Cerebrolysin Decreases Amyloid-β Production by Regulating Amyloid Protein Precursor Maturation in a Transgenic Model of Alzheimer’s Disease

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Cerebrolysin is a peptide mixture with neurotrophic effects that might reduce the neurodegenerative pathology in Alzheimer’s disease (AD). We have previously shown in an amyloid protein precursor (APP) transgenic (tg) mouse model of AD-like neuropathology that Cerebrolysin ameliorates behavioral deficits, is neuroprotective, and decreases amyloid burden; however, the mechanisms involved are not completely clear. Cerebrolysin might reduce amyloid deposition by regulating amyloid-β (Aβ) degradation or by modulating APP expression, maturation, or processing. To investigate these possibilities, APP tg mice were treated for 6 months with Cerebrolysin and analyzed in the water maze, followed by RNA, immunoblot, and confocal microscopy analysis of full-length (FL) APP and its fragments, β-secretase (BACE1), and Aβ-degrading enzymes [neprilysin (Nep) and insulin-degrading enzyme (IDE)]. Consistent with previous studies, Cerebrolysin ameliorated the performance deficits in the spatial learning portion of the water maze, followed by RNA, immunoblot, and confocal microscopy analysis of full-length (FL) APP and its fragments, β-secretase (BACE1), and Aβ-degrading enzymes [neprilysin (Nep) and insulin-degrading enzyme (IDE)]. Consistent with previous studies, Cerebrolysin ameliorated the performance deficits in the spatial learning portion of the water maze and reduced the synaptic pathology and amyloid burden in the brains of APP tg mice. These effects were associated with reduced levels of FL APP and APP C-terminal fragments, but levels of BACE1, Notch1, Nep, and IDE were unchanged. In contrast, levels of active cyclin-dependent kinase-5 (CDK5) and glycogen synthase kinase-3β [GSK-3β; but not stress-activated protein kinase-1 (SAPK1)], kinases that phosphorylate APP, were reduced. Furthermore, Cerebrolysin reduced the levels of phosphorylated APP and the accumulation of APP in the neuritic processes. Taken together, these results suggest that Cerebrolysin might reduce AD-like pathology in the APP tg mice by regulating APP maturation and transport to sites where Aβ protein is generated. This study clarifies the mechanisms through which Cerebrolysin might reduce Aβ production and deposition in AD and further supports the importance of this compound in the potential treatment of early AD.

Key words: APP; amyloid-β; Cerebrolysin; CDK5; GSK-3β; phosphorylation

Alzheimer’s disease (AD) continues to be a leading cause of dementia in the aging population. Neuropathologically, AD is characterized by synaptic injury (Terry et al., 1994; Masliah et al., 1997) and neuronal loss (Terry et al., 1981), accompanied by amyloid deposition (Selkoe, 1989), astroglisis (Beach et al., 1989), and microglial cell proliferation (Rogers et al., 1988; Masliah et al., 1991). Although the precise mechanisms leading to neurodegeneration in AD are not completely clear, most studies have focused on the role of amyloid-β protein (Aβ) precursor (APP) and its products in AD pathogenesis (Selkoe, 1989; Vassar, 2005).

Cleavage of APP by β-secretase results in the secretion of a large N-terminal ectodomain (Esler and Wolfe, 2001). In an alternative pathway, β-secretase (BACE1) generates a shorter N-terminal fragment and a 12-kDa C-terminal fragment (CTF; C99), which remains membrane bound (Esler and Wolfe, 2001). C99 is then further cleaved by γ-secretase, resulting in the production of Aβ peptides that might aggregate to form toxic oligomers and fibers (Esler and Wolfe, 2001). Recent studies suggest that Aβ oligomers (rather than fibrils associated with the plaques) might accumulate in the...
neurons as well as at the synaptic sites leading to neuronal dysfunction and cell death (Lue et al., 1999; Walsh et al., 2000, 2002; Kayed et al., 2003). Accumulation of Aβ is most likely the result of alterations in APP processing or in Aβ clearance and degradation. In this context, recent efforts have focused at developing treatments aimed at reducing Aβ deposition by decreasing Aβ production or increasing proteolysis (Morelli et al., 2005). Some studies have tested the efficacy of antinflammatory agents (Lim et al., 2000; Gasparini et al., 2004), and others have investigated the effects of inhibitors of the β- and γ-secretase pathways (Moore et al., 2000) and the effects of antibodies or other compounds that might increase Aβ clearance (Schenk et al., 1999). Alternative therapies might include the use of neurotrophic agents that might both protect neurons and modify APP metabolism. In this regard, we have previously shown that the nootrophic agent Cerebrolysin (a mixture of peptides and amino acids obtained from porcine brain tissue) ameliorates the neurodegenerative alterations and reduces amyloid burden in an APP model of AD-like pathology (Rockenstein et al., 2002). Furthermore, this compound improves memory in patients with mild to moderate cognitive impairment (Ruther et al., 1994a,b) and has been shown to display neurotrophic activity in vitro (Mallory et al., 1999) and in animal models of neurodegeneration (Francis-Turner and Valouskova, 1996; Masliah et al., 1999; Veinbergs et al., 2000).

The mechanisms through which Cerebrolysin might reduce the neurodegenerative pathology associated with Aβ production are not completely clear. In this respect, it is possible that Cerebrolysin reduces amyloid deposition by regulating Aβ degradation or by modulating APP expression, maturation, or processing. To investigate these possibilities, human (h)APP transgenic (tg) mice (3 months old) were treated for 6 months with Cerebrolysin and analyzed in the water maze, followed by RNA, immunoblot, and confocal microscopy analysis of full-length (FL) APP and its fragments, APP phosphorylation (Thr668), β-secretase (BACE1), γ-secretase, and Aβ-degrading enzymes [neprilysin (Nep) and insulin-degrading enzyme (IDE)]. We found that Cerebrolysin regulates the activity of cyclin-dependent kinase-5 (CDK5) and glycogen synthase kinase-3β [GSK-3β; but not stress-activated protein kinase-1 (SAPK1)], kinases that phosphorylate APP at Thr668 (Aplin et al., 1996; Iijima et al., 2000). Furthermore, Cerebrolysin reduced the levels of phosphorylated APP (APP-p) and the accumulation of APP in the neuritic processes. Taken together, these results suggest that Cerebrolysin might reduce the AD-like pathology by modulating APP maturation and transport to sites where Aβ protein is generated.

MATERIALS AND METHODS

Generation of APP Tg Mice, Treatment Regimen, and Tissue Processing

The tg mice generated express mutated hAPP751 under the control of the mTly–1 promoter (mTly1–hAPP751), and, for this study, the highest expresser (line 41) tg mice were used (Rockenstein et al., 2001). These tg mice are unique in that, compared with other tg models, amyloid plaques are found in the brain at a much earlier age (beginning at 3 months; Masliah and Rockenstein, 2000; Rockenstein et al., 2001, 2005). Genomic DNA was extracted from tail biopsies and analyzed by PCR amplification, as described previously (Rockenstein et al., 1995). Transgenic lines were maintained by crossing heterozygous tg mice with nontransgenic (non-tg) C57BL/6 × DBA/2 F1 breeders. All mice were heterozygous with respect to the transgene. Twenty-four 3-month-old mice were utilized for the present study; for each group, 12 mice received daily intraperitoneal (IP) injections of saline or daily IP injections of Cerebrolysin (batch 802772, 5 ml/kg) for 6 months. At the end of this period, Cerebrolysin treatment was discontinued, and mice were then tested in the water maze and sacrificed for neuropathological analysis.

Water Maze Testing

Briefly, as previously described (Rockenstein et al., 2003), to evaluate the functional effects of treatment, at the end of 6 months of treatment with Cerebrolysin the animals were trained in the water maze, beginning with a 3-day exposure to the visible platform. Then, mice were tested for learning and memory with the hidden platform for 4 days, followed by one visible test trial and 1 day of probe testing. All experiments described were approved by the animal subjects committee at the University of California at San Diego (UCSD) and were performed according to NIH recommendations for animal use.

Tissue Processing

In accordance with the NIH guidelines for the humane treatment of animals, mice were anesthetized with chloral hydrate and flush perfused transcardially with 0.9% saline. Brains were removed and divided sagitally. One hemibrain for this study, the highest expresser (line 41) tg mice were used (Rockenstein et al., 2001). These tg mice are unique in that, compared with other tg models, amyloid plaques are found in the brain at a much earlier age (beginning at 3 months; Masliah and Rockenstein, 2000; Rockenstein et al., 2001, 2005). Genomic DNA was extracted from tail biopsies and analyzed by PCR amplification, as described previously (Rockenstein et al., 1995). Transgenic lines were maintained by crossing heterozygous tg mice with nontransgenic (non-tg) C57BL/6 × DBA/2 F1 breeders. All mice were heterozygous with respect to the transgene. Twenty-four 3-month-old mice were utilized for the present study; for each group, 12 mice received daily intraperitoneal (IP) injections of saline or daily IP injections of Cerebrolysin (batch 802772, 5 ml/kg) for 6 months. At the end of this period, Cerebrolysin treatment was discontinued, and mice were then tested in the water maze and sacrificed for neuropathological analysis.

RNA Analysis

Total RNA was extracted with Tri reagent (Molecular Research Center, Cincinnati, OH) from snap-frozen hemibrains or dissected brain regions (neocortex and hippocampus) and stored in formazol buffer (Molecular Research Center) at −20°C. RNA was analyzed by solution hybridization ribonuclease protection assay (RPA), essentially as described elsewhere (Rockenstein et al., 1995). Samples were separated on 5% acrylamide/8 M urea Tris/borate/EDTA gels, and dried gels were exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). mRNA levels were quantitated from PhosphorImager readings of probe-specific signals corrected for RNA content/loading errors by normalization to β-actin signals (Rockenstein et al., 1995). The following 32P-labeled antisense riboprobes were used to identify specific mRNAs [protected nucleotides (GenBank accession No.)]: total hAPP [nt 780–1009 of exon 5–6 (No. NM_000484)], total murine (m)APP [nt 702–881 of
Determination of APP Levels, APP Products, and Aβ-Degrading Enzymes

Levels of hAPP immunoreactivity were determined in brain homogenates by Western blot and in vibratome sections by immunocytochemistry, as previously described (Masliah et al., 1989, 1992; Rockenstein et al., 2005). For Western blot analysis, 15 μg per lane of cytosolic and particulate fractions, assayed by the Lowry method, was loaded into 10% SDS-PAGE gels and blotted onto nitrocellulose paper. Blots were incubated with antibodies against FL APP (mouse monoclonal, clone 22C11; 1:20,000; Chemicon, Temecula, CA), Aβ (mouse monoclonal, clone 6E10; 1:1,000; Signet Laboratories, Dedham, MA), C-terminal APP (rabbit polyclonal, CT15; 1:2,500; courtesy of Dr. E. Koo), APP-p (1:1,200; Cell Signaling, Beverly, MA), Nep (mouse monoclonal, clone CD10; 1:1,000; Abcam, Cambridge, MA), IDE (rabbit polyclonal; 1:1,000; Calbiochem, San Diego, CA), BACE1 (1:1,000; Pro-Sci, Inc., Poway, CA), or Notch1 (rabbit polyclonal; 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), followed by secondary antibodies tagged with horseradish peroxidase (HRP; 1:2,500; courtesy of Dr. E. Koo), APP-p (1:1,200; Cell Signaling Technology), total CDK5 (rabbit polyclonal; 1:1,000; Santa Cruz Biotechnology), the CDK5 activator p35 (1:1,000; Santa Cruz Biotechnology), and total GSK-3β (mouse monoclonal; 1:1,000; Santa Cruz Biotechnology). Whereas the antibodies against APPK1-p (phosphorylated at amino acid residues threonine 183 and tyrosine 185) recognize the activated forms of these enzymes, the antibody against GSK-3β-p (phosphorylated at amino acid residue serine 9) identifies the inactivated kinase. Additional immunoblot analysis was performed with antibodies against total SAPK1 (rabbit polyclonal; 1:1,000; Cell Signaling Technology), total CDK5 (rabbit polyclonal; 1:1,000; Santa Cruz Biotechnology), and total GSK-3β (mouse monoclonal; 1:1,000; Santa Cruz Biotechnology). After overnight incubation with primary antibodies, membranes were incubated with secondary antibodies tagged with HRP (1:5,000; Santa Cruz Biotechnology), visualized by enhanced chemiluminescence, and analyzed with a Versadoc XL imaging apparatus (Bio-Rad, Hercules, CA). Analysis of actin levels was used as loading control. Results were expressed as signal/actin ratio.

For immunocytochemical analysis of hAPP distribution, 40-μm vibratome sections were doubly labeled with antibodies against hAPP (clone 8E5; courtesy of Elan Pharmaceuticals, San Francisco, CA) and the dendritic marker microtubule-associated protein-2 (MAP2; mouse monoclonal; Chemicon) or the axonal marker antineurofilament (mouse monoclonal, clone SM1312; Sternberger Monoclonals, Baltimore, MD), as previously described (Masliah et al., 2001). Briefly, sections were incubated overnight at 4°C with anti-hAPP (1:20,000) and developed with the Tyramide Signal Amplification–Direct (Red) system (1:100; NEN Life Sciences, Boston, MA). Sections were then incubated overnight with antibodies against MAP2 (1:50) or neurofilament (SM1312; 1:100), followed by incubation with fluorescein isothiocyanate (FITC)-tagged secondary goat anti-mouse (1:75; Vector Laboratories, Burlingame, CA) and imaging by laser scanning confocal microscopy (LSCM; MRC 1024; Bio-Rad).

Analysis of BACE1 Enzymatic Activity

BACE1 activity was determined by adapting the BACE1 Quenched Fluorescence Assay Kit (Panvera, Madison, WI) that uses a fluorescent peptide substrate derived from the Swedish (sw) mutant APP as previously described (Vassar et al., 1999). Briefly, as previously described (Rockenstein et al., 2005), brain homogenates from the tg mice were incubated with the BACE1 substrate, for a final concentration of 1X for each of the reagents. Then, stop buffer (containing a 2.5 M sodium acetate) was added, and the signal was determined at 545 nm by using a spectrofluorometer. Control experiments and standard curves were performed using the baculovirus–expressed BACE1 and the BACE1 product standard (Rh-EVNL, a BACE1 inhibitor) provided with the kit.

Immunoblot Analysis of Kinases That Phosphorylate APP

Briefly, as previously described (Rockenstein et al., 2005), levels of activation of enzymes that phosphorylate APP and regulate APP maturation and processing was determined by Western blot analysis with antibodies that detect the phosphorylated forms of SAPK1 (SAPK1-p; 1:1,000; Cell Signaling Technology), CDK5 (CDK5-p; rabbit polyclonal, 1:1,000; Santa Cruz Biotechnology), and GSK-3β (GSK-3β-p; mouse monoclonal, 1:1,000; Cell Signaling Technology). Whereas the antibodies against SAPK1-p (phosphorylated at amino acid residues threonine 183 and tyrosine 185) recognize the activated forms of these enzymes, the antibody against GSK-3β-p (phosphorylated at amino acid residue serine 9) identifies the inactivated kinase. Additional immunoblot analysis was performed with antibodies against total SAPK1 (rabbit polyclonal; 1:1,000; Cell Signaling Technology), total CDK5 (rabbit polyclonal; 1:1,000; Santa Cruz Biotechnology), the CDK5 activator p35 (1:1,000; Santa Cruz Biotechnology), and total GSK-3β (mouse monoclonal; 1:1,000; Santa Cruz Biotechnology). After overnight incubation with primary antibodies, membranes were incubated with secondary antibodies tagged with HRP (1:5,000; Santa Cruz Biotechnology), visualized by enhanced chemiluminescence, and analyzed with a Versadoc XL imaging apparatus (Bio-Rad). Analysis of actin levels was used as loading control.

Neuropathological Analysis and Detection of Aβ Deposits

Vibratome sections were incubated overnight at 4°C with the mouse monoclonal antibody 4G8 (1:600; Senetek, Napa, CA), which specifically recognizes Aβ. Two methods were used to detect primary antibody binding: 1) Vector ABC Elite kit and DAB/H2O2 and 2) FITC-conjugated anti-mouse IgG (Vector Laboratories). Sections reacted with DAB/H2O2 were examined with a ×2.5 objective for the Olympus Vanox light microscope. The percentage area of the hippocampus covered by 4G8-immunoreactive material (“plaque load”) was assessed, as previously described (Mucke et al., 2000a), with the Quantiplet 570C. The FITC-labeled sections were imaged with the LSCM (Bio-Rad 1024), as described previously (Mucke et al., 2000a). Digitized images were analyzed with the NIH Image 1.43 program to determine the number of plaques per unit area and the plaque size. Three immunolabeled sections were analyzed per mouse, and the average of individual measurements was used to calculate group means. Additional analysis of the neuropathological alterations in the APP tg mice was performed in sections immunolabeled with the antibody against glial fibrillary acidic protein (GFAP; 1:500; Chemicon), a marker of astroglial cells (Mucke et al., 2004b).

To evaluate the integrity of the synaptic terminals, blind-coded 40-μm-thick vibratome sections from mouse brains fixed in 4% paraformaldehyde were immunolabeled with the mouse monoclonal antibody anti-neurofilament (SMI312; 1:100,000; Sternberger Monoclonals, Baltimore, MD), as previously described (Masliah et al., 1989, 1992; Rockenstein et al., 2005). For Western blot analysis, 15 μg per lane of cytosolic and particulate fractions, assayed by the Lowry method, was loaded into 10% SDS-PAGE gels and blotted onto nitrocellulose paper. Blots were incubated with antibodies against FL APP (mouse monoclonal, clone 22C11; 1:20,000; Chemicon, Temecula, CA), Aβ (mouse monoclonal, clone 6E10; 1:1,000; Signet Laboratories, Dedham, MA), C-terminal APP (rabbit polyclonal, CT15; 1:2,500; courtesy of Dr. E. Koo), APP-p (1:1,200; Cell Signaling, Beverly, MA), Nep (mouse monoclonal, clone CD10; 1:1,000; Abcam, Cambridge, MA), IDE (rabbit polyclonal; 1:1,000; Calbiochem, San Diego, CA), BACE1 (1:1,000; Pro-Sci, Inc., Poway, CA), or Notch1 (rabbit polyclonal; 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), followed by secondary antibodies tagged with horseradish peroxidase (HRP; 1:2,500; courtesy of Dr. E. Koo), APP-p (1:1,200; Cell Signaling Technology), total CDK5 (rabbit polyclonal; 1:1,000; Santa Cruz Biotechnology), the CDK5 activator p35 (1:1,000; Santa Cruz Biotechnology), and total GSK-3β (mouse monoclonal; 1:1,000; Santa Cruz Biotechnology). After overnight incubation with primary antibodies, membranes were incubated with secondary antibodies tagged with HRP (1:5,000; Santa Cruz Biotechnology), visualized by enhanced chemiluminescence, and analyzed with a Versadoc XL imaging apparatus (Bio-Rad). Analysis of actin levels was used as loading control.
with the mouse monoclonal antibody against synaptophysin (SYN; 1:40; Chemicon), as previously described (Mucke et al., 1995). After an overnight incubation with the primary antibodies, sections were incubated with FITC-conjugated horse anti-mouse IgG secondary antibody (1:75; Vector Laboratories), transferred to SuperFrost slides (Fisher Scientific, Tustin, CA), and mounted under glass coverslips with antifading media (Vector Laboratories). All sections were processed under the same standardized conditions. The immunolabeled blind-coded sections were imaged with the LSCM (MRC1024; Bio-Rad) and analyzed with the Image 1.43 program (NIH), as previously described (Toggas et al., 1994; Mucke et al., 1995) to determine the percentage area of the neuropil covered by SYN-immunoreactive terminals.

### Statistical Analysis

Analyses were carried out with the StatView 5.0 program (SAS Institute Inc., Cary, NC). Differences among means were assessed by one-way ANOVA with post hoc Dunnet’s. Comparisons between two groups were made with the two-tailed unpaired Student’s t-test. Correlation studies were carried out by simple regression analysis and the null hypothesis was rejected at the 0.05 level.

### RESULTS

#### Neuroprotective and AntiAmyloidogenic Effects of Long-Term Cerebrolysin Treatment in APP Tg Mice

To confirm the neuroactive effects of Cerebrolysin in the APP tg mice, animals were tested in the water maze at the end of the 6 month period of treatment. Compared with untreated non-tg control mice, saline-treated APP tg mice showed significant performance deficits in the spatial learning portion of the test (Fig. 1A). These alterations were not related to motor impairments but rather to spatial learning deficits, because, on the final day of testing, when the platform was made visible, there was no difference in the swim distances between the groups (not shown). Cerebrolysin treatmentameliorated the performance deficits in the APP tg mice during the testing period with the hidden platform compared with the saline-treated group (Fig. 1A). Consistent with the water maze results, analysis of the nerve terminals by confocal microscopy showed that, in the Cerebrolysin-treated APP tg mice, levels of SYN immunoreactivity in the frontal cortex and hippocampus were comparable to those of non-tg control mice, whereas saline-treated APP tg mice showed decreased levels of SYN immunoreactivity (Fig. 1B–D). Neuropathological analysis of the APP tg mice showed that, compared with the saline group, after 6 months of Cerebrolysin treatment, there was a significant reduction in the percentage area of the neuropil covered by Aβ-immunoreactive plaques in the frontal cortex (Fig. 1E–G) and hippocampus (Fig. 1G).

#### Effects of Long-Term Cerebrolysin Treatment on APP Processing and Aβ-Degrading Enzymes

To understand better whether the effects of Cerebrolysin in tg mice were related to decreased APP processing or increased Aβ protein degradation, immunoblot analysis and BACE1 activity assays were performed. Western blot analysis showed that, compared with the saline-treated group, Cerebrolysin treatment significantly reduced the levels of FL APP and CTFs (C99, C89, and C83) of APP (Fig. 2). High-resolution Western blot analysis showed that Cerebrolysin reduced the levels of Aβ in brain homogenates from APP tg mice (Fig. 2A,B). These effects were independent of the β- and γ-secretase pathways, insofar as the respective levels of BACE1 immunoreactivity and Notch1 proteolysis were not different between groups (Fig. 3A,B). Levels of BACE1 enzymatic activity and mRNA were not affected by Cerebrolysin treatment (Fig. 4A,B). Furthermore, the levels of the Aβ-degrading enzymes Nep and IDE were similar between the saline- and Cerebrolysin-treated groups (Fig. 3A,B). Taken together, these findings suggest that Cerebrolysin’s effects on APP/Aβ−42 might be related to regulation of APP expression or intracellular maturation and distribution, rather than modulation of APP-processing enzymes or Aβ degradation.

#### Long-Term Cerebrolysin Treatment Regulates Kinases Involved in APP Phosphorylation

To investigate whether Cerebrolysin’s effects on FL APP levels were related to the regulation of APP mRNA expression or to posttranscriptional modifications, RPA and immunoblot analyses were performed. RNA analysis showed that Cerebrolysin did not affect the total levels of APP (Fig. 4B), suggesting that Cerebrolysin might alter the subcellular distribution of APP, localizing it to a compartment where it might be less prone to being cleaved via the β-secretory pathway. Recent studies suggest that APP maturation, subcellular distribution, and transport to the axonal terminal where proteolysis and generation of Aβ occurs is phosphorylation dependent (Koo et al., 1990; Ando et al., 1999; Iijima et al., 2000; da Cruz e Silva and da Cruz e Silva, 2003), so immunoblot analysis with an antibody against APP-p-threonine 668 was performed. This study showed that cerebral levels of FL APP-p and C99-p were lower in Cerebrolysin-treated mice compared with the saline-treated APP tg mice (Fig. 5A,B). Consistent with this possibility, double-labeling confocal analysis showed that, whereas in the saline-treated tg mice a significant proportion of neurites displayed abundant APP immunolabeling (Fig. 6A–C), in the Cerebrolysin-treated mice APP immunoreactivity was reduced in the neuronal cell body and absent in the neuritic processes (Fig. 6D,E). Taken together, these findings suggest that Cerebrolysin might modulate the activity of kinases involved in APP phosphorylation. To test this possibility, brain homogenates were analyzed by Western blot with antibodies against total and phosphorylated CDK5, GSK-3β, and SAPK1
Whereas the antibodies against CDK5-p and SAPK1-p recognize the activated kinase, the antibody against GSK-3β-p identifies the inactivated kinase (via the Akt pathway). This study showed that Cerebrolysin treatment reduced the levels of CDK5-p (active form) and CDK5 activators p35 and p25 and increased the levels of GSK-3β-p (inactive form), whereas the levels of SAPK1-p were unchanged (Fig. 7A,B). These results support the notion that, by regulating phosphorylation, Cerebrolysin might retain APP in the early secretory
pathway, depleting mature APP-p before it is transported to the axon terminals, where a large proportion of Aβ generation by the γ-secretase complex takes place (Lee et al., 2005).

DISCUSSION

The present study shows that Cerebrolysin might decrease Aβ production and accumulation by reducing the levels of APP available for processing. Interestingly, in Cerebrolysin-treated tg mice, levels of APP mRNA, proteolytic enzyme activity, and Aβ degradation were not affected, but rather the cellular distribution and phosphorylation of APP. Previous studies have shown that APP maturation and targeting for proteolysis involves phosphorylation at position threonine 668 (da Cruz e Silva and da Cruz e Silva, 2003; Lee et al., 2003). After phosphorylation, p-APP undergoes fast axonal transport to the synapse, where β- and γ-secretase cleavage occurs, resulting in Aβ generation (Kamal et al., 2000; Lazarov et al., 2002; Lee et al., 2005). This suggests that Cerebrolysin’s reduction of APP phosphorylation might in turn result in a depletion of mature APP-p in the trans-Golgi compartment and a lesser amount of APP targeted to the distal axon, which would preclude Aβ generation at synaptic sites.
The present study also shows that the reduced phosphorylation of APP by Cerebrolysin was associated with decreased activation of CDK5 and GSK-3β (but not SAPK1). Previous studies have shown that APP phosphorylation is regulated by CDK5 (Iijima et al., 2000), GSK-3β (Aplin et al., 1996), and SAPK1 (Standen et al., 2001; da Cruz e Silva and da Cruz e Silva, 2003; Kimberly et al., 2005). Both GSK-3β and CDK5 interact with presenilin-1 and regulate proteolysis of APP by activating this component of the γ-secretase complex (Tesco and Tanzi, 2000; Ryder et al., 2003). In addition to phosphorylating APP, GSK-3β is known to regulate a diverse array of cellular functions (Frame and Cohen, 2001), including cell survival and tau phosphorylation (Anderton et al., 2001). Similarly, CDK5 has now been identified as a kinase that phosphorylates tau (Maccioni et al., 2001) and that might be involved in tangle formation. The mechanisms through which Cerebrolysin might modulate the state of activation of these kinases is unclear and will require further investigation; however, GSK-3β activity is regulated by, among others, phosphatidylinositol-3 kinase (PI3K)/Akt, and CDK5 is regulated by p35 (Tsai et al., 1994), suggesting that these upstream molecules could be possible primary targets for Cerebrolysin’s effects.

By modulating the activity of CDK5 and GSK-3β, Cerebrolysin may regulate the production of Aβ not
only by decreasing the phosphorylation of APP but also by controlling axoplasmic flow (Sisodia et al., 1990; Lee et al., 2003; Lazarov et al., 2005). Recent studies have shown that CDK5 and GSK-3\(\beta\) regulate kinesin-driven fast axonal transport (Morfini et al., 2002, 2004), and APP processing to A\(\beta\) is dependent on this process. In conclusion, Cerebrolysin-associated reduced A\(\beta\) production might be the result of reduced kinase activity, 

Fig. 6. Double-labeling confocal analysis of the effects of Cerebrolysin treatment on amyloid protein precursor (APP) neuronal distribution. Sections from the brains of APP transgenic (tg) mice were immunolabeled with antibodies against neurofilament (SMI312, green panels) and human (h)APP (red panels). Yellow indicates colocalization of the markers; all images are from the frontal cortex. A–C: In the saline-treated APP tg mice, hAPP was distributed as granular structures (arrows) or diffusely (asterisks) in the neuritic processes. D–F: In the Cerebrolysin-treated APP tg mice, there was decreased hAPP immunoreactivity in the neuritic processes and to some extent in the neuronal cell body. Scale bar = 10 \(\mu\)m.

Fig. 7. Immunoblot analysis of the effects of Cerebrolysin on the levels of activation of amyloid protein precursor (APP)-phosphorylating kinases. A: Representative Western blot of the patterns of phosphorylated cyclin-dependent kinase-5 (CDK5-p), CDK5 activators p25 and p35, phosphorylated glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\)-p), and phosphorylated stress-activated protein kinase-1 (SAPK1-p) immunoreactivity in homogenates from the frontal cortex of APP transgenic (tg) mice treated with saline or Cerebrolysin. B: Image analysis of the levels of CDK5-p, GSK-3\(\beta\)-p, and SAPK1-p in the brains of APP tg mice. *\(P < 0.05\) compared with saline-treated APP tg mice by unpaired two-tailed Student’s \(t\)-test; \(n = 12\) mice per group.
which in turn might lead to decreased APP maturation and APP transport.

Consistent with previous studies (Rockenstein et al., 2003), the effects of Cerebrolysin on APP/Ab1–42 production were associated with amelioration of the cognitive deficits and the neurodegenerative pathology in the APP tg mice. However, for previous studies we analyzed the short (1 month)- and intermediate (3 months)-term effects of Cerebrolysin on APP tg mice, but for the present study we investigated long-term (6 months) neuroprotective and antiamyloidogenic effects of this compound. This is also in agreement with previous studies showing that this compound can exert a neurotrophic-like activity by 1) promoting synaptic formation in 6-week-old (Windholz et al., 2000) and aged (Reinprecht et al., 1999) rats, 2) protecting against excitotoxicity (Veinbergs et al., 2000), and 3) ameliorating cognitive deficits in apolipoprotein E (apoE)-deficient mice (Masliah et al., 1999). Furthermore, in a manner similar to that of nerve growth factor (NGF; Rossner et al., 1998), Cerebrolysin promotes neuritic outgrowth and cholinergic fiber regeneration (Francis-Turner and Valouskova, 1996; Satou et al., 2000).

Studies in patients with mild to moderate AD have shown that Cerebrolysin improves cognitive performance and that these effects are maintained even 6 months after termination of therapy (Ruther et al., 2000). Taken together, these studies suggest that Cerebrolysin might reduce amyloid formation and neurodegeneration by regulating kinases involved in the phosphorylation of APP. This, in turn, might decrease the amount of mature APP available for proteolysis at the synaptic site. In conclusion, Cerebrolysin might have beneficial effects in AD patients both by decreasing amyloid production and by promoting synaptic repair.

REFERENCES


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