

Electromagnetic Field Treatment Protects Against and Reverses Cognitive Impairment in Alzheimer's Disease Mice

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ABSTRACT

Despite numerous studies, there is no definitive evidence that high-frequency electromagnetic field (EMF) exposure is a risk to human health. To the contrary, this report presents the first evidence that long-term EMF exposure directly associated with cell phone use (918 MHz; 250 mW/kg) provides cognitive benefits. Both cognitive-protective and cognitive-enhancing effects of EMF exposure were discovered for both normal mice and transgenic mice destined to develop Alzheimer's-like cognitive impairment. The cognitive interference task utilized in this study was designed from, and measure-for-measure analogous to, a human cognitive interference task. In Alzheimer's disease mice, long-term EMF exposure reduced brain amyloid- β ($A\beta$) deposition through $A\beta$ anti-aggregation actions and increased brain temperature during exposure periods. Several inter-related mechanisms of EMF action are proposed, including increased $A\beta$ clearance from the brains of Alzheimer's disease mice, increased neuronal activity, and increased cerebral blood flow. Although caution should be taken in extrapolating these mouse studies to humans, we conclude that EMF exposure may represent a non-invasive, non-pharmacologic therapeutic against Alzheimer's disease and an effective memory-enhancing approach in general.

Keywords: Alzheimer's disease, amyloid- β , electromagnetic fields, memory, transgenic mice

INTRODUCTION

After reviewing an extensive literature, the World Health Organization and other health councils/organizations have concluded that there are no adverse health risks to adults or children associated with electromagnetic fields (EMFs) generated by cell phone use [1-3]. However, there is little data concerning the long-term effects of EMFs on brain physiology and function. Epidemiologic studies have suggested that occupational “low frequency” EMF exposure (such as that associated with power/telephone line maintenance) may increase risk of Alzheimer’s disease (AD) [4]. Other studies have investigated *acute* exposure to “high frequency” EMFs, such as that associated with cell phone use [5-7]. A number of these studies have, in fact, reported small beneficial effects of acute EMF exposure on attention and/or working memory in normal individuals, although other studies report no cognitive effects of acute EMF exposure (See Barth et al. [7] for a recent meta-analysis/review). To date, no controlled *long-term* studies of high frequency/cell phone EMF effects on cognitive function have been done in humans, mice, or animal models for AD.

Several earlier studies have involved short-term (7-14 days) EMF exposure at cell phone frequencies (around 900 MHz) to normal rodents. These studies all reported no effects of short-term EMF exposure on cognitive performance. For example, normal mice exposed for 10 days (45 minutes per day) to a relatively low specific energy absorption rate (SAR) of 0.05 W/kg exhibited normal performance in an 8-arm radial maze [8]. In another study, head-only exposure of normal rats to a SAR of 1 or 3.5 W/kg for 45 minutes/day over 7-10 days had no effect on performance in either spatial or non-spatial memory tasks [9]. These and other studies were largely investigating the premise that EMF exposure may induce cognitive dysfunction.

To elucidate effects of long-term (7-9 months) EMF exposure on AD-like cognitive impairment and neuropathology, we exposed AD transgenic (Tg) mice and littermate non-transgenic (NT) mice to the same high frequency EMF level that the human head is exposed to during two 1-hour periods of cell phone use (918 MHz; 250 mW/kg \pm 2 dB) each day. Here we show that such long-term intermittent EMF exposure: 1) protects adolescent/young adult Tg mice from later cognitive impairment; 2) reverses cognitive impairment and AD-like brain pathology in older Tg mice; and 3) increases cognitive performance of normal NT mice. The novel behavioral task utilized to reveal these cognitive benefits was designed and implemented to closely mimic (measure-for-measure) a human “cognitive interference” task, which very effectively discriminates AD, mild cognitive impairment (MCI), and non-demented individuals [10].

MATERIALS AND METHODS

Animals

A total of 96 mice, derived from the Florida Alzheimer’s Disease Research Center’s colony, were included in these studies. Each mouse had a mixed background of 56.25% C57, 12.5% B6, 18.75% SJL, and 12.5% Swiss-Webster. All mice were derived from a cross between heterozygous mice carrying the mutant A β PPK670N, M671L gene (A β PPsw) with heterozygous PS1 (Tg line 6.2) mice, which provided off-spring consisting of A β PP/PS1, A β PPsw, PS1, and non-transgenic (NT) genotypes. After weaning and genotyping, A β PPsw and NT mice were selected for behavioral studies, while temperature-monitoring studies also included A β PP/PS1 mice. All mice were maintained on a 12-hour dark and 12-hour light cycle with *ad libitum* access to rodent chow and water. All animal procedures were performed in AAALAC-certified facilities

under protocols approved by the University of South Florida Institutional Animal Care and Use Committees.

Young adult long-term study

A total of 24 A β PPsw (Tg) mice and non-transgenic (NT) littermates, aged 2-2½ months, were divided into the following four groups: Tg controls, Tg+EMF, NT controls, NT+EMF (n=6 per group). Tg and NT mice exposed to EMFs were housed in cages within a large Faraday cage, which also housed the antenna of an EMF generator providing two 1-hour periods of electromagnetic waves per day (early morning and late afternoon) at standard cell phone levels/frequencies (918 MHz, 0.25 W/kg, 2 dB). Initial behavioral testing involved radial arm water maze (RAMW) testing for working memory at 5 months of age (2½ months into EMF exposure). At 6½ and at 9 months of age (4-5 and 6-7 months into EMF exposure), all mice were then evaluated in a cognitive interference task that closely parallels, and was designed from, a cognitive interference task utilized in humans to differentiate aged non-demented, MCI, and AD patients from one another [10]. Behavioral testing always occurred during “OFF” periods of EMF exposure cyclicity (e.g., during the lights on period between any two exposure periods). After cognitive interference testing at 9 months of age, all animals were tested for general mnemonic function in the Y-maze task of spontaneous alternation, as well as for sensorimotor function and anxiety (See Behavioral Testing Protocols below for details of all behavioral testing). Following completion of behavioral testing at 9½ months of age, all mice were euthanatized and perfused with physiologic saline. The rostral hippocampus and posterior cortex were dissected out bilaterally, quick frozen, and stored at -80°C for later neurochemical analysis of A β and antioxidant enzyme levels.

Aged adult long-term study

At 4 months of age, A β PPsw Tg mice (n=12) and NT littermates (n=16) were first evaluated in the RAWM task of working memory (see Behavioral Testing Protocols) to establish that Tg mice were cognitively impaired prior to EMF exposure. Based on pre-treatment performance in the RAWM task, Tg and NT groups were each divided into two balanced sub-groups as follows: Tg controls, Tg+EMF, NT controls, and NT+EMF (n=5-8 mice/group). At 5 months of age, Tg and NT mice to be exposed to EMFs had their cages placed within a large Faraday cage, which contained an EMF generator antenna providing the same exposure of two 1-hour periods of electromagnetic waves per day at standard cell phone levels (918 MHz, 0.25 W/kg, 2 dB) as in the Young Adult study. At 7 months of age (2 months into EMF exposure), all mice were re-tested in the RAWM task. Then at 10 and 13 months of age (5 and 8 months into EMF exposure), all mice were evaluated in the same cognitive interference task that was utilized in the Young Adult study, with all behavioral testing being performed during “OFF” periods in EMF exposure cyclicality.

A few days prior to euthanasia at 13½ months of age (8½ months into EMF exposure), body temperature measurements were taken on a single day with a rectal probe during both early morning and late afternoon EMF exposures, as well as at 2 hour intervals between those exposures. At euthanasia, brains were perfused with isotonic phosphate-buffered saline (PBS). The caudal forebrain was then paraffin-embedded and processed for A β immunohistochemical staining, while the remaining forebrain was sagittally bisected and dissected into hippocampus and cortical areas that were quick-frozen for neurochemical analyses.

Aged adult acute study

Results from body temperature reading of animals in the Aged Adult Long-Term Study at 8½ months into EMF exposure revealed significant increases in body temperature for Tg mice selectively during EMF “On” periods. To follow-up on this finding, an acute study was performed in naïve Tg and NT mice to monitor both body temperature (via rectal probe) and brain temperature (via temporalis muscle probe) during and between EMF exposures. Prior studies have demonstrated that temporalis muscle temperature very accurately reflects brain temperature [11,12]. For the present acute study, 10 and 15 month old AβPPsw mice, 15 month old AβPP+PS1 mice, and 10-13 month old NT mice were utilized, with each mouse having the same background as mice in both long-term EMF studies. Mice at each age and genotype were divided into two groups of 4-5 mice/group for acute EMF-exposed or non-exposed. On a single day, body and brain temperatures were taken simultaneously at the following time points: Pre-treatment, during first EMF exposure/sham, 2 hours and 4 hours following exposure/sham, and during a second EMF exposure/sham. The same EMF generator equipment and setting were utilized as for the long-term EMF studies.

EMF exposure protocol

For long-term EMF exposure, the cages of single-housed mice were maintained within a Faraday cage (4 meter height x 4 meter width x 4 meter length) and arranged in a circular pattern, with each cage approximately 26 cm from a centrally-located EMF-emitting antenna. The antenna was connect to an Hewlett Packard ESG D4000A digital signal generator (Houston, TX) set to automatically provide two 1-hour exposures per day at 918 MHz and a whole body SAR (specific absorption rate) of 0.25 W/kg +/- 2 dB. The resulting EMF transmission to the

mice of these studies is equivalent to the head transmission occurring for standard cell phone use in humans. With a 12-hour light On/Off cycle, the 1-hour daily exposures occurred in early morning and late afternoon of the lights on period. For acute EMF exposure, mice were similarly placed into the Faraday cage and provide a single day's EMF exposure (e.g., two 1-hour EMF periods). Sham-treated animals were located in a completely separate room, with identical room temperature as in the EMF exposure room and cages arranged in the same circular pattern.

Behavioral testing protocols

Radial Arm Water Maze. To assess working (short-term) memory, an aluminum insert was placed into a 100 cm circular pool to create 6 radially distributed swim arms emanating from a central circular swim area. Although described in detail elsewhere [15,25], the number of errors prior to locating which one of the 6 swim arms contained a submerged escape platform (9 cm diameter) was determined for 5 trials per day. The platform location was changed daily to a different arm, with different start arms for each of the 5 trials semi-randomly selected from the remaining 5 swim arms. The numbers of errors during trials 4 and 5 are both considered indices of working memory and are temporally similar to the standard registration/recall testing of specific items used clinically in evaluating AD patients. For animals in the Young Adult Long-term Study, 10 days of testing were done, T1 (naïve initial trial), T4, and T5 being statistically evaluated overall and for the last 2-day block. For animals in the Aged Adult Long-term Study, the same three trials were evaluated over all 6 days (pre-exposure testing) or all 14 days (post-exposure testing), as well as during the final 2-day block.

Cognitive Interference Task. We designed this task measure-for-measure from a cognitive interference task used to discriminate normal aged, MCI, and AD patients from one another [10].

The interference testing protocol in humans consists of four tasks. The first task, three-trial recall, is a modified version of the Fuld object memory examination [13], in which the subject is presented with ten familiar objects (Bag A) and asked to recall the objects following a brief distraction task, repeated three times. In the second task, proactive interference, the subject is presented with ten novel objects (Bag B) and asked to recall them; this, to determine whether previous learning (Bag A objects) intrudes upon present learning (Bag B objects). The third task, short-delay recall, wherein the subject is asked to recall the original set of ten items (Bag A), provides a measure of retroactive interference (difficulty recalling previous learning due to intrusion by present learning). Finally, long-delay recall is evaluated by asking the subject to recall the original set of ten items (Bag A) after a 20-minute delay. A verbal fluency task is used as a distractor between successive trials of the three-trial recall task, as well as immediately preceding the proactive interference task.

Our analogous interference task for mice involves two radial arm water maze set-ups in two different rooms, each with different sets of visual cues. The task requires animals to remember a set of visual cues, so that following interference with a different set of cues, the initial set of cues can be recalled to successfully solve the radial arm water maze task. A set of four behavioral measures were examined. Behavioral measures were: A1-A3 (Composite three-trial recall score from first 3 trials performed in RAWM “A”), “B” (proactive interference measure attained from a single trial in RAWM “B”), A4 (retroactive interference measure attained during a single trial in RAWM “A”), and “A5” (delayed-recall measure attained from a single trial in RAWM “A” following a 20 minute delay between A4 and A5). As a distractor between trials, animals are placed in a Y-maze and allowed to explore for 60 seconds between successive trials of the three-trial recall task, as well as immediately preceding the proactive interference task. As with the

standard RAWM task, this interference task involves the platform location being changed daily to a different arm for both of the RAWM set-ups utilized, and different start arms for each day of testing for both RAWM set-ups. For A1 and B trials, the animal was initially allowed one minute to find the platform on their own before they were guided to the platform. Then the actual trial was performed in each case. As with the standard RAWM task, animals were given 60 seconds to find the escape platform for each trial, with the number of errors and escape latency recorded for each trial. Given the very close correspondence between error and latency scores in individual animals for both the RAWM and cognitive interference tasks, only error scores are presented in this report. Animals were tested for cognitive interference performance on four successive days, with statistical analysis performed for the two resultant 2-day blocks. We have recently demonstrated the cognitive interference task's utility in aged A β PPsw transgenic mice, which show clear impairment in the task, as well as cognitive benefit in this task from treatment with the cRaf-1 inhibitor "Sorafenib" [14].

Y-maze alternation task. To measure basic memory function, mice were allowed 5 minutes to explore a black Y-maze with three arms, each measuring 21x4 cm. Basic mnemonic function was measured as a percentage of spontaneous alternation (the ratio of arm choices different from the previous two choices divided by the total number of entries)

Sensorimotor/Anxiety tasks. Open field activity, balance beam, string agility, and elevated plus maze anxiety were evaluated according to the methodology of Arendash et al. [15].

Neurochemical and immunohistochemical analysis

A β ELISA analysis. The hippocampal and cerebral cortex tissues were processed for soluble A β ₁₋₄₀ and A β ₁₋₄₂ determinations by ELISA according to our established methodology [16].

A β immunohistochemistry and image analysis. At the level of the hippocampus (bregma - 2.92 mm–3.64 mm), five 5- μ m sections (150 μ m apart) were made from each paraffin-embedded mouse brain. Following immunohistochemical staining with a biotinylated human A β monoclonal antibody (clone 4G8; 1:200, Covance Research Products, Emeryville, CA), quantitative image analysis was done based on previous methods [17]. For A β burden analysis, data are reported as percentage of immunolabeled area captured (positive pixels) relative to the full area captured (total pixels). It should be noted that there was no evidence of histopathologic findings (e.g., neuronal degeneration, gliosis, subarachnoid hemorrhage, intracerebral hemorrhage, perivascular micro-hemorrhage, or abnormal cell growth such as brain tumors) in any EMF-exposed mouse examined in these studies.

Oxidative measurements. For oxyguanosine glycosylase (OGG1) activity, the method for DNA glycosylase extraction by Cardozo-Pelaez et al. [18] was utilized, with slight modification. The colorimetric assay for PARP (poly ADP-ribose polymerase) activity was performed in 96-well plates (Trevigen, Inc., Gaithersburg, MD) according to the manufacturer's protocol. Results were normalized to equal concentration of protein measured using the bicinchoninic acid assay [19]. Determination of superoxide dismutase (SOD) activity was based on the inhibition of nitrite formation that results from oxidation of hydroxylammonium by superoxide anion radical [20]. The activity of mitochondrial SOD was calculated as a difference between total and cytosolic SOD. For determination of total and oxidized glutathione, tissue samples were homogenized in cold assay buffer and de-proteinized. Supernatants were assayed for total glutathione (GSH) according to the method of Tietze et al. [21]. For determination of glutathione, samples were mixed with 10 mM of 2-vinylpyridine as GSH scavenging agent and reaction was monitored

after 1 h of incubation. The procedure for determination of protein carbonyl content was similar to that described by Levine et al. [22].

In vitro A β aggregation studies

Hippocampus tissue was isolated from 14 month-old A β PPsw Tg mice and homogenized in RIPA buffer with sonication according to Cao et al. [16]. Tissue homogenates were aliquoted at 42 μ g per vial in 30 μ l volume and stored at -80°C. For each time point, two vials were thawed, with one placed into a rotor for EMF treatment and the other put in a rotor in the same room without EMF treatment. Vial thawing was staggered (from 0 to 6 days) so that all tissue samples completed EMF treatment at the same time. EMF treatment was identical to that in the *in vivo* studies (two 1-hour exposures per day at 918 MGH and SAR of 0.25 W/kg +/- 2 dB.). Thus, EMF treatment for 3 days involved a total of 6 one-hour exposures, EMF treatment for 6 days involved 12 one-hour exposures, etc. Samples remained in the rotor at room temperature for the entire duration of the exposure period (0 to 6 days). Immediately following treatment, samples of 14 μ l from all exposure periods were loaded onto 4-12% Bis-tris gel (Invitrogen, Carlsbad, CA) and probed with 6E10 (Covance Research Products) detection after being transferred onto PVDF membranes. Membranes were then stripped with stripping buffer (Thermo Scientific, Rockford, IL) and re-probed with anti-mouse β -actin by following the standard western protocol.

Statistical analysis

Following ANOVA analysis of RAWM and cognitive interference behavioral data (2-day blocks), *post-hoc* pair-by-pair differences between groups were determined through the Fisher LSD test. In the Young Adult Long-Term Study, paired t-tests were used to compare

performance between Interference 1 and Interference 2 testing. Data analysis of neurohistologic and neurochemical measurements, as well as in all remaining (e.g., one-day) behavioral measures, were performed using ANOVA followed by Fisher's LSD post-hoc test. All data are presented as mean \pm SEM.

RESULTS

Young adult long-term study

In an initial study, 2-month old A β PPsw Tg and NT mice were started on daily EMF exposure for the next 7 months. RAWM testing was done at 2½ months into EMF exposure (Figure 1A). As exemplified by the final block of RAWM testing shown, there were no effects of EMF treatment in either NT or Tg mice compared to their respective controls for Trial 1 (naïve trial) or for working memory Trials 4 and 5. This was also the case for performance over all 10 days of testing. Thus, at 2½ months into EMF exposure, young adult NT/EMF and Tg/EMF mice were no different from genotypic controls in cognitive performance. As well, there were no significant differences in performance between NT and Tg groups for this early RAWM testing.

Ensuing cognitive interference testing performed at 4-5 (Test 1) and 6-7 months (Test 2) into EMF exposure revealed a much different profile of performance (Figure 1B). When cognitive performance was compared between the first and second test periods (e.g., at 4-5 and 6-7 months into treatment), obvious beneficial effects of EMF exposure were evident for Tg mice, as shown for the retroactive interference measure (A4; Figure 1B). Cognitive performance of Tg controls deteriorated between Tests 1 and 2, while Tg/EMF mice maintained or improved their performance over the same time period. EMF treatment to NT mice had no significant effect across both cognitive interference tests (Figure 1B).

Beneficial effects of EMF exposure in Tg mice were also apparent when evaluating performance during the second test period alone (6-7 months into EMF exposure; Figure 2). A clear impairment of Tg control mice compared to NT controls was evident as exemplified by Block 1 of testing, wherein Tg control mice were impaired not only in 3-trial recall, but also in retroactive interference (Figure 2). By contrast, Tg mice that had been receiving chronic EMF exposure for 7 months showed significantly better performance than Tg controls—not only at the end of recall (A3), but also for “overall” 3-trial recall (A1-A3) and retroactive interference (A4). Although all groups performed well in 3 of the 4 interference measures during Block 2 (data not shown), Tg controls were substantially impaired in the remaining measure, proactive interference (Figure 2); this impairment in Tg mice was completely eliminated by EMF treatment. In a final task of general mnemonic function, normal NT mice that had been given chronic EMF exposure for 7 months showed much higher Y-maze spontaneous alternation levels than control NT mice, which performed similar to Tg mice (Table 1).

Thus, EMF exposure begun in young adulthood protected Tg mice from certain cognitive impairment and even enhanced cognitive performance of normal NT mice. It is important to indicate that the beneficial cognitive effects of chronic EMF exposure to both Tg and NT mice of this Young Adult Long-Term study did not occur through non-cognitive effects on sensorimotor function or anxiety. Just prior to euthanasia at 9½ months of age, all mice were tested in a battery of sensorimotor/anxiety tasks (open field activity, balance beam, string agility, and elevated plus-maze). Compared to NT and Tg controls, there were no differences in performance of NT/EMF or Tg/EMF mice, respectively. Thus, non-cognitive effects of EMF exposure can be ruled out for significantly contributing to the beneficial cognitive effects provided by long-term EMF exposure

Following completion of all behavioral testing, animals were euthanized at 9.5 months of age (e.g., prior to overt A β deposition). In both hippocampus and frontal cortex, EMF-exposed Tg mice exhibited nearly significant increases in levels of soluble A β (Table 2). In addition, hippocampal tissues were analyzed for oxidative markers to determine any effects of long-term EMF exposure on oxidative stress (Figure 3). For Tg mice, EMF exposure had essentially no effect on hippocampal DNA repair enzymes (OGG1, oxoguanine glycosylase; PARP, poly ADP ribose polymerase), antioxidant enzyme markers (cytosolic and mitochondrial SOD, GSH/GSSH), or protein oxidative damage (protein carbonyl content). Although NT mice exposed to EMFs exhibited decreased PARP, SOD, and glutathione levels in hippocampus (Figure 3), this constellation of EMF effects in NT mice can actually be interpreted as a decrease in oxidative stress. Cerebral cortex tissue from NT mice (and that of Tg mice) revealed no effects of EMF exposure on any oxidative markers analyzed (data not presented). Additionally, no group differences in DNA oxidation (8-hydroxyguanine) were seen in striatal tissues from all four groups.

Aged adult long-term study

To determine if EMF exposure can reverse cognitive impairment and arrest brain A β pathology in older AD Tg mice, we exposed 5 month-old A β PPsw and NT mice to daily EMF exposure for the following 8 months. In this "aged adult" long-term study, cognitive testing was performed before the start of EMF exposure, as well as at 2 months, 5 months, and 8 months into EMF exposure. During pre-exposure cognitive testing at 4 months of age, naïve Tg mice were clearly impaired in the RAWM task of working memory (Figure 4A). For the last 2-day block of pre-treatment testing, NT mice nicely reduced their errors between Trial 1 (T1; the naïve trial)

and combined working memory Trials 4+5; however, Tg mice could not do so. Indeed, combined T4+5 errors during this block were much higher in Tg mice compared to NT mice. This cognitive impairment extended across all 6 days of RAWM pre-treatment testing, as evidenced by the substantially higher number of working memory errors by Tg mice on both T4 and T5 overall (Figure 4A). Thus, aged Tg mice were cognitively impaired prior to EMF exposure in this study.*

Animals were re-evaluated in the RAWM task at 2 months into EMF exposure (at 7 months of age). As depicted in Figure 4B, EMF exposure had no positive or negative effects on working memory for either Tg or NT mice over all 14 days of testing. Indeed, Tg mice in both groups were near identical in continuing to be impaired during working memory Trials 4 and 5. Thus, the initial two months of EMF exposure did not provide cognitive benefits to impaired Tg mice. This was also the case for Tg mice during cognitive interference testing performed 3 months later (e.g., 5 months into EMF exposure, at 10 months of age). As shown in Figure 5B, there were no differences between Tg and Tg/EMF mice on any measure of cognitive interference testing during the final two-day block of testing. By contrast, NT mice at 5 months into EMF exposure exhibited improved performance on several measures of cognitive interference testing (Figure 5A), particularly on the retroactive interference trial. Thus, during initial cognitive testing performed at 2 and 5 months into EMF exposure, there were no deleterious or beneficial effects observed in Tg mice, while NT mice actually showed some cognitive benefit at 5 months into exposure.

After 8 months of EMF exposure, all mice were re-evaluated in the cognitive interference task of working memory (Figure 6). At this 13 month age, non-treated Tg control mice were noticeably impaired while the cognitive performance of Tg mice receiving EMF exposure was

strikingly better (Figure 6B). On 3-trial recall, Tg/EMF mice performed significantly better than Tg controls overall and even on the initial recall trials. In addition, Tg/EMF mice showed vastly superior retroactive interference performance compared to Tg controls. Even NT mice continued to show cognitive benefits from ongoing EMF exposure through 8 months (Figure 6A).

Because it is well-known that EMF exposure can increase body/tissue temperature, we monitored body temperature via rectal probe during a single day of EMF exposure just prior to euthanasia of mice (i.e., at 8½ months into EMF exposure). Compared to animals in all other groups, AβPPsw Tg mice being given EMF exposure had significantly higher body temperature (over 1°C higher) during both early morning and late afternoon EMF exposures (Figure 7). During the “off” period between the two EMF exposures, no group differences in body temperature were observed. Thus, body temperatures of Tg mice were elevated only during “on” periods of EMF exposure.

After euthanasia at 13½ months of age (8½ months into EMF exposure), Aβ immunostaining from AβPPsw Tg mice revealed substantially lower Aβ burdens in both hippocampus (↓35%) and entorhinal cortex (↓32%) of EMF-exposed Tg mice compared to Tg controls (Figure 8A and B). These same EMF-exposed Tg mice exhibited nearly significant increases in hippocampal and cortical levels of soluble Aβ (Figure 8C). The results collectively suggest an ability of EMF exposure to suppress brain Aβ aggregation and/or to disaggregate pre-existing Aβ plaques in Tg mice. To explore this anti-Aβ aggregating potential of EMFs further, we exposed hippocampal homogenates from Tg mice to the same EMF strength/parameters as in our *in vivo* studies. By four days into EMF exposure, substantially less aggregated (oligomeric) Aβ was evident in Western blots compared to non-exposed hippocampal homogenates (Figure 9). We have confirmed, through both brain lysis and Aβ peptide aggregation studies, that the 80 kDa band

displayed is indeed oligomeric A β (and not A β PP or some other A β fragment). As such, this is the first evidence that cell phone-level EMF exposure can decrease brain A β aggregation.

* Parenthetically, similarly-aged Tg mice in the Young Adult study were not significantly impaired by the end of RAWM testing because of a longer 10-day test period that allowed them more time to reach the performance level of NT mice.

Aged adult acute study

To determine if the hyperthermic response seen in Tg mice during “On” periods of the Aged Adult Long-Term study (Figure 7) is also induced by *acute* EMF exposure, an additional study was performed over a single day in naïve aged mice. No effects of acute EMF exposure on brain temperature (as measured by temporal muscle probe) or body temperature were evident for Tg (A β PPsw, A β PPsw+PS1) mice or NT mice of several ages (Figure 10A), indicating that long-term EMF exposure was required for the increased body temperature seen in Tg mice during “on” periods. To determine if body temperature accurately reflects brain temperature, we compared temperature readings from both body and brain (as measured by skull temporal muscle). Given the very close correlation between body and brain temperature present in this acute study (Figure 10B), it is apparent that the EMF-induced elevation in body temperature of Tg mice in the adult long-term study (Figure 7) reflected similarly elevated brain temperature during “on” periods.

DISCUSSION

We report here profound effects of long-term EMF exposure to protect against/reverse cognitive impairment and A β neuropathology in AD Tg mice. Several complementary

mechanisms may be involved, most notably a remarkable action of EMF exposure to decrease brain A β aggregation, as we demonstrated in both *in vitro* and *in vivo* studies. Collective, our results, attained in AD transgenic mice, suggest that high frequency EMF exposure could be a non-invasive, non-pharmacologic therapeutic against AD, as well as a means to enhance memory in general. It is important to emphasize that the A β PPsw mouse model for AD utilized in these studies is only a partial, albeit well-established, animal model for the disease—a model that does not recapitulate aspects of AD such as neuronal loss and neurofibrillary tangle formation. As such, care should be taken in extrapolating our results to cell phone use and EMF exposure in humans.

The presently reported beneficial effects of high frequency EMF exposure in both NT and Tg mice were observed after months of exposure to cell phone-level EMFs. Whether or not more acute exposure would have provided similar cognitive benefits is not known. In contrast to the cognitive improvement shown by EMF-exposed “normal” NT mice in our study, prior studies involving acute (7-14 days) EMF exposure to normal rodents failed to show any effects on cognitive performance [8,9]. A limited daily (15-45 minute/day) and total EMF exposure length, different SAR levels, or use of different cognitive assessments could have been confounding factors in these earlier studies. In normal humans, some “acute” EMF exposure studies have demonstrated small beneficial effects on attention/response time or working memory, while others failed to demonstrate any enhanced cognitive performance [5-7]. In a recent meta-analysis of 10 such acute studies, Barth and colleagues [7] concluded there may be small beneficial or negative effects of EMF exposure on human attention and working memory, although any impact on everyday life was essentially ruled out. Perhaps most pertinent to our findings, a recent epidemiologic-based study reported that heavy cell phone use over several years resulted

in better performance on a word interference test [23]. In view of these and our present findings, we propose that only long-term EMF exposure may provide consistent and significant cognitive benefits to humans at cell phone-level EMF strengths. Second, we propose that such EMF exposure may have the capacity to enhance cognitive function in normal, non-demented individuals.

To date, there is no evidence that “high frequency” electromagnetic fields, such as those emitted by cell phones, affect the risk of AD. Indeed, the present study provides striking evidence for both protective and disease-reversing effects of long-term EMF exposure, and at cell phone-level intensities. The precise mechanisms of EMF benefit, though currently also explored to some extent, are beyond the scope of this initial work and will require more extensive research. What the novel findings of this present work do establish is that EMF exposure: 1) provides beneficial cognitive effects in an animal model of AD and normal mice; 2) reduces A β deposition in the same AD mice; 3) suppresses A β aggregation *in vitro*; and 4) induces these effects without increasing indices of oxidative stress in the brain. Moreover, the cognitive benefits of long-term EMF exposure were demonstrated in two separate and well-controlled behavioral studies, thus minimizing any potential that the results are spurious.

There are several mechanisms, separately or in combination, that are most likely involved in the beneficial impact of EMF exposure on AD-like cognitive impairment/A β neuropathology in Tg mice and on cognitive performance in normal mice (Figure 11A). Certainly, one probable mechanism in AD mice would be suppression of A β aggregation and/or disaggregation of pre-existing A β plaques. Consistent with such an “anti-A β aggregation” mechanism is the presently-reported EMF-induced: 1) decrease in brain A β deposition accompanied by the nearly significant increase in soluble brain A β levels; and 2) suppression of A β aggregation/oligomerization in

hippocampal homogenates *in vitro*. Whether the robust anti-aggregating ability of EMF exposure is dependent or independent of the increased brain temperatures induced by long-term EMF exposure requires additional studies (Figure 11A). Irrespective, the delayed ability of EMF exposure to benefit cognitive performance in adult Tg mice (e.g., manifesting itself at 8 months into exposure), may reflect the time required for the currently-used EMF parameters to substantially decrease the pool of deposited/insoluble A β by chronically decreasing flux of soluble \rightarrow insoluble A β (Figure 11B). This decreased flux toward insoluble A β would increase brain soluble A β levels, and presumably result in greater clearance of that soluble A β from the brain. Different EMF parameters that may clear brain A β more quickly are currently being investigated.

It should be noted that several other reported therapeutics that decrease brain A β aggregation also result in the combination of less A β deposition and unchanged or greater soluble A β levels in the brain. For example, the A β peptide 12-28P prevents binding of ApoE to full-length A β , thus acting as an anti-A β aggregating agent. A β 12-28P has been report to decrease brain A β deposition, to not affect soluble A β levels in the brain, and to improve cognitive function in A β PPsw mice [24]. Similarly, we have recently shown that long-term administration of the anti-A β aggregating agent melatonin to A β PP+PS1 transgenic mice reduced extent of A β deposition, increased or did not affect brain soluble A β levels, and protected Tg mice from cognitive impairment [25]; this same behavioral/brain A β profile was provided in the present study by long-term EMF exposure. Compared to other therapeutic approaches against AD, an advantage of anti-A β aggregators such as A β 12-28P, melatonin, and possibly EMF exposure, is that they target the abnormal deposited/insoluble form of A β and do not disrupt possible normal functions of the soluble A β peptide.

A second possible mechanism of action involves the ability of EMF exposure to increase neuronal/EEG activity (Figure 11A) [5, 26, 27]. This ability is underscored by studies showing that cell phone-level EMF exposure increases cortical PET signaling [28,29]. With regard to A β and AD Tg mice, A β PP in pre-synaptic neuronal membranes is internalized via endocytosis, after which A β is cleaved and available for release during neuronal activity (Figure 11B) [29,30]. Increased neuronal activity has been shown to result in greater synaptic release of this intracellular A β into brain interstitial fluid (ISF) [30,31], which would make it available for transcytotic transport out of the brain. Since elevated temperature generally increases neuronal activity [32], the increased brain temperature evident in Tg mice months into EMF exposure could be important for such increased neuronal/synaptic activity (Figure 3A), with resultant A β released into the ISF and consequent removal from the brain via transcytosis. Supportive of this cascade, a recent human study found a strong association between increased brain temperature and elevated brain ISF levels of A β [33].

In Tg mice, the 1°C increase in body/brain temperature present during ON periods of “long-term” EMF exposure may play a key role in several mechanisms involved in cognitive enhancement (Figure 11A), most notably, the EMF-induced removal of A β from the brain. This temperature increase was not observed with an acute (single day) EMF exposure in Tg or NT mice, indicating that a protracted period of intermittent EMF exposure is required to elicit increases in brain/body temperature during ON periods. Our results involving acute EMF exposure are consistent with a human study that found “acute” EMF exposure (at the cell phone levels used in the current study) induces only a small 0.1°C increase in brain temperature during the ON period [34]. Whether long-term EMF exposure at cell phone frequencies increases brain temperature in humans (as our study would predict) is an important question that remains to be

determined. In the present study, it should be underscored that: 1) brain/body temperature in Tg mice during ON periods almost never exceeded 38°C, which is well below the 41°C level that begins to result in mammalian brain damage [35]; and 2) fluctuations of 2°C or higher in mammalian brains occur regularly, depending on behavioral and metabolic state [32]. Thus, our observed 1°C elevation in temperature during EMF exposure would appear to be safe and, in fact, cognitively beneficial.

In addition to the aforementioned A β -specific actions, two generalized mechanisms of EMF action could be providing cognitive benefits to both Tg and NT mice (Figure 11A), namely, EMF-induced increases in cerebral blood flow [29, 29] and glucose utilization [36]. Both of these mechanisms appear to be mediated by EMF-induced increases in neuronal activity (Figure 11A). It is important to underscore that all of the potential A β -dependent and generalized mechanisms of EMF action to benefit cognitive performance will require appreciable follow-up study. As is often the case for such surprising and profound findings as those in the current report, many new questions arise from the initial work in a new therapeutic area.

Ironically, we began these studies with the hypothesis that long-term EMF exposure would be deleterious to cognitive function in Tg and/or NT mice, most probably through increased oxidative stress. Indeed, previous *in vitro* and “acute” (hours,days) *in vivo* studies had found EMF exposure to increase oxidative stress/damage in various organ systems/animals [37, 38]. However, our analysis of oxidative markers from brains of mice in the young adult long-term study (exposed to EMFs for 6-7 months) revealed minimal or no EMF-induced effects on DNA repair enzymes, antioxidant enzymes, or extent of protein oxidative damage. These results are consistent with a prior study involving cell phone EMF exposure to rabbits for 7 days, wherein no effects on brain oxidative markers were seen [39]. We infer that minimal/no brain oxidative

damage results from chronic cell phone-level EMF exposure or that compensatory mechanisms come into play during long-term EMF exposure that largely negate any acute EMF-induced increases in oxidative stress/damage.

Several caveats should be mentioned in view of this report's findings. First, animals received full body EMF exposure, not the head-only exposure that humans experience with cell phone use. Daily head-only exposure to mice over many months would have burdened the mice with daily immobilization stress and would have been extremely labor-intensive. Parenthetically, it is unclear whether EMF penetrate the skull comparably in humans and mice; therefore, the actual EMF "dose" and penetration of brain tissue may be different for these two species. Second, AD Tg mice are only a partial model for the disease, as indicated earlier. Therefore, the therapeutic impact of EMF exposure to human AD patients may be different. Nonetheless, our use of a novel cognitive task designed to closely mimic a human cognitive interference task that discriminates AD, MCI, and non-demented individuals [10] could heighten the potential human relevance of our findings. Indeed, we have previously utilized this cognitive interference task for mice in demonstrating impairment of A β PPsw mice and the ability of a cRaf-1 inhibitor (Sorafenib) to alleviate that impairment [14]. Finally, the optimal EMF parameters for cognitive benefit in humans may have other consequences on health not presently evident. Irrespective, we believe that the current lack of an effective therapeutic against AD, in concert with this study's surprising findings, justifies EMF exposure as a non-invasive, non-pharmacologic approach worthy of vigorous investigation.

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Table 1. Y-maze spontaneous alternation in Young Adult study at 6-7 months into EMF exposure

<u>Group</u>	<u>Percent Alternation</u>
NT	55 ± 6
NT/EMF	75 ± 5*
Tg	60 ± 3
Tg/EMF	49 ± 4

*p<0.05 or higher level of significance versus all other groups

Table 2. Effects of 7-months EMF exposure on brain soluble A β levels (pg/ml) of Tg mice in Young Adult study

	<u>Tg</u>	<u>Tg/EMF</u>	<u>% Change</u>	<u>“p” value</u>
<u>Hippocampus</u>				
A β ₁₋₄₀	4022 \pm 359	4750 \pm 208	+18%	0.11
A β ₁₋₄₂	808 \pm 116	1000 \pm 40	+24%	0.15
<u>Frontal Cortex</u>				
A β ₁₋₄₀	2785 \pm 245	4241 \pm 743	+52%	0.09
A β ₁₋₄₂	751 \pm 88	1107 \pm 281	+47%	0.26

FIGURE LEGENDS

Figure 1. Behavioral testing of young adult mice in the radial arm water maze (RAWM) task of working memory (A) at 2½ months into EMF exposure and in the cognitive interference task (B) at 4-5 months (Test 1) and 6-7 months (Test 2) into EMF exposure. (A) As shown for the final 2-day block of testing, no differences in RAWM working memory were present between NT controls and NT/EMF mice for Trial 1 (naïve trial) or the final two working memory Trials 4 and 5. This was also the case for Tg controls and Tg/EMF mice. No genotypic effect was evident as well. (B) Across both cognitive interference tests, EMF-exposed Tg mice exhibited stable/improved cognitive performance (as did both NT groups), while performance of control Tg mice worsened substantially. Data from the retroactive interference measure (A4) is depicted, wherein the final block of Interference 1 was compared to first block of Interference 2 for indexing memory retention between tests. * $p < 0.05$ by paired t-test; ** $p < 0.01$ for Tg vs. Tg/EMF.

Figure 2. Cognitive interference testing at 6-7 months into EMF exposure to young adult mice showed cognitive protection of Tg/EMF mice during Block 1 (3 trial recall, retroactive interference), as well as during Block 2 (proactive interference). * $p < 0.05$ vs. all other group(s) for the same measure; † $p < 0.05$ vs. Tg/EMF group.

Figure 3. Following 7 months of EMF exposure to young adult Tg mice, markers of oxidative damage and antioxidant enzymes/compounds in hippocampus were largely unaffected. In NT mice, EMF exposure induced decreased levels of the DNA repair enzyme PARP and suppression in some antioxidant enzymes/compounds, but no changes in protein oxidative damage. * $p < 0.05$

vs. NT controls; † $p < 0.05$ vs. Tg controls. Abbreviations: GSH, reduced glutathione; GSH/GSSG, ratio of reduced to oxidized glutathione; OGG1, 8-oxoguanine glycosylase; PARP, poly ADP-ribose polymerase; SOD, superoxide dismutase.

Figure 4. Aged adult Tg mice were impaired in working memory performance prior to the start of EMF exposure (A), as well as following the first 2 months of EMF exposure (B). (A) Radial arm water maze (RAWM) working memory performance during the last of three 2-day test blocks and over all 3 blocks for NT and Tg mice prior to EMF exposure at 4 months of age. Left graph: * $p < 0.0005$ for T1 vs. T5 (t-test); † $p < 0.005$ vs. NT group. Right graph: * $p < 0.05$ or higher level of significance vs. NT group. (B) At 2 months into EMF exposure, there were no effects on RAWM working memory performance of either NT or Tg mice over all 14 days of testing. Both groups of Tg mice were impaired on working memory trials T4 and T5 compared to both groups of NT mice. * $p < 0.02$ or higher level of significance for both Tg groups vs. both NT groups.

Figure 5. At five months into EMF exposure, no deleterious or beneficial effects were evident in cognitive interference testing of Tg mice (B), although normal (NT) mice showed EMF-induced cognitive benefits in several measures (A). Data for the final two-day block of testing are presented. * $p < 0.05$ vs. NT control.

Figure 6. Cognitive interference testing at 8 months into EMF exposure of aged adult NT (A) and Tg (B) mice. Tg mice given long-term exposure to EMF were superior to Tg controls in both 3-trial recall and retroactive interference measures (B); * $p < 0.025$ vs control. Even NT mice receiving 8 months of EMF exposure showed better recall performance than NT controls,

particular early in recall testing (A); * $p < 0.05$ or higher level of significance vs. control. The final 2-day block of testing is shown from four days of testing.

Figure 7. During both 1-hour EMF exposure periods in a given day at 8 months into exposure, aged Tg mice exhibited significantly higher body temperatures compared to mice in all other groups. NT/EMF exhibited marginally-higher body temperatures during the morning exposure. * $p < 0.05$ or higher levels of significance vs. all other groups; † $p < 0.05$ vs. NT group.

Figure 8. Long-term (8½ months) EMF exposure to aged Tg mice significantly reduced total A β deposition in entorhinal cortex and hippocampus. (A) Photomicrographic examples of typical A β immunostained-plaques from Tg and Tg/EMF showing the substantial reduction in A β deposition present in brains of age mice chronically exposed to EMF. Scale bar = 50 μ m. (B) Quantification of A β burdens in both entorhinal cortex and hippocampus of aged Tg mice following 8½ months of EMF exposure. * $p < 0.02$ vs. Tg control group. (C) Long-term EMF exposure nearly increased soluble A β_{1-40} and A β_{1-42} levels in hippocampus and cerebral cortex.

Figure 9. *In vitro* EMF exposure of hippocampal homogenates from 14 month old Tg mice resulted in progressively decreased A β aggregation (oligomerization) between 3 and 6 days into exposure. Western blots display the 80 kDa A β oligomer on top and the β -actin protein control on bottom. Left panel shows non-treated Tg controls of A β aggregation, while right panel shows the same homogenates exposed to EMF treatment through 6 days.

Figure 10. Brain temperature (as measured by temporal muscle probe) before, during, and between acute EMF exposure (A) or non-exposure (B) in naïve mice of various genotypes and ages. Measurements were all recorded during a single day, with identical results attained on several other single day EMF exposures (not shown), all done within a two-week period. Each measurement represents the mean of 4-5 mice per group. (C) Strong correlation between body temperature and brain temperature in an acute 1-day study. Each symbol represents readings from one mouse during pre-treatment measurements.

Figure 11. (A) Proposed mechanisms of action for the beneficial cognitive effects of long-term EMF exposure in normal and AD Tg mice. (B) Diagrams depicting the proposed inhibitory action of long-term EMF exposure on A β aggregation and enhanced neuronal A β release in aged Tg mice, resulting in higher interstitial fluid A β levels and increased brain clearance of A β .

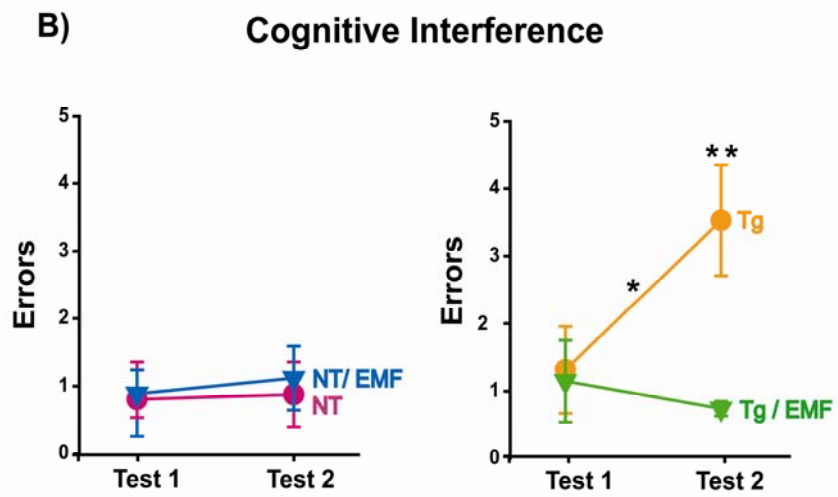
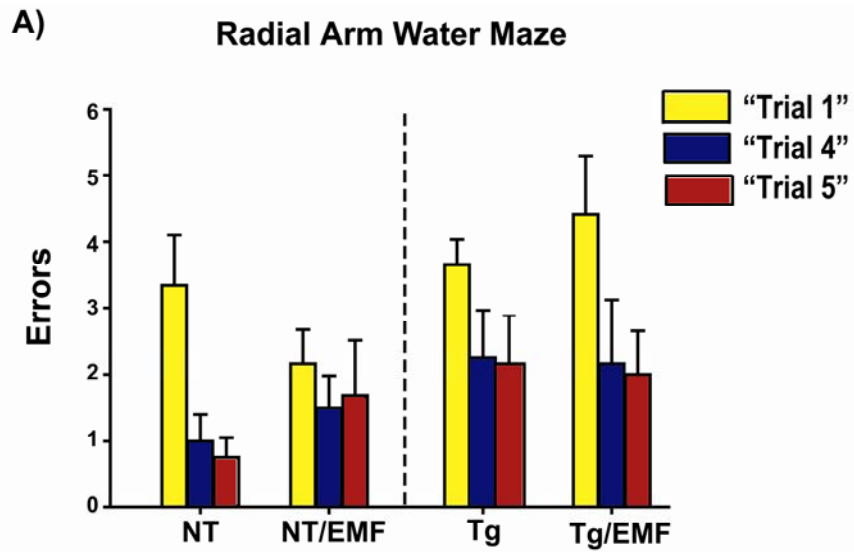


Figure 1

Young Adult : 6-7M into EMF exposure

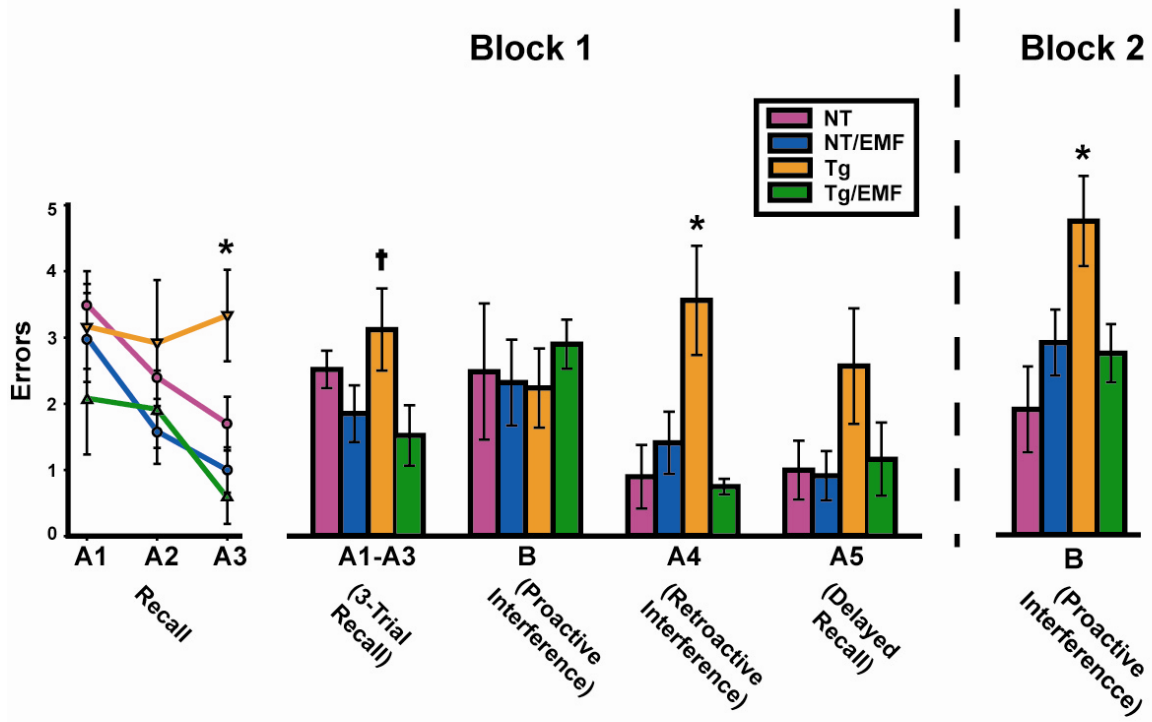


Figure 2

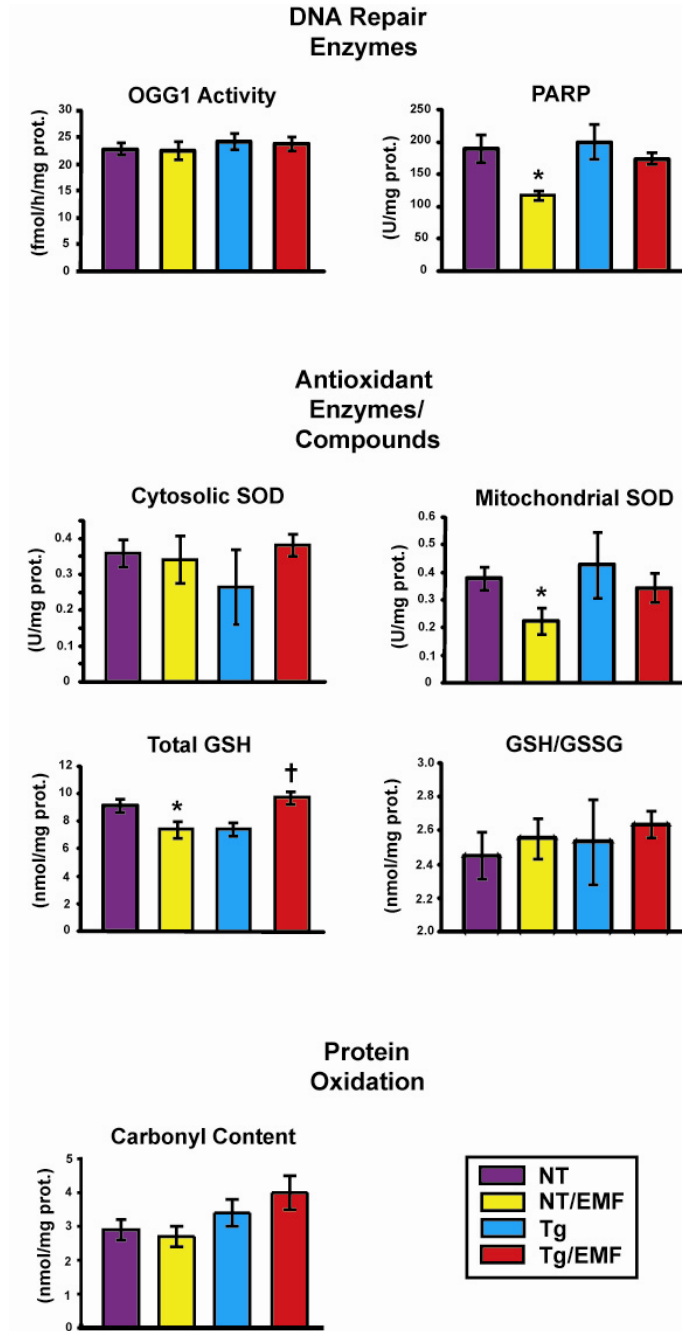


Figure 3

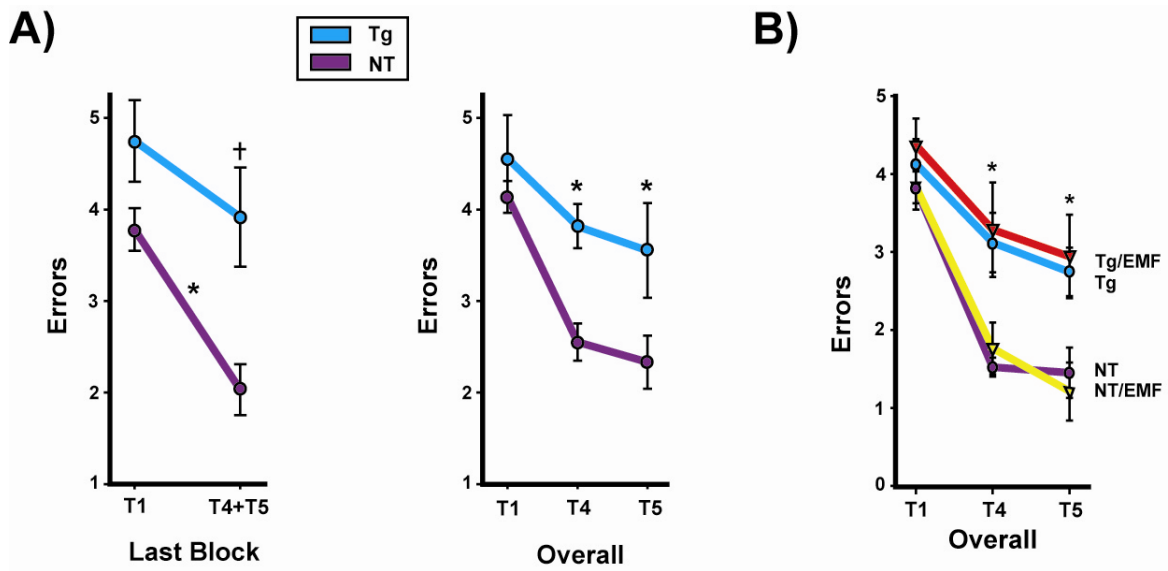


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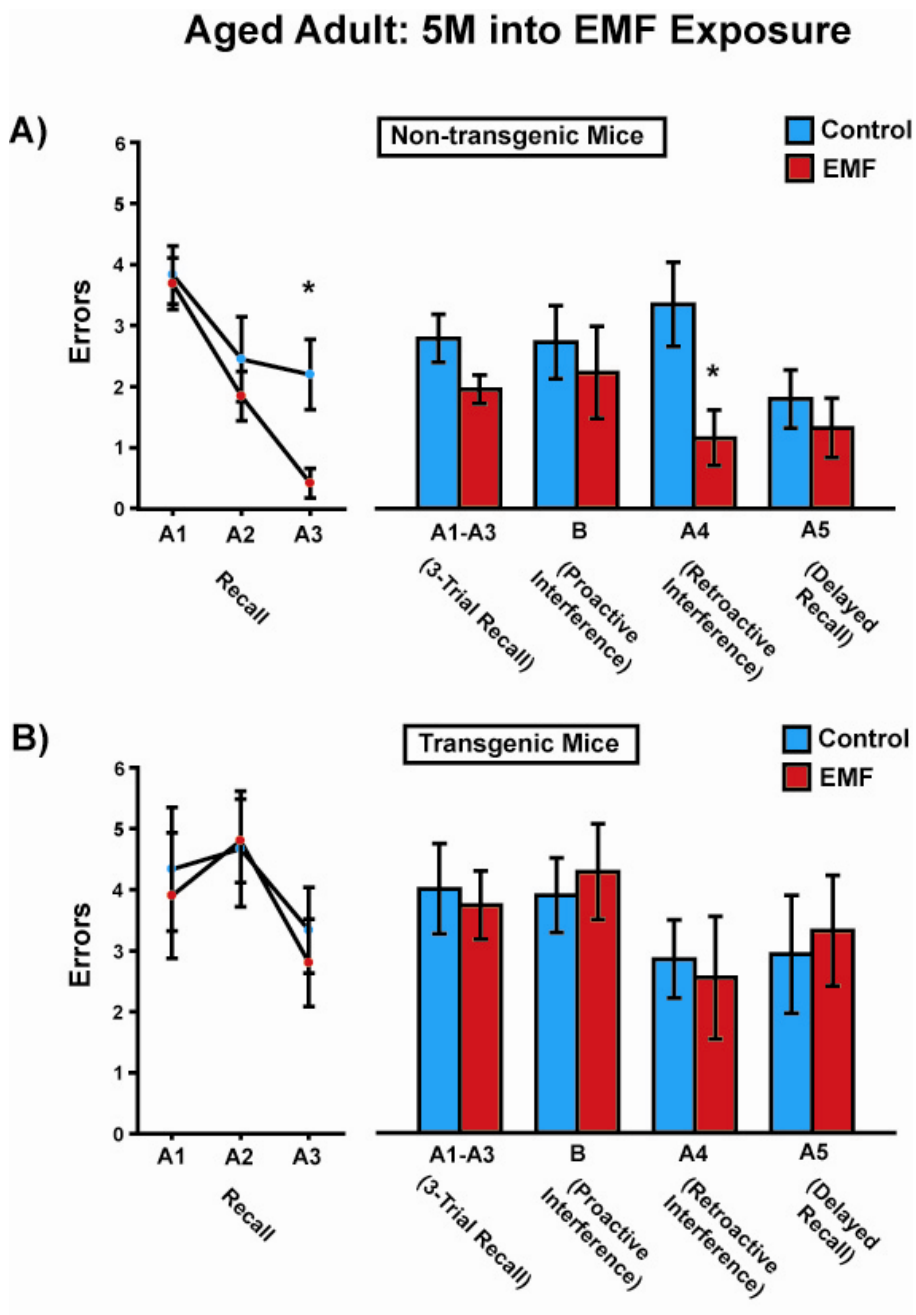


Figure 5

Aged Adult: 8M into EMF Exposure

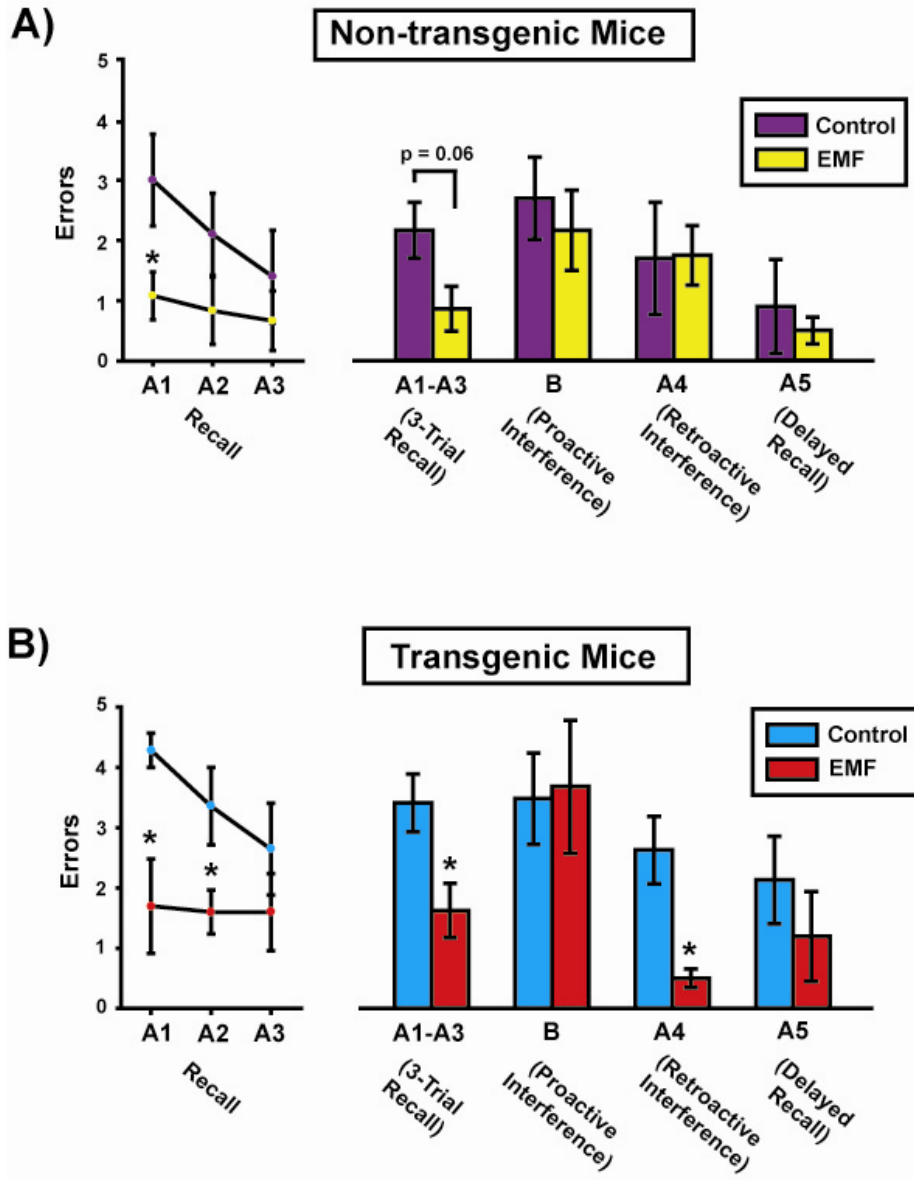


Figure 6

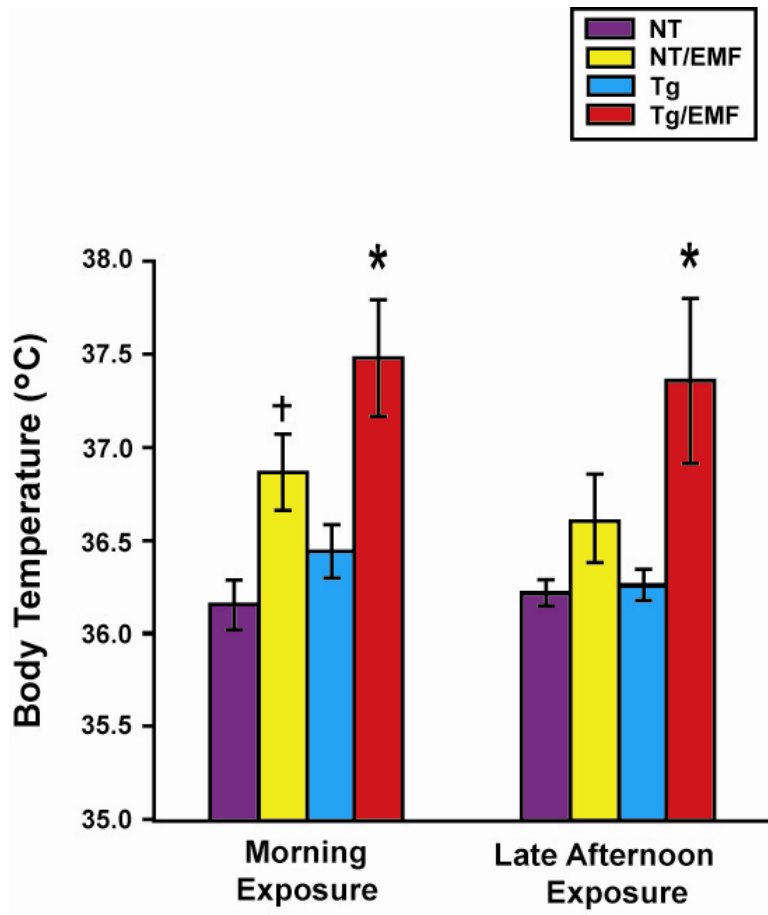


Figure 7

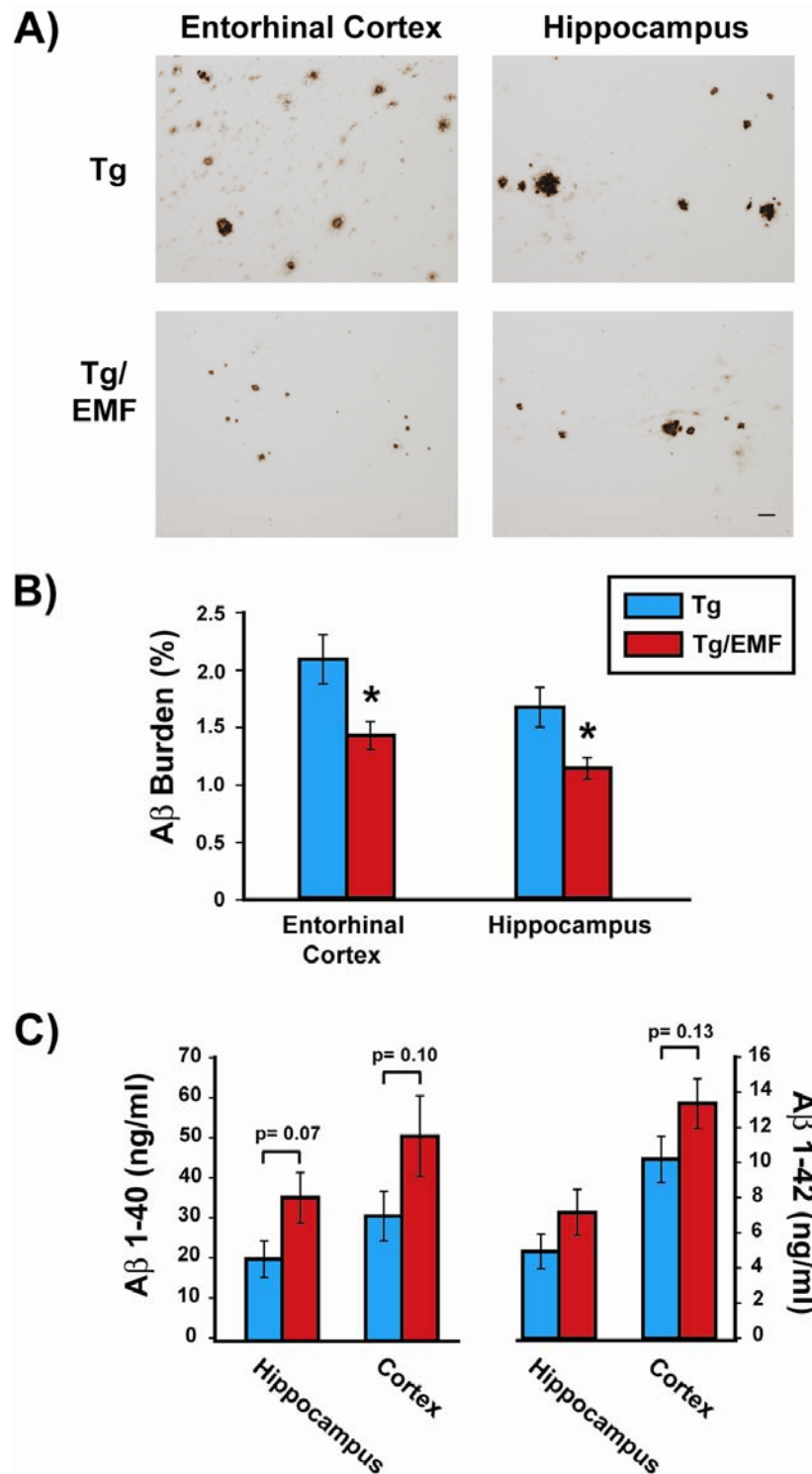


Figure 8

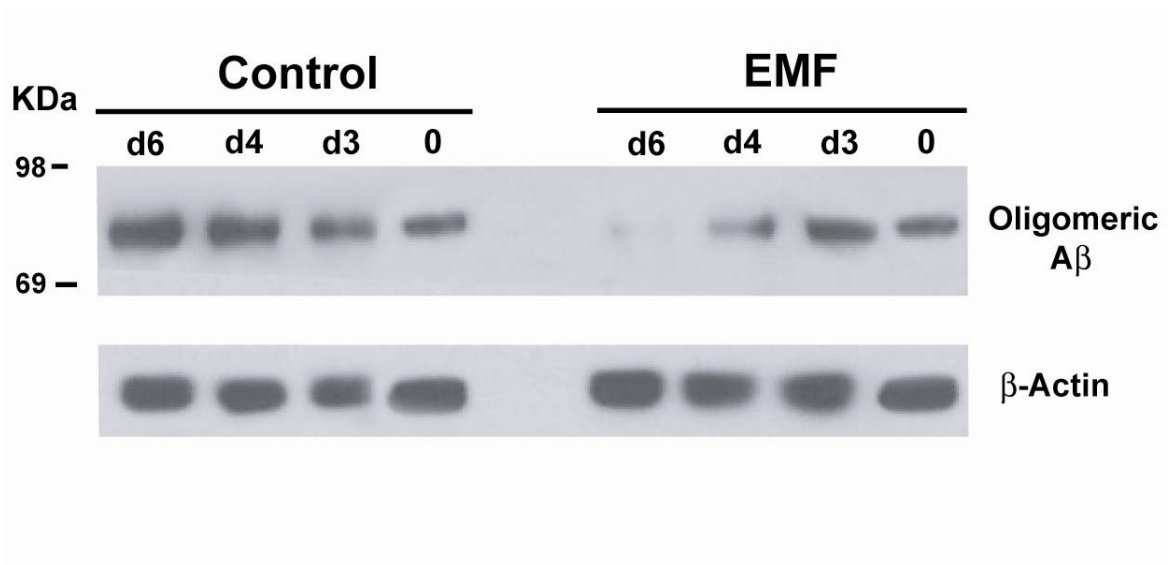


Figure 9

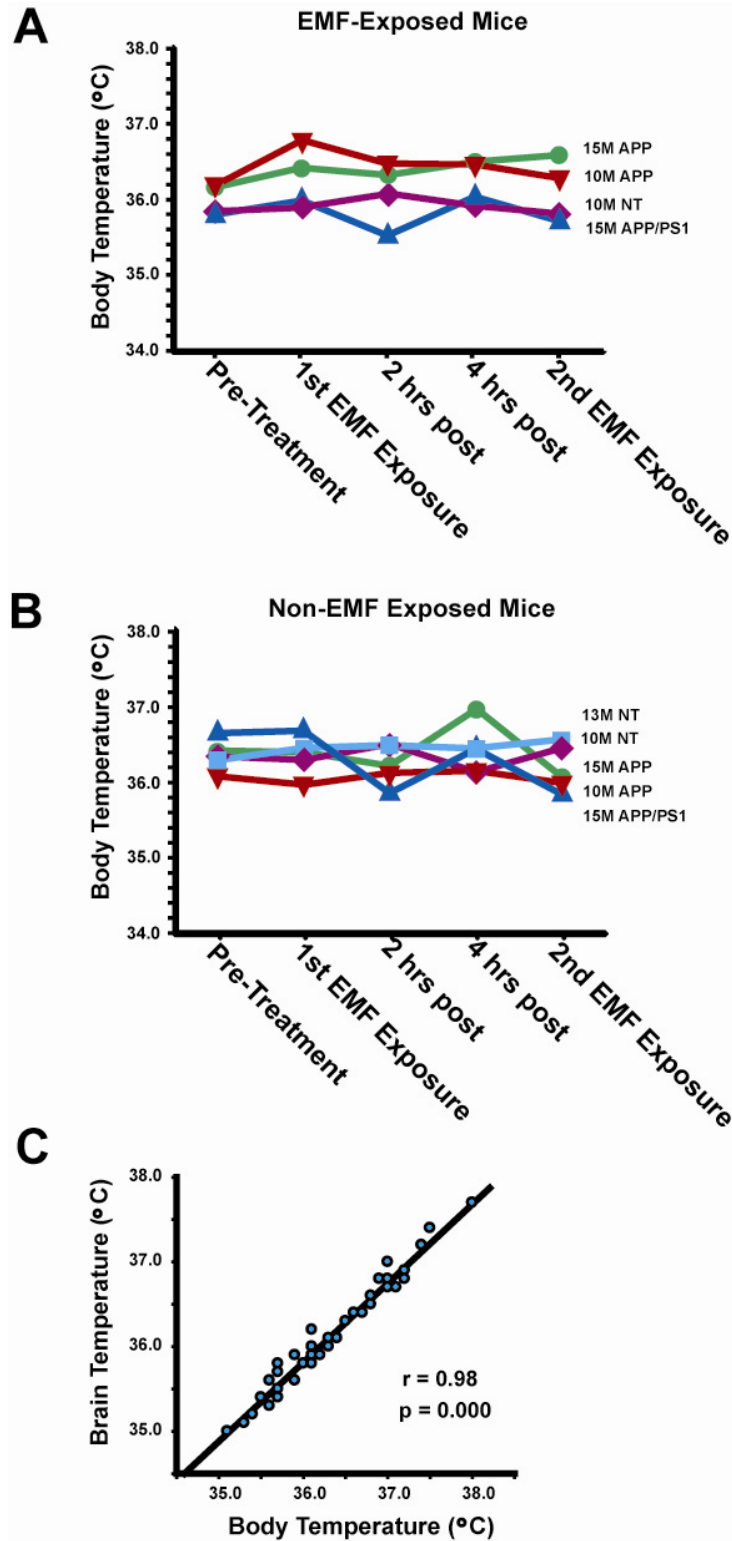


Figure 10

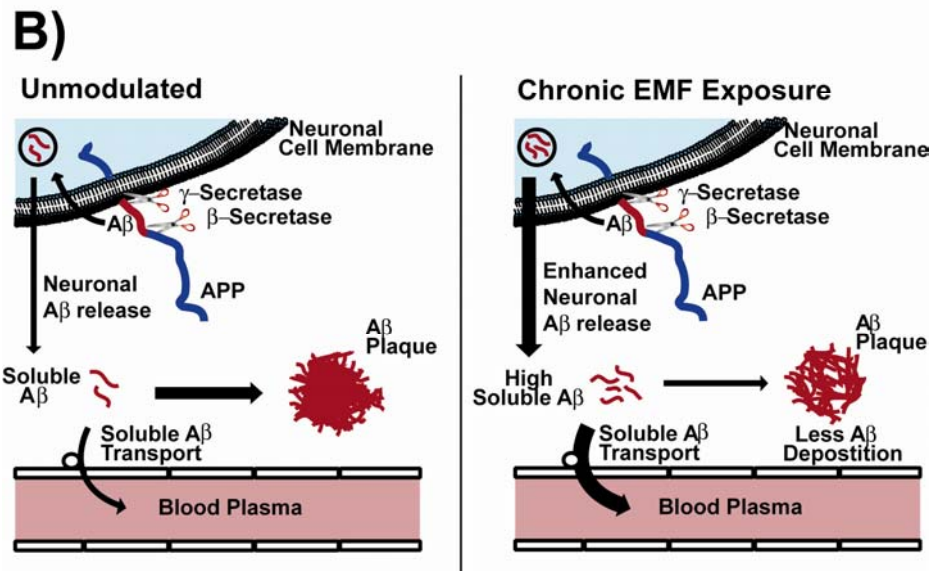
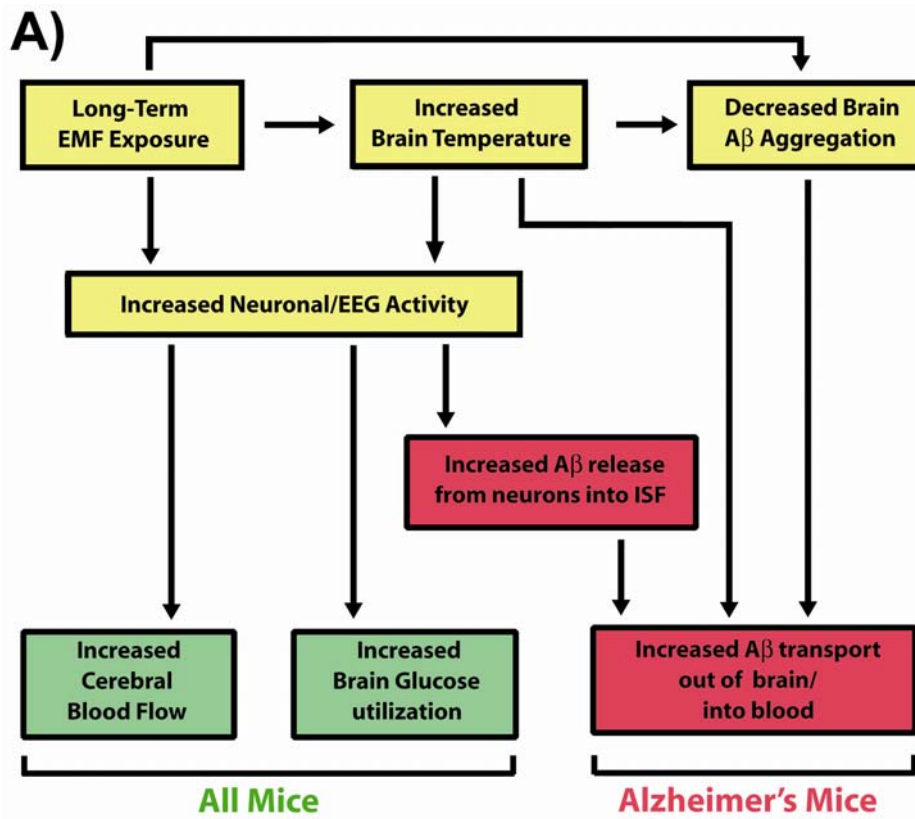


Figure 11