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The Chronic Administration of Cerebrolysin Induces Plastic Changes in the Prefrontal Cortex and Dentate Gyrus in Aged Mice

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KEY WORDS aging process; dendritic morphology; prefrontal cortex; nucleus accumbens; hippocampus; neuronal plasticity; cerebrolysin

ABSTRACT Cerebrolysin (Cbl) is a mixture of neuropeptides with effects similar to the endogenous neurotrophic factors and is considered one of the best drugs used in the treatment of dementias such as Alzheimer's disease (AD). In brains with AD, morphological changes in the dendrites of pyramidal neurons of the prefrontal cortex (PFC) and hippocampus have been reported. These changes are reflected particularly in the decrement of both the dendritic tree and spine density. Here we evaluated the effect of this drug on the dendrites of pyramidal neurons of the PFC and CA1 dorsal hippocampus and granule cells from the dentate gyrus (DG) and medium spiny neurons of the nucleus accumbens (NAcc) of aged mice. Cbl (5 ml kg^{-1}, i.p.) was administered daily for 60 days to 6-month-old mice. Dendritic morphology was studied by the Golgi-Cox stain procedure followed by Sholl analysis at 8 months ages. In all Cbl-treated mice a significant increase in dendritic spine density and dendritic length in pyramidal neurons of the PFC and granule cells of the DG was observed. Interestingly, the enhancement in dendritic length was close to the soma in pyramidal neurons of the PFC whereas in granule neurons of the DG the increase in dendritic length was further from the soma. Our results suggest that Cbl induces plastic modifications of dendritic morphology in the PFC and DG. These changes may explain the therapeutic effect seen in AD patients treated with Cbl. **Synapse 65:1128–1135, 2011.**

INTRODUCTION

Cerebrolysin (Cbl) is a mixture of low molecular weight biologically active peptides and free amino acids which has proposed neuroprotective and neurotrophic properties. The compound is produced by a biotechnological process using enzymatic breakdown of purified porcine brain proteins. This drug is widely used in the treatment of dementias such as Alzheimer's disease (AD). However, Cbl has also been successfully used in the treatment of acute ischemic stroke, vascular dementia and autism, among other neurodegenerative disorders. In dementias, Cbl has been shown to improve memory in patients with mild to moderate cognitive impairment (Alvarez et al., 2006; Ruther et al., 1994; Wei et al., 2007) and to reduce amyloid pathology by decreasing the β amyloid precursor protein (βAPP) production and proteolysis in a mouse model of AD (Rockenstein et al., 2006). In addition, it has been demonstrated that Cbl might ameliorate the clinical symptoms of dementia in AD. It has been suggested that this effect is caused by stimulation of neurogenesis in the adult hippocampus, which has been found in animal models. In this regard, Tatebayashi et al. (2003) demonstrated that Cbl enhances neurogenesis in the dentate gyrus (DG)

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of the hippocampus in adult animals. This finding correlated with improvements in spatial memory which were also observed in these animals. The hippocampus and the prefrontal cortex as well as their interconnected neural circuits are implicated in cognitive and memory processes (for review see Thierry et al., 2000). In addition, the two former areas send excitatory projections to the nucleus accumbens (NAcc) (for review see Sesack and Grace, 2010).

The large number of clinical trials using Cbl in AD patients (for review see Plosker and Gauthier, 2010) contrasts with the poor information regarding the effects of Cbl on neuronal morphology (Doppler et al., 2008; Hartbauer et al.,. 2001; Satou et al., 2000; Wenzel et al., 1981). In AD, the β amyloid peptide is able to induce either the loss or alteration of neuronal dendritic spines (for review see Knobloch and Mansuy, 2008). In AD and other dementias it has also been demonstrated that there are morphological changes in dendritic spine density in the prefrontal cortex and the hippocampus (Knobloch and Mansuy, 2008; Uylings and de Brabander, 2002). AD brains are characterized by reduced cell proliferation in area CA1 of the hippocampus (Einstein et al., 1994; Ferrer and Gullotta, 1990; Scheff et al., 2007) and prefrontal cortex (PFC) (Shim and Lubec, 2002). Recently, alterations in neuronal morphology have been reported in an AD mouse model (Aoki et al., 2007; Knafo et al., 2009; Spires-Jones et al., 2007).

In the present study, we assessed the effect of Cbl on the dendritic morphology of neurons from different limbic regions associated with learning and memory in aged mice (8-months old). Our results showed that Cbl leads to significant changes in dendritic length and dendritic spine density in the PFC and DG of the hippocampus in aged animals. These observations may be relevant to the understanding of the clinical effects of Cbl in Alzheimer-type dementias.

MATERIALS AND METHODS

Animals and cerebrolysin administration

NIH male mice 6 months of age were obtained from our facilities (University of Puebla). Animals were individually housed in a temperature and humidity controlled environment on a 12-h light-dark cycle with free access to food and water. Animals were grouped and each mouse was assigned to either a vehicle (control) or cerebrolysin group. All procedures described in this study were approved by the BUAP Animal Care Committee and the governmental guidelines (Mexican Council for Animal Care, Norma Oficial Mexicana NOM-062-ZOO-1999) and by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. Cbl (5 ml kg^{-1} from Ebewe Pharma, Austria) or an equal volume of vehicle (0.1 M phosphate-buffered saline, PBS, pH 7.4) was administered by i.p. (i.p.) injection every day in the morning (between 10:00 and 12:00 am) for a period of 2 months (9 weeks).

Abbreviations

AD	Alzheimer's disease
BDNF	brain derived neurotrophic factor
Cbl	cerebrolysin
CNS	central nervous system
DG	dentate gyrus
NAcc	nucleus accumbens
NGF	nerve growth factor
PFC	prefrontal cortex
Veh	vehicle

Spontaneous locomotor activity in a novel environment

After Cbl protocol, the locomotor activity induced by a novel environment was assessed in vehicle and Cbl (10 mice per group, 8-months old). The locomotor activity score was recorded in eight-photocell activity boxes ($20 \times 40 \times 30$ cm^3) connected to a computer to count beam crosses (Tecnología Digital, Mexico). Unacclimatized mice were placed in the activity boxes, and beam crosses were recorded for 60 min and analyzed statistically using Graph Pad 4.0. All animals were tested between 10:00 to 12:00 am.

Golgi-cox stain method

Immediately after locomotor activity tests, mice were deeply anesthetized with sodium pentobarbital (75 mg kg^{-1} body weight, i.p.) and perfused intracardially with 0.9% saline solution. The brains were removed and stained using a modified Golgi-Cox method following a previously described protocol (Alquicer et al., 2008; Flores et al., 2005; Solis et al., 2009). Two hundred-μm thick coronal sections from the PFC, hippocampus and NAcc were obtained using a vibratome (Campden Instrument, MA752, Leicester, UK). Sections were collected on clean gelatin-coated microscope slides and treated with ammonium hydroxide for 30 min, followed by 30 min in Kodak Film Fixer, and finally rinsed with distilled water and mounted with resinous medium (Gibb and Kolb, 1998; Robinson and Kolb, 1997).

Microscopic observation and sholl analysis

Pyramidal cells from layer 3 of the PFC (area Cg1 and prelimbic cortex, Plate 7–9 of Paxinos and Watson Atlas, 1986), area CA1 of the dorsal hippocampus (Plate 27–32 of Paxinos and Watson Atlas, 1986), granule cells of the DG of the hippocampus (Plate 27—32 of Paxinos and Watson Atlas, 1986), and medium spiny neurons of the NAcc (Plate 10–14 of Paxinos and Watson Atlas, 1986) were selected for study.

Five neurons from each region of each hemisphere per animal were drawn using a camera lucida at a magnification of 250× (DMLS, Leica Microscope) by a trained observer who was blind to the experimental conditions (Kolb et al., 1998). Pyramidal neurons were readily identified by their characteristic triangular soma shape, apical dendrites extending toward the pial surface and numerous dendritic spines. Medium spiny neurons of the NAcc core and shell were recognized by their soma size and dendritic extensions. In the case of CA1 and PFC pyramidal neurons, the present analyses were performed on the basal dendrites since these run parallel to the coronal plane. Sequential two-dimensional reconstructions of the entire dendritic tree were generated for each neuron and the dendritic tracings were quantified by Sholl analysis (Sholl, 1953), as follows. A transparent grid with equidistant (10 μm) concentric rings was centered over the dendritic tree tracings. The number of ring intersections was used to estimate the total dendritic length and dendritic arborization (Flores et al., 2005; Kolb et al., 1998; Martínez-Tellez et al., 2005; Silva-Gomez et al., 2003; Solis et al., 2007; Vega et al., 2004). Dendritic arborization was also measured by counting the total number of dendritic branches (branching, indicated, Y bifurcated) at each order away from the cell body or dendritic shaft. To estimate the spine number, a length of dendrite (at least ≥10-μm long) was traced (at 1000×). The exact length of each dendritic branch was calculated, and the number of spines along the length was counted (to yield spines/10 μm).

Statistical analysis

The mean values from each brain region of each animal were treated as a single measurement for the data analysis. Data on dendritic length and spine number were analyzed by two-way ANOVA, followed by the Newman-Keuls test for posthoc comparisons, with Cbl and region as independent factors ($P < 0.05$ was considered significant). Data of the length per branch order also was analyzed by two-way ANOVA, followed by the Newman-Keuls test for posthoc comparisons, with Cbl and branch order as independent factors ($P < 0.05$ being significant).

RESULTS

Locomotor activity

The effect of Cbl on spontaneous locomotor activity in a novel environment is illustrated in Figure 1. All animals (vehicle-treated and Cbl-treated) initially showed increases in locomotion; reflecting an active exploratory behavior in a novel environment. The locomotor activity then gradually declined in 40–60 min to a stable level (Fig. 1). The Mann-Whitney test showed that the spontaneous locomotor activity of Cbl-treated mice was significantly higher compared to vehicle-treated mice ($P < 0.05$, Fig. 1).

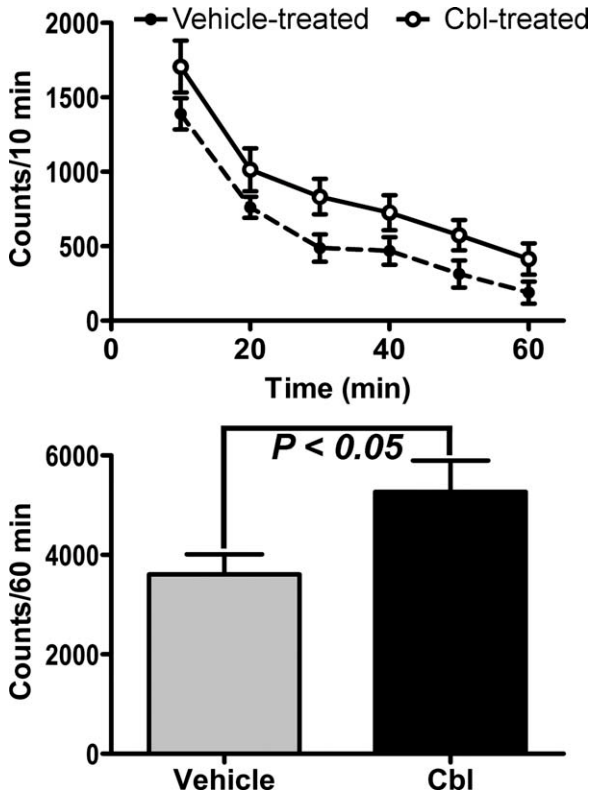

Fig. 1. Locomotor activity (mean number of beam interruptions per 10 min ±SEM, $n = 10$ per group) in a novel environment of vehicle-treated and cerebrolysin-treated mice tested at 8 months. Temporal profile of the locomotor activity at 8 months (upper panel). Analysis of total activity scores (bottom panel) reveals that cerebrolysin (Cbl)-treated mice are more active compared to corresponding vehicle-treated animals.

Dendritic morphology

The morphological analysis presented here is based on a total of 800 neurons from 20 animals. Estimates of dendritic length and spine number were obtained from 200 PFC and 200 hippocampal CA1 pyramidal neurons, 200 DG granule cells and 200 NAcc medium spiny neurons. The effects of Cbl on dendritic morphology in the prefrontal cortex, hippocampus and NAcc of the mice at 8 months of age are illustrated in Figures 2–6.

Dendritic branching and number of dendritic spines on neurons in Layer 3 of the PFC, area CA1 of the dorsal hippocampus, granule cells of the hippocampus and medium spiny neurons of the NAcc were measured by Golgi-Cox stain. The dendritic length for each branch order, spine number, and total dendritic length was obtained as reported previously (Alquicer et al., 2008; Flores et al., 2005; Juarez et al., 2008; Martínez-Tellez et al., 2009; Silva-Gomez et al., 2003; Solis et al., 2009). Golgi-Cox staining clearly filled the dendritic shafts and the spines of neurons from

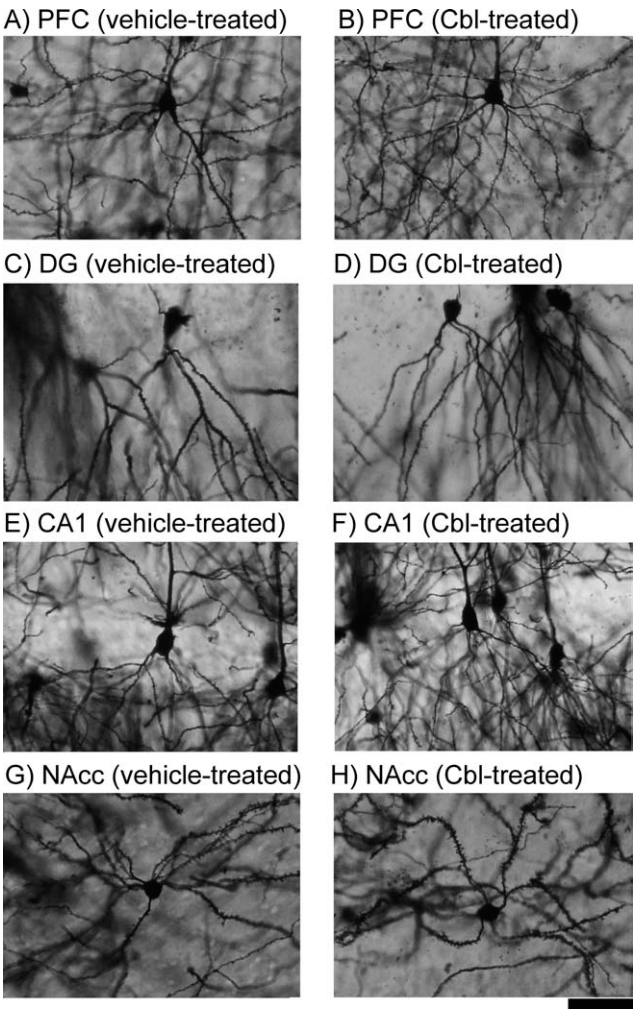

Fig. 2. Representative Golgi-Cox-impregnated neurons at 8 months of age. Layer 3 of the prefrontal cortex of the vehicle-treated (**A**) and cerebrolysin-treated mice (**B**); dentate gyrus of the vehicle-treated (**C**) and cerebrolysin-treated mice (**D**); CA1 of the dorsal hippocampus of the vehicle-treated (**E**); cerebrolysin-treated mice (**F**); nucleus accumbens of the vehicle-treated (**G**); and cerebrolysin-treated mice (**H**). Bar = 50 μm.

Layers 3 of the PFC, areas CA1 and DG of the hippocampus and the medium spiny neurons of the NAcc (Figs. 2 and 3).

A two-way ANOVA of dendritic length (Fig. 4) revealed significant main effects of Cbl ($F_{1,\ 72} = 21$, $P < 0.01$) and Cbl by region interaction ($F_{3,\ 72} = 3.34$, $P = 0.02$); it appeared, however, that dendrites of the PFC ($P < 0.01$) and DG ($P < 0.05$) of Cbl animals were larger than those of vehicle-treated animals. Although less evident, a similar trend ($P = 0.08$) was seen in the CA1 region of Cbl-treated animals.

Similarly, we observed in the PFC, DG spine densities were generally greatest in Cbl-treated animals when compared to vehicle-treated rats (Fig. 5), whereas no changes were observed in CA1 and NAcc [two-way ANOVA, Cbl ($F_{1,\ 72} = 10.4$, $P < 0.01$); region ($F_{3,\ 72} = 10.9$, $P < 0.01$)].

Another measure obtained from the Sholl analysis was the length per branch order. The branch-order analysis also revealed that dendritic length of the PFC [two-way ANOVA, Cbl ($F_{1,\ 126} = 25$, $P < 0.01$); branch order ($F_{6,\ 126} = 175$, $P < 0.01$); Cbl by branch order interaction ($F_{6,\ 126} = 4.1$, $P < 0.01$)] was larger in the Cbl-treated animals at the level of the second and third ($P < 0.01$) order compared to vehicle-treated mice (Fig. 6A). Whereas the same analysis of the DG [two-way ANOVA, Cbl ($F_{1,\ 144} = 9.1$, $P < 0.01$); branch order ($F_{6,\ 144} = 152$, $P < 0.01$)] revealed that Cbl-treated animals showed an increase in dendritic length at the level of the fourth and fifth ($P < 0.05$) orders compared to vehicle-treated mice (Fig. 6B). Finally, branch-order analysis of the CA1 [two-way ANOVA, Cbl ($F_{1,\ 119} = 9.1$, $P < 0.01$); branch order ($F_{6,\ 119} = 152$, $P < 0.01$)] revealed that Cbl-treated animals showed an increase in dendritic length only at the level of the fourth ($P < 0.05$) order compared to vehicle-treated mice (Fig. 6C).

DISCUSSION

The major aim of the present study was to investigate the effects of Cbl after 2 months of administration on locomotor activity in aged mice. In addition, we tested the potential effects of Cbl on the dendritic morphology of the PFC, areas CA1 and DG of the hippocampus, and NAcc neurons. Our results indicate that the chronic continuous administration of Cbl results in a significant increase in the locomotive performance of animals. Also, Cbl appeared to promote neuronal plasticity reflected in morphological changes that were found in the PFC and DG. These plastic changes included the enhancement in both the dendritic spine density and dendritic length. In agreement with the present results, i.p. administration of Cbl enhanced DG neurogenesis, synaptophysin immunoreactivity and density of glutamate receptor subunit 1 in hippocampus and improved the spatial learning and memory of aged rats (Eder et al., 2001; Francis-Turner and Valousková, 1996; Gschanes and Windisch, 1999; Reinprecht et al., 1999; Tatebayashi et al., 2003), reduced the amyloid burden in the frontal cortex of 5-month-old APP transgenic mice (Rockenstein et al., 2006) and improved the motor function in rats and transgenic mice (Doppler et al., 2008; Sharma et al., 2010a, 2010b).

The use of Cbl in the treatment of dementias started over 30-years ago. Cbl has also been widely used in the treatment of acute ischemic stroke (for review see Ziganshina et al., 2010). The main mechanism of action through which it influences cognition and function is presumed to be by neurotrophic and neuroprotective properties (Mallory et al., 1999). However, Cbl may also impact the pathophysiology of AD at several other levels. Although the precise

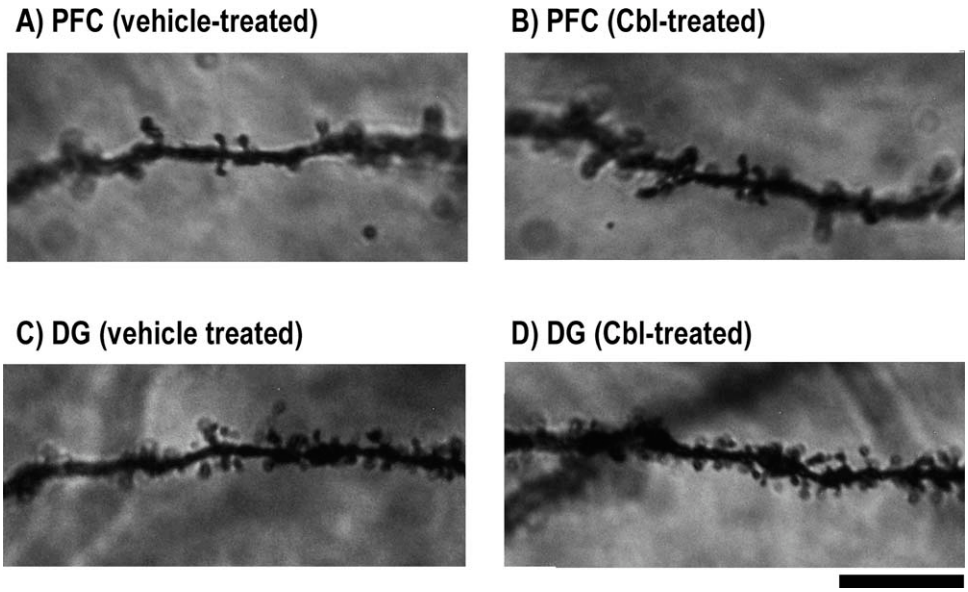

Fig. 3. Photomicrographs showing a representation of Golgi-Cox-impregnated dendritic spines of the neurons. The prefrontal cortex of the vehicle-treated (**A**) and cerebrolysin-treated mice (**B**); dentate gyrus of vehicle-treated (**C**) and cerebrolysin-treated mice (**D**). Bar = 10 μm.

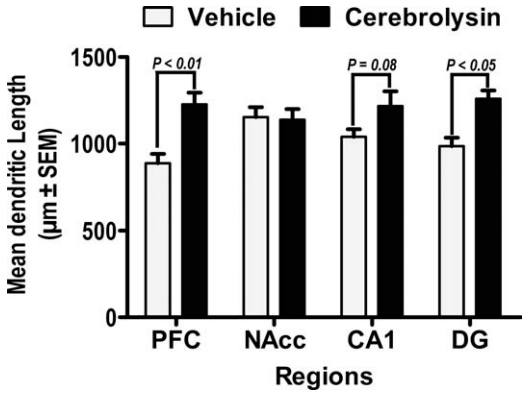

Fig. 4. Analysis of the effect of cerebrolysin (Cbl) on the total dendritic length. The total dendritic length of the prefrontal cortex (PFC), CA1, and dentate gyrus (DG) of the hippocampus were increased in the 8-month-old Cbl-treated mice compared to their corresponding vehicle-treated mice. However, the Cbl treatment did not have an effect on the medium spiny neurons of the nucleus accumbens (NAcc).

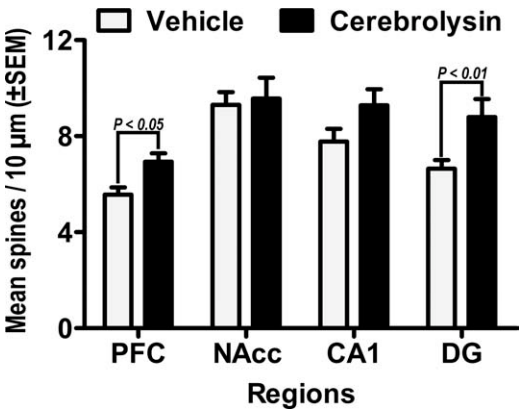

Fig. 5. Analysis of the effect of cerebrolysin (Cbl) on dendritic spine density. Dendritic spine density increased in the prefrontal cortex (PFC) and in the dentate gyrus (DG) of 8-month-old mice treated with Cbl compared to their corresponding vehicle-treated control group.

mechanisms leading to neurodegeneration in AD are not completely understood, some reports suggest that alterations in the processing of βAPP somehow result in the progressive accumulation of amyloid-β (Aβ) and βAPP C-terminal products. These events may play a key role in the pathogenesis of AD (Kamenetz et al., 2003; Koo et al., 1999; Sinha et al., 2000). In animal models of neurodegeneration such as the βAPP transgenic animal model, treatment with Cbl appears to ameliorate the neurodegenerative alterations by reducing βAPP production and proteolysis (Rockenstein et al., 2006, 2007). Cbl may be regulating the signaling pathways involved in the phosphorylation of the βAPP namely, glycogen synthase kinase-3β (GSK3β) and cyclin-dependent kinase-5 (CDK5) activity (Rockenstein et al., 2002, 2005, 2006). Interestingly, a recent report (Sharma et al., 2010a) found that in traumatic brain injuries, Cbl may reduce permeability changes in the blood-brain barrier (BBB) and blood-CSF barrier (BCSFB) and attenuate brain pathology and brain edema. Also, treatment with Cbl mitigated some functional deficits which suggest that Cbl is also involved in neuroprotection.

On the other hand, dendritic morphology of individual neurons, including dendritic length, arborization, and spine number can be assessed by using the

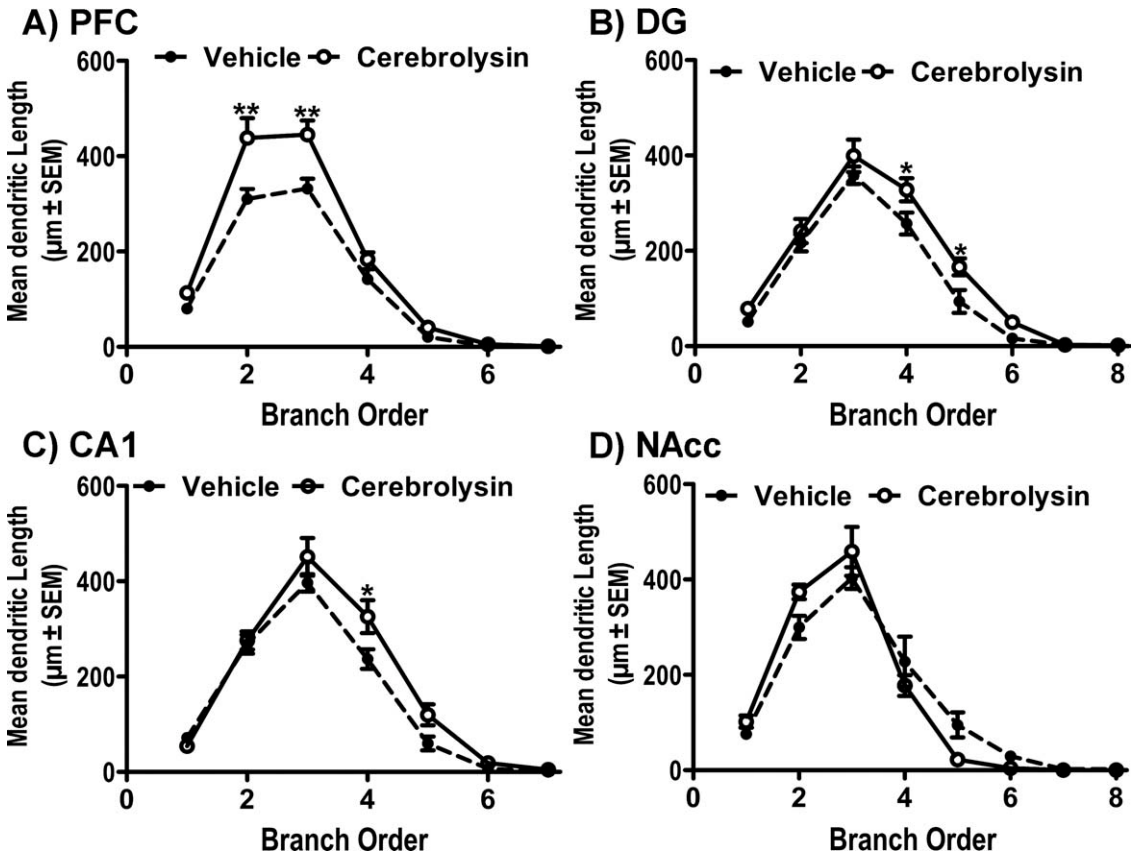

Fig. 6. Effect of cerebrolysin (Cbl) on the length of dendritic branches. The pyramidal cells of layer III from the prefrontal cortex (PFC) of Cbl-treated mice showed an increase in the dendritic length only at the level of the second and third order compared to vehicle-treated animals. Whereas granule cells of the dentate gyrus (DG) of Cbl-treated mice also displayed an increase but at the level of the fourth and fifth branch orders compared to vehicle-treated animals. Pyramidal neurons of area CA1 of the hippocampus of Cbl-treated mice also showed an increase in dendritic length but only at the fourth branch order compared to vehicle-treated animals. Finally, Cbl treatment did not have an effect on medium spiny neurons of the nucleus accumbens (NAcc). * $P < 0.05$; ** $P < 0.01$.

Golgi-Cox-impregnation method (Gibb and Kolb, 1998). This technique is commonly used to determine the dendritic surface which receives more than 95% of the excitatory synapses of a given neuron (Kolb et al., 1998). Interestingly, adult cortical neurons receive approximately 15,000 synaptic inputs (Huttenlocher, 1984). In addition, dendritic length and spine number are related to the degree of connectivity and afferent activity (McAllister, 2000). Therefore, it is possible to make inferences about connectivity from dendritic structure by measuring dendritic length, dendritic length per branch order, arborization, and spine number. The majority of our results suggest that Cbl may cause dendritic changes in aged mice especially in the pyramidal neurons of the PFC and granule cells of the DG. These morphological changes (dendritic length) occur close to the soma of neurons located in the PFC but far from the neuronal soma in the areas CA1 and DG of the hippocampus. In contrast to this, in aged mice, Cbl does not appear to affect neurons from the NAcc. The enhancement in the communication between PFC and hippocampus neurons may be playing a role in the improvement in memory performance which has been observed in Cbl-treated βAPP transgenic mice (Rockenstein et al., 2003).

As this study suggests, Cbl may also play a major trophic role which was evident in the increase in dendritic length and dendritic spine density in aged mice. At present, there is no direct evidence to associate this effect with neurotrophins. However, a recent report suggests that Cbl may be indirectly related to an increase in the neurotrophins such as ciliary neurotrophic factor (CNTF), glial-derived neurotrophic factor (GDNF), and insulin-like growth factors-1 and -2 (IGF-1, IGF-2) (Chen et al., 2007). It is well known that neurogenesis persists in the dentate gyrus of the adult mammalian brain. Another recent report suggests that AD pathogenesis might involve an abnormally elevated fibroblast growth factor-2 (FGF-2)-associated dysregulation of dentate gyrus neurogenesis (Tatebayashi et al., 2003). Interestingly, neurotrophic activities of the CNTF, GDNF, IGF-1 and IGF-2 counteract the effect of FGF-2 in inducing neuronal lineage (early neurogenesis) (Chen et al., 2007).

Therefore Cbl also appears to be related to DG hippocampal neurogenesis (Tatebayashi et al., 2003). Neurotrophins are expressed in almost all neuronal populations in the central and peripheral nervous system. It is intriguing that cerebrolysin treatment appears to induce an increase in the spine density and dendritic length in PFC and DG in 8-month-old mice. The latter areas are critical for learning and memory (Mimura, 2008). Therefore, the increase in communication among hippocampus and PFC neurons may be, at least in part, beneficial for brain function in all these degenerative processes.

Excitotoxicity might play an important role in neurodegenerative disorders such as Alzheimer's disease. In in vivo studies of the mouse brain, kainic acid (KA) lesioning results in neurodegeneration patterns similar to those found in human diseases. Interestingly, Cbl ameliorates the alterations associated with KA administration, suggesting a neuroprotective effect against excitotoxicity (Veinbergs et al., 2000). In vitro studies using cortical neurons in culture suggest that after acute glutamate exposure, Cbl reduces the number of apoptotic cortical neurons (Gutmann et al., 2002). In another model of excitotoxic cell death, brain ischemia or cytotoxic hypoxia induced by iodoacetate, Cbl treatment resulted in a dose dependent and significant rescue of neurons (Gutmann et al., 2002; Schauer et al., 2006). In addition, overactivated calpain might be a key factor in the destruction of cytoskeletal proteins involved in the pathophysiology of ischemia and disorders like AD. Interestingly, Cbl may produce an inhibitory effect on calpain, a Ca^{2+}-dependent protease (Wronski et al., 2000). Furthermore, it is known that glutamate lesion or ischemia results in a disturbance of Ca^{2+} homeostasis. Therefore Cbl is able to stabilize Ca^{2+} homeostasis to protect protein synthesis and to counteract neuronal death in different in vivo and in vitro models of ischemia (Gutmann et al., 2002).

Cbl has also been used in the treatment of autism (Radzivil et al., 2006) and diabetic neuropathy (Biesenbach et al. 1997). Previous reports (Martinez-Tellez et al., 2005) suggest that rats with diabetes mellitus induced by streptozotocin showed a decrease in dendritic length and dendritic spine density of pyramidal neuron of the PFC and area CA1 of the hippocampus. It is well known that lipid peroxidation increases under stress conditions such as hypoxia, ischemia or acidosis as well as in metabolic diseases, e.g., diabetes mellitus (for review see Mattson, 2009) and can lead to degradation of essential components of membrane lipids followed by disturbances of membrane permeability (for review see Mattson, 2009). Interestingly, Cbl inhibits lipid peroxidation induced by hypoglycemia in the brain mice (Patockova et al., 2003).

In summary, Cbl appears to play a role in the aging process which is reflected in the plastic changes observed in the neuronal dendritic morphology in limbic structures such as the DG and the PFC. In conclusion, Cbl has a potent and specific effect on neuronal plasticity in the brains of aged mice. Therefore, our results suggest that Cbl is a neurotrophic agent with a high potential to be used not only in the treatment of AD but in the aging process as well.

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