Published in final edited form as:

Neurochem Res. 2015 November ; 40(11): 2333–2347. doi:10.1007/s11064-015-1723-x.

Sodium benzoate, a metabolite of cinnamon and a food additive, upregulates ciliary neurotrophic factor in astrocytes and oligodendrocytes

Khushbu K. Modi^a, Malabendu Jana^a, Susanta Mondal^a, and Kalipada Pahan^{a,b}

^aDepartment of Neurological Sciences, Rush University Medical Center, Chicago, USA

^bDivision of Research and Development, Jesse Brown Veterans Affairs Medical Center, 820 South Damen Avenue, Chicago, USA

Abstract

Ciliary neurotrophic factor (CNTF) is a promyelinating trophic factor that plays an important role in multiple sclerosis (MS). However, mechanisms by which CNTF expression could be increased in the brain are poorly understood. Recently we have discovered anti-inflammatory and immunomodulatory activities of sodium benzoate (NaB), a metabolite of cinnamon and a widely-used food additive. Here, we delineate that NaB is also capable of increasing the mRNA and protein expression of CNTF in primary mouse astrocytes and oligodendrocytes and primary human astrocytes. Accordingly, oral administration of NaB and cinnamon led to the upregulation of astroglial and oligodendroglial CNTF *in vivo* in mouse brain. Induction of experimental allergic encephalomyelitis (EAE), an animal model of MS, reduced the level of CNTF in the brain, which was restored by oral administration of cinnamon. While investigating underlying mechanisms, we observed that NaB induced the activation of protein kinase A (PKA) and H-89, an inhibitor of PKA, abrogated NaB-induced expression of CNTF. The activation of cAMP response element binding (CREB) protein by NaB, the recruitment of CREB and CREB-binding protein to the CNTF promoter by NaB and the abrogation of NaB-induced expression of CNTF in astrocytes by siRNA knockdown of CREB suggest that NaB increases the expression of CNTF via the activation of CREB. These results highlight a novel myelinogenic property of NaB and cinnamon, which may be of benefit for MS and other demyelinating disorders.

Keywords

Sodium benzoate; CNTF; Astrocytes; Oligodendrocytes; PKA; CREB

Introduction

Ciliary neurotrophic factor (CNTF) is a neurotrophic cytokine belonging to the interleukin-6 (IL-6) family. Although it was characterized as a survival factor for neurons [1], later on it was found to support the survival of oligodendrocytes and as a result, to control myelination.

While neurotrophins (nerve growth factor, neurotrophin-3 (NT-3), NT-4/5, and brain-derived neurotrophic factor) and glial cell line-derived neurotrophic factor (GDNF)-related factors (GDNF and neurturin) do not increase myelinogenesis, CNTF induces a strong promyelinating effect [2]. It protects oligodendrocytes from various death signals [3], mediates the maturation of oligodendroglial progenitor cells (OPCs) into mature myelin-forming cells and helps differentiated oligodendrocytes to synthesize myelin [4]. Multiple sclerosis (MS) is the most common human demyelinating disorder of the CNS in which promoting remyelination remains a crucial therapeutic challenge [5-8]. Experimental allergic encephalomyelitis (EAE) is an animal model of MS. According to Kuhlmann et al [9], continued administration of CNTF protects mice from inflammatory pathology in EAE. Similarly, mesenchymal stem cells over-expressing CNTF reduce demyelination and induce clinical recovery in EAE mice [10]. Consistently, induction of EAE in *Cntf*^{-/-} mice by MOG35–55 peptide resulted in earlier onset of symptoms and increased disability [11]. Proteomic analysis has identified enhanced oligodendrocyte apoptosis and increased axonal injury in EAE in *Cntf*^{-/-} mice [12]. The clinical relevance of CNTF deficiency for MS is corroborated by the recent preliminary observation that MS patients with the *Cntf*^{+/+} alleles may have a significantly earlier onset of disease [13]. However, clinical application of CNTF has been limited because of difficulties in delivery because CNTF does not readily diffuse across the blood-brain barrier (BBB) or ventricular lining and have limited or unstable bioavailability [14]. Gene delivery [15] and/or protein delivery by stereotactic injection is definitely an option but it has several limitations. Therefore, there are reasons to believe that increasing the level of CNTF in the CNS or restoring its level is beneficial for MS and other demyelinating diseases.

Cinnamon, a commonly used natural spice and flavoring material used for centuries throughout the world, is metabolized into sodium benzoate (NaB). NaB is also of medical importance as it is a component of Ucephan, a FDA-approved drug used in the treatment for hepatic metabolic defects associated with hyperammonemia such as urea cycle disorder in children [16, 17]. It has been reported that minor amount of NaB is also excreted in the urine of human [18, 19]. It is non-toxic and can be administered as a solution in drinking water. It has been reported that 2% solution of NaB in drinking water is safe for lifelong treatment in mice without any noticeable side effects [20]. Recently, we have delineated that cinnamon and NaB are capable of protecting mice from EAE [21, 22]. Because NaB readily enters into CNS [23, 24], we tested its efficacy in stimulating CNTF from astrocytes and oligodendrocytes. Here we provide the first evidence about the myelinogenic effect of NaB. NaB treatment increases the expression of CNTF in astrocytes and oligodendrocytes in cell cultures as well as *in vivo* in the brain. Although CNTF level decreases in the brain of EAE mice, cinnamon treatment is capable of increasing the level of CNTF in EAE mice. Our findings raise a possibility that cinnamon and its metabolite NaB may find application in MS and demyelinating disorders via increased production of CNTF.

Materials and Methods

Reagent

Cell culture materials (DMEM/F-12, L-Glutamine, Hank's balanced salt solution, 0.05% trypsin, and antibiotic-antimycotic) were purchased from Mediatech (Washington, DC). Fetal bovine serum (FBS) was obtained from Atlas Biologicals. Original Ceylon cinnamon (*Cinnamomum verum*) in ground form was obtained from Indus Organics (San Ramon, CA). Sodium benzoate and sodium formate were obtained from Sigma. H-89 was purchased from Enzo Life Sciences. Primary antibodies, their sources and concentrations used are listed in Table 1. Alexa-fluor antibodies used in immunostaining were obtained from Jackson ImmunoResearch and IR-dye-labeled reagents used for immunoblotting were from Li-Cor Biosciences.

Isolation of Primary Mouse Astrocytes and Oligodendrocytes

Astrocytes and oligodendrocytes were isolated from 2-3 d old mouse pups as described earlier [25-28]. Briefly, on day 9, the mixed glial cultures were subjected to shaking at 240 rpm for 2 h at 37°C on a rotary shaker to remove loosely attached microglia. On day 11, the oligodendrocytes were detached after shaking for 18 h at 200 rpm. To purify oligodendrocytes from astrocytes and microglia, the detached cell suspension was plated in tissue culture dishes (2×10^6 cells/100 mm) for 60 min at 37 °C. This step was repeated twice for non-adherent cells to minimize the contamination. The non-adhering cells (mostly oligodendrocytes) were seeded onto poly-D-lysine-coated culture plates in complete medium (DMEM/F-12 supplemented with 10% heat inactivated FBS) at 37 °C with 5% CO₂ in air. Earlier we [29, 30] have shown that oligodendrocytes isolated through this procedure are more than 98% pure.

The attached cells, mostly astrocytes, were washed and seeded onto new plates for further studies. About ninety-eight percent of this preparation was found to be positive for GFAP, a marker of astrocytes.

Isolation of Primary Human Astroglia

Primary human astroglia were prepared from fetal brains as described by us in many studies [31-33]. All of the experimental protocols were reviewed and approved by the Institutional Review Board of the Rush University Medical Center. Briefly, 11- to 17-week-old fetal brains obtained from the Human Embryology Laboratory (University of Washington, Seattle, WA, USA) were dissociated by trituration and trypsinization. On 9th day, these mixed glial cultures were placed on a rotary shaker at 240 rpm at 37°C for 2 h to remove loosely attached microglia. Then on 11th day, flasks were shaken again at 190 rpm at 37°C for 18 h to remove oligodendroglia. The attached cells remaining were primarily astrocytes. These cells were trypsinized and subcultured in complete media at 37°C with 5% CO₂ in air to yield more viable and healthy cells. More than 98% of these cells obtained by this method were found to be positive for GFAP, a marker for astrocytes.

Semi-quantitative RT-PCR analysis

To remove any contaminating genomic DNA, total RNA was digested with DNase. Semi-quantitative RT-PCR was carried out as described earlier [26, 27, 34, 35] using a RT-PCR kit from clontech. Briefly, 1 µg of total RNA was reverse transcribed using oligo(dT)₁₂₋₁₈ as primer and MMLV reverse transcriptase (Clontech). The resulting cDNA was appropriately-diluted, and diluted cDNA was amplified. Amplified products were electrophoresed on a 1.8% agarose gels and visualized by ethidium bromide staining.

CNTF (mouse):	Sense: 5'- GGGACCTCTGTAGCCGCTCTATCTG -3'
	Antisense: 5'- GTTCCAGAAGCGCCATTAACCTCCTC -3'
CNTF (human):	Sense: 5'- TCACAGAGCATTCACCGCTGACCCC-3'
	Antisense: 5'- CTGCTGGTCTTCTAAGAGCCTGGCC-3'
GAPDH (mouse):	Sense: 5'- GGTGAAGGTCGGTGTGAACG3'
	Antisense: 5'-TTGGCTCCACCTTCAAGTG-3'
GAPDH (human):	Sense : 5'- GGTGAAGGTCGGAGTCAACG-3'
	Antisense: 5'- GTGAAGACGCCAGTGGACTC-3'

Real-time PCR Analysis

It was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) as described earlier [26, 27, 34, 35]. The mRNA expressions of respective genes were normalized to the level of GAPDH mRNA. Data were processed by the ABI Sequence Detection System 1.6 software and analyzed by ANOVA.

ELISA

Amount of CNTF was quantified in supernatants of human astrocytes using a high-sensitivity sandwich ELISA (R&D) following manufacturer's protocol. Briefly, human astrocytes were plated in 24 well plates and supernatants were directly added to microplates precoated with monoclonal antibodies against CNTF. Plates were analyzed spectrophotometrically with a Thermo-Fisher Multiskan MCC plate reader.

Assay of PKA

Assay for PKA was purchased from Enzo Life Sciences and performed according to manufacturer's protocol as described [35]. Values were normalized to purified kinase controls.

Immunoblotting

Western blotting was conducted as described earlier [26, 35]. Briefly, cells were scraped in lysis buffer, transferred to microfuge tubes and spun into pellet. The supernatant was collected and analyzed for protein concentration via the Bradford method (Bio-Rad). SDS sample buffer was added to 40-60 µg total protein and boiled for 5 min. Denatured samples were electrophoresed on NuPAGE® Novex® 4-12% Bis-Tris gels (Invitrogen) and proteins transferred onto a nitrocellulose membrane (Bio-Rad) using the Thermo-Pierce Fast Semi-Dry Blotter. The membrane was then washed for 15 min in TBS plus Tween 20 (TBST) and blocked for 1 hr in TBST containing BSA. Next, membranes were incubated overnight at

4°C under shaking conditions with primary antibodies. The next day, membranes were washed in TBST for 1 h, incubated in secondary antibodies for 1 h at room temperature, washed for one more hour and visualized under the Odyssey® Infrared Imaging System (Li-COR, Lincoln, NE).

Densitometric Analysis

Protein blots were analyzed using ImageJ (NIH, Bethesda, MD) and bands were normalized to their respective β -actin loading controls. Data are representative of the average fold change with respect to control for three independent experiments.

Chromatin immunoprecipitation

Recruitment of CREB to the *Cntf* promoter was determined using the EZ ChIP kit from Millipore as described before [26, 35, 36]. Briefly, 1×10^6 astrocytes were treated with NaB and after 4 h of stimulation, cells were fixed by adding formaldehyde (1% final concentration), and cross-linked adducts were resuspended and sonicated. ChIP was performed on the cell lysate by overnight incubation at 4°C with 2 μ g of Abs against CREB and CBP followed by overnight incubation with protein G-agarose (Santa Cruz Biotechnology). The beads were washed and incubated with elution buffer. To reverse the cross-linking and purify the DNA, precipitates were incubated in a 65°C incubator overnight and digested with proteinase K. DNA samples were then purified, precipitated, and precipitates were washed with 75% ethanol, air-dried, and resuspended in Tris-EDTA buffer. The following primers were used to amplify the mouse *Cntf* promoter flanking the only CRE: sense: 5'-GTCACCACAAGCAAGTTGGAGAGA-3', antisense: 5'-GGCTGGTAGTCCTGGGTTCTCT-3'. PCR products were electrophoresed on 2% agarose gels.

Induction of EAE

EAE was induced in male C57BL/6 mice by MOG35-55 (100 μ g/mouse) immunization as described by us [22]. Mice also received two doses of pertussis toxin (150 ng/mouse) on 0 and 2 dpi.

Cinnamon treatment

Cinnamon (*Cinnamomum verum*) powder was mixed in 0.5% methylcellulose (MC) and EAE mice were gavaged 100 μ L cinnamon-mixed MC powder once daily using gavage needle as described [22, 23]. Therefore, control EAE mice received only MC as vehicle.

Immunofluorescence Analysis

It was performed as described earlier [31, 33]. Briefly, cover slips containing 100-200 cells/mm² were fixed with 4% paraformaldehyde followed by treatment with cold ethanol and two rinses in phosphate-buffered saline (PBS). Samples were blocked with 3% bovine serum albumin (BSA) in PBS-Tween-20 (PBST) for 30 min and incubated in PBST containing 1% BSA and rabbit anti-CNTF or goat anti-GFAP. After three washes in PBST (15 min each), slides were further incubated with Cy2 (Jackson ImmunoResearch Laboratories, Inc.). For negative controls, a set of culture slides was incubated under similar

conditions without the primary antibodies. The samples were mounted and observed under a Bio-Rad MRC1024ES confocal laser-scanning microscope.

Statistics

Statistical comparisons were made using one-way analysis of variance followed by Student's *t* test.

Results

Upregulation of CNTF by NaB in primary mouse astrocytes and oligodendrocytes

Although CNTF plays an important role in myelination, little is known about the drugs and associated mechanisms that upregulate CNTF. We examined if NaB could stimulate the expression of CNTF in mouse primary astrocytes. NaB indeed increased the mRNA expression of CNTF within 6 hour of treatment in a dose-dependent manner (Fig. 1A-B). Time-course study showed that NaB could increase the expression of CNTF gene as early as 2h with maximum upregulation noted at 12 h (Fig 1C-D). We further checked the protein level of CNTF in astrocytes by Western blot. Similar to mRNA expression, NaB strongly induced the protein level CNTF at different doses in astrocytes (Fig. 1E-F). These results were specific as sodium formate (NaFO) having a similar structure but without the benzene ring was unable to increase CNTF in astrocytes (Fig. 1E-F). The increase in CNTF protein in astrocytes by NaB, but not NaFO, was further confirmed by immunofluorescence analysis (Fig. 1G).

Next, we examined if NaB could stimulate the expression of CNTF in primary mouse oligodendrocytes. Consistent to mouse astrocytes, NaB, but not NaFO, increased the expression of CNTF mRNA in oligodendrocytes (Fig. 2A-B). Although we have not seen any induction of CNTF by NaB at a dose of 50 μ M in astrocytes (data not shown), this dose of NaB was sufficient to increase CNTF mRNA expression in oligodendrocytes (Fig. 2A-B). Similarly NaB also strongly increased the expression of CNTF protein in oligodendrocytes at a dose of 50 μ M or higher (Fig. 2C-D). Immunofluorescence analysis also demonstrated upregulation of CNTF in oligodendrocytes by NaB, but not NaFO (Fig. 2E).

NaB increases CNTF in primary human astrocytes

Drug responses seen in rodent cells are not always replicated in human cells. Therefore, next, we examined if NaB could stimulate the expression of CNTF in primary human astrocytes. Consistent to mouse astrocytes, NaB strongly increased the expression of CNTF mRNA (Fig. 3A-B for dose-dependent effect and Fig. 3C-D for time-dependent effect) in primary astroglia isolated from human fetal brains. Specificity was examined by using NaFO (Fig. 3A-B). Immunofluorescence analysis also indicated induction of CNTF protein by NaB, but not NaFO, in human astrocytes (Fig. 3E). ELISA kit is available only for human CNTF. Therefore, we examined if CNTF was secreted from human astrocytes upon NaB treatment. As evident from figure 3F, NaB markedly increased the production of CNTF in primary human astrocytes.

Oral administration of NaB and cinnamon increases CNTF in vivo in the brain

Once we confirmed the upregulation of CNTF by NaB in cultured astrocytes and oligodendrocytes, we further checked whether the same results could be replicated in *in vivo* settings. After 30 days of oral administration, NaB markedly increased the mRNA expression of CNTF *in vivo* in the cortex of male C57/BL6 mice (Fig. 4A for RT-PCR; Fig. 4B for real-time). This result was specific as NaFO feeding did not increase the mRNA expression of CNTF (Fig. 4A-B). Next, we examined if cinnamon itself could also increase these invaluable molecules *in vivo* in the brain. Two major types of cinnamon that are available in the US are Chinese cinnamon (*Cinnamomum cassia*) and original Ceylon cinnamon (*Cinnamomum verum* or *Cinnamomum zylencum*). Recently by mass spectrometric analysis, we have found that *Cinnamomum verum* is much more pure than *Cinnamomum cassia* [23]. Although both *Cinnamomum cassia* and *Cinnamomum verum* contain cinnamaldehyde as the major peak, *Cinnamomum cassia* contains more styrene, benzene, 1,1'-(2-butene-1,4-diyl)bis-, benzene, 1,1'-(1,2-cyclobutanediyl)bis-, palmitic acid, stearic acid, 4-phenylbutyl chloride, and (2,3-diphenylcyclopropyl)methyl phenyl sulfoxide than *Cinnamomum verum* [23]. Most importantly, *Cinnamomum cassia*, but not *Cinnamomum verum*, contains small amount of toxic 1-benzopyran-2-one or coumarin [23]. Therefore, here, we used *Cinnamomum verum* for oral treatment of mice. We have already seen that oral administration of ground *Cinnamomum verum* increases the level of NaB in different parts of the brain [23, 24]. Similar to NaB, oral administration of *Cinnamomum verum* powder also increased the mRNA expression of CNTF *in vivo* in the cortex (Fig. 4C for RT-PCR; Fig. 4D for real-time). Western blot results showed that cinnamon feeding markedly increased the level of CNTF protein *in vivo* in the cortex (Fig. 4E-F). Immunofluorescence analysis also showed increase in CNTF protein *in vivo* in the cortex upon NaB and cinnamon treatment (Fig. 5A-C). Double-labeling with cell-specific markers indicated that NaB and cinnamon increased the level of CNTF in both GFAP-positive astrocytes (Fig. 5A) and GalC-positive oligodendrocytes (Fig. 5B).

Does oral administration of cinnamon increase CNTF in vivo in the CNS of EAE mice?

Promoting remyelination appears to be a crucial challenge for MS. Since CNTF is a promyelinating factor and oral treatment of normal mice with cinnamon increased CNTF in the CNS, next, we examined if the same could be achieved in mice with EAE, an animal model of MS. EAE was induced in male C57/BL6 mice by MOG35-55 immunization and from 8 days post-immunization (onset of acute phase), mice were treated with cinnamon powder orally. After 8 days of treatment, level of CNTF was monitored in the cerebellum by double-label immunofluorescence. Control brains expressed CNTF and many of the CNTF-positive cells were astrocytes (Fig. 6A). Consistent to a critical role of CNTF in MS [13], we found that the level of CNTF decreased drastically in the cerebellum of EAE mice (Fig. 6A-B). However, oral cinnamon treatment markedly increased the level of CNTF in the cerebellum of EAE mice (Fig. 6A-B). These results were specific as vehicle treatment had no such protective effect (Fig. 6A-B).

NaB requires CREB for the upregulation of CNTF

Next, we investigated mechanisms by which NaB upregulated the expression of *cntf* gene in astrocytes. Upon analysis of the *cntf* gene promoter by using MatInspector, we found that mouse *cntf* promoter harbors one consensus cAMP response element (CRE) between 35 and 55 base pairs upstream of the transcriptional start site (Fig. 7A). Therefore, we were prompted to investigate if NaB required CREB for the transcription of CNTF in mouse primary astrocytes. First, we examined if NaB alone was capable of inducing the activation of CREB in astrocytes. Activation of CREB was monitored by levels of phosphorylated CREB (p-CREB). NaB alone induced the phosphorylation of CREB as depicted by Western blot (Fig. 7B-C). On the other hand, we did not see any change in the level of total CREB upon NaB stimulation (Fig. 7B; lower panel).

Next, we monitored the recruitment of CREB to the CNTF promoter. At first, we used ChIP analysis to study if NaB was capable of inducing the recruitment of CREB to the CRE present in the CNTF promoter. After immunoprecipitation of NaB-treated astroglial chromatin fragments by Abs against CREB, we were able to amplify 192 bp fragment flanking the CRE and this amplification was missing in control astrocytes (Fig. 7D-E), suggesting that NaB induces the recruitment of CREB to the CNTF promoter. Similarly, NaB also induced the recruitment of CREB-binding protein (CBP) to the CNTF promoter (Fig. 7D-E). In contrast, no amplification product was observed in any of the immunoprecipitates obtained with control IgG (Fig. 7D-E), suggesting the specificity of these interactions.

Next, we investigated if NaB required CREB for the upregulation of CNTF in astrocytes. We employed the siRNA approach to study the involvement of CREB. As evident from figure 7F and G, CREB siRNA, but not control siRNA, decreased the expression of CREB protein in aspirin-treated astrocytes. Accordingly, CREB siRNA, but not control siRNA, abrogated NaB-mediated upregulation of CNTF protein (Fig. 7H-I). These results suggest that CREB is required for NaB-mediated upregulation of CNTF.

NaB increased the activation of CREB and the expression of CNTF via protein kinase A (PKA)

Next, we decided to elucidate mechanisms by which NaB induced the activation of CREB and increased the expression of CNTF in astrocytes. Recently we have demonstrated that aspirin increases CNTF via the cAMP – PKA pathway [37]. Therefore, we examined the role of PKA in NaB-mediated upregulation of CNTF. At first, we monitored if NaB alone was capable of activating PKA. As evident from figure 8A, NaB alone induced the activation of PKA in astrocytes in a time-dependent manner and this activation was significant as early as 15 min of stimulation. Next, we examined if NaB required PKA for the activation of CREB. Suppression of NaB-mediated phosphorylation of CREB (Fig. 8B-C) by H-89, a specific inhibitor of PKA, suggests that NaB induces the activation of CREB via PKA. Consistent to the involvement of CREB in NaB-mediated upregulation of CNTF, H-89 also inhibited NaB-mediated expression of CNTF protein in astrocytes (Fig. 8D-E). Next, we examined if H-89 was also capable of inhibiting NaB-induced recruitment of CREB and CBP to the CNTF promoter. Consistent to the inhibition of CNTF mRNA

expression, H-89 inhibited the recruitment of both CREB and CBP to the CNTF promoter in NaB-treated astrocytes (Fig. 7D-E), demonstrating that NaB increases the recruitment of CREB and CBP to the CNTF promoter in mouse astrocytes via PKA. These results suggest that NaB requires PKA for the activation of CREB and the transcription of CNTF gene in astrocytes.

Discussion

Increasing the level of CNTF and/or maintaining its physiological level in the CNS of patients with MS is/are an important area of research as CNTF is a pluripotent trophic factor capable of signaling oligodendrocytes to survive, differentiate, or grow [9-12]. Furthermore, CNTF has also been suggested as a rescuer of vulnerable oligodendrocytes in MS, in which the level of CNTF is significantly reduced in the brain [9-12]. Therefore, CNTF is considered as a promyelinating trophic factor. However, mechanisms to upregulate CNTF in the CNS are poorly understood. Cinnamon, the brown bark of cinnamon tree, is a commonly used spice and flavoring material for deserts, candies, chocolate, etc. It has a long history as a medicine as well. Medieval physicians used cinnamon in medicines to treat a variety of disorders including arthritis, coughing, hoarseness, sore throats, etc. In addition to containing manganese, dietary fiber, iron, and calcium, cinnamon contains a major compound, cinnamaldehyde, which is converted into cinnamic acid by oxidation. In the liver, this cinnamic acid is β -oxidized to benzoate that exists as sodium salt (NaB) or benzoyl-CoA. Several lines of evidence presented in this study clearly support the conclusion that NaB is capable of upregulating CNTF in astrocytes and oligodendrocytes. Furthermore, oral administration of cinnamon powder also augmented astroglial and oligodendroglial expression of CNTF *in vivo* in the CNS of normal mice. Because EAE serves as an animal model for MS, we also examined the efficacy of cinnamon in increasing the level of CNTF *in vivo* in the CNS of EAE mice. Although induction of EAE decreased the level of CNTF, cinnamon feeding upregulated CNTF in the cerebellum of EAE mice. Because the loss of CNTF has been implicated in the pathogenesis of MS, our results provide a potentially important mechanism whereby cinnamon may ameliorate demyelination. This is further supported by our recent finding that oral administration of cinnamon and its metabolite NaB improve remyelination in EAE mice [21, 22].

The signaling mechanisms for the transcription of CNTF in glial cells are poorly understood. According to Eto et al [38], the Sox10 is necessary and sufficient for regulating the expression of *Cntf* in Schwann cells. The cAMP-protein kinase A (PKA) pathway, one of the most common and versatile signal pathways in eukaryotic cells, is involved in the regulation of multiple cellular functions including cell growth, differentiation, and survival; and synaptic plasticity [39-41]. Recently we have delineated that aspirin, one of the widely-used analgesic, requires PKA for the induction of CNTF in astrocytes [37]. Abrogation of NaB-induced expression of CNTF in astrocytes by H-89, a specific inhibitor of PKA, suggest that NaB also induces CNTF in astrocytes via PKA. While investigating the mechanism further, we have found the presence of a consensus CREB-binding site in the proximal region of the *Cntf* promoter and induction of CREB phosphorylation by NaB in cultured astrocytes. Recruitment of CREB and CREB-binding protein (CBP) to the CRE of the CNTF promoter in NaB-treated cells and abrogation of the ability of NaB to induce the

transcription of CNTF gene by siRNA knockdown of CREB suggest that NaB increases the level of CNTF in astrocytes via CREB. Inhibition of PKA by H-89 suppressed NaB-induced activation of CREB, inhibited the recruitment of CREB to the CNTF promoter and stopped the upregulation of CNTF, indicating that NaB increases the expression of CNTF in glial cells via PKA-mediated activation of CREB. Although we found NF- κ B-binding sites in the promoter of *Cntf*, NaB alone does not induce the activation of NF- κ B. Furthermore, in activated glial cells, NaB inhibits the activation of NF- κ B [31]. Therefore, NaB does not upregulate the expression of CNTF via NF- κ B.

There are several advantages of NaB and cinnamon over other proposed anti-demyelinating and anti-neurodegenerative therapies. *First*, both NaB and cinnamon are fairly nontoxic. Cinnamon has been widely used as flavoring material and spice throughout the world for centuries. Cinnamon is metabolized to NaB. NaB is excreted through the urine, if in excess. NaB is an FDA-approved drug against Urea cycle disorders in children. *Second*, cinnamon and NaB can be taken orally, the least painful route. We have already demonstrated that NaB treatment of mice with relapsing-remitting EAE, an animal model of MS, via drinking water suppressed the disease process of EAE [21]. We have also found that oral administration of cinnamon powder exhibits neuroprotective effect in a mouse model of AD [24] and protects mice from EAE [22]. *Third*, cinnamon and NaB are very economical compared to other existing anti-demyelinating therapies. *Fourth*, after oral administration, NaB rapidly diffuses through the BBB. Similarly, after oral administration of cinnamon, we also detected NaB in the brain. *Fifth*, glycine toxicity is a problem in different neurological diseases because for movement disorders, glycine is one of the factors for inhibiting motor neurons [42]. When impaired, glycinergic inhibition leads to spastic and hypertonic disorders such as featured in MS. NaB is known to combine with glycine to produce hippurate, a compound that is readily excreted in the urine [43]. Because MS patients exhibit significant elevation in plasma level of glycine [44], NaB and cinnamon may have added benefits for MS.

In summary, we have demonstrated that cinnamon metabolite NaB upregulates CNTF via PKA-mediated activation of CREB. These results highlight an unexplored property of NaB and cinnamon and indicate that these compounds may have therapeutic value in MS and other demyelinating conditions.

Acknowledgments

This study was supported by National Institutes of Health grant (AT6681) and Veteran Affairs Merit Award (I01BX002174).

References

1. Varon S, Manthorpe M, Adler R. Cholinergic neuronotrophic factors: I. Survival, neurite outgrowth and choline acetyltransferase activity in monolayer cultures from chick embryo ciliary ganglia. *Brain Res.* 1979; 173:29–45. [PubMed: 487083]
2. Stankoff B, Aigrot MS, Noel F, Wattilliaux A, Zalc B, Lubetzki C. Ciliary neurotrophic factor (CNTF) enhances myelin formation: a novel role for CNTF and CNTF-related molecules. *J Neurosci.* 2002; 22:9221–9227. [PubMed: 12417647]

3. Louis JC, Magal E, Takayama S, Varon S. CNTF protection of oligodendrocytes against natural and tumor necrosis factor-induced death. *Science*. 1993; 259:689–692. [PubMed: 8430320]
4. Barres BA, Schmid R, Sendtner M, Raff MC. Multiple extracellular signals are required for long-term oligodendrocyte survival. *Development*. 1993; 118:283–295. [PubMed: 8375338]
5. Hafler DA, Weiner HL. MS: a CNS and systemic autoimmune disease. *Immunol Today*. 1989; 10:104–107. [PubMed: 2472810]
6. Martin R, McFarland HF, McFarlin DE. Immunological aspects of demyelinating diseases. *Annu Rev Immunol*. 1992; 10:153–187. [PubMed: 1375472]
7. Steinman L. Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell*. 1996; 85:299–302. [PubMed: 8616884]
8. Pahan K. Neuroimmune pharmacological control of EAE. *J Neuroimmune Pharmacol*. 2010; 5:165–167. [PubMed: 20414732]
9. Kuhlmann T, Remington L, Cognet I, Bourbonniere L, Zehntner S, Guilhot F, Herman A, Guay-Giroux A, Antel JP, Owens T, Gauchat JF. Continued administration of ciliary neurotrophic factor protects mice from inflammatory pathology in experimental autoimmune encephalomyelitis. *Am J Pathol*. 2006; 169:584–598. [PubMed: 16877358]
10. Lu Z, Hu X, Zhu C, Wang D, Zheng X, Liu Q. Overexpression of CNTF in Mesenchymal Stem Cells reduces demyelination and induces clinical recovery in experimental autoimmune encephalomyelitis mice. *J Neuroimmunol*. 2009; 206:58–69. [PubMed: 19081144]
11. Linker RA, Maurer M, Gaupp S, Martini R, Holtmann B, Giess R, Rieckmann P, Lassmann H, Toyka KV, Sendtner M, Gold R. CNTF is a major protective factor in demyelinating CNS disease: a neurotrophic cytokine as modulator in neuroinflammation. *Nat Med*. 2002; 8:620–624. [PubMed: 12042814]
12. Linker RA, Brechlin P, Jesse S, Steinacker P, Lee DH, Asif AR, Jahn O, Tumani H, Gold R, Otto M. Proteome profiling in murine models of multiple sclerosis: identification of stage specific markers and culprits for tissue damage. *PLoS One*. 2009; 4:e7624. [PubMed: 19865482]
13. Giess R, Maurer M, Linker R, Gold R, Warmuth-Metz M, Toyka KV, Sendtner M, Rieckmann P. Association of a null mutation in the CNTF gene with early onset of multiple sclerosis. *Arch Neurol*. 2002; 59:407–409. [PubMed: 11890844]
14. Emerich DF, Thanos CG. Intracompartmental delivery of CNTF as therapy for Huntington's disease and retinitis pigmentosa. *Curr Gene Ther*. 2006; 6:147–159. [PubMed: 16475952]
15. Aebischer P, Schluep M, Deglon N, Joseph JM, Hirt L, Heyd B, Goddard M, Hammang JP, Zurn AD, Kato AC, Regli F, Baetge EE. Intrathecal delivery of CNTF using encapsulated genetically modified xenogeneic cells in amyotrophic lateral sclerosis patients. *Nat Med*. 1996; 2:696–699. [PubMed: 8640564]
16. Gropman AL, Summar M, Leonard JV. Neurological implications of urea cycle disorders. *J Inherit Metab Dis*. 2007; 30:865–879. [PubMed: 18038189]
17. Scaglia F, Carter S, O'Brien WE, Lee B. Effect of alternative pathway therapy on branched chain amino acid metabolism in urea cycle disorder patients. *Mol Genet Metab*. 2004; 81(Suppl 1):S79–85. [PubMed: 15050979]
18. Bridges JW, French MR, Smith RL, Williams RT. The fate of benzoic acid in various species. *Biochem J*. 1970; 118:47–51. [PubMed: 4990586]
19. Kubota K, Ishizaki T. Dose-dependent pharmacokinetics of benzoic acid following oral administration of sodium benzoate to humans. *Eur J Clin Pharmacol*. 1991; 41:363–368. [PubMed: 1804654]
20. Toth B. Lack of tumorigenicity of sodium benzoate in mice. *Fundam Appl Toxicol*. 1984; 4:494–496. [PubMed: 6745539]
21. Brahmachari S, Pahan K. Sodium benzoate, a food additive and a metabolite of cinnamon, modifies T cells at multiple steps and inhibits adoptive transfer of experimental allergic encephalomyelitis. *J Immunol*. 2007; 179:275–283. [PubMed: 17579047]
22. Mondal S, Pahan K. Cinnamon ameliorates experimental allergic encephalomyelitis in mice via regulatory T cells: implications for multiple sclerosis therapy. *PLoS One*. 2015; 10:e0116566. [PubMed: 25569428]

23. Jana A, Modi KK, Roy A, Anderson JA, van Breemen RB, Pahan K. Up-regulation of neurotrophic factors by cinnamon and its metabolite sodium benzoate: therapeutic implications for neurodegenerative disorders. *J Neuroimmune Pharmacol.* 2013; 8:739–755. [PubMed: 23475543]
24. Modi KK, Roy A, Brahmachari S, Rangasamy SB, Pahan K. Cinnamon and Its Metabolite Sodium Benzoate Attenuate the Activation of p21^{rac} and Protect Memory and Learning in an Animal Model of Alzheimer's Disease. *PLoS One.* 2015; 10:e0130398. [PubMed: 26102198]
25. Jana M, Pahan K. Down-regulation of Myelin Gene Expression in Human Oligodendrocytes by Nitric Oxide: Implications for Demyelination in Multiple Sclerosis. *J Clin Cell Immunol.* 2013; 4
26. Ghosh A, Pahan K. Gemfibrozil, a lipid-lowering drug, induces suppressor of cytokine signaling 3 in glial cells: implications for neurodegenerative disorders. *J Biol Chem.* 2012; 287:27189–27203. [PubMed: 22685291]
27. Khasnavis S, Jana A, Roy A, Mazumder M, Bhushan B, Wood T, Ghosh S, Watson R, Pahan K. Suppression of nuclear factor-kappaB activation and inflammation in microglia by physically modified saline. *J Biol Chem.* 2012; 287:29529–29542. [PubMed: 22753407]
28. Brahmachari S, Fung YK, Pahan K. Induction of glial fibrillary acidic protein expression in astrocytes by nitric oxide. *J Neurosci.* 2006; 26:4930–4939. [PubMed: 16672668]
29. Jana M, Jana A, Pal U, Pahan K. A simplified method for isolating highly purified neurons, oligodendrocytes, astrocytes, and microglia from the same human fetal brain tissue. *Neurochem Res.* 2007; 32:2015–2022. [PubMed: 17447141]
30. Jana M, Mondal S, Gonzalez FJ, Pahan K. Gemfibrozil, a lipid-lowering drug, increases myelin genes in human oligodendrocytes via peroxisome proliferator-activated receptor-beta. *J Biol Chem.* 2012; 287:34134–34148. [PubMed: 22879602]
31. Brahmachari S, Jana A, Pahan K. Sodium benzoate, a metabolite of cinnamon and a food additive, reduces microglial and astroglial inflammatory responses. *J Immunol.* 2009; 183:5917–5927. [PubMed: 19812204]
32. Jana A, Pahan K. Fibrillar amyloid-beta-activated human astroglia kill primary human neurons via neutral sphingomyelinase: implications for Alzheimer's disease. *J Neurosci.* 2010; 30:12676–12689. [PubMed: 20861373]
33. Khasnavis S, Pahan K. Sodium benzoate, a metabolite of cinnamon and a food additive, upregulates neuroprotective Parkinson disease protein DJ-1 in astrocytes and neurons. *J Neuroimmune Pharmacol.* 2012; 7:424–435. [PubMed: 21701815]
34. Roy A, Pahan K. Myelin Basic Protein-primed T Helper 2 Cells Suppress Microglial Activation via AlphaV β 3 Integrin: Implications for Multiple Sclerosis. *J Clin Cell Immunol.* 2013; 7:158. [PubMed: 24575330]
35. Corbett GT, Roy A, Pahan K. Gemfibrozil, a lipid-lowering drug, upregulates IL-1 receptor antagonist in mouse cortical neurons: implications for neuronal self-defense. *J Immunol.* 2012; 189:1002–1013. [PubMed: 22706077]
36. Ghosh A, Corbett GT, Gonzalez FJ, Pahan K. Gemfibrozil and fenofibrate, FDA-approved lipid-lowering drugs, upregulate tripeptidyl-peptidase 1 in brain cells via peroxisome proliferator-activated receptor- α : implications for late infantile neuronal ceroid lipofuscinosis therapy. *J Biol Chem.* 2012
37. Modi KK, Sendtner M, Pahan K. Up-regulation of ciliary neurotrophic factor in astrocytes by aspirin: implications for remyelination in multiple sclerosis. *J Biol Chem.* 2013; 288:18533–18545. [PubMed: 23653362]
38. Ito Y, Wiese S, Funk N, Chittka A, Rossoll W, Bommel H, Watabe K, Wegner M, Sendtner M. Sox10 regulates ciliary neurotrophic factor gene expression in Schwann cells. *Proc Natl Acad Sci U S A.* 2006; 103:7871–7876. [PubMed: 16684879]
39. Taylor SS, Kim C, Cheng CY, Brown SH, Wu J, Kannan N. Signaling through cAMP and cAMP-dependent protein kinase: diverse strategies for drug design. *Biochim Biophys Acta.* 2008; 1784:16–26. [PubMed: 17996741]
40. Kandel ER. The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. *Mol Brain.* 5:14. [PubMed: 22583753]
41. Waltereit R, Weller M. Signaling from cAMP/PKA to MAPK and synaptic plasticity. *Mol Neurobiol.* 2003; 27:99–106. [PubMed: 12668903]

42. Iwasaki Y, Ikeda K, Shiojima T, Kinoshita M. Increased plasma concentrations of aspartate, glutamate and glycine in Parkinson's disease. *Neurosci Lett.* 1992; 145:175–177. [PubMed: 1361223]
43. Mitch WE, Brusilow S. Benzoate-induced changes in glycine and urea metabolism in patients with chronic renal failure. *J Pharmacol Exp Ther.* 1982; 222:572–575. [PubMed: 7108765]
44. Barkhatova VP, Zavalishin IA, Askarova L, Shavratskii V, Demina EG. Changes in neurotransmitters in multiple sclerosis. *Neurosci Behav Physiol.* 1998; 28:341–344. [PubMed: 9762701]

Figure 1. Sodium benzoate stimulates the expression of CNTF in primary mouse astrocytes Astrocytes isolated from 2-3 d old mouse pups were treated with different concentrations of NaB for 6 h followed by mRNA analysis of CNTF by semi-quantitative RT-PCR (A) and real-time PCR (B). Results are mean±SD of three independent experiments. ^b $p < 0.05$ vs control; ^a $p < 0.001$ vs control. Cells were treated with 200 μM NaB for different time periods followed by mRNA analysis by semi-quantitative RT-PCR (C) and real-time PCR (D). ^a $p < 0.001$ vs control. After 24 h of NaB treatment, the protein level of CNTF was monitored by Western blot (E). Sodium formate (NaFO) was used as a negative control for NaB. Bands were quantified and presented as relative expression (F). ^a $p < 0.001$ vs control. Double labeling for GFAP & CNTF was also performed (G). DAPI was used to visualize nucleus. Results represent three independent experiments.

Figure 2. NaB increases the level of CNTF in primary mouse oligodendrocytes

Oligodendrocytes isolated from 2-3 d old mouse pups were treated with different concentrations of NaB and NaFO for 6 h followed by mRNA analysis of *Cntf* by semi-quantitative RT-PCR (A) and real-time PCR (B). Results are mean±SD of three independent experiments. $^a p < 0.001$ vs control. After 24 h of NaB treatment, the protein level of CNTF was monitored in cells by Western blot (C). Bands were quantified and presented as relative expression (D). $^a p < 0.001$ vs control. Double labeling for GalC & CNTF was also performed (E). DAPI was used to visualize nucleus. Results represent three independent experiments.

Figure 3. NaB stimulates the expression of CNTF in primary human astrocytes

Astrocytes isolated from human fetal brain tissues were treated with different concentrations of NaB for 6 h followed by mRNA analysis of *Cntf* by semi-quantitative RT-PCR (A) and real-time PCR (B). NaFO was used as a negative control for NaB. Results are mean $\pm$ SD of three independent experiments. $^a p < 0.001$ vs control. Cells were treated with NaB for different time periods followed by mRNA analysis by semi-quantitative RT-PCR (C) and real-time PCR (D). $^a p < 0.001$ vs control. After 24 h of treatment with NaB and NaFO, double labeling for GFAP & CNTF was also performed (E). DAPI was used to visualize nucleus. The protein level of CNTF was monitored in supernatants by ELISA (F). Results are mean $\pm$ SD of three independent experiments. $^a p < 0.001$ vs control.

Figure 4. Oral treatment of NaB and cinnamon increases the level of CNTF *in vivo* in mouse brain

Male C57/BL6 mice (6-8 week old) were treated with different doses of NaB daily via gavage. NaFO was used as a negative control. After 30 d of treatment, the mRNA expression of CNTF was monitored in the cortex by semi-quantitative RT-PCR (A) and real-time PCR (B). Results are mean $\pm$ SEM of four mice per group. $^ap < 0.001$ vs control. After 30 d of cinnamon treatment, the mRNA expression of CNTF was monitored in the cortex by semi-quantitative RT-PCR (C) and real-time PCR (D). Results are mean $\pm$ SEM of four mice per group. $^ap < 0.001$ vs control. After cinnamon treatment, the protein level of CNTF was monitored in the cortex by Western blot (E). Bands were quantified and presented as relative expression (F). $^ap < 0.001$ vs control.

Figure 5. Oral treatment of NaB and cinnamon increases astroglial and oligodendroglial CNTF *in vivo* in mouse brain

Male C57/BL6 mice (6-8 week old) were treated with NaB (100 mg/kg body wt/d) and cinnamon (100 mg/kg body wt/d) via gavage. After 30 d of treatment, cortical sections were double-labeled for CNTF and either GFAP (A) or GalC (B). CNTF-positive cells were counted in four sections (two images per section) of each of four mice per group in an Olympus IX81 fluorescence microscope using the MicroSuite imaging software (C). Results are mean±SEM of four mice per group. ^a $p < 0.001$ vs control.

Figure 6. Oral administration of cinnamon increases CNTF *in vivo* in the brain of EAE mice Male C57/BL6 mice (6-8 week old) were induced EAE by MOG35-55 immunization. From 8 days post-immunization, mice were treated with cinnamon (100 mg/kg body wt/d) via gavage. After 8 days of treatment, cerebellar sections were double-labeled for CNTF and GFAP (A). CNTF-positive cells were counted in four sections (two images per section) of each of four mice per group (B). Results are mean±SEM of four mice per group. ^a $p < 0.001$ vs control.

Figure 7. NaB increases the expression of CNTF in primary mouse astrocytes via CREB
A) CNTF gene promoter harbors one consensus CRE. Cells were treated with 200 μ M NaB for different minute intervals followed by monitoring the levels of phospho-CREB and total CREB by Western blot (B). Bands were quantified and presented as relative to control (C). Results are mean $\pm$ SD of three independent experiments. ^a $p < 0.001$ vs control. Cells pretreated with 2 μ M H-89 for 30 min were stimulated with 200 μ M NaB for 4 h under serum-free condition followed by monitoring the recruitment of CREB and CBP to the CNTF promoter by ChIP analysis (D). Normal IgG was used as control. The results were confirmed by real-time (E). Results are mean + SD of three different experiments. ^a $p < 0.001$ vs control; ^b $p < 0.001$ vs NaB. Cells were transfected with CREB-siRNA and control siRNA. After 48 h of transfection, cells were treated with NaB and after 24 h of treatment the protein level of CREB was monitored by Western blot (F). Bands were quantified and presented as relative to control (G). Results are mean $\pm$ SD of three independent experiments. ^a $p < 0.001$ vs control; ^b $p < 0.001$ vs cont siRNA-NaB. Under similar treatment condition, the protein level of CNTF was monitored by Western blot (H). Bands were quantified and presented as relative to control (I). Results are mean $\pm$ S.D. of three different experiments. ^a $p < 0.001$ vs control; ^b $p < 0.001$ vs cont siRNA-NaB.

Figure 8. Involvement of protein kinase A (PKA) in NaB-induced activation of CREB and expression of CNTF in primary mouse astrocytes

Cells were incubated with 200 μM NaB for different minute intervals followed by monitoring the activation of PKA (A) as described under “Materials and Methods”. Results are mean ± SD of three independent experiments. ^a*p* < 0.05 vs control. Cells pretreated with different concentrations of H-89 for 30 min were stimulated with NaB for 60 min followed by monitoring the activation of CREB by Western blot for phospho-CREB and total CREB (B). Bands were quantified and presented as relative to control (C). Results are mean±SD of three independent experiments. ^a*p* < 0.001 vs control; ^b*p* < 0.05 & ^c*p* < 0.001 vs NaB. Cells pretreated with different concentrations of H-89 for 30 min were stimulated with NaB for 6 h followed by monitoring the protein level of CNTF by Western blot (D). Bands were scanned and values are presented as relative expression (E). Results are mean ± S.D. of three different experiments. ^a*p* < 0.001 vs control; ^b*p* < 0.05 & ^c*p* < 0.001 vs NaB.

Table 1

Antibodies used for this study

Antibody	Manufacturer	Catalog #	Host	Application	Dilution/Amount
CNTF	Santa Cruz	sc-13996	Rabbit	WB, ICC/IF	1:200
CNTF	Santa Cruz	Sc-365210	Mouse	ICC/IF	1:200
β-actin	Abcam	Ab6276	Mouse	WB	1:6000
CREB	Cell Signaling	9197S	Rabbit	WB	1:500
CREB	Millipore	CS203204	Rabbit	ChIP	1:50
pCREB ^{S133}	Cell Signaling	9198L	Rabbit	WB	1:500
GFAP	Dako	Z0334	Rabbit	ICC/IF	1:5000
CBP	Santa Cruz	sc-369	Rabbit	ChIP	2 µg
IgG	Santa Cruz	sc-3888	Rabbit	ChIP	2 µg

WB, Western blot; ICC, immunocytochemistry; IHC, immunohistochemistry; IF, immunofluorescence; ChIP, chromatin immunoprecipitation