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Neurosphere Development from Hippocampal and Cortical Embryonic Mixed Primary Neuron Culture: A Potential Platform for Screening Neurochemical Modulator

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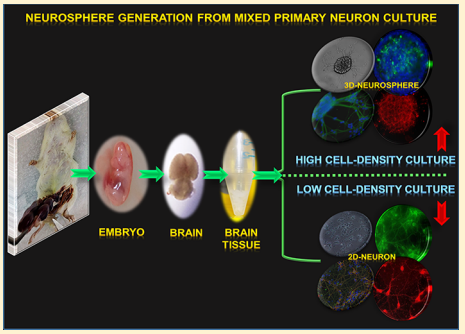
Supporting Information

ABSTRACT: Reconstitution of a complex biological structure or system following a simple and facile strategy using minimum physiochemical cues is challenging for an in-depth understanding of the system. In particular, the brain is a highly sophisticated and complex network of trillions of neurons and glial cells that controls function of our body. Understanding this complex machinery requires an innovative and simple bottom-up approach. In this venture, we report an easy and efficient strategy to culture cortical and hippocampal primary neurons from the E14-E16 embryo of Sprague−Dawley rat. This generates spontaneous neurospheres within 6−7 days of primary neuron culture of E14-E16 embryo. It further proliferates and forms radial glia-like structures, which are known to be the primary neural progenitor cells that differentiate into neurons, astrocytes, and oligodendrocytes. Interestingly, neurospheres lead to the formation of large projection neurons and radial glia, which mimic the early stage of cortical development in an in vivo system. Overall, this new, facile, strategic mixed primary neuron culture method offers a potential platform for understanding the effect of neurochemical modulators, which has tremendous future implications in the screening of neurotherapeutics.



KEYWORDS: *Embryonic rat brain, hippocampus, cortical brain, primary neuron culture, neurosphere*

1. INTRODUCTION

The brain consists of a complex network of trillions of neurons interspersed with non-neuronal cells. In order to study the mechanisms that control the various regulatory parameters of this machinery, in vitro reconstitution of this system is needed. Cell culture is an extremely important technique, which provides us a snapshot of the underlying mechanistic processes that govern the principles of life and are comparatively difficult to study in vivo. For decades, researchers around the globe have relied on cell lines for most of their studies, given their high proliferation rate and easier culture methods.[1,2] However, contaminated cell lines and a significant genotypic and phenotypic departure from their tissues of origin have reignited interest in the primary cells. Primary cells are certainly not easy to culture, but given their normal cell morphology and closer resemblance to the in vivo environment, primary cell culture has become a quintessential technique for various in vitro cell-based assays.[3,4] In the recent excitement surrounding the discovery and synthesis of a number of neuroprotective and neuroregenerative molecules, it is pertinent to develop cellular platforms for their screening, and that has increased the onus to develop a simpler primary neuron culture methodology. The primary neuron culture from rats holds a prominent area in neuroscience, especially in domains of neural differentiation, synapse formation, and neuronal communication. Most methods concerning primary neuronal culture so far have relied on a prolonged culture of hippocampal neurons for 3−5 weeks with a low-density plating in a chemically defined serum-free medium.[5−8] However, the yield of neurons in such methods has been difficult chiefly, due to the lack of paracrine trophic support from the surrounding neurons.[9] Therefore, a few more methods have been reported to yield a coculture of primary neurons with a glial feeder layer in order to support neuronal survival.[10−13] Even the glial cells are not a defined variable and do not provide a consistent formulation for neuronal culture. All of these issues and more, point toward the development of a relatively easier and easily reproducible primary neuron culture protocol that can be used for regular screening of drug candidates. Although a primary neuron culture would bear a better resemblance to the in vivo system, it would still lack the intrinsic spatial integrity and development of brain tissue. This called for the construction of a system with a closer resemblance to the

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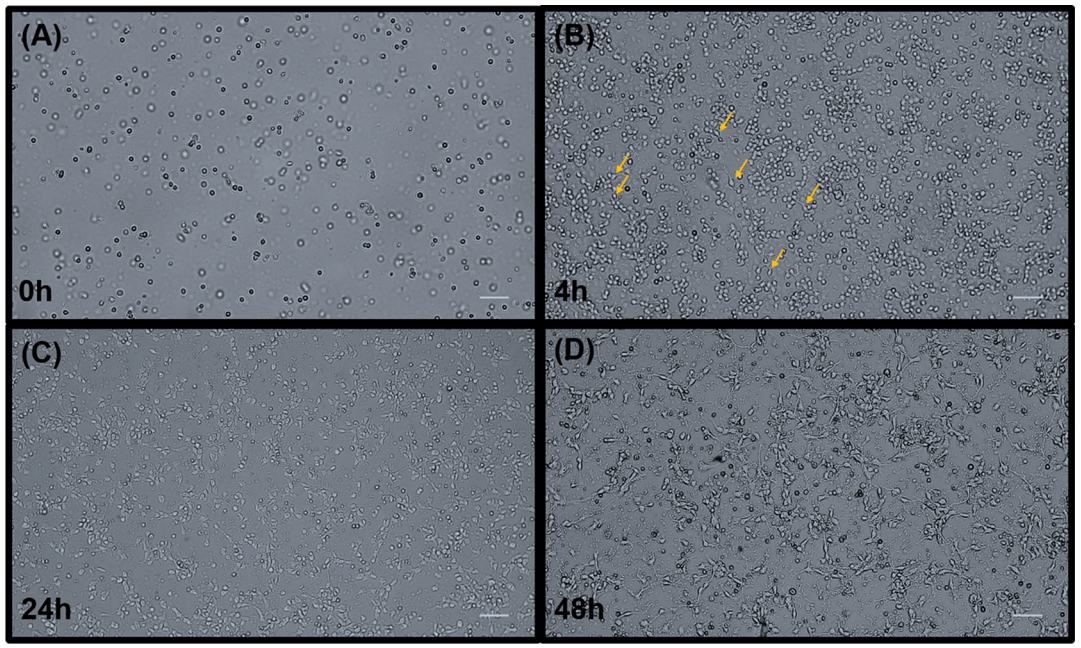

Figure 1. Primary cortical and hippocampal cells. (A) Appearance of cells just after plating. (B) Adherence of cells seen after 4 h of plating shown by arrows. At this stage plating medium is changed with maintenance medium. (C) Cortical and hippocampal cells after 24 h of culturing. (D) Cortical and hippocampal cells after 48 h of culturing. Scale bars correspond to 200 μm.

brain consisting of astrocytes, neural progenitor cells (NPCs), and neurons.[14] Hence, three-dimensional (3D) culture systems called neurospheres have emerged as new tools in neuroscience research. Neurospheres are free-floating cultures of neural progenitor cells (NPCs) which are believed to constitue a culture system more representative of the spatial cellular environment with features such as neuronal communication, various chemical and biochemical cues, and tissue heterogeneity.[15−17] The paramount importance of neurospheres comes with its increased expression of neural progenitor cells (NPC), which are self-renewing multipotent cells that have the capacity to differentiate into neurons as well as glial cells. Due to the presence of NPCs, the neurosphere culture system has gained immense importance in studies investigating differentiation, neurotoxicological studies, and biological studies of developmental processes, as a functional assay to test the neural stem cells characteristics, to model specific neurodegenerative diseases such as Alzheimer's disease[18] and Parkinson's disease[19] in vitro for screening neuroprotective drug candidates and to study glial cell differentiation.[20,21] Some models for the construction of consistent neurospheroids have been developed such as that where the neurospheroid formation has been controlled in vitro in fabricated concave microwells which have been surface modified with typical ECM molecules.[22] In addition, it was observed that the neurite outgrowth in this case was tightly regulated due to the cell−ECM interactions in such a confined area. Some other groups have also developed human neurospheroid arrays and three-dimensional human neurospheroid models for studying Alzheimer's disease with ease.[23,24] However, the major problem with the neurosphere culture is its sensitivity to the variable culture methods, which has made it difficult to formulate a unified culture procedure and therefore it has been difficult to merge discoveries from different groups to gain a complete understanding of a particular phenomenon.[25−31] In the present paper, we have formulated an easy and quick protocol with limited reagents for primary neuron culture from E14-E16 Sprague−Dawley rat embryo plated differentially at low and high densities. While the low-density culture provides a neuronal interface for screening of neurochemical modulator, at high density we observe the spontaneous generation of neurospheres after 6−7 days of culture without any change in medium or addition of growth supplements. Both the primary neuron culture and neurospheres have been characterized well through a set of neuronal and non-neuronal markers. The neurospheres have been found to contain a rich population of inherent NPCs, which further differentiate into radial glial cells that mimic the early stage of cortical development. It is important to note here that this method is a further advancement in comparison to the previous methods/protocols for fast and facile generation of high yields of neurons to facilitate research advances in chemical neurosciences.

2. RESULT AND DISCUSSION

2.1. Isolation of Cortical and Hippocampal Neurons from Embryonic Rat Brain (E14-E16). All of the experiments were carried out in a sterilized environment from the isolation of the embryos to the microdissection of the hippocampus and cortex to avoid chances of contamination, which is the prime reason for the failure of neuron culture (Figures S1 and S2, Supporting Information). Cortical and hippocampal neurons are studied to know their structural, histological, and physiological attributes, as they are more prone to be affected in various neurodegenerative diseases. Therefore, there is a need to study the functioning of these neurons in a Petri plate to analyze the changes taking place in vivo so that a therapeutic approach can be formulated. After the isolation of the uterus they are to be provided with a condition that would resemble that of its in vivo condition; therefore, an artificial environment is prepared for the survival of the cell, tissue, or organ, for which HBSS buffer solution supplemented with glucose is used to ensure that the cells receive all the appropriate growth factors for their survival. It is very important to see that the

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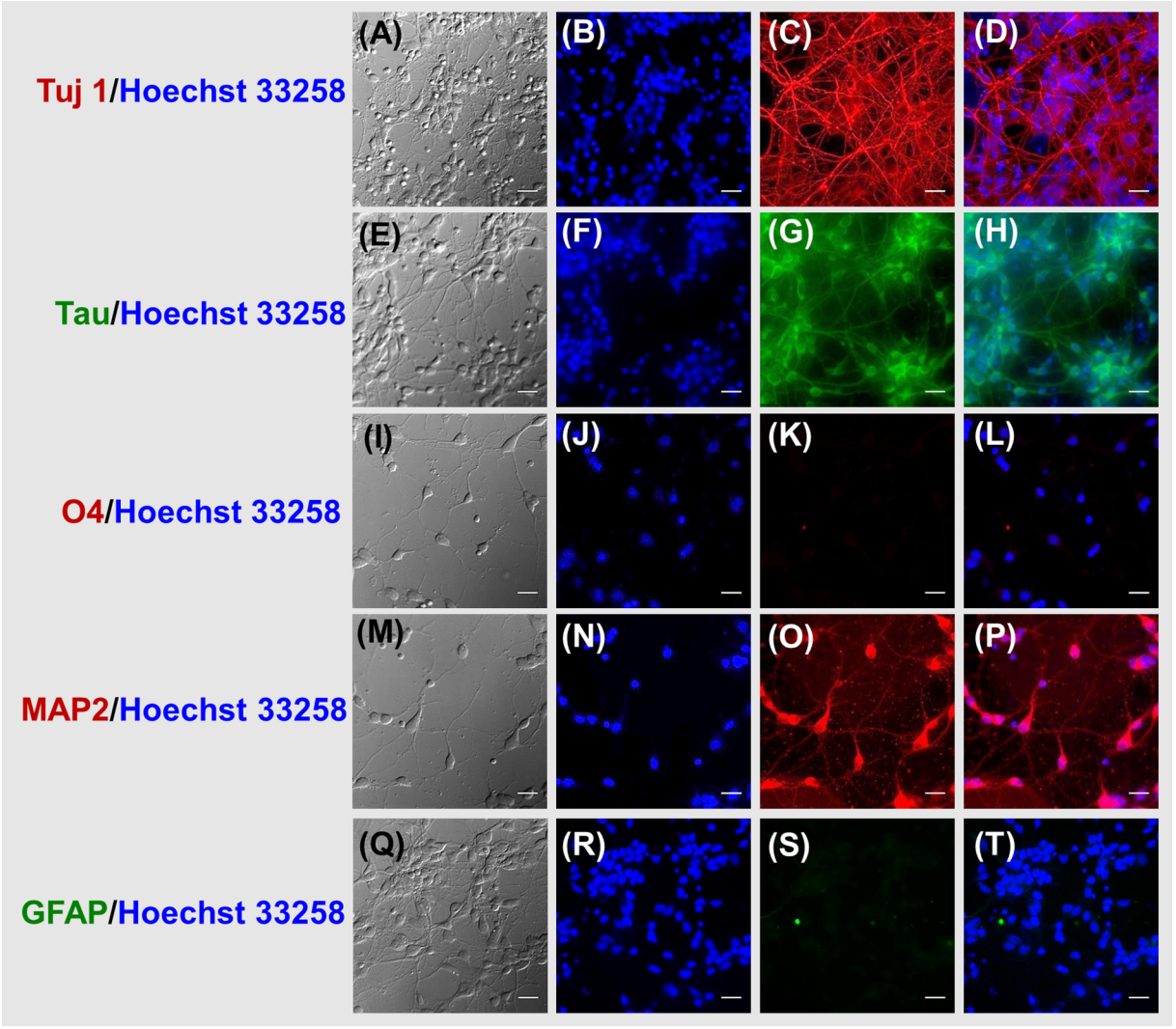

Figure 2. Immunostaining of a mixed culture of cortical and hippocampal neurons: (A–D) Tuj 1 for neurons; (E–H) Tau for axons; (I–L) O4 for oligodendrocytes; (M–P) MAP2 for dendrites; (Q–T) GFAP for astrocytes. Nuclei were stained with Hoechst 33258 (blue). The average cell number is 64 ± 5. Scale bars correspond to 20 μm.

tissue is constantly submerged in the medium and kept over ice so that there is minimal loss of cells due to unavailability of growth factors and optimal temperature. The embryos are taken out from the placental sac and decapitated for the isolation of the brain. An embryonic brain, being small in size, is difficult to handle. The brain is exposed from the skull by using a very easy method of pinching off the brain using forceps, which very easily pops up without any damage to it. The meninges of the embryo are very important to remove so that the culture does not get contaminated with meningeal fibroblasts, as it is seen that the proliferation of fibroblasts is far more aggressive than that of non-neuronal cells. It is easier to remove the meninges of the embryo as they are in their developing stage and very loosely attached to the brain surface. The cortical and hippocampal region along with the lateral ventricle region was very carefully microdissected and collected in the medium to prepare a homogenate.

2.2. Culturing the Isolated Cells. The homogenate was prepared using plating medium and was seeded on a poly-D-lysine-coated plate in low and high density and left undisturbed for approximately 4 h for better adherence of the cells. After 4 h the culture dishes were checked for cell adherence (Figure 1B) and then the cells were given a media change with neurobasal media supplemented with B27 factor. This media helps in the maintenance and maturation of the embryonic cell population without the need of any other supplement. B27 supplement helps in the long-term viability of neuronal cells taken from any origin. Figure 1C,D shows the neuronal growth after 1 and 2 days of culturing, respectively. The axonal and dendritic arborization is very prominently visible. The degree of formation of new synapses is in process, and the connections are intertwined among them, forming a dense network of outgrowths which help in the survival of the neurons.

2.3. Immunocytochemical Studies of the Primary Neuron Culture. The isolated cells were plated in low density (250000 cells/mL) in poly-D-lysine-coated dishes and were kept in culture for 7 days consecutively, providing them with the appropriate environment for their growth. The neurons were immunostained with various markers to see the presence of neuronal and non-neuronal population of cells present in the culture. Immunostaining of the cells was done to detect the population of immature differentiated neurons using Tuj 1 marker (Figure 2A–D), neuronal axon with Tau marker (Figure 2E–H), oligodendrocytes with O4 marker (Figure 2I–-L), neuronal

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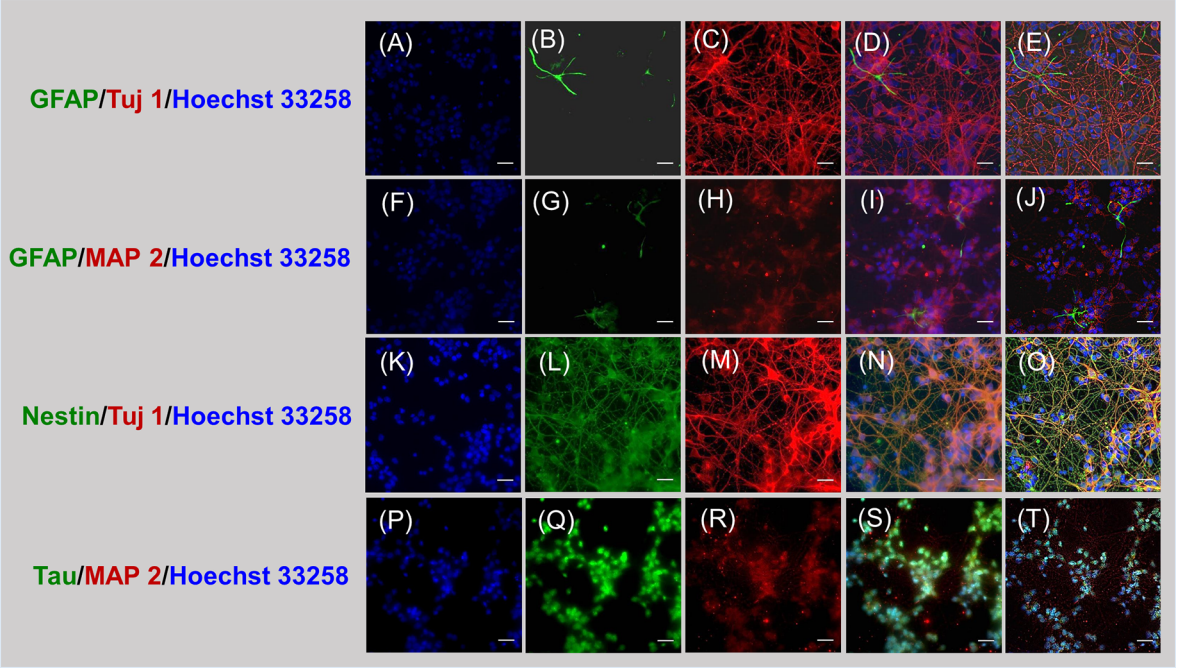

Figure 3. Double immunostaining of a mixed culture of cortical and hippocampal neurons: (A–E) GFAP/Tuj 1 for astrocytes and neurons; (F–J) GFAP/MAP 2 for astrocytes and neuronal dendrites; (K–O) Nestin/Tuj 1 for neural progenitor cells and immature differentiated neurons; (P–T) Tau/MAP 2 for neuronal axon and neuronal dendrites; (D, I, N, S) merged images; (E, J, O, P) deconvolution of the merged images; (A, F, K, P) nuclei counter stained with Hoechst 33258. The average cell number is 92 ± 5. Scale bars correspond to 20 μm.

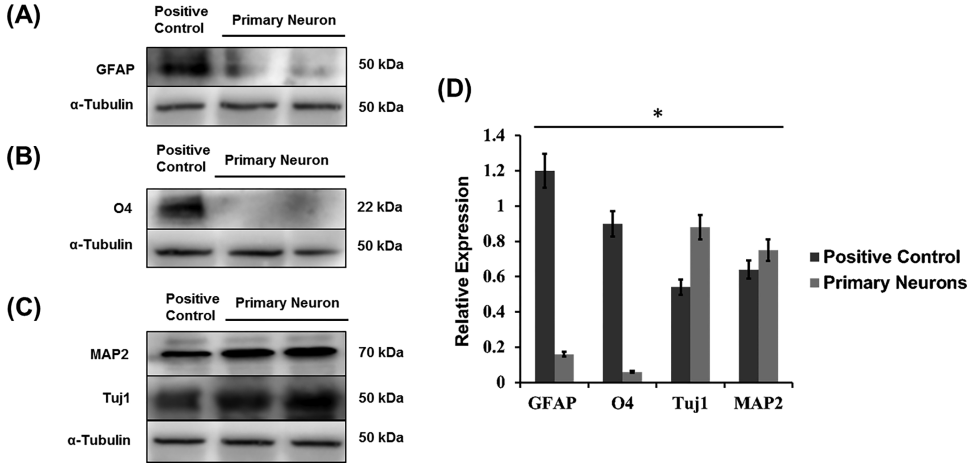

Figure 4. Western blot analysis of neuronal and non-neuronal markers isolated from mixed primary neurons, adult mice brain cortex lysate, differentiated PC12 cell line, and C6 cell line. (A) Immunoblot representing the relative expression of astrocyte marker GFAP in cultured primary neurons and C6 cell line taken as a positive control. (B) Immunoblot representing the relative expression of oligodendrocytes marker O4 in cultured primary neurons and adult mice brain cortex taken as a positive control. (C) Immunoblot representing the relative expression of neuronal dendrites and immature neuronal markers MAP2 and Tuj1, respectively, in cultured primary neurons and differentiated PC12 cell line taken as a positive control. (D) Histogram representing quantification of neuronal (Tuj1 and Map2) and non-neuronal (O4 and GFAP) markers of positive control and primary neurons. All experiments have been performed in triplicate. The error bar corresponds to the standard deviation of the value (*p < 0.05, from two-way Student's t test).

dendrites using MAP 2 marker (Figure 2M–P), and astrocytes with GFAP marker (Figure 2Q–T), and nuclei of the complete population of the cells were stained with Hoechst 33258 to detect the total number of cells present in the culture. Non-neuronal cell presence was very low in the population, showing almost a pure neuronal culture of the cortex and hippocampus. To check the connection between the neuronal and non-neuronal cells, double immunostaining was performed (Figure 3). For astrocytes and neurons the cells were immunostained against GFAP and Tuj 1, respectively (Figure 3A–E). To detect the presence of the population of neural progenitor cells and the recently formed immature differentiated neurons, the cells were immunostained against Nestin and Tuj 1, respectively (Figure 3K–O). The immunostain showed a higher population of neuronal cells in comparison to the non-neuronal cells (astrocytes and oligodendrocytes). Thus, to see the axonal and dendritic connection, the neuronal cells were stained with Tau and MAP 2, respectively (Figure 3P–T).

2.4. Spontaneous Formation of Neurospheres and Their Neural Differentiation. A high-density cell seeding

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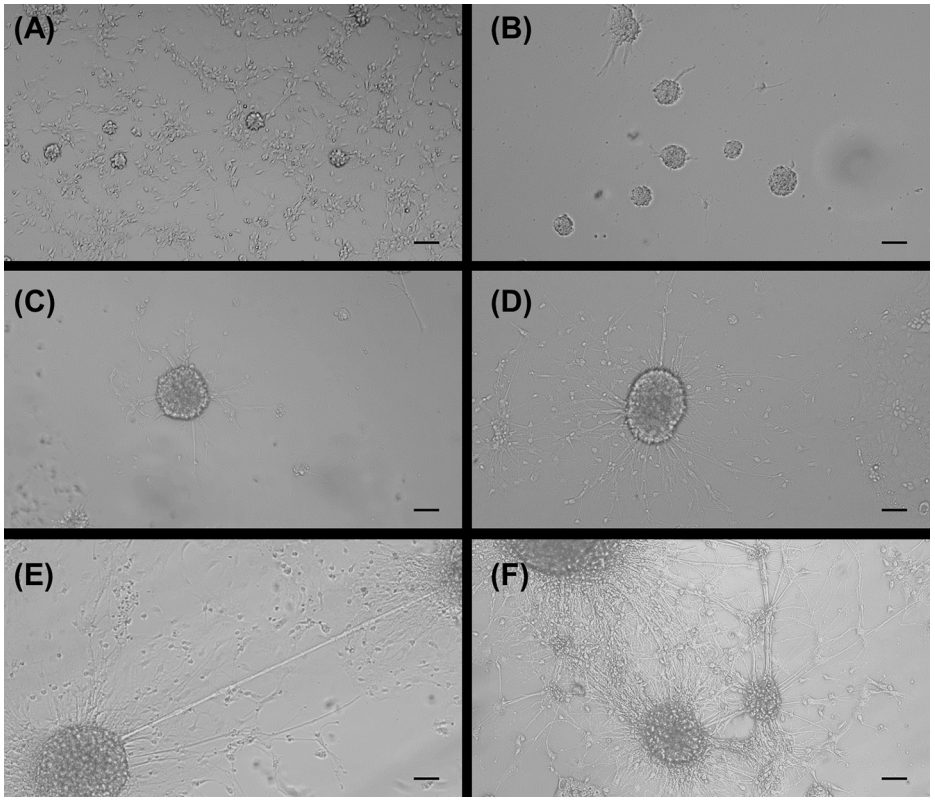

Figure 5. Bright field images revealing spontaneous generation of neurospheres from primary cortical and hippocampal cell culture. (A) After the 5th day of culture, individual cells starts to aggregate to form spheres. (B) On the 6th day of culture no individual cells were visible. (C, D) On the 7th day of culture individual neurospheres were seen sprouting out. (E) On the 8th day of culture a bridge was seen between two spheres. (F) On the 10th day of culture the neurospheres started differentiating into different individual cells. Scale bars correspond to 200 μm.

was done using 150000 cells on poly-D-lysine-coated 12 mm coverslips placed in a 24-well plate; this showed signs of aggregation of the cells and formation of small spheres during the fifth day of the culture with dimensions of approximately 3.3×10^4 μm^3 (Figure 5A) with a population of single cells. On the sixth day individual cells were no longer visible and the number of spheres increased, showing complete aggregation of the cells, with the formation of neurospheres with sprouting of processes from the outer surface of the neurosphere with dimensions of 6.8×10^5 μm^3 (Figure 5B). On the seventh day the sprouting was more pronounced (Figure 6C). Neurosphere formation is a tedious and demanding process requiring special medium formulations (some even commercially available) and a number of growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), etc. However, in our experiments we dismiss this myth by repeatedly obtaining neurospheres with no special formulation or growth factors, thus giving a greatly simplified and cost-effective neurosphere development for chemists wanting to screen their molecules in an in vivo environment more resembling the brain. As the neurospheres attain a certain size, they start to sprout out and differentiate into single cells. The size of the neurospheres formed has a nearly comparable size range of approximately 1.9×10^6 μm^3 and an efficiency of formation of approximately 360−410 neurospheres per 12 mm coverslip of diameter ∼170 μm. A bridge between two neurospheres was seen, which is the glial-like extension, on the eighth day of culture (Figure 5D and Figure S3, Supporting Information). The bridging between the neurospheres increased, and later it started to differentiate into individual cells in a centrifugal manner. Cells migrating from one neurosphere to the other could be very visibly seen in the phase contrast images (Figure S4, Supporting Information). The neurospheres showed their enrichment in NPCs through expression of nestin, which could be exploited for neural differentiation studies (Figures S5 and S6, Supporting Information).

2.5. Expression of Neuronal and Non-Neuronal Markers in Primary Neurons and Neurospheres through Immunoblotting. In addition to immunocytochemistry, we further checked the expression of key neuronal and non-neuronal markers through Western blotting to affirm our data. For the primary neurons, as neuronal markers we chose beta-III Tubulin (Tuj1) and microtubule associated protein 2 (MAP2), and for non-neuronal markers, astrocyte marker glial fibrillary acidic protein (GFAP) and oligodendrocyte marker O4 were chosen. We observed a pronounced expression in the case of both neuronal markers, while in the case of non-neuronal markers no expression was observed. In the case of neurospheres, we additionally used Nestin as a marker for neuronal progenitor cells (NPCs), wherein we observed an increased expression of NPC markers Nestin and Tuj1 while the non-neuronal markers almost showed no expression. These data corroborate our previous immunocytochemistry data and prove the identity and purity of our cultured neurons. In addition, for each of the markers, immunoblots of the respective positive controls were performed to validate our results. For neuronal markers Tuj1 and MAP2, proteins isolated from cell lysates of PC12-derived neurons were taken as a positive control.[32] In the case of Nestin and GFAP, proteins were isolated from C6 glioma cell lysate,[33] whereas for oligodendrocyte marker O4, proteins were

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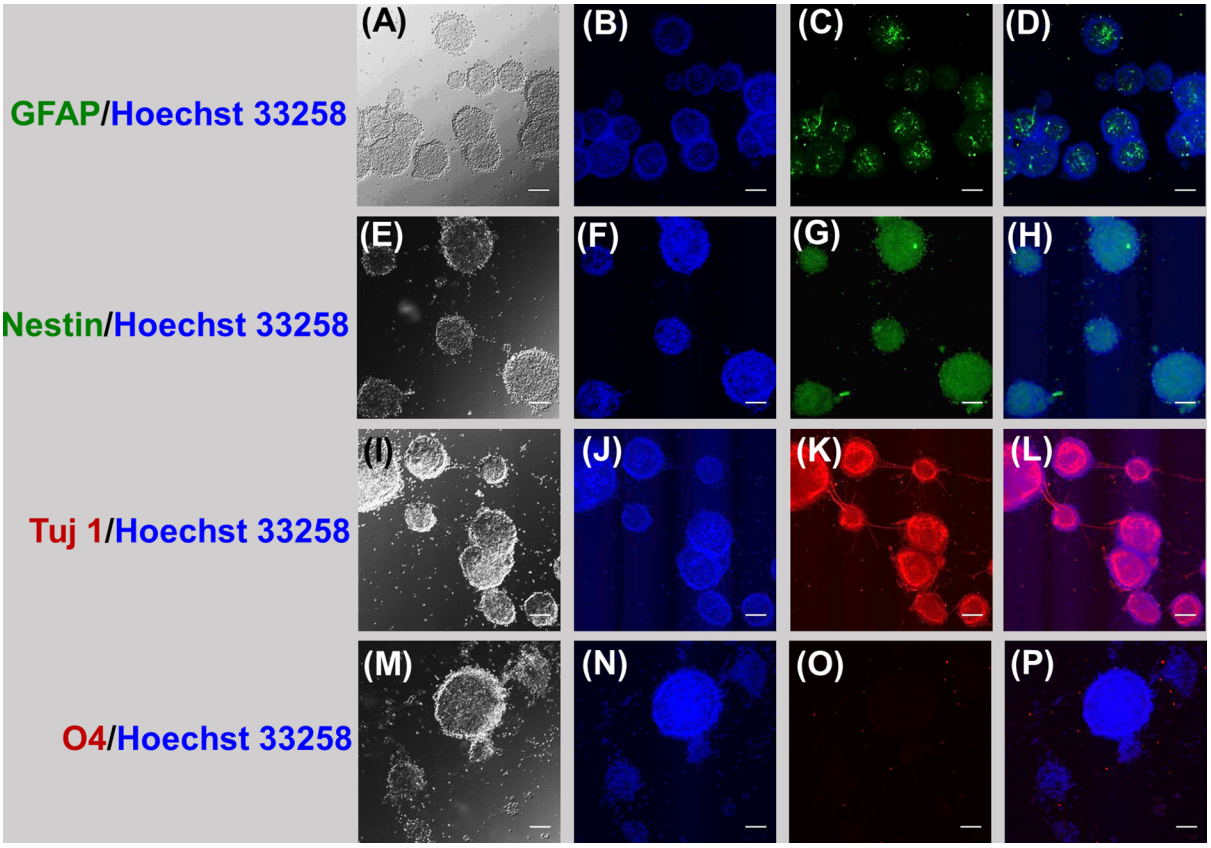

Figure 6. Immunostaining of nonadherent floating undifferentiated neurospheres. (A–D) GFAP immunostaining for astrocytes localized in the core of the neurosphere. (E–H) Nestin for neural progenitor cells present throughout the neurosphere. (I–L) Tuj 1 for immature neurons seen to be densely populated at the outer surface of the neurosphere. (M–P) O4 negative for oligodendrocytes since the neurospheres have not differentiated. Scale bars correspond to 100 μm.

collected from homogenate of mice brain cortex as a positive control.[34]

3. CONCLUSION

In the present paper, we have formulated a simple recipe to culture primary neurons derived from rat embryo, which has been plated in two variable densities—one low and another high. The low-density-seeded primary culture could be used for a prolonged period and is most suited to screening neuroprotective and neuroregenerative therapeutic molecules. On the other hand, the high-density-seeded culture leads to spontaneous generation of neurospheres, which will again be a suitable platform for neural differentiation studies due to their high enrichment of NPCs. The neurospheres generated could be used for screening of a number of different potential neural differentiating agents, as they are rich in neural progenitor cells (NPCs), and they could also be used to screen drugs for various neurodegenerative diseases as have been discussed before.[22,24] In conclusion, the highlights of our work are as follows:

- one single strategy to generate both 2D and 3D neuronal platforms for screening of neuro-therapeutic candidates
- cost-effective and simple strategy reducing the number of reagents and steps generally reported so far.
- generation of 3D neurospheres with enriched NPCs that could be used to study neural differentiation into neurons and radial glial cells reflecting the early stage of cortical development in vivo.

4. METHODS

4.1. Chemicals. Poly-D-lysine, potassium phosphate monobasic (KH_2PO_4) sodium phosphate dibasic (Na_2HPO_4) and 1 M concentrated HCl were purchased from Merck Millipore. Sodium chloride, potassium chloride, and sodium bicarbonate 1 M HEPES were purchased from SRL. Trypsin-EDTA solution was purchased from Sigma. D-Glucose was purchased from SDFCL. MEM Eagle's with Earle's BSS was purchased from Sigma-Aldrich. Horse serum was purchased from HiMedia. Penicillin/streptomycin, neurobasal medium, and 200 mM GlutaMAX-I supplement were purchased from Gibco Life Technologies. B27 serum free supplement was purchased from Invitrogen. All of the primary antibodies (anti-Tuj 1, anti-MAP 2, anti-Tau, anti-GFAP, anti-O4, and anti-Nestin) were obtained from Abcam. All secondary antibodies were purchased from Merck Millipore. RIPA lysis buffer was obtained from Thermo-Fisher Scientific. Protease inhibitor cocktail, ammonium persulfate, TEMED, Acrylamide/Bis-acrylamide solution, SDS, bromphenol blue, and bovine serum albumin (BSA) powder were all procured from Sigma-Aldrich. GLyseMT was purchased from GCC BIOTECH.

4.2. Animals. Timed-pregnant Sprague–Dawley rat, embryonic day 14 or 16 (E14-E16) and C57BL/6J mice were procured from our institute's animal house facility. All of the animal experiments have been performed following the institutional animal ethics guidelines. All of the animals used in the experiments have been approved by the institutional animal ethics committee (IICB/AEC/Meeting/Apr/2018/1).

4.3. Preparation for Plating. For cleaning of the packaged glass coverslips 1 M HCl solution was prepared in a glass beaker; the required amount of coverslips was submerged in this solution, and the beaker was covered with aluminum foil and left undisturbed for 4 h. With the help of a pair of forceps coverslips were transferred to a fresh

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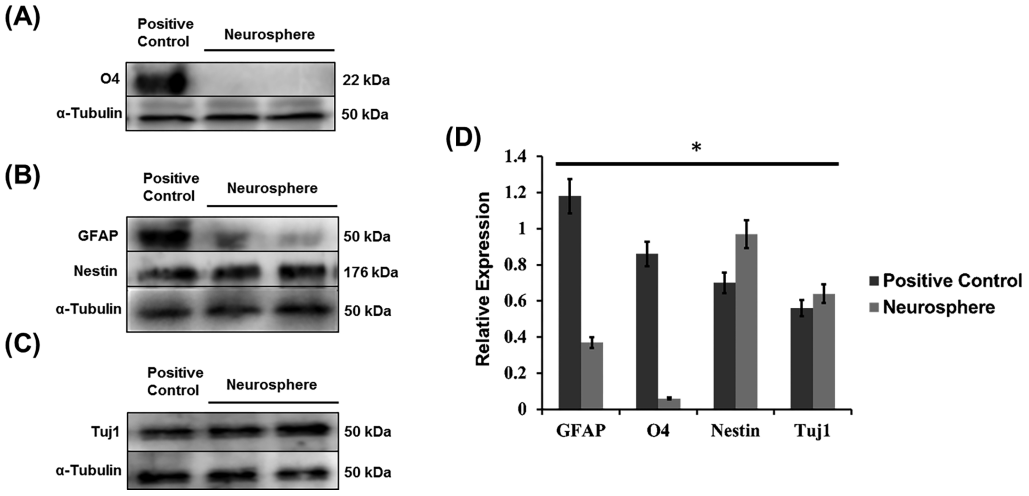

Figure 7. Western blot analysis of neuronal and non-neuronal markers isolated from neurospheres, adult mice brain cortex lysate, differentiated PC12 cell line, and C6 cell line. (A) Immunoblot representing the relative expression of oligodendrocytes marker O4 in cultured neurospheres and adult mice brain cortex taken as a positive control. (B) Immunoblot representing the relative expression of astrocytes and neural progenitor cell markers GFAP and Nestin, respectively, in cultured neurospheres and C6 cell line taken as a positive control. (C) Immunoblot representing the relative expression of immature neuronal marker Tuj1 in cultured neurospheres and differentiated PC12 cell line taken as a positive control. (D) Histogram representing quantification of neuronal (Tuj1 and Nestin) and non-neuronal (O4 and GFAP) markers of positive control and neurospheres. All experiments have been performed in triplicate. The error bar corresponds to the standard deviation of the value (*$p < 0.05$, from a two-way Student's t test).

beaker containing distilled water to wash off the acid between slides. The coverslips were thoroughly washed by swirling the beaker several times. A minimum of three washes with distilled water were made to get rid of the acid completely. The coverslips were given a rinse first with Milli-Q water before they were transferred into a beaker containing 100% ethanol. After that, coverslips were dried between two Kimwipes inside the cell culture laminar air flow hood.

4.4. Coating of Glass Coverslips and Confocal Dishes. To coat the cleaned and dried glass coverslips, a minute drop of Milli-Q water using a pipet was placed in the center of each well of a 24-well culture plate and then coverslips were placed carefully on the water droplet with the help of a sterile forceps. Then the coverslips were centered by moving them in such a way that they were equidistant from the rim of the well. Then for proper coating of the coverslips an adequate amount of poly-D-lysine (PDL) solution was added so as to cover the coverslips and confocal dishes. For 24-well 300 μL and for confocal dishes 150 μL of poly-D-lysine (PDL) solution was required for proper coating. The culture plates and dishes were wrapped with aluminum foil so as to prevent the drying of the PDL solution and left in the incubator overnight.

4.5. Washing of Glass Coverslips after Coating. The next day PDL solution was aspirated and immediately sterile water was placed into each well and aspirated for washing. This process was repeated three times. After washing, maintenance medium was added and left in the plates and dishes in the incubator until use.

4.6. Removal of Foetus. Before the experiment was started, a 100 mm sterile Petri plate filled with cold HBSS solution was placed over ice. The Sprague–Dawley pregnant rat was deeply anesthetized by injecting ketamine/xylazine (i.p.). The anesthetized rat was killed by cervical dislocation and placed on an absorbent sheet. The abdominal skin was then disinfected with 70% ethanol and the abdominal cavity was exposed by pinching the abdominal skin with sterile forceps and making a V-shaped incision using a pair of blunt end scissors. Very carefully the uterus was removed, which appeared as a string of "bubbles", without damaging the intestines of the rat to minimize bacterial contamination. The detached uterus was immediately placed onto a 100 mm sterile Petri dish containing cold HBSS present over the ice. The uterus was dissected, and the embryo was taken out from the embryonic sac and kept in fresh cold HBSS solution (Table 1).

4.7. Removal of Brains from Embryos. All of the steps were performed inside the laminar air hood in cold HBSS solution.

Table 1. Medium Composition

Reagent	Final concn
Dissection/Dissociation Medium (in Milli-Q Water, Filter Sterile)	
Sodium Chloride	8 mg mL^{-1}
Potassium Chloride	0.4 mg mL^{-1}
Potassium Phosphate Monobasic [KH_2PO_4]	0.06 mg mL^{-1}
D-glucose	1 mg mL^{-1}
Sodium Phosphate Dibasic [Na^2HPO^4]	0.479 mg mL^{-1}
1 M HEPES	10 mM
Plating Medium	
MEM Eagle's with Earle's BSS	88.4%
D-glucose	0.6%
Horse Serum	10%
Penicillin/Streptomycin	1%
Maintenance Medium	
Neurobasal Medium	97%
200 mM GlutaMAX-I Supplement	0.5 mM
B27 Serum Free Supplement	2%
Penicillin/Streptomycin	1%

To remove the brain from the embryos, the head was decapitated with the help of a sterile scissors. The head was then held from the snout region (Figure 2C) using a sterile serrated forcep, and with the help of a pointed forceps the skin and skull were cut open at the level of the parietal bone by sliding the tip of the forceps along the sagittal axis to uncover the brain. With the help of a forceps the brain was flipped by sliding the forceps beneath the olfactory bulb so that the brain came out easily from the head cavity by detaching the optic nerves and other cranial nerve connections. The olfactory bulb was left intact to facilitate the removal of the meninges. All of the embryo brains were collected in a similar manner in a separate sterile Petri plate containing cold HBSS.

4.8. Dissection of the Pia Mater and Blood Vessels. By clamping of the olfactory bulb the meningeal layer was removed from the hemisphere and very gently from the inner midbrain and between the hemispheres, avoiding any damage to the brain. The same was done for the other hemispheres as well. The hemispheres were very carefully opened by cleaning the meningeal layer from the forebrain.

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4.9. Microdissection of the Cortex and Hippocampus. The two hemispheres were separated, which resembled a "mushroom cap", and the remaining meninges on the underside of the cap were also carefully removed to expose the hippocampus. The distinct hippocampus was then dissected out from the cortex, and the outer rim of the cortex was also dissected out and collected in a 15 mL conical tube containing 10 mL of fresh dissociation media.

4.10. Cell Dissociation. The dissected hippocampal and cortical tissues were allowed to settle down. Then the dissociation media was aspirated out, leaving 5−10% of the medium in it. To it was then added 10 mL fresh dissociation medium, the tissues were again allowed to settle down; this was repeated twice. The tissue was then suspended in 5 mL of fresh dissection medium containing 10% trypsin-EDTA solution and the conical tube was given a gentle swirl to mix. It was then incubated at 37 °C in the incubator for 20 min. Then the medium was aspirated very gently at this point and the tissue was washed three times with 10 mL of fresh dissociation medium in every wash.

4.11. Cell Plating. The digested hippocampi or cortices were carefully poured in the base of a 100 mm sterile dish in the corner base of a 100 mm dish such that they occupied the least volume (by keeping the plate over the inverted cover of the dish). Then by using a 1000 μL pipet tip trituration of the tissue was done a few times with avoidance of any air bubbles to dissociate the cell to obtain a homogeneous cell suspension. After the homogeneous cell suspension was obtained, it was passed through a 70 μm cell strainer to exclude chunks of tissue, if any. The density of viable cells was determined using trypan blue. The cells were plated in the desired density by diluting the cell suspension with the plating medium. The cells were incubated at 37 °C for 4 h. After 4 h we examined the plates under the microscope to check if cells had adhered to the poly-D-lysine-coated base. Once cells had adhered gently, the plating medium was aspirated from each well and fresh maintenance medium was added (Table 1). The cell culture was incubated at 37 °C.

4.12. Maintenance of Neurons. Half the medium was aspirated and replaced with fresh maintenance medium at room temperature, twice a week. These neurons were maintained in culture for up to 7 days.

4.13. Immunocytochemistry, Mounting of Coverslips, and Fluorescence Microscopy Image Analysis. For determination of the presence of various neuronal markers on the obtained neurospheres and the 2D cultured primary neurons, immunocytochemistry was performed. The cells or the neurospheres were first fixed with 4% formaldehyde for 1 h and then washed with PBS. Thereafter, the fixed cells and neuropsheres were permeabilized using 0.2% Triton-X. After this the cells or neurospheres were stained with the respective primary antibodies and incubated overnight at 4 °C. The primary antibodies used here chiefly were anti-MAP2 (M13), anti-O4 (clone 81), anti-Tubulin beta III isoform (clone TU-20), anti-Nestin, anti-Tau (EP2456Y), and anti-GFAP antibody. Once the primary antibody step was over, the cells or the neurospheres were again washed with PBS. In the case of double staining, the cells were again incubated with another primary antibody of choice, while those for single immunostaining were incubated with mouse or rabbit fluorochrome-labeled secondary antibody for 2 h at 37 °C. The cells and the neurospheres were again washed with PBS; their nuclear staining was performed with 1% Hoechst 33258 for 1 h at 37 °C, and microscopy was performed by mounting them in clean glass slides. The microscopic images were captured with an Olympus (IX83) microscope equipped with an Andor iXon3 897 EMCCD camera, and the images were processed in ImageJ software.

4.14. Cell Culture. PC12 (pheochromocytoma) cells and C6 glioma cells were purchased from NCCS, Pune, India. PC12 cells were cultured in RPMI medium supplemented with 10% horse serum and 5% fetal bovine serum (FBS), while C6 glioma cells were cultured in low-glucose DMEM with 10% FBS at 37 °C in 5% CO_2 incubator. PC12 cells were differentiated with 100 ng/mL NGF (nerve growth factor) induction in serum-free medium for 5 days.

4.15. Immunoblot Analysis. Proteins were isolated on ice from the cultured neurons, neurospheres, differentiated PC12 cell line, and C6 cell line using RIPA lysis buffer containing 1% protease inhibitor cocktail, whereas protein from adult mice (C5BL/6J) cortex was isolated using GLyseMT mammalian tissue lysis buffer following the manufacturer's protocol. The concentrations of the proteins were quantified using Bradford reagent, and around 30 μg of proteins was loaded in 12% SDS-polyacrylamide gel. After electrophoresis, the proteins were blotted on the PVDF membranes. The membranes were first blocked by 5% BSA (blocking solution) and then probed with appropriate primary antibodies (anti-Nestin, anti-Tuj1, anti-GFAP, anti-O4, and anti-MAP2) at 4 °C overnight. Membranes were consequently washed with 1× TBST buffer and incubated with antimouse or antirabbit HRP-conjugated secondary antibody for 2 h at room temperature. The detection of the proteins was then performed through chemiluminescence using Luminata Forte reagent. The data were analyzed, and absolute expression levels of the various markers were quantified using ImageJ software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.8b00414.

> Reagents for preparation of coverslip and culture, equipment for dissection, poly-D-lysine and trypsin EDTA solution preparation, PBS and HBSS preparation method, preparation method for plating and maintenance medium, cell viability and counting method using Trypan blue exclusion principle, microscopic images of the initial formation of neurospheres, pictorial representation of the removal of embryos and microdissection of cortex and hippocampus, phase contrast images of the neurosphere development with the formation of the radial glia like structure between two neurospheres, and immunostaining of the neurosphere for neural progenitor cells (NPC) using nestin (PDF)

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Author Contributions

J.K. and G.D. jointly prepared the reagents, performed the microdissection of the Sprague−Dawley rat embryo, standardized the culture protocol of primary neuron culture and neurosphere culture, and performed immunocytochemistry and microscopy. V.G. helped J.K. and G.D. in reagent preparation, primary neuron culture, and microscopy. S.M. helped in obtaining microscopy and image processing. S.G. helped J.K and G.D. in experiments as well as in photographic documentation during the whole process. S.G. conceived the idea, supervised the project, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATION

NPC, neural progenitor cells; i.p., intraperitoneal; PDL, poly-D-lysine; E14-E16, embryonic day 14-embryonic day 16; GFAP, glial fibrillary acidic protein; MAP2, microtubule associated protein 2; Tuj-1, neuron-specific class III beta-tubulin

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