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Antiapoptotic effects of the peptidergic drug Cerebrolysin on primary cultures of embryonic chick cortical neurons

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Summary. Cerebrolysin (EBEWE Arzneimittel, Austria, Europe) is a widely used drug relieving the symptoms of a variety of neurological disorders, particularly of neurodegenerative dementia of the Alzheimer's type. It consists of approximately 25% of low molecular weight peptides (<10 k DA) and a mixture of approximately 75% free amino acids, this being based on the total nitrogen content.

In this study we used a low serum (2% serum supplement) cell stress in-vitro model to assess drug effectiveness on neuronal viability and programmed cell death (PCD). In this in-vitro model the type of cell death was previously shown to be primarly apoptotic, which was verified by DNA-laddering and TUNEL-staining. For evaluation of neuronal viability a MTT-reduction assay was performed after 4 DIV and 8 DIV and the percentage of apoptotic neurons was determined by bis-benzimide staining of nuclear chromatin.

To differentiate between possible effects of the free amino acids and the peptide fraction of Cerebrolysin an artificial amino acid mixture (AA-mix) was used as a control.

Cerebrolysin, the AA-mix and 10% foetal calf serum (FCS) caused a similar increase in viability after 4 DIV, whereas the effects of the growth factors BDNF and FGF-2 were less pronounced. After 8 DIV Cerebrolysin, but not the AA-mix, was able to ameliorate neuronal viability, which could reflect a neuro-protective effect or an increased activity of the mitochondrial dehydrogenase measured in a MTT-reduction assay. The percentage of cells showing apoptotic chromatin changes was significantly reduced ($p < 0.01$) in cultures treated with Cerebrolysin, whereas the AA-mix failed to decrease the percentage of cells showing apoptotic chromatin changes. These findings ascertain an anti-apoptotic effect of the peptide fraction of Cerebrolysin and

reveal a transient viability promoting effect of the amino acid fraction, which is most likely due to improved nutritional supply.

Keywords: Cerebrolysin, growth factors, apoptosis, chick neuron, neuro-protection, serum supplement.

Introduction

The porcine brain-derived peptide preparation Cerebrolysin (EBEWE Arzneimittel, Austria, Europe) has been used for the treatment of dementia and sequels of stroke for more than 40 years (Barolin et al., 1996; Rüther et al., 1994; Vereschagin et al., 1991). The neuro-protective efficacy of this drug has been reported after different types of lesions in-vitro (Hutter-Paier et al., 1996a,b) and in-vivo (Akai et al., 1992; Masliah et al., 1999; Schwab et al., 1997). In spite of these reports about the therapeutic efficacy and pre-clinical results demonstrating the neuro-protective function of Cerebrolysin, the underlying molecular mechanism of action still needs to be determined. Cerebrolysin is an injection solution of a porcine brain-derived peptide preparation produced by standardised enzymatic breakdown of lipid-free brain proteins. It consists of approximately 25% of low molecular weight peptides (<10 k DA) and a mixture of approximately 75% free amino acids, based on the total nitrogen content. To elucidate the active fraction of Cerebrolysin, involved in the prevention of programmed cell death (PCD), and to obtain information about the possible effects of improved nutritional support of the cultures, we used an artificial amino acid mixture (AA-mix) as a control that reflects exactly the free amino acid composition of Cerebrolysin.

A part of the therapeutic effect of Cerebrolysin can be attributed to the neurotrophic activity which resemble the properties of naturally occurring neurotrophic factors (Satou et al., 1993, 1994; Windisch et al., 1998). In this regard, neuro-protection could be a direct outcome of increased neuronal survival caused by a hindrance of PCD. An increased viability measured in a MTT-reduction assay can reflect an increase in neuronal survival and/or an increased activity of the mitochondrial dehydrogenase (metabolic drug effect). Therefore, we additionally performed an apoptosis assay to rule out a possible neuro-protective function of Cerebrolysin due to a hindrance of PCD.

PCD plays a crucial role in cerebral ischemia and neurodegenerative disorders, i.e. Parkinson's or Alzheimer's disease (Anderson et al., 2000; Cotman, 1998; Kitamura et al., 1998; MacGibbon et al., 1997; Sugaya et al., 1997) and is documented as being involved in the ischemic stroke as well (Guglielmo et al., 1998; Martinou et al., 1994; Raff et al., 1994; Tarkowski et al., 1999). Serum deprivation (0% FCS) of mature cerebellar granule neurons can be used to study mechanisms of oxidative stress-induced apoptosis (Atabay et al., 1996). Serum, containing unknown growth promoting substances (Uto et al., 1994), is able to reduce delayed ischemic cell death of cultured embryonic neurons (Kusumoto et al., 1997). The use of serum deprived (0% FCS) neuronal telencephalon cultures from chick embryos provides the possibility of ruling out PCD preventative effects as has been

demonstrated for Bay ×3702 and the 5-HT1A receptor agonist 8-OH-DPAT (Ahlemeyer et al., 1999; Ahlemeyer and Krieglstein, 1997). Recently, low serum supplementation (2% FCS) was found to cause PCD of primary embryonic chick telencephalon neurons, mimicking slow progressive neuro-degeneration (Reinprecht et al., 1998). In the current study we used this chronic low serum (2% FCS) culture model to investigate possible antiapoptotic effects of Cerebrolysin. To differentiate between short and long term viability promoting effects, MTT reduction assays were performed after 4 days and 8 days in vitro (DIV). Additionally isolated neurons were plated at two densities to investigate the correlation between the initial cell number and MTT results used in the present study for the quantification of neuronal viability. To assess PCD we differentiated between apoptotic and normal cells by means of nuclear morphology following Hoechst 33258 staining, which is a widely used method for the assessment of PCD (Ahlemeyer and Krieglstein, 1997; Lizard et al., 1995; Ratan et al., 1994; Regan et al., 1995; Simonian et al., 1996). In order to verify the method used in the current study for the assessment of PCD we additionally investigated the effects of BDNF and serum supplement on the occurrence of PCD by comparing them with results recently obtained using TUNEL-staining and DNA-laddering (Reinprecht et al., 1998). Reinprecht et al. could demonstrate that serum, but not BDNF addition, could rescue chick cortical neurons from PCD after 3 DIV using the same low serum model.

Material and methods

Neuronal culture

Primary neuronal cultures from 9 days old white Leghorn chick embryo telencephalons were prepared as described by Pettmann et al. (1979). Cerebral hemispheres were mechanically dissociated and the resulting cell suspension was centrifuged at 400 rpm for 5 min to remove cell debris. Cortical cells were suspended in Minimum Essential Medium Eagle (EMEM; Bio Whittaker), containing 2 mM L-glutamine (Bio Whittaker) and gentamycin (0.1 mg/ml; Bio Whittaker). The nutrition medium was supplemented with 2% (v/v) heat-inactivated fetal calf serum (FCS; Bio Whittaker). The number of neurons was evaluated by counting the cells with a hemocytometer and viability was determined by using Trypan Blue exclusion method. 6-well plates (Costar) were coated with poly-D-Lysin (0.1 mg/ml; Boehringer Mannheim) for 15 min and carefully washed. Cells were plated at a low density of 100.000 cells/ml which was enough to guarantee neurite network formation in cultures raised for 4 days and facilitates manual cell counting to determine the percentage of apoptotic neurons. To investigate cell viability values using a twofold cell density, neurons were plated at 200.000 cells/ml. Cultures were maintained in an incubator at 37°C, 5% CO_2 and 95% humidity. After 4 and 8 days in vitro (DIV) neuronal viability and the percentage of apoptotic neurons was assessed as described below.

The growth factors BDNF and FGF-2 were purchased form Sigma Chemicals. We used 50 ng/ml BDNF and 670 pg/ml FGF-2 in our study, determined as the optimal dose to support neuronal viability after 5 DIV under similar culture conditions (Reinprecht et al., 1998).

Cerebrolysin® (EBEWE Pharmaceuticals, Austria; Batch: 802772) was added to cultures in concentrations from 0.025 mg/ml to 0.8 mg/ml (lyophilized dry weight) from the first day on. To determine the optimal dose for promoting viability, a dose response curve by means of a MTT-reduction assay (described below) was made. For the investigation of amino acid related effects concerning viability and PCD, free amino acids

naturally found in Cerebrolysin were added to cultures using an artificial amino acid mixture (AA-mix; Batch: 902753; EBEWE Pharmaceuticals). For control purposes phosphate buffered saline (PBS, Bio Whittaker) was used.

Neuronal survival

To determine neuronal viability a MTT-reduction assay was performed as described by Mosmann (1983): The yellow reagent MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is reduced by succinate dehydrogenase in active mitochondria causing the formation of dark blue formazan crystals. It has been shown that dead cells are unable to cleave MTT and resting cells produce less formazan. Therefore, this assay can be used for quantification of cell viability. 200 µl MTT were added to culture wells containing 2 ml nutrition medium. After 2 hours of incubation, the medium was aspirated and 200 µl sodium dodecyl-sulphate (SDS) was added to lyse the cells. Formazan crystals were dissolved by addition of 1,200 µl Isopropanol/HCl (100 ml Isopropanl/4 ml 1 M HCl) and shaking the plates for 15 min. 111 µl of this solution was transferred to 96-well plates and the optical density was measured at 550 nm with a plate reader (Labsystems Multiscan MS).

Assessment of neurons undergoing PCD

Condensation of chromatin and nuclear chromatin fragmentation, visible with the fluorescent DNA intercalating dye Hoechst 33258, are hallmarks of apoptotic cell death (Simonian et al., 1996). Therefore, we assessed the percentage of apoptotic neurons at the level of light microscopy using bis-benzimide staining of nuclear chromatin. Neurons undergoing apoptosis are characterised by chromatin clumping and nuclear fragmentation (Dux et al., 1996; Regan et al., 1995).

Cells, grown in 6-well plates for 4 and 8 days, were fixed in carnoy's fixative (3 parts methanol and 1 part pure acetic acid) and stained with Hoechst 33258 (0.5 µg/ml; Sigma) for 15 min. With the help of UV-fluorescence microscopy stained chromatin was visualised and microphotographs were taken using a digital photo-camera (Eos1-DCS5; CANON, Kodak) mounted on an inverted microscope (Axiovert 35; Zeiss). Each microscopic field (size = 0.312 mm^2) was visualised and photographed using UV-fluorescence (λ Exitation max. 346 nm; λ Emission max. 460 nm) and phase contrast microscopy (200× magnification). Exposure times of UV-fluorescent images was fixed at 1,3 sec (100 ASA). Digital microphotographs (1.6 mio pixel/image) of at least 3 randomly selected fields in two separate wells per experiment were evaluated.

Normal cells showed a normally sized nucleus and a dimmer, diffusely stained chromatin. In contrast, apoptotic cells were identified by the appearance of brightly stained, hyper-condensed, and often fragmented chromatin in spherical or irregular shapes (Fig. 1). The percentage of cells with normal, condensed, or fragmented nuclei were evaluated by manually counting of at least 900 cells in each test group. In addition, we separately evaluated the percentage of brightly fluorescent spherical nuclei showing a single mass of spherical and condensed chromatin (termed condensed), since this type of apoptotic chromatin change dominated after 8 days. Necrotic cells, exhibiting diffuse and irregular nuclei, were only rarely observed (less than 1%) and therefore excluded from counting. The percentage of apoptotic cells was quantified, with the observer being blind as to which was the treatment group. Experiments were carried out in duplicate.

Statistical analyses

Differences between means of individual groups were evaluated by a Kruskal-Wallis one way analysis of variance with STATISTICA for windows (StatSoft Inc.). Groups were considered as significantly different at a level of $p < 0.05$.

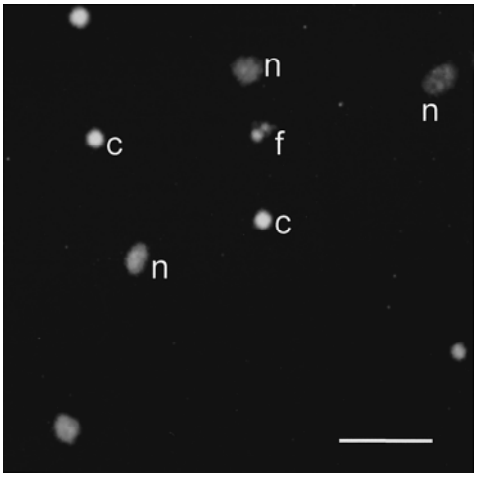

Fig. 1. Representative fluorescent photomicrograph of cultured neurons grown for 4 DIV in control medium. Cells were fixed and stained with the fluorescence intercalating dye Hoechst 33258. Nuclear chromatin was visualised by means of UV fluorescence. Normal cells (n) are visible by a diffuse and dimmer staining. Apoptotic cells were defined as cells having brightly stained, condensed and/or fragmented (f) chromatin. Cells showing a single condensed mass are defined as condensed (c). Bar = 20 μm

Results

Dose dependent effects of Cerebrolysin on the viability of cortical chick neurons

For the microscopic identification of the neurons undergoing apoptosis the cell density can be limiting. Therefore, we looked for the lowest cell density applicable for the establishment of a loose neurite network of neurons after 4 DIV. This was found to be at 100.000 neurons/ml. To determine the optimal effectiveness of Cerebrolysin under these culture conditions a dose dependent response curve was made (Fig. 2). The highest response in viability was achieved at 0.4 mg/ml which is equal to the addition of 10 μl Cerebrolysin per ml nutrition medium. This volume (10 μl/ml) of Cerebrolysin and AA-mix was added once at the beginning of each of the subsequent experiments to the appropriate groups. For control purposes we added 10 μl phosphate buffered saline (PBS) per ml medium to each group investigated.

Viability effects of Cerebrolysin and AA-mix on isolated cortical neurons from chick telencephalon

For the evaluation of viability effects of Cerebrolysin and AA-mix 100.000 neurons/ml (Fig. 3) and 200.000 neurons/ml (Fig. 4), maintained in EMEM supplemented with 2% FCS, were raised for 4 and 8 DIV. 10 μl/ml Cerebrolysin and 10 μl/ml AA-mix were added to different groups from the first day on. Results assessed with the MTT-reduction assay demonstrate a significant ($p < 0.05$) higher viability after 4 DIV under influence of Cerebrolysin and the AA-mix compared to controls (10 μl/ml PBS treated). Both treatments attenuated neuronal cell death compared to control cultures

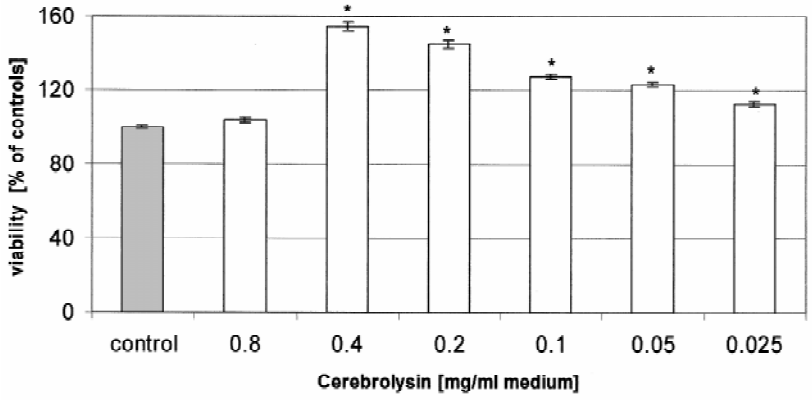

Fig. 2. Dose dependent effects of Cerebrolysin on viability. Viability of cortical cells from 9 days old chick embryos assessed by means of MTT-reduction assay after 4 DIV supplemented with 2% FCS. Cortical cells were plated at a density of 100.000 cells/ml in 6-well plastic culture dishes treated with different Cerebrolysin concentrations from the first day on. OD values (570 nm) are given as mean and standard error of mean in percent of controls from 2 independent experiments ($n = 10$). Differences vs. controls: $^*p < 0.001$. Verified by a Kruskal-Wallis one way ANOVA

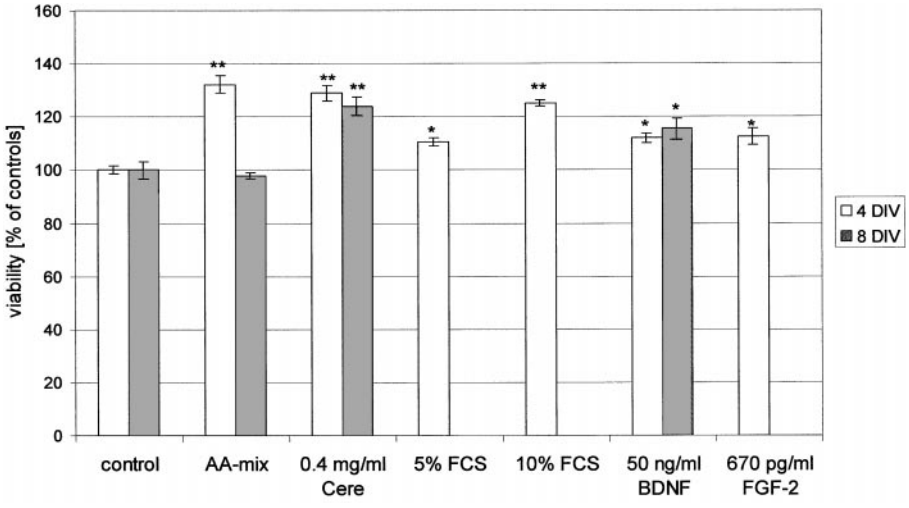

Fig. 3. Influence of Cerebrolysin, the AA-mix, serum supplementation and growth factors on the viability of chick cortical neurons. After 4 DIV and 8 DIV in culture the percentage of viability of cortical cells from 9 days old chick embryos was assessed using the MTT-reduction assay. Cortical cells were plated at a density of 100.000 cells/ml in 6-well plastic culture dishes and treated with Cerebrolysin, the AA-mix and growth factors from the first day on. FCS concentration was 2% in all cultures with the exception of those groups supplemented with 5% and 10% serum. OD values (550 nm) are given as arithmetic mean and standard error of mean in percent of controls from 2–4 independent experiments ($6 \leq n \leq 14$). Differences vs. controls: $^*p < 0.05$, $^{**}p < 0.01$. Verified by a Kruskal-Wallis one way ANOVA

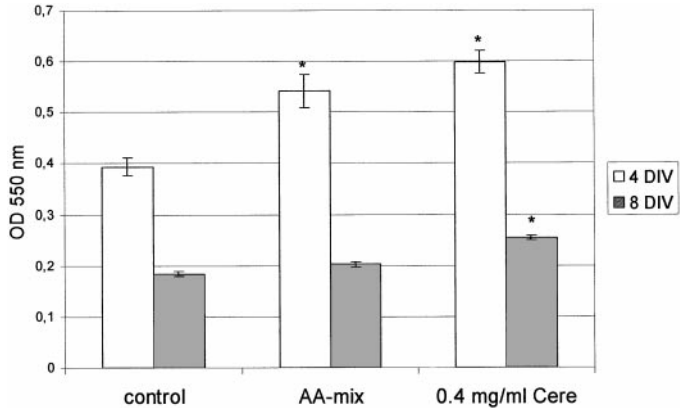

Fig. 4. Influence of Cerebrolysin and the AA-mix on the viability of chick cortical neurons plated at a density of 200.000 cells/ml. The viability of chick cortical neurons cultured for 4 and 8 DIV was assessed by use of the MTT-reduction assay. OD values (550 nm) are given as arithmetic mean and standard error of mean from 2 independent experiments (n = 6). Differences vs. controls: *$p < 0.05$. Verified by a Kruskal-Wallis one way ANOVA

resulting in an ameliorated viability after 4 DIV. After treatment with Cerebrolysin an elevated viability is still apparent in cultures raised for 8 DIV, whereas the AA-mix treated group is not different from controls.

After a cultivation period of 4 days O.D. values were doubled using 200.000 cells/ml in the control group and the groups treated with Cerebrolysin and the AA-mix compared to the experiment using 100.000 cells/ml (control: 100.000 cells/ml: 0.21 ± 0.006, 200.000 cells/ml: 0.39 ± 0.018; AA-mix: 100.000 cells/ml: 0.28 ± 0.005, 200.000 cells/ml: 0.54 ± 0.03; Cerebrolysin: 100.000 cells/ml: 0.28 ± 0.006, 200.000 cells/ml: 0.60 ± 0.02, mean ± s.e.m). After 8 DIV O.D. values of controls (0.184 ± 0.005, mean ± s.e.m) showed no significant difference ($p > 0.05$) compared to the experiment using 100.000 neurons/ml (0.184 ± 0.031, mean ± s.e.m).

Influence of growth factors and serum supplement on the viability of isolated cortical neurons

MTT-reduction assay was performed using chick telencephalon neurons (100.000 cells/ml) cultured for 4 DIV supplemented with 2% (controls), 5% and 10% FCS. This led to a steady increase in viability compared to control cultures demonstrating a neuro-protective function of serum supplement (Fig. 3). The addition of the growth factors BDNF and FGF-2 to the nutrition medium supplemented with 2% FCS resulted in a significant increase in viability compared to controls. Although, BDNF and FGF-2 were investigated at concentrations known to exert the most pronounced viability (Reinprecht et al., 1998), Cerebrolysin and the AA-mix were more effective in elevating viability compared to the growth factors investigated after 4 DIV ($p < 0.05$).

Effect of Cerebrolysin and the AA-mix on the prevention of programmed cell death

Predominantly two types of cells were found after 4 and 8 days in culture: Normal neurons were found to have phase bright, round cell bodies and neurites forming a well defined neurite network. Neuronal death was characterised by degraded neurites, a shrunken cytoplasm and brightly stained condensed chromatin, in contrast to chromatin of normal neurons, which appeared larger and dimmer. After 8 days neurite network was better preserved under the influence of Cerebrolysin or 10% serum compared to controls and AA-mix treated groups (data not shown).

Nuclear morphology was observed under fluorescence microscopy and the percentage of apoptotic neurons was determined by counting cells with apparent chromatin changes (Fig. 1). Apoptotic cells were identified by the appearance of brightly stained, hypercondensed, and often fragmented chromatin in spherical or irregular shapes. We separately counted apoptotic cells with brightly fluorescent spherical nuclei showing a single condensed mass of chromatin (termed condensed).

In cultures treated with Cerebrolysin for 4 days a significant reduction ($p < 0.05$) of apoptotic neurons compared to PBS treated controls was found (Fig. 5A). Serum addition caused a significant decrease ($p < 0.01$) in cells showing apoptotic chromatin changes, whereas the AA-mix was inefficient preventing cells from PCD. Interestingly, 10% serum supplement could not drop the percentage of apoptotic cells below 35%. After 8 DIV the percentage of apoptotic cells showing a single mass of spherical condensed chromatin increased further. Cerebrolysin treatment and serum supplement significantly reduced ($p < 0.01$) the percentage of cells with fragmented and condensed chromatin after 8 DIV (Fig. 5B) demonstrating a long term antiapoptotic effect of Cerebrolysin. In contrast, the AA-mix treated cultures showed a significant increase ($p < 0.05$) of cells with chromatin changes compared to PBS controls.

In cultures raised for 8 days, BDNF caused a small but significant reduction ($p < 0.05$) of apoptotic cells (Fig. 5B). FGF-2 significantly ($p < 0.05$) increased the percentage of cells showing chromatin changes after 4 DIV. The mean cell number per field counted after 8 days was smaller in all groups tested compared to the mean cell number counted per field after 4 days (Table 1). With the exception of Cerebrolysin this decrease was significant ($p < 0.05$) in all groups evaluated after 4 and 8 DIV. Additionally, after 8 DIV significantly more ($p < 0.05$) cells per field survived in Cerebrolysin treated cultures compared to the control group.

Discussion

It is known that Cerebrolysin is protecting cortical chick neurons from cell death and cytoskeletal breakdown after different types of lesions in-vitro (Hutter-Paier et al., 1996a,b). However, it was not shown before whether a reduction of apoptosis is involved in the long lasting protection of Cerebrolysin treated neurons and which fraction of Cerebrolysin is

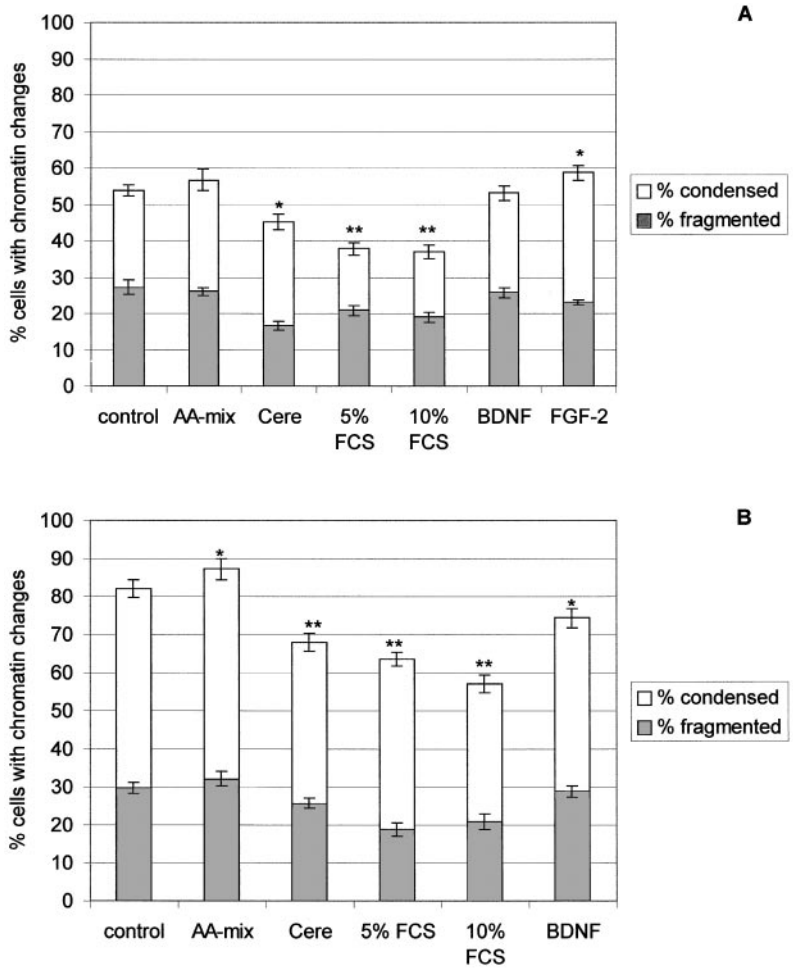

Fig. 5. The influence of Cerebrolysin, AA-mix, serum supplement and growth factors on the percentage of apoptotic cells. Isolated chick telencephalon neurons, plated at a density of 100.000 cells/ml, were cultured for 4 days (**A**) and 8 days (**B**) and the percentage of fragmented cells (hatched bars) and condensed cells (open bar) typical for apoptosis was determined for each microscopic field by evaluating Hoechst 33258 stained nuclear chromatin. Intact cells showed a normal sized nucleus and a dimmer, diffusely stained chromatin. In contrast, apoptotic cells were identified by the appearance of brightly stained, hypercondensed, and often fragmented chromatin in spherical or irregular shapes (Fig. 1). We additionally counted the percentage of brightly fluorescent spherical nuclei showing a single mass of condensed chromatin (termed condensed), since this type of chromatin change dominated after 8 days. Values are given as means ± s.e.m. from 9–12 photomicrographs (taken from 4 different wells) obtained by two individual experiments. At least 900 cells were manually counted in each group. Difference to controls: *$p < 0.05$, **$p < 0.01$ as verified by a Kruskal-Wallis one way ANOVA

responsible for these neuro-protective effects. Results of the current investigation provide evidence for a complex survival promoting action of Cerebrolysin. The AA-mix transiently increased viability to a similar extent as Cerebrolysin up to 4 DIV, which is most likely due to enhanced nutritive support of the cultures with amino acids. This is conceivable since the culture

Table 1. To determine the percentage of cells showing apoptotic chromatin changes (see Fig. 5) nuclear chromatin was stained with Hoechst 33258 (Fig. 1). The number of apoptotic and normal cells of at least 9 randomly selected microscopic fields per group were counted. Values representing the mean number of cells evaluated per microscopic field (size = 0.312 mm^2) and are given as means ± s.e.m. Difference to controls: *$p < 0.05$ as verified by a Kruskal-Wallis one way ANOVA. Mean cell number evaluated per microscopic field after treatment with the AA-mix, Cerebrolysin, serum and different growth factors after 4 and 8 DIV

Treatment	4 DIV mean cell number	8 DIV mean cell number
control	103.3 ± 5.0	74.9 ± 7.6
AA-mix	108.2 ± 5.0	71.3 ± 4.0
Cere	115.9 ± 6.5	94.1 ± 4.8*
5% FCS	120.1 ± 6.9	56.1 ± 3.0*
10% FCS	100.7 ± 5.3	68.7 ± 5.5
BDNF	125.9 ± 6.9	82.0 ± 4.6
FGF-2	69.3 ± 6.4*	

medium (EMEM) used in the current study has a low amino acid content compared to dulbecco's modified eagle medium (DMEM). After 8 days an ameliorated viability could only be found in Cerebrolysin treated cultures, determining the peptide fraction of Cerebrolysin to be responsible for a long lasting viability promoting effect. This effect was independent from cell density as shown by a doubling of the initial cell number. This experiment additionally verifies the expected tight correlation between cell number and viability determined by use of the MTT-reduction assay (Fig. 4). The drop of the OD values after 8 days to the levels corresponding 100.000 cells/ml, indicates a massive loss of neurons in the second period of the experiment. This result verifies the low serum culture model as a model of progressive neurodegeneration, also useful for the identification of neuro-protective effects.

Reinprecht et al. (1998) could show that any addition of serum reduces the number of TUNEL-positive cells without showing a dose dependency, whereas the addition of 50 ng/ml BDNF was ineffective in preventing neurons from PCD after 3 DIV. The addition of serum, but not 50 ng/ml BDNF, rescued neurons from apoptosis in the present study after 4 DIV. These similar results were obtained by DNA staining of neurons with Hoechst 33258, thereby verifing the method used for the assessment of apoptosis in the current study.

According to ultrastructural morphology of neurons, cells showing diffuse and irregular nuclear size together with chromatin condensation are regarded as necrotic (Ahlemeyer et al., 1999; Lizard et al., 1995). Necrotic cells were only rarely found in the present study, which may be related to specific culture

conditions (2% FCS, EMEM), since the type of cell death (apoptotic or necrotic) depends also on the intensity of lesion (Bonfoco et al., 1997; Bredesen, 1995).

The presented results indicate an antiapoptotic effect of Cerebrolysin in neurons cultured for 4 and 8 DIV. This is in contrast to the effect of the AA-mix, which was ineffective in rescuing cells from PCD. A survival promoting effect of Cerebrolysin was additionally demonstrated by the significant higher ($p < 0.05$) cell number counted per field compared to the control group after 8 days. Since damaged cells detach from the dish, thereby escaping cell counting, this method underestimates the percentage of cells with altered chromatin found in the control group and the AA-mix treated group. Approximately 20% less cells have been counted in the control and the AA-mix group (Table 1) compared to Cerebrolysin after 8 DIV. However, regarding the lost cells as apoptotic contributes to a higher percentage of apoptotic neurons in the control and AA-mix group, thereby scaling up the antiapoptotic effect of Cerebrolysin. Because of this mathematical problem of the method used for the assessment of apoptosis, we discuss the results obtained by this method in a more qualitative than quantitative way.

Together with the results from the MTT-reduction assay, we suggest a transient trophic influence of the AA-mix promoting cell viability independent from cell death after 4 DIV. Even 10% FCS addition could not rescue all neurons from PCD which corroborates similar findings of Reinprecht et al. (1998). These cells most likely are those which, at the time of isolation, have already been committed to undergo a cell death program. Possible reasons for PCD in-vivo are a deficiency of growth factor supply (Lo et al., 1995), which is only to some extend counteracted by serum addition in the present study.

The ineffectiveness of the AA-mix in rescuing neurons from PCD points to the peptide fraction of Cerebrolysin to be responsible for the neuroprotective effect. Although it has never been shown before, in-vivo effects of the amino acids are unlikely as Cerebrolysin application only leads to a minimal increase of the amino acid pool of the organism. In a previously published study (Reinprecht et al., 1998) the addition of 5% and 10% FCS increased the viability of isolated chicken telencephalon neurons to 200% and 319% after 7 DIV. Although, the viability of cultures supplemented with 5% and 10% FCS was not measured after 8 DIV in the current study, it can be concluded from the results of Reinprecht et al. that the addition of serum is at least as effective in ameliorating the viability of chicken telencephalon neurons than Cerebrolysin treatment. This, however, is conceivable since serum reduction (2% FCS) was used for the induction of apoptosis in the current study and serum withdrawal was shown to induce PCD in many different types of neuronal cultures (Atabay et al., 1996; Eves et al., 1996; Howard et al., 1993; Miller and Johnson, 1996; Yu et al., 1997).

In accordance to Reinprecht et al. (1998) the addition of the naturally occurring growth factor FGF-2 (670 pg/ml) improved the viability of chick cortical neurons about 10% in both studies. Although FGF-2 elevated the viability in the present study, it was found to increase PCD compared to

controls. This contradicting finding is further supported by the fact that after 4 DIV the number of cells per field (Table 1) was significantly lowered compared to the control group. This suggests a positive effect of FGF-2 on metabolism counteracted by cell death. Moreover, this result could explain the fact why higher concentrations of FGF-2 diminished neuronal viability (Reinprecht et al., 1998). Furthermore, these data are consistent with an in-vivo study demonstrating the failure of FGF-2 to improve survival and differentiation of developing neurons in the CNS of avian embryos (Oppenheim et al., 1992).

The viability of BDNF treated neurons is still increased after 8 DIV together with a small decrease of PCD. This argues for an increased metabolism of surviving neurons and a late onset of apoptosis prevention, consistent with studies demonstrating BDNF to successfully rescue rat cerebellar granule neurons from apoptosis caused by a glucose deprivation and elevated K^+ levels (Kubo et al., 1995; Suzuki and Koike, 1997; Tong and Perez-Polo, 1998).

In conclusion, our results provide evidence for a long lasting neuroprotective efficacy of Cerebrolysin elicited by a decrease of PCD of cultured chick embryonic neurons. This result can be helpful in understanding in-vivo findings in fimbria-fornix transected rats (Francis-Turner and Valouskova, 1996), showing reduced spatial memory impairment after Cerebrolysin treatment similar to the effect of NGF. Akai et al. (1992) demonstrated, using the same model, that treatment with intraperitoneal injections of Cerebrolysin for 14 days rescues the cholinergic neurons in the medial septum from degeneration. The mechanism of action leading to an antiapoptotic effect of Cerebrolysin still needs to be determined. A possible explanation of PCD preventing effect of Cerebrolysin is provided by Wronski et al. (2000) demonstrating an inhibition of both calpain types under influence of Cerebrolysin in cell free in-vitro assays. The activation of the calcium–dependent cysteine protease, calpain, has been implicated in excitotoxic and in some types of apoptotic cell death (Siman and Noszek, 1988; Squier et al., 1994) arguing for an involvement of drug mediated protease inhibition. The PCD preventing action of Cerebrolysin has to be investigated in further studies to find out the exact underlying mechanism and the active compound of the peptide fraction of Cerebrolysin responsible for the current findings.

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