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Isolation, Culture, and Characterization of Cortical and Hippocampal Neurons from Prenatal Mice: Evaluation Study of Technique and Applications

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Abstract: **Primary cultures of rat and murine cortical and hippocampal neurons are widely used to study and reveal cellular and neuro-molecular mechanisms in medical researches and neurobiology. By isolating, culturing, chatacterization and growing individual neurons, researchers in medicine and biology are able to analyze properties related to cellular functions, trafficking, cellular structure and individual protein localization using a variety of biochemical reactions.**

Results from such experiments in the molecular neurobiology and molecular neurology are critical for testing theories addressing the neural basis of memory and learning, diseases of several parts of the central nervous system (CNS) / peripheral nervous system (PNS) / autonomic nervous system (ANS, Sympathetic and Parasympathetic) and development of treatment / medication testing and therapy of several diseases affecting CNS. However, unambiguous results from these forms of experiments are predicated on the ability to grow neuronal cultures with minimum contamination by other brain cell types.

In this protocol, specific media were used, which were designed for neuron growth and careful dissection of embryonic cortical and hippocampal tissue to optimize growth of healthy neurons while minimizing contaminating cell types (such as astrocytes).

Embryonic mouse cortical and hippocampal tissue can be more difficult to isolate than similar rodent tissue due to the size of the sample for dissection.

Detailed dissection techniques are reported of cortex and hippocampus from embryonic day 18 and 19 (E18 and 19) of the mouse. Once cortical and / or hippocampal tissue is isolated, gentle dissociation of neuronal cells is achieved with a dilute concentration of trypsin and mechanical disruption designed to separate cells from connective tissue while providing minimum damage to individual cells.

A detailed description of how to prepare pipettes to be used in the disruption is included. Optimal plating densities are provided for immuno-fluorescence protocols to maximize successful cell culture. This protocol provides a fast (approximately 2-3 hours) and efficient technique for the culture of neuronal cells from mouse cortical and hippocampal tissues. These cells were used successfully in many further experiments and examinations.

Keywords: **Central and peripheral nervous system (CNS, PNS), Autonomic Nervous System (ANS, Sympathetic and Parasympathetic), Neuroscience, Neuro- Physiology, Neuronal Cell Culture, Cortical Neurons, Hippocampal Neurons, synaptogenesis, gap-43, Synaptophysin.**

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1. INTRODUCTION

The protocol for culturing of embryonic hippocampal and cortical mouse neurons was developed as a modification of the Banker protocol, which used rat neurons (1-2). This protocol was used for culturing mouse and hamster neurons as well (3-12) and it worked equally well for both hippocampal and neocortical neurons and was similar to a protocol published by Meberg and Miller (10-14, 19-29, 33-42). Generally, hippocampal neurons were used for long-term culture because they were well characterized and a more established model system. Furthermore, they were likely to contain a more homogeneous population of neurons than neocortex. However, neocortical neurons cultured using this protocol also survived and differentiated similarly (unpublished data). Hippocampal and neocortical neurons were routinely used for short term culture. Dissection of neocortex also resulted in substantially more neurons (1.5x106 neurons per pair of cortices) than hippocampal dissection (2.5x105 neurons per pair of hippocampi), which made it a better choice of material for Western blotting, for example.

As with any primary culture, it was essential to minimize the time that it takes from the death of the animal to the plating of the cells. Generally it has taken 10-20 dissections to become consistently fast at dissection and plating. Also, when working with the Lonza Nucleofector, it was critical to work quickly during the electroporation procedure, as the viability of the neurons decreased rapidly if they were left in the nucleofection buffer.

The ability to culture primary neurons under serum-free conditions facilitates tighter control of neuronal studies. Some serum-free media and supplements allow for the low-density neuronal cultures, which in turn enables the study of individual neurons and synapses. This has not been possible using serum–supplemented media without a feeder layer of glial cells. In serum-supplemented media, glial cells continue to multiply, necessitating the use of cytotoxic mitotic inhibitors. Serum also contains unknown and variable levels of growth factors, hormones, vitamins, and proteins and in this study details were demonstrated to obtain the isolation and culture of neural cells in serum-free media and supplements (9-22).

2. OPTIMIZED PROTOCOL FOR PRIMARY MOUSE HIPPOCAMPAL AND CORTICAL NEURONS

Required Materials:

Isolating Rat Brain Cells

Hibernate®-E Medium (Cat. no. A12476-01)

B-27® Serum-Free Supplement (Cat. no. 17504)

GlutaMAX™-I (Cat. no. 35050)

Hibernate®-E Medium, without Ca2+ (BrainBits LLC, Cat. no. HE-Ca)

Papain (Worthington, Cat. no. LS003119)

Neurobasal® Medium (Cat. no. 21103-049)

Trypan Blue Stain (Cat. no. 15250-061)

Pasteur pipettes

Hemacytometer, cell counter and trypan blue, or the Countess® Automated Cell

Counter (Cat. no. C10227)

Conical tubes (15-mL, 50-mL)

Culturing Embryonic Neurons

Poly-D-lysine hydrobromide (Sigma, Cat. no. P-6407)

48-well plate or 8-chambered slides

Distilled water (Cat. no. 15230-162)

Dulbecco’s Phosphate-Buffered Saline (D-PBS) (Cat. no. 14040-141)

Neurobasal® Medium (Cat. no. 21103-049)

B-27® Serum-Free Supplement (Cat. no. 17504)

GlutaMAX™-I (Cat. no. 35050)

Immunocytochemistry:

Goat serum (Cat. no. 16210-064)

Mouse anti-MAP2 antibody (Cat. no. 13-1500)

Rabbit anti-GFAP antibody (Cat. no. 08-0063)

Alexa Fluor® 488 goat anti-mouse IgG (H+L) (Cat. no. A-11029)

Alexa Fluor® 594 goat anti-rabbit IgG (H+L) (Cat. no. A-11037)

4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Cat. no. D1306)

ProLong® Gold antifade reagent (Cat. no. P36930)

EM grade paraformaldehyde (Electron Microscopy Services, Cat. no. 19208)

Preparing Media:

Hibernate®-E Complete Medium

Hibernate®-E is a serum-free, nutrient basal medium for the short-term maintenance of cultured rat neurons and long-term storage of viable brain tissue in ambient CO_2 (0.2%) conditions. The complete medium consists of Hibernate®-E medium supplemented with B-27® Serum-Free Supplement and GlutaMAX™-I. Hibernate®-E complete medium is stable for 2 weeks when stored in the dark at 2–8°C.

To prepare 100 mL of Hibernate®-E complete medium aseptically, mix the following components. For larger volumes, increase the component amounts proportionally. (Table 1)

Table 1: Preparation of components of Hibernate®-E complete medium

COMPONENT	FINAL CONCENTRATION	AMOUNT
HIBERNATE®-E MEDIUM	1X	98 ML
B-27® SERUM-FREE SUPPLEMENT	2%	2 ML
GLUTAMAX™-I SUPPLEMENT	0.5 MM	250 ML

Neurobasal® Complete Medium:

Neurobasal® complete medium requires supplementation of Neurobasal® medium with B-27® Serum-Free Supplement and GlutaMAX™-I. Complete medium is stable for 2 weeks when stored in the dark at 2–8°C. To prepare 100 mL of Neurobasal® complete medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. (Table 2).

Table 2: Preparation of Neurobasal® complete medium

COMPONENT	FINAL CONCENTRATION	AMOUNT
NEUROBASAL® MEDIUM	1X	98 ML
B-27® SERUM-FREE SUPPLEMENT	2%	2 ML
GLUTAMAX™-I SUPPLEMENT	0.5 MM	250 ML

Preparing Matrix:

1) Coating Culture Vessels with Poly-D-Lysine
2) Prepare a 2-mg/mL poly-D-lysine stock solution in distilled water.
3) Dilute the poly-D-lysine stock solution 1:40 in D-PBS to prepare a 50 μg/mL working solution (i.e., 125 μL of poly-D-lysine stock solution into 5 mL of D-PBS).
4) Coat the surface of the culture vessel with the working solution of poly-D-lysine (150 μL/cm2, i.e., 100 μL per well for a 48-well plate).
5) Incubate the culture vessel at room temperature for 1 hour.
6) Remove the poly-D-lysine solution and rinse 3 times with distilled water. Make sure to rinse the culture vessel thoroughly, because excess poly-D-lysine can be toxic to the cells.
7) Leave the coated vessels uncovered in the laminar hood until the wells have completely dried. You may use the dry plates immediately or store them at 4°C, wrapped tightly with Parafilm®, for up to one week.

Isolating Neurons:

i. Dissect cortex or hippocampus pairs from ten E-18 rat embryo brains. Remove all the meninges thoroughly.
ii. Collect all the tissue in a conical tube containing Hibernate®-E supplemented with 2% B-27® Serum-Free Supplement and 0.5 mM GlutaMAX™-I at 4°C.
iii. Allow the tissue to settle to the bottom of the tube and then carefully remove the supernatant leaving only the tissue covered by the medium.
iv. Enzymatically digest the tissue in 4 mL of Hibernate®-E medium without Ca2+ containing 2 mg/mL of filter-sterilized papain for 30 minutes at 30°C. Gently shake the tube every 5 minutes.
v. Add 6 mL of complete Hibernate®-E medium to the tube and centrifuge for 5 minutes at 150 × g.
vi. Remove the supernatant and resuspend the tissue in 5 mL of complete Hibernate®-E medium by pipetting up and down with a fire-polished glass Pasteur pipette.
vii. Let the tube stand undisturbed for 2 minutes to allow for the cell debris (if any) to settle down.
viii. Transfer the cells to a new tube leaving behind all the debris.
ix. Count the cells using a hemacytometer, cell counter and trypan blue, or the Countess® Automated Cell Counter.
x. Centrifuge the tube for 4 minutes at 200 × g.
xi. Remove the supernatant and resuspend the cell pellet in Neurobasal® medium with 2% B-27® Serum-Free Supplement and 0.5 mM GlutaMAX™-I for culturing.

Note: Plate the cells immediately after resuspension. If you need to store the cells longer, store them in Hibernate®-E medium supplemented with 2% B-27® Serum-Free Supplement and 0.5 mM GlutaMAX™-I at 4°C for up to 48 hours. Do not expose the neurons to air at any time.

Culturing Neurons:

a) Plate ~1 × 105 cells per well in poly-D-lysine coated 48-well plate or an 8-chambered slide. Bring the cell suspension volume to 500 μL per well by adding complete Neurobasal® medium.
b) Incubate the cells at 37°C in a humidified atmosphere of 5 % CO2 in air.
c) Feed the cells every third day by aspirating half of the medium from each well and replacing it with fresh medium.

Characterizing Neural Cells:

a) Preparing Paraformaldehyde Fixing Solution, prepare 20% paraformaldehyde (PFA) stock solution.

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b) Add PBS to 20 g of EM grade paraformaldehyde, and bring the volume up to 100 mL.

c) Add 0.25 mL of 10 N NaOH and heat the solution at 60°C using a magnetic stirrer until the solution is completely dissolved.

d) Filter the solution through a 0.22-µm filter, and cool on ice. Make sure the pH is 7.5–8.0.

e) Aliquote 2 mL in 15-mL tubes, freeze the tubes on dry ice, store them at –20°C.

4% PFA for fixing:

- Add 8 mL of PBS into each 15-mL tube containing 2 mL of 20% PFA, and thaw each tube in a 37°C water bath.
- Once the solution has dissolved, the tubes cool on ice.

Fixing Cells:

i. Remove the culture medium and gently rinse the cells without dislodging them twice with D-PBS containing Ca2+ and Mg2+.

ii. Fix the cells with 4% fresh Paraformaldehyde Fixing Solution (PFA) at room temperature for 15 minutes.

iii. Rinse the cells three times with D-PBS containing Ca2+ and Mg2+.

iv. Permeabilize the cells with 0.3% Triton®-X (diluted in D-PBS with Ca2+ and Mg2+) for 5 minutes at room temperature.

v. Rinse the cells three times with D-PBS containing Ca2+ and Mg2+.

Staining Cells:

(1) Incubate cells in 5% goat serum diluted in D-PBS with Ca2+ and Mg2+ for 60 minutes at room temperature.

(2) Remove the 5% goat serum solution and incubate the cells overnight with the primary antibody (Mouse anti-MAP2 at 10 µg/mL and/or Rabbit anti-GFAP at 4 µg/mL) diluted in 5% goat serum at 4°C. Ensure that the cell surfaces are covered uniformly with the antibody solution.

(3) Wash the cells three times for 5 minutes with D-PBS containing Ca2+ and Mg2+ (if using a slide, use a staining dish with a magnetic stirrer).

(4) Incubate the cells with fluorescence-labeled secondary antibody (Alexa Fluor® 488 goat‑anti mouse (H+L) at 10 µg/mL and/or Alexa Fluor® 594 goat-anti rabbit (H+L) at 10 µg/mL) diluted in 5% goat serum solution for 60 minutes at room temperature.

(5) Wash the cells three times with D-PBS containing Ca2+ and Mg2+. In the last wash, counter-stain the cells with DAPI solution (3 ng/mL) for 10 minutes.

(6) Rinse the cells with D-PBS, and if desired, mount using 3 drops of ProLong® Gold antifade reagent per slide and seal it with the cover slip. You may store the slides in the dark at 4°C.

(7) Observe the cells under the microscope using filters for FITC, Cy5, and DAPI.

Cell Plating:

1. Count the dissociated cells using a hemocytometer.
2. As a general rule, once cell numbers have been determined, subtract 20% from that final number to account for any cell death that may occur after plating.
3. Cells can be plated using the recommendations below: For coverslips in a 24 well plate - 6 x 104 cells/well in 0.5 ml For 60 mm Tissue Culture plates - 4 x 105 cells/plate in 3 ml For 100 mm Tissue Culture plates - 6 x 106 cells/plate in 6 ml.

4. Mix appropriate cell numbers with indicated volume of Neurobasal Plating Media (Neurobasal Media containing B27 Supplement [1 ml / 50 ml], 0.5 mM Glutamine Solution, 25 µM Glutamate (Mr 147.13 g / mol), Penicillin (10,000 units / ml)/Streptomycin (10,000 µg / ml) [250 µl / 50 ml], 1mM HEPES (Mr 238.3 g / mol), 10% Heat Inactivated Donor Horse Serum) and add cells to plates. Swirl plates gently to distribute cells evenly.
5. HI-Donor Horse Serum is added to the Plating Media to enrich the cells during the first 24 hours of growth. The cells are subsequently weaned from the serum and returned to a Serum-Free environment by serial reduction of the serum at each media replacement.
6. It should also be noted that while glutamate at higher concentrations is toxic to neuronal cell cultures, at the lower concentrations added here, it will inhibit the attachment of non-neuronal cells11.
7. However, it should only be added to the plating media for the first 24 hours in culture and subsequently left out of any Feeding Media to prevent neurotoxicity of the cells.
8. Place neurons in a 37 °C, 5% CO2 incubator overnight.
9. Remove half the volume of media from the cells and replace with same volume of Neurobasal Feeding Media (Neurobasal Media containing B27 Supplement [1 ml / 50 ml], 0.5 mM Glutamine Solution, Penicillin (10,000 units / ml) / Streptomycin (10,000 µg / ml) [250 µl / 50 ml], 1 mM HEPES (Mr 238.3 g / mol).
10. Neurons should be fed every 4 days by removing half of the old media and replacing it with the same volume of fresh Neurobasal Feeding media. Neuronal processes should begin to be visible on Day 1 (Figure 5a) and become prevalent by Day 10 (Figure 5b).

Special techniques of preparation, isolation and culturing of hippocampal neurons are shown below. (Figures 1 – 9).

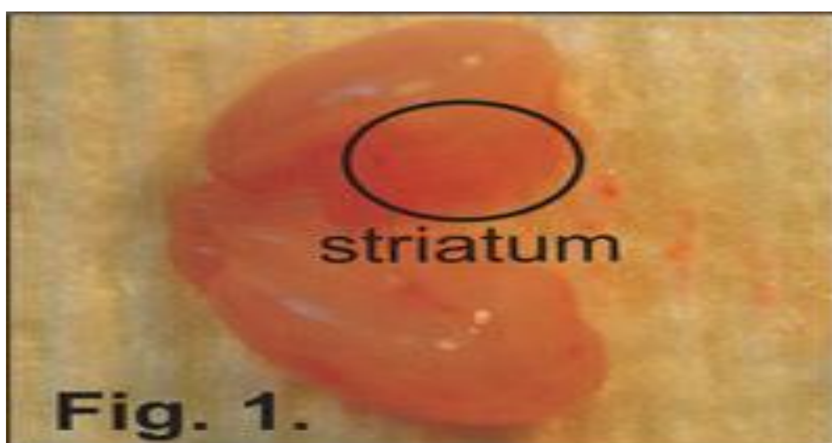

Figure 1. Dissection of the prenatal mouse brain. The first incision is down the midline of the brain separating it into two hemispheres

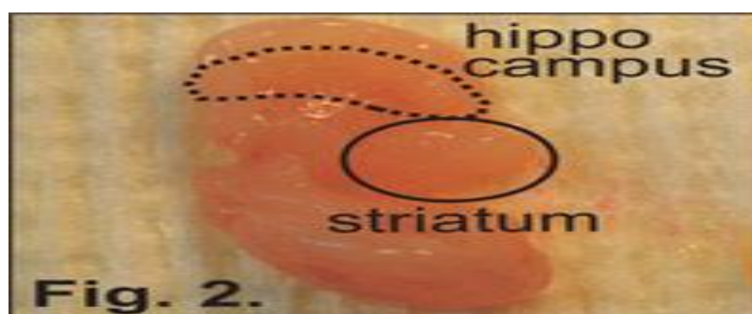

Figure 2. Location of the hippocampus in the prenatal mouse brain. The striatum is moved aside to visualize the hippocampus and is noted by the curved "kidney bean" type structure in the distal region of each hemisphere.

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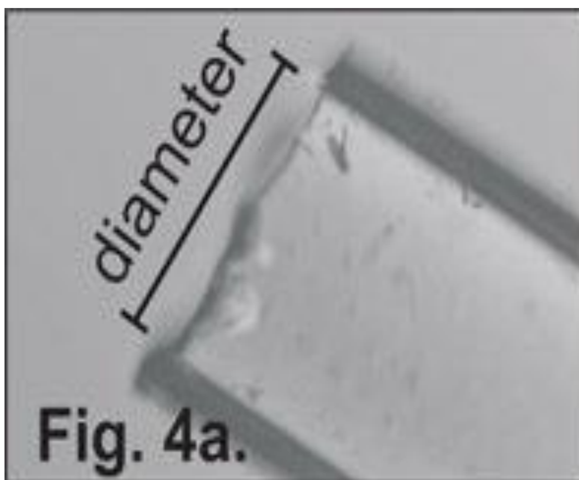
Figure 3. Dissociation of hippocampal tissue in trypsin solution.



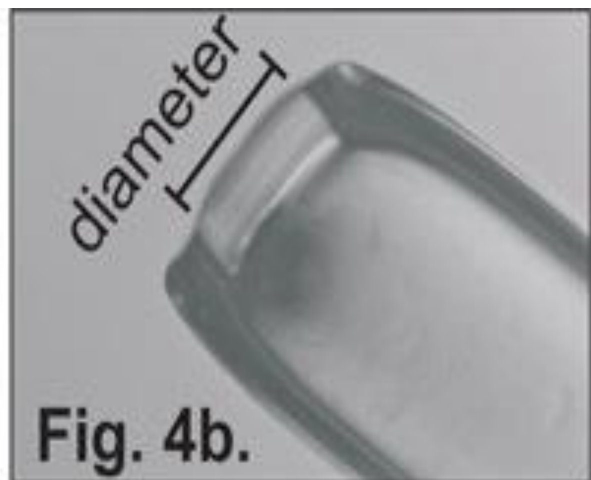
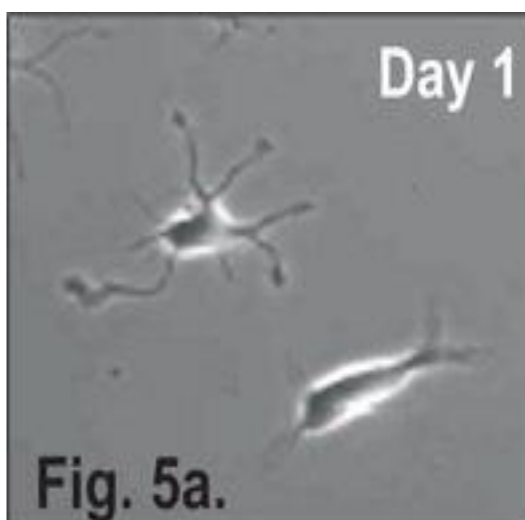
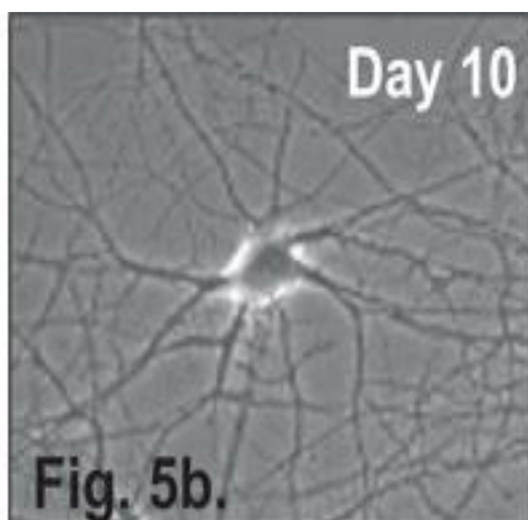


Figure 4. Pasteur pipette tips used in trituration of hippocampal tissue: (a) Normal Pasteur pipette tip. (b) Fire-polished Pasteur pipette tip. Take note of the rounded edges and the approximate 50% decrease in pipette opening size.





Figures 5 a & b. Hippocampal neurons isolated using this procedure and plated in NB Media. (a) Cell growth 1 day post-plating. Neuronal processes begin to be visible during Day 1. (b) Cell growth 10 day post-plating, neurites are branched and overlapping.

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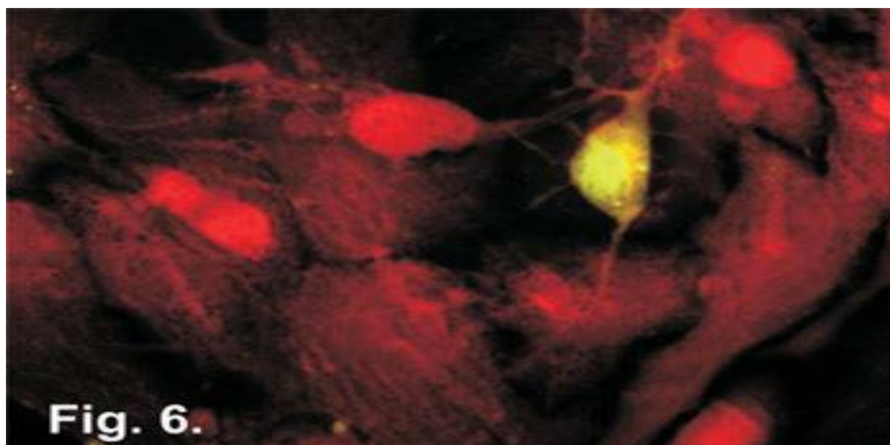

Figure 6. Hippocampal neurons contaminated with glial cells grown for 7 days and stained with the organelle marker MitoTracker Red CM-H2XRos (Invitrogen #M7515) and transfected with GFP-LC3 using Lipofectamine 2000 (Invitrogen #11668019). Mitochondria are visible in all cells however only a single neuron was successfully transfected with the fluorescent construct. Contamination with glial cells makes analysis of GFP-LC3 expression in the neuronal processes difficult to visualize.

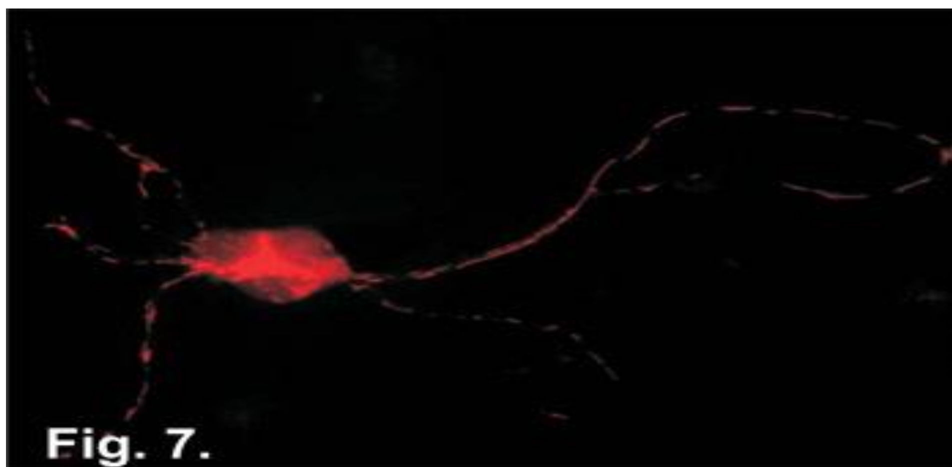

Figure 7. Hippocampal neurons grown for 7 days and stained with the organelle marker MitoTracker Red CM-H2 XRos (Invitrogen #M7513). This vital dye is used to stain active mitochondria in tissue culture cells. The cells were fixed in 4% paraformaldehyde/PBS and visualized by fluorescent microscopy. The dye itself is non-fluorescent until oxidized in the mitochondria. Active mitochondria can be seen throughout the neuronal processes.

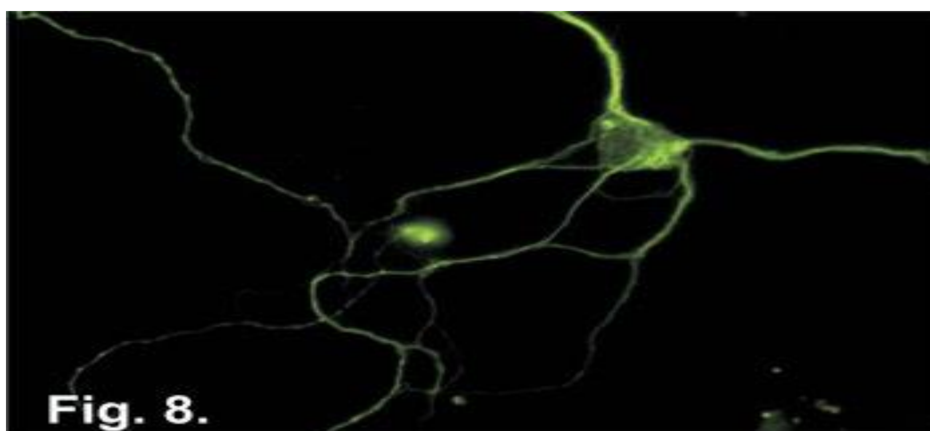

Figure 8. Hippocampal neurons grown for 7 days, fixed with 4% paraformaldehyde/PBS and immuno-stained with monoclonal anti-tubulin β antibody (Sigma #T0198). Following primary antibody, Oregon Green labeled goat-anti-mouse secondary antibody (Invitrogen #O11033) was added and fluorescence visualized by microscopy.

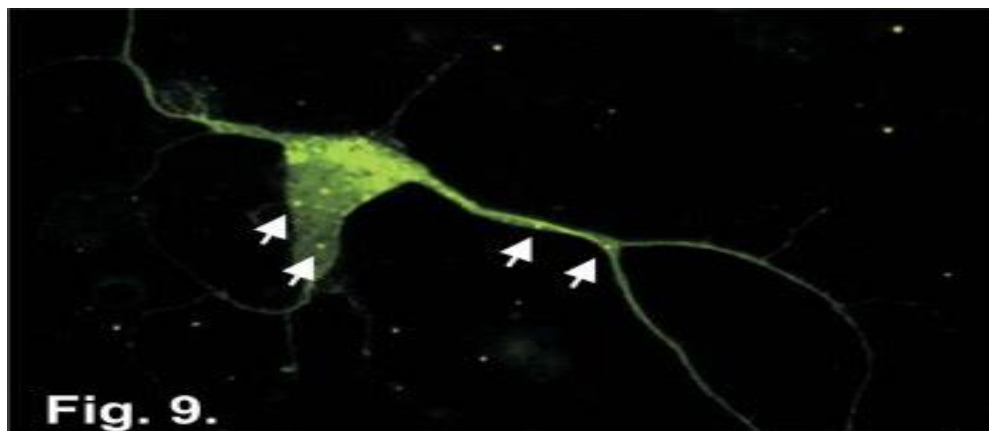

Figure 9. Hippocampal neuronal cultures were grown for 5 days and transfected with GFP-LC3β DNA construct using Lipofectamine 2000 (Invitrogen #11668019). At Day 7, cells were fixed using 4% paraformaldehyde/PBS and aggresomes with GFP tagged LC3β incorporated into their outer membrane were visualized using fluorescent microscopy. Aggresomes are located throughout the cell body and neurites and are denoted with arrows.

Expected Results For Cortical Neurons:

The cortical neurons cultured in Neurobasal® medium supplemented with 2% B-27® Serum-Free Supplement and 0.5 mM GlutaMAX™-I show > 90% neuronal population stained with MAP2 antibody and a minimum number of astrocytes. (See figure 10 below)

Within 3–4 days in culture, the neurons display extensive neurite outgrowth that keeps on increasing as long as the neurons are kept healthy in culture. Note that results vary if the neurons are cultured in the presence of serum.

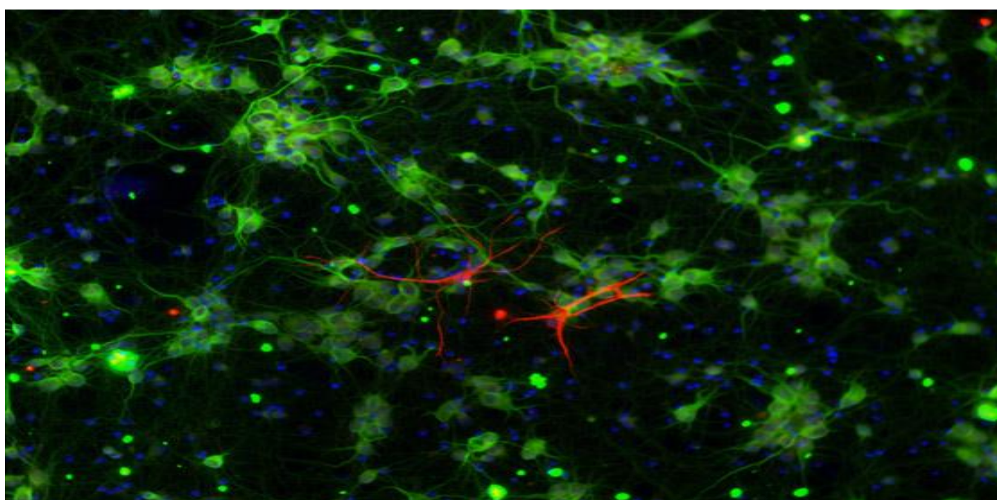

Figure 10. Primary Rat Cortical neurons. Immunofluorescence detection of primary neuronal cells stained with mouse anti-MAP2 antibody (Green) and presence of astrocytes as detected by rabbit anti-GFAP marker (Red). Nuclei are stained with DAPI (blue)

3. RESULTS

Hippocampal and cortical neurons have been used extensively to study central nervous system (CNS) neuronal polarization, axon/dendrite outgrowth, and synapse formation and function. An advantage of culturing these neurons is that they readily polarize, forming distinctive axons and dendrites, on a two dimensional substrate at very low densities.

This property has made them extremely useful for determining many aspects of neuronal development. Furthermore, by providing glial conditioning for these neurons they will continue to develop, forming functional synaptic connections and surviving for several months in culture.

The ability to grow and culture primary neuronal cells has become an indispensible part of neuroscience. Primary cultures allow the researcher to analyze specific cellular pathways, chemical modification and treatment, target localization and growth patterns in a controlled environment. Many of these procedures utilize sophisticated methodology to visualize specific changes in cell responses. In this case, hippocampal neurons are used to study specific neuronal pathways that would prove difficult, if not impossible to analyze in the intact brain. Preparation of near homogeneous populations of neurons from specific areas of the brain is critical for studying brain function. Molecular effects in individual neurons can be instrumental in delineating higher order pathways such as memory or learning. As this protocol yields relatively pure cultures of hippocampal neurons, without the need of a feeder layer of glial cells, these neurons are easily utilized for immunofluorescence studies. However, as with all primary culture from organs containing multiple cell types, some contamination by less desired cells can occur. In isolation of neuronal cells, contamination by glial cells can be a common problem. Glial cells can be easily detected upon microscopic visualization of the culture as their morphology differs significantly from the target neurons (Figure 6). The impact of glial cell contamination will depend on the planned use of the cultures. If cells are being used for immuno-fluorescence examination, glial contamination can be nothing more than an inconvenience when trying to photograph individual neurons. However, if the neuronal cultures are to be used for biochemical analysis, any significant contamination by glial cells could cause major changes in the results. Ways to address glial cell contamination are outlined further in the Discussion. Once neurons have been successfully isolated and grown in culture, one typical application is to examine cellular processes immuno-fluorescence techniques.

As illustrated in Figure 7, organelles, such as the mitochondria, can be stained using vital dyes added to the culture media prior to fixation. Endogenous cellular proteins can be visualized from fixed cells using standard immuno-fluorescence techniques (Figure 8). Once neuronal cells are fixed, specific antibodies for proteins of interest can be introduced to the cell and these proteins can be visualized using a fluorescence microscope. Cultured neurons also provide the researcher with the means to examine individual protein effects on neuronal functions. Using a variety of techniques including DNA transfections, electroporation or viral transduction, proteins can be overexpressed in neuronal cells (Figure 9). How neural cells respond to the effects of over-expressed proteins can have direct inferences on how the brain may respond and offers the possibility of identifying cellular targets for drug treatments. The details of these types of experiments go beyond the scope of this paper but they do illustrate that cultures prepared by this technique are suitable for a wide array of down-stream applications.

However, the overall simplicity of this protocol, as well as, the short time period required to prepare these neuronal cultures make this an ideal method for use in today's neuroscience laboratory.

Cultures of cortical and hippocampal cells were kept / incubated under optimal / antiseptic conditions for further examination and experiments. Average of survival period of time was 13 -21 days. Cells were used successfully in many experiments.

Primary cultured cortical and hippocampal neurons were traced their neurite outgrowth under the phase contrast microscope. Neurons were also tested synaptogenic activities along the time courses of early periods in vitro by using one of the well known neurite outgrowth and synaptogenesis markers and the detailed results are as follows. (19-33)

Morphological changes of primary cultured neurons along the time courses:

Morphological changes of primary cultured neurons were investigated day by day from day 1 in vitro. Hippocampal neurons showed rather fast growing pattern so we could find definite neurite sprouting and outgrowth even from day 1 in vitro. Neurite outgrowth was faster in the areas of relatively high neural populations whereas some isolated neurons in the areas with very low neural populations showed apoptotic features (data not shown). Cultured hippocampal neurons of neighborhood gradually moved to each other showing patterns of aggregating groups and at this stage, neurites of various lengths radiated toward all directions to establish huge meshwork of neurites that were forming numerous synapses to each other (Fig. 11A & B). Neurite sprouting and outgrowth of cortical neurons were slower than hippocampal neurons. In many cases, only limited degree of neurite sprouting from cortical neurons in early days in vitro (Fig. 11C) was found. Furthermore, we could fi nd more frequent apoptotic features in case of cortical neurons than hippocampal neurons (data not shown). Although the neurite sprouting and outgrowth of primary cultured cortical neurons were slow than those of hippocampal neurons, cortical neurons showed almost same morphological features with hippocampal neurons toward the

end of second week in vitro (Fig. 11D). Neurite outgrowth of primary cultured cells stopped from the day 14 or 18 in vitro and decayed slowly thereafter in both of cortical and hippocampal neurons.

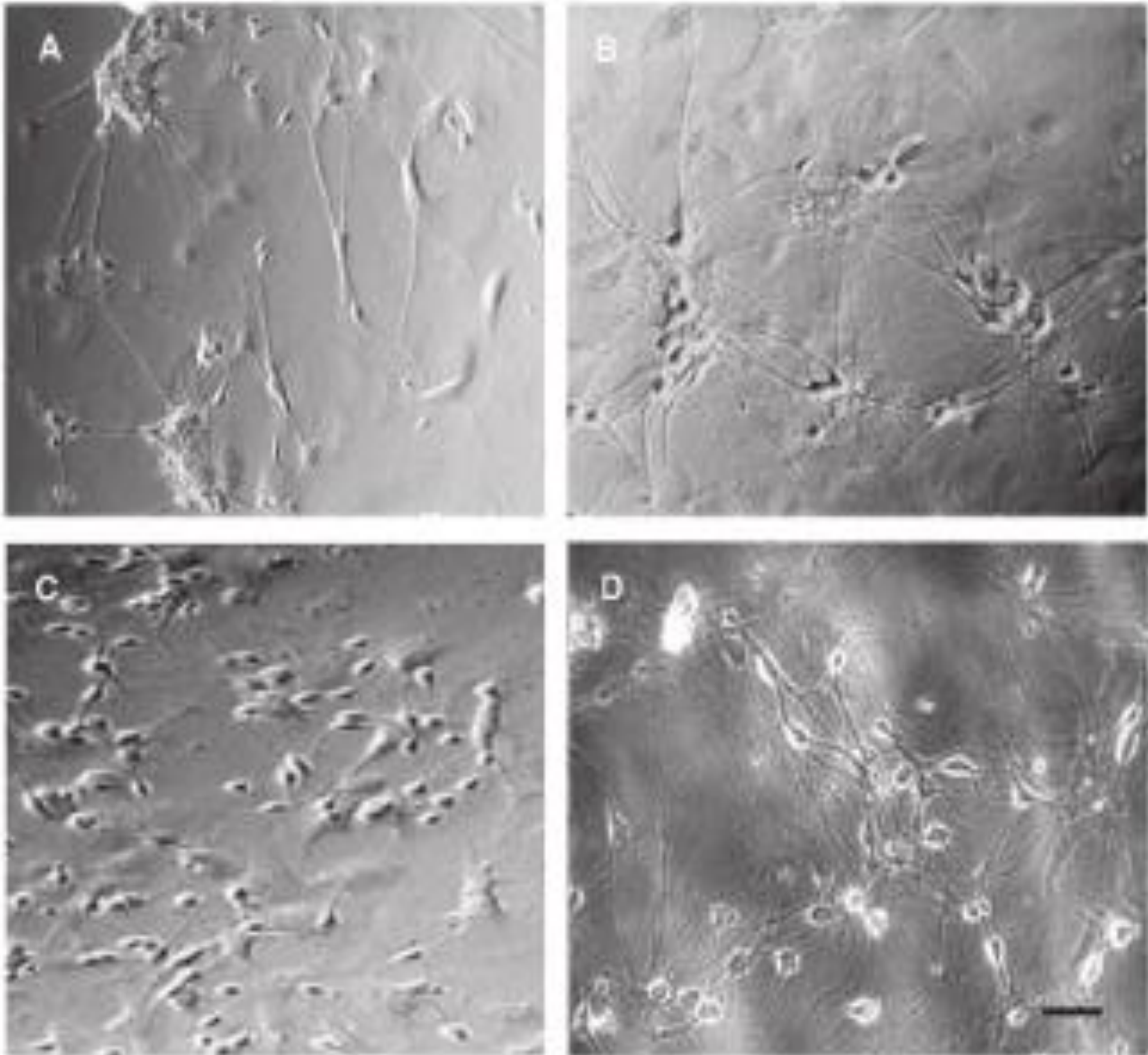

Fig. 11. Phase contrast microscopy of primary cultured hippocampal (A, B) and cortical (C, D) neurons. (A) Hippocampal neurons of day 4 in vitro. Cultured neurons started neurite sprouting from the early days in vitro. (B) Hippocampal neurons of day 10 in vitro. Neurons are more likely to be aggregated and fully grown neurites shows spider web pattern. (C) Cortical neurons of day 4 in vitro. Neurites are still very short compared to hippocampal neurons of same day in vitro. (D) Cortical neurons of day 13 in vitro. Note that neurites outgrowth of cultured cortical neurons are relatively slow than those of hippocampal neurons. Scale bar is 50 µm.

Immunofluorescence findings:

GAP-43 immunoreactivities were gradually increased according to these morphological changes from day 2 to day 8 in vitro. In day 2 in vitro, GAP-43 expressions still remained relatively low and hard to find granular immunofluorescence patterns along the outgrowing neurites. GAP-43 immunoreactivities increased rapidly thereafter as neurites extend and make connections with neighboring neurons.

Typical granular patterns of GAP43 immuno-fluorescence along the outgrowing neurites (Fig. 12D, E) could be found from day 4 in vitro and the intensity of immunofluorescence already reached maximum during the early stages of culture (day 8 in vitro).

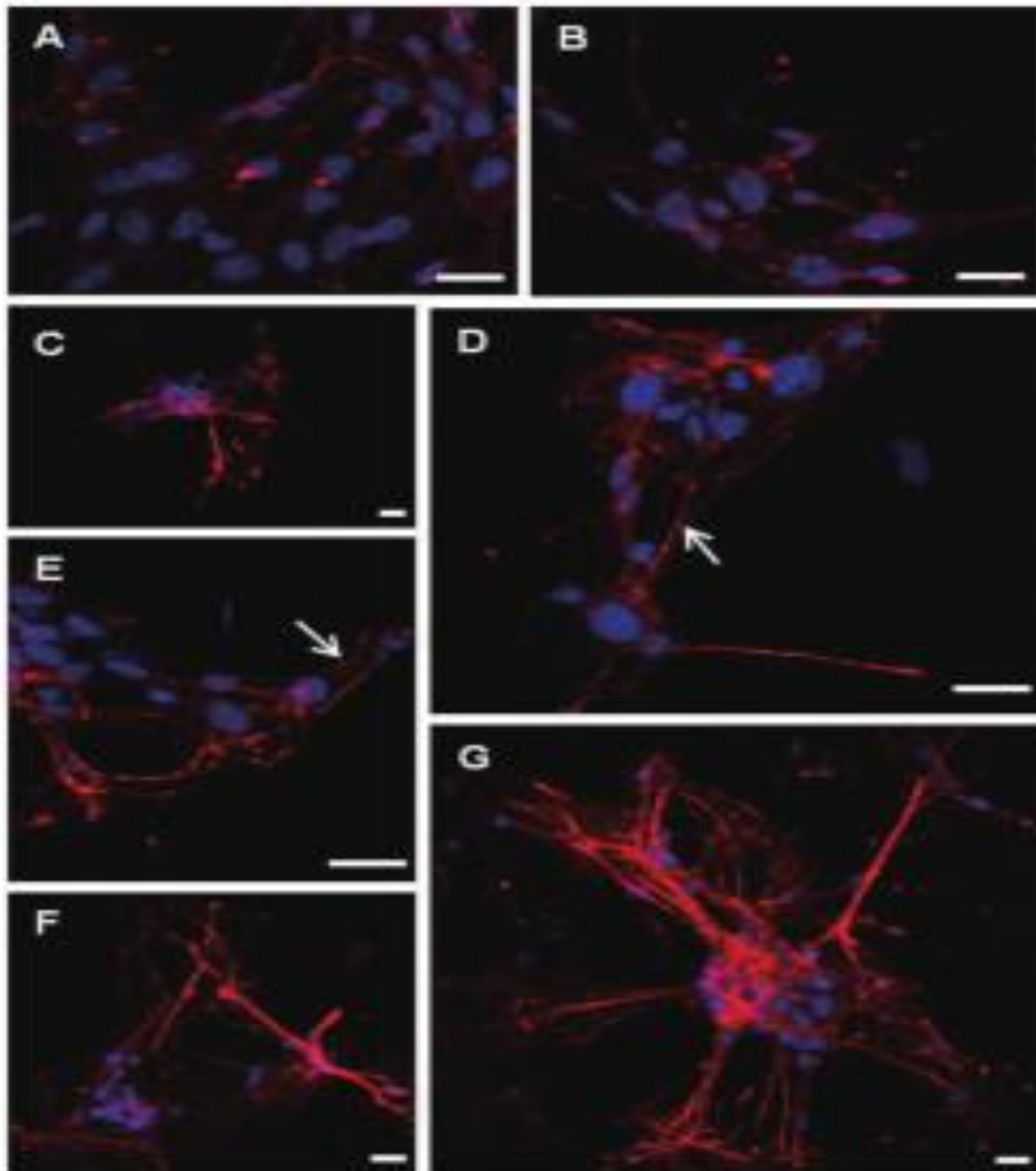

Fig. 12. Confocal microscopic deteciton of GAP-43 immunopositive cells in pirmary cultured hippocampal neurons at day 2 (A, B) day 4 (C~E) and day 8 (F, G). Nuclei are counterstained with DAPI (blue color). Note granular pattern of GAP-43 immunofluorescence along the neurites (arrows) and gradual increase of immunoreactivities according to time courses. Scale bars are 20 μm each.

Unlike Gap-43, granular immunoreactive patterns of synaptophysin along the outgrowing neurites (Fig. 13A, C, D) were clearly found from day 2 in vitro with relatively high immunoreactive levels (Fig. 13A).

These granular findings of GAP-43 and synaptophysin immunoreactivities also resembled synaptic button like structures when considered only in morphology. (11, 14, 15-20, 26, 29, 33, 36, 40)

Patterns of GAP-43 and synaptophysin immunoreactivities were similar in both types of cortical and hippocampal neurons. Morphological synaptic connections among the neighboring neurites could confirmed more clearly especially under high magnifications. (29, 35-39, 40-42)

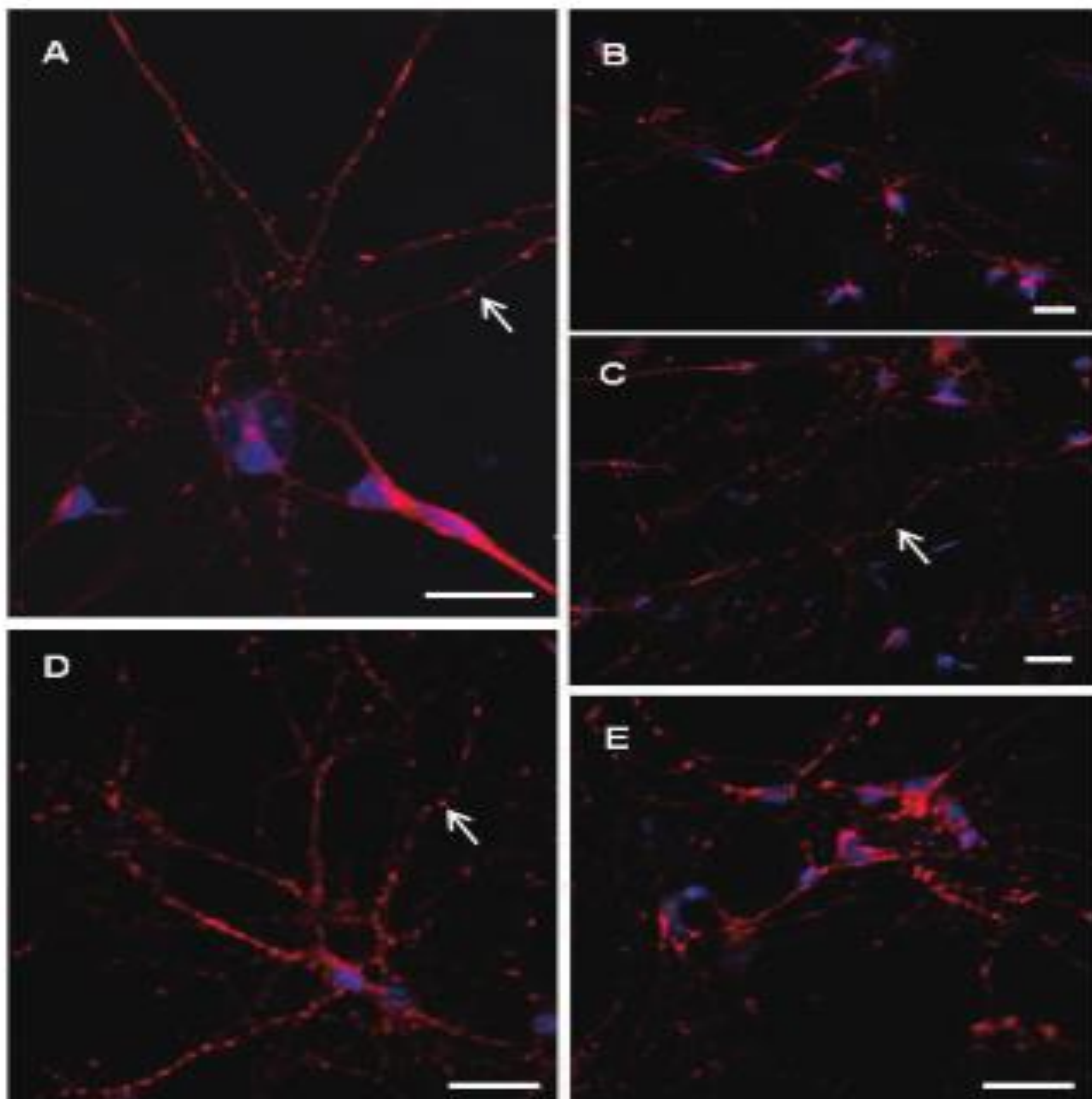

Fig. 13. Confocal microscopic deteciton of synaptophysin immunopositive cells in primary cultured neurons at day 2 (A) day 4 (B, C) and day 8 (D, E). Nuclei are counterstained with DAPI (blue color). Note revelation of granular synaptophysin immunofl uorescence along the neurites (arrows) from day 2 in vitro with relatively high immunofl uorescence levels. Scale bars are 20 μm each.

Western blotting findings:

Along with the immunofluorescence, quantification of synaptic marker expressions was examined by western blotting through the wide range of in vitro periods. GAP-43 and synaptophysin were detected from day 4 in vitro but the expression levels between hippocampal and cortical neurons were different with the time courses. In cortical neurons, GAP-43 and synaptophysin expressions reached highest levels within 1 week (day 5) in vitro. However in hippocampal neurons, both GAP-43 and synaptophysin expressions reached highest levels after 1 week (day 10) in vitro. In cortical neurons, the amounts of GAP-43 and synaptophysin expressions were considerably higher when they reached to highest levels than those in hippocampal neurons.

The differences between cortical and hippocampal neurons were more prominent in case of synaptophysin (Fig. 14B).

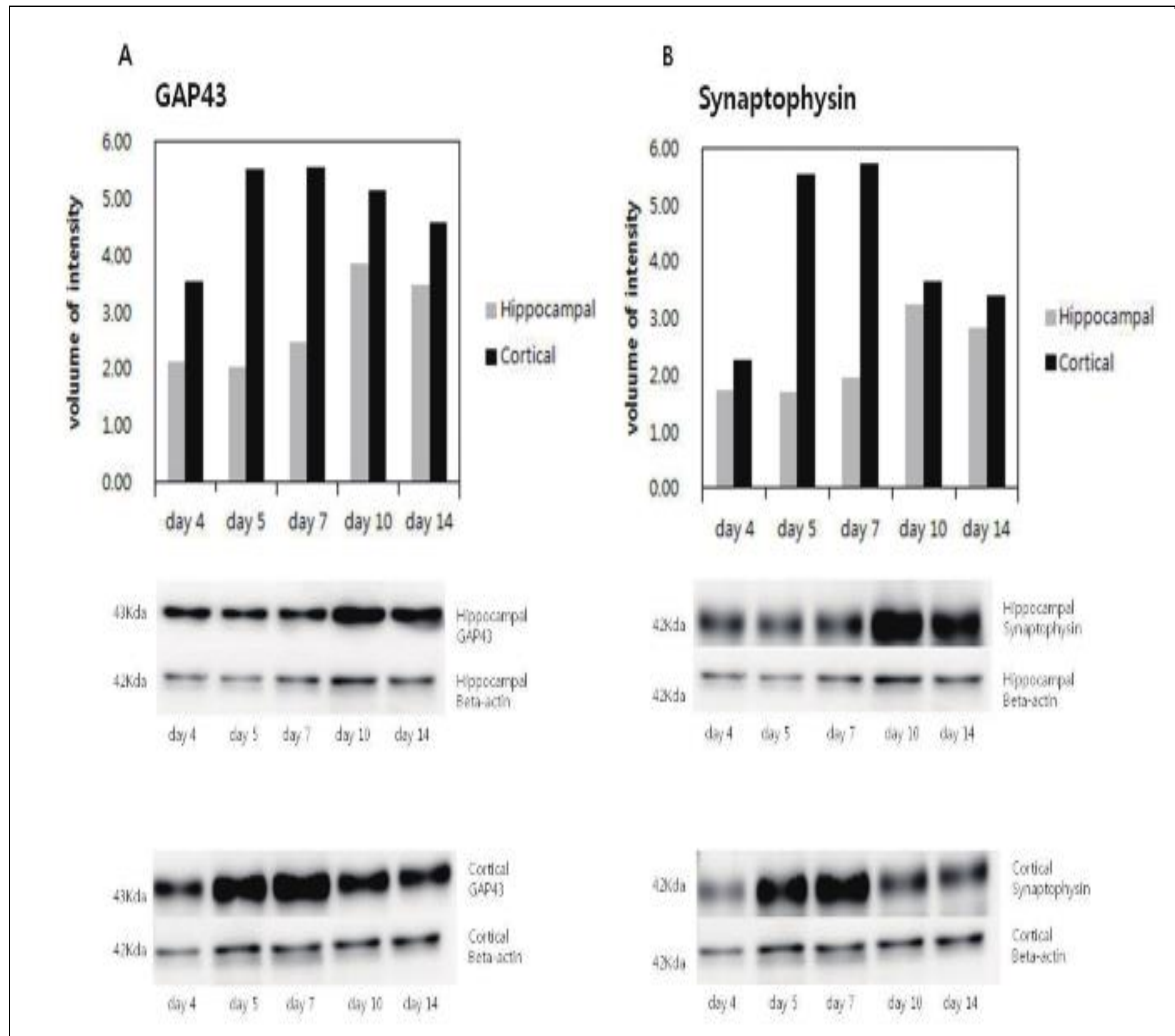

Fig. 14. Western blotting of GAP43 and synaptophysin in cortical and hippocampal neurons. In this figure, GAP43 and synaptophysin bands are digitized and appeared on the graphs as volules of intensities according to beta actins respectively. Expression levels of GAP43 (A) and synaptophysin (B) according to the time courses of in vitro are shown in hippocampal and cortical neurons.

Representative results:

In this protocol it was outlined, that a technique can be developed to dissect, culture and transfect embryonic mouse hippocampal and cortical neurons. (See figure 15 below) Transfection was accomplished by electroporating DNA into the neurons before plating via nucleofection. This protocol has the advantage of expressing fluorescently-tagged fusion proteins early in development (~4-8hrs after plating) to study the dynamics and function of proteins during polarization, axon outgrowth and branching.

In this study, the technique to dissect, culture and transfect embryonic mouse hippocampal and cortical neurons was performed. Transfection is accomplished by electroporating DNA into the neurons before plating via nucleofection. This protocol has the advantage of expressing fluorescently-tagged fusion proteins early in development (~4-8hrs after plating) to study the dynamics and function of proteins during polarization, axon outgrowth and branching. It was discovered, that this single transfection before plating maintains fluorescently-tagged fusion protein expression at levels appropriate for imaging throughout the lifetime of the neuron (> 2 months in culture). Thus, this methodology was useful for studying protein localization and function throughout CNS development with little or no disruption of neuronal function.

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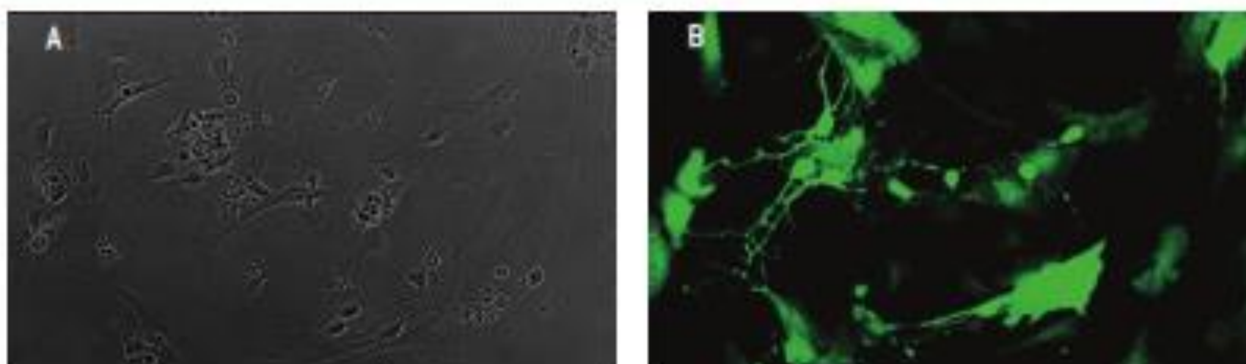
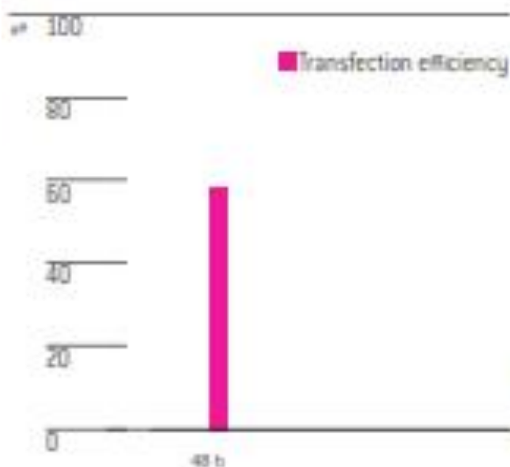
Primary dissociated mouse hippocampal and cortical neurons, isolated from embryonic [E18] or neonatal [P1] mice and cultured as mixed glial cells.

Example for Nucleofection® of mouse hippocampal neurons



Primary dissociated hippocampal neurons of mixed glial cultures were transfected using the Mouse Neuron Nucleofector® Kit, program O-005 and a plasmid encoding enhanced green fluorescent protein eGFP. 48 hours post Nucleofection®, the cells were analyzed by light [A] and fluorescence microscopy [B]. Photograph courtesy of A. Dityatev, Center for Molecular Neurobiology, Hamburg, Germany.



Transfection efficiency of primary mouse hippocampal neurons 48 hours post Nucleofection®. Cells were transfected with program O-005 and 3 µg of a plasmid encoding the enhanced green fluorescent protein eGFP.

Figure 15. Nucleofection of mouce hippocampal neurons

Much of the imaging was conducted with total internal reflectance fluorescence microscopy (TIRFM). This type of microscopy was only capable of imaging several hundred nanometers beyond the coverslip. Therefore, the areas of the neurons that were imaged frequently, the axonal growth and dendritic spines, needed to be adhered directly to the coverslip.

Thus, low density cultures that require glial feeding for long-term culture. Higher density cultures (>2x104 cells/cm2) were used, without glial feeder layers, for long-term cultures and found that they survive very well with little feeding. However, the dendritic spines of these neurons were oftentimes too far away from the substrate to image in TIRFM, although they could be readily detected with wide-field microscopy or confocal microscopy.

Living hippocampal neurons in successive stages of development were shown in paired images of representative living hippocampal neurons both a differential interference contrast image and a corresponding fluorescent micrograph (see figure 16 below).

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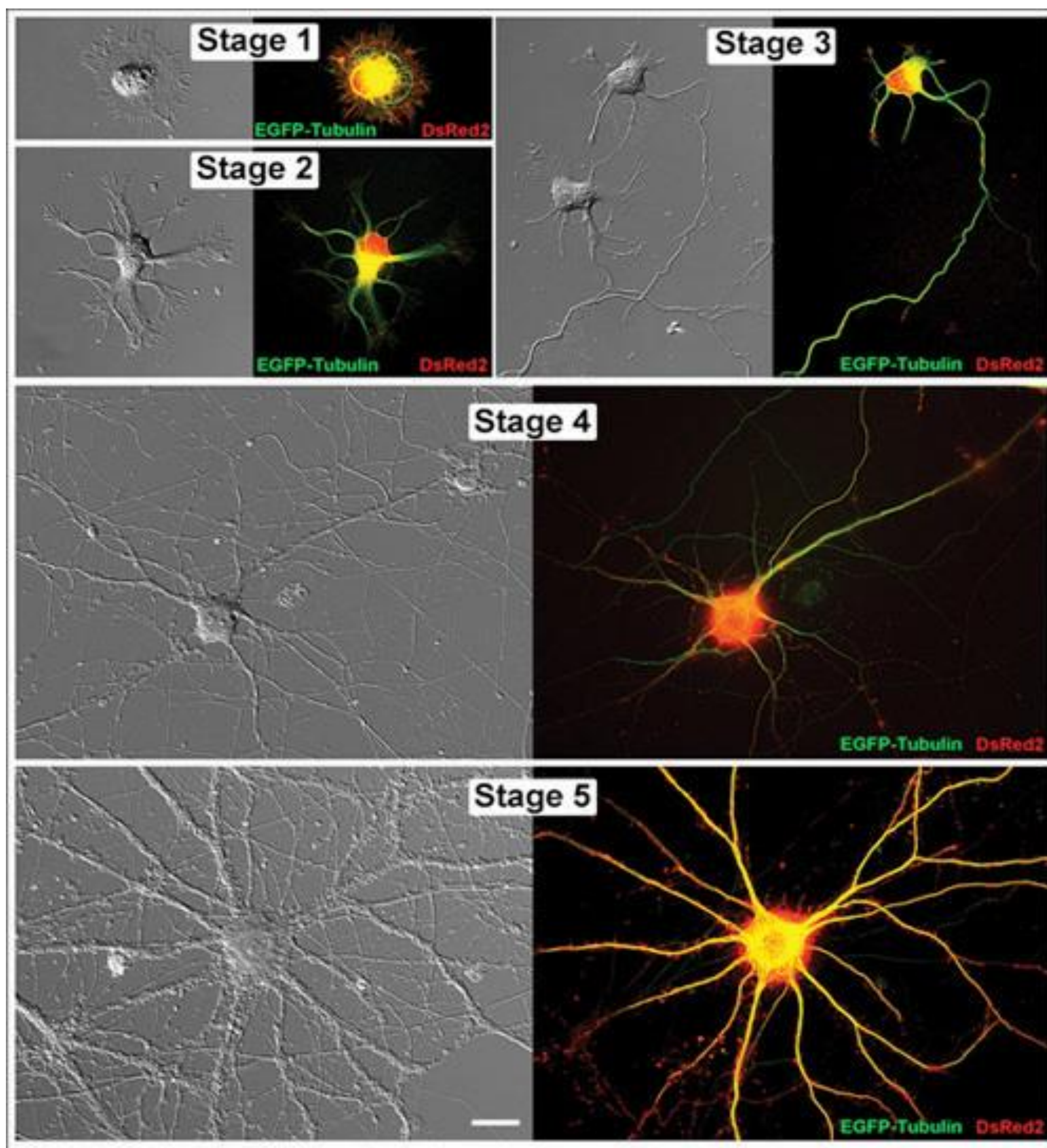

Figure 16. Living hippocampal neurons in successive stages of development. Paired images of representative living hippocampal neurons are shown as both a differential interference contrast image and a corresponding fluorescent micrograph. Each of these cells has been transfected with EGFP-Tubulin and DsRed2 in pCAX vectors. The neurons were imaged at the following days in vitro (DIV): Stage 1(1DIV), Stage 2 (1DIV), Stage 3 (2DIV), Stage 4 (11DIV) and Stage 5 (32DIV). Scale bar is 20µm.

4. DISCUSSION

Cortical and hippocampal cultures have been used in molecular biology for more than 20 years. While in principle, neuronal cultures can be made from any part of the brain, hippocampal cultures have proven to be the most popular due to the relatively simple architecture of the nerve cell population in the hippocampus (1-5, 7, 9, 11-15, 20-24, 26-33, 36, 39, 40-42).

Hippocampal cultures are typically made from late-stage embryonic tissue. This tissue is easier to dissociate and contains fewer glial cells than does mature brain tissue (1, 2, 14, 18, 22-32). Isolation of hippocampal neurons from embryonic tissue also decreases shearing damage to axons and dendrites due to fewer adhesion contacts (3).

While hippocampal cultures are most often generated from rats due to the relatively easier isolation of the hippocampus, mice can also be used with the same protocols if appropriate care is taken during tissue isolation. Once neurons are cultured, the ability to use advanced molecular techniques to analyze subcellular localization and trafficking can be employed. This can be especially advantageous when analyzing embryonic lethal transgenic mice as it provides the ability to study protein interactions that would result in the death of the embryo (27-33, 36-40).

The hippocampus has been implicated in both spatial and contextual learning (5-11) and memory (1-6, 17-21). Growth of primary cultures from the hippocampus can allow a correlation between subcellular biological events and their effects on the brain's ability to learn and remember.

As with all neural cells, neurons grown from hippocampal cultures require critical growth factors, hormones and amino acids. In the brain, these factors are provided by glial cells. This symbiotic relationship can also be carried into a culture environment by growing a "feeder" layer of glial cells along with the cultured neurons. However, glial cells will also produce cytotoxic factors during their lifespan (11, 15, 19, 22, 29-33, 39-42) which can be toxic to cultured neurons. To circumvent this, neurons have been grown in serum-free media such as Neurobasal medium supplemented with B27.

The B27 supplement is optimized for survival of hippocampal neurons but will support growth of other neuronal cultures as well (4). L-glutamine is an essential amino acid for energy production and protein synthesis in cell culture (34-39, 41-42).

However, glutamine can be labile over time, degrading into ammonia and carboxylic acid byproducts once added to culture media. Glutamax, a cell culture supplement from Invitrogen, can be used as a direct substitute for L-glutamine if desired. Glutamax is more stable in media but slightly more expensive. Growth of neurons in serum-free media allows the study of effects of growth factors and hormones on neuronal growth and differentiation (19, 22, 35, 37, 39, 41-42).

In this study, Nucleofection of mouce hippocampal neurons:was studied and demonstrated as well as neurite outgrowth and synaptogenic activities were characterized in primary cultured cortical and hippocampal neurons using immunofluorescence and Western blotting. The main features of survival, proliferation, and neurite outgrowth were similar to those cultured in a mixture of MEM and B27-supplemented neurobasal medium from rat embryos (19, 23-26, 29, 31-32, 34, 37, 39, 41).

Interestingly, the expression of synaptic markers from cortical neurons reached a peak level earlier (around 5 days in vitro) than that from hippocampal neurons (10 days in vitro), although neurite outgrowth from hippocampal neurons was faster than that of cortical neurons. Furthermore, the peak amounts of expressed synaptic markers were also higher in cortical neurons than those in hippocampal neurons.

Mice embryos at embryonic days 16~18 were used, although postnatal new-born rodents could be also used for primary neuronal or neural stem cell cultures (1-5, 11-19, 21-22, 24-30, 33, 35, 41). The matrigel, which was used for coating the glass cover slips, creates more optimal conditions for survival and neurite outgrowth than coating with laminin or other substrates alone.

The majority of plated neurons did not survive on non-coated cover slips and clumped heavily (data not shown). A gradual increase was detected in GAP-43 immunoreactivity that was associated with extension and branching of neurites. Synaptophysin immunoreactivity was already strong enough from day 2 in vitro. Although it was impossible to confirm whether they are true synaptic buttons, these findings could be considered important because the more synaptic buttons secured, the better the synaptic functional or morphological study. GAP43 is a marker of synaptic plasticity that is critical for normal development of serotonergic innervation (32-42), and synaptophysin is a well-known synaptic vesicular protein. (17, 20, 30-35, 37-40)

Considering prompt neurite outgrowth and abundant branching, synaptic marker expression levels should be clarified, particularly during the early period in vitro. The Western blotting results seemed to be a useful reference for experimental designs on synaptic activity and plasticity.

5. CONCLUSIONS

Delayed neurite sprouting and outgrowth of primary cultured cortical neurons was confirmed in comparison to those of hippocampal neurons. However, the expression levels of neurite outgrowth and synaptogenesis markers showed reversed patterns between cortical and hippocampal neurons.

These results suggest that primary cultured cortical neurons could be utilized rather earlier (within 1 week in vitro) in functional studies associated with synaptogenesis. Although it was not possible to suggest an explanation for this differential expression, the expression of synaptic vesicular associated proteins from cortical neurons seemed to be accelerated within the first week in vitro as a preparatory stage for explosive synaptogenic processes thereafter. (1-4, 7-11, 14, 17, 22, 34-39, 42)

To explain these discrepancies between neuronal groups during the early in vitro period, primary cultured neurons should be studied, that lack synaptic markers or synaptic vesicular associated proteins (possibly synaptobrevin, as a small integral membrane protein of secretory vesicles with molecular weight of 18 kilodalton (kDa), which is part of the vesicle-associated membrane protein (VAMP) family; and Munc-18 as a key regulator of neurosecretion) as a next step.

The transfection before plating maintains fluorescently-tagged fusion protein expression at levels appropriate for imaging throughout the lifetime of the neuron (> 2 months in culture). Thus, this methodology is useful for studying protein localization and function throughout CNS development with little or no disruption of neuronal function.

Although only findings based on morphological changes were demonstrated and synaptic markers expression at this time, to identify more detailed clues or mechanisms of growth cone formation and synaptogenesis during neuronal growth and differentiation should be performed by adding neurotrophic factors such brain derived neurotrophic factor (BDNF). Findings from peripheral nerve cell and neuronal stem cell cultures should also be compared to determine whether they have different mechanisms according to their origin.

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