Article *NIMHANS Journal*

Neuronal Aging in-vitro - An immunofluorescence Study using Chick Embryo Spinal Cord Cultures and Antibodies against Non-phosphorylated and Phosphorylated Neurofilaments

Volume: 11 Issue: 01 January 1993 Page: 49-54

T R Raju.

Reprints request
&, B L Meti,
- *Department of Neurophysiology, National Institute of Mental Health & Neuro Sciences, Bangalore 560 029, India*

Abstract

Dissociated eleven day old chick embryonic spinal cord cells were cultured and grown up to six days in-vitro. Temporal expression of non-phonsphorylated ands phosphorylated neurofilament (NF) epitopes was studied using appropriate antibodies. Antibodies against non-phosphorylated NF stained both soma and processes over different time periods in culture. However, antibodies against phosphorylated epitope generally stained only processes. The interesting finding was that on day six, about 10-15% of neuronal soma were also labelled by this antibody. The occurrence of phosphorylated NF in the soma of few neurons may represent an aging process in culture in the absence of any other toxic factors or stress which would induce aberrant phosphorylation.

Key words -
Spinalcord,
Neurons culture,
Neurofilament phosphorylation,
Aging

Research into defining the biological basis of neuro-degenerative diseases such as Alzhemer's, Parkinson's and Amyotropic Lateral Sclerosis (ALS) is handicapped by the non-availability of suitable animal models [1]. The lack of suitable animal models has seriously hampered the search for agents present in these patients which are capable of inducing pathological changes in the neurons. One of the hallmarks of these diseases is the formation of neurofibrillary tangles (NFT) which may block the normal axonal transport. In fact Gadjusek [2] has hypothesized that interference with axonal transport of NF could be a common pathogenic mechanism in certain diseases of the central nervous system. NF is a major component of the cytoskeleton of neurons; it ismade up of three peptides of molecular weights 200 kd, 150 kd and 70 kd [3]. Antibodies against NF have been successfully used to study neuronal differentiation in - vitro as well as in-vivo [4].

The largest of the NF triplet peptides appears much later in the development [5], [6]. Although all three peptides are phosphorylated, 200 kd and 150 kd peptides have more phosphorylation sites compared to 70 kd peptide [7]. NF peptides are phosphorylated at the site close to the origin of axons and transported to the terminals. However in certain diseases such as Alzheimer's, ALS, Progressive Supra Nuclear Palsy and Creutzfeldt Jacob's NF are phosphorylated within the

perikaryon [8], [9], [10], [11]. This abnormal phosphorylation leads to cross linking of NF with other cytoskeletal elements resulting in the formation of NFT. Even in normal aging process NF appears as phosporylated in the perikaryon of some neurons [1].

In this study wehave used cultured eleven day old chick embryonic spinal cord cells and compared the expression of non-phosphorylated and phosphorylated forms of NF in neurons over a six day period in culture. We hope this study would lead us to create a model for testing any agent(s) present in the serum or CSFor muscles of patients suffering from motor neuron disease which would cause accelerated aging of spinal cord neurons. This would be reflected by the appearance of phosphorylated epitopes of NF in the perikarya of neurons.

Material and Methods

Culturing of spinal cord cells

Spinal cord cells were dissociated from eleven day old embryonic chicks (white leghorn) after decapitation. The cords were freed of meninges and cut into small pieces. The tissue was then incubated with 0.025% trypsin (Sigma, USA) in Ca^{++} and Mg^{++} free Hank's balanced salt solution. Incubation with trypsin was carried out at 37° C for 30 minutes. The activity was stopped by the addition of culture medium containing 10% fetal calf serum (Flow Laboratories, U.K.). The tissue was then dissociated into single cells by a syringe fitted with a 19g needle. Dissociated cells were plated on 13mm glass coverslips placed in 24 wellculture plates (Laxbro, India). Seeding density was around 2,50,000 cells per coverslip. Culture medium was Dulbecco's modified Eagle's medium (GIBCO, USA) with 10% fetal calf serum and cultures were grown at 37° C in a 5% CO2 incubator. Cells were fixed after 2, 4 and 6 days of culturing with 95% ethanol - 5% acetic acid mixture.

Immunofluorescence labelling of spinal cord neurons with NF antibodies

Monoclonal antibody JJ8 against nonphosphorylated epitopes of 150 kd and 200 kd peptides were kind gifts from Doctors Dahl and Bignami [6]. Monoclonal antibody SMI 31 against phosphorylated NF was obtained from Stemberger-Myer immunocytochemical Inc. (USA). Before staining, the fixed cells were thoroughly washed with Phosphate Buffered Saline (PBS) and incubated with primary antibodies JJ8 and SMI 31 at 1:20 and 1:1000 respectively for 60 minutes at room temperature. After washing again with PBS, cover slips were incubated with fluorescence conjugated anti mouse immunoglobulins (Cappel, anti mouse immunoglobulins (Cappel, USA) for 60 minutes. Cells were viewed with a Leitz microscope equipped for epifluorescence illumination. Photographs were taken with a 400 ASA colour positive film.

The number of experiments carried out in this study was four; six coverslips were used for each experiment. Cells stained with second antibody alone, served as control. Counting of NF positive cells with both antibodies was carried out over the entire field of coded coverslips. For quantification of neurons, transmitted light was used in conjunction with fluorescence illumination. This enabled us to identify the soima of neurons which were not labelled by phosphorylated antibody.

Results

Immunofluorescence labelling with the antibody against non-phosphorylated epitopes of NF

The 2, 4 and 6 day old cultures contained neurons whichwere stained with this particular antibody. As shown in figure 1, soma as well as processes were positively labelled in 2 day spinal cord cultures. Some neurons were multipolar with 4 to 5 processes emanating from the soma. The neuronal soma diameter varied from 8 to 22 microns and the neuron shown in this figure with a soma size of 20 microns is probably a motor neuron. However this can be confirmed only by using specific antibodies against motor neurons or by injecting the target with a retrograde tracer before the dissociation of spinal cords. No difference in the staining pattern was observed at all time points (2, 4 and 6 days) tested. Soma as well as processes of neurons were stained by this antibody.

- ***Two day old spinal cord culture immunofluorescently labelled with non-phosphorylated NF antibody. Note soma as well as processes stained with this antibody ~500x.***

Immunofluorescence labelling with the antibody against phosphorylated epitopes of NF

When cultured neurons were stained with the monoclonal antibody SMI 31, which is against the phosphorylated 150 kd and 200 kd NF peptides, there was a difference in the pattern of staining over a time period in culture. In 2 and 4 day cultures only the processes of neurons were labelled by this antibody (figures 2 and 3). The interesting finding was that in 6 day cultures, in about 13% (± 2.16 s.d) of the total neurons stained, neuronal soma were also labelled (figure 4). This is quantitatively represented in figure 5. Even those neurons whose soma were stained by this antibody did into have dystrophic neurites, as determined by the absence of any intermittent staining pattern with NF antibody or any terminal swelling of the processes.

- ***Two and four day old culture immunofluorescently labelled with phosphorylated NF antibody showing only processes labelling ~500x.***
- ***Six day old culture showing labelling of both perikaryon and neurites with phosphorylated NF antibody ~800x.***
- ***Quantitative comparison of neurons with soma staining over a 6 day culture period with antibodies against non-phosphorylated and phosphorylated epitopes of NF. (Error bar represents standard deviation of the mean with n=4)***

Discussion

Our study shows that spinal cord neurons in normal culture conditions continue to express non-phosphorylated form of NF both in perikarya and neurites; phosphorylated form of NF both in perikarya and neurites; phosphorylated form of NF was present only in neurites during early stages of culture. This finding is consistent with previous report [5]. However phosphorylated NF appears in perikarya of 10-15% neurons after 6 days in culture. A recent study using an antibody against phosphorylated forms of 68 kd and 150 kd NF peptide in rat spinal cord cultures also showed that soma of a few neurons were stained by this phosphorylated antibody after 14 days in culture [13]. However no quantification has been reported in this work. The changes that are observed in-vitro are not present in-vivo in the developing chick embryo spinal cord between embryonic day 13 and 16. (Bignami personal communication).

The neurons with phosphorylated NF in the cell soma did not have any vacuolation or condensation of

the nucleineurons were also phase bright without any dystrophic neurites. Accordingly, it can be concluded that although these neurons expressed phosphorylated NF expression can also be referred to as a sign of aging in culture, since cells were not subjected to any specific stress such as heat shock or withdrawal of serum or any other essential component of the medium. Phosphorylation of NF is an intial event in the formation of neurofibourillary tangles. It has been reported that in normal aging also, neurofibrillary tangles are present in he soma of some neurons [8]. It is possible that some kind of altered gene expression is taking place in culture situation.

The possibility that phosphorylation of NF in the soma may also be due to toxins such as excitatory amino acids (EAA) and not due to aging in-vitro must be considered. The observations reported here could be a response of neurons to injury.The toxic effect of EAA on rat primary cultures has been investigated [14], [15]. In the former study, the authors have observed that 75% of neurons dies after 20 minutes exposure to glutamate. The toxicity could be detected into ehlater study only when glutamate concentration reached 100μ M to 1mM in the culture media, and by 48 hours 90% of the neurons were dead. However the changes observed in our work occurred only after 6 days in culture indicating the absence of any endogenous glutamate like substances induced toxicity.

This work underscores the fact that if in-vitro culture model is used for studying degenerative disorders of the nervous system as determined by the phosphorylation of NF inthe soma, it must be taken into consideration that a small but significant population of neurons exhibit these changes over a time period in culture, even under normal conditions.

Acknowledgements

The authors are indebted tot he Director of NIMHANS, for the support and Dr. V. Ravi & Dr. A. Chandramuki for allowing us to use the laboratory facility. Many thanks are also due to Mr.c. Narayan Kutty for his assistance.

1.Cole G M & Timiras P S, Aging related pathology in human neuroblastoma and teratocarcinoma cell lines
In: Vernadakis A (Ed). Model System. Martinus Nijhoff Publishing Page: 453-473, 1987
2.Gadjusek D C, Hypothesis: Interference with axonal transport of neurofilament as a common pathogenic mechanism in certain diseases of the central nervous system
New England Journal of Medicine Page: 312: 714-719, 1985
3.Hoffman P N & Lasek R J, The slow component of axonal transport. Identification of major structural polypeptides of the above and the generality among mammalian neurons
Journal of Cell Biology Page: 66: 351-356, 1975
4.Raju T, Bignami A & Dahl D, In vivo and in vitro differentiation of neurons and astroccytes in the rat embryo. Immunofluorescence study with neurofilament and glial filament antisera
Developmental Biology Page: 85: 344-357, 1981
5.Dahl D, Early and late appearance ofneurofilament phosphorylated in rate nervous system development: In vivo and in vitro study with monoclonal antibodies
Journal of Neurosciences Research Page: 20: 431-441, 1989
6.Dahl D, Labkovsky B & Bignami A, Early and late appearance of neurofilament phosphorylation events in nerve regeneration
Brain Research Bulletin Page: 22: 225-232, 1989

7.Nixon R A & Sihag R K, Neurofilament phosphorylation: A new look at regulation and function
TINS Page: 14: 501-506, 1991
8.Cork L C, Sternberger N H, Sternberger A A, Casanova N F, Struble R G & Prince D L, Phosphorylated neurofilament antigens in neurofibrillary tangles in Alzheimer's disease
Journal of Neuropathology & Experimental Neurology Page: 45: 54-64, 1986
9.Munoz D C, Greene C, Perl D P & Selkoe D J, Accumulation of phosphorylated neurofilaments in anterior motoneurons of amyotropic lateral sclerosis patients
Journal of Neuropathology & Experimental Neurology Page: 47: 9-18, 1988
10.Shankar S K, Nagaraja D & Liberski P P, Evidence for interference to axoplasmic flow in Creutzfeldt-Jacob disease; Ultrastructural and immunochemical study
Neurology (India) Page: 37: 555-564, 1989a
11.Shankar S K, Yangihara R, Garrutto R M, Grundke-Iqbal I, Kosik K S & Gadjsek D C, Immunocytochemical characterization of neurofibrillary tangles in amyotropic lateral sclerosis and Parkinson's dementia of Guam
Annals of Neurology Page: 25: 146-151, 1986b
12.Cole G M, Wu K & Timiras P S, A culture model for age related human neurofibrillary pathology
International Journal of Developmental Neurosciences Page: 3 (1): 23-32, 1985
13.Bigot D & Hunt S P, The effects of quisqulate and nocodazone on the organisation of MAP 2 and neurofilament in spinal cord neurons invitro
Neurosciences Letter Page: 131: 21-26, 1991
14.Michaels R L & Rothman S M, Glutamate neurotoxicity in-vitro: antagonist pharamacology and intracellular calcium concentration
Journal of Neuroscience Page: 10: 283-292, 1990
15.Mattson M P, Murrian M, Cuthrie P B & Kater S B, Fibroblast growth factor and glutamate opposing roles in the generation and degeneration of hippocampal neuroarchitecture
Journal of Neuroscience Page: 9: 3728-3740, 1989