Journal of Alzheimer's Disease xx (20xx) x–xx
DOI 10.3233/JAD-2012-121309
IOS Press

Brain Cholinergic Markers and Tau Phosphorylation are Altered in Experimental Type 1 Diabetes: Normalization by Electroacupuncture

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Accepted 29 August 2012

Abstract. Diabetes often correlates with tau phosphorylation and the development of Alzheimer's disease. Both are associated with brain cholinergic dysfunction that could benefit from nerve growth factor (NGF)-based therapies. Electroacupuncture (EA) improves brain NGF availability and action. Here we assessed the variations of NGF and tau phosphorylation in the cortex and hippocampus, as well as the expression of choline acetyltransferase in the basal forebrain following diabetes induction and EA in adult rats. We found that EA counteracts diabetes-associated tau hyperphosphorylation and decreases in NGF and choline acetyltransferase, suggesting a possible beneficial effect of EA on brain cholinergic system in diabetes.

Keywords: Choline acetyltransferase, diabetes, electroacupuncture, nerve growth factor, tau phosphorylation

Supplementary data available online: http://www.j-alz.com/issues/33/vol33-3.html#supplementarydata04

INTRODUCTION

A link is emerging between diabetes and brain pathologies, such as Alzheimer's disease (AD), associated with cholinergic dysfunctions [1, 2] and altered metabolism of the microtubule-associated protein tau [2, 3]. Higher AD incidence in diabetic patients [1] and hyperphosphorylation of tau in diabetic animals [4–6] have been reported.

Physical therapies with sensory fibers activation [7], such as aerobic exercise, electroacupuncture (EA), and transcutaneous electrical nerve stimulation, improve learning-memory [8–10], modulate brain neurotransmitters [11] and neurotrophins [12], induce brain neurogenesis [13, 14], and decrease amyloid plaques deposition [15].

The efficacy of EA on central cholinergic deficit could be correlated to its effects on the modulation of nerve growth factor (NGF) [16], a neurotrophin indicated as a possible pharmacological tool in AD [17]. The activation of receptor tyrosine-kinase A (TrkA) by NGF promotes the expression of cholinergic markers [18] and regulates tau phosphorylation [19, 20]. However, the clinical use of NGF has been hampered by side-effects [21] that can be avoided by the activation of endogenous NGF activity, like that promoted by EA [16].

We investigated the variations of TrkA and choline acetyltransferase (ChAT) expression in neurons of the

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ISSN 1387-2877/12/$27.50

basal forebrain complex (BFC), which receive NGF as trophic support from their projection nuclei in the cortex and hippocampus, as well as of NGF content and tau phosphorylation in the cortex and hippocampus after induction of type 1 diabetes mellitus and EA treatments in adult rats.

MATERIALS AND METHODS

Experimental plan

Type 1 diabetes mellitus was induced in adult female Sprague-Dawley rats (Harlan-Nossan, Italy) by an i.p. injection of 65 mg/kg streptozotocin (STZ) (Sigma-Aldrich, Italy) dissolved in 20 mM citrate buffer pH 4.5 (vehicle) [22]. One week later, hyperglycemia was checked by Accutrend®GC (Roche Diagnostic Gmbh, Germany). Rats with blood glucose above 300 mg/dl were enrolled in STZ groups. Forty-four rats were divided as follows ($n = 12$ each group): Controls were injected once with vehicle; STZ rats received STZ as described above; EA and STZ + EA rats received low frequency EA for 3 consecutive weeks starting 1 week after STZ. One day after the last EA session, 8 rats for each group were killed by decapitation, tissues collected, and stored at −80°C. Four rats for each group were trans-cardially perfused with 4% paraformaldeyde dissolved in PBS, the brain removed and processed for immunohistochemistry. All procedures were in compliant with European regulations and approved by intramural Ethical Committee.

Electroacupuncture

Rats of the EA groups received 30 min sessions of EA twice a week for 3 weeks as described [23]. Details about the EA procedure are given in the Supplementary data (available online: http://www.j-alz.com/issues/33/vol33-3.html#supplementarydata04).

NGF assay and western blot

Samples were ultra-sonicated in extraction buffer as described [23], centrifuged and supernatants recovered. NGF content was assessed by commercial ELISA (R&D Systems DY556, Space ImportExport, Italy) following manufacturer's instructions.

For western blot, 20 μg of total protein were separated by SDS-PAGE and transferred to PVDF membrane. The membranes were incubated 1 h with 5% non-fat dry milk in TTBS (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20), then washed in TTBS and incubated with primary antibodies (summarized in Table 1) at 4°C overnight. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling Technology, USA) were used as secondary antibodies. Blots were developed with ECL substrate (Millipore Corporation, USA). Gel densitometry was performed as described at http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/. The density of GAPDH bands was used as normalizing factor.

Immunohistochemistry

Coronal brain sections (20 μm) were obtained from Control, STZ, and STZ + EA rats. Those corresponding to Bregma 1.00 to 0.60, containing BFC nuclei according to Paxinos atlas [24], were processed for immunohistochemistry. Slides were pre-incubated with 10% normal goat serum in PBS + 0.1% Triton X-100 (PBST) for 2 h and then incubated overnight at 4°C with a mix of antibodies against ChAT (Clone 17, previously described [25]) and TrkA (sc118, SantaCruz

Table 1
Summary of antibodies used for western blot and immunofluorescence analysis

Antibody	Specificity	Type	Source
25778	GAPDH	Polyclonal	Santa Cruz Biotech.
Clone 15	Tau (total)	Monoclonal	Transduction Lab.
AD2	phospho$_{Ser396/404}$-Tau	Monoclonal	Courtesy of Prof. A. Delacourte
AT8	phospho$_{Ser202}$-Tau	Monoclonal	Innogenetics
pT262	phospho$_{Ser262}$-Tau	Polyclonal	Anaspec
pT231	phospho$_{Thr231}$-Tau	Polyclonal	Anaspec
9315	Gsk3β (total)	Polyclonal	Cell Signaling Technology
9336	phospho$_{Ser9}$-GSK3β	Polyclonal	Cell Signaling Technology
9212	p38 (total)	Polyclonal	Cell Signaling Technology
4631	phospho$_{Thr180\text{-}Tyr182}$-p38	Polyclonal	Cell Signaling Technology
Clone 17	Choline acetyltransferase (ChAT)	Monoclonal	Courtesy of Dr. C. Cozzari
sc-118	TrkA	Polyclonal	Santa Cruz Biotech

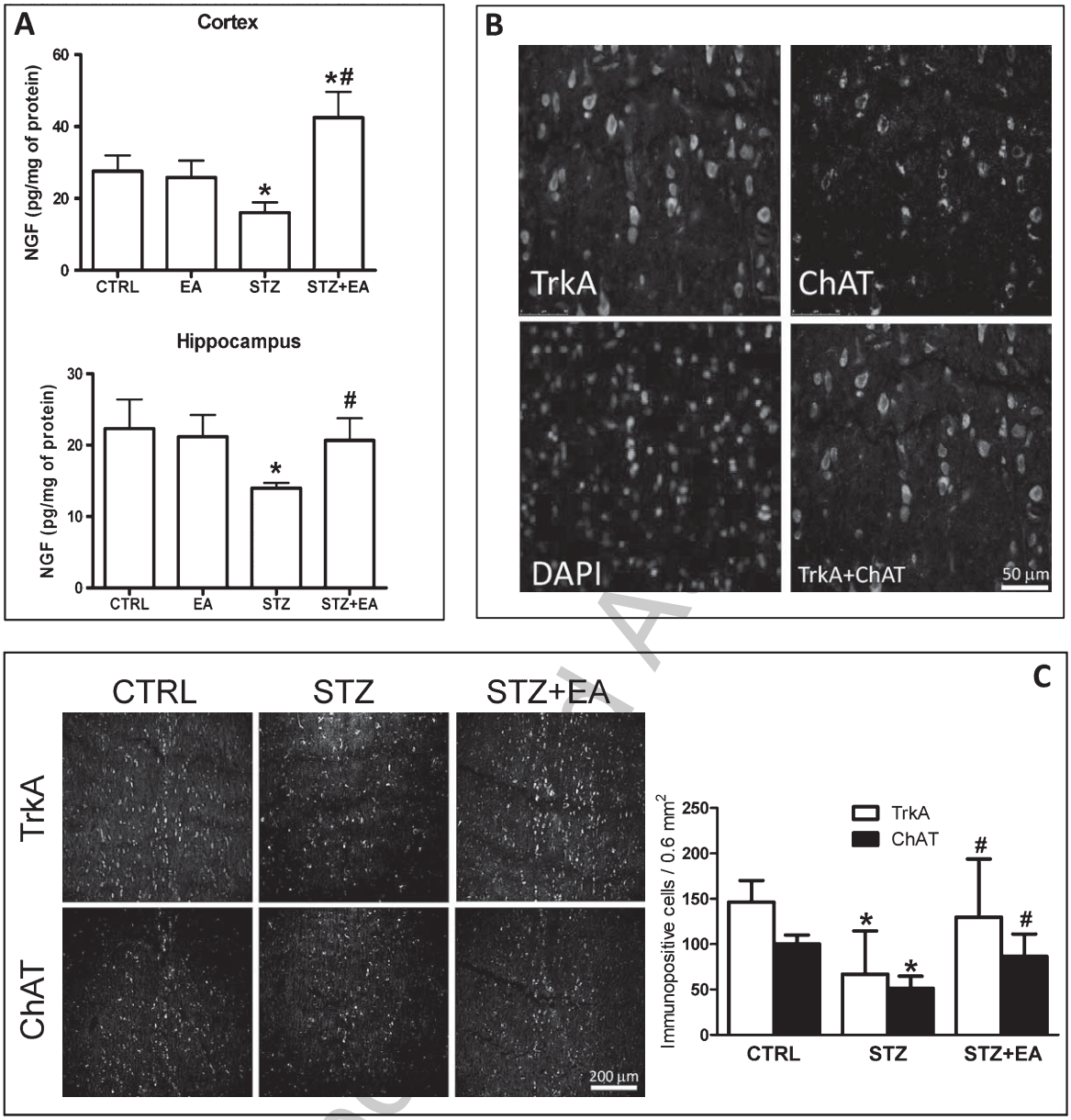


Fig. 1. Nerve growth factor (NGF), choline acetyltransferase (ChAT), and tyrosine-kinase A (TrkA) expression in the brain cholinergic system of diabetic rats are modulated by electroacupuncture (EA). The NGF levels (A) in the cortex and hippocampus of diabetic rats are decreased compared to Controls (STZ versus Controls, $p < 0.05$). Three weeks treatment with low-frequency EA did not exert significant effects on NGF brain levels in healthy rats (EA versus Controls, $p > 0.05$). EA treatments in diabetic rats counteracted STZ-induced NGF decrease (STZ versus STZ + EA, $p < 0.05$), increasing cortex and hippocampus NGF protein content toward Control levels. Results obtained by ELISA are presented as pg of NGF/mg of total tissue protein (mean ± S.D., $n = 8$, $^{*}p < 0.05$). Protein concentrations were determined by Biorad DC Protein assay (Life Science Group, Italy). (B) The expression of TrkA, ChAT, DAPI (for nuclei visualization), and colocalization of TrkA and ChAT in neurons of the medial septum (MS) is depicted (scale bar: 50 µm). TrkA immunopositive cells (C) are decreased in the MS of STZ-treated rats when compared to Controls. EA restores normal TrkA expression in the MS of STZ-treated rats. The same brain sections were also immunostained against ChAT. ChAT-stained neurons in the MS of STZ-treated rats are greatly reduced, when compared to Controls. The amount of ChAT immunostaining in the STZ + EA group is not different from Controls. For production of figures, brightness and contrast of images were adjusted by taking care to leave a light tissue fluorescence background for visual appreciation of the lowest fluorescence intensity features and to help comparison among the different experimental groups. (Scale bar: 200 µm). Cell count was performed on 2 non-adjacent sections for each brain ($n = 8$ sections each group) by NIH ImageJ software equipped with a plugin (NeurphologyJ) specifically developed for automatic quantification of morphological features (neurite length, neural soma quantification) in neuroscience. Data are expressed as mean immunopositive cell number ± S.D. $^{*}p < 0.05$ versus Control group. $^{\#}p < 0.05$ versus STZ group.

Biotech, USA) dissolved in PBST + 1% goat serum. In control slides, primary antibodies were replaced by purified rabbit and mouse IgG. After washing with PBST, slides were incubated for 1 h with a mixture of Alexa Fluor® 488 goat anti-rabbit IgG and Alexa Fluor® 594 goat anti-mouse IgG (Invitrogen Italy, Italy). Sections were examined under a confocal laser-scanning microscope (Leica SP5, Leica Microsystems, Germany). Two non-consecutive sections for each animal (8 sections/group) were analyzed for automated cell count by ImageJ software (http://rsbweb.nih.gov/ij/) and NeurphologyJ plugin [26].

Data analysis

Statistics were performed by the GraphPad 5 software (GraphPad Software Inc., USA) and data expressed as mean ± SD. Western blot, NGF-ELISA, and computerized image analysis data ($n = 8$ each group) were evaluated by one-way ANOVA and Tukey's HSD test. A $p < 0.05$ was considered significant. A summary of ANOVA results is presented in Supplementary Table 1.

RESULTS

NGF and BFC cholinergic markers

STZ decreased NGF in the cortex and hippocampus, while EA counteracted this effect (Fig. 1A). TrkA and ChAT almost completely co-localized in the medial septum (Fig. 1B), a nucleus of the BFC projecting to hippocampus and cortex [27]. STZ dramatically decreased the number of TrkA and ChAT positive cells, while EA significantly counteracted this effect (Fig. 1C).

Tau hyperphosphorylation

Western blots for tau and four different phospho-tau epitopes (see Table 1) are shown in Fig. 2A. STZ increased AD2 and AT8 phosphorylation relative to total tau in the cortex, while pT262 and pT231 were unaffected. EA was able to decrease the levels of AD2 and AT8 as well as pT262 in the cortex of diabetic animals (STZ versus STZ + EA: $p < 0.05$). Moreover, AD2, but not AT8, was increased in the hippocampus of STZ-treated rats while EA reduced both AD2/tau and AT8/tau ratios in diabetic rats.

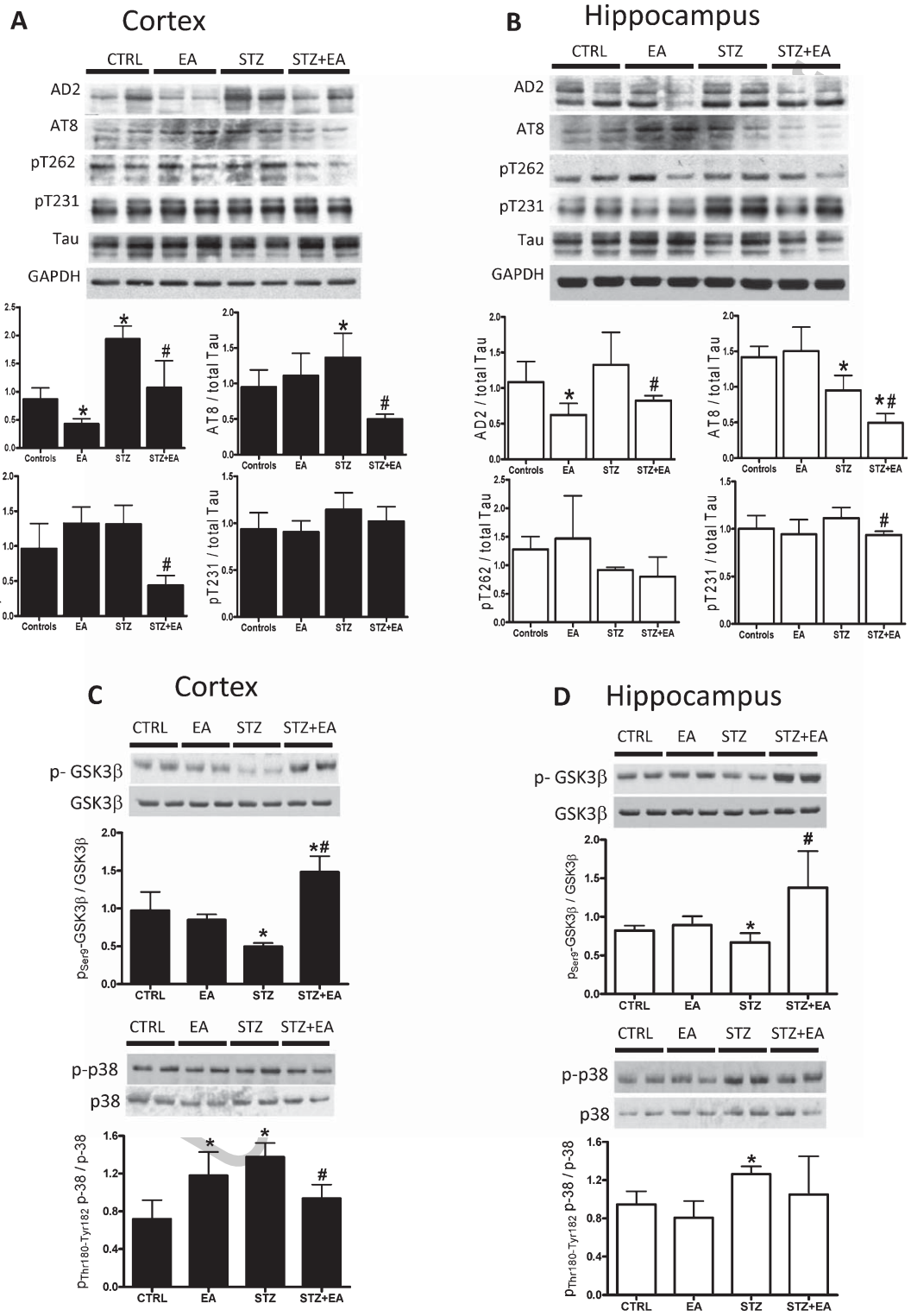
Tau kinases phosphorylation

Western blots for total and phospho$_{Ser9}$-GSK3β and total and phospho$_{Thr180\text{-}Tyr182}$-p38 kinases are shown in Fig. 2B. Both the active kinases (de-phosphorylated GSK3β or phosphorylated p38) have been associated with tau phosphorylation in diabetes [4, 28]. We found that phospho-GSK3β decreased and phospho-p38 increased in cortex and hippocampus after STZ, suggesting a connection with observed tau hyperphosphorylation. EA counteracted this STZ-induced effect in the cortex and hippocampus. Other kinases involved in tau phosphorylation, i.e., ERK1-2, JNKs and Akt, have been found not modulated by STZ or EA, while only ERK1-2 activation was increased in STZ + EA group (data not shown).

DISCUSSION

We used experimental type 1 diabetes mellitus to investigate the effect of EA on NGF, on NGF-related biomarkers TrkA and ChAT, and on tau phosphorylation in the brain cholinergic system, which is known to degenerate in AD and in diabetes [29]. EA, modulating

Fig. 2. EA counteracts the STZ-induced variation in tau and tau kinases phosphorylation in the brain. Representative western blots for total tau, phosphorylated tau (see Table 1 for a list of epitopes investigated) and GAPDH as loading control in the cortex (A) and hippocampus (B) of Controls, diabetic (STZ), EA-treated healthy (EA), and EA-treated diabetic rats (STZ + EA). Representative western blots for total and phosphorylated (Ser9)-GSK3β (p-GSK3β) and phosphorylated (Thr180Tyr182)-p38 (p-p38) kinases and GAPDH in the cortex (C) and hippocampus (D) of experimental groups. Graphs show the results of quantitative densitometry with GAPDH integrated optical density used as normalization factor. Statistics were performed on four separate gel run/blots over different sets of two samples for each experimental group ($n = 8$). Results are presented as mean ± S.D., $^{*}p < 0.05$ versus Controls, $^{\#}p < 0.05$ versus STZ. Cortex: The ratio between AD2, AT8, and total tau (A) increased after diabetes induction, indicating hyperphosphorylation of the two epitopes, and reverted to baseline by EA (STZ + EA versus STZ, $p < 0.05$; STZ + EA versus Controls, $p > 0.05$). STZ also induces a decrease of the p-Gsk3β/Gsk3β and an increase of p-p38/p38 ratios in the cortex (C, STZ versus Controls, $p < 0.05$), indicating a diabetes-associated increase in the activity of the two kinases. Both of the mentioned alterations in kinases phosphorylation were counteracted by EA (STZ + EA versus STZ, $p < 0.05$). Hippocampus: The ratio between AD2 and total tau (B) was not affected by STZ (STZ versus Controls, $p > 0.05$) but was decreased by EA in both healthy (EA versus Controls, $p < 0.05$) and diabetic rats (STZ + EA versus STZ, $p < 0.05$). The AT8/tau ratio (B) was decreased by diabetes (STZ versus Controls, $p < 0.05$) and further decreased by EA in STZ rats (STZ + EA versus STZ, $p < 0.05$). STZ induces a decrease of the p-Gsk3β/Gsk3β and an increase of p-p38/p38 ratios in the hippocampus (D, STZ versus Controls, $p < 0.05$), indicating a diabetes-associated increase in the activity of the two kinases. Both of the mentioned alterations in kinases phosphorylation were counteracted by EA (STZ + EA versus STZ, $p < 0.05$).


A
Cortex
CTRL
EA
STZ
STZ+EA
AD2
AT8
pT262
pT231
Tau
GAPDH
AD2 / total Tau
AT8 / total Tau
pT262 / total Tau
pT231 / total Tau
Controls
EA
STZ
STZ+EA
B
Hippocampus
C
Cortex
p- GSK3β
GSK3β
pSer9-GSK3β / GSK3β
p-p38
p38
pThr180-Tyr182 p-38 / p-38
D
Hippocampus

NGF synthesis/activity, might improve the function of damaged neurons in the central nervous system [16]. Similar to that observed in AD, STZ-induced diabetes has been associated with central cholinergic dysfunctions [29] and to altered NGF and NGF signaling in the brain [30]. We found that type 1 diabetes mellitus decreased NGF in the cortex and hippocampus and impaired the expression of TrkA and ChAT in BFC. We also found that EA-treatment can enhance the content of NGF in diabetic brain, with potential improvement in the function of BFC neurons. This is supported by the EA-induced increase of ChAT in the BFC, with ChAT expression in such nuclei directly regulated by the NGF [31] produced in the cortex and hippocampus.

Both AD and experimental diabetes are characterized by tau hyperphosphorylation [1, 4, 6, 32]. Though restricted to four phosphorylation sites among several described in tauopathies [32], our data confirm that experimental type 1 diabetes mellitus correlates with disturbance of tau metabolism [4] that we found in the cortex of diabetic rats. Other studies also found tau hyperphosphorylation in the hippocampus of diabetic male rats [28]. We only found STZ-induced alterations in tau kinases in the hippocampus, and it is conceivable that the lack of tau hyperphosphorylation depends on experimental timeframe and/or on gender difference in the response to STZ [33]. We also demonstrated that EA counteracted STZ-induced increase in tau phosphorylation, likely by modulating the activity of GSK3β and p38 kinases, which have been previously found deregulated in the STZ model [4, 28] and have been correlated with tauopathy in AD [32]. However, we cannot exclude that phosphatases involved in tau metabolism could also be modulated by STZ [28] and/or EA.

Our data suggest the possibility that EA normalizes tau kinases activity by improving cholinergic neurotransmission from BFC to the cortex and hippocampus [34]. It is known that physical exercise, sharing with EA common physiological substrates [7], improves cholinergic functions by stimulating NGF action in STZ-treated rats [12]. It has also been demonstrated that acupuncture reversed the corticosterone-induced decrease of ChAT in the BFC [35] and that EA improves cholinergic-related behavioral tasks in stressed mice [36], indicating that peripheral needling could influence the phenotypic features of BFC neurons. Here we postulate that EA induces an enhancement of NGF delivery from cortex and hippocampus to their afferent nuclei in the BFC. The NGF-driven augmentation of BFC cholinergic neurotransmission could in turn result in an activity-dependent decrease of tau hyperphosphorylation in the cortex and the hippocampus. Further studies using cholinergic antagonists and/or NGF blockers will clarify the proposed mechanism.

We cannot exclude that tau dysmetabolism in our experimental model could be secondary not only to hypoglycemia but also to decrease of body temperature induced by STZ, though this seems unlikely in our experimental conditions, given that reduction of body temperature has been reported to occur 30 days after STZ treatment [37]. Nevertheless, we demonstrated the effectiveness of EA in counteracting tau hyperphosphorylation in experimental diabetes suggesting a therapeutic link among EA, the NGF system, and cholinergic neurotransmission.

ACKNOWLEDGMENTS

We acknowledge Prof. A. Delacourt (Inserm, Lille Cedex, France) for providing AD2 antibody and Dr. C. Cozzari (Institute of Cellular Biology and Neurobiology, CNR, Italy) for ChAT clone 17 antibody. This work was supported by PRIN 2009KP83CR to NC.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1505).

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