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Brain-Derived Neurotrophic Factor Facilitates Functional Recovery from ALS-Cerebral Spinal Fluid-Induced Neurodegenerative Changes in the NSC-34 Motor Neuron Cell Line

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Key Words

Brain-derived neurotrophic factor · Receptor tyrosine kinase B · Choline acetyl transferase · Phosphorylated neurofilaments · Calbindin-D28K · Electron microscopy · Immunochemistry · Laser scanning confocal microscopy

Abstract

Background: The survival of motor neurons is dependent upon neurotrophic factors both during childhood and adolescence and during adult life. In disease conditions, such as in patients with amyotrophic lateral sclerosis (ALS), the mRNA levels of trophic factors like brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), and vascular endothelial growth factor are downregulated. This was replicated in our in vivo experimental system following the injection of cerebral spinal fluid (CSF) of sporadic ALS (ALS-CSF) patients. ***Objective:*** To evaluate the protective role of BDNF in a model of sporadic ALS patients. ***Methods:*** The expressions of endogenous BDNF, its receptor TrkB, the enzyme choline acetyl transferase (ChAT), and phosphorylated neurofilaments were studied in NSC-34 cells. The calcium-buffering and proapoptotic effects were assessed by calbindin-D28K and caspase-3 expression, respectively. ***Results:*** ALS-CSF considerably depleted the endogenous BDNF protein, while its effect on IGF-1 and FGF-2 was inconsequential; this indirectly indicates a key role for BDNF in supporting motor neuronal survival. The exogenous supplementation of BDNF reversed autocrine expression; however, it may not be completely receptor mediated, as the TrkB levels were not restored. BDNF completely revived ChAT expression. It may inhibit apoptosis by restoring Ca^{2+} homeostasis, since caspase-3 and calbindin-D28K expression was back to normal. The organellar ultrastructural changes were only partially reversed. ***Conclusion:*** Our study provides evidence that BDNF supplementation ameliorates most but not all degenerative changes. The incomplete revival at the ultrastructural level signifies the requirement of factors other than BDNF for near-total protection of motor neurons, and, to an extent, it explains why only a partial success is achieved in clinical trials with BDNF in ALS patients.

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Introduction

Neuronal growth and survival are largely dependent on trophic support, and, aptly, neurotrophic factors are key molecules in developmental, maintenance, and differentiation processes as well as functional synapse formation [1–3]. The survival of motor neurons, too, is linked to the levels of trophic molecules as well as of their receptors [4–6]. The depletion of trophic support explains the motor neuron degeneration seen in amyotrophic lateral sclerosis (ALS) patients, which is an adult-onset disease, leading to the loss of both upper and lower motor neurons. ALS occurs either in the less prevalent, autosomal, dominant, inherited, familial form or the predominant (90%), sporadic form.

The major growth factors involved in motor neuron survival include vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), and fibroblast growth factor-2 (FGF-2) [7–10]. IGF-1 mediated motor neuronal survival in in vitro models of ALS; this is ascribed to its ability to prevent glutamate-mediated cell death [11]. Although neuroprotective in animal studies [12–14], limited benefits were seen in clinical trials with patients receiving IGF-1 subcutaneously [15]. Reports on another neurotrophic factor expression, FGF-2, appear to be contradictory. Kage et al. [16] found its levels to decline in the spinal cord tissue of ALS patients, whereas Petri et al. [17] showed that both FGF-2 and FGFR1 were preserved, even at the end stage of the disease. VEGF has recently emerged as a key neurotrophic factor for the growth, survival, and plasticity of motor neurons [18–20].

BDNF, which belongs to the family of neurotrophins, is critical for motor neuron survival [21–23]. It is well known for its range of functions, such as in regeneration, synaptic modulation, and neuroprotection [24], emphasizing its potential role in pathological conditions. Genetically modified fibroblasts, which secrete BDNF, enable the regeneration of rubrospinal, reticulospinal, and vestibulospinal tract axons in a rat model of chronic spinal cord injury [25]. The intravitreous administration of BDNF protects axotomized retinal ganglion cells [26]. Nishio et al. [27] demonstrated a shift of trophic dependence from BDNF to NGF in ALS patients, showing decreased BDNF expression contrasted by increased NGF and TrkA levels in autopsied spinal cord tissue. Despite being extensively studied, the role of BDNF in ALS is less well understood. Its limited success in clinical trials [28, 29] underlines the requirement for detailed studies to investigate its mechanism of neuroprotection.

Our present study follows up our previous findings of a significant downregulation of BDNF mRNA in the spinal cord of rats injected with cerebral spinal fluid (CSF) of sporadic ALS (ALS-CSF) patients, as evidence of insufficient trophic support contributing towards motor neuron degeneration [30]. Here, we report the effect of ALS-CSF on the endogenous expression of BDNF and its receptor TrkB in the NSC-34 motor neuron cell line. We further examined whether exogenously supplemented BDNF affects the expression of choline acetyl transferase (ChAT) and abnormal phosphorylation of neurofilaments (NF) in the degenerating cells. Apoptosis, the key pathway of motor neuron death, was evaluated by immunochemistry of the downstream caspase, i.e. caspase-3. Calcium dysregulation, one of the key mechanisms of neurodegeneration [31], was assessed by calbindin-D28K expression. Further, the ultrastructural changes of cellular organelles were evaluated as a marker of cellular homeostasis by electron microscopy.

Materials and Methods

CSF Collection

Our study has been approved by the Human Ethics Committee of the National Institute of Mental Health and Neurosciences (Bangalore, India). We used CSF from 5 drug-naive patients [20] who had been diagnosed with definite or probable ALS according to the modified Airlie House El Escorial criteria [32, 33]. These patients were assessed for their functional status using the modified Norris scoring system and the ALS Functional Rating Scale (ALSFRS) [34]. The patients' mean age and disease duration matched well with our earlier studies [19, 20]. A neurologist (A.N.) tapped approximately 3 ml of CSF by lumbar puncture following written informed consent from the patients or their kin. The CSF was snap frozen in liquid nitrogen and stored until use at –80°C. We also collected CSF from 5 controls with neurological diseases and named this group NALS. The patients were matched for age and gender and suffered from diseases such as peripheral neuropathy, idiopathic intracranial hypertension, and normal pressure hydrocephalus.

In vitro Experiments

A mouse motor neuron-like hybrid cell line (NSC-34) was used for the in vitro experiments, since these cells largely resemble the human motor neurons [35]. The cells were cultivated in petri dishes containing poly-L-lysine-coated circular coverslips at a concentration of approximately 4.2×10^4 cells/ml. The cells were maintained in a medium consisting of DMEM, 10% FBS (Gibco; Invitrogen Corporation, Carlsbad, Calif., USA), 0.11% HEPES, 0.11% sodium bicarbonate, and antibiotics (100,000 IU penicillin and 0.05% streptomycin; Sigma-Aldrich, St. Louis, Mo., USA). On day 5, the cells were exposed to the CSF from ALS or NALS patients, attaining a final concentration of 10% v/v (CSF) in the growth medium. The control cultures (normal control, NC) were not supplemented with CSF. After 48 h of CSF exposure, the cultures were

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Table 1. Details of antibodies used in the study

Type of labeling	Immunolabeled proteins	Primary antibody	Dilution	Incubation time	Secondary antibody (1:200 dilution)	Incubation time
Triple labeling	Caspase-3	Mouse anti-caspase-3 (Millipore, Temecula, Calif., USA)	1:500	36 h at 4°C	Anti-mouse Cy5	12 h at 4°C
	Phosphorylated NF	SMI-31 (Sternberg Monoclonals Inc., Baltimore, Md., USA)	1:1,800	15 h at 4°C	Anti-mouse Cy3	2 h at RT
	ChAT	Goat anti-ChAT (Chemicon International, Temecula, Calif., USA)	1:500	24 h at 4°C	Anti-goat FITC	2 h at RT
Double labeling	BDNF	Rabbit anti-BDNF (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA)	1:500	24 h at 4°C	Anti-rabbit FITC	2 h at RT
	TrkB	Rabbit anti-TrkB (Santa Cruz Biotechnology, Inc.)	1:500	24 h at 4°C	Anti-rabbit Cy3	2 h at RT
Double labeling	IGF-1	Goat anti-IGF-1 (Santa Cruz Biotechnology, Inc.)	1:500	Overnight at 4°C	Anti-goat FITC	2 h at RT
	FGF-2	Rabbit anti-FGF-2 (Santa Cruz Biotechnology, Inc.)	1:500	Overnight at 4°C	Anti-rabbit Cy3	2 h at RT
Single labeling	Calbindin-D28K	Mouse anti-calbindin-D28K (Abcam, Cambridge, Mass., USA)	1:500	24 h at 4°C	Anti-mouse Cy5	12 h at 4°C
Single labeling (in vivo)	BDNF	Rat-anti BDNF (Chemicon-Millipore)	1:100	72 h at 4°C	Anti-rat Cy3	Overnight at 4°C

RT = Room temperature.

supplemented with human recombinant BDNF (10 ng/ml; Sigma-Aldrich). The survival protocol lasted for 24 h, following which the cells were methanol fixed for immunocytochemistry and grouped as NC+B, NALS+B and ALS+B.

Immunocytochemistry

Methanol-fixed NSC-34 cells on coverslips were equilibrated in 0.1 M phosphate-buffered saline (PBS) pH 7.4. Bovine serum albumin was used as a blocking agent (3% w/v solution) against non-specific epitopes. Subsequently, the cells were incubated in the first primary antibody. Table 1 lists the antibody dilutions and other details. The incubation in the primary antibody was followed by appropriate FITC-/Cy3-/Cy5-conjugated secondary antibodies. For the localization of multiple proteins, i.e. the colabeling of BDNF with TrkB, IGF-1, and FGF-2 as well as ChAT with SMI-31 and caspase-3, the cells were stained sequentially. After completing the immunocytochemistry protocol, the cells were washed in 0.1 M PBS and mounted in PVA-DABCO (Sigma-Aldrich). The observation and analysis of staining were performed using a confocal laser scanning microscope and its inbuilt software (Leica TCS-SL; Leica, Wetzlar, Germany).

MTT Assay

The efficacy of the individual (BDNF or VEGF) and combinatorial therapy (both BDNF and VEGF) was assessed by estimating the cell viability of NSC-34 cells using the MTT assay [36]. Briefly, following 48 h of exposure to ALS-CSF, the cultures were supplemented with human recombinant BDNF, VEGF, or both at different concentrations (BDNF: 5 and 10 ng/ml; VEGF: 75 and 150 ng/ml) for 24 h. These cultures were then treated with MTT solution (5 mg/ml, 37°C) and incubated for 2 h in darkness in a CO_2 incubator. After 2 h, di-methylsulphoxide (DMSO) was added to solubilize the resultant MTT formazan, following which the absorbance was measured at 570 nm in an ELISA plate reader. Finally, the percentage of viable cells was statistically analyzed for all groups. The study groups on the basis of different concentrations of growth factor treatment are listed in table 2.

Electron Microscopy

Cultured NSC-34 cells were pelleted and fixed in 2.5% glutaraldehyde fixative in phosphate buffer (pH 7.2) for 24 h and subjected to routine transmission electron microscopy. Briefly, the pellets were postfixed in 1% osmium tetroxide (for 1 h), dehydrated in grades of ethyl alcohol, and washed in propylene oxide, after which they were embedded in Araldite CY212 resin (TAAB, Reading, UK) and polymerized at 60°C for 48 h. The blocks were cut on a Leica EM UC6 ultramicrotome (M/S Leica Mikrosysteme, Vienna, Austria). Then, 1-μm-thick semithin sections were stained with 1% toluidine blue, and, after initial screening, several ultrathin sections (600–700 Å) were collected on copper grids and stained a using double-staining method with uranyl acetate and lead citrate [36]. Later, the sections were scanned using a Tecnai G2 Spirit Bio-twin (FEI, Eindhoven, The Netherlands) at 80 kVA. The images were captured using a Megaview-III digital CCD camera [20]. The ultrastructural alterations were studied in the NC, NC+B, ALS, ALS+B, ALS+B^5+V^{75} and ALS+B^{10}+V^{150} groups.

In vivo Experiment

The downregulatory effect on the BDNF protein was confirmed in vivo. A total of 5 μl of CSF from ALS or NALS patients was injected intrathecally into Wistar rat pups on days 3 and 14, using a previously described protocol [30]. Briefly, the rat pups in the infusion groups (ALS-CSF or NALS-CSF) were anesthetized with halothane and 5 μl of CSF was injected intrathecally into the

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Table 2. Details of groups included for MTT assay and electron microscopic analysis

Groups	Details of treatment/exposure done	Analysis done
NC	NSC-34 cells with no treatment	MTT assay
NC+B^5	NSC-34 cells with BDNF treatment at the concentration of 5 ng/ml	MTT assay
NC+B^{10}	NSC-34 cells with BDNF treatment at the concentration of 10 ng/ml	MTT assay
NC+V^{75}	NSC-34 cells with VEGF treatment at the concentration of 75 ng/ml	MTT assay
NC+V^{150}	NSC-34 cells with VEGF treatment at the concentration of 150 ng/ml	MTT assay
NC+B^5+V^{75}	NSC-34 cells with BDNF and VEGF treatment at the concentrations of 5 and 75 ng/ml, respectively	MTT assay
NC+B^{10}+V^{150}	NSC-34 cells with BDNF and VEGF treatment at the concentrations of 10 and 150 ng/ml, respectively	MTT assay
ALS	NSC-34 cells with exposure to ALS-CSF treatment	MTT assay
ALS+B^5	NSC-34 cells with exposure to ALS-CSF treatment and with BDNF treatment at the concentration of 5 ng/ml	MTT assay
ALS+B^{10}	NSC-34 cells with exposure to ALS-CSF treatment and with BDNF treatment at the concentration of 10 ng/ml	MTT assay
ALS+V^{75}	NSC-34 cells with exposure to ALS-CSF treatment and with VEGF treatment at the concentration of 75 ng/ml	MTT assay
ALS+V^{150}	NSC-34 cells with exposure to ALS-CSF treatment and with VEGF treatment at the concentration of 150 ng/ml	MTT assay
ALS+B^5+V^{75}	NSC-34 cells with exposure to ALS-CSF treatment and with BDNF and VEGF treatment at the concentrations of 5 and 75 ng/ml, respectively	MTT assay, electron microscopic analysis
ALS+B^{10}+V^{150}	NSC-34 cells with exposure to ALS-CSF treatment and with BDNF and VEGF treatment at the concentrations of 10 and 150 ng/ml, respectively	MTT assay, electron microscopic analysis

subarachnoid space. The rate of CSF infusion was maintained at 1 μl/2.5 min using a microinjector. Then, the incision was sutured, cleaned, and sprayed with Healex (Rallis, Mumbai, India). In the sham group, a needle without sample was inserted into the subarachnoid space of the rat pups for approximately 12 min in order to be comparable with the time taken for CSF infusion. Five CSF samples were used in duplicate. One group of animals (NC) was spared of any procedure. All animals of the study groups (NC, sham, NALS-CSF, and ALS-CSF) were sacrificed at the age of 16 days.

Immunohistochemistry

The rat pups were anesthetized with halothane and perfused transcardially with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer 48 h after the intrathecal injection. The spinal cords were dissected and postfixed in 4% paraformaldehyde for 48 h. Vibrotome sections (40 μm thick) were taken from the lumbar region, and antigen retrieval was performed in a microwave oven for 10 min with 0.1 M sodium citrate solution. Free-floating tissue sections were equilibrated in PBS pH 7.4 with 0.1% Triton X-100 for approximately 1 h. Nonspecific epitopes were blocked using 5% bovine serum albumin in PBS for 2.5 h. The tissue sections were incubated with primary antibody rat anti-BDNF (table 1) followed by secondary antibody tagged with Cy3. Immunofluorescence labeling was captured using a laser scanning confocal microscope. The staining intensity was quantified using the in-built software, as per our previously described methodology [37].

Statistical Analysis

Five different CSF samples in duplicate were used for both the in vivo and in vitro experiments. Each experimental group consisted of 10 rats/culture. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare between and within more than two groups. The values are expressed as means ± SEM, and $p < 0.05$ was considered significant. Student's t test was used when a comparison between two groups was intended.

Results

We found a significant downregulation of BDNF expression in NSC-34 cells following exposure to ALS-CSF; however, IGF-1 and FGF-2 expressions remained unaltered. Therefore, further investigations were focused on the role of BDNF in reversing the neurodegenerative changes induced by ALS-CSF.

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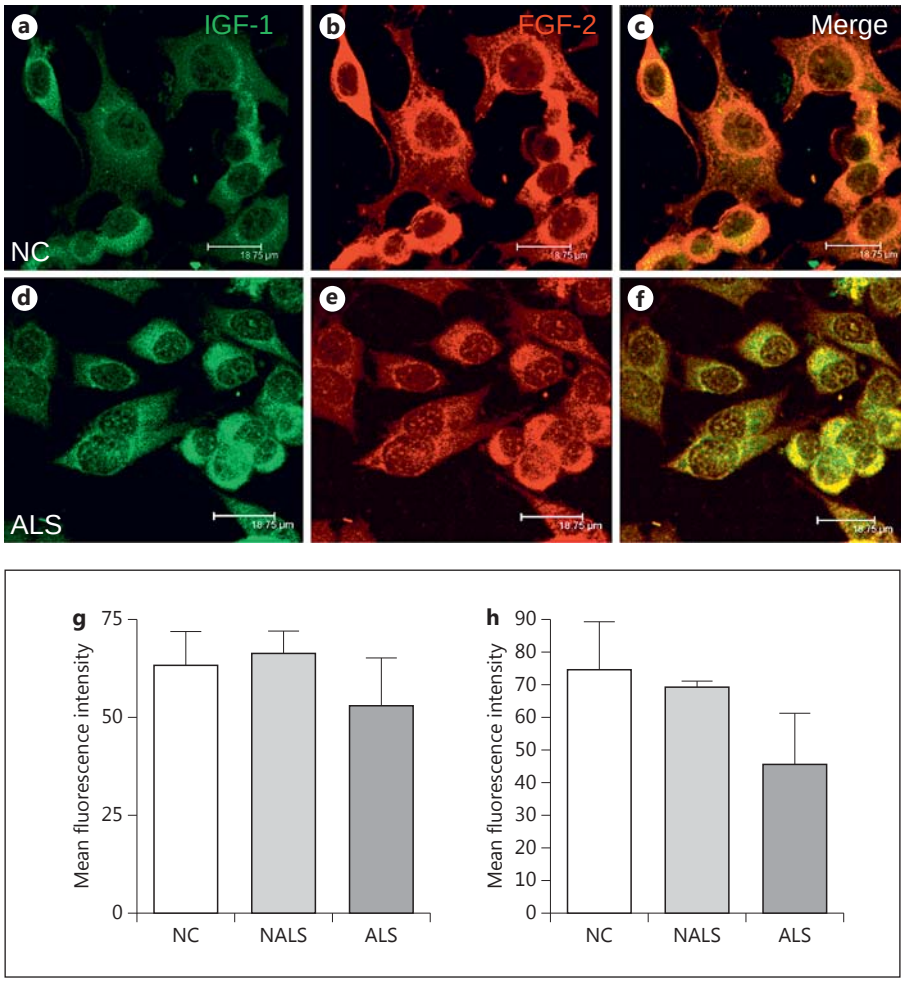

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Fig. 1. Effect of ALS-CSF on IGF-1 and FGF-2 expression in NSC-34 cells. **a–f** Laser scanning confocal photomicrographs of NSC-34 cells showing the expression of IGF-1 (FITC) and FGF-2 (Cy3), respectively, in the NC (**a**, **b**) and ALS (**d**, **e**) groups. IGF-1 and FGF-2 expression was localized to the cytoplasm of NSC-34 cells. FGF-2 expression was more prominently seen in the perinuclear region of the differentiated cells. Of note, ALS-CSF did not induce any significant changes in IGF-1 and FGF-2 levels compared to the control groups (NALS and NC groups). **c**, **f** Merged images of the NC and ALS groups, respectively. Scale bar = 18.75 µm. **g**, **h** There was a marginal reduction in the immunofluorescence intensity (0–255) of IGF-1 (**g**) and FGF-2 (**h**) without any statistical significance. The data were analyzed by one-way ANOVA followed by Tukey's post hoc test.

Effect in ALS-CSF on FGF-2 and IGF-1 Expression in NSC-34 Cells

We observed diffused cytoplasmic localization for both proteins (FGF-2 and IGF-1), with a relatively higher intensity in the perinuclear region. ALS-CSF exposure did not result in any statistically significant quantitative changes; however, a trend toward reduced expression was evident (fig. 1).

Alterations of BDNF and TrkB Expression in NSC-34 Cells

The expression of both proteins (BDNF and TrkB) was pancytoplasmic. BDNF-and TrkB-immunoreactive punctates were seen scattered in the normally diffuse immunostaining texture (fig. 2a–c). The processes of the differentiated cells were also labeled for both proteins (fig. 2c). The BDNF expression was decreased in NSC-34 cells exposed to ALS-CSF (fig. 2d, m; $p < 0.0001$ vs. NC), and a concomitant reduction was evident in the TrkB expression as well (fig. 2e, n; $p < 0.01$ vs. NC). In agreement with our earlier observations, the reduction in fluorescence intensity of BDNF was more pronounced in the differentiated cells. Supplementation of the ALS-CSF group with BDNF resulted in elevated levels of endogenous BDNF (fig. 2j, m; $p < 0.01$ vs. ALS), while those in the NC group were not affected (fig. 2g, m). Contrary to this, BDNF supplementation did not influence the TrkB protein expression in the NC or ALS-CSF-exposed cells (fig. 2h, k, n).

BDNF Expression in Spinal Cord Sections

The expression of BDNF in spinal cord sections was assessed in the lumbar segment. In those injected with ALS-CSF, a significant reduction in BDNF expression was evident in motor neurons (fig. 3a, d, e; $p < 0.01$ vs. NC). A decrease in BDNF staining was also noted in the extracellular matrix of the ventral horn (fig. 3a, d; $p < 0.05$ vs. NC).

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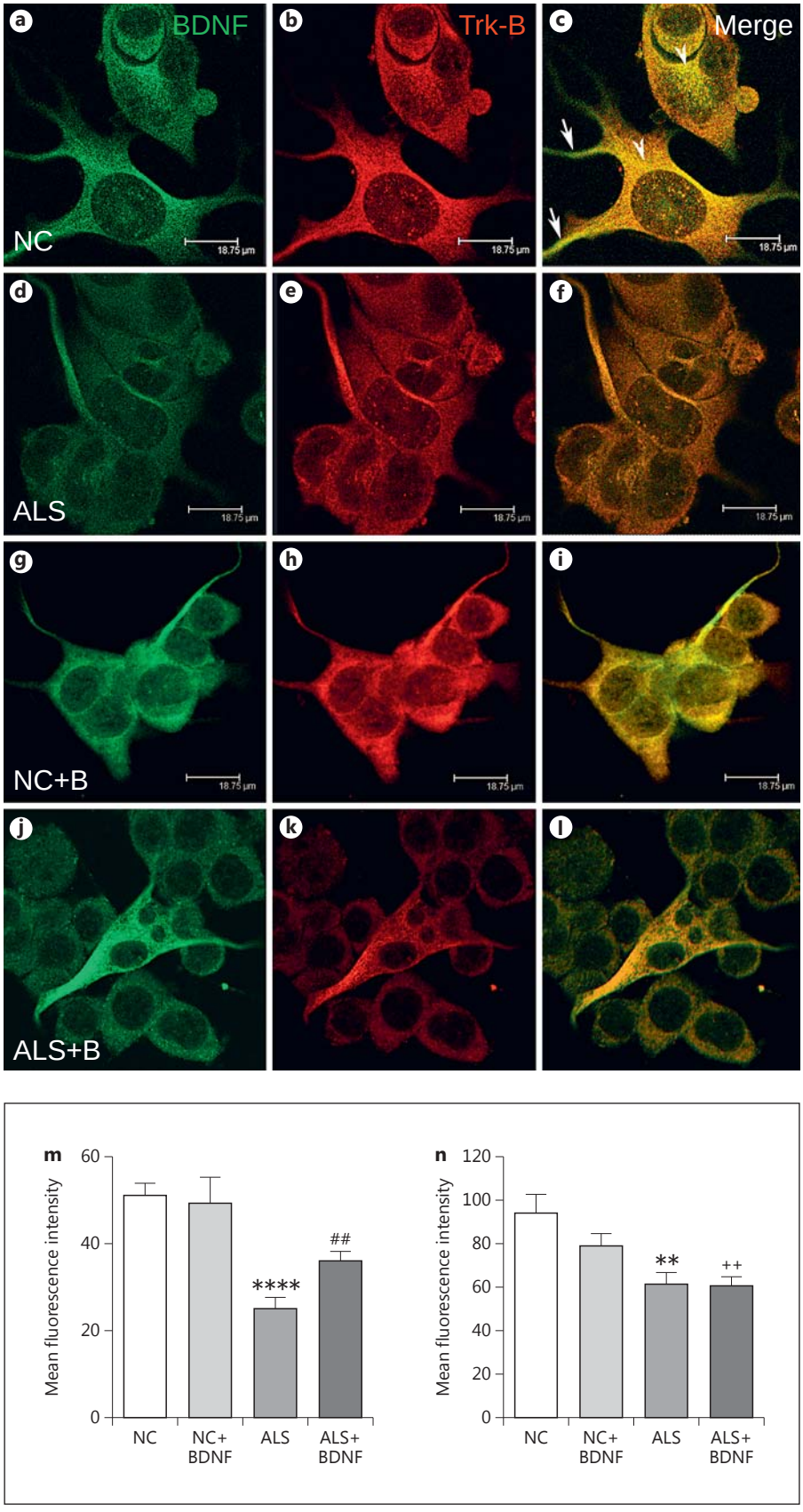

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Fig. 2. Effect of ALS-CSF and exogenous BDNF supplementation on endogenous BDNF and TrkB expression in NSC-34 cells. **a–l** Confocal photomicrographs of NSC-34 cells representing the expression of BDNF (FITC) and TrkB (CY3), respectively, in the NC (**a**, **b**), ALS (**d**, **e**), NC+B (**g**, **h**), and ALS+B (**j**, **k**) groups. **c**, **f**, **i**, **l** Merged images of the respective groups. The expression of both proteins was found to be pancytoplasmic and was punctate to diffuse (**c**, arrowheads). It was also seen in the processes of the differentiated cells (**c**, arrows). Note the decreased expression of endogenous BDNF and TrkB in ALS-CSF-exposed cells (**d** vs. **a**; **e** vs. **b**). BDNF supplementation (10 ng/ml) restored the endogenous BDNF expression but did not influence the altered TrkB expression (**j** vs. **d**; **k** vs. **e**). Scale bar = 18.75 µm. **m**, **n** Quantifications of the immunofluorescence intensity (0–255) of BDNF (**m**) and TrkB (**n**), respectively, confirming the restoration of endogenous BDNF levels in the ALS+B group compared to the ALS group. However, a significant reduction in the TrkB level caused by ALS-CSF was not restored by exogenous BDNF supplementation. ** $p < 0.01$, **** $p < 0.0001$, NC vs. ALS; $^{\#\#}$ $p < 0.01$, ALS vs. ALS+B; $^{++}$ $p < 0.01$, NC vs. ALS+B. The data were analyzed by one-way ANOVA followed by Tukey's post hoc test.

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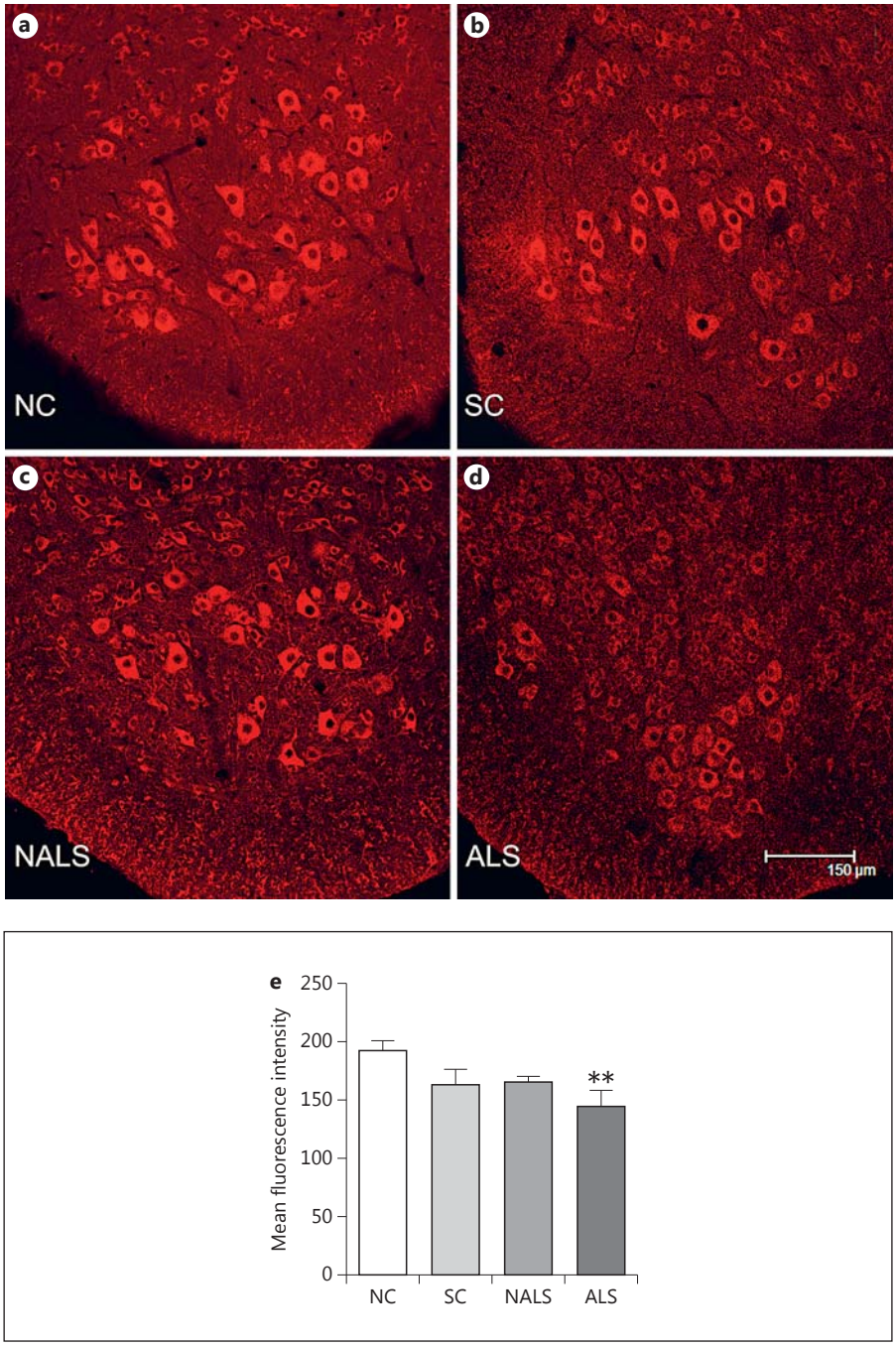

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Fig. 3. Decreased BDNF levels in the lumbar spinal cord of rat neonates intrathecally injected with ALS-CSF. SC = Sham control. **a–d** Laser scanning confocal photomicrographs showing the BDNF expression in the lumbar spinal cord sections of rat neonates intrathecally injected with ALS-CSF (CY3). **a** NC. **b** Sham control. **c** NALS. **d** ALS. Reduced BDNF expression was evident in the spinal cord of rat neonates intrathecally injected with ALS-CSF (**d** vs. **a**, **b** vs. **c**). Scale bar = 150 µm. **e** Quantification of the immunofluorescence (0–255), confirming the reduced expression of BDNF in the ALS group. ** $p < 0.01$, NC vs. ALS. The data were analyzed by one-way ANOVA followed by Tukey's post hoc test.

Effects of ALS-CSF on ChAT and SMI-31 Expression in NSC-34 Cells

A significant reduction in the expression of ChAT was observed in the differentiated motor neurons in the ALS-CSF-exposed group as against the control groups (fig. 4a, e, q; $p < 0.001$ vs. NC, $p < 0.01$ vs. NALS), whereas the expression of phosphorylated NF showed a marginal decrease (fig. 4b, f, r). Many cells in the group exposed to ALS-CSF had SMI-31-immunoreactive aggregates, which were not observed in the control groups (fig. 4f). BDNF supplementation not only restored the ChAT expression levels in the ALS-CSF-exposed groups, it also facilitated the normal localization of phosphorylated NF to neurites (fig. 4m, n, q, r; $p < 0.001$ vs. ALS).

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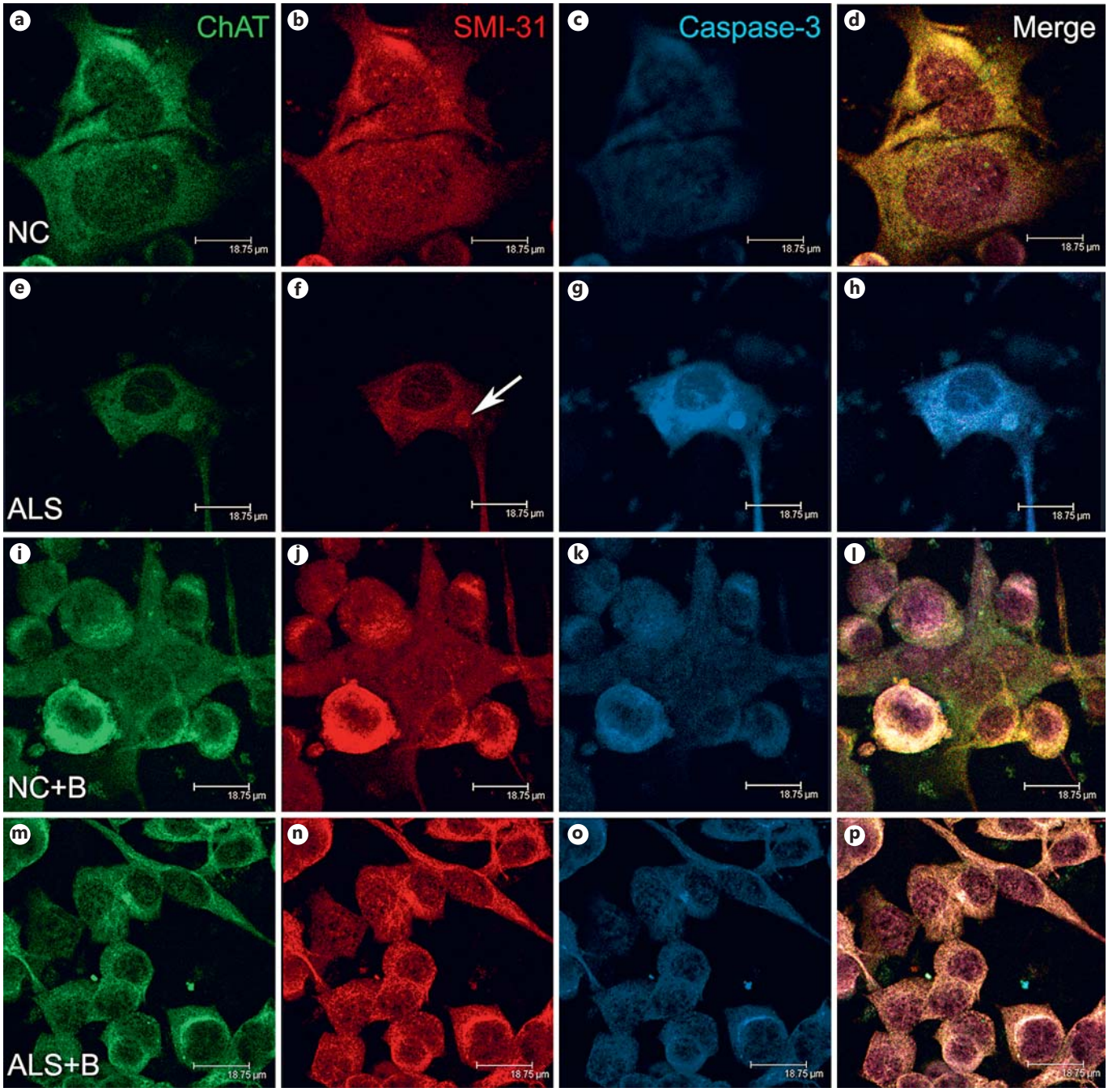

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Fig. 4. Neuroprotective effects of BDNF treatment on NSC-34 cells exposed to ALS-CSF. **a–p** Representative photomicrographs of NSC-34 cells triple labeled for ChAT (FITC), phosphorylated NF, SMI-31 (Cy3) and caspase-3 (Cy5). **a–c** NC group. **e–g** ALS group. **i–k** NC+B group. **m–o** ALS+B group. **d**, **h**, **l**, **p** Merged images of the NC, ALS, NC+B, and ALS+B groups, respectively. All three proteins were localized to the cytoplasm of the cells. Note the decreased expression of ChAT, aggregation of phosphorylated NF (**f**, arrow) and enhanced expression of caspase in cells exposed to ALS-CSF (**e–g** vs. **a–c**). BDNF supplementation reinstated the ChAT and caspase-3 levels (**m**, **o** vs. **e**, **g**, respectively). Reduced aggregation of phosphorylated NF was evident in the ALS+B group (**n** vs. **f**). Scale bar = 18.75 µm. **q–s** Quantification of immunofluorescence intensity (0–255) of all 3 proteins confirmed the qualitative observations. **q** ChAT. **r** SMI-31. **s** Caspase-3. * $p < 0.05$, *** $p < 0.001$, NC vs. ALS; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, ALS vs. NALS; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$, ALS vs. ALS+B. The data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Of note, the NC+B group was comparable to the NC group.

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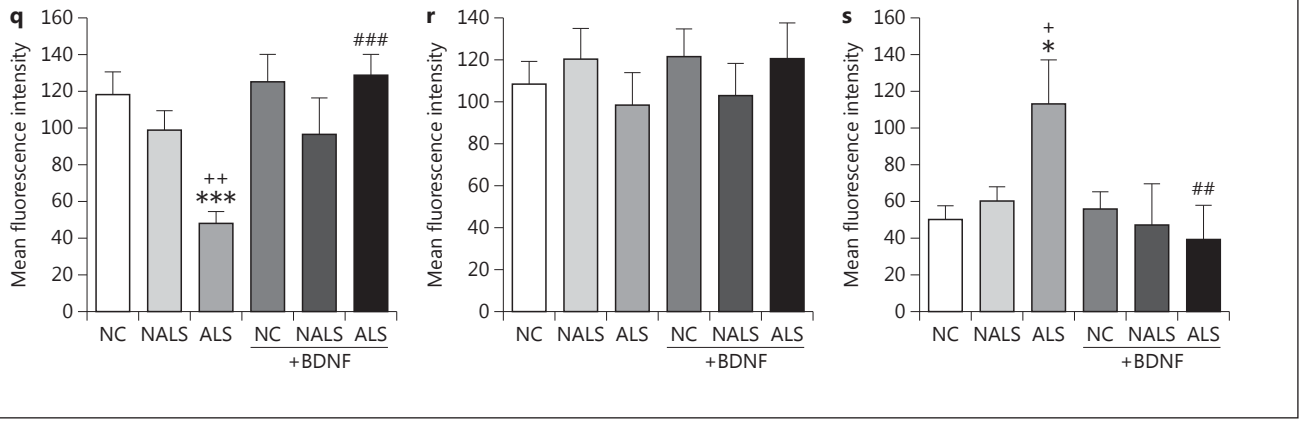

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Effect of ALS-CSF on Caspase-3 Expression

The expression of the antiapoptotic protein caspase-3 was upregulated in the ALS-CSF group compared to the NC group (fig. 4c, g, s; $p < 0.05$ vs. NC). Exogenous BDNF supplementation significantly reduced the caspase-3 levels in the ALS-CSF group (fig. 4o, s; $p < 0.01$ vs. ALS).

Effect on Calbindin-D28K Expression

The calcium-binding protein calbindin-D28K was localized to the cytoplasm (fig. 5). The cellular staining intensity declined upon exposure to ALS-CSF (fig. 5a, b, e; $p < 0.0001$ vs. NC). Calbindin-D28K returned to almost normal levels by BDNF supplementation (fig. 5d, e; $p < 0.001$ vs. ALS).

MTT Assay

The MTT assay revealed a notable reduction in the viability of NSC-34 cells upon exposure to ALS-CSF ($p < 0.05$ vs. ALS). ALS-CSF caused an approximately 40% reduction in the viability of the cells compared to the cells in the control group. The supplementation (nanograms/milliliter) of the cultures with trophic factor(s) resulted in the increased viability. In particular, the ALS+V^{150}, ALS+B^5+V^{75} and ALS+B^{10}+V^{150} groups showed a significant increase in the cell viability compared to ALS (fig. 6). Thus, the ALS+B^5+V^{75} group showed maximum survival.

CSF-Induced Organellar Damage and the Effect of BDNF Supplementation

Under control conditions, the mitochondria of NSC-34 cells were electron dense and possessed clearly defined cristae. In NSC-34 cells exposed to ALS-CSF, the mitochondria were either highly swollen or shrunken, and, in some cases, the outer membrane was fractured and the morphological crista was lost (fig. 7a vs. c). In addition (fig. 7c, d), there was fragmented endoplasmic reticulum (ER) and apoptotic micronuclei along with reduced/shrunken cytoplasm. BDNF supplementation reversed most of the degenerative changes induced by ALS-CSF except for the mitochondrial damage (fig. 7e, f).

Therapy with a mixture of both BDNF and VEGF showed interesting findings. Both concentrations showed better reversal of ALS-CSF-induced mitochondrial damage than BDNF supplementation alone (fig. 7g). Interestingly enough, the ALS+B^5+V^{75} group showed much better recovery of the other cell organelles and a near-normal status of the mitochondria, suggesting the efficacy of combinatorial therapy at half of the original dosage (fig. 7h). More dosage combinations or therapy durations would need to be tried to arrive at an optimum dosage with maximal recovery.

Discussion

The present study substantiates the significant dependence of motor neurons on BDNF rather than on IGF-1 and FGF-2. We found a notable reduction in the endogenous expression of BDNF in NSC-34 cells exposed to ALS-CSF, whereas the expression of IGF-1 and FGF-2 showed only marginal declines. A reduced BDNF expression was also evident in the rat spinal motor neurons following the intrathecal injection of ALS-CSF. Exogenous supplementation of BDNF effectively reversed the ChAT and endogenous BDNF levels in ALS-CSF-exposed NSC-34 cells without any restoration of the TrkB

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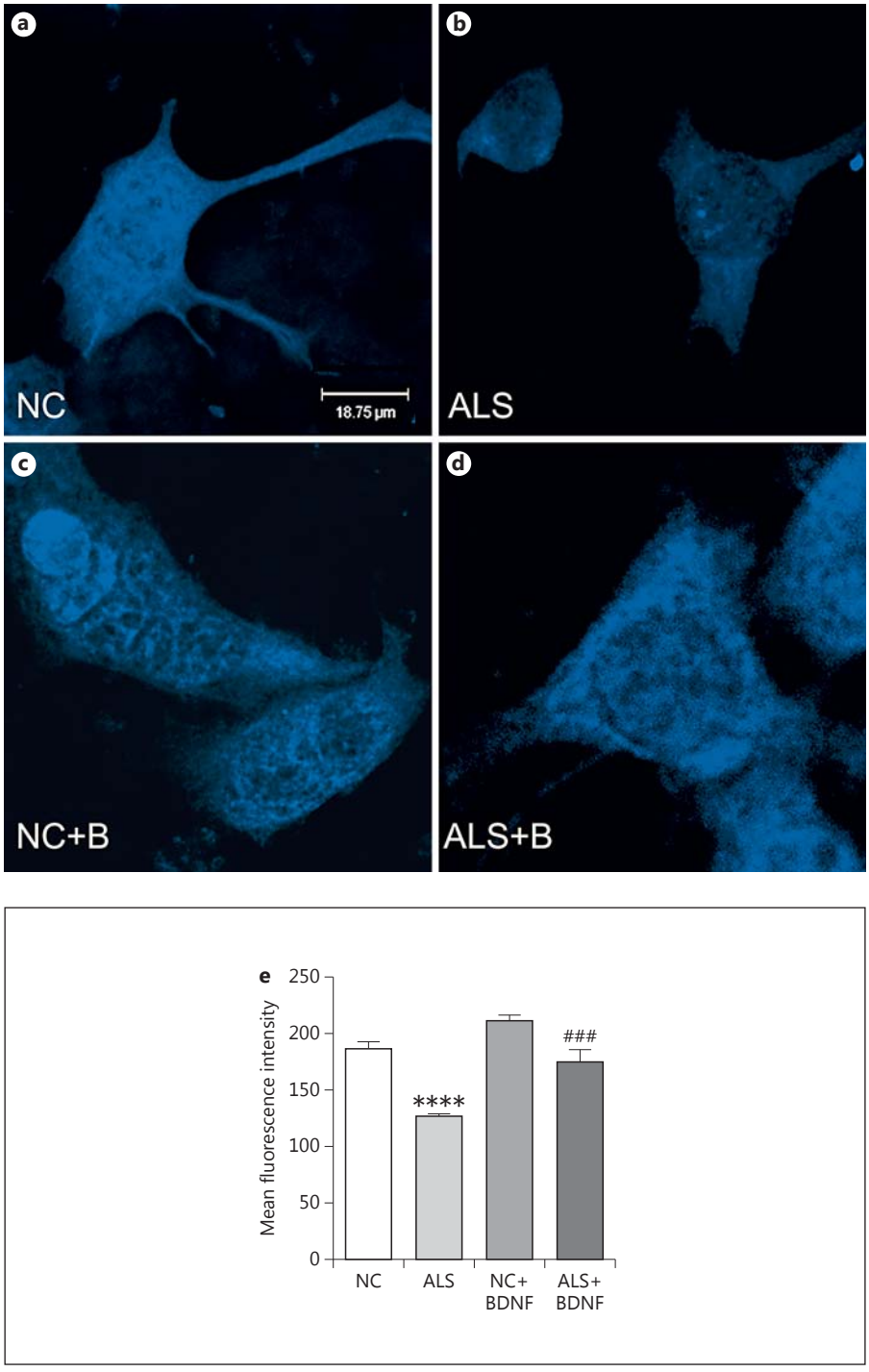

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Fig. 5. Altered expression of calbindin-D28K in response to ALS-CSF and its revival upon BDNF treatment. **a–d** Representative photomicrographs of immunofluorescently stained NSC-34 cells illustrating the expression of calbindin-D28K (Cy5). **a** NC. **b** ALS. **c** NC+B. **d** ALS+B. Note the decrease in the intensity of immunostaining in cells exposed to ALS-CSF (**b** vs. **a**) and its restoration upon BDNF supplementation (**d** vs. **b**). Scale bar = 18.75 µm. **e** Quantification of the immunofluorescence intensity (0–255) confirmed the enhanced expression of calbindin-D28K upon BDNF treatment. **** $p < 0.0001$, NC vs. ALS-CSF; ### $p < 0.001$, ALS vs. ALS+B. The data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Of note, the NC+B group was comparable to the NC group.

receptor levels. A reversal of the expression pattern was noted even with an aggregation of phosphorylated NF, caspase-3, and calbindin-D28K. BDNF supplementation remarkably reversed the 'aggregatory/clustered cell' pattern of cells exposed to ALS-CSF in vitro to that of well-differentiated and well-dispersed cells. The decrease in IGF-1 and FGF-2 protein levels was not significant or commensurate to the depleted levels of mRNA reported earlier [30], which hints at an attempted recovery during translation.

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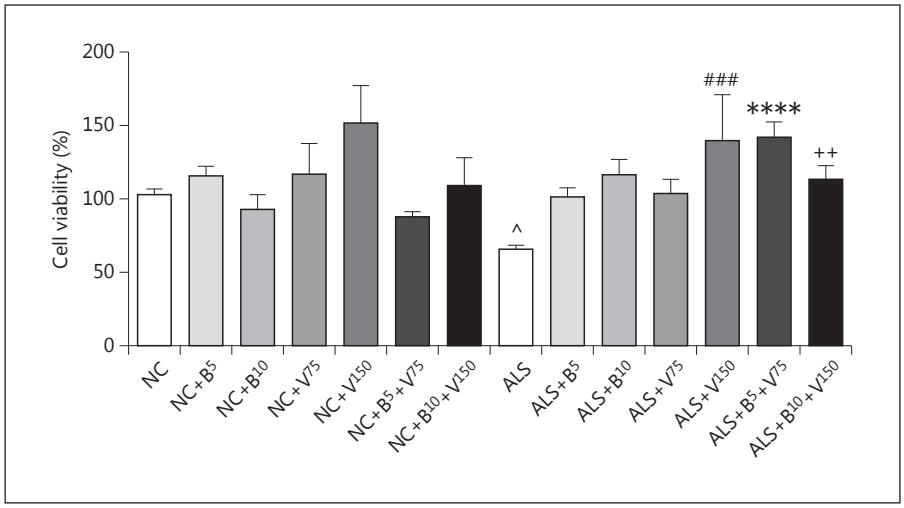

Fig. 6. Increased survival of NSC-34 cells exposed to ALS-CSF after treatment with a single trophic factor or a combination of trophic factors. The histogram represents the percentage of cell viability of NSC-34 cultures supplemented with trophic factor(s). The MTT assay revealed a significant reduction in the viability of NSC-34 cells upon exposure to ALS-CSF compared to the control groups. The exposure of NSC-34 cultures to factor(s) after ALS-CSF exposure resulted in increased viability. In particular, the ALS+V^{150}, ALS+B^5+V^{75} and ALS+B^{10}+V^{150} groups showed a significant increase in cell viability compared to the ALS-CSF-treated group. ^ $p < 0.05$, vs. NC; $^{++}$ $p < 0.01$, $^{###}$ $p < 0.001$, **** $p < 0.0001$, vs. ALS.

The endogenous synthesis of proneurotrophins is directed and sequestrated in the ER [38, 39]. Thereafter, the posttranslational modification of nascent neurotrophins into mature homodimeric proteins, prior to their release into the extracellular space, occurs in the membrane stacks of the trans-Golgi network and the Golgi apparatus. Earlier, we have shown that ALS-CSF induces fragmentation of the Golgi apparatus and ER stress [40, 41]. A similar fracturing of these organelles is seen in a transgenic SOD1 mouse model [42, 43]. Thus, these structural defects in the ER, ER-Golgi networks, and the transport system may be the underlying causes of lower endogenous BDNF protein levels. It is also likely that ALS-CSF destabilizes the local cellular neuritic cAMP/PKA activity to dysregulate its self-amplifying autocrine actions responsible for promoting axon differentiation and growth [44].

The receptors TrkB and p75 mediate the effects of endogenous BDNF on motor axonal regeneration in distinct ways [45]. Binding of BDNF to TrkB activates phospholipase Cγ/Ras/MEK/MAPK and the PI3K/Akt pathways [46], while p75 stimulates the JNK-mediated proapoptotic cascade [47, 48]. The induction of excitotoxicity reduces TrkB levels, leading to neuronal death [49]. The downregulation of TrkB levels observed in our study may result from similar phenomena. Our results are also comparable with studies on cultured neurons, wherein the TrkB protein levels drop rapidly following glutamate supplementation [50]. Earlier, we have reported that ALS-CSF induced a reduction in the expression of the glia-specific glutamate transporter Glt-1, which resulted in glutamate-mediated excitotoxicity in the rat spinal cord cultures [37].

The role of the TrkB receptor in mediating the survival-promoting activity of BDNF is well accepted and has been demonstrated in primary cortical cultures, wherein BDNF supplementation increases the phosphorylation of the cAMP response element-binding protein (CREB), leading to the expression of the CREB target genes Arc, c-fos, and BDNF exon IV. The application of TrkB-specific inhibitor K252a (200 nM), prior to BDNF treatment, blocks the downstream events since BDNF induces phosphorylation of ERK1/2 and CREB, whereas the p75 NTR inhibitor does not [51]. Paradoxically, reduced TrkB expression in motor neurons provides a beneficial effect in a mutant SOD1 mouse model, as it retards disease progression [52]. Functional blocking studies in pure motor neuron and mixed rat spinal cord cultures show that either exogenous or endogenous BDNF increases the sensitivity of motor neurons to excitotoxic insults mediated through the activation of TrkB [53]. BDNF increases TrkB expression initially; however, a reduction ensues following long-time exposure [54]. In our experimental model, no significant reversal was noted in TrkB levels following BDNF supplementation.

BDNF imparts neurotrophic effects, even through the TrkB-independent pathway, by involving calcium-calmodulin (CaM)-dependent protein kinases [55]. CaM activates protein kinase B-promoting survival of spinal motor neurons [56]. The restoration of calbindin-D28K levels upon exogenous BDNF supplementation observed in our study possibly supports this hypothesis, since it can regulate calcium homeostasis.

The expression of ChAT, the functional marker for motor neurons, is reduced in the anterior horn of the spi-

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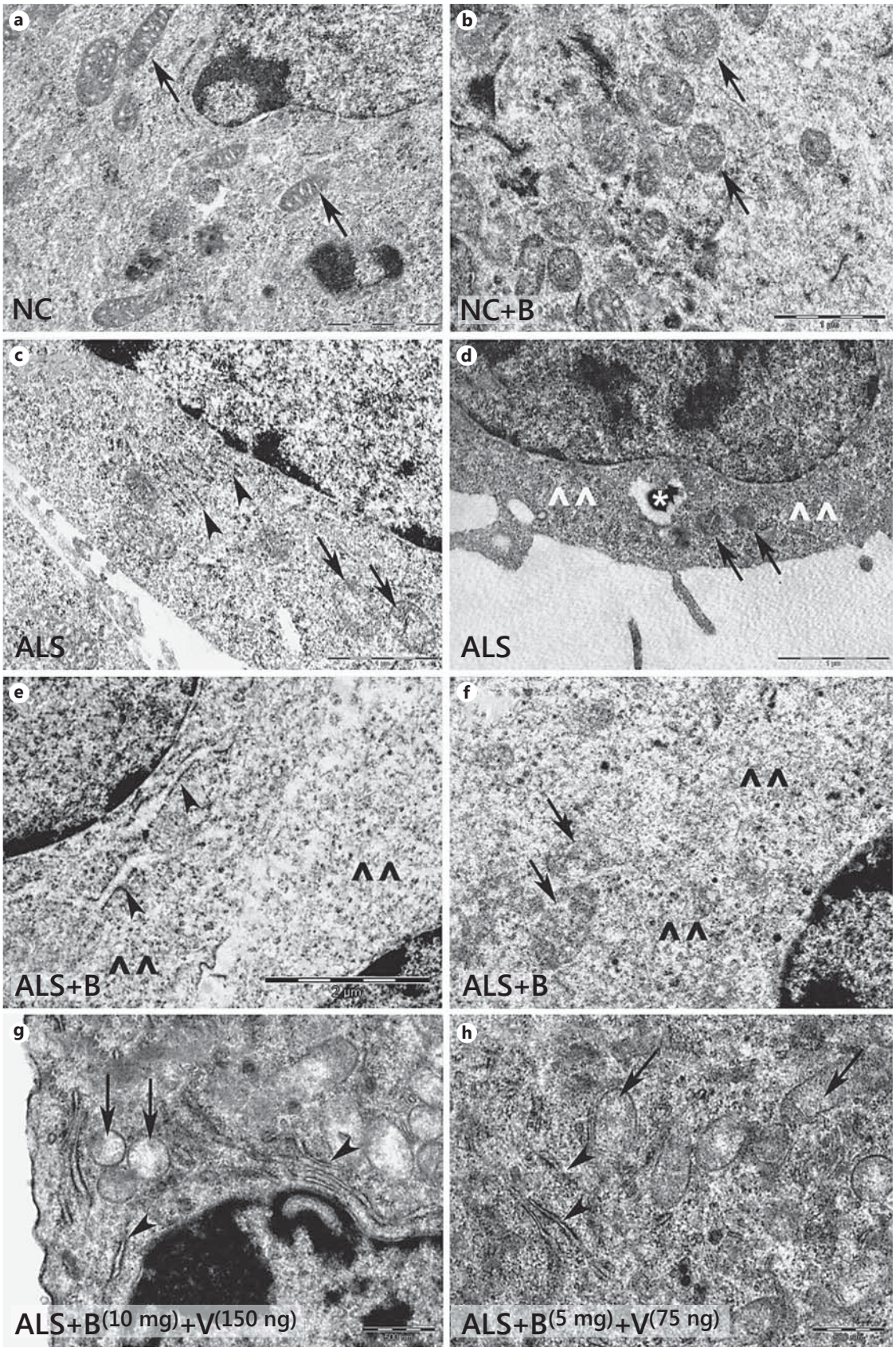

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nal cord of ALS patients [57]. NF, too, accumulate abnormally in the perikaryon and the proximal region of spinal motor neuron axons in ALS cases [58]. Earlier, we have proposed that these events may be concurrent, as a reduction in ChAT levels and an aggregation of NF were noted in NSC-34 cells upon exposure to ALS-CSF [59]. In the present study, exogenous BDNF supplementation reversed the ChAT levels and facilitated the differentiation of motor neurons. In an earlier study, we found that VEGF supplementation was instrumental in reversing ALS-CSF-induced defects [20]. BDNF supplementation promoted ChAT expression in adult spinal motor neurons in a sciatic nerve transaction model [60] as well as in axotomized motor neurons [61]. Therefore, the restoration of cellular homeostasis in the case of spinal motor neurons is trophic factor responsive.

Caspases are a family of cysteine proteases that modulate apoptosis during the development of the central nervous system and in diseases [62, 63]. The neurorestorative potential of BDNF is driven by its ability to block the expression of proapoptotic proteins, while inducing the expression of prosurvival genes [64]. Thus, it targets two interlinked pathways. It reduces glutamate-induced toxicity in cultured hippocampal neurons by decreasing caspase-3 activity, indicating its protective role upstream in the apoptotic pathway [65]. This neuroprotective activity is lost when the cells are treated with inhibitors of MAPK/ERK and Akt Kinase pathways, emphasizing the neuroprotective effect of BDNF through the activation of PI3K and MEK1/2 intracellular signaling pathways [66, 67]. PI3K prevents mitochondrial cytochrome C-dependent apoptosis by inactivating caspase [68]. In accordance with this, BDNF supplementation significantly downregulates the ALS-CSF-induced increase in caspase-3 levels to normalcy, thereby rescuing NSC-34 cells from apoptotic death.

In our experience, BDNF completely reversed caspase-3 levels but only partially restored the structural defects in the mitochondria. Swollen and shrunken mitochondria with altered crista structure were seen in NSC-34 cells exposed to ALS-CSF, and these defects persisted to a certain extent despite BDNF supplementation. Since most degenerative parameters as well as organellar defects involving ER, apoptotic bodies, and cytoplasmic shrinkage were appreciably retraced, although some structural mitochondrial defects were retained, it might be critical to evaluate the events of mitochondrial bioenergetics, mitophagy, mitochondrial fission, and fusion in this model.

Restoration of cellular BDNF expression upon BDNF supplementation, without a dramatic reversal of the TrkB receptors, suggests that the cells may apply alternative or receptor-independent mechanisms like the regulation of calcium homeostasis. The restoration of ChAT expression, abnormal NF phosphorylation, and calcium homeostasis as well as the improvement in cellular morphology with reduced apoptosis in response to BDNF underline its neuroprotective role in ALS. The partial recovery of ultrastructural defects appears to be the only limitation of BDNF-mediated neuronal regeneration. Nonetheless, this appears to be a reliable clue into the limited success of BDNF in clinical trials, and adjuvant therapies to address these deficits are needed.

We and others have noted a parallel downregulation of VEGF and BDNF in ALS, although the levels of IGF-1 and FGF-2 were not significantly affected in our study. Some studies have also demonstrated the effect of other trophic factors like glial cell line-derived neurotrophic factor or ciliary neurotrophic factor [4, 6]. Thus, the ventral horn motor neurons are undoubtedly supported by more than one trophic factor, and this is probably the reason why BDNF and even VEGF in isolation are unable to completely revive the critical defects at the ultrastructural levels. Similar observations may be envisaged for other growth factors, too.

Fig. 7. Partial recovery of the damaged organelles following BDNF supplementation in NSC-34 cells exposed to ALS-CSF and better recovery with a combinatorial approach. Representative electron photomicrographs of NSC-34 cells showing the ultrastructural changes after exposure to ALS-CSF, BDNF treatment, and combinatorial therapy with BDNF and VEGF at two different dosages. **a** NC. **b** NC+B. **c**, **d** ALS. **e**, **f** ALS+B. **g** ALS+B^{10}+V^{150}. **h** ALS+B^{5}+V^{75}. Note the normal mitochondria (**a**, black arrows) and near normal mitochondria (**b**, black arrows), the swollen mitochondria with damaged cristae (**c**, black arrows), shrunken mitochondria (**d**, black arrows), fragmented ER (**c**, black arrowheads), apoptotic micronuclei (**d**, asterisk), and cytoplasmic shrinkage (**d**, white open arrowheads) in cells exposed to ALS. **e**, **f** Partly restored ER cisternae (**e**, black arrowheads), reversal of cytoplasmic shrinkage (**e**, **f**, black open arrowheads), and the absence of apoptotic bodies but no recovery of damaged mitochondria upon BDNF supplementation (**f**, black arrows). Of note, the NC+B group was similar to the NC group. **g**, **h** Combinatorial therapy with restored ER cisternae (**g**, black arrowheads), reversal of cytoplasmic shrinkage, and the absence of apoptotic bodies and partial recovery of damaged mitochondria (**g**, black arrows) upon BDNF and VEGF (ALS+B^{10}+V^{150}) supplementation. A lower concentration of BDNF and VEGF (ALS+B^{5}+V^{75}) resulted in a better recovery of organelles (**h**, black arrowheads) and mitochondrial recovery to near-normal state (**h**, black arrows).

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The simultaneous application of two growth factors appeared to be relatively more efficacious, as seen from the significant survival adjudged by the MTT assay. The reversal of ultrastructural damages was also better, although the combinations of the most optimum dosage and therapy duration need to be investigated in detail. Hence, it might be critical to consider a combination of trophic factors to provide a composite revival in experimental models and probably even in clinical conditions.

Conclusion

In summary, ALS-CSF affects the expression of the endogenous BDNF protein, but not those of IGF-1 and FGF-2, which suggests that BDNF expression is vital in supporting motor neuronal survival. Exogenous BDNF supplementation was instrumental in reviving its autocrine expression as well as that of ChAT; however, the TrkB levels remained low, and it had only a small effect on organelles. Since the calbindin-D28K expression and caspase-3 level were back to normal after supplementation, a role in blocking apoptosis via the restoration of Ca^{2+} homeostasis may be contemplated.

Our study provides conclusive evidence that BDNF supplementation reverted most of the neurodegenerative effects, but BDNF alone may not be sufficient as a therapeutic intervention, which is reflected in the partial success of clinical trials with BDNF in ALS patients.

Acknowledgments

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