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In vitro synaptotrophic effects of Cerebrolysin in NT2N cells

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Abstract Recent studies have shown that Cerebrolysin can enhance synaptic function and ameliorate synapto-dendritic alterations in animal models of neurodegeneration, suggesting a synaptotrophic effect. We hypothesize that Cerebrolysin might exert this effect, in part, by regulating the expression of amyloid precursor protein (APP). We studied the patterns of expression of synaptic proteins during differentiation of human teratocarcinoma cell line NTera 2 (NT2) in the presence or absence of Cerebrolysin. This study showed that the terminally differentiated neurons (NT2N) expressed a wide variety of synaptic markers and that expression of these synaptic-associated proteins coincided with the shift in expression from APP770/751 to APP695. Furthermore, APP immunoreactivity was colocalized with synaptophysin-immunoreactive neuritic varicosities in NT2N neurites, and Cerebrolysin treatment of NT2N cells resulted in an augmented and earlier expression of synaptic-associated proteins. This increased synaptic protein expression coincided with an increase in APP695 over APP770/751. These results support the possibility that synaptotrophic effects of Cerebrolysin might be mediated via regulation of APP expression.

Key words Cerebrolysin · Synaptotrophic effect · Amyloid precursor protein · NT2N cells

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Introduction

Cerebrolysin (EBEWE Pharmaceuticals, Austria) is a brain-derived peptide preparation, which consists of a fraction of free amino acids and a fraction of low molecular weight biologically active peptides [28] and has been used to treat, among others, patients with mild Alzheimer's disease (AD) [29]. Recent studies have shown that Cerebrolysin is capable of enhancing synaptic function and ameliorating the synapto-dendritic alterations in animal models of neurodegeneration [8, 9, 14, 18, 32], suggesting a synaptotrophic effect. The mechanisms through which Cerebrolysin exerts these effects are not fully understood; however, it is possible that Cerebrolysin might regulate the expression of molecules involved in synaptic maintenance and formation. Among them recent studies have shown that amyloid precursor protein (APP), which is a molecule centrally involved in the pathogenesis of AD [19, 33], might play a neuroprotective and synaptotrophic role in the central nervous system (CNS) after injury [20, 23, 24, 35].

The three major CNS APP isoforms are encoded by the same gene on chromosome 21 and alternative mRNA splicing generates APP695, APP751 and APP770 [7, 12, 38]. In AD, the balance between APP isoforms is altered and there is a relative shift toward an increase in APP770/751 versus APP695 [27]. Furthermore, altered APP balance has been liked to synaptic pathology in AD [15]. Therefore, regulation of the mechanisms of alternatively spliced APP might be important in synaptic maintenance. To explore the potential role of Cerebrolysin in promoting synaptotrophic effects via regulation of APP expression, we analyzed the patterns of synaptic protein and APP expression in a human teratocarcinoma cell line NT2, which after treatment with retinoic acid commits irreversibly to a neuronal phenotype (NT2N) [25]. This human cell line offers a unique opportunity to study this possibility because previous studies have shown that during NT2 cell differentiation there is a shift in APP expression from APP770/751 to APP695 [42]. In the present study, treatment of NT2N cells with Cerebrolysin

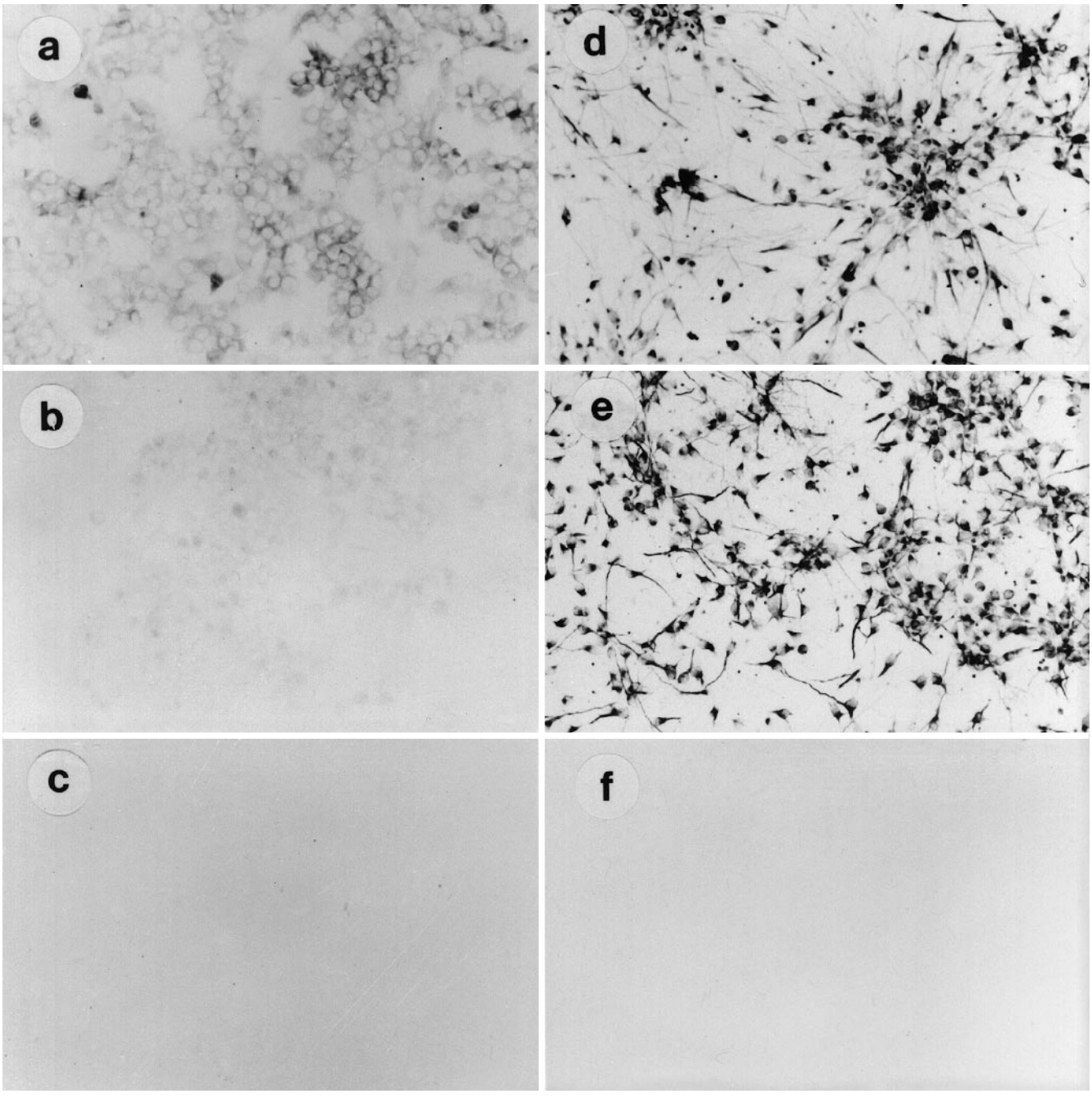

Fig. 1a–f Immunocytochemical studies of cytoskeletal proteins in NT2/NT2N cells. NT2 cells showed mild anti-neurofilament immunoreactivity (**a**), but were negative for the dendritic marker MAP2 (**b**) and the astroglial marker GFAP (**c**). NT2N cells displayed intense anti-neurofilament (**d**) and anti-MAP2 (**e**) immunoreactivity, and were anti-GFAP negative (**f**) (*MAP* microtubule-associated protein, *GFAP* glial fibrillary acidic protein)

resulted in an augmented and earlier expression of synaptic-associated proteins. This increased synaptic protein expression coincided with an increase in APP695 over APP770/751. These results support the possibility that synaptotrophic effects of Cerebrolysin might be mediated via regulation of APP expression.

Materials and methods

Cell culture and Cerebrolysin treatment

The NT2 cells were maintained in Dulbecco's modified Eagle medium (DMEM, high glucose) with 10% fetal bovine serum and 1% gentamycin. Treatment of NT2 with retinoic acid (RA) results in 99% pure NT2N cultures. To begin differentiation, 2.3×10^6 cells were seeded in T75 flasks and fed twice a week with DMEM containing 10 μM RA. After 5 weeks of treatment, the cells were plated at a lower density into two T225 flasks (for each T75 flask) for up to 10 days (replate). During the 10 days, the cells were treated with mitotic inhibitors (1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 10 μM uridine) to eliminate the remaining proliferating epithelial-like precursor cells, followed by differential harvesting of NT2N neurons, which at this stage were loosely attached to the flat undifferentiated cells.

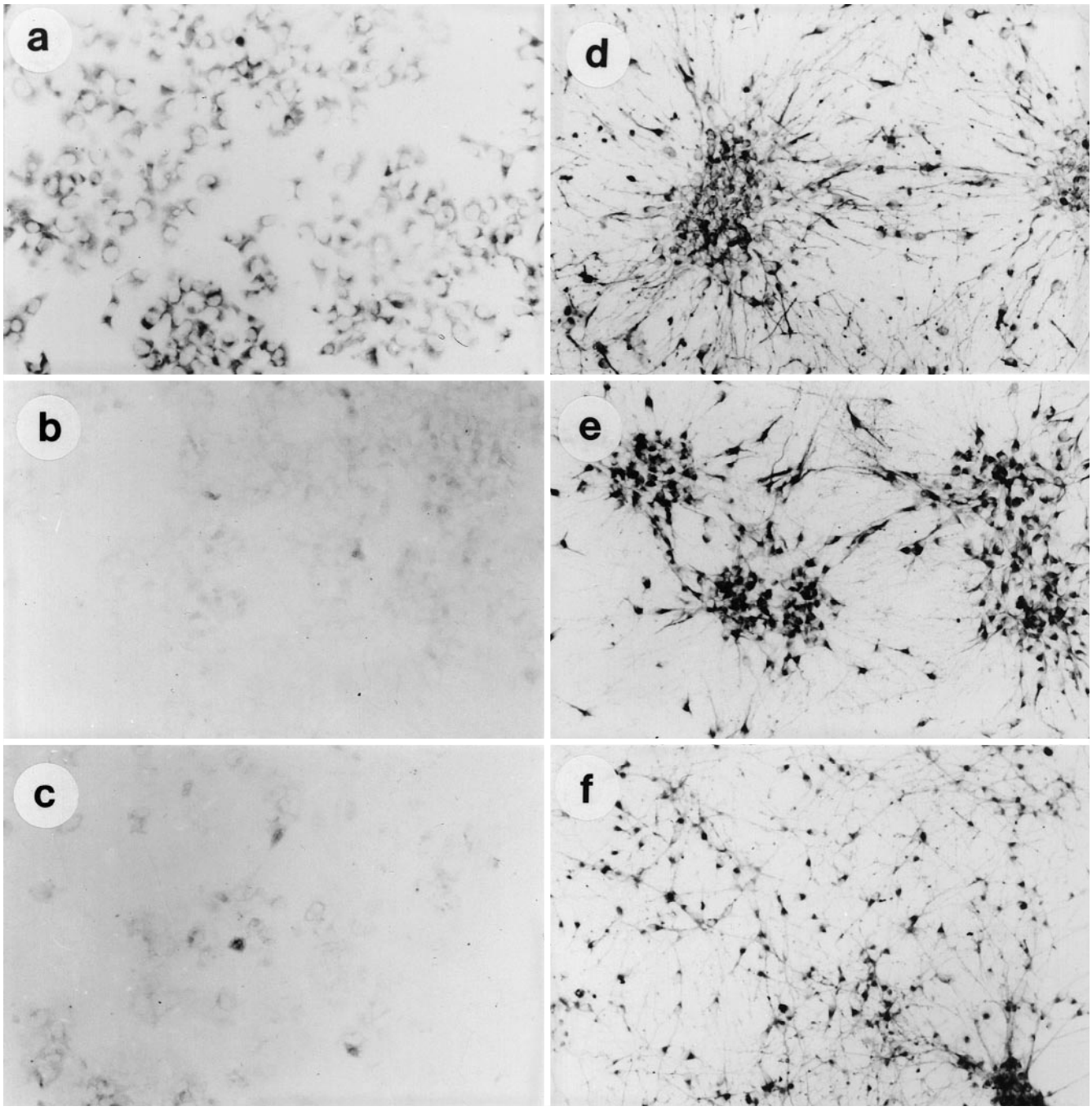

Fig. 2a–f Immunocytochemical studies of synaptic-associated proteins in NT2/NT2 N cells. NT2 cells showed moderate anti-GAP43 immunoreactivity (**a**) and mild anti-NACP (**b**) and syntaxin (**c**) immunoreactivity. NT2N cells presented intense immunostaining of neuronal cell bodies and processes with anti-GAP43 (**d**), α-synuclein (**e**) and syntaxin (**f**) (GAP growth associated protein, α-synuclein)

For treatment with Cerebrolysin, cells were given 10 μl/ml Cerebrolysin in addition to RA for 5 weeks, twice a week and collected for analysis. Following the differential replate, the flasks were divided into ones receiving mitotic inhibitors only or mitotic inhibitors plus 10 μl/ml Cerebrolysin and these cells were collected for analysis at days 2, 4, 7 and 10 post-replate. These treatment conditions resulted in generation of the following groups of cells: (1) RA alone, no Cerebrolysin after replate, (2) RA alone, Cerebrolysin after replate, (3) RA + Cerebrolysin, no Cerebrolysin after replate, and (4) RA + Cerebrolysin, Cerebrolysin after replate. Each experiment was performed in triplicate to confirm the reproducibility of the results.

Cell preparation

For immunocytochemical evaluation, NT2N cells were plated onto poly-L-lysine/Matrigel-coated chamber slides (1×10^5 cells/ml per chamber). The neurons were cultured for 7–10 days in medium consisting of 50:50 DMEM/48-h neuron conditioned medium, followed by fixation for 25 min at room temperature (RT) in 4% paraformaldehyde and three washes with phosphate-buffered saline (PBS).

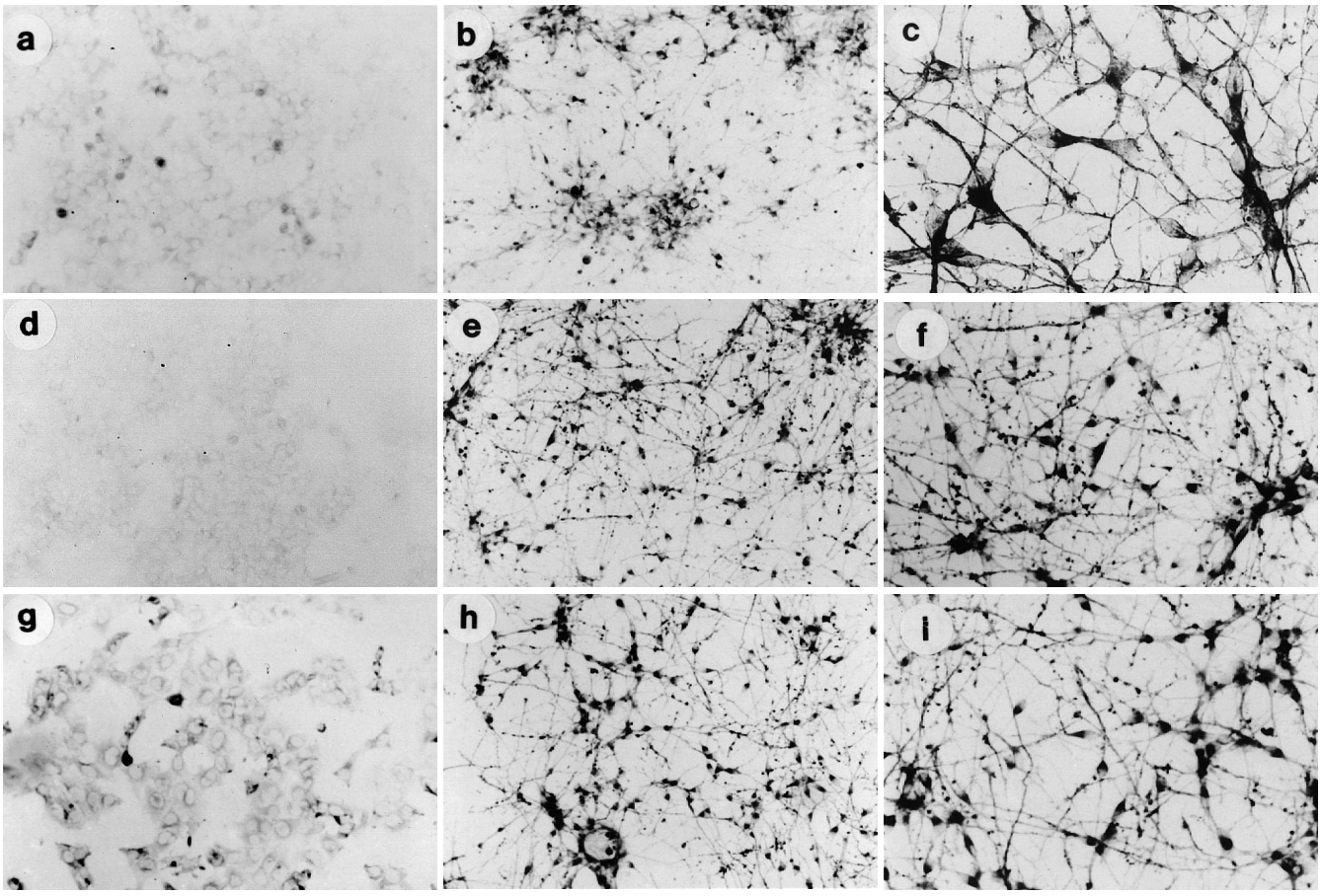

Fig. 3a–i Immunocytochemical studies of synaptic proteins in NT2/NT2N cells. NT2 cells showed low levels of immunoreactivity with the antibody against synaptotagmin (**a**), no reactivity with anti-SYN (**d**) and moderate levels of immunoreactivity with SP18 (**g**). In contrast, NT2N cells showed strong immunoreactivity for synaptotagmin (**b, c**), SYN (**e, f**) and SP18 (**h, i**) (SYN synaptophysin)

To study the patterns of synaptic protein and APP expression by Western blot, cells were harvested as follows: (1) NT2 cells before RA treatment, after 1 week (w1), 3 weeks (w3) and 5 weeks (w5) of RA treatment with or without Cerebrolysin, and (2) NT2N cells at day 2 (d2), day 7 (d7) and day 10 (d10) post-replating with or without Cerebrolysin. The harvesting of cells was performed as recommended by Stratagene. Briefly, for w1, w3 and w5, cells were trypsinized for 5 min at RT, followed by inactivation with DMEM and centrifugation at 1,200 g for 5 min. The cells were then washed twice in PBS and the final cell pellet re-suspended in HEPES homogenization buffer. For d2, d7 and d10, the replated cells were lightly trypsinized (2.5 min at RT) and the sides of the flask were struck three times to dislodge the attached NT2N cells. After inactivation of trypsin with the culture medium, the cells were centrifuged and processes as described above for w1, w3 and w5. Each experiment was performed in triplicate to assess the reproducibility of the results.

Table 1 Summary of antibodies used

Antibody clone	Antigen	Antibody type	Source	Concentrations	
				Western blot	ICC
22C11	APP	Monoclonal	Boehringer-Mannheim, Indianapolis, IN	0.2 µg/ml	1.25 µg/ml
SP18	Synaptic protein	Monoclonal	Dr. William Honer	0.2 µg/ml	1.0 µg/ml
SP6	Syntaxin	Monoclonal	Dr. William Honer	0.2 µg/ml	1.0 µg/ml
SP11	Synaptobrevin	Monoclonal	Dr. William Honer	0.2 µg/ml	1.0 µg/ml
SP12	SNAP-25	Monoclonal	Dr. William Honer	0.2 µg/ml	1.0 µg/ml
SYA-130	p65	Monoclonal	Stressgen Biotech., Victoria, BC Canada	0.3 µg/ml	1.0 µg/ml
SY38	Synaptophysin	Monoclonal	Boehringer-Mannheim, Indianapolis, IN	0.1 µg/ml	0.2 µg/ml
GAP-7B10	GAP43	Monoclonal	Sigma Chemical Co., St. Louis, MO	0.7 µg/ml	3.5 µg/ml
Alpha-synuclein	aa131-140	Polyclonal	In house		
G-A-5	GFAP	Monoclonal	Boehringer-Mannheim, Indianapolis, IN	N/A	2.0 µg/ml
AP23	MAP2	Monoclonal	Boehringer-Mannheim, Indianapolis, IN	N/A	2.0 µg/ml
SMI32	Neurofilament	Monoclonal	Sternberger Monoclonals, Baltimore, MD	N/A	0.5 µg/ml

Antibodies, immunocytochemistry and laser scanning confocal microscopy

For single immunocytochemical analysis, NT2 and NT2N cells were fixed in 4% paraformaldehyde for 20 min at RT, pretreated with 0.03% hydrogen peroxide in PBS, blocked with normal serum and incubated overnight at 4 °C with each of the antibodies listed in Table 1. The next day cells were incubated with the primary antibody-specific biotinylated antibody (Vector Laboratories, Burlingame, Calif.), followed by avidin D-horseradish peroxidase (ABC Elite, Vector). Sections were then reacted with diaminobenzidine (DAB; 0.2 mg/ml) in 50 mM TRIS buffer pH 7.4 with 0.001% hydrogen peroxide. Chambers were removed from the slides, coverslipped, analyzed and photographed with an Olympus Vanox photomicroscope.

For evaluation of colocalization of APP with synaptic proteins, NT2N cells were fixed, pretreated, blocked and then incubated with a combination of mouse monoclonal anti-APP (22C11, Boehringer) and rabbit polyclonal anti-synaptophysin (SYN, DAKO). After overnight incubation, the cells were immunoreacted with a mixture of Texas red-conjugated anti-rabbit IgG (Vector) and FITC-conjugated anti-mouse IgG (Vector). Chambers were then removed from the slides, which were then coverslipped with antifading medium (Vector), and analyzed with the Bio-Rad 1024 laser scanning confocal microscope. This system allowed the simultaneous imaging of the patterns of APP (FITC channel) and SYN (Texas red channel).

Western blot analysis

Whole cell homogenates of NT2 and NT2N cells were prepared by washing the cell pellets twice with PBS. The final pellet was resuspended in a HEPES homogenization buffer (1 mM HEPES, 5 mM benzamidine, 2 mM 2-mercaptoethanol, 3 mM EDTA, 0.5 mM magnesium sulfate, 0.05% sodium azide, 0.01 mg/ml leupeptin) and sonicated for 30 s. Protein concentration was determined by the method of Lowry [16]. Between 10–15 μg of protein was loaded onto 10% TRIS-glycine ready gels (for SYN, SP18 and syntaxin) or 4–15% TRIS-glycine ready gels (for APP) (Bio-Rad, Hercules, Calif.). The samples were subsequently electroblotted to nitrocellulose membranes, incubated overnight at 4 °C with the primary antibodies, followed by rabbit anti-mouse secondary antibody, and ^{125}I-labeled protein A (see Table 1 for antibody specificities and concentrations).

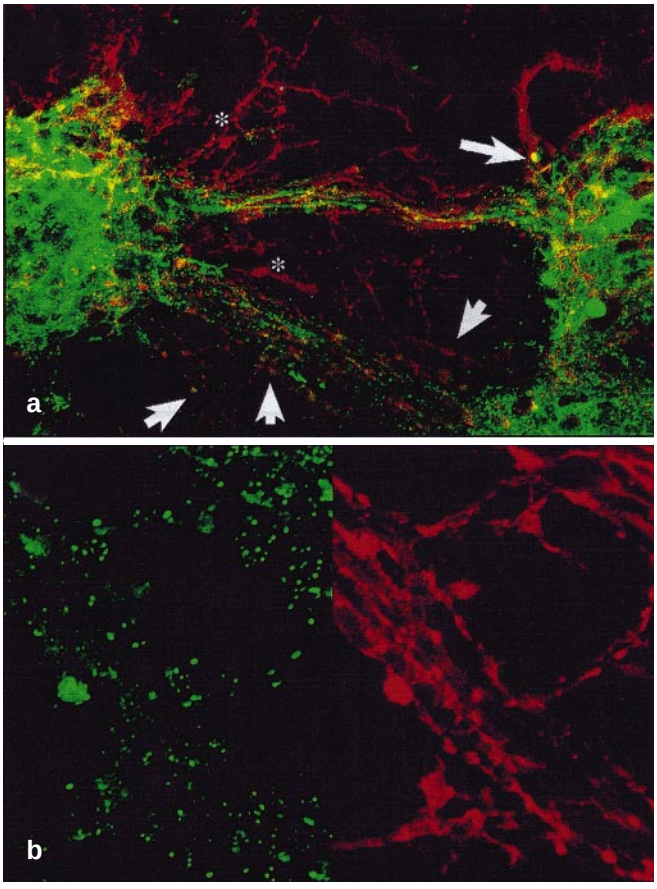

Fig. 4a, b Laser scanning confocal studies of APP and SYN localization in NT2N cells. **a** In the merged image, NT2 N cells double-immunolabeled with anti-APP (green) and anti-SYN (red) showed that APP was colocalized with SYN in the neuritic varicosities (arrows). APP was also abundant in the cell bodies and neurites. **b** The split image view of APP (green) and SYN (red) (APP amyloid precursor protein)

Results

Patterns of synaptic protein expression in NT2 and NT2N cells

Consistent with previous studies, the immature precursor NT2 cells expressed low levels of neuronal cytoskeletal markers such as the intermediate molecular weight neurofilaments (Fig. 1a) and were negative for the dendritic microtubule-associated protein 2 (MAP2) (Fig. 1b) and glial cytoskeletal marker-GFAP (Fig. 1c). NT2 cells also showed low levels of immunoreactivity for growth-associated protein 43 (GAP43) (Fig. 2a) and the synaptic calcium sensor, synaptotagmin (p65) (Fig. 3a), and were negative for other synaptic-associated markers including: SYN (Fig. 3d), syntaxin (Fig. 2c), synaptobrevin and SNAP-25 (not shown), and SP18 (Fig. 3g) and NACP/α-synuclein (Fig. 2b). In contrast, after RA treatment the mature NT2N cells showed intense neurofilament (Fig. 1d) and MAP2 (Fig. 1e) immunoreactivity and were GFAP negative (Fig. 1f). Furthermore, NT2N cells showed intense immunoreactivity for SYN (Fig. 3e, f), GAP43 (Fig. 2d), synaptotagmin (Fig. 3b, c), syntaxin (Fig. 2f), SNAP-25 (not shown), SP18 (Fig. 3h, i) and NACP/α-synuclein (Fig. 2e), but not for synaptobrevin (not shown). While immunoreactivity for MAP2 (Fig. 1e) and neurofilament (Fig. 1d) was mainly localized to the neuronal cell bodies and to a lesser extent to their neuritic processes, immunoreactivity for synaptic proteins was more intense in the processes where varicosities and fine neuritic branches were more prominent (Fig. 3c, f, i). Both NT2 and NT2N cells displayed immunoreactivity with the antibodies against APP (22C11) (Fig. 4) and β-amyloid (3D6 and 10D5) (not shown). However, while in the undifferentiated NT2 cells APP immunoreactivity was observed early in the cell body, after RA differentiation APP immunoreactivity was redistributed to the neuritic processes (Fig. 4). Double-labeling studies showed that, in fact, APP (green) immunoreactivity was closely colocalized with SYN (red) in the neuritic varicosities, indicating that translocation of APP to the distal aspects of the neurites might coincide with synapse formation (Fig. 4).

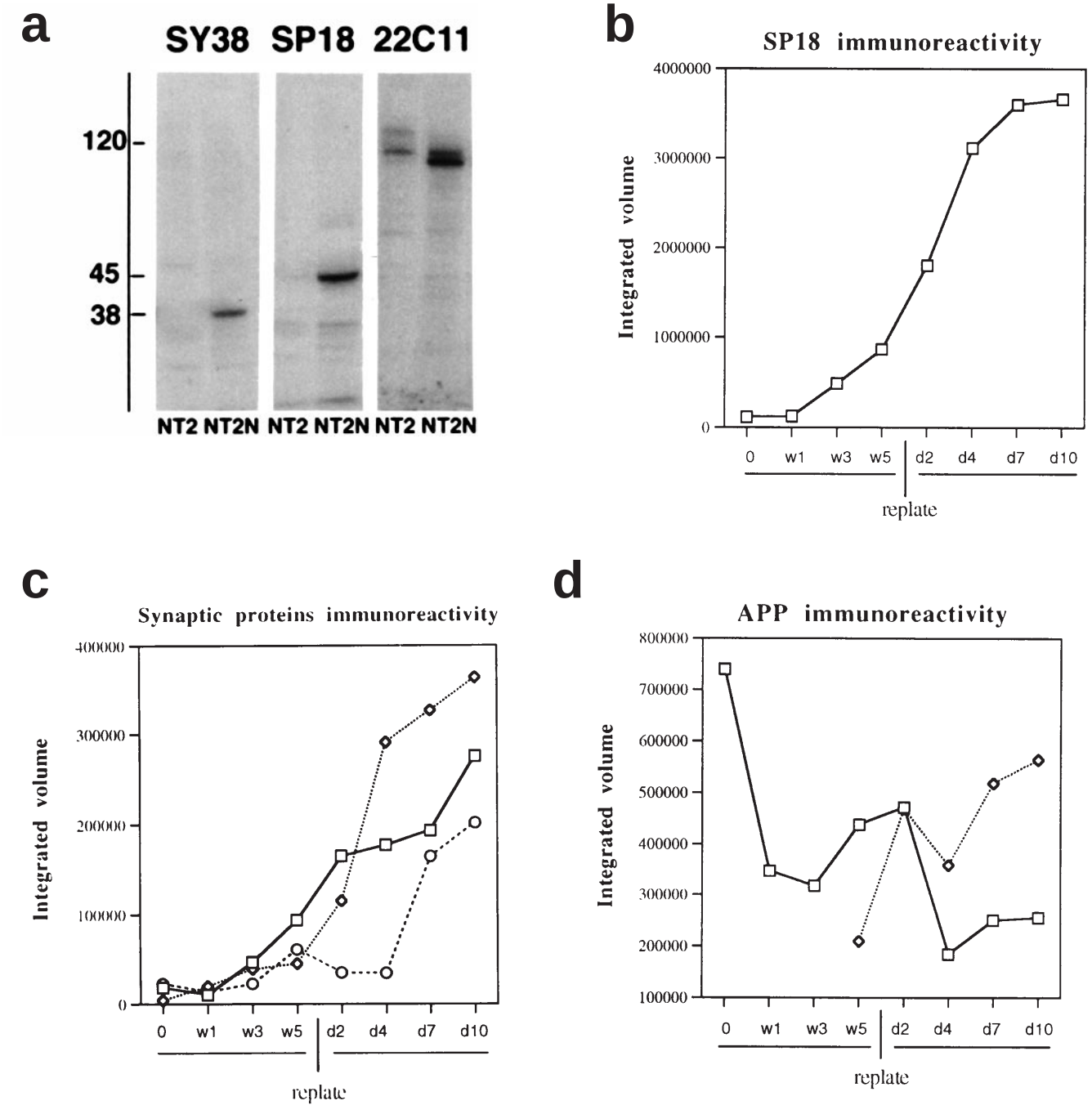

Fig. 5a–d Western blot expression of synaptic-associated proteins in NT2/NT2N cells. **a** Undifferentiated and differentiated cells labeled with antibodies against SYN (SY38), SP18 antigen and APP (22C11). For APP there was a characteristic shift from higher (120–130 kDa) to lower molecular (110–120 kDa) molecular mass bands. **b** SP18 immunoreactivity increased during NT2 cell differentiation. **c** Patterns of syntaxin, SYN and synaptotagmin expression and **d** patterns of APP immunoreactivity during NT2 cell differentiation (—□— syntaxin, ···◇··· synaptophysin,···○···synaptotagmin). **d** —□— APP higher molecular mass band, ···◇··· APP lower molecular mass band

APP expression in NT2N cells is accompanied by expression of synaptic proteins

To better understand the relationship between APP expression and synaptic formation, the patterns of expression of synaptic proteins during NT2 cell differentiation were analyzed by Western blot. These studies showed that SP18 was one of the earliest synaptic markers to be expressed (Fig. 5b). This protein was expressed in NT2 cells at low levels after 1–3 weeks of RA induction (Fig. 5b). At replate there was a progressive increase in the levels of SP18 expression in NT2N cells (Fig. 5b). Other synaptic-associated proteins like syntaxin and synaptotagmin were

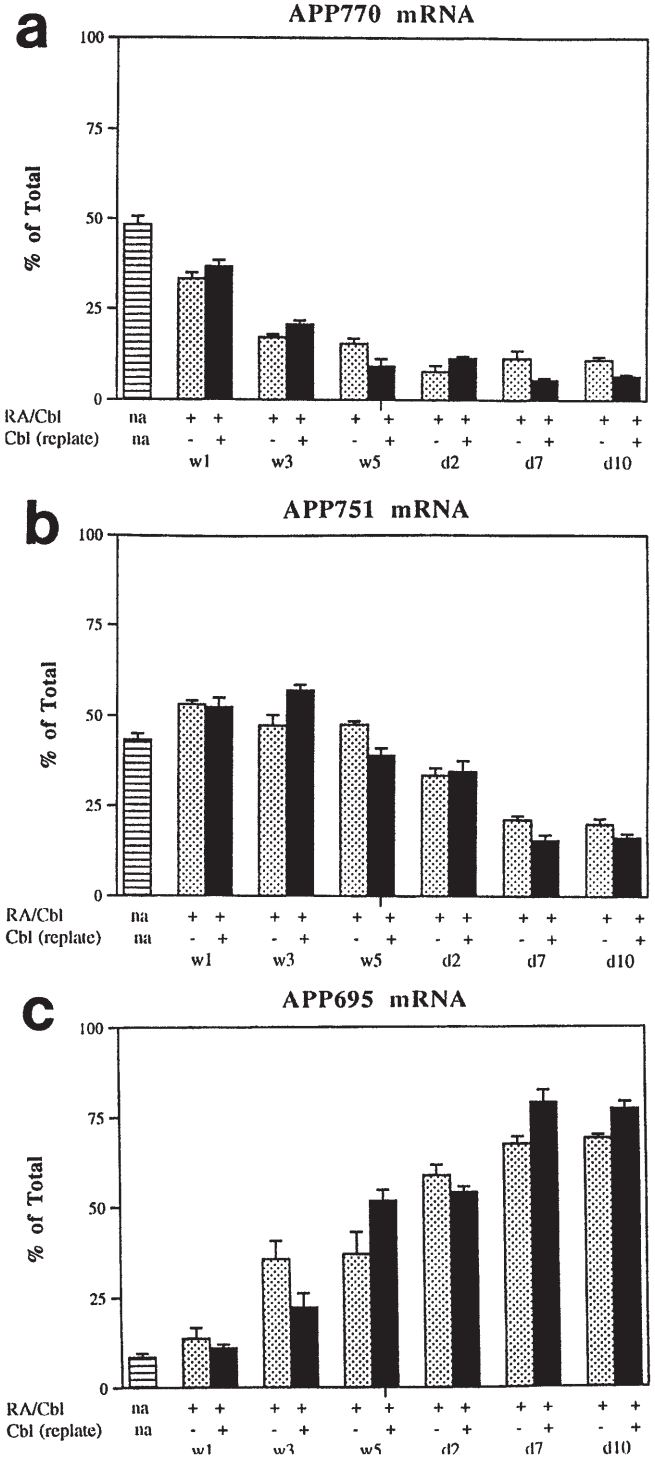

Fig. 6a–c APP mRNA expression during NT2 cell differentiation and after Cerebrolysin administration. Ribonuclease protection assay showed that in the NT2 cells the predominant APP isoforms were 770/751, followed by APP695. Compared to RA treatment alone, cells treated with Cerebrolysin showed a decrease in the levels of APP 770 (**a**) and 751 (**b**) and an increase in the levels of APP695 (**c**) (RA retinoic acid)

expressed at much lower levels during the 5-week period of RA treatment. At 4–7 days after replating, the levels of these two proteins were increased (Fig. 5c). Synaptophysin levels were very low or undetectable during the 5 weeks of RA treatment, followed by a dramatic increase at 2–4 days post-replating (Fig. 5c). SNAP-25 was not detectable in NT2 cells until 5 weeks after the initiation of RA treatment (not shown). After replating, NT2N cells showed a steady increase in SNAP-25 levels which was more prominent at days 7–10 (not shown).

Western blot analysis showed that the 22C11 antibody against APP predominantly detected a broad band at approximately 120–125 kDa in the NT2 cells; in contrast, in the NT2N cells a band with lower molecular mass at approximately 100–110 kDa was more prominent (Fig. 5a). Image Quant-assisted analysis showed that during the NT2 cell differentiation, the APP higher molecular mass band (presumably APP770/751) decreased during the 5 weeks of RA treatment and at day 4 post-replate (Fig. 5d). In contrast, the lower molecular mass band (presumably APP695) appeared at a detectable level at week 5 and increased during replating (Fig. 5d). This is consistent with previous studies showing that NT2 cells express predominantly APP770/751, while NT2N cells express mostly APP695 [42], and indicates that the higher molecular mass, broader band might correspond to APP770/751, while the lower molecular mass band could be APP695.

To corroborate the Western blot data with levels of APP expression at the mRNA level, a ribonuclease protection assay (RPA) was performed [15] (Fig. 6). This study showed that both in undifferentiated NT2 cells and at weeks 1–3 of the RA treatment, the predominant APP isoforms were 770 (Fig. 6a) and 751 (Fig. 6b). At week 5, all three APP isoforms increased and after replating APP695 was the predominant mRNA species (Fig. 6c).

Cerebrolysin treatment promotes enhanced and early expression of synaptic proteins in NT2N cells

NT2 cells were treated with RA in the absence or presence of Cerebrolysin for 5 weeks. At 1 week, the combination of RA and Cerebrolysin resulted in a moderate increase in the expression of the synaptic protein-SP18 when compared to cells treated with RA alone (Fig. 7a). In contrast, 5 weeks after RA and Cerebrolysin treatment the expression of SP18 doubled compared to controls treated with RA alone (Fig. 7a). Treatment of NT2 cells with Cerebrolysin alone in the absence of RA did not promote significant differentiation of the cells into a neuronal phenotype (not shown). The cells treated with Cerebrolysin during the 5-week RA induction, continued to receive Cerebrolysin for the 10 days post-replate period. In contrast, cells treated with RA alone did not receive Cerebrolysin at replating. In the absence of Cerebrolysin, cells showed a steady level of SP18 immunoreactivity, while cells treated with Cerebrolysin showed stronger SP18 immunoreactivity at days 7 and 10 post-replate (Fig. 7a). Withdrawal of Cerebrolysin treatment at replate resulted

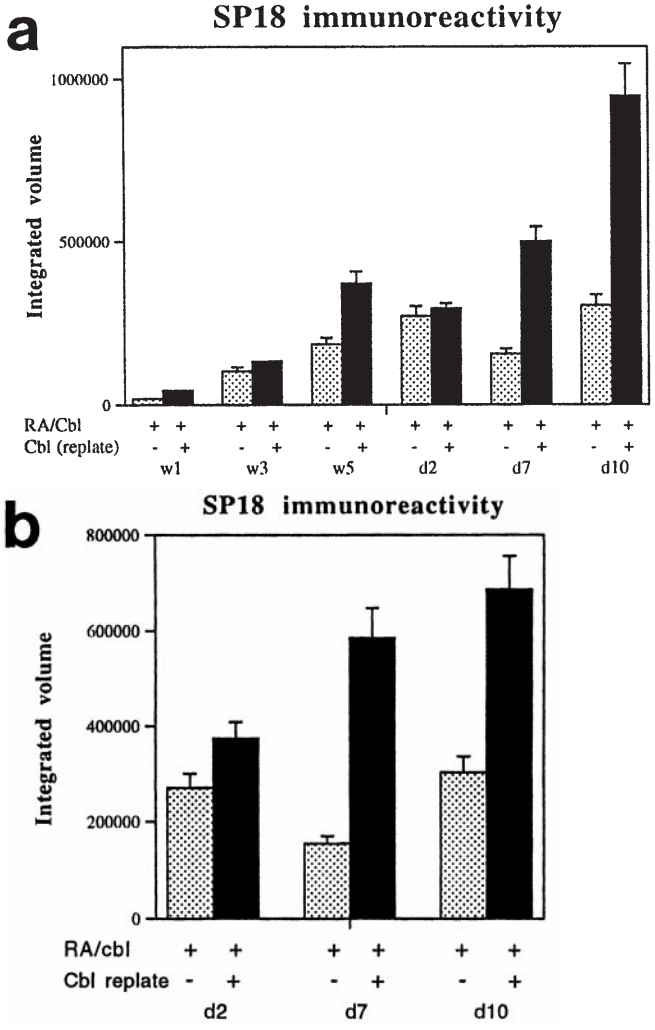

Fig. 7a, b Western blot synaptic protein expression after Cerebrolysin treatment of NT2N cells. **a** At 5 weeks after RA + Cerebrolysin treatment SP18 expression doubled compared to RA treatment alone and this difference was highest at day 10 post-replate. (**b**) Withdrawal of Cerebrolysin treatment at replate resulted in a reversion of the effects in terms of SP18 immunoreactivity

in a reversion of the effects in terms of SP18 immunoreactivity (Fig. 7b).

To determine if the enhanced expression of synaptic proteins observed after Cerebrolysin treatment was accompanied by enhanced APP expression, an RPA was performed. This type of assay was favored over Western blot because of its ability to distinguish with greater accuracy the various APP isoforms. The RPA analysis showed that, compared to cells treated with RA alone, after replating Cerebrolysin-treated cells expressed higher levels of APP695 (Fig. 6c), while the levels of APP770/751 were decreased (Fig. 6a, b).

Discussion

The present study investigated the relationship between levels of APP expression and synaptic proteins during NT2 cell differentiation and whether the synaptotrophic effects of Cerebrolysin might be associated with altered expression of APP isoforms. The first part of this study showed that in the NT2/NT2N model system, the SP18 antigen and GAP43 were the first synaptic proteins to be expressed, followed by SYN and SNAP-25 In contrast, synaptotagmin expression was observed later. Moreover, while in the undifferentiated cells synaptic antigens were found in the cell body, in the differentiated cells there was redistribution to the synapses and axons. Consistent with these findings, previous in vitro studies utilizing primary tissue cultures from embryonic day (E) 14 rat diecephalon [30], E18 rat hippocampus [43], postnatal day 1 rat hippocampus [4] and mouse hypothalamic and cerebellar E15 cells [1] have shown that at early stages the synaptic proteins rab3A, synapsin I, SYN and synaptotagmin were present in the neuronal soma, but not in the axons [36]. Supporting the possibility that the sequential expression of synaptic proteins shown here is physiologically relevant for NT2N cells, previous studies have shown that approximately at the time point when synaptic proteins are redistributed to axons, primary hippocampal cultures exhibit synaptic activity.

The precise mechanisms involved in synaptic formation in the NT2N cells are not completely known. Studies have suggested that NT2N cells have the potential to express functional synapses, as well as several neurotransmitters. In vitro electrophysiological studies utilizing patch clamp recordings from NT2N cells have shown synaptic currents that exhibited both biophysical and pharmacological characteristics of glutamergic synaptic transmission [37]. Furthermore, consistent with the present study, mature NT2N cells were also immunopositive for the synaptic vesicle protein SYN [37]. In vivo transplantation studies have shown that during NT2 cell differentiation there is formation of synaptic structures [13] that might coincide with a shift in levels of APP isoforms from APP770/751 to APP695. Consistent with these findings, the present study showed that at the time of this shift there is a considerable increase in synaptic protein expression. Furthermore, double-immunolabeling studies showed that at this point APP was redistributed to the SYN-immunoreactive varicosities of the NT2N cells, suggesting that APP might play a role. Consistent with these in vitro findings, previous in vivo studies have shown that overexpression of APP in transgenic mice [23] or infusion of APP peptides [26] into rat brain results in increased synaptic density. In vitro studies suggest that APP synaptotrophic effects might be mediated via neuritic outgrowth [10, 34]; however, other studies suggest that APP might affect synapses directly [17, 23, 31]. Supporting this possibility, a recent study by Morimoto et al. [22] showed a correlation between the number of neurons forming synapses and APP expression.

As to the mechanisms by which APP might promote synaptic formation, several lines of investigation propose a possible role for APP in cell-cell and/or cell-matrix interactions [6], as well as enhancing the neurotrophic activity of growth factors such as nerve growth factor (NGF) [5,

21, 40]. Similarly, recent studies have shown that Cerebrolysin might exhibit NGF-like trophic activity [2, 3, 8, 18]. This assumption is based on previous studies showing that NGF stimulation induces up-regulation and translocation of GAP43 [39]. Interestingly, it has also been suggested that APP might participate in the neurotrophic effects of NGF [5] and that Cerebrolysin promotes synaptic repair after injury in animal models of neurodegeneration [8, 9, 14, 18, 32]. Previous work has shown that APP secretion by NT2N cells can be regulated by activation of phosphatidylinositol-linked metabotrophic glutamate receptor signaling pathway and the muscarininc phospholipase C [11, 41]. To date no data has been reported as to the possible effects of Cerebrolysin on these pathways.

Furthermore, previous studies have shown that Cerebrolysin ameliorates the cognitive impairment in mild forms of AD [29], suggesting the possibility that this neurotrophic effects of Cerebrolysin in AD patients might be mediated, at least in part, via modulation of APP expression. In summary, Cerebrolysin treatment of NT2N cells resulted in an augmented and earlier expression of synaptic-associated proteins. This increased synaptic protein expression coincided with an increase in APP695 over APP770/751. These results support the possibility that synaptotrophic effects of Cerebrolysin might be mediated via regulation of APP expression.

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