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Lumbar spinal cord explants from neonatal rat display age-related decrease of outgrowth in culture

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Abstract

Lumbar spinal cord explants, harvested from neonatal rat pups aged between postnatal day 0 (P0) and P7, were cultured for a period of 48 h in the chemically defined medium R_{12} [17] (Romijn, H.J., van-Huijen, F., Wolters, P.S., Neurosci Biobehav Rev, 8 (1984) 301–334), embedded in a collagen matrix. The outgrowth into the surrounding matrix was quantified. Age-matched cortical explants were used as controls. Despite adaptations of the culture protocol, outgrowth remained variable. Statistical analysis demonstrated a clear relation between the age of the explant (at the time of explantation) and the number of neurites in the corona surrounding the explant. The number of outgrowing neurites decreased sharply with age. The average number of neurites per explant obeyed to the expression $log(N) = -0.652\ A + 17$ (N: the number of neurites per explant; A: the age expressed in gestational days; $A \in$ [G23–G30]; G23 signifying gestational day 23, or P0). The observed age-related decrease of outgrowth could not be explained by progressive myelination of the spinal cord white matter, nor by the absence of trophic support from muscle, but may be related to a progressive inability of the spinal neurites to interact with collagen. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Collagen; Culture; Culture medium; Gestational age; Integrin; Muscle; Myelin; Neonate; Outgrowth; Rat; Spinal cord

In the present study we will describe a protocol for the culture of neonatal rat spinal cord explants in a collagen matrix. We adapted a previously described method for the culture of cortical explants [11] to culture spinal explants. This protocol is a preliminary step towards more specific investigations into the regenerative properties of motoneurons.

For this aim the culture of explants from the adult spinal cord would be preferable. Adult spinal cord, like adult nervous tissue in general, is notably difficult to maintain in culture, as opposed to fetal nervous tissue [4]. Fetal spinal cord is not appropriate for the study of regeneration, as many of the motor axons have not been generated yet, or have not attained their target [16]. Explant cultures from young postnatal spinal cord provide an alternative to adult spinal cord, as the peripheral axons have developed fully, while the cord tissue is still amenable to culture conditions. Attempts to culture the postnatal spinal cord have long been unsuccessful [7], and only a few studies exist that describe a protocol for the culture of postnatal spinal cord explants. Delfs and coworkers [3] cultured postnatal spinal cord explants with the roller tube method for periods up to 8 weeks, and described motoneuronal development and outgrowth inside, but not outside the explant. Zhu and coworkers [21] did describe the effect of anti-motoneuron serum and muscle extract on the outgrowth outside neonatal rat spinal cord explants after 5 days of treatment, but did not provide a comprehensive culture protocol.

We cultured a series of lumbar spinal cord explants of increasing ages, each for a period of exactly 48 h. We quantified the outgrowth and confirmed the presence of motor-axons among the outgrowing neurites.

Initial findings indicate that the window for regeneration in vitro is limited to the first 2 postnatal days. Since explants were cultured in a collagen matrix, we propose that the decrease of the regenerative capacity of the spinal explants may be related to a down-regulation of membrane-bound

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integrins, that mediate interactions with the collagen substrate molecules [1].

All procedures involving the husbandry, handling and sacrifice of experimental animals were performed in accordance with national laws.

The culture protocol was adapted from a previously described method for the culture of cortical explants [11]. Initial cultures demonstrated that the amount of outgrowth from the spinal explants was in general less profuse as compared to the cortical control explants, and more variable. We varied a number of parameters that we knew (from experience) to influence the quality of explant cultures, and the quantity of outgrowth. None of the parameters appeared to influence the number of neurites in the corona from the spinal explants, but it was noted that some parameters influenced the quality of the culture, while others facilitated the preparation. Therefore, the procedure was optimized for the culture of spinal explants.

Wistar Albino Glaxo rats in oestro were mated between 10:00 and 11:00 h. [20]. The end of this period was taken as the start of gestational day 0 (G0) [14]. We used neonates born at G23 and sacrificed at ages ranging from G23–G30.

All procedures for the preparation of spinal and cortical explants took place under antiseptic conditions. The spinal cord from the pups was collected in a chemically defined serum free medium R_{12} [17] and the meningial covering was stripped away. The lumbar enlargement was chopped immediately into 500 μm slices. These slices were separated into lateral halves. Seven of the resulting slices were selected. Individual rats were processed sequentially, and each batch of slices was stored in R_{12} in a separate 1" Petri dish. Cortical explants, used as controls, were prepared similarly [11].

A collagen solution was prepared extemporaneously from: (1) 800 μl of Vitrogen®, (3 mg/ml bovine type I collagen solution; Collagen Corporation, Fremont, CA); (2) 100 μl of a solution containing 38 mg/ml $NaHCO_3$ and 135 mg/ml Dulbecco's modified Eagle's medium (DMEM) (ICN Biomedicals, Zoetermeer, NL); (3) 100 μl of 0.1 N NaOH. The pH of this solution was adjusted with a few microliters of 1 N HCl using the colorimetric properties of DMEM. The right amount of acid was the one that immediately resulted in a yellow color of the collagen solution and in a change of color to gold within 5 s. Usually this was achieved with 24 μl of 1 N HCl.

The collagen solution was stored in melting ice. Each explant was cultured in a separate 1" Petri dish. Two 40 μl drops of the collagen solution were placed on the bottom of each culture dish. The explant was washed through one of these drops immediately before insertion into the center of the other drop. Using a fine blunt needle the explant was then gently coaxed into a central and horizontal position. Each dish was marked with a number and a separate list was kept to link these numbers to the origin of the explant (pup and culture conditions). The culture dishes were placed in a CO_2 incubator (37°C, 5.5% CO_2). Gelation of collagen occurred within 2 h and after this time 1 ml of R_{12} medium was added to each culture dish. The cultures were maintained for exactly 48 h. Antibiotics were never used.

The cultures were then fixed, stained with osmiumtetroxide, dehydrated with alcohol and mounted with Entellan® [11].

A few explants were randomly chosen to ascertain the presence of motor axons among the outgrowing axons. After fixation a tiny incision was made in the collagen drop, at the periphery of the corona. A crystal of 1,1' dioctadecyl-3,3,3',3' tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Oregon) was inserted through the incision. After 4 days photomicrographs of the retrogradely labeled somata in the explants were taken with the appropriate filter set.

Outgrowth was quantified by light-microscopical examination. If only a few neurites were present all were counted. If many were present they were only counted in a radial sector of the field and the total amount was calculated. For the present study 231 spinal explants and 63 cortical controls were resected from 76 pups, and cultured. After completion of the quantification the values were correlated to the separately listed data on the age of the pup from which they originated.

Values were expressed as the logarithm of the mean number of neurites from the explants of each pup. The distribution was normal for each age. An analysis of variance was used to compare the means among different populations consisting of different ages. Values of P less than 0.05 were considered significant. The correlation between the number of neurites and the age of the pups was calculated.

Neurites emerged mostly from the sectioned surface of the explant and extended horizontally. New neurites were continuously added, and at the end of the culture period explants appeared surrounded by an evenly distributed radiate corona of neurites (Fig. 1). Growth cones and bundled neurites could be observed throughout the axonal corona.

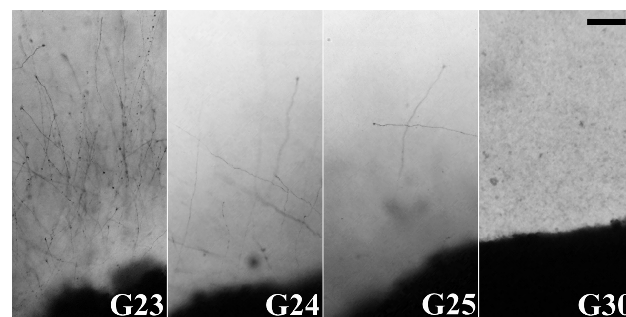

Fig. 1. Photomicrographs of representative explants harvested from neonatal rats of four different ages (G23, G24, G25, G30), and cultured for 48 h. Explants harvested from the G23 pups generated robust outgrowth into the surrounding collagen matrix. Explants harvested from older pups generated progressively less outgrowth, and from G30 onwards no outgrowth was observed into the matrix. A part of the explant is visible in the lower right corner. Bar is 50 μm.

Near the explant the density of neurites precluded the discrimination of individual neurites along their entire length (from explant surface to growth cone).

Labeling with DiI ($n = 11$) demonstrated the presence of prominent retrogradely labeled stellate somata in the ventral horn of the explant (Fig. 2). Size, position and shape suggest that these are motoneurons. In all instances labeled somata displayed several neurites restricted to the explant (presumably dendrites) and only one (labeled) neurite extending into the collagen (presumably the axon). This suggests that the corona consists mainly if not entirely of axonal neurites.

The number of neurites surrounding the spinal explants ranged from 0 to 10,000. The length of the neurites ranged from 0 to 1.5 mm within 48 h. The thickness of the spinal neurites ranged (on estimate) from 0.6 to 1.2 μm. Cortical control explants grew well (up to 100,000 neurites) under the same culture conditions, and neurites from cortical explants were (on estimate) twice as thick. Likewise the growth cones on neurites from spinal explants were much less obvious as compared to growth cones from cortical explants. Many spinal neurites appeared to terminate abruptly without a light-microscopically discernable growth cone.

Age was the only parameter that did show an effect on the number of outgrowing neurites (Fig. 1). With increasing age the number of neurites diminished radically. We computed the logarithm of the number of neurites per explant and then for all explants from each pup the mean of this logarithm. Subsequently an analysis of variance was performed. The number of neurites per explant from individual rats displayed a normal distribution. The age at which the pup was sacrificed has a strong effect on the number of neurites ($P < 0.001$). The number of neurites per explant obeyed $\log(N) = -0.652\,A + 17$, with N the number of neurites per explant, and A the age expressed in gestational days, and $A \in$ [G23–G30] (Fig. 3).

Consequently the mean number of neurites per explant would be 200 should the culture be started at birth. Because

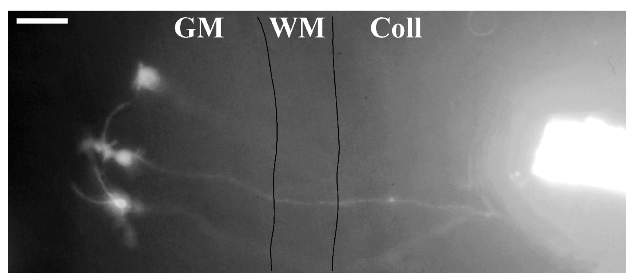

Fig. 2. Representative photomicrograph of three retrogradely labeled (DiI) neurons in the ventral horn of a fixated explant (G24). The lines indicate the border between gray matter (GM), white matter (WM) and the collagen matrix (Coll). Only the lowermost neuron is in the focal plane, demonstrating the presence of a stellate soma, three dendritic neurites (at 6, 9 and 10 o'clock) and one axonal neurite (at 3 o'clock, and out of focus). Only the axonal neurites leave the explant, while the dendritic neurites are confined to the explant. Bar is 100 μm.

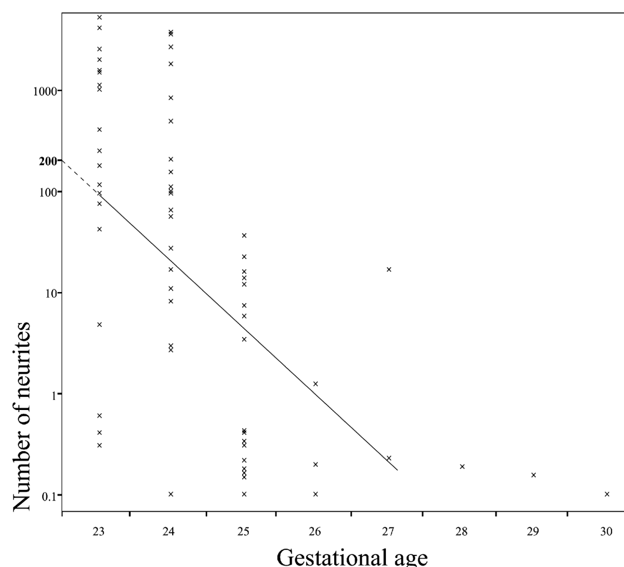

Fig. 3. Regression line describing the decrease of the average number of neurites from the explants of one pup in function of the gestational age of that pup at the time of harvesting of the explants.

birth occurs at night, the number will be 100 at the actual time of explantation at P0. The mean number of neurites per explant will decrease by a factor 4 every day. As the variability around the mean value is large, some explants will display profuse outgrowth, with up to 10,000 neurites at P0, while others will be almost devoid of outgrowth. A large standard variation persisted in the number of neurites growing out of explants taken from pups of the same litter, though not in the number of neurites growing from the explants of each individual pup (data not shown). Since the gestational age of individual pups within a litter may vary up to 24 h [5,10,12] and since the outgrowth capacity of the explants diminishes quickly with age, the observed variability in outgrowth is, in all probability, a reflection of the differences in gestational age of the pups in each litter.

Explant cultures from postnatal rat lumbar spinal cord were cultured in a collagen matrix. While explants harvested from G23 (P0) pups exhibited fair outgrowth, this outgrowth diminished sharply with increasing age at explantation. Outgrowth was much less profuse as compared to the age-matched cortical explants even at G23, while the outgrowth from cortical explants did not diminish with increasing age at explantation. As these cortical explants grew well under the same culture conditions as the lumbar spinal explant we conclude that the culture protocol per se is not responsible for the observed diminishment in the outgrowth from the lumbar spinal explants.

Myelin exerts a local inhibitory influence on the outgrowth of axons. Though significant myelination of the white matter at the lumbar level is not to be expected during the relevant period (G23–G30; P0–P7), some descending tracts are partly myelinated [19]. Thus it seems possible that the development of myelination of the white matter

progressively prevents the (axonal) neurites from leaving the explants. The neurites, however, mainly emerge from the sectioned surface of the explant, which is primarily gray matter. Furthermore, when the white matter was removed or when only the ventral horn was cultured, the same influence of age could be observed (data not shown).

Co-cultured with muscle either spinal cord explants or dissociated spinal (moto)neurons harvested from fetal or neonatal rats will develop persistent outgrowth [8,15,18], and the trophic support of motoneurons and their axons by muscle is a well established fact [13]. The absence of such trophic support is not sufficient to explain the age-related decrease of outgrowth from the spinal explants in our cultures, as motoneurons represent only a fraction of the neurons in the spinal explants. Apparently all the neurons present in the explants share a property that determines the ability of their axons to grow outside the explant in an age-related fashion.

Fetal spinal cord explants do generate axons into a surrounding collagen matrix [6]. Postnatal spinal cord explants lose the capacity for such outgrowth in an age-related fashion (present study). This phenomenon is not related to myelination of the spinal cord, nor can it be explained by the absence of trophic support from muscle. In order to extend the axonal growth cone must attach to the substratum [9]. Growth cones interact with collagen (the substratum in the present culture system) through integrins. These glycoproteins mediate cell adhesion, play a role in neuritic outgrowth [2] and display a spatio-temporally regulated expression pattern during development. The observed age-related decrease of outgrowth may be related to a progressive inability of the spinal axons to interact with collagen in the absence of other cues. The involvement of integrins in this phenomenon merits further investigation.

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