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Cinnamon converts poor learning mice to good learners: Implications for memory improvement

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Abstract

This study underlines the importance of cinnamon, a commonly used natural spice and flavoring material, and its metabolite sodium benzoate (NaB) in converting poor learning mice to good learning ones. NaB, but not sodium formate, was found to upregulate plasticity-related molecules, stimulate NMDA- and AMPA-sensitive calcium influx and increase of spine density in cultured hippocampal neurons. NaB induced the activation of CREB in hippocampal neurons via protein kinase A (PKA), which was responsible for the upregulation of plasticity-related molecules. Finally, spatial memory consolidation-induced activation of CREB and expression of different plasticity-related molecules were less in the hippocampus of poor learning mice as compared to good learning ones. However, oral treatment of cinnamon and NaB increased spatial memory consolidation-induced activation of CREB and expression of plasticity-related molecules in the hippocampus of poor-learning mice and converted poor learners into good learners. These results describe a novel property of cinnamon in switching poor learners to good learners via stimulating hippocampal plasticity.

Keywords

Cinnamon; Sodium benzoate; Poor learners; CREB; Memory and learning

Introduction

Improvement in memory and learning is of great value in human life. Although some people are inborn good learners and some become so in association to appropriate environments, some find it hard in learning new tasks. The hippocampal neurons are often associated during early memory performance and preservation. During memory impairments, hippocampal neurons are often compromised and exhibit down-regulation of many functional genes (Colangelo et al., 2002) involved in ion conductance (Myers et al., 1999;

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Jacob et al., 2007), synapse formation (Selkoe, 2002), dendritic arborization (Shim and Lubec, 2002), long term potentiation (Clayton et al., 2002; Malenka and Bear, 2004), and long term depression (Collingridge et al.; Malenka and Bear, 2004). Impaired calcium influx through ionotropic glutamate receptors including NMDA and AMPA receptors is directly linked to the loss of hippocampal learning and memory (Olney et al., 1998). Accordingly, postmortem analysis of dementia brains shows alteration in NMDA receptor subunits including NR1, NR2A, and NR2B in susceptible brain regions including the hippocampus (Mishizen-Eberz et al., 2004). Down-regulation of immediate early genes (IEGs) (Desjardins et al., 1997; Mishizen-Eberz et al., 2004) including arc, zif-268, homer-1, c-fos and inhibition of synapse-associated genes (Proctor et al.; Reddy et al., 2005; Bell et al., 2008) including psd-95, synpo, adam-10 have also been reported in demented brains. Therefore, increasing neuronal plasticity is an important area of research as it may eventually help us in improving memory in poor learners and treating different forms of dementia.

Here, we have undertaken a simple and natural approach to upregulate hippocampal plasticity. Cinnamon is a commonly used natural spice and flavoring material used for centuries throughout the world. We have already demonstrated that after oral administration, cinnamon is metabolized into sodium benzoate (NaB), a food-additive and an FDA-approved drug for hyperammonemia, and that the level of NaB increases in different parts of the brain after cinnamon intake (Jana et al., 2013; Modi et al., 2015). We have also delineated that NaB is anti-inflammatory (Brahmachari et al., 2009), immunomodulatory (Brahmachari and Pahan, 2007), neurotrophic (Jana et al., 2013), anti-oxidant (Modi et al., 2015), and neuroprotective (Jana et al., 2013). Here, we delineate that NaB stimulates calcium influx via NMDA- and AMPA-sensitive ionotropic glutamate receptors, increases the expression of plasticity related molecules and enhances morphological plasticity in hippocampal neurons. NaB stimulates the expression of plasticity-related molecules in hippocampal neurons via activation of CREB. Furthermore, we describe that spatial memory consolidation-induced activation of CREB and expression of different plasticity related molecules are less in the hippocampus of poor-learning mice than good-learning mice. However, oral treatment of poor-learning mice with cinnamon and NaB increases memory consolidation-induced DNA-binding activity of CREB and the expression of plasticity-related molecules in the hippocampus and converts poor-learning mice into good-learning ones. These results suggest that cinnamon may be used to improve memory and learning in poor learners.

Materials and Methods

Reagents

Fetal bovine serum (FBS) and DMEM/F-12 were from Mediatech (Washington, DC). Sodium benzoate (NaB), sodium formate (NaFO), N-Methyl-D-aspartic acid (NMDA), and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) were purchased from Sigma Aldrich (St. Louis, MO). Original Ceylon cinnamon (*Cinnamomum verum*) in ground form was obtained from Indus Organics (San Ramon, CA). Antibodies against NR2A (catalog # 4205), GluR1 (catalog # 8850) and CREB (catalog # 9197S) were purchased from

Cell Signaling. Antibodies against tubulin (catalog # Ab6046), Gabra5 (catalog # Ab10098) and PSD95 (catalog # Ab2723) were purchased from Abcam. 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 and Maintenance of Mouse Hippocampal neurons

Hippocampal neurons were isolated from fetuses (E18) of pregnant female mice as described by us (Jana et al., 2007; Saha et al., 2009). Briefly, dissection and isolation procedures were performed in an ice-cold, sucrose buffer solution (sucrose 0.32 M, Tris 0.025 M; pH 7.4). The skin and the skull were carefully removed from the brain by scissors followed by peeling off the meninges by a pair of fine tweezers. A fine incision was made in the middle line around the circle of Willis and medial temporal lobe was opened up. Hippocampus was isolated as a thin slice of tissue located near the cortical edge of medial temporal lobe. Hippocampal tissues isolated from all fetal pups ($n > 10$) were combined together and homogenized with 1 ml of Trypsin for 5 min at 37°C followed by neutralization of trypsin (Jana et al., 2007; Saha et al., 2009). The single cell suspension of hippocampal tissue was plated in the poly-D-lysine pre-coated 75 mm flask. Five minutes after plating, the supernatants were carefully removed and replaced with complete neurobasal media. The next day, 10 μ M AraC was added to remove glial contamination in the neuronal culture. The pure cultures of hippocampal neurons were allowed to differentiate fully for 9-10 days before treatment.

Measurement of spine density and size

Spine density and size were measured as described before by us (Roy et al., 2014) with minor modification. Briefly, E18 hippocampal neurons were stained with Alexa-647 conjugated phalloidin together with anti-Tuj1 antibody. Only densely stained neurons were counted. The total length of each dendrite was measured at 400X magnification using an Olympus BX-51 fluorescence microscope. The number of spines on the dendrites was counted under oil immersion. As some of the spines were hidden under the dendrite, only spines that protruded laterally from the shafts of the dendrites into the surrounding area of clear neuropil were counted. The spine density of a pyramidal neuron was calculated by dividing the total number of spines on a neuron by the total length of its dendrites, and was expressed as the number of spines/10 μ m dendrite. The size of the dendritic spines was measured by calculating the ratio of mean fluorescent intensity (MFI) of the spine head and MFI of the dendritic shaft.

Calcium Influx Assay in Primary Mouse Hippocampal Neurons

Calcium influx was monitored in cultured hippocampal neurons as described by us earlier (Roy et al., 2013; Roy et al., 2014). Briefly, neurons were loaded with Fluo4-fluorescence conjugated calcium buffer (Invitrogen Molecular Probes, Cat# F10471) and incubated at 37°C for 60 mins following manufacturer's protocol. After that, fluorescence excitation and emission spectra were recorded in a Perkin-Elmer Victor X2 Luminescence Spectrometer in the presence of 50 μ M NMDA and AMPA solutions. The recording was performed with 300 repeats at 0.1ms intervals.

Immunofluorescence analysis

Immunofluorescence analysis was performed as described earlier (Mondal et al.; Roy et al., 2008). Briefly, cells cultured in 8-well chamber slides (Lab-Tek II) were fixed with 4% paraformaldehyde for 20 min followed by treatment with cold ethanol (-20°C) for 5 min and 2 rinses in PBS. Samples were blocked with 3% BSA in PBST for 30 min and incubated in PBST containing 1% BSA and rabbit anti-NR2A (1:100), anti-GluR1 (1:100), anti-PSD95 (1:100) and anti-CREB (1:100). After three washes in PBST (15 min each), slides were further incubated with cy2- and cy5-conjugated secondary antibodies (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 an Olympus IX81 fluorescent microscope. For tissue staining, brains kept in 4% paraformaldehyde were sectioned in cryostat machine with $30\text{ }\mu\text{m}$ thickness followed by the immunostaining as described before (Ghosh et al., 2007).

Assay of PKA and PKC

Assays for PKA and PKC were purchased from Enzo Life Sciences and performed according to manufacturer's protocol as described (Corbett et al., 2012; Modi et al., 2013). Values were normalized to purified kinase controls.

EMSA

DNA-binding activity of CREB was analyzed by non-radioactive electrophoretic mobility shift assay (EMSA) as described (Corbett et al., 2012; Ghosh and Pahan, 2012). Briefly, nuclear extracts were complexed with a cocktail of binding buffer (Tris-HCl, KCl, EDTA, DTT, 10x TGE, glycerol and Triton-X 100), custom designed fluorescent CREB-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 1X TGE for 2 hrs. The shift was visualized under the Odyssey® Infrared Imaging System (Li-Cor).

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 (Corbett et al., 2012; Ghosh and Pahan, 2012; Khasnavis et al., 2012) using a RT-PCR kit from Clontech. Briefly, $1\text{ }\mu\text{g}$ 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.

Immunoblotting

Western blotting was performed as described earlier (Corbett et al.; Khasnavis and Pahan, 2012; Jana et al., 2013) with modifications. 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 30-50 μg total protein and the sample was 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 h in TBST containing BSA. Next, membranes were incubated overnight at 4°C under shaking conditions with primary antibody. The next day, membranes were washed in TBST for 1 h, incubated with secondary antibody (Li-Cor Biosciences) for 1 h at room temperature, washed for one more hour and visualized under the Odyssey® Infrared Imaging System (Li-COR, Lincoln, NE).

Animals and cinnamon/NaB treatment

Male C57/BL6 mice (8-10 week old; purchased from Harlan, Indianapolis, IN) were treated with cinnamon (50 mg/kg body wt/d) and NaB (50 mg/kg body wt/d) via gavage for 30 d followed by monitoring memory and learning and hippocampal histochemical and biochemical assays. NaFO was used as a negative control of NaB. While NaB and NaFO were solubilized in water, cinnamon (*Cinnamomum verum*) powder was mixed in 0.5% methylcellulose (MC). Mice were gavaged 100 µL cinnamon-mixed MC and 100 µL NaB- and NaFO-mixed water once daily using gavage needle as described (Jana et al., 2013; Khasnavis and Pahan, 2014; Mondal and Pahan, 2014; Modi et al., 2015). Another group of control mice also received only MC as vehicle.

Barnes Maze

Barnes maze was performed as described by us (Corbett et al., 2013; Roy et al., 2013; Modi et al., 2014; Modi et al., 2015). Briefly, for Barnes maze, mice were trained for 2 consecutive days followed by examination on day 3. During training, the overnight food-deprived mouse was placed in the middle of the maze in a 10 cm high cylindrical black start chamber. After 10 s, the start chamber was removed to allow the mouse to move around the maze to find out the color food chips in the baited tunnel. The session was ended when the mouse entered the baited tunnel. The tunnel was always located underneath the same hole (stable within the spatial environment), which was randomly determined for each mouse. After each training session, maze and escape tunnel were thoroughly cleaned with a mild detergent to avoid instinctive odor avoidance due to mouse's odor from the familiar object. On day 3, the maze was illuminated with high wattage light that generated enough light and heat to motivate animals to enter into the escape tunnel (Prut et al., 2007), allowing us to measure latency (duration before all four paws were on the floor of the escape box) and errors (incorrect responses before all four paws were on the floor of the escape box).

T Maze

T maze experiments were performed as described by us (Roy et al., 2013; Modi et al., 2014; Modi et al., 2015). Briefly, mice were habituated in the T-maze for two days under food-deprived conditions so that animals can eat food rewards at least five times during 10 minutes period of training. During each trial, mice were placed in the start point for 30 s and then forced to make a right arm turn which was always baited with color food chips. On entering the right arm, they were allowed to stay there for 30-45 s, then returned to the start point, held for 30 s and then allowed to make right turn again. As described above, after each training session, T maze was thoroughly cleaned with a mild detergent. On day 3, mice were tested for making positive turns and negative turns. The reward side is always associated

with a visual cue. Number of times the animal eats the food reward would be considered as a positive turn.

Statistical Analysis

All values were expressed as means $\pm$ SD of three independent experiments. Statistical differences between means were calculated by the Student's *t*-test. A *p*-value of less than 0.05 ($p < 0.05$) was considered statistically significant. Differences in behavioral measures were examined by independent one-way ANOVA using SPSS. Homogeneity of variance between test groups was examined using Levene's test. *Post-hoc* analyses were conducted using Tukey's or Games-Howell tests, where appropriate. $p < 0.05$ was considered statistically significant.

Results

NaB stimulated the expression of plasticity-related molecules in hippocampal neurons

To understand the effect of cinnamon metabolite NaB on plasticity, we examined the expression of different plasticity-related molecules in NaB-treated hippocampal neurons. NMDA receptor subunit NR-2A, AMPA-receptor subunit GluR1 and PSD-95, a membrane-associated guanylate kinase and a potent regulator of synaptic strength, are some of the major plasticity-related molecules in the hippocampus. NaB treatment stimulated the expression of NR2A, GluR1 and PSD95 proteins in hippocampal neurons (Fig. 1A-B). This result was specific as sodium formate (NaFO) having similar chemical structure as NaB except the benzene ring had no such stimulatory effect (Fig. 1A-B). Moreover, in contrast to the stimulation of plasticity-related molecules, NaB was unable to stimulate the expression of GABRA5, receptor subunit of γ amino butyric acid and a molecule that is involved in long-term depression (Atack, 2010), in hippocampal neurons (Fig. 1A-B). Dose-dependent studies showed that NaB was capable of upregulating the expression of NR2A, GluR1 and PSD95 at a dose of 50 μ M and the maximum increase was found at either 100 or 200 μ M (Fig. 1C-D). Again, NaB did not increase the expression of GABRA5 at any of the doses tested (Fig. 1C-D). We corroborated our Western blot results with immunofluorescence analysis of NR2A, GluR1 and PSD95 in hippocampal neurons. Double-label immunofluorescence of NR2A (Fig. 1E), GluR1 (Fig. 1F) and PSD95 (Fig. 1G) with TuJ1 showed that NaB strongly upregulated these proteins in the projections of hippocampal neurons.

NaB induced morphological plasticity in cultured hippocampal neurons

Since the formation and maturation of dendritic spines contribute directly to the long-term enhancement of synaptic efficacy of hippocampal neurons involved in learning and memory, we studied the effect of NaB on the number, size, and maturation of dendritic spines. Fluorescently tagged phalloidin was used to label filamentous actin in cell bodies and axons and throughout the dendrites. Interestingly, NaB, but not NaFO, increased the density of dendritic spines in cultured hippocampal neurons (Fig. 2A). Detailed morphological analyses further revealed that NaB, but not NaFO, stimulated the number (Fig. 2B) and size (Fig. 2C) of dendritic spines in hippocampal neurons. Furthermore, NaB, but not NaFO, increased the maturation of dendritic spines (Fig. 2D) and NaB-treated hippocampal neurons

exhibited greater number of mushroom (Fig. 2E) and stubby (Fig. 2F) spines than either untreated or NaFO-treated neurons. These results suggest that NaB enhances the synaptic maturation of hippocampal neurons by enriching the density and size of dendritic spines.

NaB stimulated inward calcium current in cultured hippocampal neurons

Since an increase in the number of matured spines directly regulates synaptic transmission in the post-synaptic neurons via NMDA- and AMPA-regulated ionotropic receptors, our next aim was to study calcium influx through NMDA and AMPA receptors after NaB treatment. Interestingly, we observed that NaB, but not NaFO, stimulated calcium influx in the presence of NMDA (Fig. 3A) or AMPA (Fig. 3B), suggesting that NaB potentiates postsynaptic membrane depolarization, eventually leading to the long term enhancement of synaptic activity in hippocampal neurons.

NaB requires CREB for the upregulation of NR2A in hippocampal neurons

Next, we investigated mechanisms by which NaB upregulated the expression of plasticity-related molecules in hippocampal neurons. Upon analysis of the promoter of *nr2a* gene, one of important plasticity-related genes, by using MatInspector, we found that mouse *nr2a* promoter harbors several consensus cAMP response element (CRE). Therefore, we were prompted to investigate if NaB required CREB for the upregulation of NR2A in mouse hippocampal neurons. First, we examined if NaB alone was capable of inducing the activation of CREB in hippocampal neurons. 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. 4A-B). NaB-induced phosphorylation of CREB was evident as early as 5 min of stimulation (Fig. 4A-B). On the other hand, we did not see any change in the level of total CREB upon NaB stimulation (Fig. 4A). Next, we investigated if NaB required CREB for the upregulation of NR2A in hippocampal neurons. We employed the siRNA approach to study the involvement of CREB. As evident from figure 4C-D, CREB siRNA, but not control siRNA, decreased the expression of CREB protein in NaB-treated neurons. Accordingly, CREB siRNA, but not control siRNA, abrogated NaB-mediated increase in NR2A protein (Fig. 4E-F). These results suggest that CREB is required for NaB-mediated upregulation of NR2A in hippocampal neurons.

NaB increased the activation of CREB and the expression of NR2A in hippocampal neurons via protein kinase A (PKA)

Next, we decided to elucidate mechanisms by which NaB induced the activation of CREB and increased the expression of NR2A in hippocampal neurons. Because kinases like protein kinase A (PKA) and protein kinase C (PKC) play an important role in the activation of CREB, we examined the role of PKA and PKC in NaB-mediated upregulation of NR2A. At first, we monitored if NaB alone was capable of activating PKA and PKC in mouse hippocampal neurons. As evident from figure 4G, NaB alone induced the activation of PKA in hippocampal neurons in a time-dependent manner and this activation was significant as early as 15 min of stimulation. In contrast, NaB remained unable to induce the activation of PKC at different time points tested (Fig. 4H), ruling out the involvement of PKC in these processes. Therefore, next, we examined if NaB required PKA for the activation of CREB. Suppression of NaB-mediated phosphorylation of CREB (Fig. 4I-J) by H-89, a specific

inhibitor of PKA, suggests that NaB induces the activation of CREB in hippocampal neurons via PKA. Consistent to the involvement of CREB in NaB-mediated upregulation of NR2A, H-89 also inhibited NaB-mediated expression of NR2A in hippocampal neurons (Fig. 4K-L). These results suggest that NaB requires PKA for the activation of CREB and the expression of NR2A in hippocampal neurons.

Reduced activation of CREB and decreased levels of plasticity-related molecules in the hippocampus of poor learners as compared to good learners

The hippocampus regulates the generation of long term memory and spatial learning (Morris et al., 1986; Roy et al., 2013; Roy and Pahan, 2015). Therefore, we investigated the levels of CREB activation of plasticity-related molecules in the hippocampus of poor learners and good learners. Based on spatial memory consolidation (Roy et al., 2013) on Barnes circular maze (Fig. 5A), male C57/BL6 mice (6-8 week old) were separated into good and poor performers. Good performing mice exhibited significantly greater memory performance on Barnes maze than poor performing mice as shown by track plot (Fig. 5B), latency (Fig. 5C) and number of errors (Fig. 5D). There was significant difference of $t_{46}=4.852$; $p < 0.001$ observed with mean difference of 66.22 between good and poor learners as calculated by unpaired t-test. Since CREB is regarded as an important regulator memory and learning, we examined the activation of CREB in the hippocampus of good and poor learning mice 24 h post trial (Fig. 5A) by EMSA. Interestingly, the level of CREB activation was much higher in the hippocampus of good learning mice than poor learning mice (Fig. 5E). These results suggest that memory consolidation-induced activation of CREB in the hippocampus is much higher in good learning mice than poor learning ones. Therefore, we further analyzed the expression of different plasticity-related molecules that depend on CREB activation. Consistent to CREB activation, we also found that the levels of NR2A (Fig. 5F & G), GluR1 (Fig. 5F & H) and PSD95 (Fig. 5F & I) were much less in the hippocampus of poor learning mice than good learning mice. Together, these results suggest that spatial memory consolidation-induced activation of CREB and expression of plasticity-related molecules in the hippocampus are much higher in good learning mice than poor learning ones.

Oral administration of cinnamon and NaB increases spatial memory consolidation-mediated expression of plasticity-related molecules in the hippocampus of poor learning mice

Since NaB induced the activation of CREB and upregulated the expression of plasticity-related molecules in hippocampal neurons, we examined if NaB and cinnamon itself could upregulate memory consolidation-induced expression of plasticity-related molecules in the hippocampus of poor learning mice. Poor learning mice were treated with NaB and cinnamon orally for a month followed by Barnes maze training and test (Fig. 5A). Twenty-four h after the test, the activation of CREB and the expression of different plasticity-related molecules were monitored in the hippocampus. Untreated poor and good learning mice as well as vehicle-treated poor learning mice were also run in parallel. As evident from CREB EMSA in Figure 6A, oral administration of cinnamon and NaB, but not vehicle, increased memory consolidation-induced DNA-binding activity of CREB *in vivo* in the hippocampus of poor learning mice. Accordingly, memory consolidation-induced expression of neuronal activity-regulated genes (Egr1, Fos, Bdnf, Homer1, Arc, Snk, & Spar) (Fig. 6B-C), synaptic

formation-related genes (Synapsin, Prosap2, Nr2a1, Nr2a3, Agrin, & CamKii α) (Fig. 6D-E) and synaptic plasticity-associated genes (GluR1, GluR4, Nr2a1, & Nr2a3) (Fig. 6F-G) was lower in the hippocampus of poor learning mice than good learning ones. However, oral treatment of poor learning mice with cinnamon and NaB significantly increased the expression of neuronal activity-regulated genes (Fig. 6B-C), synaptic formation-related genes (Fig. 6D-E) and synaptic plasticity-associated genes (Fig. 6F-G) *in vivo* in the hippocampus. To further confirm these results, we performed Western blot of hippocampal extracts for some of these plasticity-related molecules. Consistent to mRNA expression, cinnamon and NaB, but not vehicle, augmented memory consolidation-induced expression of NR2A, GluR1 and PSD95 in the hippocampus of poor learning mice (Fig. 6H-K). Cinnamon and NaB were capable of enhancing the DNA-binding activity of CREB (Fig. 6A) and the expression of different plasticity-related genes (Fig. 6B-G) and proteins (Fig. 6H-K) in the hippocampus of poor learning mice to a level very similar to that found in good learning mice.

Oral administration of cinnamon and NaB improves spatial learning and memory in poor learning mice

Since cinnamon and NaB treatment increases memory consolidation-induced activation of CREB and the expression of different plasticity-related molecules in the hippocampus of poor learning mice, we analyzed spatial learning and memory of poor learners after cinnamon and NaB treatment. First, we analyzed the performance in Barnes maze. As expected, poor learning mice exhibited more latency and greater number of errors than good learners (Fig. 7A-C). However, after cinnamon and NaB treatment, poor learning mice exhibited significantly improved memory performance on Barnes maze as shown by track plot (Fig. 7A), latency [$F_{5,42}=17.598$, $p<0.001$ (=0.00002)] (Fig. 7B) and number of errors [$F_{5,42}=10.570$, $p<0.05$ (=0.000012)] (Fig. 7C) as compared to vehicle-treated poor learning mice, confirmed by *one way ANOVA* test. *Post hoc* tests of multiple comparisons using Tukey HSD analyses showed that poor learning mice took longer time to find the reward hole and exhibited more latency $p<0.01$ (=0.00018) and greater number of errors $p<0.05$ (=0.038) in the Barnes maze as compared to good learning mice. However, cinnamon-treated poor learning mice were as capable as good learning mice in finding the target hole (Fig. 7A-C) and exhibited significantly less latency $p<0.001$ (=0.001) and fewer errors $p<0.05$ (=0.006). Similarly, NaB-treated poor learning mice were also effective in finding the target hole (Fig. 7A-C) with reduced latency ($p=0.01$) and fewer errors $p<0.01$ (=0.0001). We also treated a group of good learning mice with cinnamon for a month to see if there were any further improvements in Barnes maze performance. However, as compared to untreated good learning mice, we did not find any significant improvement in latency and errors in cinnamon-fed good learning mice (Fig. 7A-C).

Next, we performed T maze to determine whether cinnamon and NaB could improve spatial memory in poor learning mice. Untreated poor performing mice exhibited less number of positive turns $p<0.05$ (=0.014) and greater number of negative turns $p<0.001$ (=0.004) than age-matched untreated good performing mice in T maze apparatus (Fig. 7D-E). However, cinnamon-treatment significantly improved hippocampus dependent memory performances as cinnamon-fed poor learning mice exhibited more positive turns $p<0.05$ (=0.0041) and less

negative turns $p < 0.01 (=0.00012)$ compared to vehicle-fed poor learning mice in T maze (Fig. 7D-E). In contrast, cinnamon treatment did not further improve T maze functions of good learning mice as compared to untreated good learning ones (Fig. 7D-E). Similar to cinnamon treatment, NaB-treated poor learning mice also exhibited greater number of positive turns $p < 0.05 (=0.0011)$ and reduced number of negative turns $p < 0.01 (=0.00031)$ as compared to vehicle-fed poor learning mice.

We also monitored if orally administered cinnamon or NaB had any effect on general locomotor activities in these mice. In fact, after open field test analyses, we did not observe any significant alteration in total distance travel (Fig. 7F), moving speed (Fig. 7G) and stereotypy (Fig. 7H) in poor learning mice, suggesting that cinnamon or NaB treatment does not modulate gross motor activities in these mice.

Discussion

Individual difference in educational performance is a global issue. For example, between two students of the same background studying in the same class, in most cases, one does better than the other academically, suggesting that inter-individual differences can have striking output in performance and achievement. While such inter-individual differences are strongly related to differences in intelligence and memory, relatively little is known on how to improve performance in poor performers. Therefore, exploring ways to boost plasticity and improve memory and learning is an important area of research as it may help switching poor learners to good learners and preventing age-related decline in memory. The hippocampus, a small organ located within the brain's medial temporal lobe, participates in generating, organizing and storing memory. Although there are other approaches for improving hippocampal function, here we introduce a widely-used natural product to augment hippocampal plasticity. 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 (Abd El-Mawla et al., 2001) that exists as sodium salt (NaB) or benzoyl-CoA. It has been reported that minor amount of NaB is also excreted in the urine of human (Bridges et al., 1970; Kubota and Ishizaki, 1991). NaB is 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 (Leonard and Morris, 2002; Scaglia et al., 2004). It is also widely used as a preservative in broad range of foods and cosmetic products (Nair, 2001). We have seen that cinnamon and NaB modify T cells and protect mice from experimental allergic encephalomyelitis, an animal model of multiple sclerosis (Brahmachari and Pahan, 2007; Mondal and Pahan, 2015; Pahan, 2015). Cinnamon and NaB also upregulate neuroprotective molecules (Parkin and DJ-1) and protect dopaminergic neurons in MPTP mouse model of Parkinson's disease (Khasnavis and Pahan, 2012, 2014). Recently, we have seen that cinnamon and NaB attenuate the activation of p21^{ras}, reduce the formation of reactive oxygen species and protect memory and learning in 5XFAD model of AD (Modi et al.,

2015). Here we delineate that NaB is also capable of improving plasticity in cultured hippocampal neurons. Our conclusion is based on the following: *First*, NaB upregulated the expression of a number of plasticity-associated molecules (NR2A, GluR1, Arc, and PSD95) in hippocampal neurons. *Second*, Gabra5 is known to support long-term depression (Isaac, 2001). It is interesting to see that NaB did not stimulate the expression of Gabra5 in hippocampal neurons. *Third*, NaB increased the number, size and maturation of dendritic spines in cultured hippocampal neurons, suggesting a beneficial role of NaB in regulating the synaptic efficacy of neurons. *Fourth*, we observed that NaB did not alter the calcium dependent excitability of hippocampal neurons, but rather stimulated inbound calcium currents in these neurons through ionotropic glutamate receptor. Together, these results clearly demonstrate that NaB is capable of increasing neuronal plasticity.

The signaling events required for the transcription of plasticity-related genes are becoming clear. Upon analysis of the NR2A promoter using the Genomatix Software Suite, we have found multiple binding sites for CREB and at least one consensus binding site for NF- κ B, C/EBP β and AP-1 in the NR2A promoter. Although these transcription factors (CREB, NF- κ B, C/EBP β , and AP-1) may play a role in the expression of various plasticity-associated genes, activation of CREB seems essential for the transcription of these molecules (Impey and Goodman, 2001; Roy and Pahan, 2015). Therefore, for a drug to stimulate plasticity, it is almost mandatory to stimulate the activation of CREB. Activation of CREB in cultured hippocampal neurons by NaB and abrogation of the ability of NaB to induce NR2A by siRNA knockdown of CREB suggest that NaB increases the levels of NR2A via CREB. However, it was unknown by which mechanisms NaB induced the activation of CREB in hippocampal neurons. 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, survival, and synaptic plasticity (Waltereit and Weller, 2003). Here we present evidence that NaB induced the activation of CREB via PKA. Although both PKA and PKC are known to phosphorylate and activate CREB, NaB specifically induced the activation of PKA, but not PKC. Accordingly, suppression of PKA by H-89 inhibited NaB-induced activation of CREB and expression of NR2A in hippocampal neurons.

While many molecules show plasticity-stimulating effect in cell culture models, very few exhibit efficacy *in vivo* in the brain. Therefore, we tested the efficacy of NaB and cinnamon itself in poor learning mice identified based on performance during spatial memory consolidation on Barnes maze. Interestingly, level of CREB activation and status of plasticity-related molecules were much less in the hippocampus of poor-performed mice than good-performed mice during memory consolidation, suggesting that memory consolidation induces the activation of CREB and increases the expression of plasticity-related genes in the hippocampus of good learning mice. Recently we have delineated that oral administration of cinnamon increases the level of NaB *in vivo* in the nigra (Jana et al., 2013) and hippocampus (Modi et al., 2015) of mice, suggesting that NaB produced from the metabolism of cinnamon is capable of entering into the CNS. In this case, oral administration of NaB and cinnamon increased the activation of CREB and CREB-associated plasticity-related molecules in the hippocampus of poor learning mice. Interestingly, hippocampus of poor learning mice expressed greater level of Gabra5 as

compared to good learning mice and treatment of poor learning mice with NaB and cinnamon decreased hippocampal Gaba5 to a level that is found in good learning mice. These results suggest that increased Gaba5 *in vivo* in the hippocampus may be linked to poor learning. Finally, cinnamon and NaB treatment also improved spatial learning and memory in poor learning mice to a level that is seen in good learning mice. We did not notice any side effect (e.g. hair loss, weight loss, untoward infection etc.) in any of the mice used during the course of the treatment with either cinnamon or NaB. Moreover, there are several advantages of NaB and cinnamon over other proposed plasticity-boosting therapies as both NaB and cinnamon are fairly nontoxic and cinnamon has been widely used as flavoring material and spice throughout the world for centuries. These results suggest that NaB and cinnamon should not cause health problems and that these compounds may have prospects in boosting plasticity in poor learners and in dementia patients.

In summary, we have demonstrated that cinnamon metabolite NaB upregulates plasticity-associated molecules and calcium influx in cultured hippocampal neurons via activation of CREB. While spatial memory consolidation-induced activation of CREB and expression of plasticity-related molecules were less in the hippocampus of poor learning mice as compared to good learning ones, oral administration of cinnamon and NaB increased memory consolidation-induced activation of CREB and expression of plasticity-related molecules *in vivo* in the hippocampus of poor learning mice and improved their memory and learning almost to the level that observed in untreated good learning ones. These results highlight a novel plasticity-boosting property of cinnamon and its metabolite NaB and suggest that this widely-used spice and/or NaB may be explored for stimulating synaptic plasticity and performance in poor learners.

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Fig. 1. Effect of NaB on the expression of plasticity-associated molecules in cultured mouse hippocampal neurons

Mouse hippocampal neurons were treated with either NaB or NaFO for 18 h followed by monitoring the protein levels of NR2A, PSD95, GluR1, and Gabra5 by Western blot (A). Bands were scanned and presented as relative to control (B). Results are mean $\pm$ SD of three independent experiments. $^a p < 0.001$ vs. control. Cells were treated with different concentrations of NaB for 18 h followed by monitoring the protein levels of NR2A, PSD95, GluR1, and Gabra5 by Western blot (C). Bands were scanned and presented as relative to control (D). Results are mean $\pm$ SD of three independent experiments. $^a p < 0.001$ & $^b p < 0.05$ vs. control. Cells were treated with either NaB or NaFO for 18 h followed by double-label immunofluorescence of Tuj1 & NR2A (E), Tuj1 & GluR1 (F) and Tuj1 & PSD95 (G). Results represent three independent analyses.

Fig. 2. Stimulation of morphological plasticity in cultured mouse hippocampal neurons by NaB
Cells were treated with either NaB or NaFO for 18 h followed by phalloidin labeling together with Tuj1 immunostaining (A). Number of spines (B), spine size (C), mature spines (D), mushroom spines (E), and stubby spines (F) were monitored in ten hippocampal neurons per group. Results are mean + SEM of 10 neurons per group.

Fig. 3. Effect of NaB on NMDA- and AMPA-dependent calcium influx in cultured mouse hippocampal neurons

Cells were treated with either NaB or NaFO for 18 h followed by treatment with 50 μ M NMDA (A) and AMPA (B) as described in the materials and methods section. Results are presented as the mean of three independent experiments.

Fig. 4. Role of CREB in NaB-mediated upregulation of NR2A in cultured mouse hippocampal neurons

Cells were treated with 100 μ M NaB for different minute intervals followed by monitoring the levels of phospho-CREB and total CREB by Western blot (A). Bands were scanned and values of P-CREB/CREB were presented as relative to control (B). Results are mean $\pm$ SD of three independent experiments. $^a p < 0.001$ vs. control. Cells were transfected with CREB siRNA and control siRNA. After 48 h, cells were treated with NaB for 18 h followed by monitoring the level of CREB by Western blot (C). Tubulin was run as loading control. Bands were scanned and values of CREB/Tubulin were presented as relative to control (D). Similarly, after siRNA knockdown of CREB and NaB treatment, the level of NR2A was monitored by Western blot (E). Bands were scanned and values of NR2A/Tubulin were presented as relative to control (F). Results are mean $\pm$ SD of three independent experiments. $^a p < 0.001$ vs. control; $^b p < 0.001$ vs. NaB. Cells were treated with 100 μ M NaB for different minute intervals followed by monitoring the activities of PKA (G) and PKC (H). Results are mean $\pm$ SD of three independent experiments. $^a p < 0.01$ vs. control; ns, not significant. Cells preincubated with different concentrations of H-89 for 30 min were treated with NaB for 30 min followed by monitoring the levels of phospho-CREB and total CREB by Western blot (I). Bands were scanned and values of P-CREB/CREB were presented as relative to control (J). Results are mean $\pm$ SD of three independent experiments. $^a p < 0.001$ vs. control; $^b p < 0.01$ vs. NaB. Cells preincubated with different concentrations of H-89 for 30 min were treated with NaB for 18 h followed by monitoring the level of NR2A by Western blot (K). Tubulin was run as loading control. Bands were scanned and values of NR2A/

Tubulin were presented as relative to control (L). Results are mean $\pm$ SD of three independent experiments. ^a $p < 0.001$ vs. control; ^b $p < 0.001$ vs. NaB.

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Fig. 5. Status of CREB activation and plasticity-related molecules in the hippocampus of good and poor learning mice

A) Schematic representing the Barnes maze procedure employed for the selection of good-performed and poor-performed 6-8 week old male C57/BL6 mice. B) Training elucidates two behaviorally distinct groups of mice. Representative track plots of good-performed (green) and poor-performed (red) mice. Latency (C) and number of errors (D) throughout the trial were quantified for good-performed (green; $n=6$) and poor-performed (red; $n=6$) mice. Results are mean + SEM of six mice per group. $^a p < 0.001$ vs. poor-performed. DNA-binding activity of CREB was monitored by EMSA in nuclear extracts of hippocampal tissues isolated from poor-performed and good-performed mice (E). Results represent analysis of four mice per group. Levels of NR2A, GluR1 and PSD95 were monitored in hippocampal tissues of poor-performed and good-performed mice by Western blot (F). Bands were scanned and values of NR2A/Tubulin (G), GluR1/Tubulin (H) and PSD95/Tubulin (I) were presented as relative to poor-performed mice. Results are mean $\pm$ SEM of four mice per group. $^a p < 0.001$ vs. poor-performed.

Fig. 6. Oral administration of cinnamon and NaB upregulates the activation of CREB and stimulates the expression plasticity-associated molecules in the hippocampus of poor learning mice

Poor-performed mice were treated with cinnamon (50 mg/kg body wt/d) and NaB (50 mg/kg body wt/d) via gavage. A group of poor-performed mice also received 100 μ l 0.5% methylcellulose as vehicle. After 30 d of treatment, mice were trained and tested on Barnes maze as described in Figure 5A. Twenty-four h after the test, the DNA-binding activity of CREB was monitored in hippocampal nuclear extracts by EMSA (A). Results represent analysis of four mice per group. The mRNA expression of neuronal activity-related molecules (Egr1, Fos, Bdnf, Homer1, Arc, Snk, & Spar) (B & C), synaptic formation-associated genes (Synapsin, ProSap2, Nr1h1, Nr1h3, Agrin, & CamkIIa) (D & E) and synaptic plasticity-related genes (GluR1, GluR4, Nr2a, & Nr2b) (F & G) was examined in the hippocampus by RT-PCR. The protein levels of NR2A, PSD95, GluR1, & Gaba5 was also monitored in the hippocampus by Western blot (H&I, cinnamon; J&K, NaB). Results are mean + SEM of four mice per group. ^a $p < 0.001$ vs. good-performed; ^b $p < 0.001$ vs. poor-performed.

Fig. 7. Oral administration of cinnamon and NaB improves memory and learning in poor learning mice

Poor-performed mice were treated with cinnamon (50 mg/kg body wt/d) and NaB (50 mg/kg body wt/d) via gavage. As control, one group of poor-performed mice received 100 μ l 0.5% methylcellulose as vehicle. For comparison, one group of good-performed mice also received cinnamon. After 30 d of treatment, mice were tested for Barnes maze (A, track plot; B, latency; C, number of errors), T maze (D, positive turns; E, negative turns) and general locomotor activities (F, distance travelled; G, speed; H, stereotypy). Eight mice ($n=8$) were used in each group. Results are mean $\pm$ SEM of eight mice per group. ^a $p<0.001$ vs. good-performed; ^b $p<0.01$ vs. poor-performed.