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Isolation and Culturing of Primary Neurons from Newborn Mouse Cortex Tissue

Yeni Doğan Farelerden İzole Edilen Korteks Dokusundan Primer Nöron Elde Edilmesi

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Abstract

Objective: This study aimed to establish a reliable protocol for obtaining healthy and long-lived cortical neurons from newborn mice, providing a valuable model for studying neuronal function.

Materials and Methods: Cortical regions of P0 mice were isolated and healthy neurons were obtained by enzymatic and mechanical dissociation. On the seventh day of incubation, the presence of neurons was detected by staining with neuron-specific antibodies. Transgenic mice expressing fluorescent proteins specific to neurons, glia and oligodendrocytes were also used for culture.

Results: Almost all the neurons had adhered to the petri dish bottom by the second hour of incubation. Most of the neurons were healthy and started to grow extension quickly.

Conclusion: Neuron cultures are an important tool in research and are invaluable for studying the behaviour of cells. Nanoparticles facilitate genetic manipulation of these cultures for various biotechnological applications. In particular, they have great potential in areas such as the delivery of genetic material into cells, drug delivery and targeted treatment methods. Such techniques have the potential to open up new avenues for the study and treatment of neurological diseases.

Keywords: Cortex, Newborn Mice, Cell Culture, Neuron Culture, Central Nervous System.

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Öz

Amaç: Bu çalışma, yeni doğmuş farelerden sağlıklı ve uzun ömürlü kortikal nöronlar elde etmek için güvenilir bir protokol geliştirmeyi ve elde edilen nöronları kullanarak nöron fonksiyonlarını incelemeyi hedeflemiştir.

Gereç ve Yöntemler: P0 farelerin korteks bölgeleri izole edilerek enzimatik ve mekanik ayrıştırma uygulanarak sağlıklı nöronlar elde edildi. İnkübasyonun yedinci gününde nörona özgü antikorlar ile boyama yapılarak nöronların varlığı gösterildi. Ayrıca nöron, glia ve oligodendrositlere özgü floresan proteinleriifade eden transgenik farelerden de kültür yapıldı.

Bulgular: İnkübasyonun ikinci saatinde nöronların neredeyse tamamının kültür kabına yapıştığı gözlemlendi. Elde edilen nöronların büyük kısmının sağlıklı olduğu ve uzantılarının hızlıca büyümeye başladığı gözlemlendi.

Sonuç: Bu protokolü diğer protokollerden ayıran temel özellik, geliştirme sürecini destekleyecek hiçbir faktör veya serum kullanılmamasıdır. Nöron kültürleri, araştırmalarda önemli bir araç olarak kullanılır ve hücrelerin davranışlarını incelemek için oldukça muazzam olanak sağlar. Nanoparçacıklar, bu kültürler üzerinde çeşitli biyoteknolojik uygulamalar için genetik manipülasyonları kolaylaştırır. Özellikle, hücrelere genetik materyal taşıma, ilaç salınımı ve hedeflenmiş tedavi yöntemleri gibi alanlarda büyük potansiyele sahipler. Bu tür teknikler, nörolojik hastalıkların araştırılması ve tedavisi için yeni yollar açabilir.

Anahtar Kelimeler: Korteks, Yenidoğan Fare, Hücre Kültürü, Nöron Kültürü, Merkezi Sinir Sistemi.

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Introduction

Damage to the nervous system often leads to irreversible degenerative processes. Injuries to the central nervous system, in particular, can lead to substantial functional impairments. The primary reason for this is the post-mitotic nature of neurons, which precludes the replacement of lost cells and damaged axonal connections through regenerative mechanisms. Consequently, there are many challenges that still require attention.

In developing treatment strategies for neurological diseases or nervous system trauma, many researchers aim to deepen their understanding of neurodegenerative processes. It would be beneficial for the mammalian central nervous system (CNS) to develop pharmacological interventions that can repair injured or compromised adult tissues (1).

It is important to note that the regeneration mechanism is complex, largely due to the unfortunate reality that regenerative capacity following CNS injuries in mammals is inherently limited (2). The regeneration process involves complex interactions. These include the role of glial cells, scar tissue formed at the site of injury, inflammatory responses and the sensitivity of neurons to growth factors. It is therefore difficult to achieve full recovery from CNS injury. Primary neuron cultures are highly appropriate for investigating the establishment of axonal networks and the molecular mechanisms that underlie cellular maturation in the CNS (3). It might be suggested that primary neuron cultures offer an opportunity to examine several processes, including polarization, neurite outgrowth, axon guidance (pathfinding), synaptogenesis, and neuronal network formation. In addition, it offers the possibility of studying physiological processes that occur in vivo in vitro with more than one neuron at a time (4). This in vitro approach is objective and avoids potential biases that may occur in in vivo settings.

Primary cell cultures, with their homogeneous cell populations, allow to understand normal physiology by simple manipulations (5). Using simple manipulations, it is possible to observe how cells respond to environmental, growth and various chemicals. In addition, these cultures allow us to create disease models, test the effects of drugs and better understand the basic mechanisms of cellular processes. Primary neurons are vulnerable to destruction within a few days, and are capable of producing separate axonal and dendritic structures (6). There are several methods for growing nerve cells. The maturation of neurons can be stimulated by the addition of growth factors (for example, nerve growth factor- NGF). The efficiency of primary neuron cultures heavily depends on the swift and precise dissection, the usage of a appropriate mediums in each step of dissociation, and proper mechanical separation methods to minimize damage (7).

Our laboratory has developed a neuronal culture protocol that ensures reproducibility and promotes rapid and robust axon regeneration. The developed protocol for cell dissociation and plating is both gentle and rapid, allowing for completion in less than two hours. Our short protocol enhances the survival of neurons. Usage of Neurobasal-A increases survival rate and regeneration rate in contrast to many other protocols for cell plating/growth and culture maintenance, respectively. A reliable and reproducible protocol has been established for the preparation of cortical neurons that can be successfully primed from newborn mice.

Materials and Methods

Animals

Newborn mice, including Balb-C and FVB-Tg (Prism)1989Hz/J coded transgenic mice aged between P0 and P3, were used. The animals were maintained in compliance with the ethical and welfare standards established by the Istanbul Medipol Institutional Animal Care and Use Committee (IMU-HADYEK) with the approved reference number [E-38828770-772.02-7790]).

Cell culture

Newborn mice (P0-P3) were euthanized via decapitation and the brain was isolated from the skull. The brain was placed in chilled L-15 medium and mixture of antibiotics and Glutamax were added in proportion as 1% respectively. The cortex was dissected in Hibernate medium under a stereomicroscope. The tissue was then placed in L-15 medium containing 1% papain. It was then incubated at +4°C for 45 minutes. Following incubation, 1% DNase was added and trituration step was carried out. After homogenisation, the tissues were

incubated in a medium containing 10% fetal bovine serum for enzyme inhibition. The homogenized tissues were then centrifuged at 1000rpm for 5 minutes, and the resulting supernatant was discarded. All petri dishes were coated with 1 % poly lysine and incubated for 2 hours at room temperature. The cell pellet was resuspended in Neurobasal Medium supplemented with 1% antibiotics, 1% glutamax and 2% B27, and seeded in each petri dish with a maximum volume of 300 µl. After two hours of waiting for adherence of cells to the petri dish bottom, the growth medium was prepared and the cells were incubated under physiological conditions (37°C, 5% CO2) throughout the experiment (8).

Table 1. Chemicals

Chemicals	Manufacturer
B27	Gibco
Glutamax	Gibco
Antibiyotik	Sigma Aldrich
NBA	Gibco
L15	Sigma Aldrich
Hibernate	Gibco
Poly-L-Lizin	Sigma Aldrich
Fetal Bovine Serum	Sigma Aldrich
Papain	Sigma Aldrich
BSA	Sigma Aldrich
Dnase	Sigma Aldrich
Paraformaldehit	Sigma Aldrich
Triton X-100	Sigma Aldrich
PBS	Sigma Aldrich
Doublecortin	Abcam
Bate III Tubulin	Cell Signaling
Alexa Flour 488 Goat anti chicken	Invitrogen
Alexa Flour 647 Goat anti Mouse	Invitrogen

Immunofluorescence

On the seventh day of the incubation of cells fixation was performed with 4% paraformaldehyde (PFA, Sigma-Aldrich) in PBS at pH 7.4 for 15 minutes at room temperature (RT, 20-22°C). After fixation, cells were washed with PBS, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS and washed again with PBS for 5 min. Cells were then blocked with 0.3% w/v BSA in PBS for 30 minutes. Incubation with primary antibodies (Doublecortin Mouse, BetaIII Tubulin Chicken) was performed overnight in a humidified chamber. This was followed by washing steps in PBS and incubation with secondary antibodies Alexa 488 goat anti-chicken and Alexa 647 goat anti-mouse for 1 hour.

The higher magnification images were obtained using a 40x objective in airscan mode of a Zeiss LSM 800 confocal microscope at Istanbul Medipol University SABITA. Sequential scanning was used for image acquisition to reduce crosstalk between different channels. 488 and 647 lasers were used (9).

The study was approved by the Istanbul Medipol University Local Ethics Committee (date: 11.12.2023 and approval number: 68).

Results

The brain tissues were dissected rapidly under sterile and cold conditions. Following two-hour incubation after seeding, it is observed that most of all neurons adhered to the culture dish, and a rapid growth of axonal extensions were seen. The cell culture medium was changed by 50% every three days to remove possible toxicity. The cells were maintained in a serum-free, factor-free environment for an extended period of time (Figure 1).

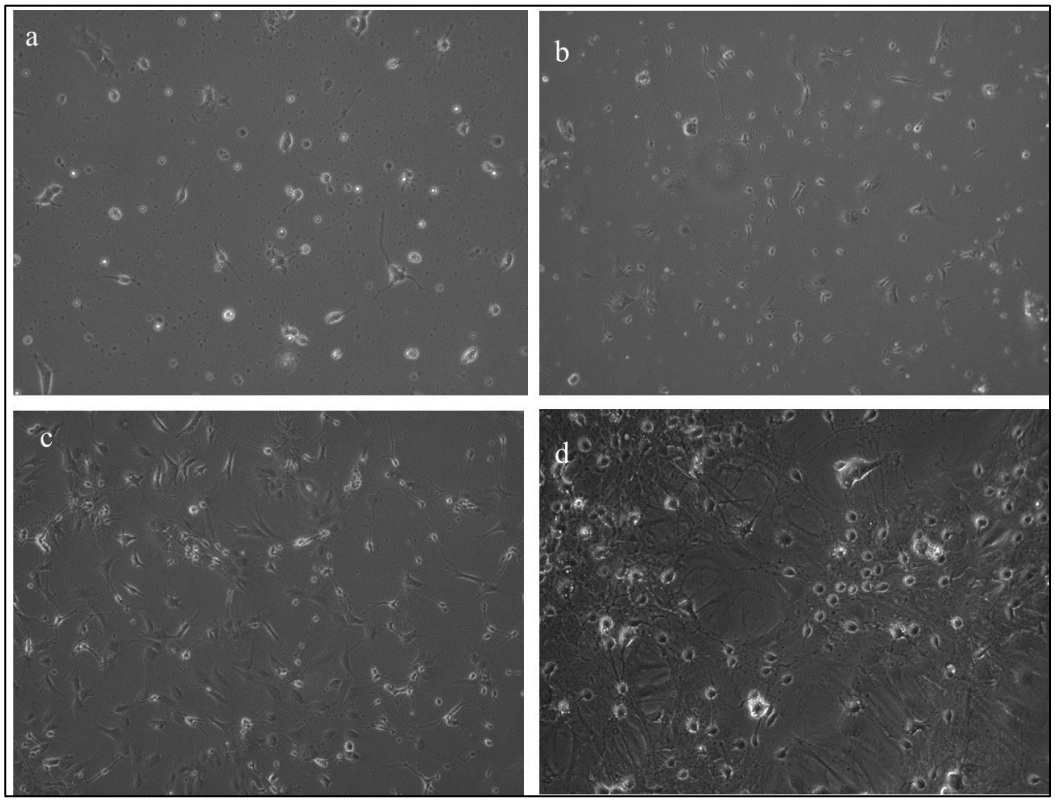

Figure 1. Photographs of the cell culture taken at different times during the incubation period. A: 1st day of cell culture B: 3rd day of cell culture C: 14th day of cell culture D: 21st day of cell culture

Beta-tubulin III, also referred to as Tuj-1, belongs to class III of beta-tubulin protein family, which is one of two structural elements that comprise the cells' microtubule network. While general tubulins participate in a variety of cellular processes, including mitosis and motility, beta-tubulin III specifically found in neurons. Cells stained for beta III tubulin, a neuronal marker, indicated successful axonal elongation (Figure 2).

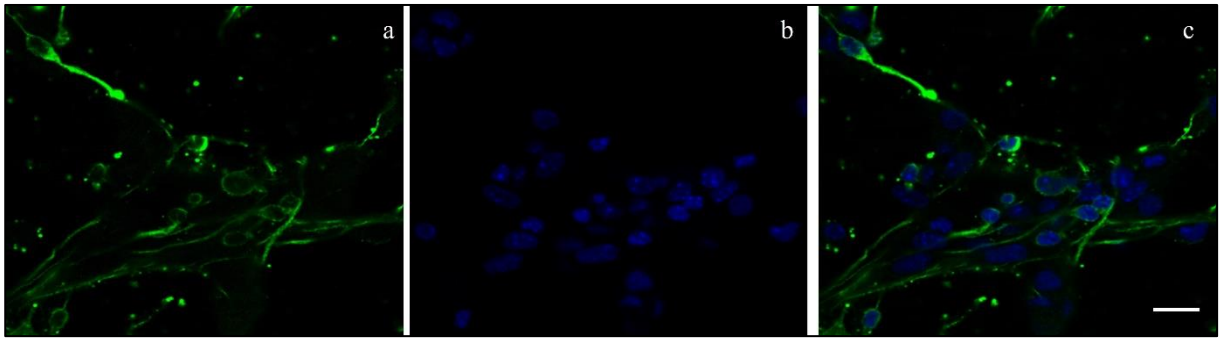

Figure 2. Image of a neuron labelled with Beta III Tubulin. A: shows primary neurons stained for beta III tubulin, a neuronal marker; B: shows a DAPI image; C: shows a merged beta III tubulin and DAPI image (scale bar 100 μm).

Doublecortin (DCX) is a protein that plays an important role in the process of neuronal development and migration. In particular, it has a critical function in the maturation and colonisation of the nerve cells. DCX supports the development of the dendritic structures of neurons and helps to position new neurons correctly in the brain.

DCX is often used as a marker for neural progenitor cells and immature neurons (Figure 3).

The transgenic mice exhibit three distinct fluorophores in specific subsets of brain cells: Mobp regulates Myc-tagged Cerulean (CFP) for blue-green fluorescent oligodendrocytes; Aldh1l1 regulates DsRedMax for red-fluorescent astrocytes; and Snap25 regulates Rpl10a (ribosomal protein L10A) tagged YFP for yellow-fluorescent neurons.YFP expression is primarily localized to the cell body and nucleolus of neurons. The intensity of neuronal YFP fluorescence is notably lower than that of glial fluorescence. Additionally, red fluorescence can be detected through the skulls of newborn pups. Upon exa mination of the distribution of

cortex cells isolated from the transgenic prism animal, it was observed that neurons, astrocytes, and oligodendrocytes were present (Figure 4).

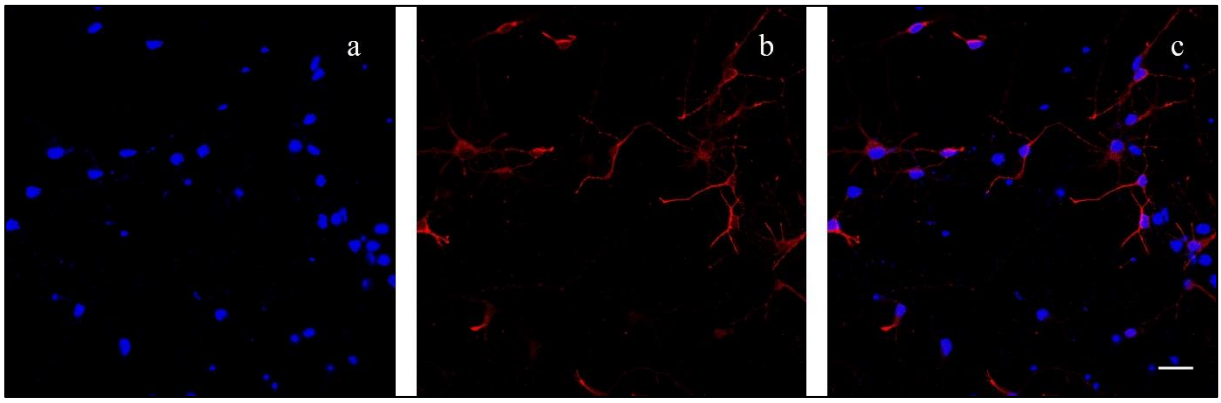

Figure 3. Cellular image showing doublecortin (DCX) immunoreactivity: A: Neurons with DCX immunoreactivity; B: DAPI image; C: Neurons with DCX immunoreactivity and DAPI merge image (scale bar 100 μm).

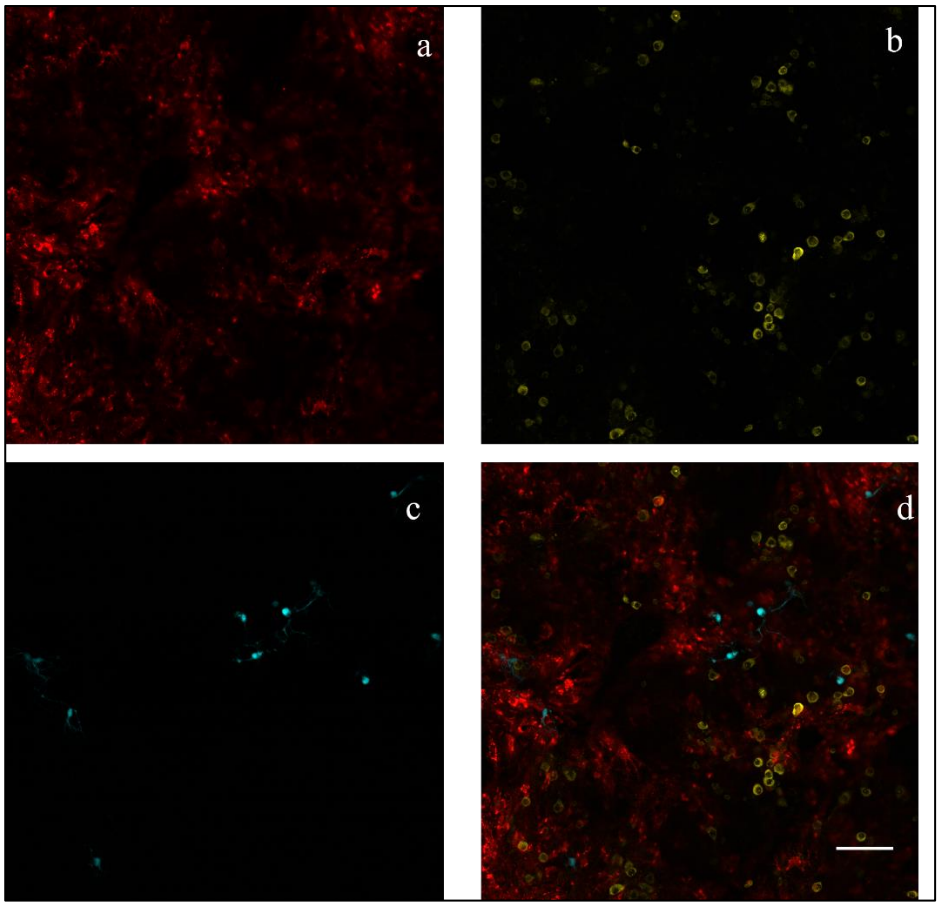

Figure 4. Cells were collected from the cortex of transgenic mice, A:Aldh1l1 controlled DsRedMax, resulting in red-fluorescent astrocytes, B:Snap25 controlled YFP-tagged Rpl10a (ribosomal protein L10A), leading to yellow-fluorescent neurons C :Mobp controlled Myc-tagged Cerulean (CFP) for blue-green fluorescent oligodendrocytes. D: merged image of all cell groups was created (scale bar 100 μm).

Discussion

The postnatal hippocampal culture model represents an appropriate in vitro system for studying neuronal pathophysiology, particularly when transgenic animals are used. The majority of current procedures utilizing

newborn mice are based on protocols originally developed for embryonic culture, which has resulted in inconsistency in culture quality or limitations to P0-P1 pups (10). However, the protocol that we developed enabled us to isolate cortex tissue from animals as young as p10 and transform it into a single cell, maintaining its viability for an extended period and allowing us to observe the rapid regeneration of axons.

Primary neuron cell culture offers a unique framework for investigating neuronal structure and function at a specific level. The protocol currently employed utilizes neuron cultures that are relatively pure, highly reproducible, and contain minimal glial cell contamination. During this process, neurons were maintained in a viable state for several months without the need for additional treatments (such as serum or growth factors).

In contrast to numerous protocols designed for embryonic or postnatal cultures, dissociation of the hippocampus with the protease trypsin has been demonstrated to promote neuronal death. The use of papain, a gentle enzymatic tissue digestion agent, is sufficient to dissociate cells while preserving neuron survival. Similarly, a reduction in mechanical stress can be achieved by minimizing the tirturation procedure, which results in a cell suspension containing a high proportion of viable cells. Furthermore, the survival of neurons was significantly enhanced when the cortex and dissociated cells were maintained in Hibernate medium, a CO_2 independent nutrient medium, throughout the initial stages of the process (11). Papain, an enzyme derived from plants, was used to dissolve the cells. The mechanical separation step is crucial in primary cell culture protocols (12). This step was carried out with great care. The highest amount of living cells were observed.

In neuron cultures, it was observed that during the first week, the number of glial cells was low. This interval served as the perfect time frame to conduct biochemical analyses, including transcriptomic and proteomic assessments, particularly on less contaminated neurons (2). On the other hand it is confirmed that glial cells aid in the maturation and plasticity of neurons through the production of factors (13). So, it is assumed that an increased number of glial cells during the second week has a beneficial impact on neurons in this context. Additionally, secondary cell lines have become a valuable resource for medical research due to their immortal nature.

However, these cell lines have been found to produce variable results over time, likely due to increasing numbers of passages. As a result, the reliability of these cell lines is reduced (14). In contrast, primary cells are genetically more stable and therefore preferred for both pharmacological and biomedical researche (15). They provide prospects for investigation that allow for stricter regulation of cellular functions and processes.

The primary success of CRISPR-Cas9 gene editing has successfully enabled the use of neuronal cultures (16). These cultures also provide demonstrating easy tracking of cellular dynamics through live imaging and electrophysiology (2).

Additionally, another significant benefit is that the technology permits the administration of neurons in drug experiments, thereby reducing the demand for animal testing. With nanoparticles, biotechnological tools designed for various purposes, it is also possible to carry out genetic manipulations in a short time.

Conclusion

The generation of primary mouse cultures followingthe described protocol allows for the undertaking of a multitude of cell biological and biochemical studies. The cultures, prepared with a certain degree of difficulty and care, are highly resilient. These cultures can offer valuable insights into neuronal cellular architecture and function.

The in vitro culture of primary mouse cortical neurons has been successfully established in this study. The viability of these neurons has been demonstrated over an extended period, with no serum or other factors required for their maintenance. The long-term culture of these neurons allows for the examination of their response to DNA damage, apoptosis and other cell death mechanisms.

Ethics Committee Approval: The study was approved by the Istanbul Medipol Institutional Animal Care and Use Committee (IMU-HADYEK) with the approved reference number [E-38828770-772.02-7790]).

Conflict of Interest: Authors declared no conflict of interest.

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