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Effects of Cerebrolysin on the outgrowth and protection of processes of cultured brain neurons

M. Hartbauer[1], **B. Hutter-Paier**[2,*], and **M. Windisch**[2,*]

[1] Institute of Zoology, Department of Neurobiology, Karl-Franzens University, and
[2] Institute of Experimental Pharmacology, Research Initiative EBEWE, Graz, Austria

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Summary. Cerebrolysin (Cere, EBEWE Arzneimittel, Austria), a peptidergic drug produced by a standardised enzymatic breakdown of porcine brain proteins, consists of a mixture of 75% free amino acids and 25% low molecular weight peptides (<10k DA). Cerebrolysin was shown to protect against MAP2 loss in primary embryonic chick neuronal cultures after brief histotoxic hypoxia and in a rat model of acute brain ischemia. Since MAP2 is involved in processes like neuronal growth, plasticity and dendritic branching, we address the question whether Cere is protecting processes against degeneration in a chronic low serum (2% FCS) cell stress model and whether the spontaneous outgrowth of axon-like processes is influenced. This was accomplished by quantification of the neurite lengths of embryonic chicken telencephalon neurons after 4 and 8 days. Additionally, time-laps video microscopy was performed to study a possible influence of Cere on the growth cone behaviour of axon-like processes. To distinguish between effects caused by the peptide fraction and the effects related to free amino acids, we used an artificial amino acid solution (AA-mix).

Results demonstrate a process outgrowth promoting effect of the AA-mix and Cere after 4 DIV. After 8 days neuronal network degeneration occurred in the AA-mix treated cultures, whereas Cere treated cultures still presented a well differentiated neuronal network. Dying neurons could release factors possibly impeding neurite outgrowth and Cere was shown to increase the viability of chicken cortical neurons. Neither the addition of BDNF nor serum supplementation (5% and 10% FCS) could protect the neuronal network against degeneration after 8 DIV, although these treatments were shown to ameliorate the viability of chicken telencephalon neurons. This result together with the finding obtained using the artificial amino acid solution points to the peptide fraction of Cere to be responsible for the protection of pro-

* Present address: JSW-Research Ltd, Graz, Austria

cesses against degeneration. Time-laps studies of Cere treated cultures revealed a significant decrease of the velocities characterising random growth cone movements, which is thought to be responsible for an increase in the length of axon-like processes after 4 DIV.

Keywords: Cerebrolysin, time-laps study, neuronal degeneration, serum, neurite outgrowth.

Introduction

Since it has been shown that cells transfected with MAP2 showed increased microtubule stability (Ferralli et al., 1994) and cells treated with MAP2 antisense oligonucleotides (Caceres et al., 1992; Dinsmore and Solomon, 1991) failed to form neuritic processes, the role of MAP2 in modulating microtubule functions and its implication on neuronal plasticity has clearly been demonstrated. Cerebolysin (Cere) (EBEWE Arzneimittel, Austria, Europe) treated rats showed a significant protection against loss of MAP2 immunoreactivity in the subiculum and CA1 region of the hippocampus after focal brain ischemia (Schwab et al., 1996, 1998). Neurodegenerative disorders like Alzheimer's disease and pathologies resulting from ischemic insults are characterised by cytoskeletal dysfunction. Cere has been used for the treatment of dementia and the sequels of stroke for more than 40 years (Barolin et al., 1996; Rüther et al., 1994; Vereschagin et al., 1991). This porcine brain-derived peptide preparation is produced by a standardised enzymatic breakdown of porcine brain proteins, containing a mixture of 75% free amino acids and 25% low molecular weight peptides (<10k DA), based on the total nitrogen content. Recent in-vitro studies using chicken telencephalon neurons demonstrated a dose dependent effect of Cere against neurodegeneration (verified by a viability assay) and MAP2 loss after brief histotoxic hypoxia and iron induced oxidative stress (Hutter-Paier et al., 1998a,b). Since MAP2 is involved in processes like neuronal growth, plasticity (Johnson and Jope, 1992) and dendrite branching (Friedrich and Aszodi, 1991) and Cere is known to reduce the translation dependent loss of MAP2 (Wronski et al., 2000a), we address the question whether Cere is altering the outgrowth and stability of neuronal processes of isolated chicken embryonic cortical neurons and whether this is accompanied by a change in the growth cone behaviour. This was accomplished by the study of the total process length after 4 and 8 DIV of neurons maintained in low serum culture (2% FCS) and by studying growth cone behaviour using time lapse video microscopy. The culture of chicken brain neurons is unusually simple and reliable, and some of these cells undergo early events of axonal-dendritic polarity. We differentiated between effects related to the peptide and amino acid fraction of Cere by use of an artificial amino acid solution (AA-mix) as a control resembling exactly the free amino acid fraction of Cere in quality as well as in quantity. Since Cere treatment is increasing the viability of chicken telencephalon neurons (Hutter-Paier et al., 1998a), the higher amount of dying neurons in control cultures may release factors possibly impeding process outgrowth. In order to elucidate a possible influence caused by dying neurons either BDNF or foetal

calf serum was added to additional control cultures. Both of these treatments were recently shown to ameliorate the viability of chicken telencephalon neurons (Reinprecht et al., 1998). Total process outgrowth per neuron was assessed by manually tracing processes of neurons in culture for 4 and 8 days using an image analysis software.

As previously shown by Chada et al. (1997) approx. 25–30% of chick telencephalon neurons formed a long uniform caliber process (in the following referred to as axon-like process or simply axons) after 2–3 days. In this process polysomes were completely absent and a similar density of microtubules and neurofilaments to that observed previously in axon-like neurites of chick sensory neurons were evident (Baas et al., 1987). The net outgrowth of axon-like processes of neurons either treated with the AA-mix or with Cere was quantified after 4 DIV. Additionally, time-lapse video microscopy was performed in order to rule out a possible influence of the peptide fraction of Cere on the growth cone behaviour of axon-like processes. Parameters describing stationary and dynamic properties of growth cones obtained at sampling intervals of 1 min revealed growth cone behaviour to be dominated by random movements leading to mean durations of continuous outgrowth, shrinkage and resting phases of about 1.3 min.

Materials 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 reduce 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 counted in a hemocytometer and viability was determined by using Trypan Blue exclusion method. Six-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 28,000 cells/cm^2, which was enough to guarantee neuronal network development in cultures raised for 4 days. Cultures were maintained in an incubator at 37°C, 5% CO_2 and 95% humidity.

Cerebrolysin® (EBEWE Pharmaceuticals, Austria; Batch: 802772), in the following referred to as Cere, was added to cultures in concentrations of 0.4 mg/ml (10 µl Cere/ml medium) from the first day onwards. This dose was shown to increase the viability of chicken cortical neurons about 55% compared to controls using low cell density and 2% serum supplement (Hartbauer et al., unpublished results). For the investigation of amino acids related effects concerning the outgrowth of processes and for the study of growth cone behaviour, 10 µl of free amino acids naturally found in Cere were added to cultures using an artificial amino acid mixture (AA-mix; Batch: 902753; EBEWE Pharmaceuticals). Since dying neurons might have influence on process outgrowth positive control groups either treated with BDNF or foetal calf serum were used. 50 ng/ml human recombinant BDNF (Sigma Chemicals) were added to the BDNF control group, determined as the optimal dose to support the viability of chicken telencephalon neurons (Reinprecht et al., 1998). This dose led to 34% increase in viability of chicken telencephalon neurons compared to control cultures (2% FCS) after 7 DIV. 5% FCS and 10% FCS supplementation led to 100% and 219% increase in viability compared to controls supplemented

with 2% FCS after 7 DIV (Reinprecht et al., 1998). In order to prevent effects due to dilution with 10 µl Cere or 10 µl AA-mix, we added 10 µl phosphate buffered saline (PBS, Bio Whittaker) per ml medium to the BDNF treated group and the cultures supplemented with 2%, 5% and 10% serum.

A newly designed, now commercially available, microincubation chamber (Fa. Gottlieb; Graz Austria) was used to maintain constant temperature (37 ± 0.1°C) and gas supply (5% CO2, 95% air). The temperature of the culture medium was continuously measured with a calibrated temperature sensor in direct contact with the culture medium. This method guaranteed the culturing of telencephalon neurons in the microincubator for up to 8 days without changing cell culture medium.

After sterilising the whole microincubator in an autoclave the glass cover slip (30 mm diameter) was coated with poly-D-lysine (Boehringer Mannheim). After carefully rinsing three times with sterile water, the cover slip was allowed to dry and freshly isolated telencephalon neurons were seeded at low density of 28,000 cells/cm^2.

Quantification of neurite morphology at different stages of differentiation

Chick embryonic neurons cultured in 6 well plates for 4 and 8 days were visualised using a colour video camera (JVC) mounted on an inverted microscope (Axiovert 35, Zeiss). Phase contrast micrographs of randomly selected microscopic fields (20X lens, NA 0.5) were taken using an analogue frame grabber (Matrox Meteor, Matrox electronic systems Ltd.) and images were stored on a personal computer. Net neurite outgrowth was measured by manually tracing processes with the mouse cursor using an image analysis software (KS 300, Zeiss). Since neurons already established a neuronal network after 4 DIV, the mean total process length per neuron was evaluated by quantifying the length of all processes visible in a microscopic field and dividing it by the number of neurite bearing neurons. This was done without differentiating between "minor" processes and long caliber processes. The number of neurite segments was used as a measure for neurite branching and ramification, thereby reflecting neuronal network complexity. A neurite segment was defined as the distance between branching points or the distance between branches and the neurite tip. The mean number of neurite segments per neuron was calculated from the number of segments per microscopic field divided by the number of neurite bearing neurons. The lengths of neurites and the number of neurite segments of about 120 neurons were analysed in each group using cells cultured for 4 and 8 DIV.

Measurement of time laps sequences of spontaneously outgrowing axons

Axonal growth of chicken embryonic telencephalon neurons was monitored at 400X magnification (40X lens, NA 0.75) on an inverse microscope (Axiovert 35; Zeiss Germany) equipped with a phase contrast optic. A colour video camera (JVC; Japan) was attached to the microscope and a video capture board (Matrox Meteor; Matrox Electronic Systems; Canada) used to grab video frames and to store them on a PC. Additionally, the camera was connected to a SVHS-video recorder (JVC; Japan), which allowed the recording of growth cone behaviour in real time. Frame grabbing and the control of a motorised microscope stage (MCP-4, Zeiss Germany) was realised with the help of an image analysis software (KS300; Zeiss Germany). This allowed to automatically generate time laps sequences lasting for 1 hour using sampling intervals of 1 minute. Damage due to light exposure was prevented by use of a heat-reducing filter and low light conditions.

Criteria for choosing axon-like processes

The axon-like processes of neurons in culture for 4 DIV were all at least 90 µm long and separated from other structures. Axons selected for the study of growth cone behaviour were in areas of minimal debris. Data was analysed only when no contact of the growth cone with other cells or processes occurred during the observation period of one hour. Axons with predominately lamellipodial growth cones were preferred.

Analysis of growth cone motility

At a high magnification considerable detail became apparent in growth cone morphology, requiring a sophisticated approach to quantitation. A macro was written (KS300; Zeiss) to analyse growth cone motility and shape parameters. After manually outlining the lamellipodial region of the growth cone, the growth cone centroid was calculated from the filled area. By tracking the motion of the growth cone centroid we calculated parameters describing growth cone dynamics. Image drift was compensated using cell debris serving as an invariant reference point.

To determine axonal elongation and shrinkage in the direction of growth, we adopted a method recently described by Tanaka and Kirschner (1991). A reference point on the neurite shaft was defined by drawing a circle with a radius of 8.2 μm around the tip of the growth cone centroid. The point where the circle crosses the neurite shaft was defined as a reference point (p_1). For each successive frame a vector (v_p) from p_1 to the tip of the growth cone was calculated. The vector magnitudes of two successive frames were subtracted ($|v_{p+1}| - |v_p|$) and the resulting vector magnitude represents the velocity in the direction of axonal growth (in the following referred to as persistence velocity). This analysis is a crude indicator of the persistent movement of the growth cone. If the growth cone retracts or moves backwards, the velocity is negative, if the growth cone wanders laterally, the velocity also approaches 0. It happened only two times that the shrinkage of the growth cone centroid exceeded 8 μm causing a conflict with the currently used method. The whole time laps sequence of these growth cones was rejected.

Phase analysis

Outgrowth was defined as the displacement of the growth cone of more than 0.5 μm in the direction of axonal growth. Shrinkage was defined as the retraction of the axon of more than −0.5 μm. Resting was defined as the growth cone centroid displacement (in the direction of axonal outgrowth) between −0.5 and 0.5 μm. We have chosen 0.5 μm as a threshold since it is 2.5 times the pixel resolution, which was shown to be sufficient to prevent errors due to the limits of the theoretical optical resolution. Using above definitions of growth, shrinkage and retraction the mean velocities and the mean duration of complete outgrowth, shrinkage and resting phases (phase analysis) was calculated.

Parameters describing stationary properties of the growth cone

– *area:* The area enclosed by the growth cone outline.
– *perimeter:* The perimeter of the growth cone outline.

To test whether the shape of the growth cones is related to growth cone dynamic a shape parameter was calculated. The fcircle ((4π*area) / (perimeter crofton^2)) describes the degree to which a shape differs from a circle. It varies from 0 to 1 (a perfect circle = 1).

Parameters describing growth cone dynamics

The instantaneous velocity defines the distance, irrespective of direction, between each successive x, y position of the growth cone centroid. The persistence velocity is the velocity of the growth cone centroid in the direction of axonal elongation. The nonelongational outgrowth velocity is the velocity of the growth cone which does not contribute to axonal elongation. This parameter was calculated by subtraction of the instantaneous velocity from the absolute value of the persistence velocity.

The sum of all growing and shrinking movements in the direction of axonal outgrowth during one hour was defined as the net outgrowth. The direction of the growth cone was calculated from the vector from p_1 to the growth cone centroid. The "change in direction" is the difference between the directions of the displacements during adjacent time intervals.

Statistical analyses

Differences between means of individual groups were evaluated by a Kruskal-Wallis one way analysis of variance. Groups were considered as significantly different at a level of $p < 0.05$. Correlations between individual parameters characterising growth cone behaviour were analysed by use of a Spearmans rank correlation with paired exclusion of missing data. Correlations between individual parameters were considered as significant at $p < 0.05$ (Bonferroni correction was performed). All statistics were carried out with STATISTICA for windows (StatSoft Inc.).

Results

Effects of Cere, the AA-mix, BDNF and serum supplement on neurite morphology in-vitro

As recently described by Chada et al. (1997) cultured cortical chick neurons undergo a stereotyped developmental sequence of neurite outgrowth that resembles that observed for rat hippocampal neurons in culture (Dotti et al., 1988). After 4 days of cultivation in media containing 2% foetal calf serum, most of the cells have developed dendrite like processes (corresponding to stage 2–3 of Dotti et al., 1988), with a few cells developing a single, long neurite of uniform caliber. The majority of control cells, however, do not develop to stage 4 and a substantial fraction of these cells did not develop a single long neurite even after 8 DIV.

As shown in Fig. 1A and 1B, after 4 DIV Cere and the AA-mix treatment significantly increased ($p < 0.01$) the mean total process length and the mean segment number calculated per neuron. Additionally, serum was able to significantly increase ($p < 0.01$) these parameters at that stage of development. After 8 DIV, however, the mean total process length per neuron was only significantly increased ($p < 0.01$) in Cere treated cultures compared to controls. The mean segment number was significantly increased ($p < 0.05$) in the Cere group as well. A significant increase ($p < 0.05$) in total neurite length per neuron was found in all groups in the late phase of the experiment. The increase in neurite length was counteracted by a loss of process segments with the exception of the Cere treated group. This loss was significant in the AA-mix and cultures supplemented with serum ($p < 0.05$ for AA-mix treated cultures, $p < 0.05$ for cultures supplemented with 5% FCS, $p < 0.05$ for cultures supplemented with 10% FCS).

Growth cone behaviour of axon-like processes of chicken brain neurons

At sampling intervals of 1 min the growth cone behaviour of axon-like processes was dominated by alternating growth and retraction movements thereby reducing phases of continuous outgrowth to about 10% of the observation period. The investigation of the average instantaneous velocity and the average velocity in the direction of axonal growth (persistence velocity) of 25 individual growth cones revealed no correlation of these parameters with the net outgrowth during an observation period of one hour. Moreover, the time spent in outgrowth and retraction determined net outgrowth. This was shown by a significant correlation ($p < 0.05$) of the ratio of growth vs. shrinkage

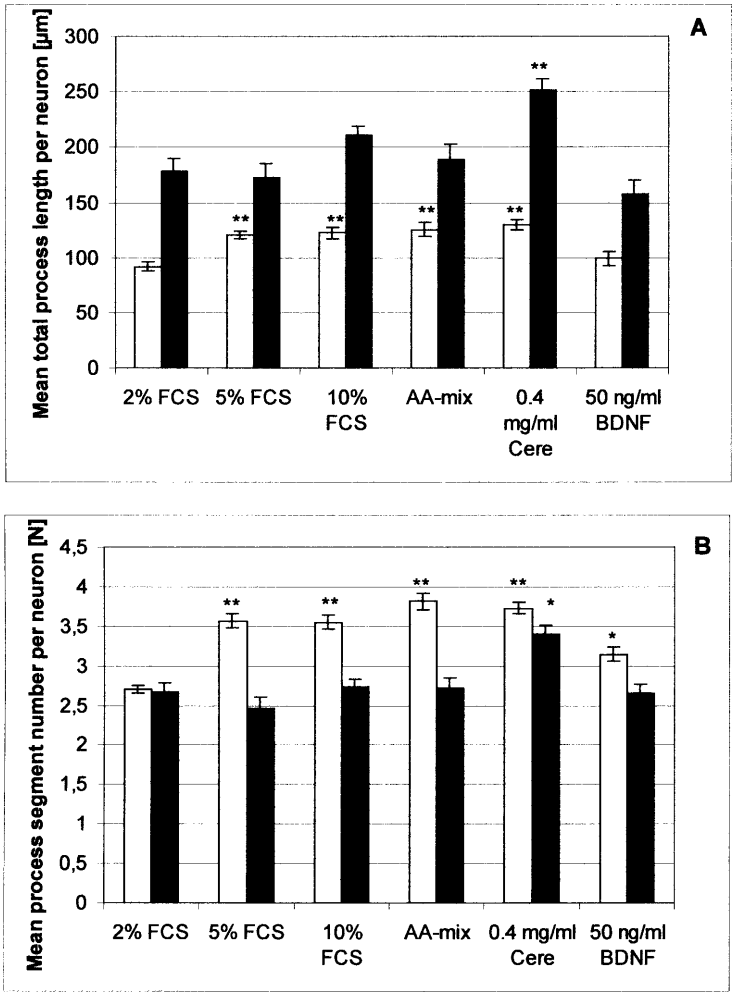

Fig. 1. Mean process length per neuron and mean number of process segments per neuron after 4 and 8 DIV. We quantified the total process length by manually tracing the processes visible on microphotographs acquired from cultures maintained in the incubator for 4 DIV (white bars) and 8 DIV (black bars). The mean process length per neuron (**A**) was calculated from the total process length per microscopic field divided by the number of process bearing neurons. The mean number of process segments per neuron (**B**) was calculated from the total number of process segments per field divided by the number of process bearing neurons. Values are given as means ± s.e.m of about 20 microscopic fields obtained from four individual experiments. A total of about 120 neurons were analysed in each group. Differences vs. controls: *$p < 0.05$, **$p < 0.01$.Verified by a Kruskal-Wallis one way ANOVA

times with the net outgrowth obtained from observation periods of one hour. Axonal outgrowth is therefore time controlled, which means that a difference in axonal outgrowth will become apparent only during longer observation periods.

Correlation analysis of individual parameters characterising the velocities of growth cones obtained during a total observation period of 564 min revealed to be independent of the area and the shape of growth cones.

Influence of the AA-mix and Cerebrolysin on the growth cone behaviour of chicken telencephalon neurons

The total length of axon-like processes after 4 DIV was significantly increased ($p < 0.05$) in Cere treated cultures compared to the AA-mix treated control group (AA-mix: 99.2 ± 35 μm; Cere: 117.3 ± 29 μm). In order to investigate whether this increase is accompanied by an altered growth cone dynamic, time-lapse studies of growth cones of axon-like processes of neurons in culture for 4 DIV were performed. The instantaneous and the persistence growth cone velocity of Cere treated neurons were significantly smaller ($p < 0.05$ for the persistence velocity, $p < 0.01$ for the instantaneous velocity) compared to the AA-mix treated cultures (Table 1). This decrease in growth cone velocities was accompanied by significant smaller changes in the growth cone direction ("change in direction"). Additionally, the form of the growth cones of Cere treated neurons was significantly different ($p < 0.01$) from the group treated with the AA-mix. Interestingly, the ratio of growth time vs. shrinkage times and the net outgrowth during one hour were similar in both groups. The mean duration of complete shrinkage and resting phases were not significantly different between the Cere and the AA-mix treated group and was very short (about 1.3 min). Therefore, the average phase velocities were similar com-

Table 1. Influence of Cere on parameters characterising stationary and dynamic properties of growth cones of neurons cultured for 4 DIV

Sampling intervals	Pooled data	AA-mix	SD	Cere	SD
1 min	number of growth cone movements analysed	564		894	
	growth time / shrinkage time	1.20		1.11	
	instantaneous velocity [μm/min]	1.83	1.30	1.62**	1.21
	persistence velocity [μm/min]	1.26	1.12	1.16*	1.12
	nonelongational outgrowth rate [μm/min]	0.57	0.80	0.44**	0.67
	change in direction [degree/min]	9.40	20.60	7.37*	16.63
	growth cone area [μm^2]	49.43	25.61	31.39**	14.24
	growth cone perimeter [μm]	55.91	21.83	40.74**	15.35
	fcircle (growth cone shape)	0.25	0.11	0.30**	0.12
	avg. net outgrowth [μm/h]	4.37	4.20	3.73	4.00
5 min	number of growth cone movements analysed	112		178	
	instantaneous velocity [μm/min]	0.51	0.33	0.42*	0.31
	persistence velocity [μm/min]	0.39	0.42	0.32	0.28

Chicken telencephalon neurons maintained in tissue culture medium supplemented with 2% FCS were either treated with the AA-mix or Cere once at the beginning of culture. After 4 DIV time laps sequences lasting for at least 1 hour of growth cones of axon-like processes (at least 90 μm long) were generated at sampling intervals of 1 min and 5 min. Parameters characterising growth cone dynamic were described in detail in the materials and methods chapter. With the exception of values shown without standard deviation (SD), values were calculated as means ± SD from pooled data of 10 individual growth cones in the AA-mix treated group and 15 individual growth cones in the Cere treated group. *$p < 0.05$, **$p < 0.01$ that the parameter is significantly different from the AA-mix treated group. Verified by a Kruskal-Wallis one way ANOVA

pared to the growth cone velocities obtained at sampling intervals of 1 min (data not shown). The estimated growth cone velocities obtained at sampling intervals of 5 min were significantly smaller ($p < 0.01$) compared to the velocities measured at 1 min sampling intervals (Table 1). Interestingly the outgrowth velocity in the direction of axonal growth was not significantly different from the shrinkage velocity obtained at sampling intervals of 1 min under Cere and the AA-mix treatment (persistence outgrowth velocity: AA-mix: 1.25 ± 1.06, Cere: 1.15 ± 1.13; persistence shrinkage velocity: AA-mix: 1.28 ± 1.20, Cere: 1.19 ± 1.11).

Discussion

Chicken cortical neurons, used in the current study, did not follow all morphological stages of development known from cultured rat hippocampal neurons (Dotti et al., 1988), because outgrowth of a single long neurite was only observed in a small fraction of cells. This finding is in line with studies of Chada et al. (1997) performing cytomechanic investigation of spontaneously initiated neurites from chicken forebrain neurons cultured on poly-L-lysine. In the present study, after 4 DIV Cere treatment, the addition of the AA-mix and serum supplement accelerated neuronal differentiation, which was reflected by an increase in the total length of processes and the number of process segments calculated per neuron. A difficulty of the present study is the fact that cell death could counteract neurite outgrowth and therefore influence the establishment of a neuronal network. Cere is increasing the viability of chicken cortical neurons (Hutter-Paier et al., 1998a) and therefore factors released by dying neurons could impede neurite outgrowth in control cultures. We tried to overcome this fact by measuring the process length and the number of neurite segments per neuron under influence of serum supplement or BDNF. Treatment with BNDF and serum supplement were shown to significantly increase the viability of chicken cortical neurons (Reinprecht et al., 1998). Since after 8 DIV only Cere treated cultures exhibited significantly longer ($p < 0.01$) processes lengths and a higher number of process segments, the peptide fraction of Cere is capable of protecting once established neurites against degeneration (Fig. 1).

After 4 DIV, however, the amino acid fraction of Cere increased process outgrowth of neurons compared to the group supplemented with 2% FCS. This effect could be mediated by a possible neurotrophic effect, although other mechanisms are conceivable. Amino acids, especially excitatory amino acids, play a key role in neurite outgrowth and synaptic plasticity. L-glutamate is known to act as dendrite outgrowth inhibitor in hippocampal cell cultures (Mattson, 1990), and L-serine was recently shown to promote process outgrowth and differentiation of chick retinal explants (Savoca et al., 1995). Since both amino acids are found in Cere, it is conceivable that they elicit a complex interaction resulting in a neurite outgrowth promoting effect visible after 4 DIV. However, little is known about the influence of amino acids on neurite outgrowth of chicken brain neurons. Although it was never shown before, in-vivo effects of the amino acids of Cere are unlikely, since the plasma level of

amino acids is much higher than those administered during Cere treatment. Axonal outgrowth was time controlled and small differences in axonal growth rate, leading to an increase of the average axonal length after 4 DIV, will not be detectable during observation periods lasting for one hour. The axonal outgrowth of hippocampal neurons was found to be intermittent (Dotti et al., 1988) with an average net outgrowth rate of approximately 6 μm/h, which is similar to the one measured in the current study (approximately 4 μm/h). Axons of hippocampal neurons in culture for 4 days established axons approximately 180 μm in length (Dotti et al., 1988). In contrast axon-like processes of chicken telencephalon neurons of the current study are shorter (about 100 μm).

The velocities measured at sampling intervals of 1 min were shown to be independent of axonal outgrowth and independent of the form of the growth cones. The short time of outgrowth, shrinkage and resting phases (about 1.3 min) of axon-like processes of chicken telencephalon neurons together with the result that the persistence outgrowth and shrinkage velocities were not significantly different at both sampling intervals, accounts for a growth cone behaviour dominated by random movements. However, the growth cone movement cannot entirely be controlled by random movements, since then axonal outgrowth would not occur. At longer sampling intervals a lot of these random movements will be filtered leading to significantly smaller growth cone velocities thereby reflecting axonal elongation better. The study of the outgrowth of axon-like processes of chicken telencephalon neurons therefore provides the possibility to study the stochastic nature of growth cone behaviour. The nonelongational velocity is about 2.5 times smaller compared to the directional persistence velocity even when the proportion of velocity contributing to net outgrowth (about 0.06–0.07 μm/min) is subtracted. This result would not be expected for a growth cone behaviour mainly dominated by random movements with no preference in direction. Microtubules assembly was found to be tightly coupled to growth cone advance (Odde and Buettner, 1995). Therefore the higher velocities in the direction of axonal growth could reflect dynamic instability of microtubules necessary for growth cone advance (Tanaka et al., 1995). The decrease of the persistence velocity, found under Cere treatment, could therefore indicate an altered microtubule dynamic. MAP2 variants are involved in neuronal growth (Johnson and Jope, 1992) and seem to be major determinants of microtubule function. Because MAP2 protection was demonstrated by an inhibition of both calpain types under the influence of Cere (Wronski et al., 2000b) a possible involvement of microtubule dynamic under influence of this drug is conceivable and should be proven in a separate study. The decrease in the instantaneous velocity, which was accompanied by a smaller change in direction of growth cone movements, accounts for a stabilisation of growth cone advance most likely responsible for longer axon-like processes after 4 DIV. Additionally, the shape and the area of growth cones was significantly ($p < 0.01$) different between Cere and AA-mix treated groups. This result indicates an influence of Cere on the actin structure of growth cones possibly changing growth cone dynamics.

Taken the results together the current study provides evidence for a protective role of the peptide fraction of Cere against neuronal network degeneration, which is accompanied by an increase in outgrowth of axon-like processes possibly related to an altered growth cone behaviour. These results account for a protective mechanism of Cere acting independent from effects related to cell death and is of interest for further studies investigating the exact mechanism of Cere action. A part of the therapeutic effect of Cere can be attributed to the neurotrophic activity, resembling the properties of naturally occurring neurotrophic factors (Satou et al., 1993, 1994; Windisch et al., 1998). In this regard the findings of the current study might be of importance for patients suffering from Alzheimer's disease which is accompanied by a breakdown of neuronal cytoarchitecture.

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References

Baas PW, Sinclair GI, Heidemann SR (1987) Role of microtubules in the cytoplasmic compartmentation of neurons. Brain Res 420: 73–81

Barolin GS, Koppi S, Kapeller E (1996) Old and new aspects of stroke treatment with emphasis on metabolically active medication and rehabilitative outcome. EUROREHAB 3: 135–143

Caceres A, Mautino J, Kosik KS (1992) Suppression of MAP2 in cultured cerebellar macroneurons inhibits minor neurite formation. Neuron 9: 607–618

Chada S, Lamoureux P, Buxbaum RE, Heidemann SR (1997) Cytomechanics of neurite outgrowth from chick brain neurons. J Cell Sci 110 (Pt 10): 1179–1186

Dinsmore JH, Solomon F (1991) Inhibition of MAP2 expression affects both morphological and cell division phenotypes of neuronal differentiation. Cell 64: 817–826

Dotti CG, Sullivan CA, Banker GA (1988) The establishment of polarity by hippocampal neurons in culture. J Neurosci 8: 1454–1468

Ferralli J, Doll T, Matus A (1994) Sequence analysis of MAP2 function in living cells. J Cell Sci 107 (Pt 11): 3115–3125

Friedrich P, Aszodi A (1991) MAP2: a sensitive cross-linker and adjustable spacer in dendritic architecture. FEBS Lett 295: 5–9

Hutter-Paier B, Steiner E, Windisch M (1998a) Cerebrolysin protects isolated cortical neurons from neurodegeneration after brief histotoxic hypoxia. J Neural Transm [Suppl] 53: 351–361

Hutter-Paier B, Grygar E, Fruhwirth M, Temmel I, Windisch M (1998b) Further evidence that Cerebrolysin protects cortical neurons from neurodegeneration in vitro. J Neural Transm [Suppl] 53: 363–372

Johnson GV, Jope RS (1992) The role of microtubule-associated protein 2 (MAP-2) in neuronal growth, plasticity, and degeneration. J Neurosci Res 33: 505–512

Mattson MP (1990) Excitatory amino acids, growth factors, and calcium: a teeter-totter model for neural plasticity and degeneration. Adv Exp Med Biol 268: 211–220

Odde DJ, Buettner HM (1995) Time series characterization of simulated microtubule dynamics in the nerve growth cone. Ann Biomed Eng 23: 268–286

Pettmann B, Louis JC, Sensenbrenner M (1979) Morphological and biochemical maturation of neurones cultured in the absence of glial cells. Nature 281: 378–380

Reinprecht K, Hutter-Paier B, Crailsheim K, Windisch M (1998) Influence of BDNF and FCS on viability and programmed cell death (PCD) of developing cortical chicken neurons in vitro. J Neural Transm [Suppl] 53: 373–384

Rüther E, Ritter R, Apecechea M, Freytag S, Windisch M (1994) Efficacy of the peptidergic nootropic drug Cerebrolysin in patients with senile dementia of the Alzheimer type (SDAT). Pharmacopsychiatry 27/1: 32–40

Satou T, Imano M, Akai F, Hashimoto S, Itoh T, Fujimoto M (1993) Morphological observation of effects of Cerebrolysin on cultured neural cells. Adv Biosci 87: 195–196

Satou T, Itoh T, Fujimoto M, Hashimoto S (1994) Neurotrophic-like effects of FPF-1070 on cultured neurons from chick embryonic dorsal root ganglia. Jpn Pharmacol Ther 22/4: 205–212

Savoca R, Ziegler U, Sonderegger P (1995) Effects of L-serine on neurons in vitro. J Neurosci Methods 61: 159–167

Schwab M, Antonow-Schlorke I, Durer U, Bauer R (1996) Effects of Cerebrolysin on cytoskeletal proteins after focal ischemia in rats. J Neural Transm [Suppl] 47: 279

Schwab M, Antonow-Schlorke I, Zwiener U, Bauer R (1998) Brain-derived peptides reduce the size of cerebral infarction and loss of MAP2 immunoreactivity after focal ischemia in rats. J Neural Transm [Suppl] 53: 299–311

Tanaka EM, Kirschner MW (1991) Microtubule behavior in the growth cones of living neurons during axon elongation. J Cell Biol 115: 345–363

Tanaka E, Ho T, Kirschner MW (1995) The role of microtubule dynamics in growth cone motility and axonal growth. J Cell Biol 128: 139–155

Vereschagin NV, Nekrasova YM, Lebedova NV, Suslina ZA, Soloview OI, Priadov MA, Altunina M (1991) Mild forms of multi-infarct dementia: efficacy of cerebrolysin. Sov Med 11: 1–6

Windisch M, Gschanes A, Hutter-Paier B (1998) Neurotrophic activities and therapeutic experience with a brain derived peptide preparation. J Neural Transm [Suppl] 53: 289–298

Wronski R, Kronawetter S, Hutter-Paier B, Windisch M (2000a) A brain derived peptide preparation reduces the translation dependend loss of a cytoskeleton protein in primary cultured chicken neurons. J Neural Transm [Suppl] 59: 263–272

Wronski R, Tompa P, Hutter-Paier B, Crailsheim K, Friedrich P, Windisch M (2000b) Inhibitory effect of a brain derived peptide preparation on the Ca++-dependent protease, calpain. J Neural Transm 107: 145–157

Authors' address: Mag. M. Hartbauer, Institute of Zoology, Department of Neurobiology, Karl-Franzens University, Universitätsplatz 2, A-8010 Graz, Austria, e-mail: hartbaue@email.kfunigraz.ac.at