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Up-regulation of Ciliary Neurotrophic Factor in Astrocytes by Aspirin

*IMPLICATIONS FOR REMYELINATION IN MULTIPLE SCLEROSIS**

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Background: An increase in ciliary neurotrophic factor (CNTF) in the brain may be beneficial for demyelinating disorders.
Results: Aspirin, a widely used analgesic, increases CNTF in astrocytes via the PKA-CREB pathway.
Conclusion: These results delineate a novel myelinogenic property of aspirin.
Significance: Aspirin may be of therapeutic benefit in demyelinating disorders.

Ciliary neurotrophic factor (CNTF) is a promyelinating trophic factor, and the mechanisms by which CNTF expression could be increased in the brain are poorly understood. Acetylsalicylic acid (aspirin) is one of the most widely used analgesics. Interestingly, aspirin increased mRNA and protein expression of CNTF in primary mouse and human astrocytes in a dose- and time-dependent manner. Aspirin induced the activation of protein kinase A (PKA) but not protein kinase C (PKC). H-89, an inhibitor of PKA, abrogated aspirin-induced expression of CNTF. The activation of cAMP-response element-binding protein (CREB), but not NF-κB, by aspirin, the abrogation of aspirin-induced expression of CNTF by siRNA knockdown of CREB, the presence of a consensus cAMP-response element in the promoter of CNTF, and the recruitment of CREB and CREB-binding protein to the CNTF promoter by aspirin suggest that aspirin increases the expression of the *Cntf* gene via the activation of CREB. Furthermore, we demonstrate that aspirin-induced astroglial CNTF was also functionally active and that supernatants of aspirin-treated astrocytes of wild type, but not *Cntf* null, mice increased myelin-associated proteins in oligodendrocytes and protected oligodendrocytes from TNF-α insult. These results highlight a new and novel myelinogenic property of aspirin, which may be of benefit for multiple sclerosis and other demyelinating disorders.

Multiple sclerosis (MS)[2] is the most common human demyelinating disorder of the CNS in which promoting remyelination remains a crucial therapeutic challenge (1–4). Although neurotrophins (nerve growth factor, neurotrophin-3 (NT-3), NT-4/5, and brain-derived neurotrophic factor) and glial cell line-derived neurotrophic factor-related factors (glial cell line-derived neurotrophic factor and neurturin) do not increase myelinogenesis, ciliary neurotrophic factor (CNTF) induces a strong promyelinating effect (5). CNTF is an important survival factor for oligodendrocytes. It protects oligodendrocytes from various death signals (6), mediates the maturation of oligodendroglial progenitor cells into mature myelin-forming cells, and helps differentiated oligodendrocytes to synthesize myelin (7). According to Kuhlmann *et al.* (8), continued administration of CNTF protects mice from inflammatory pathology in experimental allergic encephalomyelitis (EAE). Similarly, mesenchymal stem cell overexpressing CNTF reduces demyelination and induces clinical recovery in EAE mice (9). Consistently, in $Cntf^{-/-}$ mice, earlier onset of symptoms and increased disability were observed after induction of EAE by MOG35-55 peptide (10). Recently, proteome analysis has also identified enhanced oligodendrocyte apoptosis and increased axonal injury in EAE in $Cntf^{-/-}$ mice (11). 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 (12). However, clinical application of CNTF has been limited because of difficulties in delivery because CNTF does not readily diffuse across the blood-brain barrier or ventricular lining and has limited or unstable bioavailability (13). Gene delivery (14) 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.

Aspirin, also known as acetylsalicylic acid, is one of the most widely used medications in the world. Being a part of the group of medications called nonsteroidal anti-inflammatory drugs, it is often used as an analgesic to relieve minor aches and pains, as an antipyretic to reduce fever, and as an anti-inflammatory medication. Because aspirin readily enters into CNS (15, 16), we tested its efficacy in stimulating CNTF from astrocytes, the major glial cells in the CNS. Here, we provide the first evidence

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[2] The abbreviations used are: MS, multiple sclerosis; CNTF, ciliary neurotrophic factor; CREB, cAMP-response element-binding protein; EAE, experimental allergic encephalomyelitis; CBP, CREB-binding protein; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; GFAP, glial fibrillary acidic protein; PLP, proteolipid protein; MOG, myelin oligodendrocyte glycoprotein.

TABLE 1

Antibodies, sources, applications, and dilutions used

The following abbreviations were used: WB, Western blot; ICC, immunocytochemistry; IHC, immunohistochemistry; IF, immunofluorescence; ChIP, chromatin immunoprecipitation.

Antibody	Manufacturer	Catalog no.	Host	Application	Dilution/amount
CNTF	Santa Cruz Biotechnology	sc-13996	Rabbit	WB, ICC/IF	1:200
CNTF	Santa Cruz Biotechnology	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
GFAP	Santa Cruz Biotechnology	sc-6171	Goat	ICC/IF	1:200
p300	Millipore	05-257	Mouse	ChIP	2 μg
CBP	Santa Cruz Biotechnology	sc-369	Rabbit	ChIP	2 μg
IgG	Santa Cruz Biotechnology	sc-3888	Rabbit	ChIP	2 μg
PLP	Santa Cruz Biotechnology	sc-23570	Goat	WB, ICC/IF	1:100
MOG	Santa Cruz Biotechnology	sc-66968	Rabbit	WB, ICC/IF	1:100
GalC	Sigma	G9152	Rabbit	ICC/IF	1:500

about the myelinogenic effect of aspirin. Aspirin increases the expression of CNTF in astrocytes via the PKA-CREB pathway. Furthermore, we demonstrate that aspirin-induced astroglial CNTF is capable of protecting oligodendroglia from TNF-α-mediated apoptosis and cell death. Our findings raise the possibility that aspirin may find further application in MS and demyelinating disorders via increased production of CNTF.

MATERIALS AND METHODS

Reagents—Cell culture materials (DMEM/F-12, L-glutamine, Hanks' balanced salt solution, 0.05% trypsin, and antibiotic/antimycotic) were purchased from Mediatech (Washington, D. C.). Fetal bovine serum (FBS) was obtained from Atlas Biologicals. Aspirin and all molecular biology-grade chemicals 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 Astroglia—Astroglia were isolated from 7- to 9-day-old old mouse pups as described earlier (17–19). 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 microglia followed by another round of shaking on day 11 at 190 rpm for 18 h to remove oligodendroglia and residual microglia. The attached cells were washed and seeded onto new plates for further studies. About 98% 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 (20–22). 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) were dissociated by trituration and trypsinization. On the 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 the 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% CO_2 in air to yield more viable and healthy cells. More than 98% of the 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 (17, 18, 23) using a RT-PCR kit from Clontech. Briefly, 1 μg of total RNA was reverse-transcribed using $oligo(dT)_{12-18}$ as primer and Moloney murine leukemia virus 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. The primers used are as follows: CNTF (mouse), sense 5′-GGGACCTCTGTAGCCGCTCTATCTG-3′ and antisense 5′-GTTCCAGAAGCGCCATTAACTCCTC-3′; LIF (mouse), sense 5′-GAACCCCACTGCCGTGAGCC-3′ and antisense 5′-CCACGTGGCCCACACGGTAC-3′; IL-11 (mouse), sense 5′-GCCGTGCAGGTGGTCCTTCC-3′ and antisense 5′-GCACGGCCCAGTCCAAGGTC-3′; CNTF (human), sense 5′-TCACAGAGCATTCACCGCTGACCCC-3′ and antisense 5′-CTGCTGGTCTTCTAAGAGCCTGGCC-3′; LIF (mouse), sense 5′-ACCTCTGGGGTGGCTTCCAGCG-3′ and antisense 5′-GGCGCGGGGCGGGTGTATTT-3′; IL-11 (mouse), sense 5′-CCGCTCTCTCCTGGCGGACA-3′ and antisense 5′- CACTCATGGCCAGGGTGGGC-3′; GAPDH (mouse), sense 5′-GGTGAAGGTCGGTGTGAACG-3′ and antisense 5′-TTGGCTCCACCCTTCAAGTG-3′; and GAPDH (human), sense 5′-GGTGAAGGTCGGAGTCAACG-3′ and antisense 5′- GTGAAGACGCCAGTGGACTC-3′.

Real Time PCR Analysis—It was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) as described earlier (17, 18, 23). 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 analysis of variance.

MTT Assay—Mitochondrial activity was measured with the MTT assay from Sigma as described (21, 23).

Lactate Dehydrogenase Measurement—The activity of lactate dehydrogenase (LDH) was measured in supernatants using the Sigma LDH kit as described (21, 23).

Fragment End Labeling of DNA—Fragmented DNA was detected *in situ* by the terminal deoxynucleotidyltransferase-mediated binding of 3′-OH ends of DNA fragments generated in response to TNF-α, using the terminal deoxynucleotidyltransferase FragEL^{TM} kit from Calbiochem as described before (21, 23, 24).

ELISA—An amount of CNTF was quantified in supernatants of human astrocytes using a high sensitivity sandwich ELISA (R&D systems) following the 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 and PKC—Assays for PKA and PKC were purchased from Enzo Life Sciences and performed according to the manufacturer's protocol as described previously (23). Values were normalized to purified kinase controls.

Immunoblotting—Western blotting was conducted as described earlier (17, 23). Briefly, cells were scraped in lysis buffer, transferred to microcentrifuge tubes, and spun into pellets. The supernatant was collected and analyzed for protein concentration via the Bradford method (Bio-Rad). SDS sample buffer was added to 40–60 μg of total protein and boiled for 5 min. Denatured samples were electrophoresed on NuPAGE® Novex® 4–12% BisTris gels (Invitrogen), and proteins were 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 h 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 1 more hour, and visualized under the Odyssey® Infrared Imaging System (Li-COR, Lincoln, NE).

Densitometric Analysis—Protein blots were analyzed using ImageJ (National Institutes of Health, Bethesda), 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.

EMSA—DNA binding activities of CREB and NF-κB were analyzed by nonradioactive electrophoretic mobility shift assay (EMSA) as described (17, 23). After treatment, cells were washed with Hanks' balanced saline solution, scraped into 1.5-ml tubes, and centrifuged at 4 °C for 5 min at 500 rpm. The supernatant was aspirated, and the pellet was resuspended in a membrane lysis buffer composed of HEPES, pH 8.0, $MgCl_2$, KCl, dithiothreitol (DTT), and protease/phosphatase inhibitors (Sigma), vortexed, and centrifuged at 4 °C at 720 × *g* for 5 min. Again, the supernatant was aspirated, and the pellet was resuspended in a high salt nuclear envelope lysis buffer composed of HEPES, pH 8.0, $MgCl_2$, glycerol, NaCl, EDTA, DTT, and protease/phosphatase inhibitors, rotated vigorously at 4 °C for 15 min, and centrifuged at 4 °C at 13,000 rpm for 15 min. The resultant supernatant was complexed with a mixture of binding buffer (Tris-HCl, KCl, EDTA, DTT, 10× Tris/glycine/EDTA, glycerol, and Triton X-100), custom-designed fluorescent CREB-specific or NF-κB-specific probes (Li-Cor Biosciences), and salmon sperm DNA (Invitrogen) for 15 min at room temperature and electrophoresed on custom-cast 6% polyacrylamide-TGE gels in 1× TGE for 2 h. The shift was visualized under the Odyssey® Infrared Imaging System (Li-Cor).

Assay of Transcriptional Activities—Cells plated at 70–80% confluence in 12-well plates were co-transfected with 0.25 μg of either pCRE-Luc (a CRE-dependent reporter construct) or pNF-κB-Luc (an NF-κB-dependent reporter construct) and 12.5 ng of pRL-TK using Lipofectamine Plus (17, 23). After 24 h of transfection, cells were treated with aspirin for 4 h. Firefly and *Renilla* luciferase activities were obtained as described above.

Immunofluorescence Analysis—Analysis was performed as described earlier (20, 22). Briefly, coverslips containing 100–200 cells/mm^2 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). 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.

Chromatin Immunoprecipitation—Recruitment of CREB to the *Cntf* promoter was determined using the EZ ChIP kit from Millipore as described before (17, 23, 25). Briefly, 1×10^6 astrocytes were treated with aspirin, and after 3 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 μg of antibodies against CREB, CBP, and p300 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 and precipitated, and precipitates were washed with 75% ethanol, air-dried, and resuspended in Tris/EDTA buffer. The following primers were used to amplify the 197-bp fragment flanking the only CRE in the mouse *Cntf* promoter: sense 5′-GTCACCACAAGCAAGTTGGAGAGA-3′ and antisense 5′-GGCTGGTAGTCCTGGGTTCTCT-3′. PCR products were electrophoresed on 2% agarose gels.

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

RESULTS

Aspirin Up-regulates CNTF in Primary Mouse and Human Astrocytes—Although there is growing knowledge about the role of CNTF in the health and survival of oligodendrocytes as well as in myelination, little is known about the drugs and associated molecular mechanisms that up-regulate CNTF. CNTF is mainly expressed by astroglia (26). We examined if aspirin could stimulate the expression of CNTF in mouse primary astroglia. We found that aspirin indeed increased the mRNA expression of CNTF within 6 h of treatment in a dose-depen-

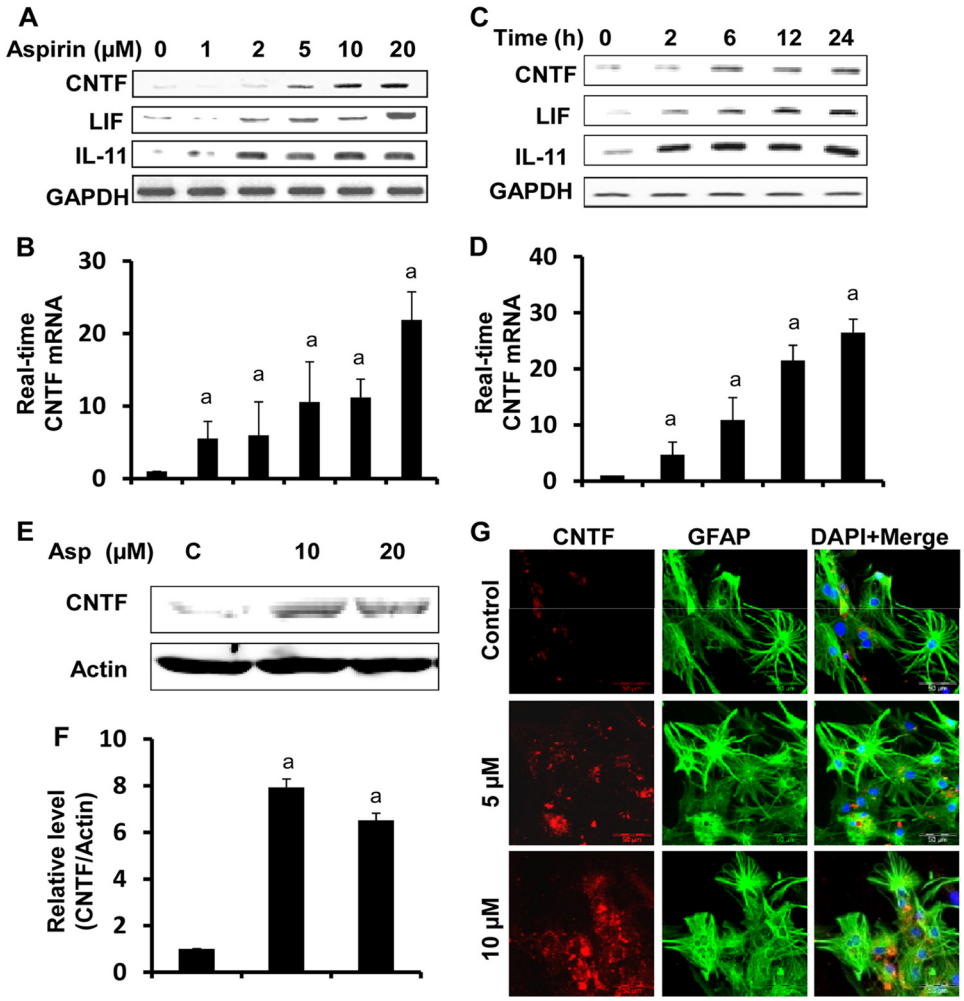

FIGURE 1. **Aspirin stimulates the expression of CNTF family of cytokines (CNTF, IL-11, and LIF) in primary mouse astrocytes.** Astrocytes were treated with different concentrations of aspirin for 6 h followed by mRNA analysis of CNTF, IL-11, and LIF by semi-quantitative RT-PCR (*A*) and real time PCR (*B*). Results are mean ± S.D. of three independent experiments. [a], $p < 0.001$ *versus control.* Cells were treated with aspirin (*Asp*) for different time periods followed by mRNA analysis by semi-quantitative RT-PCR (*C*) and real time PCR (*D*). [a], $p < 0.001$ *versus* control. After 24 h of aspirin treatment, the protein level of CNTF was monitored by Western blot (*E*). Bands were quantified and presented as relative expression (*F*). [a], $p < 0.001$ *versus* control. Double labeling for GFAP and CNTF was also performed (*G*). DAPI was used to visualize nucleus. Results represent three independent experiments.

dent manner (Fig. 1, *A* and *B*). Time course study showed that induction of the CNTF gene started from as early as 2 h and increased with time (Fig. 1, *C* and *D*). We investigated if aspirin was capable of up-regulating other members (LIF and IL-11) of the CNTF family of cytokines in astrocytes. Similar to CNTF, the mRNA expression of both LIF and IL-11 was also stimulated by aspirin treatment (Fig. 1, *A* and *C*). We further checked the protein level of CNTF in astrocytes. We observed about a 6–8-fold increase in the protein levels of CNTF in astrocytes (Fig. 1, *E* and *F*) when treated with aspirin, which is consistent with our mRNA findings. The increase in CNTF protein in astrocytes by aspirin was further confirmed by immunofluorescence analysis (Fig. 1*G*).

Next, we examined if aspirin could stimulate the expression of CNTF in primary human astrocytes. Consistent with mouse astrocytes, aspirin strongly increased the expression of CNTF mRNA (Fig. 2, *A* and *B,* for dose-dependent effect; Fig. 2, *C* and *D,* for time-dependent effect) and protein (Fig. 2, *E* and *F,* for Western blot; Fig. 2*G* for immunofluorescence) in primary astroglia isolated from human fetal brains. ELISA kit is available only for human CNTF. Therefore, we examined if aspirin-stimulated CNTF was secreted from human astrocytes. As appeared from Fig. 2*H*, aspirin markedly increased the production of CNTF in primary human astroglia.

Aspirin Increased the Expression of CNTF via PKA—Next, we decided to elucidate mechanisms by which aspirin increased the expression of CNTF in astrocytes. Although it has not been demonstrated for CNTF, in general the activation of the cAMP-PKA pathway increases the expression of neurotrophic factors (27–29). Therefore, we examined the role of PKA in aspirin-mediated up-regulation of CNTF. At first, we monitored if aspirin alone was capable of activating PKA. As evident from Fig. 3*A*, aspirin alone induced the activation of PKA in astrocytes in a time-dependent manner, and this activation was evident from as early as 5 min of stimulation. However, aspirin had no effect on the activation of PKC (Fig. 3*B*), indicating the specificity of its effect. Because aspirin was very potent in inducing the expression of CNTF at later hours of stimulation, we also monitored the activation of PKA in astrocytes at different hours after aspirin stimulation. Similar to minute intervals, aspirin

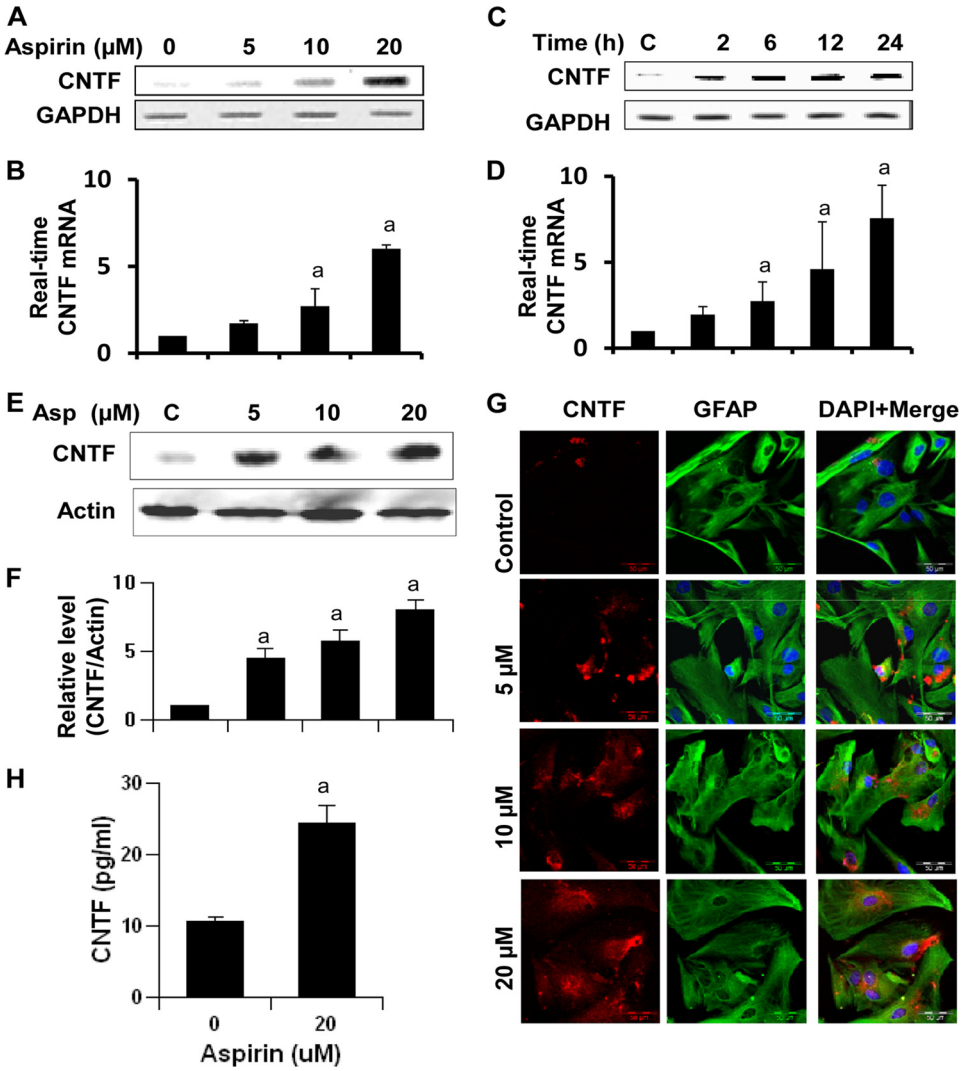

FIGURE 2. **Aspirin stimulates the expression of CNTF in primary human astrocytes.** Astrocytes isolated from human fetal brain tissues were treated with different concentrations of aspirin (*Asp*) for 6 h followed by mRNA analysis of *Cntf* by semi-quantitative RT-PCR (*A*) and real time PCR (*B*). Cells were treated with aspirin for different time periods followed by mRNA analysis by semi-quantitative RT-PCR (*C*) and real time PCR (*D*). [a], $p < 0.001$ *versus* control. After 24 h of aspirin treatment, the protein level of CNTF was monitored in cells by Western blot (*E*). Bands were quantified and presented as relative expression (*F*). [a], $p < 0.001$ *versus* control. Double labeling for GFAP and CNTF was also performed (*G*). DAPI was used to visualize nucleus. After 24 h of aspirin treatment, the protein level of CNTF was monitored in supernatants by ELISA (*H*). Results are mean ± S.D. of three independent experiments. [a], $p < 0.001$ *versus* control.

markedly increased the activity of PKA in astrocytes at 6, 12, and 24 h of stimulation (Fig. 3*C*). Suppression of aspirin induced expression of *Cntf* mRNA (Fig. 3*D*) and protein (Fig. 3, *E* and *F*) in astrocytes by H-89, a specific inhibitor of PKA, and suggested that aspirin up-regulates CNTF in astrocytes via PKA. This was further confirmed by siRNA knockdown of PKA. PKA siRNA, but not control siRNA, suppressed the level of PKA (Fig. 3, *G* and *H*) and abrogated aspirin-mediated increase in *Cntf* mRNA (Fig. 3*I*) and protein (Fig. 3, *J* and *K*).

Aspirin Requires CREB for the Up-regulation of CNTF—Next, we investigated mechanisms by which the PKA signaling pathway coupled CNTF up-regulation in aspirin-treated astrocytes. Upon analysis of the CNTF promoter using MatInspector, we observed binding sites for many transcription factors, including one consensus cAMP-response element (CRE) near the transcriptional start site. Therefore, we were prompted to investigate if aspirin required CREB for the transcription of CNTF in astrocytes. First, we examined if aspirin alone induced the activation of CREB in astrocytes by monitoring levels of phosphorylated CREB (p-CREB), DNA binding activity by EMSA, and transcriptional activity using a luciferase reporter construct. Aspirin alone induced the phosphorylation of CREB as depicted by Western blot (Fig. 4, *A* and *B*). This CREB activation was very rapid and evident as early as 5 min after aspirin stimulation (Fig. 4, *A* and *B*). Moreover, we did not see any change in the level of total CREB within 60 min of stimulation (Fig. 4*A, lower panel*). Because aspirin-induced expression of CNTF was very prominent after 6, 12, or 24 h of stimulation, we examined if CREB remained active in these time periods in aspirin-treated cells. As evident from Fig. 4, *C* and *D*, CREB remained activated in astrocytes even after 6, 12, and 24 h of stimulation. Next, we examined the DNA binding activity of CREB. As seen in Fig. 4*C*, aspirin treatment induced the DNA binding activity of CREB. Consistent with the activation of PKA and the phosphorylation of CREB, the DNA binding activity of CREB was also evident in mouse astrocytes as early as 5 min

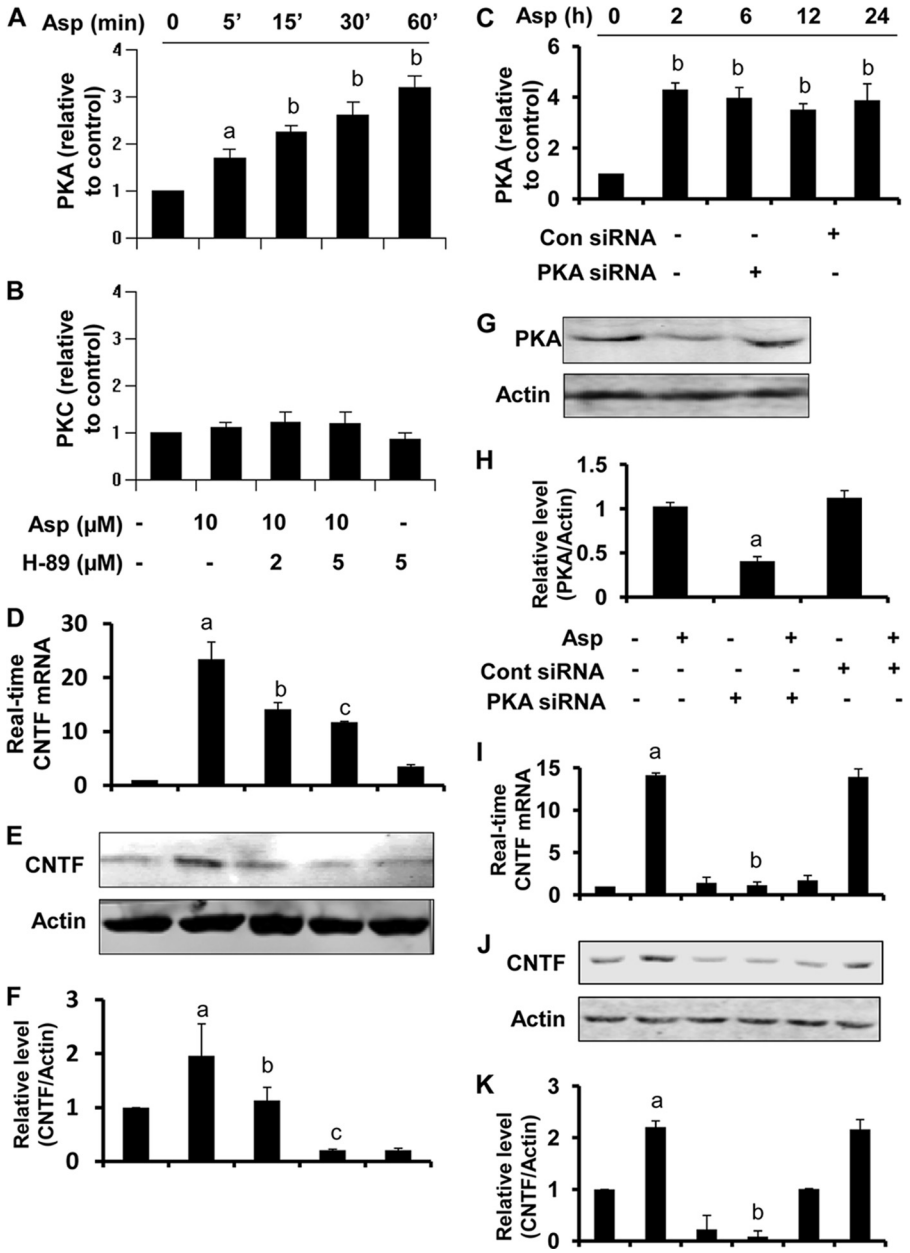

FIGURE 3. **Involvement of PKA in aspirin-induced expression of CNTF in primary mouse astrocytes.** Cells were incubated with 10 μM aspirin (*Asp*) for different minute intervals followed by monitoring the activation of PKA (*A*) and PKC (*B*) as described under "Materials and Methods." Cells were incubated with aspirin for different hour intervals followed by monitoring the activation of PKA (*C*). Results are mean ± S.D. of three independent experiments. [a], $p < 0.05$; [b], $p < 0.001$ *versus* control (*Con*). Cells pretreated with different concentrations of H-89 for 30 min were stimulated with aspirin followed by monitoring the mRNA expression of *Cntf* by real time PCR (*D*) and the protein level of CNTF by Western blot (*E*). Bands were scanned and values presented as relative expression (*F*). Results are mean ± S.D. of three different experiments. [a], $p < 0.001$ *versus* control; [b], $p < 0.05$; [c], $p < 0.001$ *versus* aspirin. Cells were transfected with PKA-siRNA and control siRNA. After 48 h of transfection, the protein level of PKA was monitored by Western blot (*G*). Bands were quantified and presented as relative to control (*H*). Results are mean ± S.D. of three independent experiments. [a], $p < 0.001$ *versus* control. Under similar treatment conditions, the mRNA expression of CNTF was monitored by real time PCR (*I*). The protein level of CNTF was monitored by Western blot (*J*). Bands were quantified and presented as relative to control (*K*). Results are mean ± S.D. of three different experiments. [a], $p < 0.001$ *versus* control; [b], $p < 0.001$ *versus* control siRNA-aspirin.

after aspirin stimulation (Fig. 4*C*). In contrast, aspirin was unable to induce the DNA binding activity of NF-κB (Fig. 4*D*), indicating the specificity of the effect. When we analyzed transcriptional activities, we also observed that aspirin specifically induced the transcriptional activity of CREB (Fig. 4*E*) but not that of NF-κB (Fig. 4*F*). Next, we examined if aspirin required PKA for the activation of CREB. Suppression of aspirin-mediated phosphorylation of CREB (Fig. 5, *A* and *B*) and inhibition of aspirin-induced DNA binding (Fig. 5*C*) and transcriptional (Fig. 5*D*) activities of CREB by H-89 suggest that aspirin induces the activation of CREB via PKA.

Next, we investigated if aspirin required CREB for the up-regulation of CNTF in astrocytes. At first, we examined if antisense knockdown of CREB was capable of suppressing the expression of CREB protein in astrocytes. As evident from Fig. 6, *A* and *B*, CREB siRNA, but not control siRNA, decreased the expression of CREB protein in control and aspirin-treated astrocytes. Accordingly, CREB siRNA, but not control siRNA,

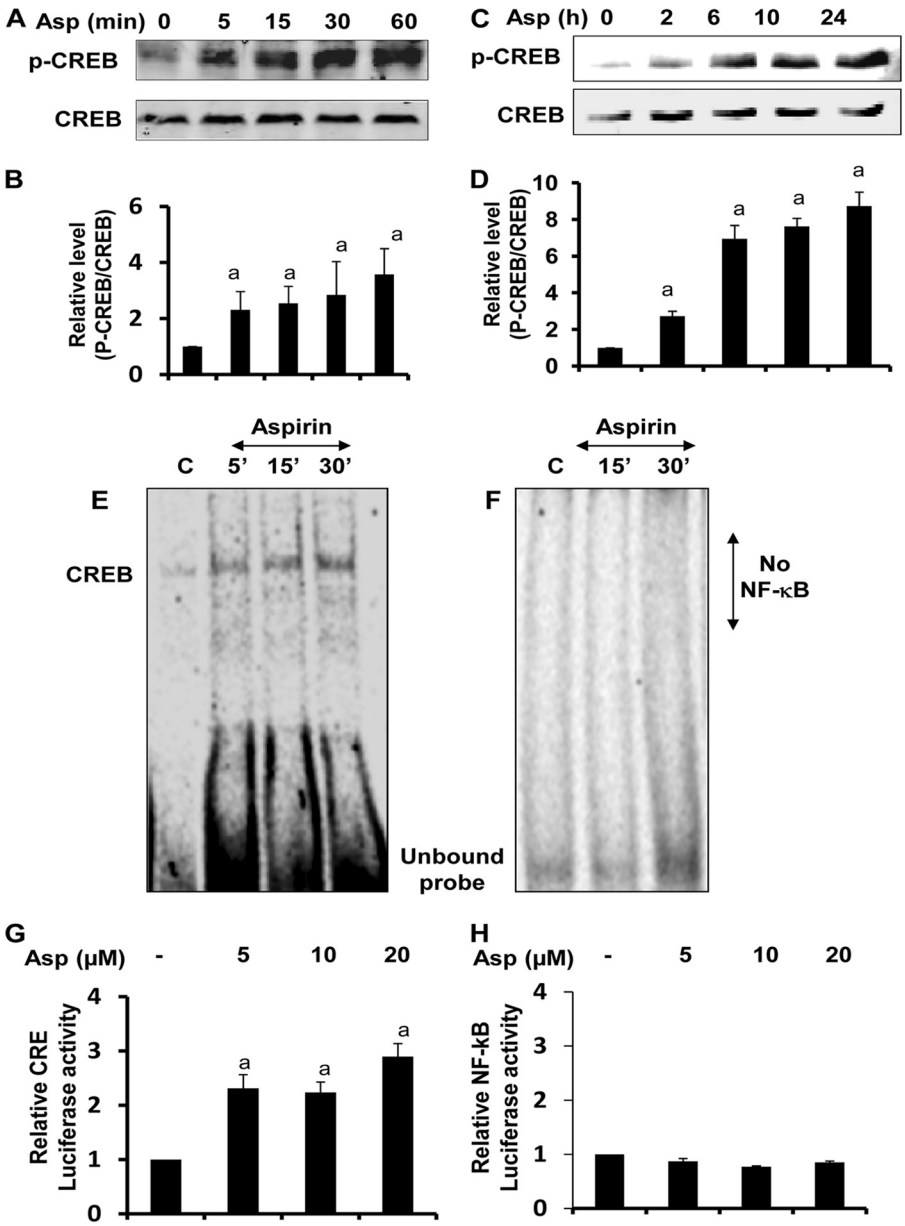

FIGURE 4. **Activation of CREB, but not NF-κB, by aspirin in primary mouse astrocytes.** Cells were treated with 10 μM aspirin (*Asp*) for different minute intervals (*A* and *B*) and hour intervals (*C* and *D*) followed by monitoring the levels of phospho-CREB and total CREB by Western blot (*A* and *C*). Bands were quantified and presented as relative to control (*B* and *D*). Results are mean ± S.D. of three independent experiments. [a], $p < 0.001$ *versus* control. Cells were incubated with 10 μM aspirin for different time periods followed by monitoring the activation of CREB (*E*) and NF-κB (*F*) by EMSA. Cells plated at 70–80% confluence in 12-well plates were transfected with 0.25 μg of either pCRE-Luc (*G*) or PBIIX-Luc (*H*). All transfections also included 12.5 ng of pRL-TK. Twenty four hours after transfection, cells were treated with different concentrations of aspirin. After 5 h of treatment, activities of firefly and *Renilla* luciferase were monitored. Results are mean ± S.D. of three different experiments. [a], $p < 0.001$ *versus* control.

also decreased the expression of CREB mRNA in control and aspirin-treated astrocytes (Fig. 6*C*) and abrogated aspirin-mediated up-regulation of CNTF mRNA (Fig. 6, *C* and *D*). Western blot analysis also shows the suppression of CNTF protein expression by siRNA knockdown of CREB in aspirin-treated astrocytes (Fig. 5, *E* and *F*). These results suggest that aspirin induces the activation of CREB via PKA and that CREB is required for increased transcription of CNTF.

Aspirin Induces the Recruitment of CREB to the cntf Promoter in Mouse Astrocytes—To further confirm the role of CREB in aspirin-induced transcription of CNTF, we monitored the recruitment of CREB to the CNTF promoter. Mouse *cntf* promoter harbors one CRE between 35 and 55 bp upstream of the transcriptional start site (Fig. 7*A*). At first, we used ChIP analysis to study if aspirin induced the recruitment of CREB to this CRE. After immunoprecipitation of aspirin-treated astroglial chromatin fragments by antibodies against CREB, we were able to amplify the 192-bp fragment flanking the CRE (Fig. 7*C*), and this amplification was missing in control astrocytes (Fig. 7*B*), suggesting that aspirin induces the recruitment of CREB to the *Cntf* promoter. Because histone acetyltransferases play an important role in transcriptional activation, we investigated which one of the two most important histone acetyltransferases (p300 and CBP), or both, are involved in aspirin-induced tran-

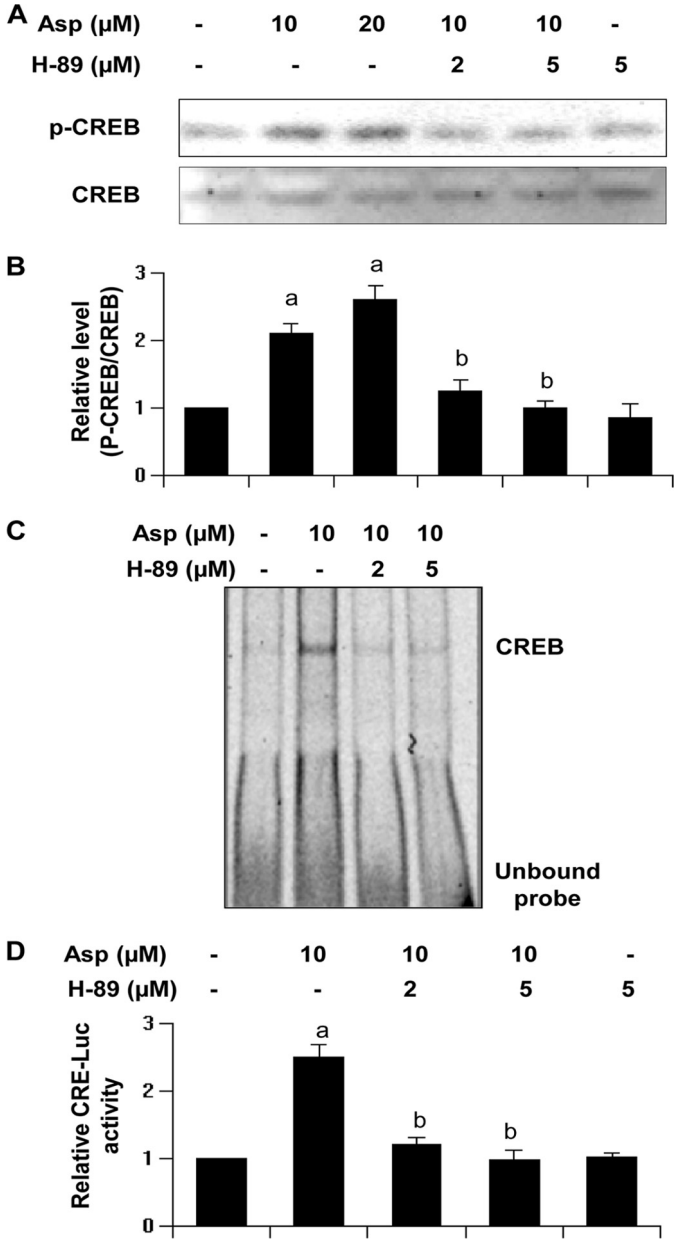

FIGURE 5. **Aspirin induces the activation of CREB in mouse astroglia via PKA.** Cells pretreated with different concentrations of H-89 for 30 min were stimulated with aspirin (*Asp*) followed by monitoring the levels of phospho-CREB and total CREB by Western blot (*A*). Bands were quantified and presented as relative to control (*B*). Results are mean ± S.D. of three independent experiments. [a], $p < 0.001$ *versus* control; [b], $p < 0.001$ *versus* aspirin 10 μM. Under similar treatment conditions, activation of CREB was monitored by EMSA (*C*) and CRE-driven luciferase activity (*D*). Results are mean ± S.D. of three different experiments. [a], $p < 0.001$ *versus* control; [b], $p < 0.001$ *versus* aspirin.

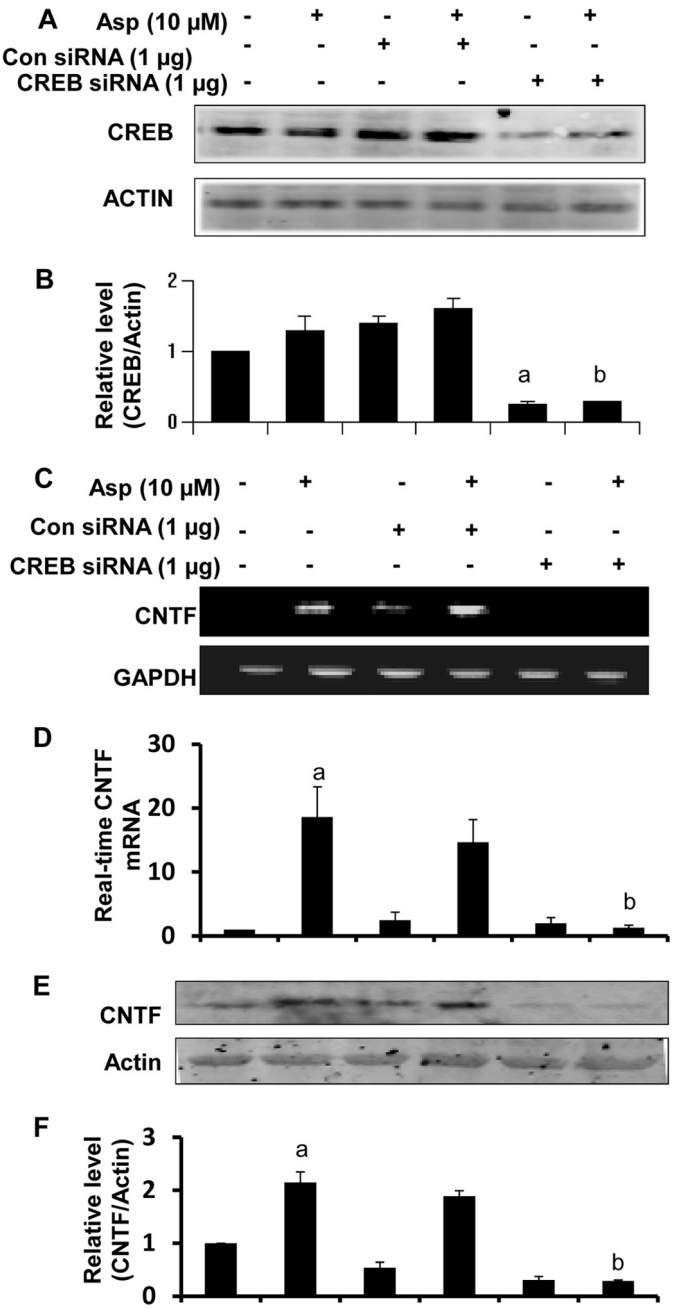

FIGURE 6. **siRNA knockdown of CREB abrogates the ability of aspirin to induce CNTF in mouse astroglia.** Primary astrocytes were transfected with CREB-siRNA and control siRNA. After 48 h of transfection, cells were treated with aspirin (*Asp*), and after 6 h of treatment the protein level of CREB was monitored by Western blot (*A*). Bands were quantified and presented as relative to control (*Con*) (*B*). Results are mean ± S.D. of three independent experiments. [a], $p < 0.001$ *versus* control; [b], $p < 0.001$ *versus* control siRNA-aspirin. Under similar treatment conditions, the mRNA expression of *Cntf* was monitored by semi-quantitative RT-PCR (*C*) and real time PCR (*D*). The protein level of CNTF was monitored by Western blot (*E*). Bands were quantified and presented as relative to control (*F*). Results are mean ± S.D. of three different experiments. [a], $p < 0.001$ *versus* control; [b], $p < 0.001$ *versus* control siRNA-aspirin.

scription of CNTF. Interestingly, aspirin was more potent in recruiting CBP than p300 to the mouse CNTF promoter (Fig. 7, *E* and *F*). In contrast, no amplification product was observed in any of the immunoprecipitates obtained with control IgG (Fig. 7, *E* and *F, last lanes*), suggesting the specificity of these interactions. These results suggest that aspirin alone is capable of increasing the recruitment of both CREB and CBP to the mouse *Cntf* promoter. Next, we examined if aspirin stimulated this recruitment via PKA. Consistent with the inhibition of *Cntf* mRNA expression, H-89 inhibited the recruitment of both CREB and CBP to the *Cntf* promoter in aspirin-treated astrocytes (Fig. 7, *C* and *D*). These results demonstrate that aspirin increases the recruitment of CREB and CBP to the CNTF promoter in mouse astrocytes via PKA. Similarly, multiple CREs

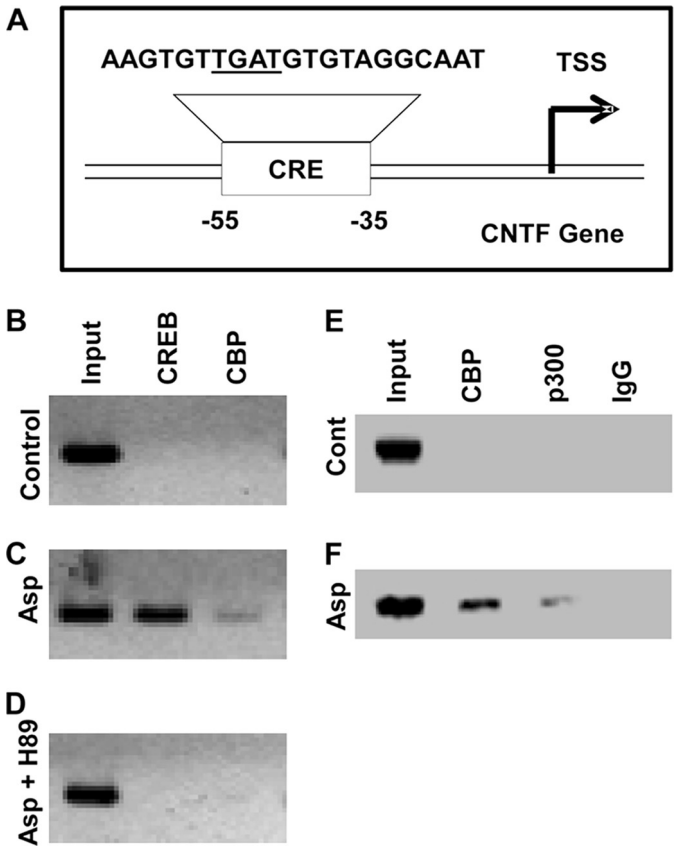

FIGURE 7. **Aspirin induces the recruitment of CREB to the *Cntf* promoter.** *A,* DNA sequence of the *CNTF* promoter region containing the CRE. Mouse astrocytes pretreated with 2 μM H-89 for 30 min were stimulated with 10 μM aspirin for 2 h under serum-free conditions followed by monitoring the recruitment of CREB, CBP, and RNA polymerase to the indicated position of the *Cntf* promoter by ChIP analysis (*B*, control (*Cont*); *C*, aspirin (*Asp*); *D*, aspirin + H-89). The recruitment of CBP was compared with that of p300 (*E,* control; *F*, aspirin). Normal IgG was used as control. Results represent three independent experiments.

(−696 to −716, −1363 to −1383, and −1440 to −1460) are also present in the human *Cntf* promoter, indicating that aspirin may transduce similar signaling mechanisms in human astrocytes as well.

Aspirin-treated Supernatants of WT, but Not CNTF$^{-/-}$, Astrocytes Increase the Expression of Myelin Proteins in Mouse Oligodendrocytes—CNTF is particularly important for promoting differentiation, maturation, and survival of oligodendrocytes (6, 10). Therefore, to understand the functional implications of aspirin-mediated up-regulation of CNTF in astrocytes, we determined the effect of aspirin-treated astroglial supernatants on the expression of myelin proteins in primary mouse oligodendrocytes. Primary astrocytes were isolated from wild type (WT) and CNTF$^{-/-}$ mice. As expected, aspirin increased the level of CNTF protein in WT, but not CNTF$^{-/-}$, astrocytes (Fig. 8, *A* and *B*). Next, aspirin-treated supernatants of WT and CNTF$^{-/-}$ astrocytes were added to mouse oligodendrocytes. As evident from double label immunofluorescence in Fig. 8*C*, aspirin-treated supernatants of WT, but not CNTF$^{-/-}$, astrocytes increased the expression of PLP and MOG in oligodendrocytes. However, supernatants of control WT and CNTF$^{-/-}$ astrocytes had no such stimulatory effect on the protein level of PLP and MOG in oligodendrocytes (Fig. 8*C*). DM20 is a smaller PLP splice isoform that differs from PLP by 35 residues in length of the cytosolic loop (30). In most cases, antibodies against PLP recognize DM20. Western blot analysis (Fig. 8, *D* and *E*) also shows the increase in PLP and DM20 in oligodendrocytes that were stimulated with supernatants of aspirin-treated WT, but not CNTF$^{-/-}$, astrocytes. These results suggest that aspirin-treated astroglial CNTF is capable of increasing myelin-associated proteins in oligodendrocytes.

Aspirin-treated Supernatants of WT, but Not CNTF$^{-/-}$, Astrocytes Protect Oligodendrocytes from TNF-α-induced Apoptosis and Cell Death—Several studies have shown that oligodendrocyte cell death plays an important role in the pathogenesis of MS and that CNTF protects oligodendrocytes from toxic insults (5, 6, 10). Therefore, we investigated if aspirin-stimulated astroglial CNTF was capable of protecting oligodendrocytes from TNF-α-induced apoptosis and cell death. As expected, the TUNEL test for DNA fragmentation revealed marked induction of terminal deoxynucleotidyltransferase-mediated labeling of DNA fragments in oligodendrocytes after 6 h of insult with TNF-α (Fig. 9, *A* and *B*). Accordingly, cell viability assays as determined by the MTT metabolism in cells and LDH release in supernatants also revealed decreased MTT and increased LDH release in oligodendrocytes by TNF-α insult (Fig. 9, *I* and *J*). However, supernatants of aspirin-treated WT, but not CNTF$^{-/-}$, astrocytes suppressed apoptosis (Fig. 9, *A*–*E*) and normalized MTT metabolism (Fig. 9*I*) and LDH release (Fig. 9*J*) in TNF-α-insulted oligodendrocytes. Conversely, supernatants of control WT and CNTF$^{-/-}$ astrocytes had no effect on TNF-α-induced apoptosis (Fig. 9, *A*–*H*), loss of MTT (Fig. 9*I*), and an increase in LDH release (Fig. 9*J*). These results suggest that aspirin-treated astroglial CNTF is capable of protecting oligodendrocytes from inflammatory insult.

DISCUSSION

CNTF is a pluripotent trophic factor capable of signaling oligodendrocytes to survive, differentiate, or grow. 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. Accordingly, CNTF exhibits protective effects in cell culture as well as the animal model of MS (8–11). Therefore, 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. However, such mechanisms are poorly understood. Several lines of evidence presented in this study clearly support the conclusion that aspirin is capable of up-regulating CNTF in mouse and human astrocytes. Aspirin-induced astroglial CNTF was also functionally active because the supernatants of aspirin-treated WT, but not CNTF$^{-/-}$, astrocytes supported oligodendroglial growth as evidenced by increased expression of PLP and MOG. Furthermore, aspirin-induced CNTF protected oligodendroglia from TNF-α-induced apoptosis and cell death. Because the loss of CNTF has been implicated in the pathogenesis of MS, our results provide a potentially important mechanism whereby aspirin may ameliorate demyelination.

The signaling events required for the transcription of CNTF in astrocytes that are the main source of CNTF in the CNS are

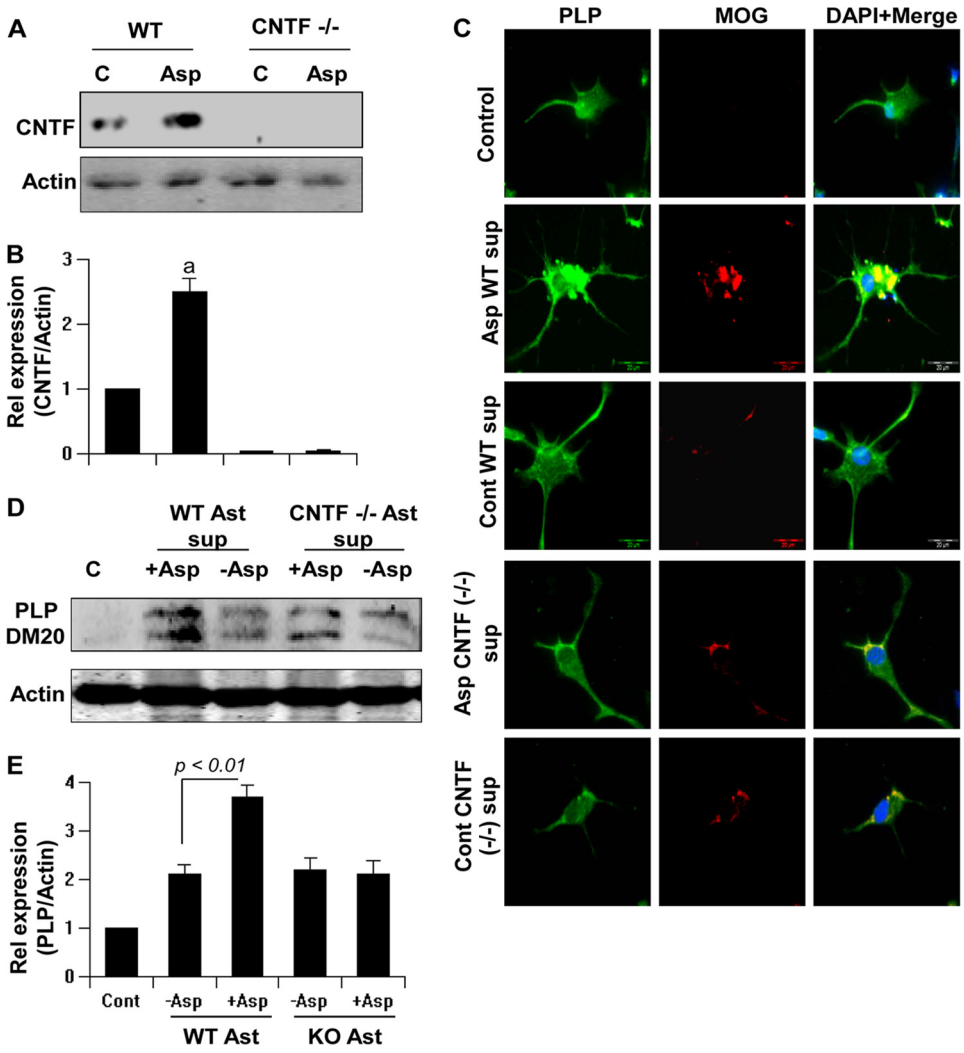

FIGURE 8. **Aspirin-treated supernatants of WT, but not CNTF$^{-/-}$, astrocytes increase the expression of myelin proteins in mouse oligodendrocytes.** Primary astrocytes isolated from wild type (*WT*) and CNTF$^{-/-}$ mice were treated with 10 μM aspirin (*Asp*) for 24 h followed by Western blot analysis of cell homogenates for CNTF (*A*). Actin was run as control (*C*). Bands were quantified and presented as relative to control (*B*). Results are mean ± S.D. of three independent experiments. [a], $p < 0.001$ *versus* WT control. Mouse oligodendrocytes were stimulated with conditioned supernatants of untreated and aspirin-treated WT and CNTF$^{-/-}$ astrocytes for 24 h followed by double label immunofluorescence of oligodendrocytes for PLP and MOG (*C*). DAPI was used to visualize the nucleus. Results represent three independent experiments. Under similar treatment conditions, protein levels of PLP and DM20 (a smaller PLP splice isoform) were monitored by Western blot (*D*). PLP bands were quantified and presented as relative to control (*E*). Results are mean ± S.D. of three different experiments.

poorly understood. Earlier studies have shown that the Sox10 is necessary and sufficient for regulating *Cntf* expression in Schwann cells (29). The cAMP-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 (27, 28, 31). PKA is a holoenzyme composed of two distinct subunits, regulatory (R) and catalytic (C). These regulatory and catalytic subunits form a tetrameric holoenzyme (R_2C_2) (31, 32). In the absence of cAMP, PKA exists as a stable inactive tetramer (31, 32). After an increase in intracellular cAMP, the regulatory PKA subunits bind to cAMP in a cooperative manner, resulting in the conformational change in the regulatory subunits and disassociation of the holoenzyme into a dimer of regulatory subunits and two monomers of catalytically active kinase (31, 32). Here, we demonstrate that aspirin induces the activation of PKA but not PKC in astrocytes, suggesting the specificity of the effect. Furthermore, abrogation of aspirin-induced expression of CNTF in astrocytes by H-89, a specific inhibitor of PKA, suggests that aspirin induces CNTF in astrocytes via PKA. However, at present, we do not know the mechanisms by which aspirin induces PKA. In general, the PKA signaling pathway is transduced from the G proteins (31, 32). Among the various Gα isoforms, $G_s\alpha$ stimulates adenylyl cyclase, and $G_i\alpha$ mediates the inhibition of adenylyl cyclase. The activation of adenylyl cyclase catalyzes the conversion of ATP to cAMP, which then serves as a second messenger and activates PKA. Because aspirin induces the activation of PKA within 5 min, it may not be surprising if aspirin uses any of the G proteins to activate PKA.

It remains to be elucidated how the PKA pathway couples the transcription of CNTF in astrocytes. Interestingly, upon analysis of the *Cntf* promoter using the Genomatix Software Suite, we have found a consensus CREB-binding site in the proximal

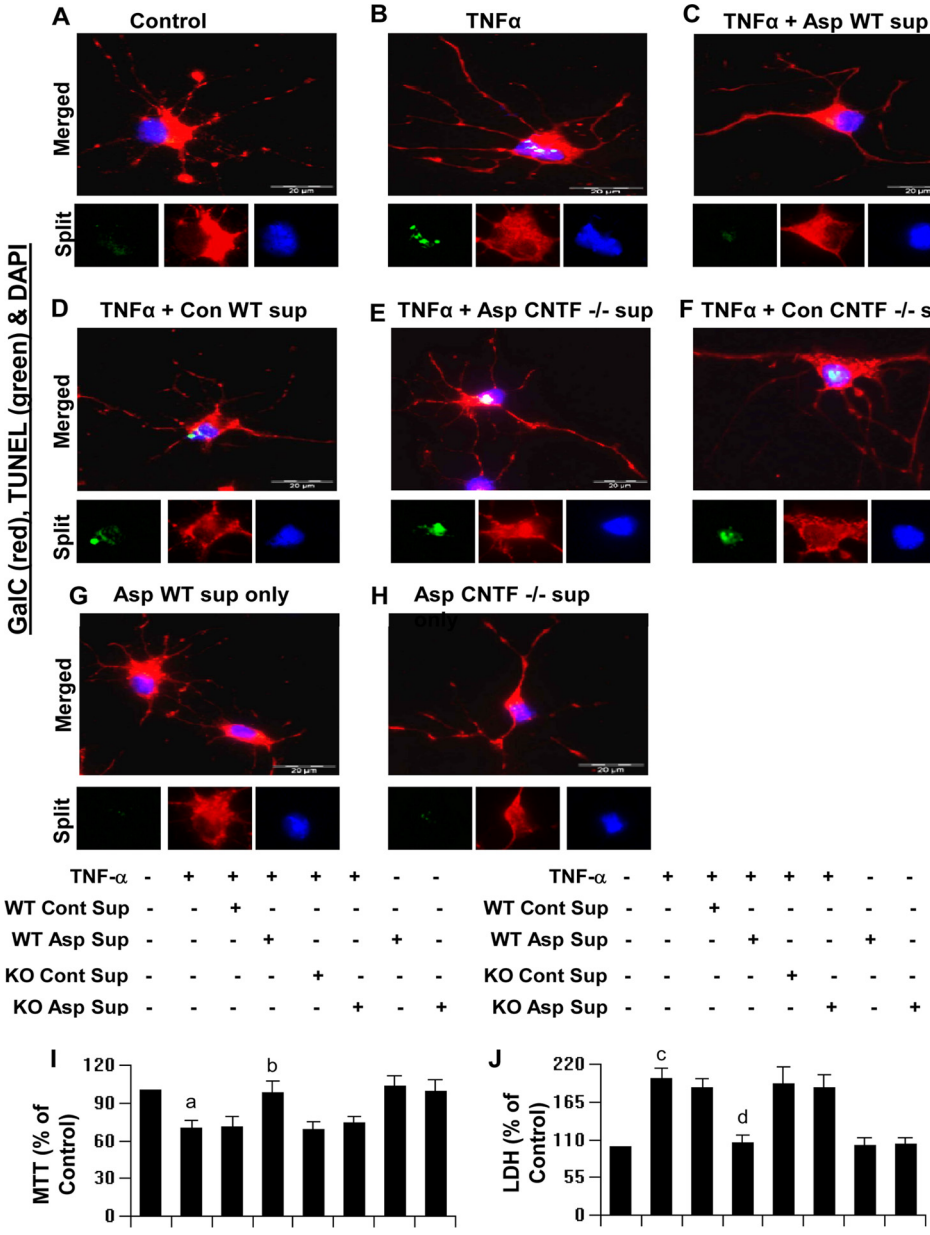

FIGURE 9. **Aspirin-treated supernatants of WT, but not CNTF$^{-/-}$, astrocytes protect mouse oligodendrocytes from TNF-α insult.** *A–H,* mouse oligodendrocytes preincubated with conditioned supernatants of untreated and aspirin (*Asp*)-treated WT and CNTF$^{-/-}$ astrocytes for 1 h were challenged with 20 ng/ml TNF-α for 6 h followed by monitoring apoptosis by TUNEL and GalC double labeling. DAPI was used to visualize the nucleus (*A*, control; *B*, TNF-α; *C*, TNF-α + aspirin-treated WT astroglial supernatant (*sup*); *D*, TNF-α + control WT astroglial supernatant; *E*, TNF-α + aspirin-treated CNTF$^{-/-}$ astroglial supernatant; *F*, TNF-α + control CNTF$^{-/-}$ astroglial supernatant; *G*, aspirin-treated WT astroglial supernatant only; *H*, aspirin-treated CNTF$^{-/-}$ astroglial supernatant only). Results represent three independent experiments. After 18 h of TNF-α challenge, cell death of oligodendrocytes was assayed by MTT (*I*) and LDH (*J*). Results are mean ± S.D. of three different experiments. [a], $p < 0.05$ *versus* control; [b], $p < 0.05$ *versus* TNF-α; [c], $p < 0.001$ *versus* control; [d], $p < 0.001$ *versus* TNF-α.

region of the *Cntf* promoter (Fig. 7*A*). Accordingly, aspirin induced the phosphorylation, DNA binding activity, and transcriptional activity of CREB in cultured astrocytes. Recruitment of CREB and CBP to the CRE of the *Cntf* promoter in aspirin-treated cells and abrogation of the ability of aspirin to induce the transcription of *Cntf* gene by siRNA knockdown of CREB suggest that aspirin increases the level of CNTF in astrocytes via CREB. Although we found NF-κB-binding sites in the promoter of *Cntf*, aspirin alone does not induce the activation of NF-κB. Furthermore, in activated glial cells, aspirin inhibits the activation of NF-κB (33). Therefore, aspirin does not up-regulate the expression of CNTF via NF-κB. Furthermore, here we present evidence that aspirin induced the activation of CREB via PKA. Our conclusion is based on the following observations. First, different protein kinases (*e.g.* PKC, PKA, etc.) are known to phosphorylate and activate CREB (27, 34). However, aspirin specifically induced the activation of PKA but not PKC. Second, inhibition of PKA by H-89 suppressed aspirin-induced activation of CREB. Third, H-89 also inhibited the recruitment of CREB and CBP to the *Cntf* promoter and the mRNA expression of CNTF in aspirin-stimulated astrocytes. Because PKA has several key functions in the cell, including glycogen, sugar, and

lipid metabolism, our results suggest that aspirin may also participate in glucose and lipid metabolism.

There are several advantages of aspirin over other available therapies for MS. On the one hand, aspirin has been widely used as an analgesic throughout the world for decades. On the other hand, it can be taken orally, the least painful route. Although aspirin has been reported to exhibit some toxic effects (gastric ulcers, stomach bleeding, and tinnitus, etc.) at high doses (35), in our study, aspirin is demonstrating a myelinotrophic effect via CNTF at low doses (5 and 10 μM), and at low doses, aspirin may not be toxic. However, if aspirin exhibits any toxic effects even at lower doses (*e.g.* gastric ulcer), there are ways to reduce its side effects. For example, enteric-coated aspirin is available for oral use and to avoid degradation in the stomach. In an open randomized trial, low doses of aspirin in a slow releasing formulation showed efficacy as an anti-platelet agent (36) without much noticeable side effects. One research study (37) used *S*-adenosylmethionine, an amino acid naturally formed in the body, and found that a dose of 500 mg of *S*-adenosylmethionine given together with a large dose of aspirin (1300 mg) reduced the amount of stomach damage by 90%.

In summary, we have demonstrated that aspirin, a widely used analgesic, up-regulates CNTF via PKA-mediated activation of CREB and that aspirin-treated astroglial supernatant protects oligodendroglia from inflammatory insult via CNTF. Although the *in vitro* situation of mouse astrocytes and oligodendrocytes in culture and its treatment with aspirin may not truly resemble the *in vivo* situation of these cells in the brain of MS patients, these results highlight an undiscovered property of aspirin and indicate that this drug may be used for therapeutic intervention in MS and other demyelinating conditions as primary or adjunct therapy.

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