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Enhancement of cultured adult motor neuron survival with cold pre-incubation

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HIGHLIGHTS

- The survival of adult spinal motor neurons were rather poor in serum free culture.
- Adult mouse spinal motor neurons survived only 24 h in control cultures.
- We pre-incubated them at 4 °C for 3 days to slow down their metabolism.
- Then we transferred to them to 37 °C and they lived up to 8 days.
- Inhibition of protein synthesis during cold pre-incubation made little difference.

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ABSTRACT

The aim of the study was to extend the survival of adult spinal motor neurons in serum free culture. Anterior half of the spinal cord was removed from young adult mice and dissociated. Cultured cells attempted to extend neurites within hours of incubation at 37 °C and died within 24 h. To prevent this early regenerative activity, thus to decrease the metabolic requirements of the neurons, cultures were transferred to 4 °C immediately after they were set and kept there for 3 days. Preparations were then taken to 37 °C where they lived up to 8 days. Some neurons continued to extend neurites until the day they died. To understand whether the enhancement of survival involves new protein synthesis, transcription and translation were blocked during cold pre-incubation, which shortened the half life of neurons but not changed the maximum survival period. In conclusion this study has shown that, in the serum-free cultures, the survival of adult spinal motor neurons can be significantly enhanced by cold pre-incubation whose effect seems to depend largely on a reduction in the metabolic activity and less on new protein synthesis.

1. Introduction

Neuron cultures are indispensible tools to understand physiological and pathological processes in the nervous system and various motor neuron diseases are among them. For example, the etiology of amyotrophic lateral sclerosis, the main motor neuron disease in adulthood can be better understood in the controlled conditions of the cell culture. However such in vitro models can not be used extensively due to poor survival of mature motor neurons in culture [1]. Though they can not exactly substitute the mature forms, embryonic cells with a higher propensity to survive are used as a resort [9,14]. Attempts have been made to keep adult motor neurons alive longer by supplementing serum, muscle extract or conditioned media together with various growth factors [17–20]. However these treatments render the culture medium "undefined" and pose restrictions on the use of the in vitro model. In defined media without any serum or extract, the survival of motor neurons are reported to be only about 24 h [1,20]. In this article, we describe a new method for culturing adult mouse spinal motor neurons in serum and growth factor-free medium with survival up to 8 days, which was achieved by pre-incubation of the neurons at low temperature.

2. Material and methods

2.1. Animals

Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with EEC Council Directive 86/609. All efforts were made to minimize animal suffering and to reduce the number of animals used. An institutional ethical committee approval was obtained prior to

Abbreviations: NBA, Neurobasal A culture medium; DRB, 5,6-dichloro-1-β-D-ribofuranosilbenzimidazol; ANI, anisomycin; PI, propidium iodide.

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experiments. Six to eight weeks old female Balb-C mice were used in the study.

2.2. Tissue dissection and cell culture

Animals were killed by cervical transection under deep anesthesia induced by an I.P. injection of ketamin (100 mg/kg, Ketalar, Pfizer). The skin was excised and the spinal canal was cut open from cervical to sacral region at the back with fine scissors exposing the spinal cord, which was gently removed and transferred into ice-cold Leibovitz's medium (L15-Sigma). The posterior half of the spinal cord was excised and discarded. The anterior half was trimmed of the attached nerves and cut into small pieces (2–3 mm), which were then transferred to a tube containing cold Neurobasal A medium supplemented with 2% B27 (NBA-B27) (Invitrogen) containing 2 mM Glutamax-I (Invitrogen), 100 units of penicillin, 100 mg streptomycin, 250 ng amphotericin B (Sigma) and 6 U papain (Sigma) per ml. The tube was kept at 4 °C for 45 min; during the last 15 min it was agitated on a custom made agitator vibrating horizontally at 50 Hz. The tissue pieces were then triturated for about 7 min by gently and repeatedly pipetting through the tips of narrowing bores (from 2 mm diameter down) and finally through a 26-gauge needle. For selective isolation of the motor neurons, gradient centrifuge technique was used. For this, the cell suspension was carefully pipetted on top of a three-layer percol (Sigma) gradient (60, 30, 20 and 10% from bottom to top) prepared with NBA-B27 and spun at 3000 × *g* for 25 min in a centrifuge cooled down to 4 °C. The neurons that were collected from 30% layer were washed with NBA-B27 and spun once more at 700 G for 3 min after which the supernatant was discarded and pellet was resuspended in NBA-B27. The neurons were seeded on 35-mm diameter Petri dishes (WPI), which had been previously coated with poly-D lysine (Sigma) (1.8 μg/cm^2, 3 h at RT). The preparations were kept in an incubator (37 °C, % 5 CO_2) 2 h to let the neurons attach to the bottom of the dish, after which they were gently washed with warm NBA-B27 to remove unattached cells and remaining debri. At this stage, some dishes were filled with NBA-B27 and directly returned to the incubator. Other dishes were filled with L15 containing 2% B27, 2 mM Glutamax-I, 100 units of penicillin, 100 mg streptomycin and 250 ng amphotericin B and kept in a refrigerator at 4 °C for 72 h after which the medium was replaced with NBA-B27 and the preparations were transferred to the incubator for further incubation. To inhibit transcription and translation 5,6-dichloro-1-β-D-ribofuranosilbenzimidazol (DRB) and anisomycin (ANI) were added to some preparations during the cold incubation, respectively. These reagents were used at 50 μM and 3.8 μM concentrations respectively and removed by replacing medium with fresh NBA-27 at the end of cold incubation.

2.3. Assessment of viability and neurite extension

The viability of cultured neurons were assessed by propidium iodide (PI), a nuclear stain that labels only dead cells with red fluorescence. For this, PI was added to the cultures at 7.5 μM concentration and the number of dead and live neurons were counted every 24 h. To ensure that the same neuronal populations were evaluated each time, coordinates of microscopic fields examined were recorded through the dedicated software (Axiovision, Zeiss) of the inverted microscope system used (Cell Observer, Zeiss).

The rates of survival on each day were compared with chi-square test. Differences were considered significant at $p < 0.05$.

For quantification of neurite outgrowth, multiple microscopic fields were digitally imaged and the length of longest extension was measured for each day. Measurements from a total of 75 neurons were taken on the first day; this number decreased to two on the eighth day of physiological incubation due to ongoing cell loss.

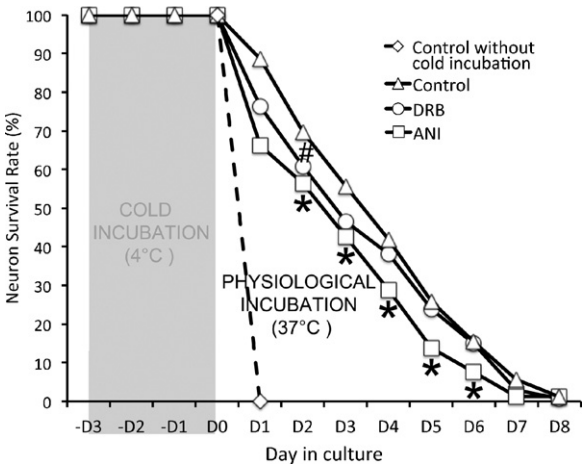

Fig. 1. Survival rate of spinal motor neurons directly cultured at physiological temperature (37 °C) and following 3-day pre-incubation at 4 °C, after which they were transferred to 37 °C. Inhibition of protein synthesis by ANI (translation inhibitor) or DRB (transcription inhibitor) led to earlier death of some neurons but did not change the maximum survival period. $^{*}p < 0.05$ ANI vs. control, $^{\#}p < 0.05$ DRB vs. control.

2.4. Immunocytochemistry and microscopy

For phenotypic identification of the isolated neurons, indirect immunofluorescence staining was performed with antibodies against motor neuron markers. These were goat anti-choline acetyltransferase (anti-ChAT, Chemicon), goat anti-peripherin (Santa Cruz), rabbit anti-calcitonin gene related peptide (anti-CGRP, Affiniti/Enzo Life Sciences) and mouse anti-p75 (Sigma) IgG's. Antibodies against neurofilament heavy (Mouse anti-NFH IgG, Sigma; Rabbit anti-NFH IgG, Chemicon) were used as general neuronal marker. The staining procedure was performed as follows: preparations were fixed with 4% paraformaldehyde for 15 min at RT and permeabilized and blocked with phosphate buffer saline (PBS) containing 0.1% Triton-X, 3% bovine serum albumin and 1% chicken serum for 30 min at 4 °C. Preparations were then incubated with primary antibodies overnight. Next day they were given three washes with PBS and for 3 h, incubated with secondary antibodies which were Alexa fluor 488 chicken anti-mouse IgG, Alexa fluor 488 chicken anti-goat IgG or Alexa fluor 594 chicken anti-rabbit IgG (all from Invitrogen). Then preparations were washed again with PBS for three times and mounted and imaged with a laser scanning confocal microscope (Zeiss LSM 510).

3. Results

The total number of viable neurons isolated from a single animal varied between 350 and 550. Almost all neurons attached to the bottom of the culture dish. The neurons that were continued to be incubated at 37 °C after attachment started to extend neurites within hours; however, all of them died within 24 h. We proposed that the cells' early attempt to regenerate neurites exhausted them and caused death within hours. To circumvent this problem, we tried lowering metabolic activity of the cells by incubation at low temperature. Indeed, when the cells were kept at 4 °C for 3 days, half of them lived for another 3 days after they were transferred to physiological temperature (Fig. 1). The maximum duration of survival under this condition was 8 days, which was true for only 1.2% of cultured neurons. To understand whether cold incubation support the cell survival simply by decreasing the metabolic need or involves new protein synthesis, we blocked transcription and