Neuroscience 262 (2014) 40–52

FUNCTIONAL REGENERATION OF INTRASPINAL CONNECTIONS IN A NEW *IN VITRO* MODEL ☆

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Abstract—Regeneration in the adult mammalian spinal cord is limited due to intrinsic properties of mature neurons and a hostile environment, mainly provided by central nervous system myelin and reactive astrocytes. Recent results indicate that propriospinal connections are a promising target for intervention to improve functional recovery. To study this functional regeneration *in vitro* we developed a model consisting of two organotypic spinal cord slices placed adjacently on multi-electrode arrays. The electrodes allow us to record the spontaneously occurring neuronal activity, which is often organized in network bursts. Within a few days *in vitro* (DIV), these bursts become synchronized between the two slices due to the formation of axonal connections. We cut them with a scalpel at different time points *in vitro* and record the neuronal activity 3 weeks later. The functional recovery ability was assessed by calculating the percentage of synchronized bursts between the two slices. We found that cultures lesioned at a young age (7–9 DIV) retained the high regeneration ability of embryonic tissue. However, cultures lesioned at older ages (>19 DIV) displayed a distinct reduction of synchronized activity. This reduction was not accompanied by an inability for axons to cross the lesion site. We show that functional regeneration in these old cultures can be improved by increasing the intracellular cAMP level with Rolipram or by placing a young slice next to an old one directly after the lesion. We conclude that co-cultures of two spinal cord slices are an appropriate model to study functional regeneration of intraspinal connections.

Key words: spinal cord, organotypic cultures, multi-electrode array, regeneration.

INTRODUCTION

The adult spinal cord of higher vertebrates fails to regenerate after injury. This has been attributed to an inhibitory environment provided, for example, by myelin associated proteins such as Nogo-A, oligodendrocyte myelin glycoprotein and myelin associated glycoprotein (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000; Kottis et al., 2002; Wang et al., 2002) or proteins associated with the extracellular matrix like chondroitin sulfate proteoglycans (CSPGs) (Levine, 1994). In addition, it has been shown that the age of a neuron can play an essential role in limiting axon regeneration. For example, Blackmore and Letourneau (2006) demonstrated with E9 and E15 brainstem explants of chicks that the axons growing from the older explants paused more often and advance more slowly than axons originating from the younger explants when grown on identical substrates. Therefore, the inhibitory environment and the state of neuronal maturation both seem to account for the failure of regeneration in the spinal cord. With their co-culture model Blackmore and coworkers found a 55% reduction in axon regeneration due to spinal cord environment inhibition while maturation of hindbrain neurons caused a 90% reduction. To which extent which factor contributes in other animals and other fiber tracts is not clear.

For many studies, descending tracts, especially the corticospinal tract have been the model systems of choice to investigate regeneration after spinal cord injury (SCI). However, recent results point to a pivotal role of propriospinal connections in the spontaneous recovery of function after incomplete spinal cord lesions (Bareyre et al., 2004; Courtine et al., 2008). Bareyre et al. (2004) demonstrated that after an incomplete SCI, severed corticospinal tract axons form connections with propriospinal neurons that are spared by the lesion and therefore still have intact axons. Thereby, a detour circuit around the lesion site can be formed allowing the transmission of descending signals below the damaged region. Furthermore, propriospinal neurons seem to respond better than descending tracts to certain treatments such as application of growth factors or tissue grafting (Houle, 1991; Xu et al., 1995; Blesch et al., 2004). Because of these characteristics, propriospinal fibers are a suitable target for therapeutic interventions to promote functional recovery after SCI. However, it is difficult to study the contribution of these fibers to regeneration in isolation without the

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Abbreviations: chABC, chondroitinaseABC; CSPG, chondroitin sulfate proteoglycans; CV, coefficient of variation; DAPI, 6-diamino-2-phenylindole; Dis, disinhibition; DIV, days *in vitro*; DRG, dorsal root ganglion; Hepes, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; MEA, multi-electrode array; NgR-1, Nogo receptor 1; PBS, phosphate-buffered saline; SCI, spinal cord injury; StdC, standard conditions; TTX, tetrodotoxin.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.12.051

interference of ascending and descending fiber tracts. Bonnici and Kapfhammer (2008) used longitudinal spinal cord slices consisting of several segments to study their structural recovery. After setting lesions they stained the cultures with an anti-neurofilament SMI-31 antibody and estimated the amount of fibers crossing through the lesion center. However, the functional implications of this axonal regrowth are not known. So far, no *in vitro* model exists that allows the investigation of functional regeneration of propriospinal fibers. Here, we present a newly developed model based on two transversal spinal cord slices from 14-day-old rat embryos (E14) co-cultured adjacently on multi-electrode arrays (MEAs). Within a few days, the two slices connect to each other with axonal fibers that can subsequently be cut for further investigation.

We have previously shown that spontaneous bursting activity appears in organotypic spinal cord cultures, originating from intrinsic spiking and recurrent excitation and reflecting the activation of a neuronal network in the slice (Tscherter et al., 2001; Darbon et al., 2002; Czarnecki et al., 2008). In this study, we use the percentage of synchronized bursts between both slices 2–3 weeks after lesions to quantify functional regeneration. Moreover, the model offers the advantage of circumventing the need for large animal cohorts often required to test the efficacy of strategies to promote regeneration after SCI *in vivo*.

EXPERIMENTAL PROCEDURES

Culture preparation

Organotypic cultures were obtained from spinal cords of 14-day-old rat embryos (E14) from either Wistar rats purchased from Janvier (Le Genest St Isle, France) or Lewis rats expressing GFP ubiquitously in most organs (Inoue et al., 2005), kindly obtained from Professor St. Leib (University of Bern). The embryos were delivered by cesarean section from deeply anaesthetized animals (0.4 ml pentobarbital i.m., Streuli Pharma SA, Switzerland) and sacrificed by decapitation. The mother animals were sacrificed by intracardiac injection of pentobarbital. This procedure guaranteed a minimal suffering of animals (grade 0). The number of animals used to prepare the cultures was kept minimal. Animal care was in accordance with guidelines approved by Swiss local authorities (Amt für Landwirtschaft und Natur des Kantons Bern, Veterinärdienst, Sekretariat Tierversuche, approval Nr. 52/11). These guidelines are in agreement with the European Community Directive 86/609/EEC. The backs of the embryos were isolated from their limbs and viscera and cut into 225-μm-thick transverse slices with a tissue chopper. After dissecting the spinal cord slices from the surrounding tissue two of them were fixed next to each other on top of each MEA (Qwane Biosystems, Lausanne, Switzerland) by using reconstituted chicken plasma coagulated by thrombin (both Sigma–Aldrich, Switzerland). The slices were arranged with the ventral parts facing each other on each side of the 300-μm-wide groove located in the middle of the MEAs. The cultures were maintained in sterile plastic tubes containing 3 ml of nutrient medium and incubated in roller drums rotating at 2 rpm in a 5% CO_2-containing atmosphere at 36.5 °C (Streit et al., 1991). The medium was composed of 79% Dulbecco's modified Eagle's medium with Glutamax, 10% horse serum (both Gibco BRL, Switzerland), 10% H_2O and 5 ng/ml 2.5S nerve growth factor (Sigma–Aldrich, Switzerland). Half of the medium was replaced once to twice per week.

Experimental design and recordings

During the next few days *in vitro* (DIV) the slices grew and fused with each other. In most cultures, we performed a complete lesion with a scalpel to separate the two slices at the location of the groove within 8–28 DIV (Fig. 1A, B). This cut split the cultures in such a way that the slices were not touching each other anymore and their distance maximally reached the size of the groove width. The cultures were incubated further for at least 2 weeks until the activity was measured.

For the recordings the MEAs were mounted on an inverted microscope and kept in a bath of extracellular solution containing (in mM) NaCl 145; KCl 4; $MgCl_2$ 1; $CaCl_2$ 2; HEPES 5; Na-pyruvate 2; glucose 5 (pH 7.4). Recordings with extracellular solution are called ''standard condition''. Under ''disinhibition'' gabazine (10 μM) and strychnine (1 μM, both Sigma–Aldrich, Switzerland) were added to the extracellular solution. The bath was exchanged after every recording session, which lasted 10 min and was made at room temperature.

The MEAs are composed of a glass substrate, indium-tin oxide electrodes and an SU-8 polymer insulation layer. The recording site consists of 68 electrodes arranged in a rectangular grid. The electrodes were 40 × 40 μm in size and were spaced 200 μm apart (center to center, Fig. 1B). The electrode grid is split into two zones by the groove that is free of electrodes, electrical leads and insulation. The amplified signals from the 68 platinum electrodes are visualized and stored using custom-made virtual instruments within Labview (National Instruments, Switzerland). The analysis is performed offline with the software package IGOR (WaveMetrics, Lake Oswego, OR, USA).

Electrical stimulation was performed under disinhibition with the MEA electrodes with monopolar biphasic stimuli (duration = 1 ms, amplitude = 1.5–2.8 V), delivered from a custom-made stimulator.

Analysis of bursting activity

The extracellularly detected signals are fast voltage transients (< 4 ms) corresponding to action potentials in neuronal somata or axons. They are represented by single time markers called events. Raster plots show the events for each individual electrode (Fig. 1C). They can be transformed into network activity plots, which display the total activity recorded by all selected electrodes, summed within a sliding window of 10 ms, shifted by 1-ms steps (Fig. 1D) (Tscherter et al., 2001). Usually, fast voltage transients appeared in clusters and on several electrodes. We call these clusters bursts.