

Auto-antibodies against proteins of spinal cord cells in cerebrospinal fluid of patients with amyotrophic lateral sclerosis (ALS)

Irena Niebroj-Dobosz¹, Dorota Dziewulska^{2,3}, Piotr Janik³

¹ Neuromuscular Unit, Medical Research Centre, Polish Academy of Sciences; ²Department of Experimental and Clinical Neuropathology, Medical Research Centre, Polish Academy of Sciences; ³Department of Neurology, Medical University of Warsaw, Warsaw, Poland

Folia Neuropathol 2006; 44 (3): 191-196

Abstract

Aetiology and pathogenesis of amyotrophic lateral sclerosis (ALS) is still a mystery. Among several hypotheses autoimmune mechanisms are also taken into account. We report here our investigations of auto-antibodies against proteins of spinal cord cells in the cerebrospinal fluid (CSF) and serum of ALS patients. The results were correlated with the severity of disease course. The subjects were 57 ALS patients (29 severe, 28 mild) and 10 normal controls. The major finding in CSF was the presence of antibodies against a 70 kD protein in the majority of ALS patients. This protein was identified as neurofilament 68. The second protein of high reactivity and frequency of appearance was a 82 kD protein, which was identified as α -actinin. Less reactive and less frequent were antibodies directed against 55 kD and 40 kD proteins. They were immunologically defined to be related to desmin and actin, resp. The difference between the reactivity of anti-neurofilament and anti-desmin related protein in the severe and mild ALS groups was significant. More frequent were the anti-neurofilament antibodies in the severe ALS cases as compared to the milder ones. In normal CSF, antibodies directed against 55 kD, 70 kD and 82 kD proteins were present in traces and appeared in 5%, 20% and 10% of cases, respectively. In the serum of 30% of severe ALS patients traces of antibodies against 70 kD protein were detected. The morphological studies in the presence of CSF of ALS patients revealed pronounced immunoreactivity of spinal cord neurons, mainly within anterior horns.

The significance of the presence of auto-antibodies in CSF of ALS patients against cellular proteins of the spinal cord is hard to define. It is conceivable that they appear as a secondary immunological consequence of neuronal death. It is also possible that they may accelerate the course of neuronal degeneration.

Key words: amyotrophic lateral sclerosis, autoimmunity, antibodies.

Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by degeneration of large pyramidal neurons in the motor cortex, the corticospinal tracts, and the motor

neurons in the spinal cord anterior horns. The pathogenesis of these changes is still uncertain. Different causes of the disease are suggested. The origin may be 1) genetic with mutations of the SOD1 gene, 2) environmental, 3) persistent viral and prion

Communicating author:

Irena Niebroj-Dobosz, MD PhD, Neuromuscular Unit, Medical Research Centre, Polish Academy of Sciences, 5 Pawińskiego St., 02-108 Warsaw, Poland, tel./fax: +48 22 658 4501, e-mail: neurmyol@cmdik.pan.pl

infection, 4) excitotoxicity, 5) oxidative damage, 6) disorganization of neurofilaments, 7) impaired mitochondrial functions, 8) deficit of neurotrophic factors and 9) autoimmunity [4, 19]. The right answer to the question of autoimmune origin, in a subset of ALS cases, is essential in, at least, efforts of therapeutic procedures in this disease. The link between the presence of auto-antibodies in ALS and neuronal injury is not clear, the more so as the presence of antigen/antibodies is so far not specific for ALS. Indications of autoimmune mechanisms are first derived from experimental models of Engelhardt et al. [10]. Autoimmune mechanisms in ALS are also suggested through the high frequency of serum monoclonal gammopathy, association of thyroid diseases, and lymphocytic infiltration of spinal cord and brain. Several antibodies, detected with variable frequency, are associated with a subset of ALS patients. These are antibodies to gangliosides [16, 17, 25], calcium channels [24], neurofilament subunits [6, 26], Fas [22], tubulin, glial fibrillary acidic protein (GFAP), β -amyloid peptide, myelin basic protein, heparan sulphate proteoglycan, S100 and the Tau protein [26], acetylcholinesterase [5] and unidentified proteins of 150-200, 70, 50 kD [3, 23]. The antibodies against neuronal antigens in the majority of reports are detected in serum. No consistent changes of auto-antibodies in CSF of ALS patients are presented yet [16, 25, 26].

The presence of several antibodies may support the suggestion of autoimmunity in a subset of ALS patients. Its role in ALS remains, however, uncertain. The immune responses evoked by some of the spinal cord antigens may induce damage to motor neurons, and/or may be the consequence of neurodegeneration and neuronal death.

The aim of this study was to characterize the proteins in the spinal cord which may be targets for auto-antibodies in the CSF and serum of ALS patients and also to check whether their presence is related to the course of this disease.

Patients and methods

A total of 57 patients with ALS (34 male, 23 female) aged 52 ± 11 , ranging from 26 to 69 years, were examined. The patients were diagnosed as classical ALS (29 cases), progressive bulbar palsy (11 cases), primary spinal muscular atrophy (8 cases), and probable upper motor neuron syndrome (9 cases). The duration was 28 ± 23 months (ranging from 5 to 192 months). The clinical course of the disease in 29 patients was severe, in 28 mild. The

control group comprised 10 age-matched healthy subjects.

CSF and blood were taken on the same day after overnight fasting and centrifuged at 3000 rpm for 10 min. The supernatant was frozen at -72°C until used.

For the morphological analyses spinal cord of a 50-yr-old non-neurologic subject was taken and frozen. The sections were incubated first with 0.5% hydrogen peroxide to block the activity of endogenous peroxidase, then overnight with ALS patients' CSF, followed by antibodies against human immunoglobulins (Rabbit anti-Human immunoglobulins, Dako 1:100), secondary antibodies labelled streptavidin-biotin (Dako LSAB2+HRP system), and 1% 3,3'-diaminobenzidine used as chromogen.

For immunochemical examination the same spinal cord was quickly frozen, weighed and homogenized in 50 mM phosphate buffer (pH 7.5) with protease inhibitor cocktail, consisting of 5 mg leupeptin, 5 mg pepstatin, 5 mg antipain and phenylmethylsulphonyl fluoride. 10 μl of 1.7% dimethyl sulphoxide was added per 1 ml of the buffer. The homogenates were frozen in small quantities to avoid repeated freezing/thawing. The concentration of proteins in the spinal cord was estimated by the method of Peterson [18]. The same spinal cord homogenate was further used for all sets of antibody analyses. 100 μg of the proteins were separated by SDS-polyacrylamide gel electrophoresis in the Mini-Protean II system (Bio-Rad) on 4-15% gradient gels and developed according to Laemmli [13]. Afterwards the proteins were blotted on nitrocellulose membranes 0.2 μm (LKB) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The nitrocellulose sheets were overlaid with a 3% blocker in PBST (Bio-Rad), then CSF/serum of patients and controls at dilutions of 1:50 in 1% BSA-PBST in Mini-Protean Multi-Screen (Bio-Rad) was applied and incubated for 1 hr. After washing secondary IgG goat anti-mouse was used. The nitrocellulose sheets were further processed by means of the GelDoc 1000/2000 system and imaging densitometer GS-700 (Bio-Rad), Multi-Analysts/PC version 1.1, Quantity One version 4 and P91 Mitsubishi Video Printer. For identification of 82 kD, 70 kD, 55 kD and 40 kD monoclonal antibodies against α -actinin, neurofilament 68, desmin and actin (Sigma) in appropriate dilutions were incubated with the separated spinal cord proteins. Their Rf values were calculated and compared to those of the immunochemically stained bands in the presence of

CSF. One lane of each blot was loaded with 1% BSA-PBST to detect non-specific binding of peroxidase-labelled second antibodies. No staining was observed.

Statistical analysis

The variances and significance analysis by Wilcoxon's and Student's test were calculated. Fisher's exact test to compare the frequency of positive results was used in the tested groups.

Results

The immunocytochemical examinations indicated an evident cytoplasmatic reactivity in neurons of spinal cord anterior horns (Fig. 1). No reactivity in the other spinal cord cell and structures was present.

In ALS there was a positive reaction for 82 kD, 70 kD, 55 kD and 40 kD in 80.7%, 80.6%, 59.7% and 19.2% of patients, respectively. There were significant differences in reactivity against the 70 kD and 55 kD proteins when the severe and mild ALS groups were compared (Fig. 2, Table I). The auto-antibodies against the 70 kDa protein (neurofilament 68) appeared also to be more frequent in the severe ALS group than in the milder cases (Table II). In control CSF traces of reactivity against the 55 kD, 70 kD and 82 kD proteins were present. They appeared in 5%, 20% and 10% of tested normal CSF samples. In the serum of 30% of the severe ALS group traces ($0.3 \pm 0.2 \text{ mm} \times 10^{-4} \text{ OD}$) of anti-70 kD protein antibodies were observed. In control serum no reactivity was observed.

The reactive bands of the spinal cord cell proteins in the presence of CSF of the ALS patients were identified immunochemically as α -actinin, neurofilament 68, desmin and actin.

No correlation between age, sex, disease duration and antibody reactivity and the frequency of their appearance was present.

Discussion

A number of reports indicate the presence of auto-antibodies in the serum of ALS patients. Less frequent are data concerning the appearance of auto-antibodies in the CSF of these patients. Several questions need to be answered: 1) Are the antibodies primarily involved in the disease pathomechanism(s)? 2) Do they appear

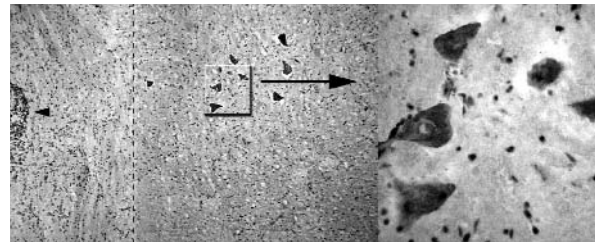


Fig. 1. Immunocytochemical examination of spinal cord sections in the presence of CSF. Visible cytoplasmatic reactivity of neurons in the anterior horn, insert – magnification of the marked area. Arrowhead – central canal

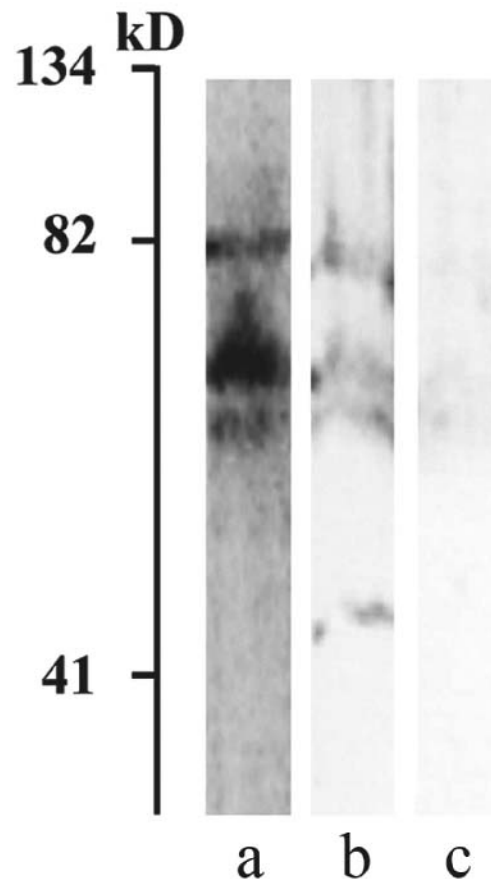


Fig. 2. Western blots showing antibodies' reactivity against spinal cord proteins in cerebrospinal fluid of a severe ALS patient (a), a mild ALS patient (b) and a control case (c). The scale on the left indicates relative molecular mass of the proteins $\times 10^3$

Table I. Staining intensity of IgG antibodies in cerebrospinal fluid against spinal cord proteins in amyotrophic lateral sclerosis patients (mm x 10⁻⁴ OD)

| Mol. wt. kD | ALS (57) | ALS severe (29) | ALS mild (28) | Controls (10) |
|-------------|-----------------------|------------------------|-----------------------|---------------|
| 82 | 25.9±3.2 ¹ | 28.6±4.3 ¹ | 23.2±2.3 ¹ | 0.2±0.1 |
| 70 | 22.5±2.5 ¹ | 29.9±2.9 ^{1a} | 15.1±2.1 ¹ | 0.8±1.8 |
| 55 | 16.5±3.7 ¹ | 22.3±3.9 ^{1b} | 11.6±2.3 ¹ | 0.4±1.0 |
| 40 | 10.2±3.7 ¹ | 15.1±5.1 ¹ | 5.3±2.2 ¹ | 0 |

The values are means ± SE. The number of examined patients and controls is given in parentheses. Comparison of ALS patients and controls: significant at ¹ P < 0.001.

Comparison of severe ALS vs mild ALS: significant at ^a P < 0.001, ^b P < 0.02

Mol. wt. – molecular weight

Table II. Frequency (%) of IgG antibodies in cerebrospinal fluid against spinal cord proteins in amyotrophic lateral sclerosis patients

| Mol. wt. kD | ALS (57) | ALS severe (29) | ALS milder (28) | Control (10) |
|-------------|-------------------|--------------------|-------------------|--------------|
| 82 | 80.7 ¹ | 82.8 ¹ | 78.6 ¹ | 10 |
| 70 | 80.6 ² | 97.0 ^{1a} | 64.3 ⁵ | 20 |
| 55 | 59.7 ² | 58.6 ⁴ | 60.7 ³ | 5 |
| 40 | 19.2 | 27.0 | 10.7 | 0 |

The values are means ± SE. The number of examined patients and controls is given in parentheses.

Comparison of the ALS total material, the severe group, and the mild group vs controls: Fisher's exact test: significant at ¹ P < 0.0001, ² P < 0.0004,

³ P < 0.0007, ⁴ P < 0.001, ⁵ P < 0.0197

Comparison of severe ALS vs mild ALS: Fisher's exact test: significant at ^a P < 0.0022.

Mol. wt. – molecular weight

as a consequence of the antigen release from damaged neural tissues? 3) Do the antibodies accelerate neuronal degeneration? 4) Are the auto-antibodies in CSF synthesized intrathecally? 5) Do the examinations of auto-antibody profiles have any diagnostic or prognostic value?

Circulating IgG/IgM antibodies against human spinal cord motor neurons are already reported in about half of ALS patients [14]. They are, however, often found also in other diseases of the nervous system, and even in a small proportion of normal controls. In about 32% of ALS patients, but also in 27% of other neurological diseases, glial and axonal reactivity in the presence of ALS serum and

CSF is described. Staining of endothelial cells by serum and CSF in 24% of ALS patients is indicated to be specific for this disease [12].

According to our results among different proteins of the spinal cord only some of them became targets for auto-antibodies. Neurofilament 68, α -actinin, desmin and actin responded immunologically to the CSF of ALS patients. Reactivity against neurofilament 68 subunit was higher in the ALS severe cases than in the milder ALS group. Also the frequency of their appearance was higher in the severe ALS group than in the milder one (97% vs 64%). In serum only traces of anti-70 kD antibodies in a subset of severe ALS cases were present.

Neurofilaments have been a subject of interest for several years. Neurofilaments are intermediate filaments of neurons and are composed of three subunits (200, 150 and 68 kD). The 68 kD protein forms the backbone of the protein, while the other subunits are located more peripherally. In morphological examinations of ALS cases an accumulation of phosphorylated neurofilaments and focal-GFAP positive astrocytosis in the precentral cortex is reported [27]. The role of intraneuronal aggregates of neurofilaments in the pathogenesis of ALS is, however, not yet settled. Antibodies against the low molecular weight neurofilament cross-react with the neuronal surface protein [20]. It is also indicated that the anti-neurofilament antibodies, which are present in 25% of ALS patients' sera, react with axons, are directed against different subunits of neurofilaments and are involved in an autoimmune process in a subgroup of ALS patients [6]. There are also data of antibodies directed against the neurofilament heavy subunit in 15% of ALS patients [26]. No relation between antibodies' presence and the age, sex or clinical form of ALS is present; however, their correlation with slow evolution of the disease has been demonstrated [6]. The presence of auto-antibodies against spinal cord 70 kD protein in ALS serum is also indicated in other reports [3, 23]. No data on the presence of anti-neurofilament 68 antibodies in the CSF of ALS patients are available. These two reports also indicate the presence of antibodies in serum directed against spinal cord proteins of other molecular weights. Except for the 70 kD protein, also antibodies against proteins of 150-200 kD and 52 kD in the brain, cerebellum, liver and spinal cord are presented. They react more frequently with ALS sera than that of controls [3, 23].

We also present data showing that α -actinin (82 kD), desmin (55 kD) and actin (40 kD) also become targets for auto-antibodies in the CSF of ALS patients.

No reports on α -actinin antibodies in CSF and serum of ALS patients have been presented yet. In our material the auto-antibodies in CSF directed against this protein were nearly of equal intensity and frequency in both tested groups of ALS patients.

Alpha-actinin links the N-methyl-D-aspartate glutamate receptors to actin cytoskeleton in NMDA subunits [9] and determines the localization of NMDA receptors and their modulation by Ca^{2+} [28]. In

conjunction with other components of the contractile and relaxing system it plays a role in the release of neurotransmitters from synaptic vesicles [21].

The antibodies directed against a 55-kD desmin-related protein were present in equal frequency in the CSF in both groups of ALS, but their reactivity was higher in the severe ALS group. No antibodies in the CSF and serum of ALS patients against this protein have been described yet. In two reports, however, in the ALS sera antibodies against an unidentified 52 kD or 50 kD protein are presented [3, 23]. There is a possibility that these proteins are desmin-related. The significance of this finding is uncertain as desmin in the spinal cord is not present. It is suggested, however, that immunostaining of astrocytes in the presence of anti-desmin antibodies is due to proteins immunologically related to desmin [7].

The reactivity presented here at 40 kD, more intense and frequent although not significant, in the severe ALS group probably belongs to an actin-related protein. No auto-antibodies against this protein and/or its degradation fragments in the body fluids of ALS patients have been described yet. Actin, a 42 kD protein, is a major cytoskeletal protein which takes part in axonal transport. When cleaved by proteases two fragments result (33 kD and 9 kD) [2]. An abnormal protein (32.5 kD), which resembles the larger actin degradation fragment, is detected in Lewy body-like inclusions in the anterior horn cells of ALS patients. It has been suggested that it is an abnormal protein related to actin or to some epitopes common to actin, which are associated with neuronal death in ALS [15].

The presence of several antibodies predominantly in CSF may indicate their intrathecal synthesis. Generally intrathecal IgG synthesis is known to be present in about 60% [8], according to others in 16% [1], of ALS patients. Chronic, compartmental intrathecal immune response of IgM type of low activity is also indicated by the fact that no correlation between the anti-ganglioside titer in serum and CSF is present [16,25]. The occurrence of anti-neurofilament antibodies in the serum in a small number of severe ALS patients may be a consequence of increased blood-CSF permeability, which is present in about 20% of ALS cases [8].

The presence of auto-antibodies is of uncertain pathogenetic significance and may not be of primary aetiological importance. They appear rather as a secondary consequence of neuronal death. It is

possible, however, that the auto-antibodies, although unspecific for ALS and unlikely to be the major cause of this disease, may accelerate the course of neuronal degeneration.

Acknowledgement

The statistical review was performed by Dr. Beata Sokołowska, Medical Research Centre, Polish Academy of Sciences.

References

1. Apostolski S, Nikolic J, Bugarski-Prokopljevic C, Miletic V, Pavlovic S, Filipovic S. Serum and CSF immunological findings in ALS. *Acta Neurol Scand* 1991; 83: 96-98.
2. Bershadsky AD, Vasilev JM. Cytoskeleton. Plenum Press, New York 1988, p. 12.
3. Brown RH, Johnson D, Ogonowski M, Weiner HL. Antineural antibodies in the serum of patients with amyotrophic lateral sclerosis. *Neurology* 1987; 37: 152-155.
4. Cluskey S, Ramsden DB. Mechanisms of neurodegeneration in amyotrophic lateral sclerosis. *Mol Pathol* 2001; 54: 386-392.
5. Conradi, Ronnevi L-O. Further studies on the occurrence of serum autoantibodies against a membrane bound AChE fraction in ALS/MND patients and controls. *J Neurol Sci* 1994; 24: 67-69.
6. Couratier P, Yi F-H, Preud'homme J-L, Clavelou P, White A, Sindou P, Vallat JM. Serum autoantibodies to neurofilament proteins in sporadic amyotrophic lateral sclerosis. *J Neurol Sci* 1998; 154: 137-145.
7. Dahl D, Crosby CJ, Bignami A. Intermediate filament proteins immunologically related to desmin in astrocytes: a study of chicken spinal cord by two-dimensional gel electrophoresis and immunoblotting. *Neurochem Res* 1989; 14: 1013-1016.
8. Domitrz I, Kendziorek A, Wiśniewska W, Niebroj-Dobosz I. Intrathecal synthesis of IgG in amyotrophic lateral sclerosis. *Materia Med Pol* 1998; 31: 44-46.
9. Dunah AW, Wyszynski M, Martin DM, Sheng M, Standaert DG. Alpha-actinin-2 in rat striatum: localization and interaction with NMDA glutamate receptor subunits. *Brain Res Mol Brain Res* 2000; 79: 77-87.
10. Engelhardt J, Joo F. An immune-mediated guinea pig model for lower motor neuron disease. *J Neuroimmunol* 1986; 12: 279-290.
11. Engelhardt JL, Appel SH, Kilian JM. Motor neuron destruction in guinea pigs immunized with bovine spinal cord ventral horn homogenate: experimental autoimmune gray matter disease. *J Neuroimmunol* 1990; 47: 21-31.
12. Greiner A, Schmausser B, Petzold K, Kruger H, Marx A. Neuronal targets of serum and cerebrospinal fluid autoantibodies in amyotrophic lateral sclerosis. *Acta Neuropathol* 1996; 91: 67-71.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-685.
14. Kiernan JA, Hudson AJ. Anti-neurone antibodies are not characteristic of amyotrophic lateral sclerosis. *NeuroReport* 1993; 4: 427-430.
15. Mather K, Watts FZ, Carroll M, Whitehead P, Swash M, Caim N, Burke J. Antibody to an abnormal protein in amyotrophic lateral sclerosis identifies Lewy body-like inclusions in ALS and Lewy bodies in Parkinson's disease. *Neurosci Lett* 1993; 160: 13-16.
16. Niebroj-Dobosz I, Jamrozik Z, Janik P, Hausmanowa-Petrusewicz I, Kwieciński H. Anti-neural antibodies in serum and cerebrospinal fluid of amyotrophic lateral sclerosis (ALS) patients. *Acta Neurol Scand* 1999; 100: 238-243.
17. Pestronk A, Adams RN, Cornblath D, Kuncl RW, Drachman DB, Clawson L. Patterns of serum IgM antibodies to GM1 and GD1a gangliosides in amyotrophic lateral sclerosis. *Ann Neurol* 1989; 25: 98-102.
18. Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* 1977; 83: 346-356.
19. Rowland LP, Shneider NA. Medical progress: Amyotrophic lateral sclerosis. *New Engl J Med* 2001; 344: 1688-1700.
20. Sadiq SA, van den Berg LH, Thomas FP, Kildireas K, Hays AP, Latov N. Human monoclonal antineurofilament antibody cross-reacts with a neuronal surface protein. *J Neurosci Res* 1991; 29: 19-25.
21. Schook W, Ores C, Puszkun S. Isolation and properties of brain alpha-actinin. *Biochem J* 1978; 175: 63-72.
22. Sengun IS, Appel SH. Serum anti-Fas antibody levels in amyotrophic lateral sclerosis. *J Neuroimmunol* 2003; 142: 137-140.
23. Shanina NA, Sveikata AP, Zavalishin IA, Gel'fand VP. Analysis of serum antibodies to nerve tissue antigens in patients with lateral amyotrophic sclerosis (in Russian). *Biull Eksp Biol Med* 1993; 115: 169-173.
24. Smith RG, Hamilton S, Hoffman F, Schneider T, Nastainczyk W, Birnbaumer L. Serum antibodies to L-type calcium channels in patients with amyotrophic lateral sclerosis. *New Engl J Med* 1992; 327: 1721-1728.
25. Stevens A, Weller M, Wietholter H. A characteristic ganglioside antibody pattern in the CSF of patients with amyotrophic lateral sclerosis. *J Neurol Neurosurg Psych* 1993; 56: 361-364.
26. Terryberry JW, Thor G, Peter JB. Autoantibodies in neurodegenerative diseases: Antigen-specific frequencies and intrathecal analysis. *Neurobiol of Aging* 1998; 19: 205-216.
27. Troost D, Sillevs Smitt PA, de Jong JM, Swaab DF. Neurofilament and glial alterations in the cerebral cortex in amyotrophic lateral sclerosis. *Acta Neuropathol (Berl)* 1992; 84: 664-673.
28. Wyszynski M, Lin J, Rao A, Nigh E, Beegs AH, Craig AM, Sheng M. Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. *Nature* 1997; 385: 439-442.