GFP+ axons within the guidance conduit. As we expected, the axons successfully followed the propagation of the IGF-1 gradient and reached the full targeted length of 5 mm at Day 5 and were held constant over 10 days (Figure 2c). In the meantime, increasingly more GFP+ neuroblasts migrated along the axons (Figure 2c). We also observed the increased axonal density over time (Figure 2d).

To verify that the temporospatial control, enabled by establishing the sequential release profiles with matching spatial...
layout of the guiding conduit, was the pivotal feature underlying the successful directional axon extension, we also examined the effects of modifying temporospatial conditions of the IGF-1 release on the characteristics of axon extension. We first examined the condition where the incorporated particle admixtures had continuous IGF-1 releasing profiles for generating a static gradient of IGF-1 over time (Figure S3) but were placed with the original spatial layout (5 mm range). The extent of the axon extension under this condition was largely limited within the range of the location of the first admixture (about 1.7 mm, Figure 2e and Figure S3). We then modified the spatial layout, while keeping the three sequential release profiles, by extending the spacing between the particle admixtures to 10 mm range. This spacing exceeded the pre-determined optimal spacing (5 mm range) with which the incorporated CSMN could effectively respond to the released IGF-1. Although we could observe some axons extended up to 10 mm in five days, both the extent of axonal growth and the neuroblast migrations were drastically weakened (Figure 2e and Figure S4). In control conditions, we observed that the incorporation of plain PLGA particles prepared with PBS instead of IGF-1 could not induce directed axon outgrowth, comparable to the case of CSMN cluster culture without any axon attractants (Figure 2f). These results imply that in order to elicit axonal extension with desired directionality and extent, it is critical to optimize the temporospatial conditions of the gradient of an axon attractant and that our guidance matrix successfully established the optimal conditions.

The biocompatible axonal guidance device developed in this work could serve as a versatile experimental platform for elucidating the axon extension mechanisms and for translating them into the development of novel treatment strategies for various neurological diseases. With the feature of temporospatial control, this approach could also be widely applicable to studying the kinetics of many other water-soluble biomolecules and controlling their behaviors to satisfy distinct clinical needs.
Experimental Section

Preparation of IGF-1 Encapsulating PLGA Microspheres: IGF-1 (R&D Systems) was encapsulated in PLGA microspheres by the double emulsion process (water-in-oil-in-water (w/o/w)) as described previously.[16] Briefly, poly (lactic-co-glycolic acid) (PLGA, LACTEL) was dissolved in the non-polar organic solvent, dichloromethane (DCM), at the designated concentrations (10% or 20% (w/v)). We used 50/50 PLGA (composed of 50/50 molar ratios of glycolide units and lactide units, 85k molecular weights), 65/35 PLGA (95k molecular weight), 75/25 PLGA (75k molecular weight), and 85/15 PLGA (80k molecular weight). IGF-1 was dissolved in PBS at 100 µg/mL. Aqueous IGF-1 solution was then added to the non-polar PLGA solution at the one to nine ratio and then emulsified for 1 min at approximately 30 000 rpm using a homogenizer (PRO200 Laboratory, Pro Scientific). The emulsion was poured into 1000 times its volume of ice-cold water. The solution was stirred at 10 000 rpm for 10 min using a homogenizer and then moved to a magnetic stirrer for continuous stirring to allow the DCM to evaporate for 24 hours. The diameters of the microparticles were 5.9 ± 4.5 µm based on the analysis of the microscopic images using AxioVision image software (Carl Zeiss). The resulting solid microspheres were collected by centrifugation, lyophilized, and stored at –20 °C. The bioactivity of the released IGF-1 was measured by assigning the theoretical amount.

Measurement of IGF-1 Releasing Profile: In this study, all IGF-1 releasing profiles were obtained after the particles were incorporated into a hydrogel matrix. The hydrogel matrices with particles were incubated in PBS for 48 hours. And the cell proliferation was measured by Alamar blue assay (Invitrogen).

Measurement of the Axonal Density and the Axon Outgrowth Length: The axonal density was determined as described in Pro stick et al. [20] with slight modification. Briefly, the individual microscopic images of the extended axonal bodies were obtained by choosing a level in the z-axis containing the most axonal profiles. Then parts of the obtained images, from randomized selection, were cropped along an angle where the direction of the extended axons was perpendicular to the cropped views. Using image softwares (PhotoShop, Adobe and PowerPoint, Microsoft), the area of the axon profiles was highlighted and the numbers of the pixels across the marked area were assessed. At least three cropped views per one whole image were measured. The length of the extended axons was determined with microscopic images using an image software (AxioVision, Carl Zeiss).

CSMN Cell Culture: The generation and culturing of the mouse ES cell line F7-GFP are described in the work of Ideguchi M. et al.[14] F7-GFP ES cells were induced to a neural precursor state by co-culture with the mouse stromal cell line MSS (Kirin Pharma). The small molecule LDN-193189 (100 nM, Stemgent), which inhibits BMP type I receptors was added to the differentiation media on day 2 of neural induction, while FGF2 (fibroblast growth factor 2) (R&D Systems) at 20 ng/mL was added for the last 2 days to increase cortical pyramidal neuron numbers. ES-NPC colonies were removed by incubation in Ca2+-, Mg2+-free HBSS (15mM HEPES) for 30–45 min. The ES-NPC colonies were then dissociated in papain (Worthington Biochemicals) for 20 min at 35 °C and triturated into a single-cell suspension. Cells were centrifuged to form small pellets that were then placed in the cell loading zone next to the axon guiding conduit. One day after placing the cell pellets into the axon guiding device, insulin was removed from the media since insulin can bind to and active IGF receptors. Therefore, all referenced time points in this work began after culture medium was replaced by the one without insulin. The chambers were then placed into a 37 °C incubator with 5% CO2.

Statistical Analysis: Statistical analysis was performed by One-way ANOVA with Bonferroni–Holm post-hoc test, unless otherwise specified. The regression analysis in Figure 2d was performed by assigning the incubation period as independent variables (day 5, 7, and 10 were assigned as 0, 2, and 5, respectively) and the axonal density as dependent variables.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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