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LONG-TERM ELECTROMAGNETIC FIELD TREATMENT ENHANCES BRAIN MITOCHONDRIAL FUNCTION OF BOTH ALZHEIMER'S TRANSGENIC MICE AND NORMAL MICE: A MECHANISM FOR ELECTROMAGNETIC FIELD-INDUCED COGNITIVE BENEFIT?

N. DRAGICEVIC,[a,b] P. C. BRADSHAW,[a] M. MAMCARZ,[a] X. LIN,[c] L. WANG,[a] C. CAO[c,d] AND G. W. ARENDASH[a,e]*

[a]*Department of Cell Biology, Microbiology, and Molecular Biology, University of South Florida, FL 33620, USA*

[b]*Department of Radiology, University of South Florida, FL 33620, USA*

[c]*USF Health/Byrd Alzheimer's Disease Research Center, Tampa, FL 33613, USA*

[d]*Department of Molecular Pharmacology and Physiology, University of South Florida, Tampa, FL 33620, USA*

[e]*The Florida Alzheimer's Disease Research Center, Tampa, FL 33613, USA*

Abstract—We have recently reported that long-term exposure to high frequency electromagnetic field (EMF) treatment not only prevents or reverses cognitive impairment in Alzheimer's transgenic (Tg) mice, but also improves memory in normal mice. To elucidate the possible mechanism(s) for these EMF-induced cognitive benefits, brain mitochondrial function was evaluated in aged Tg mice and non-transgenic (NT) littermates following 1 month of daily EMF exposure. In Tg mice, EMF treatment enhanced brain mitochondrial function by 50–150% across six established measures, being greatest in cognitively-important brain areas (e.g. cerebral cortex and hippocampus). EMF treatment also increased brain mitochondrial function in normal aged mice, although the enhancement was not as robust and less widespread compared to that of Tg mice. The EMF-induced enhancement of brain mitochondrial function in Tg mice was accompanied by 5–10 fold increases in soluble Aβ1-40 within the same mitochondrial preparations. These increases in mitochondrial soluble amyloid-β peptide (Aβ) were apparently due to the ability of EMF treatment to disaggregate Aβ oligomers, which are believed to be the form of Aβ causative to mitochondrial dysfunction in Alzheimer's disease (AD). Finally, the EMF-induced mitochondrial enhancement in both Tg and normal mice occurred through non-thermal effects because brain temperatures were either stable or decreased during/after EMF treatment. These results collectively suggest that brain mitochondrial enhancement may be a primary mechanism through which EMF treatment provides cognitive benefit to both Tg and NT mice. Especially in the context that mitochondrial dysfunction is an early and prominent characteristic of Alzheimer's pathogenesis, EMF treatment could have profound value in the disease's prevention and treatment through intervention at the mitochondrial level. © 2011 Published by Elsevier Ltd on behalf of IBRO.

Key words: Alzheimer's, mitochondrial function, electromagnetic fields, transgenic mice.

Mitochondria are considered essential organelles for life and death of a cell. They are responsible for ATP production via oxidative phosphorylation and have major roles in many cellular functions, including reactive oxygen species generation, apoptosis, and Ca^{2+} homeostasis (Nicholls, 2002). There is accumulating evidence from *in vitro*, *in vivo*, and human neurologic tissue studies supporting the concept that mitochondrial abnormalities are early and common events in Alzheimer's disease (AD) progression and pathogenesis (Galindo et al., 2010; Morais and De Strooper, 2010; Reddy et al., 2010; Chen and Yan, 2010). Mitochondrial dysfunction in AD brains is in part characterized by increased oxidative stress, altered activity of key enzymes of the Krebs cycle (α-ketoglutarate dehydrogenase and pyruvate dehydrogenase), and by decreased Complex IV activity (Parker, 1991; Maurer et al., 2000; Reddy and Beal, 2005; Lin and Beal, 2006). In some AD studies, the decreased Complex IV activity has been accompanied by increased free-radical generation, apoptosis, disruption of Ca^{2+} homeostasis, impaired energy metabolism, reduced levels of ATP, and decreased mitochondrial membrane potential (Offen et al., 2000; Reddy and Beal, 2005; Cardoso et al., 2004; Keil et al., 2004; Lin and Beal, 2006).

A hallmark of AD is the presence of neuritic plaques, primarily in cognitively-important brain areas such as the cortex and hippocampus. The major component of these plaques is a core comprised of aggregated amyloid-β peptide (Aβ) in two most abundant forms, Aβ40 and Aβ42.

Well before Aβ aggregates extracellularly to form the core of neuritic plaques, it is present intraneuronally in high amounts due to proteolytic cleavage of amyloid precursor protein (APP). In Aβ-producing transgenic mice (see below), brain mitochondrial dysfunction and cognitive impairment occur early in adulthood, when Aβ is only present intraneuronally (Eckert et al., 2008; Hauptmann et al., 2009). This is consistent with mounting evidence indicating Aβ-induced mitochondrial dysfunction as an early and central event in AD pathogenesis (Devi et al., 2006; Galindo et al., 2010; Morais and De Strooper, 2010; Reddy et al., 2010). Both monomeric and oligomeric "soluble" forms of Aβ have been found in mitochondrial membranes from AD brains (Anandatheerthavarada et al., 2003; Caspersen et al., 2005; Manczak et al., 2006; Devi et al., 2006; Petersen

*Correspondence to: G. W. Arendash, Department of Cell Biology, Microbiology, and Molecular Biology, University of South Florida, Tampa, FL 33620, USA. Tel: +1-813-732-9040; fax: +1-813-974-1614.
E-mail address: arendash@cas.usf.edu (G. W. Arendash).

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β peptide; EMF, electromagnetic field; FCCP, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; GMSK, Gaussian minimal-shift keying; Tg, transgenic; NT, non-transgenic; RCR, respiratory control ratio; SAR, specific absorption rate; TMPD, tetramethylphenylenediamine.

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et al., 2008; Yao et al., 2009). In neurons of AD brains, Complex IV of the electron transport chain may be particularly vulnerable since it is a direct target of Aβ (Price and Sisodia, 1998; Muller et al., 2010). Moreover, Aβ administration to isolated mitochondria induces mitochondrial dysfunction (Casley et al., 2002) and facilitates mitochondrial permeability transition opening (Parks et al., 2001), the latter of which is a key event in cell death. Although the mechanism through which Aβ accumulation in mitochondria causes their dysfunction is not known, Aβ oligomers (and not monomeric Aβ) are thought to mediate the mitochondrial dysfunction of AD (Mattson et al., 2008).

Genetically-manipulated mouse models for AD, based on brain production and aggregation of human Aβ (monomers → oligomers → fibrils), have been utilized in recent years as the primary vehicle for developing potential therapeutics against AD (Olcese et al., 2009; Sanchez-Ramos et al., 2009; Arendash et al., 2009). For the two most utilized of these transgenic models (e.g. the APPsw and APPsw+PS1 mouse), this process of Aβ production/aggregation occurs sooner and to a greater extent in APPsw+PS1 mouse, resulting in earlier/more robust cognitive impairment. We have recently analyzed Aβ levels and mitochondrial function in "synaptic mitochondria" from 12-month old APPsw, APPsw+PS1, and normal (non-transgenic) mice (Dragicevic et al., 2010). Similar to human AD brains, we found highly significant mitochondrial dysfunction/hypofunction in APPsw and APPsw+PS1 mice. Moreover, this mitochondrial dysfunction was directly linked to Aβ levels and cognitive dysfunction in that: (1) APPsw+PS1 mice had higher synaptic Aβ levels and higher mitochondrial dysfunction (compared to APPsw mice) for any given brain area, and (2) APPsw+PS1 mice had greater and more extensive cognitive impairment compared to APPsw mice (Dragicevic et al., 2010). Our study is consistent with earlier studies showing mitochondrial dysfunction in individual AD transgenic lines (Smith et al., 1998; Casley et al., 2002; Li et al., 2004) and the likelihood that Aβ within synaptic brain mitochondria is involved in this dysfunction (Mungarro-Menchaca et al., 2002; Gillardon et al., 2007).

Given the above findings, therapeutics that target enhancement of mitochondrial function and/or decrease mitochondrial oxidative stress may provide substantive cognitive benefits. A purported mitochondrial-enhancing drug called Dimebon (latrepiridne) was initially reported to provide surprising cognitive benefits to AD patients in Russia (Doody et al., 2008). However, a large multi-site Phase III follow-up study in the US recently reported no cognitive benefits of Dimebon on multiple measures evaluated over a 26 weeks period in AD patients (NCT00838110, CONNECTION trial). Although *in vitro* cell culture studies have suggested that Dimebon enhances mitochondrial function (Bachurin et al., 2003; Zhang et al., 2010), the concentrations of Dimebon needed to affect mitochondrial function *in vitro* are far above physiologic range (Supnet and Bezprozvanny, 2010). Rather, Dimebon in physiologic concentrations appears to exert only non-mitochondrial actions, such as inhibition of brain serotoninergic receptors (Wu et al., 2008; Okun et al., 2009). Thus, any future studies to determine if Dimebon has any cognitive efficacy in AD transgenic mice would not entail mitochondrial enhancement as a possible mechanism.

To maximize the prospects that a proposed AD therapeutic will enhance mitochondrial function and provide true clinical benefits against AD in humans, it is highly desirable to demonstrate the ability of that therapeutic to enhance mitochondrial function in an established animal model for AD wherein the therapeutic has been found to provide cognitive benefit. The current study forwards electromagnetic field (EMF) treatment into that prospectus. We have recently published the first evidence that high frequency EMF treatment (two 1-h periods daily at 918 MHz, 0.25–1.05 specific absorption rate (SAR), pulsed and modulated) over months protects AD transgenic (Tg) mice from cognitive impairment, reverses established cognitive impairment in aged Tg mice, and boosts the memory of normal mice (Arendash et al., 2010). Mechanistically, we found both *in vitro* and *in vivo* evidence for EMF-induced suppression of brain Aβ aggregation in these AD mice, presumably resulting in increased Aβ clearance from the brain (Arendash et al., 2010).

Although the effects of EMF exposure on mitochondrial function have been essentially unexplored, we rationalized that if EMF treatment benefits both Tg and non-transgenic (NT) mice, some common/generalized mechanism could be involved and that mitochondrial enhancement could be that common mechanism of cognitive benefit. Therefore, the present study was designed to determine the long-term effects of EMF treatment on brain mitochondrial function in both aged AD transgenic mice and littermate normal mice. In this first study to investigate high frequency EMF effects on brain mitochondrial function in any animal, we report that long-term EMF treatment enhances the impaired brain mitochondrial function of aged APPsw+PS1 transgenic mice, as well as brain mitochondrial function of aged normal mice. Moreover, these EMF-induced benefits occur through non-thermal mechanisms and, for transgenic mice, apparently through disaggregation of Aβ oligomers associated with brain mitochondria.

EXPERIMENTAL PROCEDURES

Animals

All mice in these studies were derived from The Florida Alzheimer's Disease Research Center's colony. Each mouse had a mixed background of 56.25% C57, 12.5% B6, 18.75% SJL, and 12.5% Swiss-Webster. Mice were derived from a cross between heterozygous mice carrying the mutant APPK670N, M671L gene (APPsw) with heterozygous PS1 (Tg line 6.2) mice to obtain F11 generation mice consisting of APPsw+PS1, APPsw, PS1, and NT genotypes. After weaning and genotyping, only APPsw+PS1 (Tg) and NT mice were selected for these studies. All mice were maintained on a 12 h dark and 12 h light cycle with *ad libitum* access to rodent chow and water. All animal procedures were performed in AAALAC-certified facilities under protocols approved by Institutional Animal Care and Use Committees at the University of South Florida. In addition, all ARRIVE guidelines were followed.

EMF treatment protocol

At 15–17 months of age, APPsw+PS1 (Tg) and NT mice were divided into the following four treatment groups, with a total of three to four mice per group: Tg+EMF, Tg controls, NT+EMF, and NT controls. Tg and NT mice exposed to EMFs were individually-housed in cages within a large Faraday cage, which also housed the antenna of an EMF generator providing two 1-h periods of electromagnetic waves per day (early morning and late afternoon). Each EMF exposure was at 918 MHz frequency, involved modulation with Gaussian minimal-shift keying (GMSK) signal, and was non-continuous with carrier bursts repeated every 4.6 ms, giving a pulse repetition rate of 217 Hz. The electrical field strength varied between 17 and 35 V/m. This resulted in calculated SAR levels that varied between 0.25 and 1.05 W/kg. SAR was calculated from the below equation, with σ (0.88 s/m) and ρ (1030 kg/m^{-3}) values attained from Nightingale et al. (1983):

$$SAR = \sigma E^2/\rho$$

σ=mean electrical conductivity of mouse brain tissue.
ρ=mass density of mouse brain.
E=electrical field strength.

For the 1-month period of EMF treatment, cages of individually housed mice were maintained within a Faraday cage (1.2×1.2×1.2 m^3) and arranged in a circular pattern, with each cage approximately 26 cm from a centrally-located EMF-emitting antenna. The antenna was connected to a Hewlett–Packard ESG D4000A digital signal generator (Houston, TX, USA) set to automatically provide two 1-h exposures per day. The resulting EMF transmission to the mice of these studies is equivalent to the head transmission occurring for standard cell phone use in humans, although any direct comparison between our "whole-body" mouse and "head-only" human EMF exposure should be made with caution. With a 12-h light On/Off cycle, the 1-h daily exposures occurred in early morning and late afternoon of the lights on period. Sham-treated control Tg and NT mice were located in a completely separate room, with identical room temperature as in the EMF exposure room and with animals individually-housed in cages that were arranged in the same circular pattern.

Body and brain temperature measurements

On the day prior to euthanasia, all mice had both body temperature (via rectal probe) and brain temperature (via temporalis muscle probe) taken mid-way "during" and midway "between" both EMF exposures. Each measurement only took a couple of minutes for each mouse. Prior studies have demonstrated that temporalis muscle temperature very accurately reflects brain temperature in rodents (Shimizu et al., 1997; Brambrink et al., 1999).

Isolation of total brain mitochondria from mice

Following the 1-month long EMF or sham treatment period (at 16–18 months of age), animals were euthanatized after their morning EMF treatment session, using CO_2 asphyxiation and decapitated as previously described (Brown et al., 2004). Total mitochondria isolation was performed using slightly modified procedures (Sullivan et al., 2003; Dragicevic et al., 2010). First, brains were quickly removed and placed on ice. The cerebral cortex, hippocampus, striatum, and amygdala were carefully dissected out bilaterally, tissues from each area/genotype/treatment of several animals from each group were pooled together, then placed in a glass Dounce homogenizer containing five times the volume of isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 1 mM EGTA, 20 mM HEPES (Na^+), pH 7.2). For each brain area and genotype/treatment group, tissue from several animals had to be combined in order to get a sufficient yield of mitochondria. The entire procedure was repeated once more (with the several remaining mice from each group). This protocol provided two different runs of pooled samples (n=2), with each run repeated three times. Following homogenization, a low-speed spin (1300×g for 5 min) to remove unbroken cells and nuclei was performed, the supernatant was carefully placed in fresh tubes, topped off with isolation buffer, and spun down again at 13,000×g for 10 min. Supernatant was discarded and resultant mitochondrial pellets were suspended in 500 μl isolation buffer with EGTA [215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES (Na^+), pH 7.2] and 0.1% digitonin (in DMSO) was added to the pellets to disrupt synaptosomes. After 5 min, samples were brought to final volume of 2 ml using isolation buffer with EGTA and centrifuged at 13,000×g for 15 min, with pellets resuspended in isolation buffer without EGTA (75 mmol/L sucrose, 215 mmol/L mannitol, 0.1% BSA, and 20 mmol/L HEPES with the pH adjusted to 7.2 using KOH) and centrifuged at 10,000×g for 10 min. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a final concentration of approximately 10 mg/ml. The tube containing the mitochondrial suspension was then submerged in ice for storage (approximately 4 °C) for up to 2 h until the experiments were performed. To normalize the mitochondrial function results, the protein concentrations were determined with all the samples on the same plate using the BCA protein assay kit and measuring absorbance at 560 nm with a Biotek Synergy HT plate reader.

Respiratory measurements

The respiratory function of isolated total mitochondria was measured using a miniature Clark type oxygen electrode (Strathkelvin Instruments, MT200A chamber, Glasgow, UK). 100 μg (0.3 mg/ml final concentration) of mitochondria were suspended in a sealed, constantly stirred and thermostatically controlled chamber at 37 °C containing 350 ml of respiration buffer (125 mM KCl, 1 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM pyruvate, 2.5 mM malate, 500 uM EGTA, 20 mM HEPES, pH 7.0) at 37 °C. State II (basal) respiration was measured directly following mitochondrial suspension in the buffer. State III respiration was assessed by the addition of 200 mM ADP. State IV respiration was achieved by addition of 1 μM oligomycin. The respiratory control ratio (RCR) was determined by dividing the rate of oxygen consumption for state III by that of state IV. Coupling of the ETC is assessed by the RCR. Values of less than 2 for RCR indicate poorly coupled mitochondria. Proton (the flow of protons back into the matrix space through pathways other than the F_1F_0 ATP synthase) increases state IV respiration and decreases the RCR. Maximum (state V) respiration was assessed by addition of 1 μM carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation.

Reactive oxygen species production

Mitochondrial reactive oxygen species (ROS) production was measured using 25 μM 2',7'-dichlorodihydrofluorescein diacetate. This compound is cleaved by intramitochondrial esterases and oxidized to fluorescent dichlorofluorescein, (DCF) (excitation 485 nm, emission 530 nm) and was measured in a Biotek Synergy 2 microplate reader as previously described (Brown et al., 2004). 100 μg (0.8 mg/ml final concentration) of isolated nonsynaptic mitochondria were added to 120 μl of KCl-based respiration buffer (see above) with 5 mM pyruvate and 2.5 mM malate added as respiratory substrates and 25 μM 2',7'-dichlorodihydrofluorescein. ROS production was expressed as the DCF fluorescence after a 20 min incubation period and presented in relative fluorescence units. Mitochondrial ROS production in the presence of oligomycin (inducer of increased ROS production) or FCCP (to decrease ROS production) was performed to ensure measurement values were within the range of the indicator.

Mitochondrial membrane potential measurements

A 200 mM solution of JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) was made using DMSO as the solvent. The assay buffer contained mitochondrial isolation buffer with the addition of 5 mM pyruvate and 5 mM malate. 150 μl of assay buffer and 20 μl (1.2 mg/ml final concentration) of mitochondria were added to the wells of a 96 well black microplate (Corning) followed by addition of 1 μM JC-1 and mixed gently. The microplate was covered with aluminium foil and left at room temperature for 20 min before reading. Fluorescence (excitation 530/25 nm, emission 590/30 nm) was measured using a Biotek Synergy 2 multi-mode microplate reader. Fluorescence was expressed in relative fluorescence units.

ATP levels

The ATP determination kit containing D-luciferin, luciferase [40 μl of a 5 mg/ml solution in 25 mM Tris·acetate, pH 7.8, 0.2 M ammonium sulfate, 15% (vol/vol) glycerol, and 30% (vol/vol) ethylene glycol], dithiothreitol (DTT), ATP, and a reaction buffer (10 ml of 500 mM tricine buffer, pH 7.8, 100 mM $MgSO_4$, 2 mM EDTA, and 2 mM sodium azide) was purchased from by Molecular Probes (Eugene, OR, USA). The reagents and reaction mixture were combined according to the protocol by Molecular Probes. In addition, 1 mM pyruvate and 1 mM malate were added to the reaction mixture as substrates for oxidative phosphorylation. Total mitochondria from brain regions of interest were isolated following standard protocol (see mitochondrial isolation in methods). For all experiments, ATP standard curves were run in the range of 0.5–50 μM (0.5, 1.0, 5.0, 10.0, 25.0, and 50.0 μM, respectively). To test the functionality of the ATP determination assay, 0, 1, and 5 mM oligomycin were added and incubated for 10 min at 37 °C to freshly isolated mitochondria. The inhibition in the rate of ATP production was determined by comparing the oligomycin-treated mitochondria to an equal portion of freshly isolated mitochondria from the same tissue. The luminescent signals for six ATP standards ranging from 0.5 to 50 μM (0.5, 1.0, 5.0, 10.0, 25.0, and 50.0 μM, respectively) were measured using Biotek Plate Reader. After the calibration curves were run, ATP levels from the tissue samples were quantified. Firefly luciferase was added to the luciferin-containing ATP-Glo™ assay solution in a ratio of 1 μl to 100 μl (25 μl luciferase for 2.5 ml of the ATP-Glo™ assay solution). The ATP-Glo™ Detection Cocktail was prepared immediately before each use according to the manufacturer's directions. Relative luminescence activity was

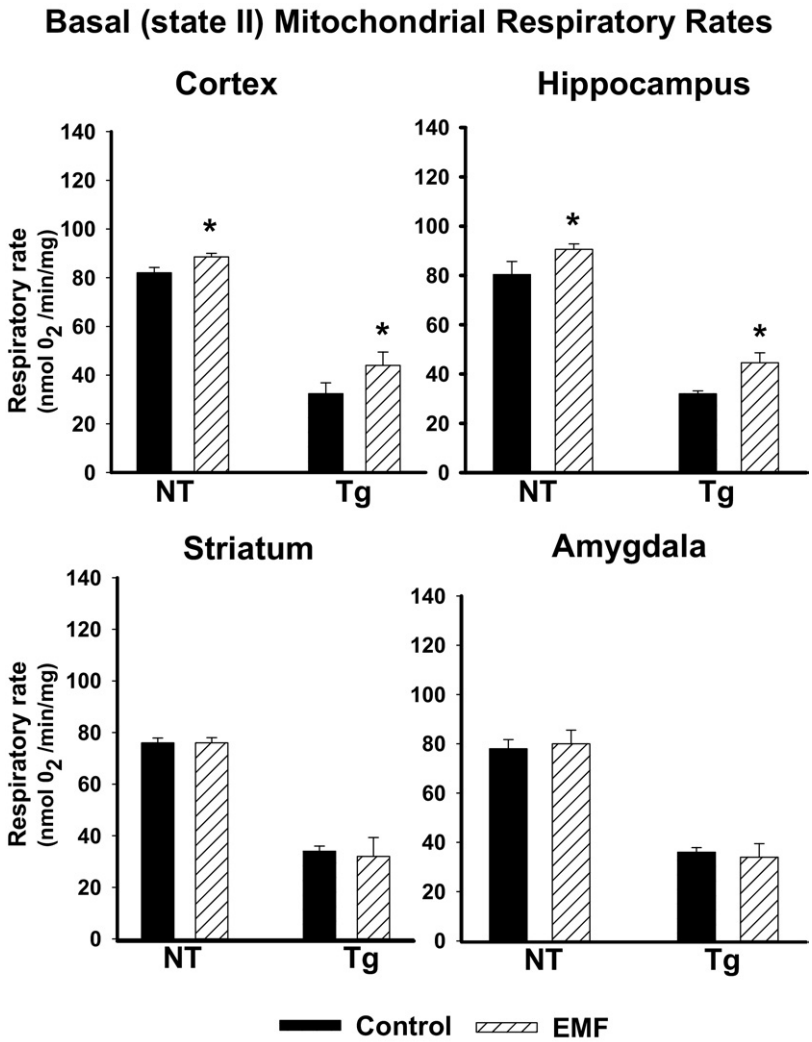

Fig. 1. Basal (state II) mitochondrial respiratory rates are increased in both NT and Tg mice by EMF treatment. Cerebral cortex and hippocampus both showed EMF-induced increases in basal mitochondrial respiration, although striatum and amygdala were not affected. * $P<0.05$ or higher level of significance vs. control mice for that genotype.

recorded and was subsequently translated into ATP concentration using the calibration curves constructed earlier. ATP levels were expressed as nmol ATP/mg protein.

Cytochrome c oxidase assay

Cytochrome c oxidase (Complex IV) activity was measured using a Clark-type oxygen electrode in the presence of tetramethylphenylenediamine (TMPD) (250 μM) and ascorbate (500 μM) in KCl-based respiratory medium (125 mM KCl, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, 20 mM HEPES, pH 7.0) in the presence of 1 μM FCCP. TMPD donates electrons directly to cytochrome c oxidase, by-passing the upstream respiratory complexes. Rates of oxygen consumption were calculated after addition of TMPD and ascorbate, and were expressed as nmol 0_2/min/mg protein.

Aβ measurement via ELISA

Brain mitochondrial preparations from cerebral cortex, hippocampus, and striatum were analyzed for soluble Aβ1-40 using an ELISA kit (KHB3482, Invitrogen, Carlsbad, CA, USA). Standard and samples were mixed with detection antibody and loaded on the antibody pre-coated plate as the designated wells. HRP-conjugated antibody was added after wash, and substrates were added for colorimetric reaction, and then stopped with sulfuric acid. Optical density was obtained and concentrations were calculated according a standard curve. Aβ1-40 levels are presented as pg/ml, with all protein concentrations the same at 2.555 mg/ml in lyses buffer.

Statistical analysis

Data analysis of neurochemical and temperature measurements were performed using ANOVA followed by Tukey or Fisher's LSD post hoc test. For each of the mitochondrial function measures, the data from both runs of pooled samples were averaged. All data are presented as mean$\pm$SEM.

RESULTS

Alzheimer's Tg mice have wide-spread impairment of mitochondrial function throughout their brains

For all six measures of mitochondrial function analyzed and in all four brain areas investigated (cerebral cortex, hip-

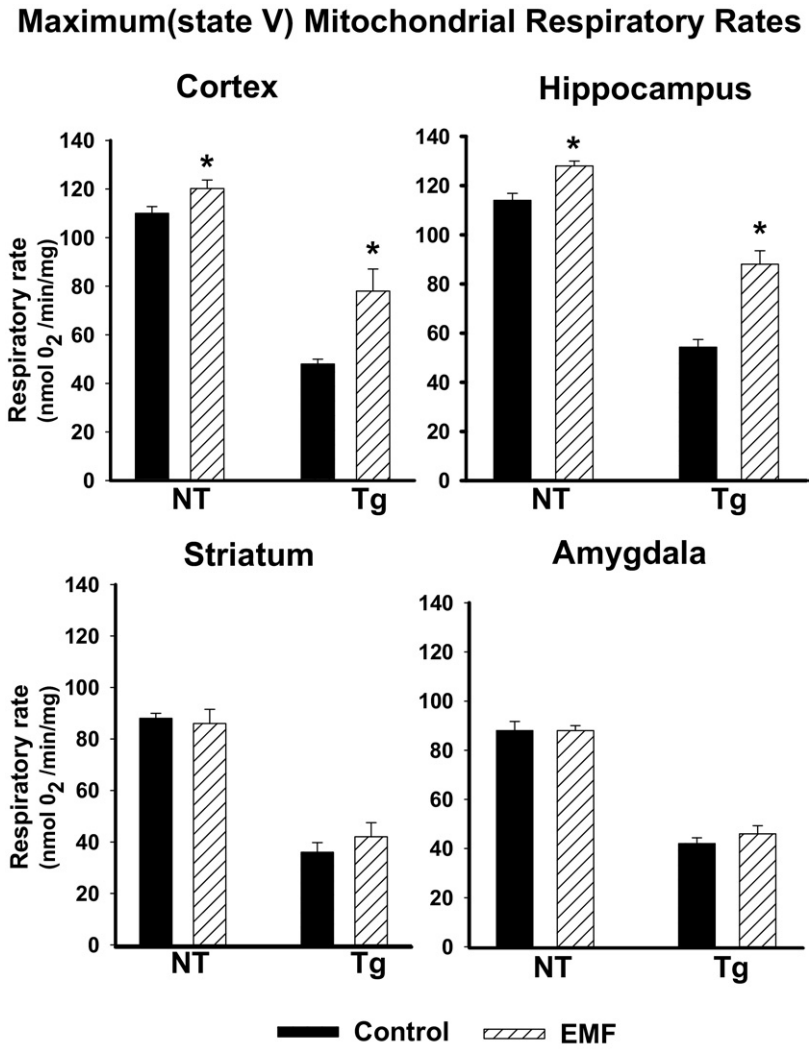

Fig. 2. Maximum (state V) mitochondrial respiratory rates are enhanced by EMF treatment in both NT and Tg mice. The cognitively-important brain areas of cerebral cortex and hippocampus in EMF-exposed mice exhibited clear increases in maximum mitochondrial respiration, while the striatum and amgydala were unaffected. * $P<0.05$ or higher level of significance vs. control mice for that genotype.

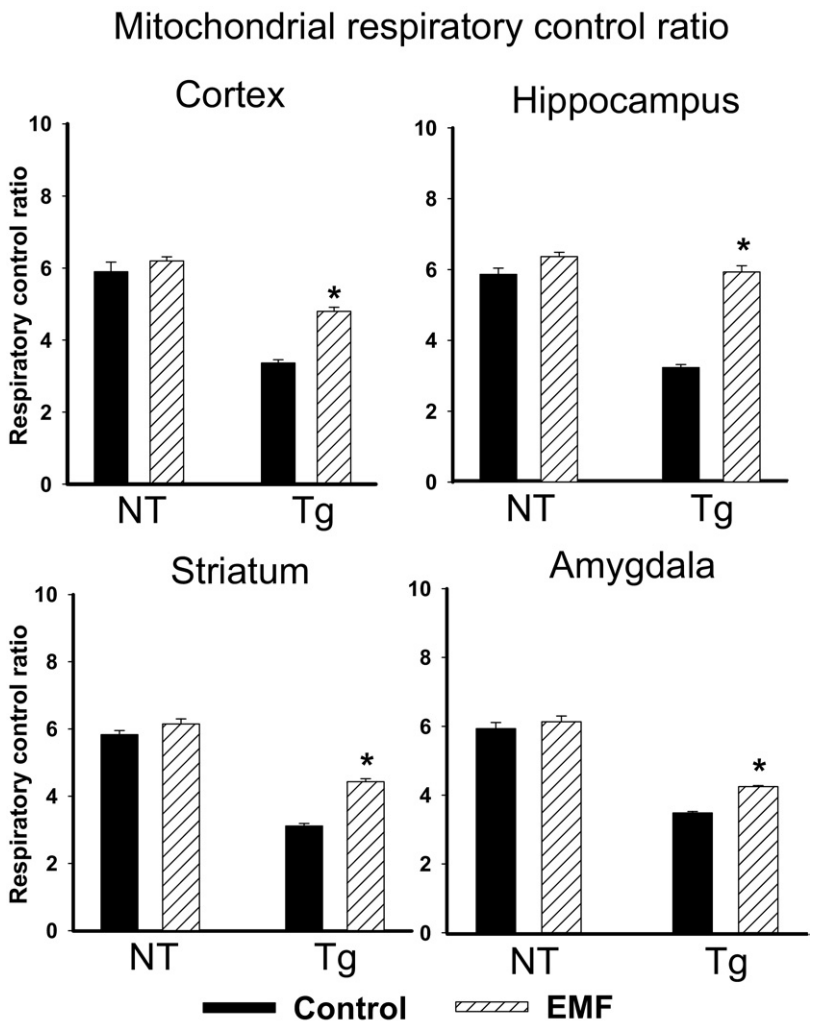

Fig. 3. EMF treatment increases the respiratory control ratio (RCR) in brains of Tg mice, but not NT mice. The RCR is calculated as state III/state IV respiratory rates. The EMF-induced increase in RCR ratio for Tg mice indicates enhanced electron transport coupling throughout the brain. * $P<0.05$ or higher level of significance vs. Tg controls.

pocampus, striatum, and amygdala), APPsw+PS1 (Tg) control mice exhibited highly significant mitochondrial dysfunction compared to NT controls (Figs. 1–6). For example, hippocampal mitochondria from Tg mice had 60% lower basal respiratory rates, 53% lower maximum respiratory rates, 216% higher reactive oxygen species levels, 56% lower membrane potential level, 71% lower ATP levels, and 71% lower Complex IV activity. Also for all six measures of mitochondrial function analyzed, there were no differences between the four brain areas taken from NT control mice.

EMF treatment enhances both basal and maximum mitochondrial respiratory rates in cognitively important brain areas

For both NT and Tg mice, long-term EMF treatment increased basal (state II) mitochondrial respiratory rates in the cerebral cortex and hippocampus, but not in the striatum or amygdala (Fig. 1). A similar pattern of EMF-induced mitochondrial enhancement was evident for maximum (state V) respiratory rates of both NT and Tg mice in cortex and hippocampus (Fig. 2). These EMF-induced increases in maximum respiratory rate were particularly impressive for Tg mice, which had 68% and 69% higher respiratory levels in their cortex and hippocampus, respectively, compared to Tg controls (Fig. 2). As was the case for basal respiratory rates, EMF treatment did not impact mitochondrial maximum respiratory rates in either striatum or amygdala of NT and Tg mice. Thus, the beneficial effects of EMF treatment on brain basal (state II) and maximal (state V) mitochondrial respiratory rates were region-specific. By contrast, EMF-induced changes in the RCR, which is calculated by dividing state III by state IV respiratory rates, were genotype-specific (Fig. 3). EMF treatment had no effect on the RCR of any brain area in normal mice, but dramatically increased the low RCR present in all four brain regions of Tg mice. Thus, EMF treatment enhanced electron transport coupling in mitochondrial selectively of Tg mice.

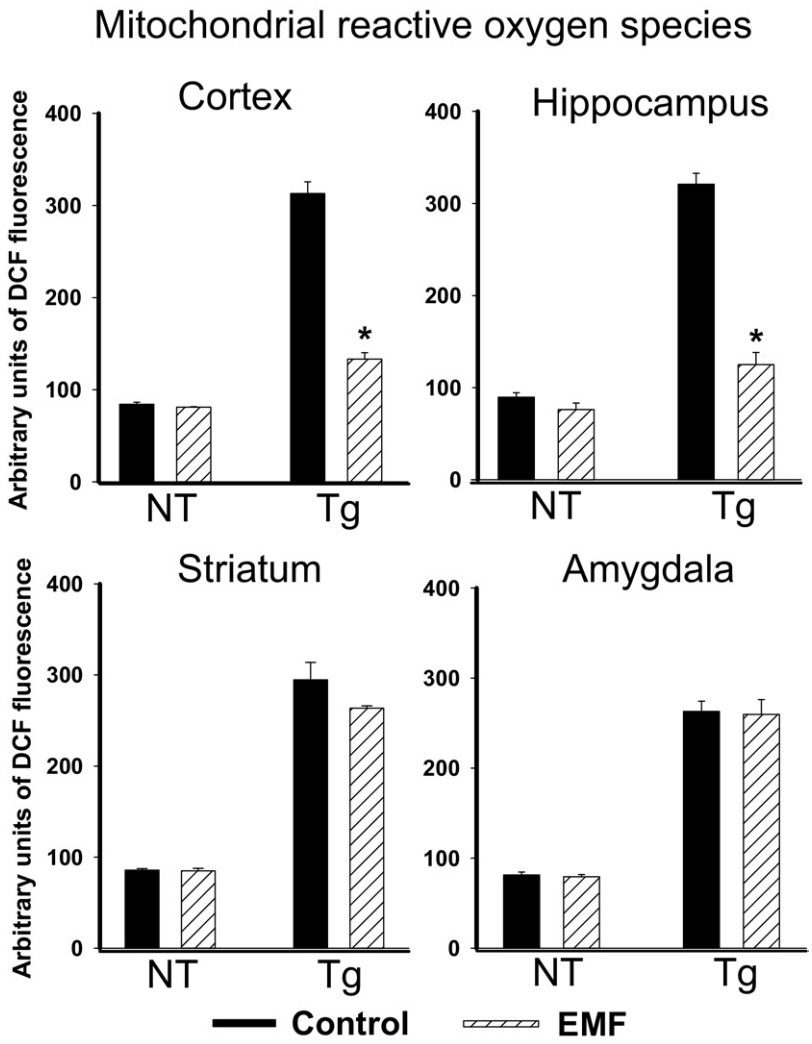

Fig. 4. Mitochondrial reactive oxygen species (ROS) are selectively reduced in cortex and hippocampus of EMF-treated Tg mice. The striatum and amygdala were not affected in Tg mice. The already low ROS levels present in all brain areas of NT mice were not further reduced by EMF treatment. * $P<0.05$ or higher level of significance vs. control mice for that genotype.

EMF treatment reduces mitochondrial reactive oxygen species in cognitively-important brain areas of Tg mice

For Tg mice, long-term EMF treatment induced a dramatic reduction in mitochondrial ROS levels in both cerebral cortex and hippocampus, but not in striatum or amygdala (Fig. 4). By contrast, NT mice given EMF treatment did not show significant changes in ROS levels within any of the four brain areas analyzed. Therefore, EMF treatment reduced ROS levels selectively in Tg mice and selectively in cognitively-important brain areas.

Mitochondrial membrane potentials in cognitively-important brain areas are enhanced by EMF treatment to Tg mice

For Tg mice, but not for NT mice, EMF treatment enhanced mitochondrial membrane potentials in both cortex (↑70%) and hippocampus (↑78%) (Fig. 5). EMF-induced increases in mitochondrial membrane potential in both striatum and amygdala did not research significance. Thus, EMF treatment enhanced mitochondrial membrane potential in cognitive-important brain areas, but only in Tg mice.

EMF treatment increases mitochondrial ATP levels selectively in Tg mice

Similar to the above effects of EMF treatment on mitochondrial membrane potential, only Tg mice exhibited enhancement of brain mitochondrial ATP levels by EMF treatment (Fig. 6). The already high brain ATP levels of NT mice were not further increased in any brain area by EMF treatment. For Tg mice, the EMF-induced increases in mitochondrial membrane potential within the cortex (↑47%) and hippocampus (↑59%) were especially large.

Mitochondrial Complex IV respiratory activity is enhanced by EMF treatment

Tg mice given EMF treatment exhibited significantly greater mitochondrial Complex IV (cytochrome c oxidase) activity in

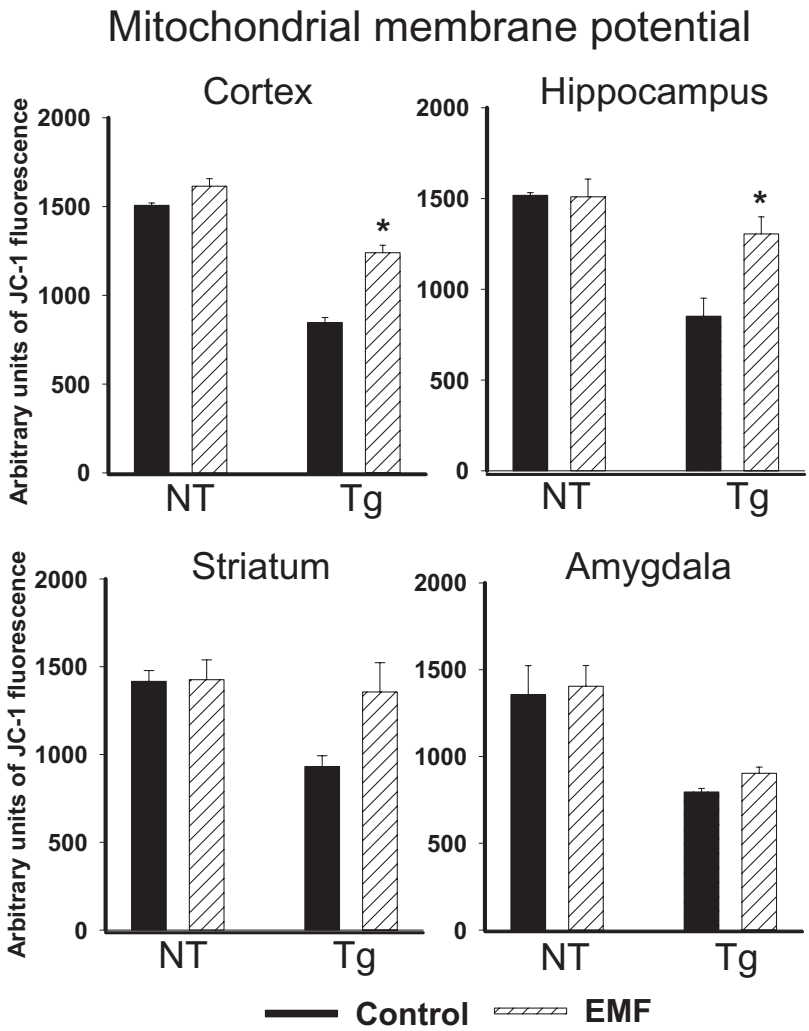

Fig. 5. EMF treatment enhances mitochondrial membrane potentials in cortex and hippocampus of Tg mice. Mitochondria from cognitively-important brain areas of EMF-treated Tg mice had elevated membrane potential; however, mitochondrial membrane potentials in these same brain areas from NT mice were not affected. * $P<0.05$ or higher level of significance vs. control mice for that genotype.

three of the four brain areas analyzed (Fig. 7). As was the case for other mitochondrial measures, the EMF-induced increase in brain Complex IV activity in Tg mice was particularly robust for cortex (↑133%) and hippocampus (↑158%). For NT mice, EMF treatment induced more modest increases in mitochondrial Complex IV activity within cortex, hippocampus, and striatum compared to NT controls. Thus, EMF treatment enhanced the inherently low Complex IV activity in brains of Tg mice, particularly in cortex and hippocampus.

Fig. 8 summarizes the wide-spread and consistent beneficial effects of long-term EMF treatment on mitochondrial function in the cerebral cortex and hippocampus of Alzheimer's Tg mice. For most mitochondrial measures, at least a 50% beneficial change was observed, although an even greater (125–150%) increase was evident for Complex IV activity in both of these cognitively-important brain areas. Parenthetically, it should be noted that, despite the widespread enhancement of brain mitochondrial function (e.g. across all measures) provided to Tg mice by EMF treatment, mitochondrial function was not normalized in EMF-treated Tg mice to the level of NT controls for any measure evaluated.

Mitochondrial soluble Aβ1-40 is greatly increased by EMF treatment

To determine what effect EMF treatment had on mitochondrial Aβ levels, the same preparations used for mitochondrial function assays were analyzed for soluble Aβ1-40 via ELISA. Compared to control Tg mice, dramatic 5–10 fold increases in mitochondrial soluble Aβ1-40 were evident in brain areas from EMF-treated Tg mice. Specifically, mitochondrial Aβ1-40 levels for control vs. EMF-treated Tg mice were: 55.1 vs. 469.8 pg/ml in cortex, 95.4 vs. 534.4 pg/ml in hippocampus, and 41.3 vs. 490.4 pg/ml in striatum.

The beneficial effects of EMF treatment on brain mitochondrial function do not involve an EMF-induced increase in brain temperature

Because EMF treatment can have both thermal and non-thermal effects in affected tissues (Van Leeuwen et al.,

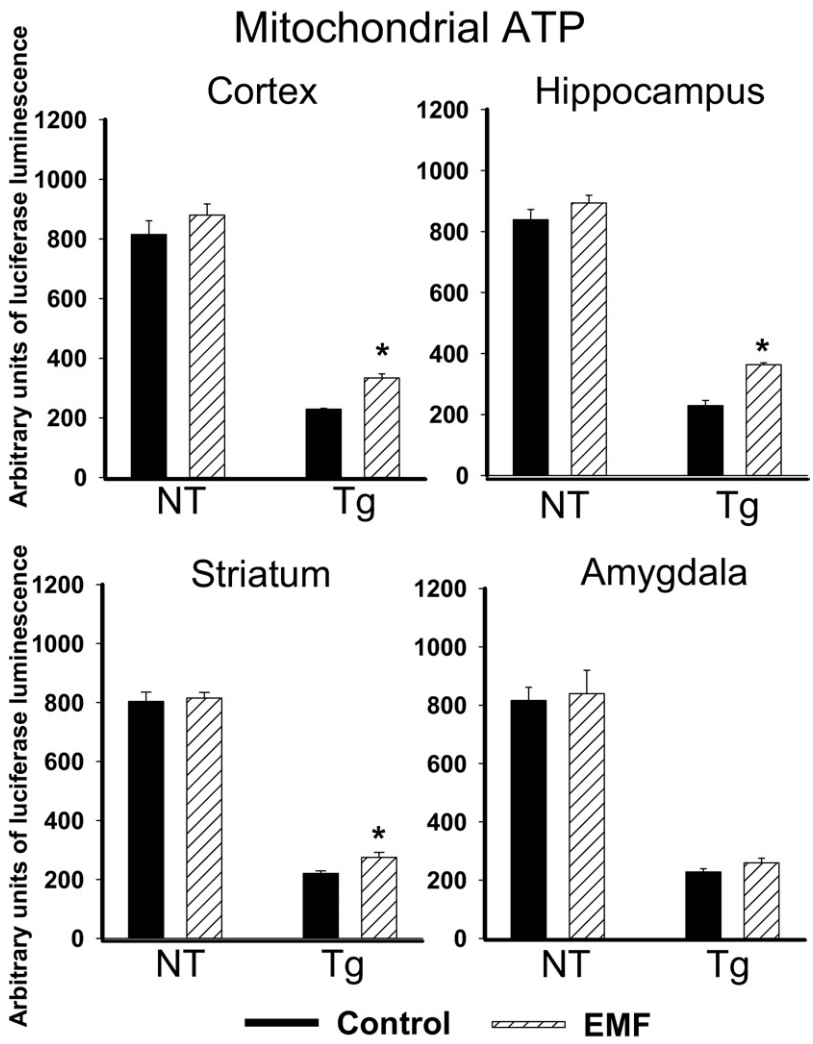

Fig. 6. Mitochondrial ATP levels in brains from Tg mice are elevated by EMF treatment. Mitochondrial ATP levels in amgydala were not affected by EMF treatment in Tg mice, nor were the already high mitochondrial ATP levels in brain regions of NT mice. * $P<0.05$ or higher level of significance vs. control mice for that genotype.

1999), both brain and body temperatures were taken for all animals on the day prior to euthanasia (1 month into EMF treatment) to determine if thermal effects were involved in the benefits of EMF treatment to brain mitochondrial function. Brain temperatures, taken during both morning and late afternoon EMF exposure periods as well as mid-day in between these exposures, revealed no significant changes induced by EMF in NT mice (Fig. 9, lower). For Tg mice, there was a significance decrease in brain temperature during the morning EMF exposure and a similar near-significant decrease in brain temperature during the late afternoon exposure. Thus, the brain mitochondrial enhancement effects of long-term EMF treatment (Figs. 1–7) did not involve thermal (e.g. increased brain temperature) mechanisms. By contrast, body temperature readings taken at the same time revealed significant increases in temperature for both NT and Tg mice being given EMF treatment compared to non-EMF treatment controls (Fig. 9, upper). Indeed, for both NT and Tg mice during EMF treatment, brain temperatures were decreased ($P<0.05$; paired *t*-tests) in comparison to body temperatures.

DISCUSSION

Mounting evidence indicates that brain mitochondrial dysfunction is not only central to AD pathogenesis, but also an early event therein (Devi et al., 2006; Manczak et al., 2006; Eckert et al., 2008; Galindo et al., 2010; Morais and De Strooper, 2010; Reddy et al., 2010; Chen and Yan, 2010). As such, therapeutic strategies that provide mitochondrial enhancement to the brain may bring about real cognitive benefit to AD patients. The present study provides clear evidence that high frequency EMF treatment to aged Alzheimer's Tg mice enhances their impaired brain mitochondrial function by 50–150% across six well-established measures. Moreover, this EMF-induced mitochondrial enhancement was greatest in cognitively important brain areas (e.g. cerebral cortex and hippocampus), which could

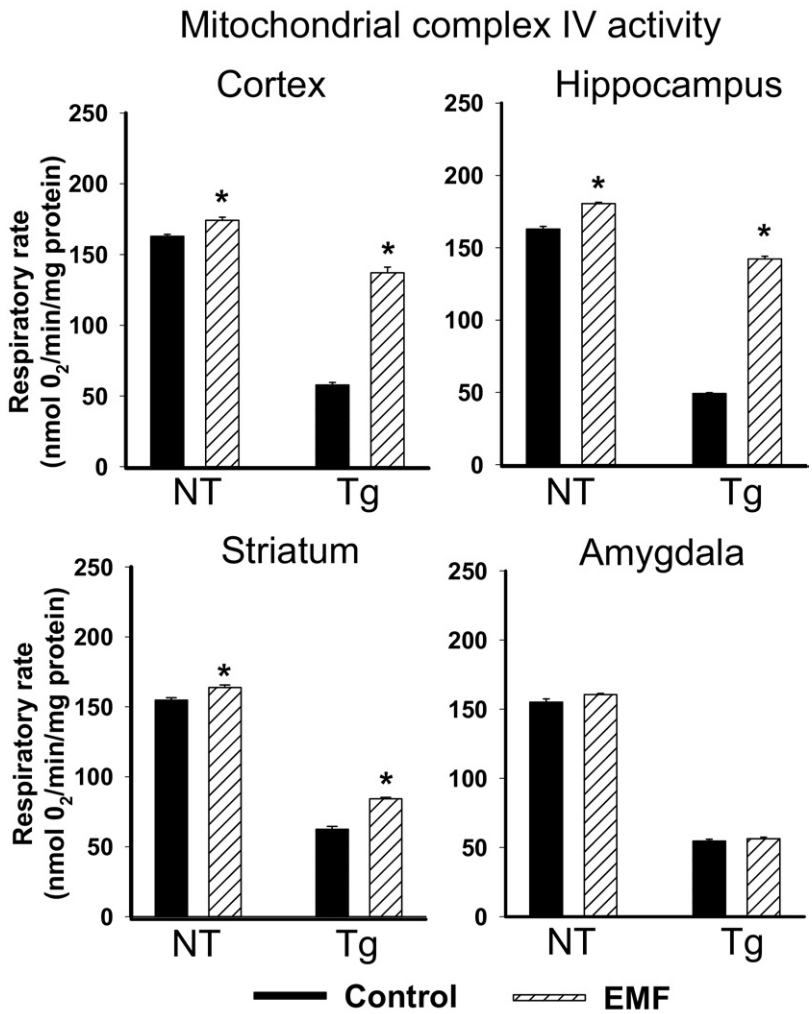

Fig. 7. EMF treatment enhances mitochondrial Complex IV respiratory activity in both Tg and NT mice. EMF-treated Tg mice showed enhancement of Complex IV activity across all brain areas except the amygdala. Similarly, NT mice given EMF treatment had significant, albeit more modest, enhancements in the same brain areas. * $P<0.05$ or higher level of significance vs. control mice for that genotype.

be key to the cognitive benefits we have previously shown to result from long-term EMF treatment to similar Alzheimer's Tg mice (Arendash et al., 2010). Importantly, EMF-induced mitochondrial enhancement in Tg mice was linked to dramatic 5–10 fold elevations in soluble Aβ within the same mitochondria, which is apparently indicative of our earlier finding that EMFs disaggregate toxic Aβ oligomers in brain tissue (Arendash et al., 2010). Although EMF treatment also increased brain mitochondrial function in normal aged mice, this effect was not as robust and less widespread in measures/brain areas affected by EMF treatment compared to EMF-treated Tg mice. Finally, the brain mitochondrial enhancement provided to Tg and normal mice by EMF treatment occurred without an increase in brain temperature, indicating involvement of non-thermal mechanisms. Our study is significant in being the first to demonstrate beneficial *in vivo* effects of EMF exposure on brain mitochondrial function in any animal.

Recently, we reported surprising cognitive benefits of long-term EMF treatment to AD transgenic mice, as well as to normal mice (Arendash et al., 2010). In Tg mice, the same high frequency EMF treatment that we used in the present study resulted in protection against otherwise certain cognitive impairment when started in young adulthood. Months of EMF treatment given to older Tg mice after onset of cognitive impairment resulted in a remarkable reversal of that impairment, as well as reversal of brain Aβ deposition through Aβ anti-aggregation actions (Arendash et al., 2010). Moreover, we found that normal (NT) mice exhibited supra-normal levels of cognitive performance when provided this same EMF treatment over months. In that initial paper, we provided direct *in vitro* and *in vivo* evidence for the ability of EMF to reverse Aβ aggregation in Tg mice and indicated how that mechanism could result in increased clearance of Aβ from the brain. Not surprisingly, the present study's finding of EMF-induced mitochondrial enhancement is mechanistically linked directly to our previous finding of EMF-induced disaggregation of oligomeric Aβ (Arendash et al., 2010).

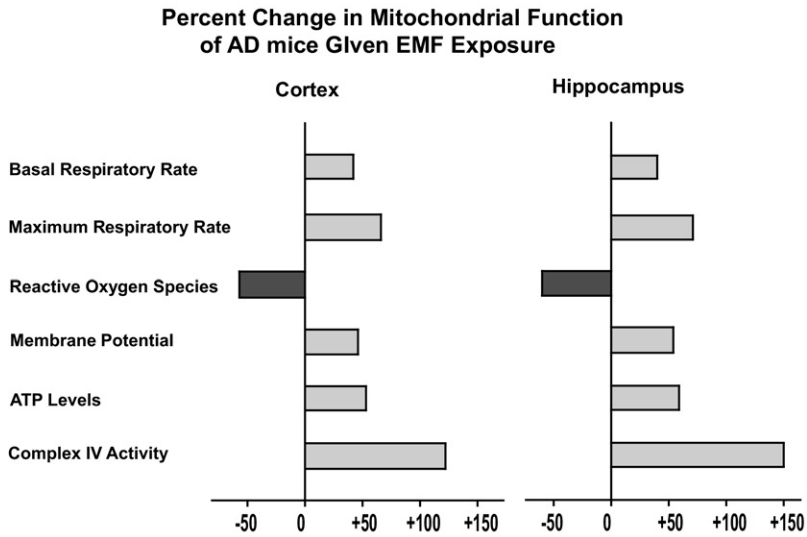

Fig. 8. Percent change in mitochondrial function within the cerebral cortex and hippocampus induced by EMF treatment in Tg mice. For both brain areas and across all six measures of mitochondrial function, 50–150% enhancements were induced by EMF treatment.

Numerous prior studies have connected the brain mitochondrial dysfunction in AD to elevated Aβ levels in mitochondria (Galindo et al., 2010; Morais and De Strooper, 2010; Caspersen et al., 2005), presumably resulting in synaptic dysfunction/loss and ensuing cognitive impairment (Reddy and Beal, 2008; Venkitaramani et al., 2007; Tampellini et al., 2007). Similar accumulations of Aβ in synaptic mitochondria and associated mitochondrial dysfunction have been reported in several mouse AD transgenic lines (Smith et al., 1998; Casley et al., 2002; Mungarro-Menchaca et al., 2002; Li et al., 2004; Devi et al., 2006; Manczak et al., 2006; Gillardon et al., 2007; Eckert et al., 2008). Our recent study adds to this association by showing a gradation of synaptic mitochondrial dysfunction in APPsw and APPsw+PS1 transgenic mice that is strongly linked to gradations in their synaptic mitochondrial Aβ levels and cognitive impairment (Dragicevic et al., 2010). Results from the present study further solidify this linkage by showing profound mitochondrial dysfunction in multiple brain areas from aged (16–18 month old) APPsw+PS1 mice. This dysfunction is most likely due to aggregated, "oligomeric" Aβ interacting with mitochondrial membrane proteins, with mitochondrial "monomeric" Aβ being relatively harmless to mitochondrial function (Mattson et al., 2008; Galindo et al., 2010; Muller et al., 2010; Reddy et al., 2010). For example, oligomeric Aβ causes mitochondrial dysfunction in cortical cells from P301L tau transgenic mice, but monomeric (diaggregated) Aβ has no such effect (Hauptmann et al., 2009). The ability of EMF treatment to disaggregate Aβ (Arendash et al., 2010), in combination with our current finding that EMF-treated Tg mice had 5–10$\times$ higher "soluble" Aβ in their brain mitochondria points to a specific mechanism of mitochondrial enhancement by EMF—namely, that EMF-induced increases in soluble Aβ represents disaggregated, monomergic Aβ that is not harmful to mitochondrial function. Definitive confirmation of this EMF effect awaits future studies in which direct measurement of oligomeric and monomeric Aβ levels in brain mitochondria are performed following long-term EMF exposure. Similar to the present 1-month study, our original study (Arendash et al., 2010) found long-term (6+month) EMF exposure to APPsw mice resulted in near-significant increases in brain soluble A-beta levels. Moreover, we have found oral melatonin treatment to also provide anti-Aβ aggregation actions in APPsw+PS1 mice, resulting in enhanced brain mitochondrial function (Dragicevic et al., 2010) and a protection of cognitive abilities (Olcese et al., 2009). To our knowledge, EMF and melatonin treatment are the first AD therapeutics that target mitochondrial dysfunction and that provide clear cognitive benefit in AD models.

The present study evaluated seven well-established measures of brain mitochondrial function in "aged" APPsw+PS1 transgenic mice—mice which we have previously documented to have substantial brain Aβ levels/deposition and associated cognitive impairment (Austin et al., 2003; Olcese et al., 2009; Sanchez-Ramos et al., 2009; Boyd et al., 2010). The impaired mitochondrial function of Tg mice across all seven measures was substantially reversed by 1 month of EMF treatment. Basal and maximum respiratory rates, RCR, membrane potential levels, ATP levels, and Complex IV activity were all increased in brain mitochondria from EMF-treated Tg mice, while mitochondrial ROS levels were decreased. A direct EMF-induced increase in brain mitochondrial ATP levels would be predicted from an earlier study by Buchachenko et al. (2006), which concluded that high frequency EMFs increase mitochondrial ATP production due to nuclear spin/magnetic effects on Mg^{2+} ions. Thus, some enhancement of mitochondrial ATP production by EMF treatment in Tg mice may have involved a direct effect of EMFs on ATP production independent of mitochondrial Aβ presence. However, the across-the-board enhancement in all mitochondrial measures provided by EMF treatment in aged Tg mice

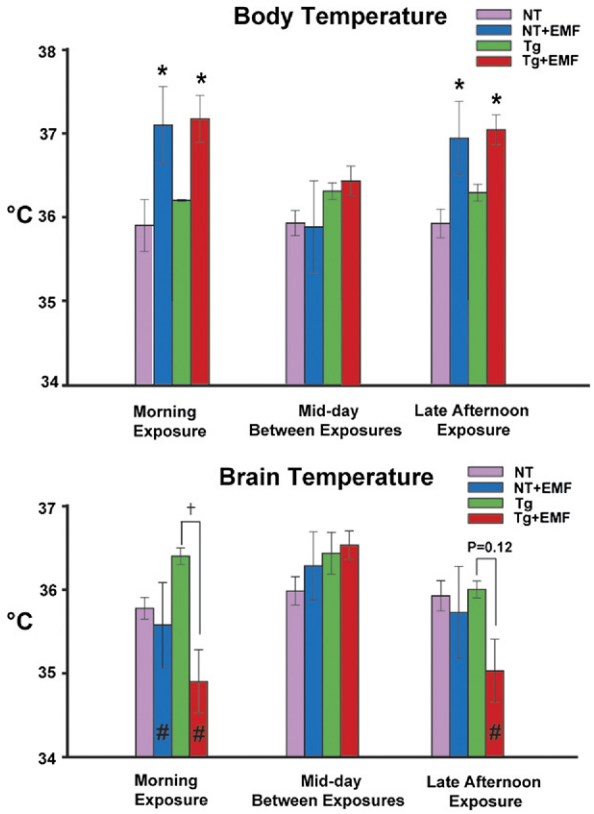

Fig. 9. Body and Brain Temperatures taken during and between the two daily EMF exposures at 1 mon into treatment. (Lower) Brain temperatures from Tg mice during EMF exposures was decreased, or strongly trended so, while brain temperatures from NT mice were unaffected by EMF exposure. (Upper) However, body temperatures for both genotypes were increased significantly during EMF exposures, resulting in brain temperatures being significantly lower vs. body temperature during exposures. * $P<0.05$ vs. control mice for that genotype; † $P<0.05$ vs. Tg control; # $P<0.05$ vs. body temperature for that group. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

argues that some central mechanism of dysfunction is being removed. We believe this is likely to be an EMF-induced disaggregation of oligomeric Aβ in mitochondria, resulting in high levels of monomeric Aβ, which are thought to be innocuous to mitochondrial function (Hauptmann et al., 2009; Mattson et al., 2008).

For all mitochondrial measures evaluated, and across the brain areas analyzed, the beneficial effects of EMF treatment were more profound for Tg mice compared to NT mice. This probably reflects the much lower and impaired mitochondrial function of Tg mice caused by high intra-mitochondrial levels of oligomeric Aβ prior to the start of EMF treatment. Nonetheless, NT mice did show EMF-induced mitochondrial enhancement (over-and-above already excellent levels) for basal/maximum respiratory rates and Complex IV activity—most consistently in the cerebral cortex and hippocampus. Thus, a direct enhancement of mitochondrial function could be contributing to the EMF-induced cognitive benefits in NT mice that we previously reported (Arendash et al., 2010). Although one mechanism for this "generalized" effect of EMF treatment on mitochondrial enhancement was previously mentioned (i.e. effects on nuclear spin/magnetic moment of Mg^{2+} ions in creatine kinase and ATPase), we are unaware of any other mechanisms that have been proposed to link the chronic, high frequency EMF exposure we administered to mitochondrial function. Prior EMF studies investigating mitochondrial function have either investigated low frequency (50–60 Hz) EMF exposure in peripheral tissues or short-term EMF exposure to cell cultures—neither of which would provide mechanistic insight to the present study's findings in NT mice.

Since EMF-induced effects on body tissues *in vivo* can involve thermal (heating) or non-thermal mechanisms (Van Leeuwen et al., 1999), it was important for us to determine any changes that EMF treatment induced to body and brain temperatures of mice during and between treatments. Although body temperatures of both Tg and NT mice increased during EMF treatment (as we had previously reported in Arendash et al., 2010), "brain" temperatures actually decreased (Tg mice) or remained unchanged (NT mice). Indeed, comparing body vs. brain temperatures during EMF exposures, brain temperatures for both Tg and NT mice were significantly below their body temperatures. We propose that these decreases in brain temperature during EMF exposures are linked to changes in cerebral blood flow (CBF), at least for Tg mice. More specifically, we have determined that the same long-term EMF exposure given to mice of the present study also decreases cerebral blood flow in similarly-aged AD transgenic mice, as measured by Doppler probes (G. Arendash, unpublished observations). Several studies involving normal humans show that acute EMF treatment (with parameters similar to those utilized in the present study) can also affect regional CBF (Huber et al., 2005; Aalto et al., 2006). Thus, our present results make modulation of cerebral blood flow a viable effect of EMF action in Tg mice, resulting in the decreased brain temperatures observed.

There are several caveats that should be mentioned in considering the novel results of this study. First, since mice received full body EMF treatment that was not localized to the brain, mitochondrial function in peripheral tissues may have also been affected. Second, although EMF treatment had robust and widespread beneficial effects on mitochondrial function in aged Alzheimer's Tg mice, the level of mitochondrial function in these aged Tg mice was not normalized to that of NT controls. This is probably because the oligomeric Aβ burdens in the brain mitochondria of Tg mice, though substantially reduced by EMF treatment, remained sizable and thus prevented a complete reversal of mitochondrial dysfunction. A longer period of EMF treatment may have resulted in a more normalized pattern of mitochondrial function in Tg mice. Third, the aged (15–17 M) animals of the present study were not behaviorally evaluated, and for several reasons: (1) the three to four mice per group were sufficient for grouped neurochemical analysis, but not for behavioral analysis, and (2) our prior study indicated that EMF treatment for 5–6+ months (not just 1–2 months) is required to attain cognitive benefit with the parameters utilized (Arendash et al., 2010). The differ-

ence in EMF exposure length between the present study (1 month) and the greater exposure length needed for cognitive benefit (5–6 months) suggests that mitochondrial enhancement is an early effect of EMF exposure and that this early enhancement is insufficient for cognitive benefit. Other differences between the present study compared to our initial study (Arendash et al., 2010) involve differences in age of mice (15–17 vs. 5 M) at the start of EMF exposure and differences in genotype (APPsw+PS1 vs. APPsw). As a final caveat, the brain mitochondrial extraction procedure isolated not only neuronal, but also glial, mitochondria. Thus, EMF effects on both mitochondrial populations are likely and contributory to the results reported.

Two long-term epidemiologic studies in humans have provided evidence that years of high frequency EMF exposure is associated with cognitive benefit. One of these studies found that heavy cell phone use over several years resulted in better performance on a word interference test (Arns et al., 2007), while the other study reported that long-term cell phone users (≥10 years) had a 30–40% decreased risk of hospitalization due to Alzheimer's disease and vascular dementia (Schuz et al., 2009). These human studies, in combination with our recent study (Arendash et al., 2010) underscore that long-term EMF treatment (daily and over months/years) will probably be required to manifest cognitive benefits against AD. Although some investigators have repeatedly asserted that long-term use of cell phones doubles the risk of brain cancer (Hardell et al., 2009; Khurana et al., 2009), the 13-nation INTERPHONE Study involving over 5000 individuals with brain cancer has recently been completed, finding that long-term (>10–20 years) exposure to cell phone EMF's does not increase risk of any brain cancer (Hours et al., 2007; Samkange-Zeeb et al., 2010). Thus, responsible use of bioelectromagnetic therapeutics may incur minimal or no health risks. Indeed, in our study administering daily EMF treatment to Tg and NT mice for 9+ months (Arendash et al., 2010), we found no evidence of any tissue abnormalities either in the brain or peripherally in EMF treatment mice. Moreover, we found no evidence of increased oxidative stress in the brains of these mice (Arendash et al., 2010), nor did we find any increase in DNA damage in their blood cells, as indexed by the sensitive AluDNA assay (Cao, unpublished observations). Indeed, this assay can be used in human EMF clinical trials as an on-going monitor of oxidative stress levels/damage, as it already is for human cancer patients receiving radiation therapy. EMF treatment could therefore be a safe, non-invasive, and "disease-modifying" approach to AD treatment that enhances mitochondrial function and likely has other beneficial actions as well.

Especially since pharmacologic approaches to the prevention and treatment of AD have been universally ineffective, we believe EMF treatment fosters in a new and promising "non-pharmacologic" therapy against AD, as well as a memory-enhancing approach in general. In view of the data presented in this and our earlier study (Arendash et al., 2010), and in the context that EMF treatment can be safely monitored, a compelling argument is forwarded for initiation of long-term clinical trials with EMF treatment in AD patients.

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