Sodium Phenylbutyrate Enhances Astrocytic Neurotrophin Synthesis via Protein Kinase C (PKC)-mediated Activation of cAMP-response Element-binding Protein (CREB)

IMPLICATIONS FOR ALZHEIMER DISEASE THERAPY

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Background: Increase in neurotrophic factors in the brain is a possible therapeutic approach for different neurodegenerative disorders.

Results: Sodium phenylbutyrate, an FDA-approved drug for hyperammonemia, increases neurotrophic factors in brain cells via the PKC-CREB pathway.

Conclusion: These results delineate a novel neurotrophic property of sodium phenylbutyrate.

Significance: Sodium phenylbutyrate may be of therapeutic benefit in neurodegenerative disorders.

Neurotrophins, such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are believed to be genuine molecular mediators of neuronal growth and homeostatic synapse activity. However, levels of these neurotrophic factors decrease in different brain regions of patients with Alzheimer disease (AD). Induction of astrocytic neurotrophin synthesis is a poorly understood phenomenon but represents a plausible therapeutic target because neuronal neurotrophin production is aberrant in AD and other neurodegenerative diseases. Here, we delineate that sodium phenylbutyrate (NaPB), a Food and Drug Administration-approved oral medication for hyperammonemia, induces astrocytic BDNF and NT-3 expression via the protein kinase C (PKC)-cAMP-response element-binding protein (CREB) pathway. NaPB treatment increased the direct association between PKC and CREB followed by phosphorylation of CREB (Ser133) and induction of DNA binding and transcriptional activation of CREB. Up-regulation of markers for synaptic function and plasticity in cultured hippocampal neurons by NaPB-treated astroglial supernatants and its abrogation by anti-TrkB blocking antibody suggest that NaPB-induced astroglial neurotrophins are functionally active. Moreover, oral administration of NaPB increased the levels of BDNF and NT-3 in the CNS and improved spatial learning and memory in a mouse model of AD. Our results highlight a novel neurotrophic property of NaPB that may be used to augment neurotrophins in the CNS and improve synaptic function in disease states such as AD.

Neurotrophins are a class of small, dimeric growth factors essential for the development, maintenance, and function of the vertebrate nervous system (1–4). They are released in an activity-dependent manner from neurons, the major producers of neurotrophins (5–7) and from glial cells, where they are up-regulated under pathophysiological conditions (8, 9). Neurotrophin knock-out animals are not viable, and alterations in neurotrophin levels have profound effects on regeneration, long term potentiation, and synaptic remodeling (10, 11). In conditions such as Alzheimer disease (AD),3 the most common progressive dementing disorder, trophic levels are reduced (12–14), probably contributing to characteristic synaptic loss, neuronal deterioration, and subsequent impairments in learning, memory, planning, and executive functioning (15–17).

To date, neurotrophin-based therapies for neurodegenerative diseases have produced variable results (18–20) and are largely invasive. Because exogenous BDNF does not cross the blood–brain barrier without the aid of saturable transport systems (21) and intracerebroventricular injections of neurotrophins have many limitations (22), it is important to identify blood–brain barrier-permeable compounds that can induce neurotrophin production within the CNS. However, few compounds capable of directly modulating CNS neurotrophin production exist (20).

Here, we describe the ability of sodium phenylbutyrate (NaPB), a Food and Drug Administration-approved drug used to treat urea cycle disorders, to induce BDNF and NT-3 in cultured astrocytes and in a mouse model of AD via direct interaction between protein kinase C (PKC) and CREB. NaPB induced the activation of CREB, the proposed “final common pathway” of neurotrophin synthesis (23). In agreement with others (24), we found that NaPB readily crossed the blood–brain...
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barrier. We show that NaPB-mediated elevations in astrocytic neurotrophins up-regulated the expression of PSD-95 (postsynaptic density-95) and glutamatergic receptor subunits GluR1 and NR2A. Because astrocytes are recruited to sites of inflammation and neurodegeneration (25), we identify a novel mechanism by which astroglia can be programmed to maintain and protect surrounding neuronal populations.

MATERIALS AND METHODS

Reagents and Antibodies—Cell culture materials (DMEM/F-12, Neurobasal, Hanks’ balanced salt solution, antibiotic-antimycotic, L-glutamine, 0.05% trypsin, and B27/B27-AO) were purchased from Invitrogen, and fetal bovine serum (FBS) was obtained from Atlas Biologicals. All molecular biology grade chemicals were obtained from Sigma. NaPB, sodium phenylacetate (NaPA), and sodium formate (NaFO) were acquired from Sigma. H-89 was purchased from Enzo Life Sciences, GF chemicals were obtained from Sigma. NaPB, sodium phenylacetate (NaPA), and sodium formate (NaFO) were obtained from Atlas Biologicals. All molecular biology grade chemicals were obtained from Sigma. NaPB, sodium phenylacetate (NaPA), and sodium formate (NaFO) were acquired from Sigma. H-89 was purchased from Enzo Life Sciences, GF chemicals were obtained from Sigma.

Animals—B6SJL-Tg(APPSwFlLon,PSEN1*M146L*L286V)-6799Vas/J transgenic (5XFAD) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male 5XFAD and non-transgenic mice were used for experimentation. Animals were maintained, and experiments were conducted in accordance with National Institutes of Health guidelines and were approved by the Rush University Medical Center Institutional Animal Care and Use Committee.

Behavioral Analyses—Five-month-old 5XFAD mice or their non-transgenic littermates were fed 100 mg of NaPB per kg of body weight every day for 30 days, followed by testing on open field and Barnes maze tasks. For the open field test, mice were placed in the Digiscan Optical Animal Monitoring System (AccuScan Instruments) and allowed to explore the chamber for 30 min. Gross distance traveled was recorded every 60 s. Barnes maze testing was performed using a standard elevated platform (92-cm diameter) consisting of 20 5-cm (diameter) holes spaced 7.5 cm apart, one of which was the target hole (26). Visual cues were maintained beyond the perimeter of the maze for the duration of testing, and light was used as a weak aversive stimulus. For the spatial acquisition phase, animals were allowed to explore the maze for 180 s for 4 days with an intertrial interval of 24 h. On the fifth day, animals were not tested. Forty-eight h after the fourth spatial acquisition trial, the probe trial was conducted. Again, mice were allowed to explore the maze for 180 s and were monitored for primary latency (duration before the first encounter with the target hole), total latency (duration before all four paws were on the floor of the escape box), primary errors (incorrect responses prior to the first encounter with the target hole), total errors (incorrect responses before all four paws were on the floor of the escape box), primary time spent in the target quadrant (duration before the first encounter with the target hole), and total time spent in the target quadrant (duration before all four paws were on the floor of the escape box).

Cell Culture and Transfection—Astrocytes and neurons were obtained from postnatal day 7 and fetal (embryonic days 16–18) mice, respectively. The optimized protocol for these procedures has been described by us (27). Hippocampal or cortical neurons were plated on poly-d-lysine (Sigma)-coated chamber slides for 5 min to allow neurons to adhere. The glia-enriched supernatant was aspirated and pelleted, and astrocytes were seeded in flasks for 12 days prior to experimentation. Neurons were seeded for varying time periods, and extended culture was achieved by replacing half of the culture volume every 3 days. Transfection of 0.25 μg of CREB or scrambled siRNA was performed using Lipofectamine 2000 and siPORT NeoFX (Invitrogen) according to the manufacturer’s instruction. After 48 h, cells were washed with Hanks’ balanced salt solution and treated.

Isolation of Primary Human Astrocytes—Primary human astroglia were prepared as described (28, 29). All experimental protocols were reviewed and approved by the Institutional Review Board of the Rush University Medical Center. Briefly, 11–17-week-old fetal brains obtained from the Human Embryology Laboratory (University of Washington, Seattle, WA) were dissociated by trituration and trypsinization. On the ninth day, mixed glial cultures were placed on a rotary shaker at 240 rpm at 37 °C for 2 h to remove loosely attached microglia. On the 11th day, the flasks were shaken again at 190 rpm at 37 °C for 18 h to remove oligodendroglia. The remaining cells were primarily astrocytes. These cells were trypsinized and subcultured in complete medium at 37 °C with 5% CO2.

Immunocytochemistry—Slides plated at 50–60% (astrocytes) or 20–30% (neurons) confluence were fixed overnight with chilled methanol, blocked with 2% bovine serum for 30 min, and incubated with primary antibodies (see supplemental Table 1) in PBST plus Triton X-100 (0.1%) for 2 h. Samples were then incubated with Alexa-fluor-tagged secondary antibodies (1:200) for 1 h, washed, and treated with DAPI (1:10,000; Sigma) for 4–5 min. For negative controls, a slide was incubated under similar conditions void of primary antibodies. Slides were dehydrated in an EtOH and xylene gradient, mounted, and imaged using an Olympus BX41 fluorescent microscope equipped with a Hamamatsu ORCA-03G camera.

Immunohistochemistry—Following treatments, animals were sacrificed, and brains were fixed and embedded. 30-μm sections were cut on a cryostat and placed in sucrose cryoprotectant at −20 °C until use. Endogenous peroxidase inhibition was performed using 0.03% H2O2 and 0.1% NaN3 in PBS. Free floating sections were blocked (2% BSA); incubated with antibodies against BDNF, NT-3, and glial fibrillary acidic protein (GFAP) (see supplemental Table 1) in PBST plus Triton X-100 (0.1%) at 4 °C overnight; washed (PBS); incubated with Alexa-fluor-tagged secondary antibodies (1:200) for 4 h; washed again; and treated with DAPI (1:10,000) for 5 min. Sections were then placed on slides, air-dried overnight, dehydrated in an EtOH/xylene gradient, mounted with Cytoseal 60 (Richard-Allan Scientific), and dried for >3 h prior to imaging with an Olympus BX41 fluorescent microscope equipped with a Hamamatsu ORCA-03G camera.
Morphometric Analysis—To characterize and quantify astrocytes, the Cy3 (GFAP) channel was isolated from each image, converted to black and white, and normalized using a spatial threshold filter. 20–40 cells in four serial sections (n = 5, all groups) were traced using the MicroSuite FIVE™ Biological Suite. Based on area distribution, astrocytes were classified as small (less than quartile 1), medium (between quartile 1 and quartile 3), or large (more than quartile 3). The isolated Cy5 (BDNF) channel was then remerged with the same Cy3 (GFAP) images, and the proportion of BDNF-immunoreactive, GFAP-positive astrocytes was quantified for each quartile.

RT-PCR and Real-time Quantitative PCR (qPCR)—Total RNA was isolated using the Qiagen RNA-Easy kit according to the manufacturer’s protocol. cDNA was created using oligo(dT), dNTP, 0.1 M DTT, Moloney murine leukemia virus reverse transcriptase, RNaseOUT, and 5× FS Buffer (all from Invitrogen) and amplified with PCR Master Mix (Promega) and the following primers (Invitrogen) for murine genes: CREB (sense), 5’-TGC AGC TGC CAC TCA GCC GG-3; CREB (antisense), 5’-TGC CAA GCC AGT CCA TTC TTC AC-3; BDNF (sense), 5’-AGG CAA CTT GCC CTA CCC AGG TGT G-3; BDNF (antisense), 5’-TGG CAC ACA CGC TCA GCT CCC C-3; NT-3 (sense), 5’-GAG AGG AGG AGG CCA GGT AGT GAG AGG CCA GGT CAG CAG AGT TCC A-3; NT-3 (antisense), 5’-GTC ATC AAT CCC CTC AAC CTT CCT GCA ACC GTT T-3; GAPDH (sense), 5’-GGT GAA GAPDH GGT CCG AGT CAA CG-3; GAPDH (antisense), 5’-GTG AAG CAG CCA GTG GAC TAC-3’. Amplified products were electrophoresed on 2% agarose gels, visualized by EtBr staining, and normalized to GAPDH. qPCR was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) using iTaq™ Fast Supermix with ROX (Bio-Rad) and the ABI-Prism7700 sequence detection system (Applied Biosystems). Data are expressed as -fold change relative to control for three or four independent experiments.

ELISA and Kinase Assays—BDNF and NT-3 were assayed using high sensitivity sandwich ELISA kits from Promega and Syd Labs, respectively, according to the manufacturers’ recommendations. Plates were analyzed spectrophotometrically with a Thermo-Fisher Multiskan MCC plate reader. Assays for protein kinase A (PKA) and PKC were purchased from Enzo Life Sciences and performed according to the manufacturer’s protocol using high sensitivity sandwich ELISA kits from Promega and ABI-Prism7700 sequence detection system (Applied Biosystems). ELISA and Kinase Assays were analyzed using the relative 2(−ΔΔCT) method (30).

DNA Binding and Transcriptional Activities of CREB—DNA binding activity of CREB was analyzed from nuclear fractionation by non-radioactive electrophoretic mobility shift assay (EMSA) as described previously by us (27). After stimulation, cells were washed, centrifuged, and resuspended in a low salt buffer. The resulting pellet was digested in a high salt, nuclear envelope lysis buffer, rotated at 4 °C for 30 min, and pelleted by centrifugation. The resultant supernatant was assayed for protein concentration via the Bradford method (Bio-Rad), complexed with a mixture of binding buffer (1 M Tris-HCl, 2.5 M KCl, 0.5 M EDTA, 1.0 M DTT, 10× Tris-glycine-EDTA), and visualized/quantified with the Odyssey infrared imaging system.

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Transcriptional activity of CREB was analyzed using the protocol outlined previously by us (27) using pCRE-Luc (a CREB-dependent luciferase reporter construct; Stratagene).

Morphological Characterization—Hippocampal neurons plated to 15–20% confluence were immunostained with anti-β-tubulin (1:1,000; Millipore) and anti-PSD-95 (1:600; Abcam) and analyzed for dendritic morphology. For each treatment condi-
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FIGURE 1. Time- and dose-dependent induction of BDNF and NT-3 mRNAs in primary mouse astrocytes by NaPB. Cultured astrocytes were immunostained with GFAP (green) and MAP-2 (A; red) or Iba-1 (B; red) to determine purity. Cells were stimulated with different doses of NaPB for 6 h and analyzed for BDNF (blue) and NT-3 (orange) mRNAs by semiquantitative RT-PCR (C) and qPCR (D). Time-dependent responses of BDNF (blue) and NT-3 (orange) mRNA to 0.2 mM NaPB were monitored by RT-PCR (E) and qPCR (F). Results represent three independent experiments. Data (mean ± S.D. (error bars)) were analyzed for statistical significance by one-way ANOVA (*, p < 0.01; **, p < 0.001). Scale bar, 50 μm. MRGD, merged image.

RESULTS

NaPB Up-regulates BDNF and NT-3 mRNA in Pure Astrocytic Cultures—At first, we determined the purity of astroglial preparations. Cultures were double-labeled for GFAP and microtubule-associated protein-2 (MAP-2), a neuronal marker (Fig. 1A), or ionized calcium-binding adaptor molecule-1 (Iba-1), a microglial marker (Fig. 1B). Astrocytes were highly pure (≥99%) because it was very difficult to find one Iba-1-positive cell among 100 GFAP-positive astrocytes (Fig. 1, A and B). It is clear from RT-PCR (Fig. 1C) and qPCR (Fig. 1D) that maximal increases in BDNF (blue) mRNA transcription were achieved by 0.2 mM NaPB. Maximal NT-3 (orange) mRNA expression was observed with 0.5 mM NaPB for the same time period (Fig. 1, C and D). Time course analyses via RT-PCR (Fig. 1E) and qPCR (Fig. 1F) of astrocytes treated with 0.2 mM NaPB revealed steady increases in BDNF and NT-3 mRNA up to 6 h.

NaPB Up-regulates Secreted and Cell-bound BDNF and NT-3—NaPB is known to be β-oxidized to NaPA, its bioactive metabolite (32). Therefore, primary mouse astrocytes were stimulated with both NaPB and NaPA. Both NaPB and NaPA significantly increased the production of BDNF (blue) and NT-3 (orange; Fig. 2, A and B) compared with unstimulated cells, as determined by ELISA. Lower concentrations and shorter treatment durations did not significantly elevate release of either neurotrophin. NaFO, similar in structure to NaPB but lacking the benzene ring, did not significantly alter BDNF or NT-3 secretion, suggesting the specificity of the effect. After 24-h exposure to NaPB and NaPA, increases in cell-bound BDNF (Fig. 2, C and D) and NT-3 (Fig. 2, E and F) were observed in mouse cortical (Fig. 2, C and E) and human primary (Fig. 2, D and F) astrocytes, as determined by immunostaining. Immunoblotting for BDNF in cell lysates yields a faint band of ∼30 kDa and a dark band at ∼15 kDa (Fig. 2G). Increasing concentrations of NaPB significantly increased expression of the 14 kDa band corresponding to mature BDNF (Fig. 2, I and J), whereas no increases were observed in the ∼30 kDa band corresponding to precursor BDNF (data not shown). Immunoblotting for NT-3 from cell lysates shows similar high and low molecular weight (MW) species as BDNF (Fig. 2H). The low molecular weight species (∼15 kDa), which corresponds to mature NT-3, was significantly elevated by NaPB (Fig. 2, K and L; 0.2 and 0.5 mM), whereas the high molecular weight species (∼29 kDa) corresponding to precursor NT-3 was unchanged (data not shown). Again, NaFO did not significantly alter the expression of either neurotrophin.

NaPB-mediated Neurotrophin Production Is Dependent upon CREB—Because neurotrophins have long been thought to be under the transcriptional control of CREB (33, 34), we analyzed the effect of NaPB on CREB expression and activation. As analyzed by qPCR (Fig. 3, A and B, top) and RT-PCR (Fig. 3, A and B, bottom), CREB expression was not significantly elevated by NaPB (Fig. 3, A and B, bottom). Although CREB activation was significantly increased by NaPB (Fig. 3, B, bottom), time- and dose-dependent induction of BDNF and NT-3 mRNAs in primary mouse astrocytes by NaPB. Cultured astrocytes were immunostained with GFAP (green) and MAP-2 (A; red) or Iba-1 (B; red) to determine purity. Cells were stimulated with different doses of NaPB for 6 h and analyzed for BDNF (blue) and NT-3 (orange) mRNAs by semiquantitative RT-PCR (C) and qPCR (D). Time-dependent responses of BDNF (blue) and NT-3 (orange) mRNA to 0.2 mM NaPB were monitored by RT-PCR (E) and qPCR (F). Results represent three independent experiments. Data (mean ± S.D. (error bars)) were analyzed for statistical significance by one-way ANOVA (*, p < 0.01; **, p < 0.001). Scale bar, 50 μm. MRGD, merged image.
and B, bottom), NaPB significantly elevated CREB transcription dose-dependently (Fig. 3A) and time-dependently (Fig. 3B) in primary mouse astrocytes. Immunoblotting for total CREB expression in lysates from mouse astrocytes treated with various concentrations of NaPB also revealed significant increases with 0.1 and 0.2 mM concentrations (Fig. 3C, densitometry, top). Additionally, analysis of cells treated with 0.2 mM NaPB for brief time periods showed significant increases in phosphorylated (Ser133) CREB by immunoblot (Fig. 3D and in-cell Western blot (Fig. 3F) without altering total CREB expression, suggesting a biphasic effect of NaPB on CREB. This increase in phosphorylated CREB was largely localized to the nucleus, as monitored by immunostaining (Fig. 3E). EMSA of nuclear extracts from astrocytes treated with NaPB revealed increased DNA-protein interactions in NaPB-treated cells (Fig. 3G). In support of this, astrocytes stimulated with various concentrations of NaPB and analyzed by a luciferase assay show increased reporter activity of CREB (Fig. 3H, blue). NaPB, however, did not significantly affect the transcriptional activity of either activator protein-1 (AP-1; orange) or nuclear factor-κB (NF-κB; navy) (Fig. 3H). siRNA knockdown of CREB (confirmed in Fig. 3, I and J) abrogated the ability of NaPB to induce BDNF and NT-3 mRNA (Fig. 3, K and L) and protein (Fig. 3, M–O), suggesting an important role of CREB in NaPB-mediated neurotrophin expression.

PKC Phosphorylates CREB to Propagate NaPB-mediated Neurotrophin Synthesis—CREB activity can be regulated by a number of signaling pathways. For example, stimuli that activate PKA and PKC result in CREB phosphorylation (35, 36). This prompted us to determine the upstream regulator of the NaPB-mediated CREB activation. At 30 min and 2 h, 0.2 mM NaPB produced ∼120 and ∼160% increases (navy), respectively, in active PKC relative to active PKA, and increases in PKA were not significant (Fig. 4A). NaPB-treated mouse astrocyte lysates immunoprecipitated with an anti-PKC antibody and immunoblotted for CREB show time-dependent increases in the PKC-CREB interaction (Fig. 4B). As determined by immunoblotting, increases in

FIGURE 2. NaPB up-regulates BDNF and NT-3 in primary mouse and human astrocytes. A, NaPB dose-dependently induces BDNF and NT-3 release. Supernatants from primary mouse astrocytes stimulated with various concentrations of NaPB or 0.2 mM NaFO for 24 h (A) were analyzed for BDNF (blue) and NT-3 (orange) expression. B, NaPB time-dependently induces BDNF and NT-3 release. Supernatants from mouse astrocytes stimulated with 0.2 mM NaPB for various time points (B) were analyzed for BDNF (blue) and NT-3 (orange) expression. C and D, NaPB elevates cell-bound BDNF. Primary mouse cortical (C) and primary human astrocytes (D) stimulated with 0.2 mM NaPB, NaPA, or NaFO for 24 h were immunostained for BDNF (red), GFAP (green), and DAPI (blue). E and F, NaPB elevates cell-bound NT-3. Primary mouse cortical (G) and primary human astrocytes (H) stimulated with 0.2 mM NaPB, NaPA, or NaFO for 24 h were immunostained for NT-3 (red), GFAP (green), and DAPI (blue). G, anti-BDNF antibody detects two bands. 50 μg of lysate from unstimulated astrocytes was resolved next to pure recombinant mouse NT-3 (I). H, anti-NT-3 antibody detects two bands. 60 μg of lysate from unstimulated astrocytes was resolved next to pure recombinant mouse NT-3 (mNT-3). K, NaPB induces expression of mature NT-3. Primary mouse astrocytes stimulated with various concentrations of NaPB or 0.2 mM NaFO for 24 h were subjected to immunoblotting with anti-BDNF and anti-β-actin antibodies and subsequent densitometry (J). H, anti-NT-3 antibody detects two bands. 60 μg of lysate from unstimulated astrocytes was resolved next to pure recombinant mouse NT-3 (mNT-3). K, NaPB induces expression of mature NT-3. Primary mouse astrocytes stimulated with various concentrations of NaPB or 0.2 mM NaFO for 24 h were subjected to immunoblotting with anti-NT-3 and anti-β-actin antibodies and subsequent densitometry (L). Each treatment condition is represented by two independent bands in I and K. ELISAs and densitometric analyses were analyzed for significance by one-way ANOVA (*, p < 0.01; **, p < 0.001). Scale bar, 20 μm. MRGD, merged image; MW, molecular weight; MPA, mouse primary astrocytes; HPA, human primary astrocytes.
phosphorylated CREB in response to 0.2 mM NaPB were abrogated by the PKC inhibitor GF 109203X (GFX; 0.5 μM) but not the PKA inhibitor H-89 (2 μM) (Fig. 4C). In support of this, GFX, but not H-89, inhibited the NaPB-mediated increases in the DNA binding (Fig. 4D) and transcriptional activities (Fig. 4E) of CREB. As determined by qPCR, increases...
in the mRNA expression of CREB (Fig. 4F) and BDNF and NT-3 (Fig. 4G) in response to 0.2 mM NaPB were abated by GFX but not H-89. The mRNA results (Fig. 4G) were supported by Western blot data (Fig. 4H; densitometry, top). Taken together, these results suggest an obligatory role for PKC in the NaPB-mediated induction of BDNF and NT-3.
NaPB Does Not Alter Neurotrophin Receptor Expression—Because precursor neurotrophins and mature neurotrophins can bind p75NTR and trigger apoptotic pathways (37) and up-regulation of both neurotrophins and their receptors may contribute to the development of neuroblastomas (38, 39), we monitored the expression of p75NTR and TrkB, the selective BDNF receptor, in neurons. As determined by immunocytocontrol (Fig. 5), NaPB does not alter p75NTR or TrkB expression in cortical neurons. Neurons seeded at 50,000 cells/mm² for 24 h; fixed; immunostained with antibodies against p75NTR, TrkB, DAPI (blue), and β-actin (red). Whole cell images and somatic images were captured using 60 objective lenses, respectively. Scale bar, 50 μm. B, NaPB does not alter p75NTR or TrkB expression in neurons. Unstimulated or treated with 0.5 mM NaPB for 24 h displayed no change in somatic p75NTR or TrkB expression (left panels; arrowheads). Both left and right panels were treated with DAPI (blue). Receptor expression was equally unaltered in NaPB (Fig. 5A2) or TrkB-blocking antibody (Fig. 5A4). As expected, neurons exposed to the TrkB blocking antibody (Fig. 5A4) failed to produce significant increases in PSD-95 expression across days (Figs. 6, B1–B3, orange), suggesting the specificity of the effect. Abrogation of NaPB-treated astroglial supernatant-mediated increase in PSD-95 immunoreactivity in neurons by TrkB blocking antibody (Fig. 6A4) suggests the involvement of NaPB-induced astroglial neurotrophins in synaptic plasticity. As expected, neurons exposed to the TrkB blocking antibody alone (Fig. 6A5) displayed the weakest PSD-95 immunoreactivity and were largely unviable at DIV 21 (Fig. 6, B1–B3, black). Because PSD-95 induces the clustering and maturation of AMPA (42) and NMDA receptors (43), we used the same culture system to determine expression of GluR1 and NR2A at DIV 21. In agreement with Fig. 6, A and B, NaPB-treated astroglial supernatant and rhBDNF up-regulated PSD-95 expression, as determined by immunoblotting (Fig. 6C) and subsequent densitometry (Fig. 6D, blue). Expression of both GluR1 and NR2A was significantly increased by NaPB-treated astroglial supernatant and rhBDNF, as determined by immunoblotting and subsequent densitometry (Fig. 6D, GluR1 (orange) and NR2A (navy)). As a whole, these results implicate the ability of NaPB-mediated inductions in astroglial BDNF to contribute to synaptic development and remodeling.

Oral Treatment of NaPB Increases the Interaction between CREB and PKC and Up-regulates BDNF and NT-3 in a Mouse Model of AD—To determine the effect of NaPB on neurotrophin induction in vivo, we used 5XFAD mice, an accelerated model of AD. At first, we examined if NaPB entered into the CNS of 5XFAD mice. NaPB is known to be β-oxidized into NaPA in the liver. Therefore, frontal-cortical homogenates from NaPB-treated mice were analyzed for NaPA by HPLC to confirm the bioavailability of NaPA in the brain (Fig. 7A). NaPA produced a spike just after 2 min in both the standard (top) and NaPB-treated (bottom), but not vehicle-treated (middle), sample. Hippocampal fractions isolated from 5XFAD mice, immunoprecipitated with antibodies against CREB and PKC and immunoblotted for PKC and CREB, respectively, indicate an increased interaction between PKC and CREB in mice fed with NaPB (Fig. 7B), supporting our cell culture finding (Fig. 4B). Immunoblotting for BDNF and NT-3 in cortical (top) and hip-
pocampal (bottom) fractions from non-transgenic (non-Tg) (Fig. 7C) and 5XFAD (Fig. 7D) mice shows significant increases in neurotphin expression in NaPB-treated mice (orange) as compared with vehicle-treated mice (blue). Each band represents independent animals. NaPB treatment also promoted PSD-95 expression in vivo in the hippocampus of 5XFAD mice (Fig. 6, E and F).

**NaPB Up-regulates BDNF Independent of Astrocytic Morphology in CA1 of the Hippocampus**—To determine colocalization of BDNF with astrocytes in vivo and verify immunoblotting data (Fig. 6), coronal hippocampal sections were double-labeled for GFAP and BDNF (Fig. 8A). The boxed area in CA1 indicates the dorsal anterior region magnified in Fig. 8B. BDNF-immunoreactive astrocytes from this area were then quantified based on rigorous morphological parameters. Administration of NaPB (100 mg of NaPB per kg of body weight every day for 30 days) or vehicle (H2O) to non-Tg and 5XFAD mice did not alter GFAP staining intensity (Fig. 8C), astrocyte area (Fig. 8D), or astrocyte size distribution (Fig. 8, E and F). NaPB also did not...
alter BDNF immunoreactivity in smaller astrocytes (less than quartile 1), but significantly elevated BDNF expression in medium (quartile 1 < x > quartile 3) and large (more than quartile 3) astrocytes in non-Tg and 5XFAD mice relative to vehicle-treated mice (Fig. 8G).

Oral Administration of NaPB Improves Spatial Memory in a Mouse Model of AD—Because neurotrophins are known to modulate mechanisms underlying learning and memory, we tested performance of NaPB-treated 5XFAD and non-Tg mice on the Barnes maze, a hippocampus-dependent cognitive task that requires spatial reference memory (26). NaPB did not alter gross motor activity or exploratory drive in either non-Tg or 5XFAD mice, as determined by open field testing (Fig. 9A). As expected, 5XFAD mice performed poorly on the Barnes maze task as compared with non-Tg mice (Fig. 9, B–D). However, oral treatment with NaPB for 30 days significantly reduced primary (F3,19 = 3.073, p < 0.05, p = 0.035) and total (F3,19 = 9.978, p < 0.001, p = 0.047) errors (Fig. 9B) and improved primary (F3,19 = 3.175, p < 0.05, p = 0.034) latency (Fig. 9C) in 5XFAD mice relative to vehicle treatment. NaPB-treated 5XFAD mice also spent significantly more time in the target quadrant before the first encounter with the target hole (primary; F3,19 = 3.292, p < 0.05, p = 0.043) as well as throughout the trial period (total; F3,19 = 3.989, p < 0.05, p = 0.029) (Fig. 9D). Similarly, NaPB treatment also improved the performance of non-Tg mice on the Barnes maze as compared with vehicle treatment (Fig. 9, B–D).

DISCUSSION

Neurotrophins are secreted small proteins that are capable of signaling neurons to survive, differentiate, and grow. Furthermore, neurotrophins have also been suggested as rescuers of vulnerable neurons in many neurodegenerative diseases, including AD, Parkinson disease, and HIV-associated dementia, in which the levels of some neurotrophic factors are significantly reduced in the brain. For example, it has been shown that the levels of BDNF and NT-3 are significantly down-regulated in the brains of patients with AD. Accordingly, these neurotrophic factors (BDNF and NT-3) exhibit protective effects in cell culture as well as animal models of different neurodegen-
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**FIGURE 8. NaPB increases astrocytic BDNF expression in CA1.** A, colocalization of BDNF with GFAP in the mouse hippocampus. Shown is a representative coronal section of a non-Tg mouse treated with vehicle (Veh) (H2O) captured with a ×4 objective lens (scale bar, 250 μm). B, NaPB induces BDNF expression in mouse astrocytes in vivo. Free floating coronal sections from non-Tg mice fed vehicle (B1) or NaPB (B2) and 5XFAD mice fed vehicle (B3) or NaPB (B4) were immunostained for BDNF (red), GFAP (green), and DAPI (blue). Images represent the boxed region of CA1 in A (scale bar, 25 μm). C–F, NaPB does not significantly alter astrocyte morphology. Three serial sections within CA1 from non-Tg mice fed vehicle (blue) or NaPB (orange) and 5XFAD mice fed vehicle (navy) or NaPB (green) (n = 4, all groups) were analyzed for GFAP fluorescence intensity (C) and area (D) using MicroSuite FIVE™ Biological Suite. Box plots in D represent area quartile (quartile 1 [Q1], median, quartile 3 [Q3]) dispersion, whiskers represent area maximums/minimums, and white insets represent area mean ± S.E. (error bars) for each group. Representative astrocytes, tracings, and areas from each quartile are represented in E. Astrocytes within each quartile range were quantified as a percentage of total cells counted in each group and presented as a 100% stacked column chart in F. G, NaPB induces expression of BDNF in astrocytes of various sizes. BDNF-immunoreactive (BDNF+) astrocytes were counted and quantified as a percentage of GFAP-immunoreactive (GFAP+) astrocytes for all quartiles. See “Materials and Methods” for detailed stereological morphometry information. Data were analyzed for statistical significance by one-way ANOVA (*, p < 0.05; **, p < 0.01). CA, cornu ammonis; DG, dentate gyrus; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.

Neurodegenerative diseases (19). Although under physiological conditions, neurons produce neurotrophic factors, glial derived neurotrophins (44) assume significance under neurodegenerative conditions, when neurons die and do not produce these invaluable factors. Therefore, increasing the levels of neurotrophins in astrocytes, major glial cells in the CNS, is an important area of research. However, such mechanisms are poorly understood.

NaPB is a sodium salt of short chain fatty acid having multiple clinical interests. Known as Buphenyl® and tributyrin® in the United States or Ammonaps® in Sweden, it is used as a medication against urea cycle disorders involving deficiencies of carbamylphosphate synthetase, ornithine transcarbamylase, or argininosuccinic acid synthetase. Although its major function is to scavenge ammonia and glutamine (45), recent studies have described NaPB as a potent inhibitor of HDAC (46). Due to its HDAC-inhibitory effects, it has been proposed as a drug against various forms of cancer (47, 48). Accordingly, it has also been clinically tested as an anticancer drug (47). In addition to ammonia removal and HDAC inhibition, Ozcan et al. (49) have shown that NaPB can also function as a chemical chaperon during endoplasmic reticulum stress. Recently, we have also demonstrated anti-inflammatory and antioxidative properties of NaPB (50). Several lines of evidence presented in this study clearly support the conclusion that NaPB and its metabolite NaPA are capable of up-regulating neurotrophins in astrocytes. Our conclusion is based on the following observations. First, NaPB dose-dependently induced the expression of BDNF and NT-3 in primary mouse and human astrocytes. The metabolite of NaPB, NaPA, similarly up-regulated BDNF and NT-3 in astrocytes. This up-regulation was specific because NaFO, a compound structurally similar to NaPA but without having the benzene moiety, had no effect. Second, oral administration of NaPB increased the level of NaPA in the brains of mice and up-regulated BDNF and NT-3 in vivo in the brain.

Because the loss of these neurotrophic factors has been implicated in the pathogenesis of various neurodegenerative diseases (13) and these neurotrophins have been shown to be required for synaptogenesis and synaptic function (10, 51, 52), our results provide a potentially important mechanism whereby NaPB may improve synaptic function associated with different neurodegenerative conditions. In fact, we found that NaPB-treated astrocytic supernatant up-regulated the expression of PSD-95, NR2A, and GluR1 in cultured hippocampal neurons. Application of rhBDNF produced similar results, whereas blocking TrkB abrogated NaPB-mediated up-regulation of PSD-95 in hippocampal neurons. As expected, we observed decreased PSD-95 and poor memory performance in 5XFAD mice, an aggressive animal model of AD. However, oral administration of NaPB recovered characteristic deficiencies in PSD-95 expression and improved spatial learning and memory.

The signaling events required for the transcription of neurotrophic factors are becoming clear. Upon analysis of the BDNF promoter using the Genomatix Software Suite, we have found multiple binding sites for CREB and NF-κB and at least one consensus binding site for C/EBPβ and AP-1 in the BDNF promoter. Although these transcription factors (CREB, NF-κB, C/EBPβ, and AP-1) may play a role in the expression of various trophic factors, activation of CREB seems essential for the transcription of these neurotrophic factors (23). Therefore, for a drug to exhibit neurotrophic effect, it is almost mandatory to stimulate the activation of CREB. Activation of CREB in cultured astrocytes by NaPB and abrogation of the ability of NaPB to induce neurotrophic factors by siRNA knockdown of CREB suggest that NaPB increases the levels of neurotrophic factors via CREB. Although the activation of NF-κB has also been shown to be involved in the expression of neurotrophic factors, NaPB alone does not induce the activation of NF-κB. Furthermore, recently, we have found that NaPB inhibits the activation...
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**FIGURE 9.** NaPB improves spatial learning and memory in non-Tg and 5XFAD transgenic mice. A, NaPB does not affect gross motor or exploratory behavior. Non-Tg mice fed vehicle (Veh) (H2O; blue) or NaPB (orange) and 5XFAD mice fed vehicle (navy) or NaPB (green) were recorded every 1 min for 30 min on the open field test 24 h after the final treatment. Values are expressed as averages of 3-min bins. B–D, NaPB improves performance on the Barnes maze task. B, 48 h following the final acquisition trial, errors made prior to the first encounter with the target hole (primary errors) and errors made prior to escape (total errors) were quantified for non-Tg mice fed vehicle (blue) or NaPB (orange) and 5XFAD mice fed vehicle (navy) or NaPB (green). C, latency to the first encounter with the target hole (primary latency) and latency to escape (total latency) were quantified for non-Tg mice fed vehicle (blue) or NaPB (orange) and 5XFAD mice fed vehicle (navy) or NaPB (green). D, time spent in the target quadrant (TQ) before the first encounter with the target hole (primary time in target quadrant) and time spent in the target quadrant prior to escape (total time in target quadrant) were quantified for non-Tg mice fed vehicle (blue) or NaPB (orange) and 5XFAD mice fed vehicle (navy) or NaPB (green). For all studies, n = 6 for non-Tg mice fed vehicle, n = 6 for non-Tg mice fed NaPB, n = 5 for 5XFAD mice fed vehicle, and n = 6 for 5XFAD mice fed NaPB. Mice were fed vehicle or 100 mg of NaPB per kg of body weight every day for 30 days. Data were analyzed for statistical significance by one-way ANOVA followed by Games-Howell post hoc tests (*, p < 0.05; **, p < 0.01). NS, not significant. Error bars, S.D.

of NF-κB in activated glial cells (50). Therefore, NaPB does not up-regulate the expression of neurotrophic factors via NF-κB.

However, it was unknown by which mechanisms NaPB induced the activation of CREB in brain cells. The PKC 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 synaptic plasticity (53, 54). Here we present evidence that NaPB induced the activation of CREB via PKC. Although both PKA and PKC are known to phosphorylate and activate CREB, NaPB specifically induced the activation of PKC and direct association between PKC and CREB. Accordingly, suppression of PKC by GF 109203X, but not of PKA by H-89, inhibited NaPB-induced activation of CREB and expression of neurotrophic factors in astrocytes. However, at present, we do not know the mechanisms by which NaPB induces PKC. In general, the PKC signaling pathway is transduced in different cell types, including brain cells, by diacylglycerol and/or Ca2+ (55, 56). Because translocation of PKC to the cell membrane is considered a hallmark of PKC activation and PKC interacts weakly or transiently with membranes in the absence of Ca2+ or diacylglycerol, it is possible that NaPB may alter cytosolic Ca2+ levels.

There are several advantages of NaPB over other proposed neurotrophic therapies. First, NaPB is fairly nontoxic. It is a Food and Drug Administration-approved drug against urea cycle disorders in children. It is rapidly metabolized in the liver and kidneys via β-oxidation to phenylacetaldehyde, the bioactive form of the drug (32). NaPB and its metabolite have been found to exhibit protection in animal models of multiple sclerosis (57), ischemic injury (58), and Parkinson disease (50) and to reduce Tau pathology in an AD mouse model (59). Second, NaPB can be taken orally, the least painful route. Third, NaPB, being a lipophilic molecule, readily diffuses across the blood-brain barrier. For example, glutamine toxicity is a problem in urea cycle disorders. After treatment of patients with urea cycle disorders, NaPB is known to combine with glutamine to produce phenylacetylglutamine, a compound that is readily excreted in the urine. Simultaneous serum and CSF sampling in those patients showed comparable levels of phenylacetylglutamine in the CSF (32). Here we have also seen that oral administration of NaPB increases the level of NaPA, its active metabolite, in the brains of 5XFAD mice.

In summary, we have demonstrated that NaPB up-regulates neurotrophic factors in astrocytes via PKC-mediated activation of CREB. These results highlight undiscovered properties of NaPB and indicate that this Food and Drug Administration-approved drug may be used to improve synaptic plasticity in neurodegenerative disorders as primary or adjunct therapy.

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