

Down regulation of trophic factors in neonatal rat spinal cord after administration of cerebrospinal fluid from sporadic amyotrophic lateral sclerosis patients

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Abstract Accumulating evidence supports neuroprotective role of trophic factors in amyotrophic lateral sclerosis (ALS). Previous studies from our laboratory report that the CSF of patients with sporadic ALS (ALS-CSF) induces degenerative changes in the rat spinal motor neurons and reactive astrogliosis in the surrounding gray matter. The present study was aimed to investigate if the ALS-CSF affected the expression of trophic factors namely, brain-derived neurotrophic factor (BDNF), fibroblast growth factor 2 (FGF2) and insulin-like growth factor 1 (IGF1) in the newborn rat spinal cords. ALS-CSF was intrathecally injected into the neonatal rats and the mRNA levels of the trophic factors were determined by quantitative real-time polymerase chain reaction. Here, we report significant down regulation in the gene expression of trophic factors

for BDNF, FGF2 and IGF1. BDNF mRNA levels were found to be reduced by 6.8-fold in the ALS-CSF injected group compared to control groups. The levels of IGF1 and FGF2 mRNA were also decreased by 3.91- and 2.13-fold, respectively, in the ALS group. We further found that exogenous supplementation of BDNF considerably reduced the aberrant phosphorylation of neurofilaments, complementing our earlier findings of restored expression of voltage gated sodium channel. Reduced expression of trophic factors indicates an altered microenvironment of the motor neurons and could possibly be one of the contributing factors in the degeneration process.

Keywords Sporadic ALS · BDNF · IGF1 · FGF2 · ALS-CSF · Rat spinal cord

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Introduction

Amyotrophic lateral sclerosis (ALS) is considered as one of the most devastating neurodegenerative diseases with a progressive loss of both upper and lower motor neurons (Rowland 2000). Insight into the molecular mechanisms involved in the pathogenesis of ALS would provide an efficient tool in designing an effective therapeutic intervention (Mitumoto and Tsuzaka 1999; Rothstein 1996; Segal and Greenberg 1996; Strong 2003).

Various studies have provided evidences regarding the application of neurotrophic factors to treat neurological diseases, including ALS. It is very well known that neurotrophic factors are primarily involved in the growth, survival and regulation of neurons during the initial development of peripheral and central nervous system and also play an important role in the functioning and survival of adult nervous system (Barde 1989; Ekester 2004). It

has been suggested that impairment of neurotrophic levels leads to disease progression by causing selective motor neuron death (Ekester 2004). In ALS, loss of trophic support could be one of the underlying factors causing motor neuron degeneration.

Brain-derived neurotrophic factor (BDNF), a member of neurotrophin family, has shown beneficial effect on the survival and differentiation of sensory and motor neurons in vitro (Kishino et al. 1997; Sendtner and Holtmann 1992; Wong et al. 1993; Yan and Elliott 1992). Skeletal muscles, the target tissue of motor neurons express BDNF. Dorsal root ganglion neurons which provide afferent inputs to spinal motor neurons, also express BDNF (Maisonpierre et al. 1990). Thus motor neurons are neurotrophically supported by both target skeletal muscle and afferent neurons (Chiu et al. 1994; Schecterson and Bothwell 1992). Treatment of chick embryo spinal cord with BDNF protects motor neurons from naturally occurring cell death in the lumbar region of spinal cord (Oppenheim et al. 1992).

Insulin-like growth factor (IGF1) is vital for the survival of many types of neurons (Adem and Ekblom 1994; Vincent and Mobley 2004; Lewis et al. 1993). Intrathecal administration of IGF1 improved motor performance and delayed the onset of clinical signs (Lewis et al. 1993). It also stimulates rapid extension of corticospinal motor neurons, which are affected in ALS (Doré et al. 1997). The IGF1 modulates choline acetyl transferase activity and attenuates loss of cholinergic phenotypes (Doré et al. 1997). It stimulates motor neuron proliferation and sprouting as well as differentiation from precursor cells (Doré et al. 1997). In muscles, it promotes acetyl choline receptor cluster formation and increases neuro-muscular function (Scicchitano et al. 2009). Fibroblast growth factor 2 (FGF2), a neurotrophic factor abundantly found in spinal cord, also supports motor neuronal survival (Cuevas et al. 1995; Grothe et al. 1991; Hughes et al. 1993; Ikeda et al. 1995; Kage et al. 2001). Altered levels of these neurotrophic factors have been reported in postmortem tissues of ALS patients (Ekester 2004).

Several studies have reported the involvement of trophic factors in the progression of the disease in familial models of ALS, yet such studies on sporadic ALS (SALS) models are remarkably fewer. We have developed an in vivo and in vitro rat model of SALS in our laboratory using CSF from SALS patients. Exposure of embryonic rat spinal cord cultures to ALS-CSF (in vitro) and intrathecal injection of the same into neonatal rat pups (in vivo)-induced degenerative changes in motor neurons along with the involvement of astrocytes (Gunasekaran et al. 2009; Rao et al. 1995; Shahani et al. 1998; Shobha et al. 2007, 2010). In the present study, we investigated the effect of ALS-CSF on BDNF, FGF2 and IGF1 mRNA expression in the rat spinal cord. We also investigated the effect of BDNF on the

degenerative changes induced by ALS-CSF in cultured spinal cord neurons.

Materials and methods

Neonatal rats used for the experiments were obtained from Central Animal Research Facility of our institute after obtaining the approval from the Institutional Animal Ethics Committee which is in accordance with NIH guidelines. Lactating mothers along with their pups (3 days old) were housed individually, under standard housing conditions with ad libitum access to food and water. CSF injections were carried out as described previously (Shahani et al. 1998). CSF samples were collected by lumbar puncture from clinically diagnosed ALS patients after obtaining their informed consent. Injection of CSF was done twice (3rd and 14th day) using micro injector at the rate of 1 μ l/2.5 min. Animals were divided into four study groups as follows.

1. Normal control (NC): pups not subjected to surgery ($n = 10$)
2. Sham control (SC): pups subjected to surgical procedure only ($n = 10$)
3. Non-ALS-CSF (NALS): pups injected with non-ALS-CSF samples ($n = 10$)
4. ALS-CSF (ALS): pups injected with ALS-CSF samples ($n = 10$).

Quantitative real-time PCR

After 48 h of the second CSF injection, animals were killed and lumbar region of the spinal cord was harvested in RNA later (Ambion, USA) and stored at -20°C to avoid RNA degradation. RNA was isolated using TRIZOL reagent (Ambion). RNA integrity was assessed by agarose gel electrophoresis and concentrations were calculated using a nano drop spectrophotometer. Approximately 3 μ g of RNA was used for reverse transcription using a High Archival cDNA kit (Applied Biosystems). Real-time polymerase chain reaction (PCR) was carried out using ABI Prism 7000 (Applied Biosystems). 18SrRNA was taken as the internal reference control. The amplification protocol consisted of initial Gold *Taq* Polymerase activation at 55°C , 40 cycles of denaturation at 95°C and annealing at 65°C . The amplification efficiency of 18SrRNA and other three gene primers were calculated using fivefold dilutions of cDNA from spinal cords of NC rats. Finally, the PCR data were obtained by Sequence Detection Software (SDS v2.0, Applied Biosystems).

The quantitative expression data for BDNF, FGF2 and IGF1 mRNAs were normalized to constitutively expressed

18SrRNA and relative to NC rats. Relative amplification method was used for quantifying the real-time results ($\Delta\Delta Ct$). Real-time PCR amplification efficiency for target genes and 18SrRNA was calculated using the formula $E = 10^{-1/\text{slope}}$. Average Ct values of target genes like BDNF, FGF2 and IGF1 were obtained in triplicates. PCR reactions were normalized to average Ct values of 18SrRNA from same cDNA samples.

$$\Delta Ct (\text{control}) = \text{mean } Ct (\text{target}) - \text{mean } Ct (18\text{SrRNA})$$

$$\Delta Ct (\text{experimental}) = \text{mean } Ct (\text{target}) - \text{mean } Ct (18\text{SrRNA})$$

$$\text{Mean } \Delta\Delta Ct = \Delta Ct (\text{control}) - \Delta Ct (\text{experimental})$$

The ratio of expression of each target mRNA between control and experimental samples was calculated as $2^{-(\Delta\Delta Ct)}$ (Livak and Schmittgen 2001). The PCR efficiency of the *Taq* man primer–probe mix was also calculated for each gene. The non-template control showed no Ct values indicating absence of genomic DNA contamination. Each reaction volume (25 μl) included 11.5 μl of universal master mix, 1 μl of *Taq* man primer–probe mix (Applied Biosystems), 10 μl of molecular grade water and 2.5 μl of cDNA. The relative gene expression was analyzed by $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001), a method of choice to assess the fold changes in gene expression in experimental groups relative to calibrator (NC), when target gene expression is normalized to endogenous control remaining unaltered after experimental treatment, e.g. 18SrRNA.

BDNF immunohistochemistry

After 48 h of the last injection, the animals were anesthetized with halothane and transcardially perfused using 0.9% saline and 4% buffered paraformaldehyde following which the spinal cords were processed for immunohistochemistry. The vertebral columns of the rats were post-fixed in the same fixative for 24 h. Later, 30 μm thick cryosections at the lumbar level of the spinal cord were thawed on gelatine coated slides. To begin with, the sections were equilibrated using 0.1 M phosphate buffered saline. Thereafter the endogenous peroxidase was quenched by incubating the sections in 70% methanol containing 0.03% H_2O_2 for 30 min at room temperature, in dark. The sections were then incubated with 3% skimmed milk protein for 4 h to block background staining. Thereafter the sections were incubated in anti-BDNF primary antibody (1:1,000, sc-546, Santa Cruz Biochemicals, USA) for 72 h at 4°C. Incubation in biotinylated anti-rabbit secondary antibody (1:200; Vector Laboratories, Burlingame, USA) was followed by application of avidin–biotin complex (1:200, Elite ABC kits; Vector Laboratories, USA). The staining was visualized using 0.05%

3'-3'-diaminobenzidine and 0.03% H_2O_2 as a chromogen as per our earlier protocol (Alladi et al. 2010).

Spinal cord cultures

Spinal cord cultures of embryonic (15–17 days) rats were established from meninges free dissected spinal cords, cultured in Dulbecco's modified Eagle's medium (DMEM). Cultures were supplemented with gastrocnemius muscle extract, 10% fetal bovine serum, antibiotics and other essential amino acids (Eagleson et al. 1985, Shobha et al. 2007; Gunasekaran et al. 2009) and grown on poly-L-lysine coated coverslips for 7 days. The cultures were then exposed to ALS-CSF. Following 48 h of ALS-CSF exposure, the cultures were supplemented with BDNF (10 ng/ml, Sigma-Aldrich Chemicals Co., USA). The cultured cells were immunostained with anti-SMI-31 antibody after 24 h of exposure to BDNF.

Immunofluorescence staining

We followed our earlier established protocol for immunolocalization of phosphorylated neurofilaments (Vijayalakshmi et al. 2009). Briefly, fixed cells on coverslips were washed with PBS and the non-specific staining was blocked using 3% BSA. Thereafter the cells were incubated in SMI-31 antibody (1:1,500; Sternberger Monoclonal Inc., USA) for 24 h at 4°C. The SMI-31 antibody purchased from Sternberger Monoclonal Inc. largely identifies the NF-H subunit and to a lesser extent the NF-M subunit. The FITC tagged anti-mouse secondary antibody was used to detect the immunofluorescence (1:200; Sigma-Aldrich Co., USA). Phosphorylated neurofilament staining was assessed using Leitz Microscope (Leica Microsystems, Germany).

Results

Effect of ALS-CSF on neurotrophic factors mRNA

BDNF showed a down regulation of 6.8-fold in ALS-CSF exposed groups compared to the control. The relative changes in the BDNF mRNA level in ALS-CSF exposed groups are significantly different (-6.868 ± 0.7) from NALS (-1.366 ± 0.466), SC (-2.361 ± 0.36) and calibrator or NC (0.0 ± 0.707 ; Table 1; Fig. 1b). Similarly IGF1 mRNA expression was down regulated significantly in ALS groups, i.e. by 3.9-fold when compared to NALS (-2.32 ± 0.366), SC (-2.54 ± 0.5020) and NC (Table 1; Fig. 1b). FGF2 mRNA levels were also down regulated by 2.19-fold when compared to control groups like NALS (-1.39 ± 0.48), SC (-1.29 ± 0.41) and NC (0.0 ± 0.40 ;

Table 1 Relative quantitation of BDNF, FGF2 and IGF1 mRNA expression by $2^{-\Delta\Delta Ct}$ method as fold changes in gene expression in neonatal rat spinal cord after exposure to CSF from sporadic ALS patients

Groups	18s rRNA average Ct			$\Delta Ct = \text{target} - \text{reference}$			$\Delta\Delta Ct \Delta Ct (\text{exposed}) - \Delta Ct (\text{normal})$			Relative to normal $2^{-\Delta\Delta Ct}$		
	BDNF	FGF2	IGF1	BDNF	FGF2	IGF1	BDNF	FGF2	IGF1	BDNF	FGF2	IGF1
NC	14.96 ± 0.1	26.93 ± 0.70	23.24 ± 0.258	21.006 ± 0.284	11.97 ± 0.707	8.28 ± 0.40	6.04 ± 0.284	0.00 ± 0.707	00 ± 0.40	00 ± 0.284	1	1
Sham	14.19 ± 0.322	27.42 ± 0.17	23.48 ± 0.265	21.581 ± 0.385	13.21 ± 0.364	8.65 ± 0.41	7.39 ± 0.5020	1.24 ± 0.364	0.37 ± 0.41	1.35 ± 0.5020	-2.361	-1.29
NALS	14.82 ± 0.31	27.17 ± 0.35	23.58 ± 0.374	21.075 ± 0.196	12.35 ± 0.466	8.76 ± 0.48	7.255 ± 0.366	0.45 ± 0.466	0.48 ± 0.48	1.215 ± 0.366	-1.366	-2.32
ALS	14.22 ± 0.53	28.975 ± 0.608	23.615 ± 0.46	22.233 ± 0.362	14.755 ± 0.73	9.395 ± 0.7	8.013 ± 0.64	2.78 ± 0.73	1.115 ± 0.7	1.973 ± 0.64	-6.868	-2.16

Table shows average Ct values (threshold cycle number) of endogenous control (18S rRNA) and target mRNA (BDNF, FGF2 and IGF1) expression in NC, SC, NALS and ALS-CSF groups. The reduction in BDNF mRNA (-6.86-fold; $\Delta Ct P < 0.001$) was highest compared to FGF2 (-3.9-fold; $\Delta Ct P < 0.001$) and IGF1 (-2.16-fold; $\Delta Ct P < 0.05$). ΔCt indicates the difference between average Ct values of target and 18S rRNA ($P < 0.001$); $\Delta\Delta Ct$ indicates the difference between ΔCt of normal control and ALS/NALS/SC. Data are represented as mean Ct ± SD that is obtained from sequence detection software of Applied Biosystems (ABI 7000 Prism). Note: since PCR is an exponential process, the relative copy number of target mRNA versus 18S rRNA, $\Delta\Delta Ct$ can be expressed as the power of PCR efficiency

Table 1; Fig. 1b). Data are presented as mean ± SD as detected by Sequence Detection Software (SDS v2.0, Applied Biosystems). Statistical significance was analysed by one-way ANOVA followed by Tukey's post hoc test to compare ΔCt values versus different groups (BDNF $P < 0.001$, IGF1 $P < 0.05$, FGF2 $P < 0.001$).

Effect of ALS-CSF on BDNF expression

The BDNF immunoreactivity was localized to the cytoplasm of the ventral horn neurons as well as in the other cell types in both the control (Fig. 2a) and ALS-CSF injected animals (Fig. 2b). Qualitative examination, however showed loss of BDNF immunoreactive neurons in the ventral horn of the animals injected with ALS-CSF (Fig. 2b).

Effect of BDNF on phosphorylated neurofilament expression

The neurons in control cultures had a healthy phase bright soma with neurites (Fig. 3a). Following exposure to ALS-CSF, the neurites appeared to be beaded and the soma appeared unhealthy (Fig. 3b). The 'beading of neurites' appeared to be reduced and neurite out growth was restored after application of BDNF to cultures (Fig. 3c). Phosphorylated neurofilaments were localized to the neurites/processes in the spinal cord cultures grown under normal conditions (Fig. 3d). In the ALS-CSF exposed cultures, in a large majority of cells the phosphorylated neurofilaments were localized to the cytoplasm (arrowhead; Fig. 3e) and only a few cells showed localization in the neurites/processes (arrow; Fig. 3e). Majority of neurons responded to exogenous supplementation of BDNF (10 ng/ml) as the phosphorylated neurofilament localization in them was restored to the processes (Fig. 3f).

Discussion

Our study provides the first experimental evidence for altered mRNA expression of three trophic factors viz. BDNF, IGF1 and FGF2 in a model of SALS. Down regulation of trophic factor expression in our study may be either the cause or a consequence of degenerative changes induced by patient CSF.

Neurotrophins are capable of antagonizing several distinct apoptotic stimuli in PNS as well as CNS neurons (Ghosh et al. 1994) by interacting with tyrosine kinase receptors (Segal and Greenberg 1996). In addition to rescuing the deprived neurons, neurotrophins can sustain the survival of neurons damaged by a variety of cellular injuries like excitotoxicity, ischemia and neurodegeneration (Apfel 1999). In the spinal cords of ALS patients, three quarters of

Fig. 1 Effect of ALS-CSF on expression of BDNF, IGF1 and FGF2 mRNA in rat spinal cord. **a** The amplification plot of real-time PCR demonstrating the detection of different growth factor mRNAs in postnatal rat spinal cord by sequence detection software using ABI Prism 7000. The graph in **b** shows fold reduction in trophic factor expression, calculated following normalization to the constitutively expressed gene 18SrRNA relative to the value of normal control (here considered as 1). Following ALS-CSF injection BDNF gene expression was decreased 6.8-fold when compared to the normal control. The levels of IGF1 and FGF2 gene expression were also decreased by 3.91 and 2.13-fold, respectively, in the ALS-CSF administered groups

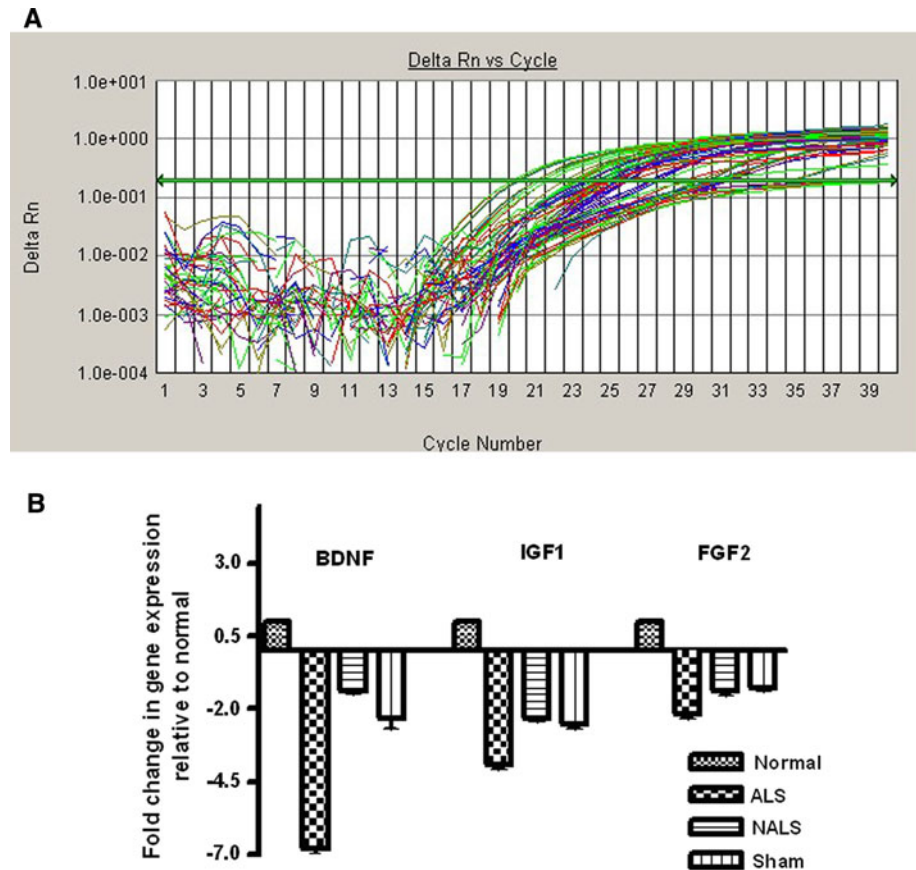
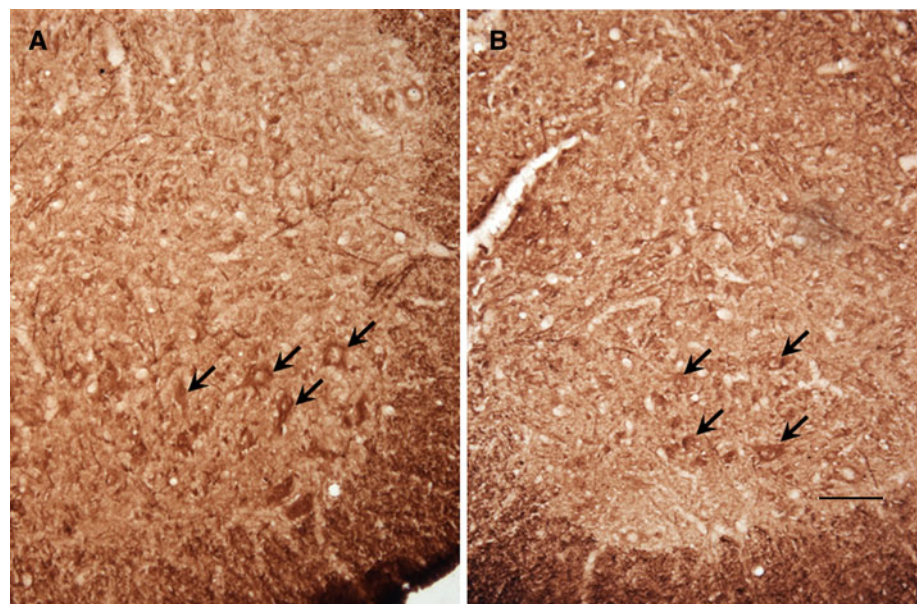


Fig. 2 Effect of ALS-CSF on BDNF expression in the ventral horn neurons of rat spinal cord. Representative photomicrographs of the spinal cord at the lumbar level from **a** normal control animal and **b** animal injected with ALS-CSF. Note the lesser number of BDNF immunoreactive neurons in the ventral horn of the animals injected with ALS-CSF



motor neurons degenerate and remaining demonstrate decreased BDNF levels (Nishio et al. 1998). A controlled trial of recombinant methionyl human BDNF in ALS patients showed an increase in the survival and retarded the pulmonary dysfunction (BDNF study group phase III, 1999, Neurology; No authors listed). Both methionine free and

endogenous BDNF produced beneficial effects in wobbler mouse, as reflected by increased grip strength and running speed (Ishiyama et al. 2002). In a rat spinal root avulsion model, BDNF rescued motor neurons from somal atrophy, prolonged their survival while reversing the loss of cholinergic functions in the motor neurons (Ikeda et al. 1995).

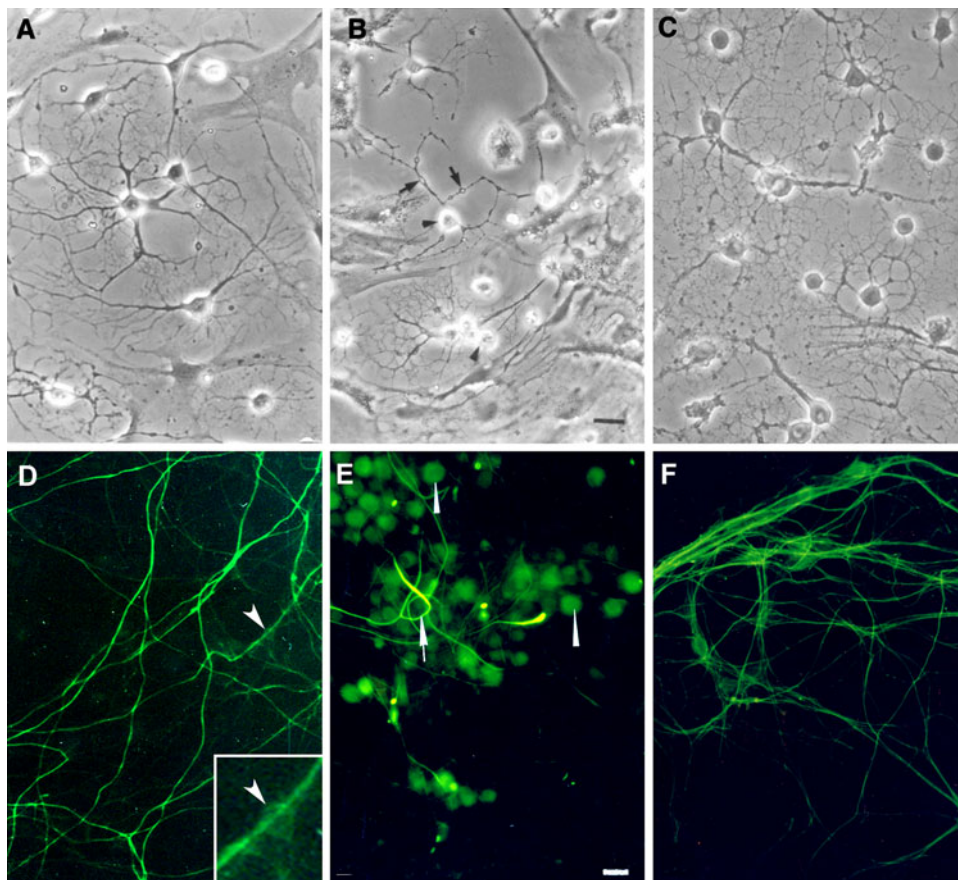


Fig. 3 Effect of ALS-CSF and BDNF on the expression of phosphorylated neurofilaments in primary cultures of the spinal cord. The phase contrast images of the primary spinal cord cultures under control condition (**a**) after exposure to ALS-CSF (**b**) and the ALS-CSF exposed cultures after treatment with 10 ng/ml BDNF (**c**). Exposure to ALS-CSF caused beading of neurites (**b** arrows), which appears to be reduced after treatment with BDNF (**c**). The immunofluorescence photomicrograph shows SMI-31 staining in motor neurons in the embryonic rat spinal cord cultures. In the normal cultures (**d**) only the neuronal processes are stained prominently for

the phosphorylated neurofilaments. An *inset* picture in **d** shows higher magnification image of a cell body (**d** arrowhead). This image was enhanced using Adobe software to highlight the cell body which is normally unstained. In the cultures exposed to ALS-CSF (**e**) the cytoplasm of the neurons is also stained, hence the cell bodies are visible. Exogenous BDNF supplementation after ALS-CSF exposure (**f**) reverses the staining pattern from cytoplasmic to neuritic in most of the cells. Also note the increase in the number of processes as well as reduced beading of the neurites in this group

Motor neurons depend on autocrine and paracrine source of trophic factors, of which astrocytes are a major paracrine source.

Administration of ALS-CSF induces reactive astrogliosis both *in vitro* and *in vivo* (Shahani et al. 1998; Shobha et al. 2010). We deciphered that *in vitro*, the reactive astrogliosis was associated with morphological transformation from flat astrocytes to process bearing ones (Shobha et al. 2010). While the flat astrocytes release trophic factors, the process bearing ones may be responsible for releasing neurotoxic substances like glutamate, nitric oxide, cytokines, etc. (Shobha et al. 2010). Thus the observed reduction in trophic factors could be a consequence of glial transformation, affecting the paracrine trophic support. It could also be due to the direct neurotoxic effect of the CSF, like the reduction of neuronal neurotrophin mRNA levels, resulting in decreased autocrine release. Our observation of fewer

BDNF immunoreactive neurons in the ventral horn of the spinal cords of animals injected with ALS-CSF, may suggest that the patient CSF affects the autocrine secretion too. Reduced expression of neurotrophic factors has been reported in postmortem tissues of ALS patients (Ekesterne 2004).

In addition to inducing astrogliosis, ALS-CSF caused aberrant phosphorylation of neurofilaments in the motor neuronal soma, in primary spinal cord cultures. Aberrant phosphorylation leading to neurofilament accumulation ends in disorganized axon transport and hence is considered as an early sign of neurodegeneration. Absence of aberrantly phosphorylated neurofilaments after exogenous supplementation with BDNF suggests reversal of such axonal disorganization in the affected motor neurons. Amongst the neurotrophins presently examined, the reduction in BDNF mRNA being maximal, studying its

neuroprotective effect was considered, yet further investigations are needed.

In spinal motor neurons, BDNF exerts its trophic effect through PI3-K pathway (Dolcet et al. 1999). BDNF mediated protection could be abolished by the treatment with the PI-3K inhibitor LY294002 (Hetman et al. 2000). In addition, Erk1/2 is also involved in neuro-protective action through growth factor signaling (Segal and Greenberg 1996). Decline in expression of trophic factors in the postnatal rat spinal cord after the exposure to ALS-CSF might result in the stimulation of pro-apoptotic proteins and DNA damage, which can be overturned by the trophic factors if they are available in sufficient quantities.

In an in vitro model of ALS utilizing enriched cultures of embryonic rat spinal cord motor neurons, IGF1 prevented motor neuronal death by blocking the DNA fragmentation and caspase-3 activation, induced by excess glutamate within the ALS-CSF (Barde 1989). Intrathecal viral delivery of IGF1 gene to spinal cord prolongs the life span and delays the disease progression in mice over expressing mutant G93A SOD1 (Kaspar et al. 2003). Thus, alterations seen in the levels of IGF1 could also affect the survival of motor neurons in the present model.

FGF prevents the death of motor neuron in rats after spinal cord injury (Cuevas et al. 1995). Decreased levels of FGF mRNA were reported in the spinal cord of ALS patients compared to controls (Kage et al. 2001). Injection of both EGF and FGF2 into symptomatic transgenic mutant SOD1 mice showed increased neurogenesis in the lumbar region of spinal cord. Similarly, Anand and Parrett (1995) detected decreased levels of ciliary neurotrophic factor (CNTF) in the postmortem tissue of ALS patients indicating that trophic support may be impaired.

In the present study, we have shown a significant decrease in trophic factor mRNA expression in neonatal rat spinal cords following exposure to ALS-CSF. Decreased neurotrophic factors could result in the degeneration of motor neurons as reported by us earlier. It is also supported by the fact that increased availability of BDNF can confer neuroprotection, as shown by the in vitro experiments.

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