



## Coexpression of Intracellular $\beta$ Amyloid, $\beta$ Actin with Partners of N-Methyl-D-Aspartate Receptors and NSE in Differentiated Rat Hippocampal H 19/7 Cells

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### Abstract

**Background.** The integrity of neuronal axons and ability to differentiate are of utmost importance to understand pathology of neurological diseases.  $\beta$ -actin is one of the cytoskeletal proteins that is important for cell integrity and synaptic formation. Previous studies showed contradictory findings concerning the effects of  $\beta$ -amyloid protein on neuronal structure and synaptic function. N-methyl-D-aspartate receptors (NMDARs) have crucial role in neuronal plasticity. Neuronal cell line (H19-7) generated from rat embryonic hippocampal neurons offers a model for screening assays. **Objective.** In this study, we used microculture system to characterize H19/7 cells in regard to their phenotypes, differentiation, synaptic connections, responsiveness to temperature and insulin like growth factor-I (IGF-I) hormone, and the ability to express growth hormone receptors (GHRs). Moreover, we examined the ability of the differentiated H19/7 cells to coexpress  $\beta$  amyloid and  $\beta$  actin with partners of NMDARs. **Results.** We found that raising temperature of cell culture to 39°C in medium supplemented with high glucose, basic fibroblast growth factors (bFGF) and IGF-I decreased the rapid cell proliferation and induced differentiation seen as elongation, multiple processes extension, thickening of cell bodies, and formation of synaptic connections. All differentiated cells express GHRs highlighting the importance of growth factors in maintaining the integrity of H19/7 cells. We further proved the differentiation of cells by testing the expression of NSE using western blots at different temperature and days of development. We found significantly higher expression seen at days 4 and 8 at 39°C compared to day 4 at 34°C. We found that H19/7 cells express cytoskeletal protein;  $\beta$ -actin as well as neuronal cytoplasmic proteins including,  $\beta$ -amyloid, and neuron specific enolase (NSE); two key molecular marker of neuronal cells. Moreover, we showed that differentiated H19/7 cells express a channel protein NR1 and NR2A; partners of N-methyl-D-aspartate receptors. We found that immune-reactivity of  $\beta$ -actin, and NSE was uniformly distributed throughout the cytoplasm in soma and neuronal processes. All differentiated cells showed co-expression of both  $\beta$  actin and NR2A;  $\beta$  amyloid and NR1 proteins especially on soma and neuronal processes suggesting a role of  $\beta$  actin and  $\beta$  amyloid in NMDAR-mediated effects at synaptic connection sites. **Conclusion.** This study suggested that H19/7 cells represent promising cell line model that reveal mechanisms regulating neuronal axon outgrowth and integrity that may help in treatment of neurological diseases in the future.

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### Keywords

- Beta-Actin
- Beta Amyloid ( $A\beta$ )
- Growth hormone receptors (GHR)
- H19/7 Cells
- Hippocampus
- Neural Cell Lines
- N-Methyl-D-Aspartate-Receptors (NMDARs)
- NR1
- NR2A
- Neuron specific enolase (NSE)
- Synaptic connections

## INTRODUCTION

Neuronal differentiation, synaptic connections and proper responsiveness to neurotrophic factors are critical in understanding neuropathological conditions associated with physiological processes such as aging or disease. Various factors are capable of modulating neurogenesis of the hippocampus including hormones, drugs, growth factors, and cytokines.<sup>1</sup> Attempts to simulate hippocampal neuronal outgrowth in vitro have included the hippocampal neuronal cell line H19/7 cells in previous studies. Growth hormone receptors (GHRs) have wide distribution both in rat and human brain and can bind both growth hormone and insulin like growth factor-1 (IGF-1).<sup>2</sup> Growth hormone and insulin like growth factors are crucial for growth, development, and repair of central neurons<sup>3</sup> through their effects on adult neurogenesis.<sup>4</sup> The role of insulin-like growth factors and temperature in differentiation and development of H19/7 cells was highlighted by previous studies.<sup>5</sup> To this end, this study is designed to examine the phenotypes, differentiation, synaptic connections of H19/7 cells. Moreover, we tested the responsiveness of H19/7 cells to temperature, bFGF, and IGF-I hormone, and the expression of GHRs in the differentiated cells.

Cytoskeletal proteins and intermediate filaments regulate synaptic and dendritic spines formation. Beta-actin is one of the two nonmuscle cytoskeletal highly conserved proteins that are involved in cell motility, structure and integrity.<sup>6</sup> Previous studies reported that actin is a cytoskeletal protein that has dynamic nature mediating changes in cellular morphology

associated with stress-induced changes in synaptic transmission.<sup>7, 8</sup> Gamma-enolase or phosphopyruvate hydratase is one of the three enolase alpha, beta, and gamma isozymes found in mammals.<sup>9</sup> The gamma isozyme forms homodimer that is found in mature neurons and cells of neuronal origin therefore, given its name neuron specific enolase (NSE).<sup>9</sup> Detection of NSE with antibodies can be used to identify neuronal cells and cells with neuroendocrine differentiation.<sup>10</sup> To validate the role of NSE in neuronal differentiation and axon arborization of H19/7 cells, we examined the expression of  $\beta$ -actin and NSE in differentiated H19/7 cells.

Moriyoshi and his group reported that N-methyl-D-aspartate receptor (NMDAR) messenger RNA is expressed in neuronal cells throughout all the brain regions.<sup>11</sup> NMDARs are formed by coexpression of the NR1 and one or more members of the NR2 family (NR2A-D) of genes.<sup>12</sup> The NR1 subunit is expressed throughout the brain and serves as a general partner for association with NR2 subunits.<sup>13</sup> NR2 subunit composition modulates channel kinetics and pharmacology.<sup>14</sup> The NMDAR has important role in synaptic development and plasticity.<sup>15</sup> NR2A subunit of NMDAR is mainly found at synapses while NR2B subunit is found at extrasynaptic receptors in adults.<sup>16</sup> Previous studies showed that soluble oligomeric forms of  $\beta$  amyloid are involved in neuronal degeneration and deteriorated synaptic function in Alzheimer's disease.<sup>17, 18</sup> Conversely, other studies suggested that  $\beta$  amyloid protein decreases neuronal hyperactivity through negative feedback mechanism to prevent progression in Alzheimer's disease.<sup>19</sup> To examine whether  $\beta$ -

amyloid protein is good for synaptic formation, we examined the coexpression of  $\beta$  amyloid protein and NR1 subunit of NMDARs. Moreover, we examined the coexpression of  $\beta$ -actin and NR2A subunits of NMDARs in differentiated H19/7 cells.

In this study, we used a microculture system of hippocampal cell line H19/7 to analyze neuronal differentiation and organization of hippocampal H19/7 cell line, the mode of neuronal connection at different temperature and days of development. We examined the expression of NSE protein at various temperature and days of development using western blots. We examined the expression of  $\beta$ -actin, NSE, NR1, NR2A, and GHR proteins in mature hippocampal H19/7 cells in culture by immunofluorescence immunocytochemistry. We also, examined the colocalization of  $\beta$ -actin and NSE;  $\beta$ -amyloid and NR1;  $\beta$ -actin and NR2A proteins in mature hippocampal H19/7 cells.

## MATERIALS AND METHODS

### *H19-7/IGF-IR cells*

This study was conducted in the laboratory of Dr. Lawrence Grover Department of Pharmacology, Physiology & Toxicology at the Joan C. Edwards School of Medicine, Marshall University, in Huntington, West Virginia, USA. The hippocampal neuronal cell line H19-7/IGF-IR (ATCC<sup>®</sup> CRL-2526<sup>TM</sup>) was purchased from the American Type Culture Collection, Manassas, VA, USA. The H19-7/IGF-IR cells were derived from hippocampi dissected from the brain of embryonic day 17 (E17) Holtzman rat embryos (*Rattus norvegicus*) and immortalized by transduction of retroviral vector at temperature-sensitive tsA58

SV40 large T antigen.<sup>20</sup> These cells have the morphology of fibroblasts, grow and proliferate at permissive temperature (34°C) in serum but differentiate at nonpermissive temperature (39°C) by addition of basic fibroblast growth factor (bFGF) in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose medium with supplements.<sup>5</sup> At 39°C, expression of the human type 1 insulin-like growth factor receptor (IGF-IR) induces neuronal differentiation in H19-7 cells.<sup>5</sup>

### *Cell culture*

H19-7/IGF-IR cells (passages 5 - 9) were included in this study. The cells were seeded at  $\sim 1 - 3 \times 10^6$  cells/25 cm<sup>2</sup> in tissue culture flasks, and were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin 100 units/ml, and 200  $\mu$ g/ml G418 (selection for the T antigen plasmid) in flasks coated with 15  $\mu$ g/ml poly-L-lysine (Sigma) at 34°C in humidified atmosphere of 5% CO<sub>2</sub> - 95% air. The culture medium was renewed every 2 days.<sup>21</sup> The experiments were performed on day 4 of subculture (60–80% confluence) to analyze cell proliferation and to study NSE expression by western blotting.

### *Analysis of Differentiation*

For neuronal differentiation, H19-7/IGF-IR cells were cultured at 39°C in DMEM-high glucose medium supplemented with 10% fetal bovine serum, 10 ng/ml bFGF, 50 ng/ml IGF-I, penicillin G sodium 100 units/ml, and 200  $\mu$ g/ml G418, a combination that is known to induce differentiation of H19-7 cells.<sup>5</sup> The experiments were performed on day 4 and day 8 of subculture for NSE expression by western blotting and to check neurite formation (processes longer than the cell body were considered neurites) with a Zeiss

microscope using a 20X lens, and photographed with a digital Nikon camera.<sup>22, 23</sup>

### ***Immunofluorescent Staining of NSE, NR1, NR2A, $\beta$ -actin, $\beta$ -amyloid, and GHR***

On differentiation, H19-7 cells show an increased expression of neuronal proteins detectable by immunofluorescence immunocytochemistry using an antibody against NSE, NR1, NR2A,  $\beta$ -actin,  $\beta$ -amyloid and GHR. Cells were plated on 25 mm circular glass coverslips (Thomas Scientific, Swedesboro, NJ, USA), coated with poly-L-lysine (Sigma-Aldrich, USA) in 6-well flat bottom Corning<sup>®</sup> Costar<sup>®</sup> cell culture plates for each experiment. On Day 4, the cells were fixed in 10% formalin (methanol free) for 10 min at room temperature and permeabilized in 2% formalin/0.2% Triton X-100 for 5 min at room temperature, and after washing with PBS, the cells were blocked in 1% goat serum (in PBS) for 30 min at 37°C. After washing with PBS, the cells were then incubated overnight (4°C) in primary antibody against NSE, NR1, NR2A,  $\beta$ -actin,  $\beta$ -amyloid or GHR. We successively incubated some sections with two primary antibodies:  $\beta$ -actin and NSE; NR2A and  $\beta$ -actin;  $\beta$ -amyloid and NR1. The cells were then washed extensively with PBS, and fluorophore-conjugated secondary antibodies were added at a 1:500 dilution and incubated for 2 h at room temperature. After washing with PBS, the cells were incubated with ProLong<sup>®</sup> Gold Antifade Reagent with DAPI for 30 min at room temperature then covered with Superfrost Plus slides, sealed around their perimeter with clear nail polish. Mounted slides were stored in the dark at 4 °C, then analyzed with a fluorescence microscope, and photographed with a digital Nikon camera. The immunofluorescence

double stained culture plates were processed using two wavelengths of exciting lights set and merged picture were synthesized by image software.

### ***Materials***

The following primary antibodies were used: Anti-beta actin Antibody (200-301-E69, Rockland Inc., P.O. BOX 5199, Limerick, Pennsylvania, USA) at 1: 500 dilution, anti-NSE antibody - a neuronal marker, (AB951, Merck Millipore, Darmstadt, Germany) at a dilution of 1:500; Anti-growth hormone receptor antibody (Anti-GHR) (AB65304, from Abcam, Cambridge, MA, USA) at dilution of 1: 500; Anti-beta Amyloid antibody (AB2539, from Abcam, Cambridge, MA, USA) to detect intracellular beta amyloid only and is negative for amyloid plaques at 1:200 dilution; Anti-NMDAR1(ab17345, Cambridge, MA, USA) at a dilution of 1:1000; Anti-NMDAR2A antibody (ab124913, Cambridge, MA, USA) at 1:250 dilution; Goat anti-rabbit IgG-peroxidase conjugate antibody, Alexa Fluor<sup>®</sup> 488-conjugated; Goat Anti-Mouse IgG, Alexa Fluor<sup>®</sup> 594-conjugated; O-phenylenediamine dichloride, bovine serum albumin (BSA); protease inhibitor cocktail, Tween 20<sup>®</sup>; dimethyl sulfoxide (DMSO); acrylamide were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). DMEM-high glucose, heat inactivated fetal bovine serum (FBS), IGF-I, bFGF, and Hank's Balanced Salt Solution (HBSS) were ordered from Invitrogen<sup>™</sup> Life Technologies, Inc (Carlsbad, CA, USA). Bradford protein reagent and low range prestained SDS-PAGE standards were from Bio-Rad Laboratories, Inc. (Hercules, CA). Protran<sup>®</sup> pure nitrocellulose transfer and immobilization membranes (0.45  $\mu$ m) were purchased from Schleicher and Schuell

BioScience (Keene, NH). SuperSignal<sup>®</sup> West Pico Chemiluminescent substrate for detection of peroxidase reaction was from Pierce Biotechnology Inc. (Rockford, IL). Medical X-ray film (Super RX) was from Fujifilm Medical Systems, Inc. (Stamford, CT), and penicillin G sodium-G418 antibiotics were purchased from Gibco BRL<sup>®</sup> Life Technologies (Grand Island, NY). 25 cm<sup>2</sup> polystyrene tissue culture flasks were from Corning Life Sciences - Mediatech Inc. (Manassas, VA, USA). Superfrost Plus slides were purchased from Fisher Scientific (Pittsburgh, PA, USA). All chemicals and reagents were of analytical grade.

#### **Western blot analysis**

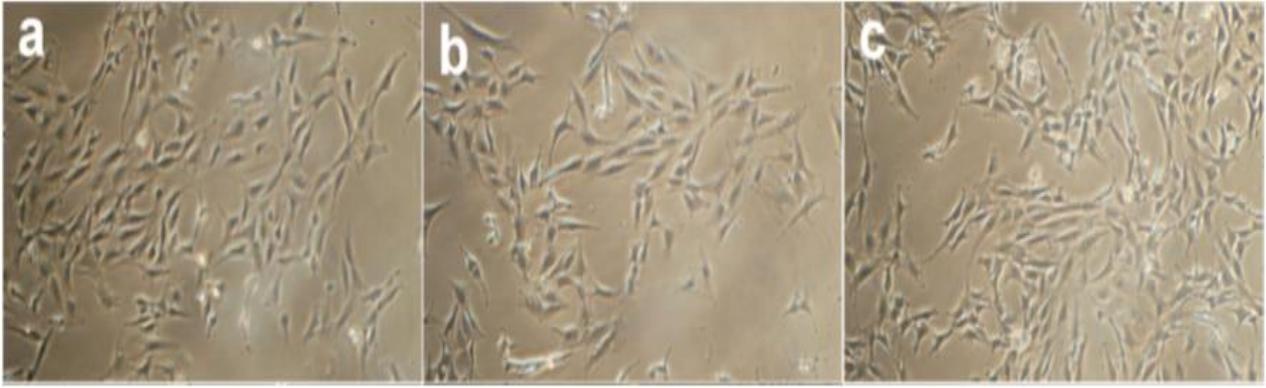
NSE protein expression in H19-7/IGF-IR cells was studied on Day 4 of culture at 34°C; and on Days 4 and 8 of culture at 39°C. H19-7/IGF-IR cells were washed thrice in HBSS to remove all traces of serum, then incubated in 3 ml of 0.25% (w/v) Trypsin-0.53mM EDTA solution and observe cells under an inverted light microscope for 5-15 min till cell layer is dispersed. A temperature of 37°C was used for Cells that are difficult to detach to facilitate dispersal, harvested from the tissue culture flasks, centrifuged, and the pellets were frozen quickly on dry ice, and stored at -80°C until used. The pellets were then placed into lysis buffer [1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, and 1% protease inhibitor cocktail]. After homogenization in protein lysis buffer, cells were disrupted by burst sonication (< 10 seconds) while cooling on ice,

and samples were centrifuged at 14,000 × g for 20 min at 4°C. The supernatant solution was obtained and total protein estimated using the Bradford method.<sup>24</sup> Equal amounts of total protein from each sample were loaded per lane in 12% SDS polyacrylamide gels. Low range prestained SDS-PAGE standards (Bio-Rad) were also used. After protein transfer<sup>25</sup>, nitrocellulose sheets were incubated in Tris-buffered saline with Tween 20<sup>®</sup> and 5% non-fat dry milk to block non-specific binding sites.<sup>26</sup> After applying the specific primary and peroxidase-conjugated secondary antibodies, the specific protein bands were visualized using SuperSignal<sup>®</sup> West Pico Chemiluminescent Substrate kit.

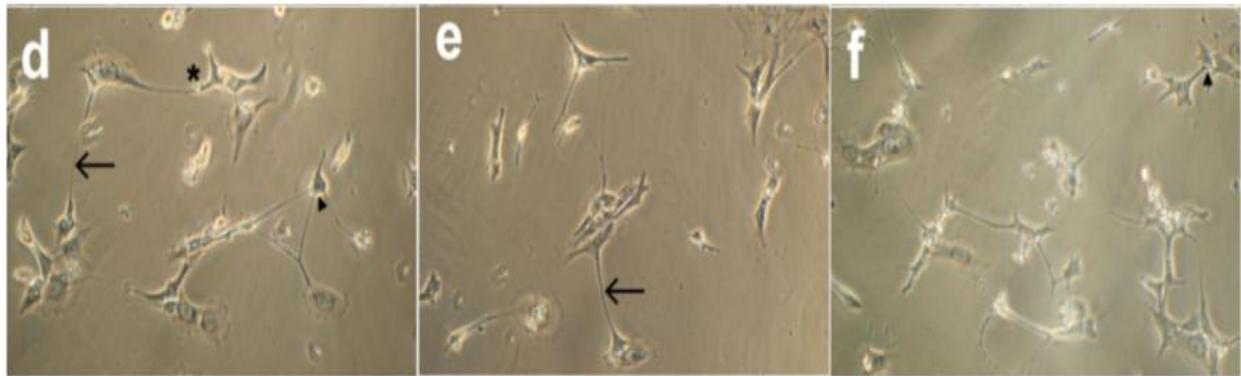
## **RESULTS**

### ***Effects of temperature and medium on the morphology of H19-7 neuronal hippocampal cell line***

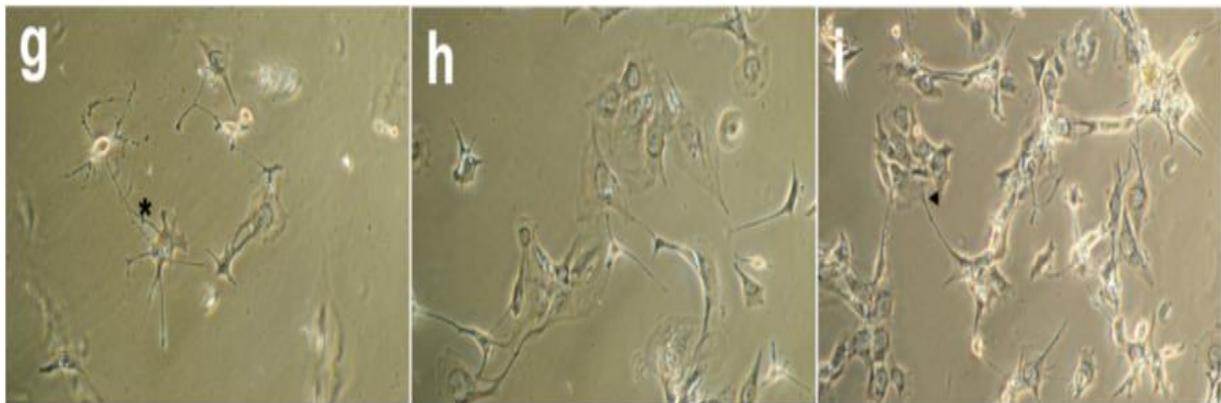
The cells proliferate when grown at 34°C and were seen undifferentiated (flat and polygonal) on Day 4 shown in phase contrast photomicrographs Fig. 1 (a-c). Raising the temperature of cell culture to 39°C in medium supplemented with high glucose, bFGF and IGF-I induced differentiation (elongation, multiple processes were extended, cell bodies become thickened, and synaptic connections were established) with neurite formation as seen on Day 4 Fig. 2 (d-f). Axonal extensions, cell-to-cell contacts and synapses were formed. The cells showed increased neurite outgrowth, more complex processes and differentiation on Day 8 of culture at 39°C Fig. 3 (g-i).



**Fig. (1):** The effects of cell culture on Day 4 at 34°C on the morphology of H19-7 neuronal hippocampal cell line were shown in phase contrast photomicrographs (a-c) [Magnification,  $\times 100$ ]. The cells proliferate when grown at 34°C and were seen undifferentiated (flat and polygonal) on Day (4).



**Fig. (2):** The effects of cell culture on Day 4 at 39°C in medium supplemented with high glucose, bFGF and IGF-I on the morphology of H19-7 neuronal hippocampal cell line were shown in phase contrast photomicrographs (d-f) [Magnification,  $\times 100$ ]. The cell culture at 39°C in medium supplemented with high glucose, bFGF and IGF-I induced differentiation (elongation, multiple processes were extended, and cell bodies become thickened) with neurite formation. Axonal extensions, cell-to-cell contacts and synapses were formed. Axosomatic synapses (arrow heads), axodendritic synapses (asterisks), and axoaxonal synapses (arrows) were indicated.



**Fig. (3):** The effects of cell culture on Day 8 at 39°C in medium supplemented with high glucose, bFGF and IGF-I on the morphology of H19-7 neuronal hippocampal cell line were shown in phase contrast photomicrographs (g-i) [Magnification,  $\times 100$ ]. The cells showed increased neurite outgrowth, more complex processes and differentiation on Day 8 of culture at 39°C. Axonal extensions, cell-to-cell contacts and synapses were more extensive. Axosomatic synapses (arrow heads), axodendritic synapses (asterisks), and axoaxonal synapses (arrows) were indicated.

***Effects of temperature and medium on the expression of neuron specific enolase (NSE) proteins of H19-7 neuronal hippocampal cell line***

The Western blots analysis showed highly significant increase in expression of NSE in cultured H19-7 cells with the rise in the temperature and the supplementation of the medium with glucose, bFGF, and IGF-1 on Day 8 and Day 4 at 39°C compared to Day 4 at 34°C and highly significant increase in the NSE protein expression on Day 4 at the higher temperature compared to Day 4 at the lower temperature. Fig. (4).

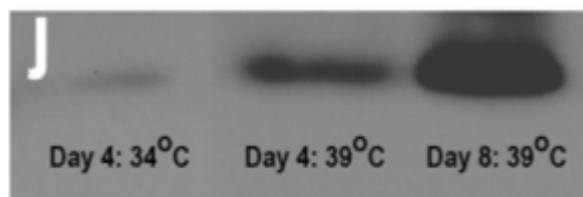


Fig. (4): The Western blots analysis of NSE on Day 4 at 34°C, Day 4 at 39°C, and Day 8 at 39°C. Western blots analysis showed highly significant increase in the expression of NSE proteins in cultured H19-7 cells on Day 8 at 39°C compared to Day 4 at 39°C and Day 4 at 34°C. It shows a highly significant increase in the NSE expression on Day 4 at 39°C compared to Day 4 at 34°C.

***Expression of  $\beta$ -actin and Neuron Specific Enolase (NSE) proteins in H19/7-IR cells on day 8 at 39°C***

The cells were cultured at 39°C and studied on Day 8. H19/7 cells showed expression of both NSE (pale blue, a) and  $\beta$ -actin (green color, b). They showed similar partially overlapping distributions throughout the cytosol (areas of

colocalizations shown as blue-green color, c). Fig. 5.

***Expression of NR2A and  $\beta$ -actin proteins in H19/7-IR cells on day 8 at 39°C***

The cells were cultured at 39°C and studied on Day 8. H19/7 cells showed expression of both NR2A (red color, a) and  $\beta$ -actin (green color, b). They showed similar partially overlapping distributions throughout the cytosol (areas of colocalizations shown in yellow, d). Fig. 6.

***Expression of NSE and DAPI proteins in H19/7-IR cells on day 8 at 39°C***

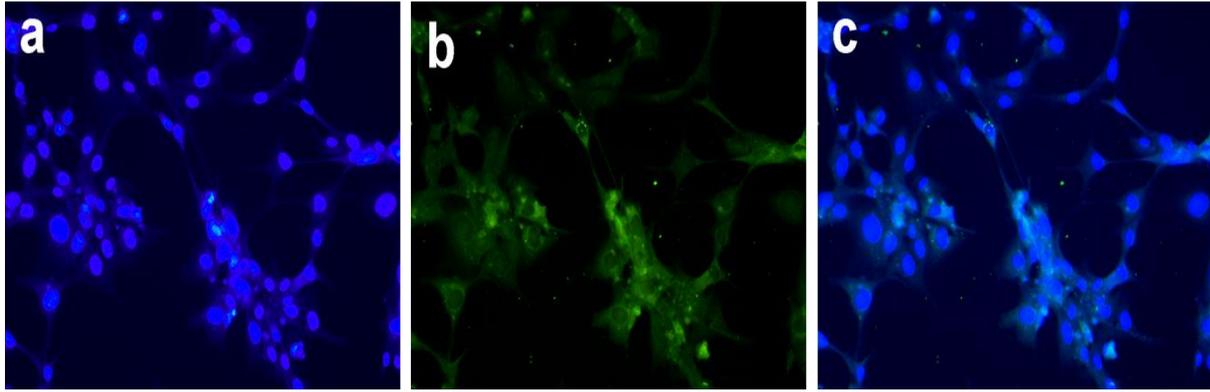
The cells were cultured at 39°C and studied on Day 8. H19/7 cells showed expression of NSE (a) and DAPI (blue) (b) merged image (c). Fig. 7.

***Expression of  $\beta$ -amyloid and NR1 proteins in H19/7-IR cells on day 8 at 39°C***

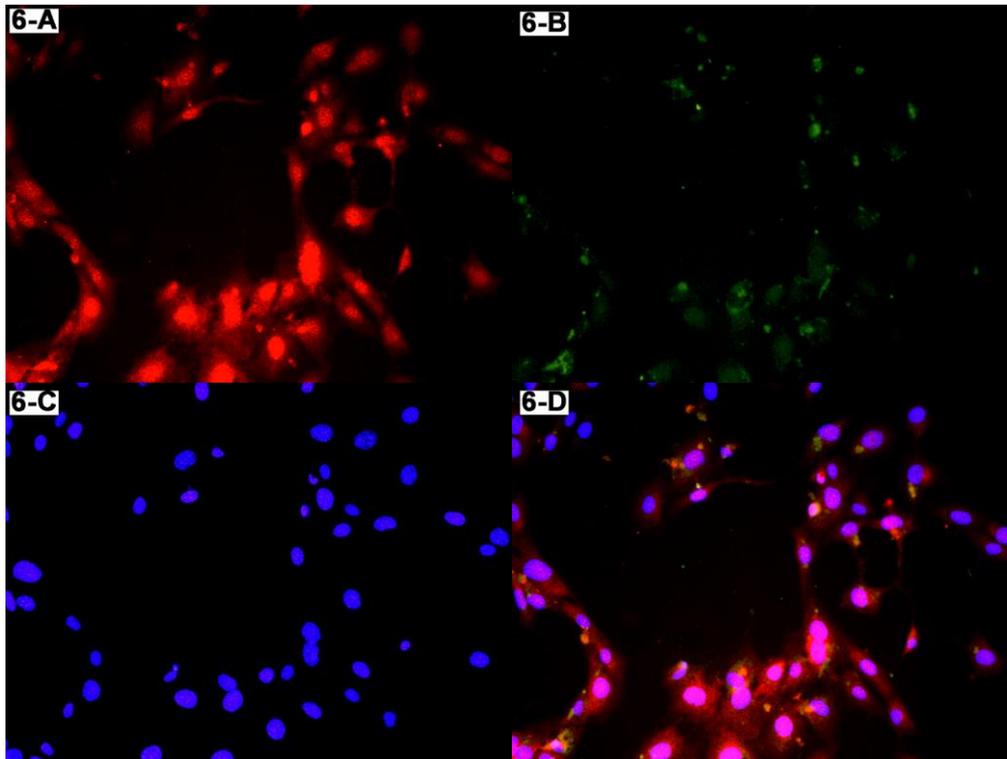
The cells were cultured at 39°C and studied on Day 8. H19/7 cells showed expression of  $\beta$ -amyloid (red) (a) and NR1 (green) (b) indicating high number of well differentiated neurons. They showed similar partially overlapping distributions throughout the cytosol (colocalizations). Colocalization was seen in the merged image (d). Fig. 8.

***Expression of Growth hormone receptor (GHR) protein in H19/7-IR cells on day 8 at 39°C***

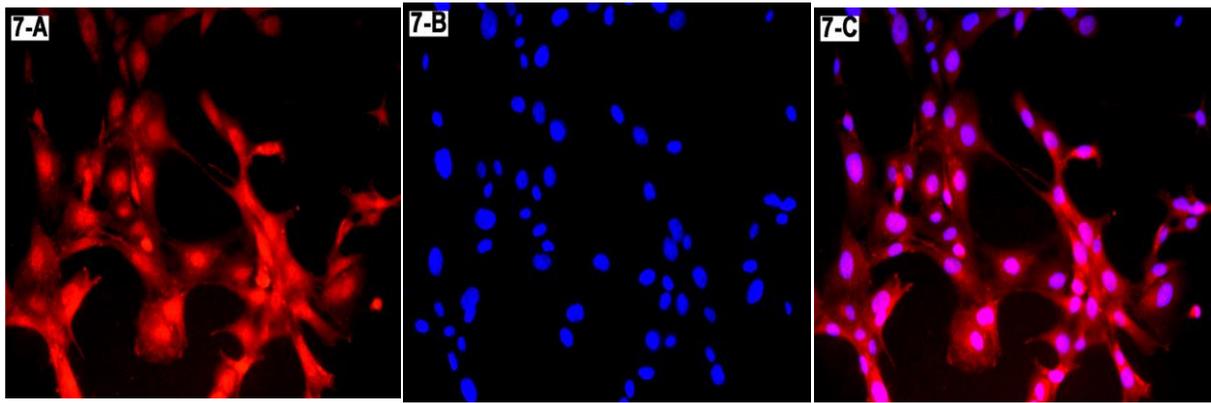
The cells were cultured at 39°C and studied on Day 8. H19/7 cells showed expression of GHR (green) (a) and the nuclei of H19-7 cells were stained blue (b) with DAPI nucleic acid stain. They showed similar partially overlapping distributions throughout the cytosol (colocalizations). No colocalization was seen in the merged image (c). Fig. 9.



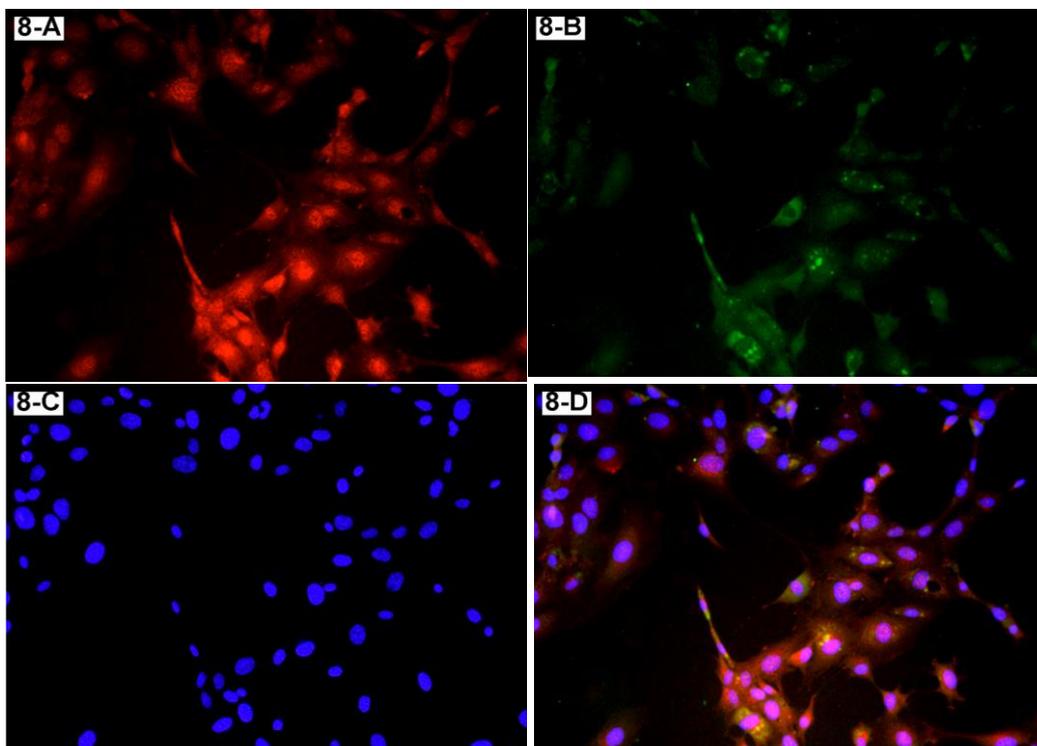
**Fig. (5):**  $\beta$ -actin and Neuron Specific Enolase (NSE) show similar partially overlapping distributions throughout the cytosol (colocalizations) in H19-7/IGF-IR cells. The cells were cultured at 39°C and studied on Day 8. Antibody against  $\beta$ -actin (shown in green) and antibody to NSE (shown in pale blue) were applied. The same field was shown in each photomicrograph. a, The nuclei of H19-7 cells were stained blue with DAPI nucleic acid stain, and the immunopositive reactivity for NSE was shown as pale blue. Immunofluorescence labeling of the H19-7 cells showed immunopositive staining for  $\beta$ -actin (b, green). Sites of colocalization exhibit a blue-green color in the merged image (c). Magnification  $\times$  400.



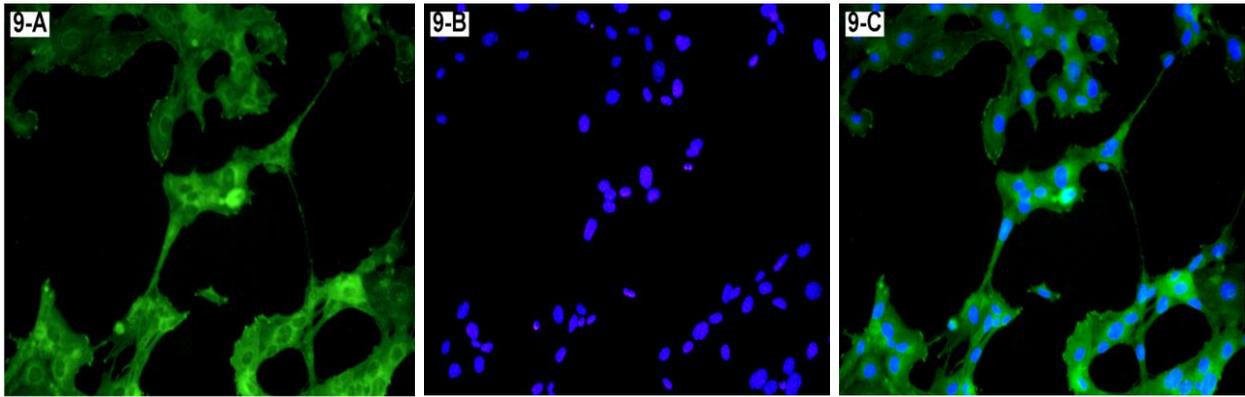
**Fig. (6):** NR2A and  $\beta$ -actin show similar partially overlapping distributions throughout the cytosol (colocalizations) in H19-7/IGF-IR cells. The cells were cultured at 39°C and studied on Day 8. Antibody against  $\beta$ -actin (shown in green) and antibody to NR2A (shown in red) were applied. The same field was shown in each photomicrograph. The nuclei of H19-7 cells were stained blue with DAPI nucleic acid stain, and unlabeled areas appear black (C). Immunofluorescence labeling of the H19-7 cells showed immunopositive staining for NR2A (a, red) and  $\beta$ -actin (b, green). Sites of colocalization exhibit a yellow color (red + green = yellow) in the merged image (d). Magnification  $\times$  400.



**Fig. (7):** Neuron Specific Enolase (NSE) and DAPI immunohistochemical localizations were studied in H19-7/IGF-IR cells. The cells were cultured at 39°C and studied on Day 8. Antibody to NSE were applied and the nuclei of H19-7 cells were stained blue with DAPI nucleic acid stain, and unlabeled areas appear black (b). Immunofluorescence labeling of the H19-7 cells showed intense immunopositive reaction for NSE (a, red). No colocalization was seen in the merged image (c). Magnification  $\times 400$ .



**Fig. (8):**  $\beta$ -amyloid and NR1 immunohistochemical localizations were studied in H19-7/IGF-IR cells. The cells were cultured at 39°C and studied on Day 8. Antibody against NR1 (shown in green) and antibody to  $\beta$ -amyloid (shown in red) were applied. The same field was shown in each photomicrograph. The nuclei of H19-7 cells were stained blue with DAPI nucleic acid stain, and unlabeled areas appear black (c). Immunofluorescence labeling of the H19-7 cells showed intense immunopositive reaction for  $\beta$ -amyloid (a, red) and mild immunostaining for NR1 (b, green). Colocalization was seen in the merged image shown in yellow (d). Magnification  $\times 400$ .



**Fig. (9):** Growth hormone receptor (GHR) and DAPI immunohistochemical localizations were studied in H19-7/IGF-IR cells. The cells were cultured at 39°C and studied on Day 8. Antibody against GHR (a, shown in green) and the nuclei of H19-7 cells were stained blue (b) with DAPI nucleic acid stain, and unlabeled areas appear black. No colocalization was seen in the merged image (c). Magnification  $\times$  400.

## DISCUSSION

The appropriate structural geometry of central axons appears to be important in ensuring optimal neuronal function. Factors regulating axonal outgrowth and connections are incompletely understood, in part because we lack the good assay systems. Primary neuronal cultures provide the principal means for assessing factors regulating axon outgrowth in vitro. This study showed that the morphology, proliferation, and differentiation of H19-7 neuronal hippocampal cell line are temperature sensitive and medium dependent. The role of growth factors in stimulation of neural stem cell (NSC) and neural progenitor cell (NPC) proliferation are highlighted by several previous studies.<sup>27, 28</sup> However, our results showed that supplementation of the H19/7 culture medium with high glucose, bFGF and IGF-I reduced proliferation and induced differentiation elongation, multiple processes extension, thickening of cell bodies, and cell-to-cell synaptic connections. In support of our results, Ulloa-Montoya et al. reported that growth factors promote differentiation, expansion and survival of specific cell types and considered to be the key in

controlling the differentiation towards specific cell lineages.<sup>29</sup> In contrast to our results, Moyses and his group demonstrated that the presence of the specific mitogens EGF and bFGF are important for proliferation but not for differentiation of neural stem cells (NSC) in primary culture of dissociated adult nervous tissue.<sup>28</sup> This difference could be explained on the basis of different cell lines used.

This study demonstrated that raising the temperature of cell culture from 34°C to 39°C caused decreased proliferation and induced differentiation and maturation of H19/7 cells to neurons on day 4 with increased neurite outgrowth, more complex processes and differentiation on day 8 of culture at 39°C. This result is supported by the work of Peng and his colleagues who showed a robust temperature-induced neurite outgrowth, high-order branching and enlarged growth cones in isolated embryonic neurons of *Drosophila* in culture that was lacking cell-cell contacts.<sup>30</sup> The decreased cell number induced by rise in temperature to 39°C is supported by a previous study demonstrating that mild hypothermia (31.5°C) results in an increase in

cell number of *in vitro* cell culture of oligodendrocyte precursor cells relative to cells cultured under normothermic conditions (37°C).<sup>31</sup> The relationship between raising temperature and IGF-1 in inducing the differentiation of H19/7 cells is proved by the work of Morrione et al. who reported neuronal differentiation and expression of the human IGF-IR in H19-7 cells at 39°C that is induced by IGF-I.<sup>5</sup>

The Western blots analysis showed significantly higher expression of NSE in cultured H19-7 cells on day 8 at 39°C compared to day 4 at 39°C as well as on day 4 and 8 at 39°C compared to day 4 at 34°C. This result is supported by previous finding that embryonic neurons express NSE at a very low level compared with mature neurons.<sup>32</sup> Previous studies found that neuron specific enolase (NSE) promotes the survival of cultured rat neocortical neurons and acts as a neurotrophic factor for a broad spectrum of CNS neurons.<sup>33,34</sup> Taken together, we may speculate that the increased expression of NSE induced by raising the temperature promoted the survival and at the same time increased differentiation and synaptic connection between those neurons.

To further examine the role of NSE in synaptic formation, we examined the expression of  $\beta$ -actin; a well known cytoskeletal protein and NSE in H19/7 on day 8 at 39°C with immunohistochemistry and the colocalization of  $\beta$ -actin and NSE proteins. We found immunoreactivity of  $\beta$ -actin protein and NSE protein were distributed throughout the cytoplasm in soma and axonal processes. In the merged image we found that all differentiated cells showed co-expression

of both NSE and  $\beta$ -actin proteins throughout the cytoplasm. This result is supported by previous work, showing that NSE is associated with the cytoskeleton and thence to play important roles in cell growth, proliferation, and survival.<sup>35</sup> Previous studies reported that detection of NSE with antibodies can be used to identify neuronal cells.<sup>10</sup> The specific mechanisms of actin regulation together with various actin cytoskeleton-binding proteins are considered as an integral part at synaptic connection sites.<sup>36</sup>

For further investigating that H19/7 cells differentiate into neurons, we looked at the expression of two well known neuronal proteins NR1 and NR2A proteins by immunohistochemistry. We found that H19/7 expresses NR1 and NR2A proteins throughout the cytoplasm and axonal arborization. Previous results showed that  $\beta$ -actin is a cytoskeletal protein that plays an important role in the formation, maturation and plasticity of dendritic spines.<sup>36</sup> To investigate this finding, we examined the colocalization of  $\beta$ -actin and NR2A proteins in H19/7 cells. In this study, we demonstrated that all differentiated cells showed co-expression of both  $\beta$ -actin and NR2A proteins especially on soma and neuronal processes suggesting a role of  $\beta$ -actin at synaptic connection sites. In line with this result, previous studies reported that actin binding protein is involved in joining NMDAR to actin filaments.<sup>37</sup> The interaction of NMDAR and actin-associated proteins play an important role in proper trafficking, anchoring, stabilization of NMDARs at dendritic spines, and synaptic transmission.<sup>38</sup>

This study showed the expression of  $\beta$ -amyloid in mature H19/7. To answer the question if  $\beta$ -amyloid expressed by the same cells that express N-methyl-D-aspartate receptors, we tested the colocalization of  $\beta$ -amyloid and NR1. We found that all differentiated cells showed co-expression of both  $\beta$ -amyloid and NR1 proteins. This result is supported by the work of Danysz and Parsons who reported that soluble oligomers of  $\beta$  amyloid protein are tightly linked with NMDARs to maintain uptake and release of glutamate neurotransmitter.<sup>39</sup> In contrast to us, several previous studies suggested the involvement of  $\beta$  amyloid protein in neuronal degeneration and deterioration of synaptic function in Alzheimer's disease.<sup>40, 41</sup> This apparent conflict could be explained on the basis of different species/forms of this peptide. Moreover, we found that the differentiated H19/7 cells express growth hormone receptors. This result is supported by previous studies showing that addition of growth factors to culture medium of H19/7 cells are known to induce differentiation of H19-7 cells<sup>5</sup> through mitogen-activated protein (MAP) kinase-dependent mechanism.<sup>42</sup>

The present data establish that H19/7 cells represent a good cell line to clearly reveal mechanisms regulating neuronal axon outgrowth and integrity. The ability of the immortalized embryonic hippocampal progenitor cells to proliferate, differentiate, extend axonal arborization, and express  $\beta$ -actin,  $\beta$ -amyloid protein as well as neuronal markers NSE, NR1, NR2A represent a promising insight to treat diseases associated with hippocampal damage as Alzheimer's disease.

## CONCLUSION

This study showed that the morphology, proliferation, and differentiation of H19-7 neuronal hippocampal cell line are temperature sensitive and medium dependent. We demonstrated that the expression of cytoskeletal protein  $\beta$ -actin, and NSE act as essential markers for axonal and synaptic development in hippocampal cell line H19/7. The colocalization of  $\beta$ -actin and NSE indicating that the cytoskeletal protein actin has an integral role in the formation and maturation of neuronal arborization. The colocalization of NR2A and  $\beta$ -actin suggesting a role of  $\beta$ -actin as NR2A guide to the synaptic sites that should be further investigated. Moreover, the colocalization of  $\beta$ -amyloid and NR1 protein suggest a role of  $\beta$ -amyloid in NMDAR-mediated synaptic transmission. We showed that differentiated H19/7 expresses GHR that has a well known effect on synaptic plasticity. Immortalized neural cell lines have provided novel and important insight into fundamental principles of the capacity to differentiate into neurons. Particularly nowadays, neural progenitor cell grafting is a promising therapeutic option in the treatment of many diseases. Further studies are needed to demonstrate if immortalized embryonic hippocampal neural cell line H19/7 can recognize microenvironmental signals as bFGF and IGF-1 from the lesioned adult hippocampus and preferentially differentiate along a lineage that is essential for functional repair.

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التكوين و التوزيع الموحد لبروتينات بيتا أميلويد ، و بيتا أكتين ، مع مستقبلات  
ن ميثيل د-أسبارتات ، و اينولاذ الخلايا العصبية فى خلايا الهيبيوكامبوس  
المستمدة من زراعة الخلايا الخطية (هاء 7/19)

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**المقدمة.** ان سلامة الأعصاب الحسية والقدرة على النضوج ذات أهمية قصوى لفهم التغيرات المرضية التى تصاحب الأمراض العصبية. البيتا أكتين هو أحد البروتينات الخلوية الهيكلية و المهمة لسلامة بنية الخلايا العصبية. كما أظهرت الدراسات السابقة نتائج متناقضة بشأن تأثير بروتين البيتا اميلويد على بنية الخلايا و سلامة الوصلات العصبية . ان مستقبلات ن-ميثيل- د-أسبارتات المخية لها دور حاسم فى تكوين الوصلات العصبية. الخلايا العصبية الخطية (هاء 7/19) والتي تم إنشاؤها من الخلايا العصبية الجنينية قرن آمون للجرذان فى اليوم ال17 من الحمل يقدم نموذجا جيدا للفحص. **الهدف.** يهدف هذا البحث، الى دراسة خلايا (هاء 7/19) فيما يتعلق بالشكل، وقدرتها على النضج والتمايز، و تكوين وصلات عصبية ، والاستجابة لدرجات الحرارة المختلفة ، و الهرمون المماثل لهرمون النمو، كما يهدف الى اختبار قدرة هذه الخلايا على تكوين بروتينات: بيتا أميلويد ، و بيتا أكتين ، و مستقبلات ن ميثيل د-أسبارتات ، و اينولاذ الخلايا العصبية. **النتائج.** لقد وجدنا أن رفع درجة حرارة الخلايا المزروعة إلى 39 درجة مئوية مع ارتفاع نسبة الجلوكوز، و العوامل الاخرى التى تساعد على النمو أدى الى خفض سرعة انقسام الخلايا مصحوبا بزيادة قدرتها على النضج والتمايز واستطالة الزوائد العصبية ، و زيادة سمك أجسام الخلايا، وتشكيل اتصالات متشابكة . جميع خلايا الناضجة كونت مستقبلات هرمون النمو مما يوضح أهمية عوامل النمو فى الحفاظ على سلامة الخلايا المزروعة. لقد أثبتنا كذلك نضج الخلايا عن طريق اختبار قدرة هذه الخلايا على تكوين بروتين الاينولاز الخاص بالخلايا العصبية فى درجات حرارة و مدد مختلفة من زراعتها معمليا. وجدنا زيادة فى قدرة هذه الخلايا على تكوين بروتين الاينولاز فى أيام 4 و 8 عند درجة حرارة 39 درجة مئوية مقارنة بيوم 4 عند درجة حرارة 34 درجة مئوية. وجدنا أن خلايا (هاء 7/19) تستطيع تكوين البروتين الهيكلى الخلوى بيتا-أكتين وكذلك البروتينات السيتوبلازمية العصبية بما فى ذلك، بيتا-أميلويد، والأينولاز اللذان يعدان اثنين من السمات التركيبية الرئيسية للخلايا العصبية. و علاوة على ذلك، أظهرت نتائج هذا البحث أن خلايا (هاء 7/19) الناضجة لها القدرة على تكوين البروتينات الخاصة بمستقبلات ن-ميثيل-د-أسبارتات مثل ن ر 1 و ن ر 2 أ. كما أظهرت نتائج الفحص الخلوى المناعى التفاعلى التوزيع الموحد لبروتينات البيتا -أكتين و الاينولاز ، البيتا -أكتين و ن ر 2 ، و البيتا أميلويد و ن ر 1 بروتين مما يشير إلى دور البيتا أكتين و البيتا اميلويد فى الوظائف الخاصة بمستقبلات ن-ميثيل-د-أسبارتات عند مواقع الاتصال العصبى. الاستنتاج. وتشير هذه الدراسة إلى أن خلايا (هاء 7/19) تمثل نموذجا مثاليا للخلايا المزروعة التى تكشف عن آليات نمو و نضج الخلايا العصبية ومن ثم استخدامها فى علاج الامراض العصبية فى المستقبل .