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Long-Term Pioglitazone Treatment Improves Learning and Attenuates Pathological Markers in a Mouse Model of Alzheimer's Disease

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Abstract

Thiazolidinediones (TZDs) are agonists at peroxisome proliferator-activated gamma-type (PPAR- γ) receptors and are used clinically for the treatment of type 2 diabetes where they have been shown to reestablish insulin sensitivity, improve lipid profiles, and reduce inflammation. Recent work also suggests that TZDs may be beneficial in Alzheimer's disease (AD), ameliorating cognitive decline early in the disease process. However, there have been only a few studies identifying mechanisms through which cognitive benefits may be exerted. Starting at 10 months of age, the triple transgenic mouse model of AD (3 $\times$ Tg-AD) with accelerated amyloid- β (A β) deposition and tau pathology was treated with the TZD pioglitazone (PIO-Actos[®]) at 18 mg/Kg body weight/day. After four months, PIO-treated animals showed multiple beneficial effects, including improved learning on the active avoidance task, reduced serum cholesterol, decreased hippocampal amyloid- β and tau deposits, and enhanced short- and long-term plasticity. Electrophysiological membrane properties and post-treatment blood glucose levels were unchanged by PIO. Gene microarray analyses of hippocampal tissue identified predicted transcriptional responses following TZD treatment as well as potentially novel targets of TZDs, including facilitation of estrogenic processes and decreases in glutamatergic and lipid metabolic/cholesterol dependent processes. Taken together, these results confirm prior animal studies showing that TZDs can ameliorate cognitive deficits associated with AD-related pathology, but also extend these findings by pointing to novel molecular targets in the brain.

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Keywords

3×Tg-AD; aging; hippocampus; long-term potentiation; microarray analysis; pioglitazone; PPAR; T2DM

INTRODUCTION

Alzheimer's disease (AD) is the most widespread cause of dementia and its incidence will continue to increase rapidly as the population ages [1]. Although intensive research efforts have provided considerable insights into the biology of AD, the underlying processes mediating the progressive decline in cognitive function are still poorly understood. However, a potentially important observation on the pathogenesis of AD has emerged from studies showing that the risk of AD and mild cognitive impairment (MCI) is increased in subjects with metabolic syndrome (characterized by insulin resistance) and Type 2 diabetes mellitus (T2DM) [2–6]. This link has suggested that drugs used to treat diabetes may also be efficacious in AD. In particular, the thiazolidinediones (TZDs), drugs that restore insulin sensitivity and reestablish glucose homeostasis, have been shown to exert multiple beneficial effects on age-related cognitive decline [7–11]. For example, the TZD rosiglitazone improved cognition in patients with AD or MCI [10], and the TZD pioglitazone (PIO) exerted positive effects on cognitive and functional outcomes in patients with MCI and diabetes [11]. Further, adjunct treatment with rosiglitazone appears to provide some relief from cognitive decline in MCI patients with T2DM [7]. In addition, factors related to lipid metabolism appear to play a role in determining sensitivity to the possible cognition enhancing effects of TZDs. Thus, rosiglitazone improves cognition in non-diabetic elderly patients with probable or mild-to-moderate AD [8] lacking the E4 allele of the apolipoprotein E (APOE-4), but not in APOE-4 carriers.

TZDs are synthetic peroxisome proliferator-activated receptor γ (PPAR- γ) agonists that regulate expression of genes that play a role in glucose handling and adipogenesis. Peripheral targets include insulin receptors, glucose transporters, fatty acid synthase, and several key regulators of adipocyte differentiation [12–15]. Importantly, however, clinical studies with AD patients have also raised the possibility that TZDs might impact cognitive function through mechanisms other than glucose regulation. That is, TZDs have been shown not only to reestablish glucose regulation and normal lipid levels, but also to reduce inflammation and amyloid- β (A β) peptide as well as increase cerebral blood flow in humans [11, 16, 17].

Studies in animal models have also identified multiple potential mechanisms of neuroprotection conferred by TZDs. In addition to improvements in glucoregulation, the beneficial effects of TZDs in several murine models of AD have been linked to reductions in inflammatory cytokines [18–21], oxidative stress [22], A β deposits [18, 23, 24], glial activation [18, 22], tau phosphorylation [23, 24], or glucocorticoid signaling [25–28]. In the Tg2576 model of AD which develops diabetes, TZDs reestablish insulin sensitivity, but also reduce soluble A β ₄₀ fragments [29] suggesting that the improvement in cognition in this model may occur independently of an effect on glucose regulation [30].

Given that the pathways through which TZDs potentially modulate cognitive decline and AD-related pathology are poorly understood, we conducted an extensive multidisciplinary analysis of the CNS effects of chronic TZD exposure in a mouse model of AD pathology. The 3×Tg-AD model was chosen because it exhibits a wide range of biomarkers of AD, including cognitive decline, synaptic impairment, elevated A β peptides and neuritic plaques, as well as hyperphosphorylated tau proteins and neurofibrillary tangles [31]. We used

behavioral, electrophysiological, biochemical, and gene microarray approaches to examine the impact of long-term TZD treatment *in vivo* on multiple markers of AD-related pathology. Of the two clinically relevant TZDs, PIO was chosen because of its greater lipophilicity and potential higher access to the brain [32].

This is one of the first multidisciplinary studies of chronic exposure to a TZD looking at both central and peripheral markers in a mouse model of AD pathology. The results showed that long-term PIO significantly attenuated several pathological markers of AD. Peripheral glucose concentrations were not altered although several major pathways in the brain were associated with the improvement of outcome measures, suggesting that PIO acted, at least in part, through mechanisms other than glucose regulation.

MATERIALS AND METHODS

Animals

Experiments were conducted in compliance with the institutional guidelines of the Animal Care and Use Committee at the University of Kentucky. Twenty-nine, 10 month-old female triple transgenic mice overexpressing A β PP_{SWE} and Tau_{p301L}, and carrying a PS1M146V knock-in mutation (3 $\times$ Tg-AD) were maintained in a 10-h dark and 14-h light cycle in groups of three or four per cage. These animals were randomly separated into two groups, one receiving a control diet ($n = 15$, CTRL), the other a PIO-enriched diet ($n = 14$, PIO). Animals were maintained on these diets for 14 weeks and were, therefore, 14–15 months of age when all experiments began. CTRL and PIO diet were sustained until all data were acquired. Animals weights and food consumption were recorded three times per week throughout the duration of the study. For analyses on animal weights, data were averaged into a weekly mean for each animal. Approximate daily food consumption per animal was determined from weekly measurements of food weight, divided by the number of days between feedings and the number of animals in the cage.

Diets

Actos[®] was purchased through the division of laboratory animal research facility at the University of Kentucky and was crushed to a fine powder. Two different diets were formulated by Harlan Laboratories, Inc. (Madison, WI) including a control diet (AIN-93G, 18.8% Kcal from protein, 63.9% Kcal from carbohydrates, and 17.2% Kcal from fat; TD. 94045), and a PIO diet formulated at 84 ppm and also combined into AIN-93G. Calculated PIO dosages based on average food consumption were approximately 18 mg/kg body weight and were determined using the daily average food intake, the concentration of drug in the diet, and the average weight of the mice. Takeda Pharmaceuticals (Osaka, Japan), the manufacturer of Actos[®], recommends doses of PIO should not exceed 20 mg/Kg in the mouse.

Behavior

After 14 weeks of PIO treatment, the one-way active avoidance paradigm, a hippocampal-dependent task [33], was used to assess learning. On the week prior to behavioral characterization, two animals housed in the same cage and on the PIO supplemented diet began to show signs of paresis in caudal extremities (hind limbs and tail) and were excluded from the behavioral analyses even though no hypermetria or proprioceptive deficits were noted. Once the condition started, no overt sign of worsening was seen (well groomed, no change in body weight). Mice (12 PIO and 15 CTRL) were thus trained in an active avoidance task composed of two adjacent chambers (a dark and a light one) separated by a guillotine door (Med Associates Inc., St. Albans, VT). The computer-controlled foot shock delivery (0.8 mA, 25 s foot shock) was always delivered in the dark chamber following a 4 s

grace period. Mice were acclimated to the dimly lit training room for 20 min before each session. On day one, the mice were placed into the light compartment for 120 s during which they could move freely to either chamber, no shock was delivered. In order to determine chamber preference (light or dark) baseline activity was recorded as time spent in each chamber. Starting on day two, mice were trained on the task with three trials per session for a total of four sessions over three days. Day two of training consisted of one morning session, day three consisted of a morning and an afternoon session and day four consisted of one morning session. During a single trial, the mice were introduced into the dark chamber and given 4 s to escape to the light chamber. The guillotine door was closed immediately following the escape to the light chamber. Time to exit the dark chamber was recorded. The mouse was then removed from the light chamber and placed in a holding cage for 1 min until subsequent trials. Avoidance learning data are presented as percent avoidance across sessions (sum of the total number of avoidances across trials divided by the total number of trials *100) or mean escape latency (raw and ranked). While some degree of sensory dysregulation has been noted in several other models of AD [34, 35], in the 3×Tg-AD, no overt sign of visuo-motor dysfunction has been noted [36] and these animals show the least amount of retinal A β deposition and pathology compared to other models [37].

Blood collection and tissue isolation

At the end of the study, eight mice from each group were anesthetized with phenobarbital (IP – 100 mg/kg) and transcardially perfused with ice-cold 0.9% saline. Blood from these non-fasted animals was collected via cardiac puncture from the right ventricle immediately prior to perfusion. Brains were removed and bisected, one hemisphere was reserved for immunohistochemical staining, while the other hemisphere was dissected in order to isolate the hippocampus (see microarrays section) and the cortex (soluble/insoluble A β measurements). The hearts and adrenal glands were harvested, weighed, and flash frozen in liquid nitrogen. Heart weights and adrenal weights were normalized to body weight (mg/g) for each mouse. Livers were harvested and flash frozen for histology.

Serum analysis

Collected blood (as above and from some of the animals used for electrophysiology) was centrifuged at 3,000 rpm for 10 min in BD Microtainer tubes. Plasma was aliquoted into centrifuge tubes and stored at –80°C until analysis. Serum from each animal was shipped on ice to the Comparative Pathology Laboratory at UC Davis, and analyzed on a Roche COBAS Mira Plus Chemistry Analyzer. Quantitative data are reported on several blood markers obtained from 10–15 samples/group.

Liver histology

To determine the extent of lipid accumulation in the liver of PIO and CTRL mice, oil red O staining was performed. Frozen livers were sectioned (10 μ m) onto glass slides and stored at –80°C until further use. The tissue was thawed and allowed to air dry for 30 min, fixed in ice-cold 10% formalin for 5 min, and then washed three times in distilled water. Sections were placed in 100% propylene glycol, stained in 5% oil red O stain (Sigma Aldrich) for 8 min at 60°C, and subsequently placed in 85% propylene glycol. Slides were rinsed in distilled water and counterstained with hematoxylin. Liver sections from four randomly selected animals from each group were imaged on a Nikon microscope (TE200) through a 20 X lens using a spectral analysis camera (Nuance, CRi, Woburn, MA). Images were analyzed with ImageJ software (NIH) using the color threshold function to identify hues of reds in the section associated with the oil red O stain pattern. The percent area stained was used for statistical analysis.

Microarrays

Hippocampi were dissected from the left hemispheres of mouse brain ($n = 8$ per group) following intracardial saline perfusion. Tissue was flash-frozen on dry ice and stored in -80°C freezer until RNA extraction. RNA was prepared using TRIzol reagent precipitation with ethanol and reconstituted in RNAase-free water. Quality control results: [RNA] = 401 ± 26 ng/ μl (Nanodrop); RNA Integrity Number 8.5 ± 0.04 (Bioanalyzer). RNA labeling was performed as described previously [38–41], and according to standard Affymetrix procedures. Labeled cRNA for each animal was individually hybridized to an Affymetrix Gene Chip Mouse Genome microarray (430v2). All arrays passed standard Affymetrix quality control: GAPDH 3'–5' ratio 0.78 ± 0.004 ; RawQ 2.48 ± 0.04 ; Background noise 72.9 ± 1.5 ; Scaling factor (based on target intensity of 500) 1.31 ± 0.33 ; % Present 62.0 ± 0.3 and were not significantly different across the two groups (T -test $p > 0.1$ for all measures). Visual inspection of residual sign images (Affy PLM [42]) revealed no major image defects. The gcRMA probe level algorithm ('justgcRMA' command run in Bioconductor in the R operating environment) calculated signal intensities [43, 44]. Only unique probe sets/genes with 'A' grade annotations and at least 3 chips with signal intensities >4.3 were retained for further analysis. Values were transferred to Excel (2007, Microsoft), Bioconductor [43], MultiExperiment Viewer [MEV, [45] and the DAVID suite of bioinformatic tools [46] for subsequent analysis. Specific statistical procedures are outlined in Results. The signal intensity (gcRMA) and images (.cel files) have been deposited to the MIAME compliant Gene Expression Omnibus (GEO) database [47] - accession # GSE32536].

CA1 A β and tau immunostaining

Right brain hemispheres from each mouse ($n = 8$ per group) were removed after saline perfusion and placed in 4% paraformaldehyde by weight in 0.4 M phosphate buffer for ~ 4 h. The brains were then switched to a 30% sucrose solution (by weight) in 0.04% phosphate buffer for ~ 24 h in the fridge. Prior to placing the brains in a -20°C freezer for storing, they were immersed in an antifreeze solution composed of sucrose (15% by weight) and ethylene glycol (33% by volume) in 0.04 M phosphate buffer. Brain sections (35 μm) were immunohistochemically stained for human A β (W0–2 antibody, The Genetics Co., Switzerland; specific for human A β_{4-10}) [48], or PHF-1 (generously provided by Dr. Peter Davies, Albert Einstein college of medicine, NY), which recognizes tau phosphorylated at Ser396 and Ser40. Secondary antibody was goat anti-mouse biotin, followed by ExtraAvidin (Sigma, USA). Sections were visualized using Ni-enhanced diaminobenzidine (DAB). Dorsal hippocampus sections were digitized on a Leica microscope equipped with a 40 $\times$ objective and an Olympus DP70 camera. Images were converted to gray scale using Paint Shop Pro V7. A β densities were measured using ImageJ and following an automatic thresholding process for each image using the "make binary" function. A region of interest was defined around stratum pyramidale in area CA1 (excluding large extracellular deposits/plaques) to obtain measures of integrated density (mean gray value $\times$ area), and of percent area covered by immunostained neurons (A β -positive cells). For the PHF-1 positive cell counts, immunostained cells were quantified in area CA1 in two sections containing the dorsal hippocampus per brain, from sections adjacent to the sections stained for W0–2.

Soluble and insoluble A β levels

RIPA extraction—Previously published techniques were used to quantify soluble and insoluble A β levels [49, 50]. Cortical tissue (50–100 mg) taken at the time of hippocampal dissection for microarrays from 7 CTRL and 8 PIO mice was frozen on dry ice for later analysis (one sample was lost). The tissue was thawed on ice and placed in enough radio-immunoprecipitation assay (RIPA) buffer to create equal concentrations of 0.1 mg/ μL of tissue. The solution was then placed under a polytron tissue homogenizer set on medium

speed. Brain homogenates (500 μ L) were aliquoted into microcentrifuge tubes and then centrifuged at 100,000 g for 1 h. The supernatant was collected and the remaining pellet was reserved for formic acid (FA) extraction.

Formic acid extraction—The pellet was sonicated in 70% FA for 30 s (500 ms pulse duration). The homogenate was then centrifuged at 100,000 g for 1 h. The aqueous layer was collected and stored at -80°C until further use.

ELISA—RIPA and FA samples were thawed on ice and vortexed. FA samples were neutralized with 1 : 20 dilution in tris phosphate buffer. Sandwich ELISA was conducted using Ab9 and 4G8 antibodies and detected with neutravidin-HRP and read with a Victor plate reader. Data are presented as A β measured in fmol/mg tissue.

Slice preparation, extracellular and intracellular recordings

The remaining mice in each group ($n = 6$ PIO and $n = 7$ CTRL) were used for electrophysiological recordings. Hippocampal slices were split across two electrophysiological setups, one monitoring field potentials before and after long-term potentiation (LTP) induction, the other measuring intracellular potentials (e.g., afterhyperpolarization (AHP), synaptic hyperpolarization). All data were acquired using pClamp 8.0 (Molecular Devices MDS, Toronto, Canada) software through a Digidata 1320A A/D converter (Molecular Devices), and quantification of potentials (e.g., amplitude and duration of AHPs, field EPSPs) was obtained with Clampfit software (Molecular Devices). Each setup used Axoclamp 2A amplifiers (Molecular Devices).

LTP measures—One mouse from each group provided unviable tissue and data could not be recorded, therefore, LTP data was obtained from 5 PIO and 6 CTRL mice. 7 slices from 4 CTRL animals and 6 slices from 3 PIO animals provided stable data for inclusion into statistical analyses of LTP induction and maintenance (see criterion for inclusion below). Mice were anesthetized in a CO_2 filled chamber prior to decapitation. Brains were quickly removed and placed in oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 114 NaCl, 3 KCl, 10 Glucose, 1.25 KH_2PO_4 , 26 NaHCO_3 , 8 MgCl_2 , 0.1 CaCl_2 . Hippocampi were transversely sectioned with a Vibratome 3000 (Bannockburn, IL) yielding 350 μm slices. Slices were transferred to an interface-type recording chamber containing oxygenated recording ACSF (rACSF) with the following composition (in mM): 114 NaCl, 3 KCl, 10 Glucose, 1.25 KH_2PO_4 , 26 NaHCO_3 , 2.5 CaCl_2 and 1.3 MgCl_2 . The recording chamber was kept at 32°C and warm moist 95% O_2 /5% CO_2 was continually pumped into the chamber. Individual slices were recorded after at least a 2 h incubation period in the chamber. A twisted, bipolar Teflon-coated stainless steel stimulating electrode was placed on the Schaeffer collaterals, while the glass recording electrode (3–15 $\text{M}\Omega$) was positioned in the stratum radiatum. Stimulation (baseline and LTP) was delivered through a pair of SD9K stimulators (Astro Med Inc., Grass Instr., Warwick, RI). Prior to initiation of data acquisition on dendritic field potentials (fEPSPs), the slice was stimulated once every 20 s for 10 min at a minimal stimulation setting (~ 10 –20% of the I/O). Current outputs (I/O) were recorded during stepwise increases in voltage at the stimulating electrode to determine maximum output responses. Prior to LTP induction, baseline fEPSPs were recorded for 20 min at a stimulation setting set to 50% of the maximum EPSP (determined by I/O). LTP was induced using a 1 s train of pulses delivered at 100 Hz using the same settings for stimulation amplitude and duration as those used during baseline. EPSPs were recorded for 30 min post-LTP induction. For each slice, baseline EPSP slopes averaged across the 20 min prior to LTP induction were used to normalize EPSP slopes after LTP induction. Data are reported on the slope of the descending phase of the EPSP and on the amplitude of the EPSP measured as the maximum deflection from baseline. Slices with responses drifting up or

down greater than 20% across the 20 min baseline were excluded from the analysis. All EPSPs obtained after LTP induction (30 min) were used for statistical analysis using a 2-way RM-ANOVA with time and drug as factors.

Intracellular recordings—Ca²⁺-dependent AHP were recorded from 11 cells from 7 CTRL animals and 13 cells from 6 PIO treated animals. Passive membrane properties were not different between the two animal groups (Table 2), suggesting that cells with comparable baseline characteristics were analyzed. The tissue preparation was shared with the LTP setup and a similar holding chamber was used to maintain the tissue. The rACSF consisted of the same solution except for final concentrations of CaCl₂ (2.0 mM) and MgCl₂ (2.0 mM). Slices were placed in a perfusion chamber (RC22C, Warner Instruments, Co., Hamden, CT) and maintained in a continuous flow of oxygenated ACSF pre-heated at 32°C using a TC2Bip/HPRE2 in line heating system (Cell Micro Controls, Northfolk, VA). This setup was mounted on the stage of a Nikon E600FN inverted microscope. As previously described [51], cells were impaled with sharp microelectrodes filled with 2 M KMeSO₄ and 10 mM HEPES, pH 7.4 (tip resistance 90–150 MΩ), pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) using a P80 pipette puller (Sutter Instruments, Novato, CA). All experiments were performed in current clamp mode with bridge balance compensation and capacitance neutralization. Signal was digitized at 2 kHz and low-pass filtered at 1 kHz. Recordings of membrane input resistance (IR) were obtained in response to 800 ms, 200 pA hyperpolarizing current injections using an Axoclamp 2B amplifier (Molecular Devices, MDS, Toronto, Canada) while holding the cell at –70 mV. To generate an afterhyperpolarization (AHP) cells were held at –65 mV (baseline) and depolarized with a 100 ms current injection in order to generate three Na⁺ action potentials. AHPs were elicited every 30 s and at least 6 AHPs were averaged for each cell. The medium AHP (mAHP) was measured as the peak hyperpolarization immediately after the offset of the depolarizing current injection, the slow AHP (sAHP) was measured 800 ms after the end of the current injection. The AHP duration was measured from the end of the depolarizing step until return to baseline. Maximal membrane hyperpolarization during repetitive synaptic activation (3, 7 and 15 Hz) at action potential threshold was also measured during a 10 s stimulation [52]. Only neurons with input resistance >40 MΩ, holding current <500 pA and overshooting action potentials were included in the analysis.

Statistics

Unless otherwise noted in Results, *T*-tests or repeated measure ANOVAs (RM-ANOVAs) were used for statistical analyses ($\alpha = 0.05$).

RESULTS

Body weights and food consumption

Analysis of animal weights in the week prior to introduction of the purified diets revealed a significant effect across groups with heavier animals seen in the CTRL group ($p = 0.04$, *T*-test). Weight data in the next 13 weeks of the study were therefore normalized to the weight of each animal during the first week. This analysis revealed no main effect of drug on weight gained across 13 weeks ($F(1,26) = 1.37$; $p = 0.25$; RM-ANOVA) and while animal weights fluctuated minimally during this period, CTRL animals always remained slightly heavier than PIO animals ($p < 0.0001$, *T*-test; Table 1). A similar analysis on food intake across the same period revealed no significant difference in grams of food consumed per week per cage in the two groups ($F(1,7) = 1.30$, $p = 0.29$, RM-ANOVA). Average body weights and food consumption across the 14 weeks are presented in Table 1.

Serum analysis

Table 1 also presents post-treatment results of the serum analysis on a selected panel of peripheral markers. No sign of diabetes, renal or liver dysfunction, calcium dysregulation, or heart damage were observed. Compared to CTRL mice, PIO mice showed significant reductions in cholesterol, HDL, and triglyceride levels. A strong trend for reductions in LDL levels was also seen ($p = 0.056$; T -test).

Organ weights and liver histology

TZDs have been associated with adverse cardiovascular events in several clinical trials [53] which prompted us to harvest and weigh animal hearts. A significant increase in heart weight was seen in the PIO group ($p < 0.0001$, T -test; Fig. 1A). While the basis for this effect is unclear, it is unlikely that the doubling in heart weight can be attributed to weight gain over the course of the study as the PIO-treated group only showed a 2% increase in body weight. Furthermore, an effect on fluid retention is unlikely because it appears higher PIO doses are necessary for this effect in rodents [54]. Clearly, further studies are necessary to address the basis for potential adverse cardiovascular effects of PIO.

Because prior studies have shown a possible link between TZD actions and stress hormones [25], we measured adrenal weights in all animals. There were no significant group differences on this measure ($p = 0.72$; Fig. 1A). Given the significant decrease in plasma cholesterol, we also tested for evidence of lipid accumulation in the liver of PIO and CTRL mice using oil red O staining. Staining of liver sections with oil red O revealed no difference in the PIO compared to the CTRL animals ($p = 0.72$; Fig. 1B).

CA1 A β staining/levels

In prior animal and culture studies, TZDs have been shown to reduce A β deposits [18, 22, 28, 29, 55]. We therefore monitored A β staining in brain sections of dorsal hippocampus. Sections were immunostained with the W02 antibody (Fig. 2) to quantify intracellular A β accumulation within area CA1 neurons (stratum pyramidale), and we also quantified soluble and insoluble A β fractions from leftover cortices obtained during tissue preparation for microarray experiments. We chose to quantify intracellular A β because it is thought to contribute importantly to disease progression [56]. PIO significantly reduced A β staining in the CA1 region of the hippocampus. The surface area analyzed (region of interest, ROI) was not different between the 2 groups ($p = 0.21$; T -test; data not shown), however, the mean gray value in this ROI ($p = 0.025$; T -test; data not shown), the integrated density (mean gray value $\times$ area; $p = 0.022$; T -test; Fig. 2A left), and the percent area covered by immunostained CA1 neurons ($p = 0.025$; Fig. 2A right) were all significantly reduced by PIO treatment. Furthermore, a trend for a decrease in the soluble A β fraction was seen in these animals compared to those fed the CTRL diet ($p = 0.07$; Fig. 2C left). While the insoluble A β fraction was also decreased, this did not approach significance in animals on the PIO diet ($p = 0.13$; Fig. 2C right). Correlation between intracellular A β density and cholesterol in 16 animals for which both variables were measured yielded a positive correlation which yielded a trend ($p = 0.076$; $r^2 = 0.21$; data not shown).

CA1 phosphorylated-tau positive neurons

Prior work has demonstrated TZDs can reduce phosphorylated tau pathology in culture and animal models of AD [23, 24, 57]. Here, we quantified phosphorylated tau-labeled cells and monitored the impact of PIO on this unique pathology in the transgenic animal. A significant and striking decrease in phosphorylated tau positive cells was seen (Fig. 2C). As such, the mean number of PHF-1-positive neurons per section was 7.81 ± 2.01 for the CTRL animals, and 1.18 ± 0.23 for the PIO-treated animals ($p = 0.005$, T -test).

Active avoidance learning

Light/dark preference—On day one of training, each mouse was allowed to explore the dark and light chambers of the active-avoidance shuttle box for 120 s. Both groups of animals showed a significant preference for the dark side of the chamber ($F(1,48) = 31.1$; $p = 0.0001$; Fig. 3A). There was no difference between treatment groups on measures of time spent in the dark chamber (in seconds, CTRL: 74.4 ± 4.48 , PIO: 66.9 ± 2.34), the mean number of crossings (CTRL: 7.60 ± 0.92 , PIO: 9.83 ± 0.99), or the time to first crossing (in seconds, CTRL: 20.9 ± 5.6 , PIO: 18.0 ± 3.6), indicating no overt drug-related difference in ambulatory behavior, fear or anxiety levels.

Avoidance learning—PIO significantly improved learning in the early phases of training (sessions 1–3), showing greater percent avoidance compared to CTRL animals ($F(1,25) = 5.08$, $p = 0.03$; RM-ANOVA; Fig. 3B), as well as a reduced mean escape latency across all sessions ($F(1,25) = 4.85$; $p = 0.04$, RM-ANOVA, data not shown). The ranked escape latency measured across all sessions (each session = 3 trials, see methods) also was significantly reduced in the PIO group compared to the CTRL group ($F(1,25) = 6.42$; $p = 0.017$, RM-ANOVA; Fig. 3C). In sixteen behaviorally characterized animals for which A β immunostaining data were available, a correlation analysis revealed that active avoidance learning was worse in animals displaying larger A β deposits ($p = 0.006$; $r^2 = 0.43$; Fig. 3D).

Electrophysiology

Membrane hyperpolarization—While measures of cell health (Table 2) and passive membrane properties were not different than those recorded in cells from F344 rats [51, 58–60], the AHPs recorded from 3 $\times$ Tg-AD animals were surprisingly small. Perhaps due to this floor effect, PIO-treated animals did not show significant reductions in the AHP. Neither the sAHP ($p = 0.84$, T -test) or mAHP amplitude ($p = 0.94$, T -test), nor the AHP duration ($p = 0.97$, T -test) were significantly modified by PIO (Fig. 4A). A subset of cells (9 cells from 6 CTRL animals and 7 cells from 7 PIO animals) was subjected to short-term plasticity stimulation protocols using repetitive synaptic activation. The degree of hyperpolarization was very similar to that seen in young adult F344 rats [59] indicating that synaptic hyperpolarization processes were intact in the 3 $\times$ Tg-AD animals. Interestingly, PIO caused a significant increase in synaptic hyperpolarization amplitude (Fig. 4B) at the three frequencies tested ($F(1, 39) = 8.57$; $p = 0.006$; ANOVA; Fig. 4B), a phenotype typically seen in younger animals [59].

Long-term potentiation—The mean baseline EPSP prior to tetanization of the pathway was not significantly different between groups (in mV, CTRL: -1.65 ± 0.13 , PIO: -1.64 ± 0.08 ; data not shown). Immediately after tetanization and for the next 30 min, slices from PIO-treated animals showed robust potentiation of the EPSP compared to CTRL animals on measures of both EPSP slopes ($F(1,11) = 14.5$; $p < 0.005$; RM-ANOVA; Fig. 5) and amplitudes ($F(1,11) = 8.55$; $p < 0.05$; RM-ANOVA, data not shown). These results are consistent with prior evidence for a deficit in LTP maintenance in 3 $\times$ Tg-AD animals [31].

Microarray analyses

Figure 6 (upper left) displays the flowchart for the microarray analysis, yielding a total of 1936 significant genes split into up and down elements that all showed sensitivity to the PIO treatment. Approximately 7% of those genes (136/1936) contained consensus PPAR β -response elements (PPREs - Motif Finder, Partek Inc., MO) and represent potential direct transcriptional targets of PIO (supplementary Table 1; available online: <http://www.j-alz.com/issues/30/vol30-4.html#supplementarydata07>). Characterization of upregulated and downregulated genes using DAVID analysis returned a series of functional

categories. These were heuristically combined into over-arching groups based on biological processes. Individual genes are shown graphically where the length of each bar represents the $-\log p$ value for that message (Fig. 6). With the exception of few extremely significant genes ($p < 0.00001$), the majority of genes sensitive to PIO falls between p values of 0.05 and 0.005. The overall list of significant genes is shown in supplementary Table 1.

From the DAVID analysis, a striking number of RNA targets associated with female hormone processes were shown to be upregulated as were DNA binding processes (Fig. 6, left). Of note, in the downregulated categories (right), decreases in lipid metabolism, myelination, cholesterol transport, energy dependent processes, and glutamatergic neurotransmission were also seen. While the DAVID analysis does not support an overall PIO-mediated suppression of inflammatory processes, several individual candidates known to play a role in inflammatory processes were identified and found to be significantly down regulated by PIO (supplementary Table 1). These included tumor necrosis factor alpha-induced protein 2 (Tnfaip2), tumor necrosis factor receptor type 1-associated DEATH domain protein (Tnfrsf1a), interleukin-1 receptor accessory protein (Il1rap), complement C1q-like proteins 3 (C1ql3) and 2 (C1ql2), and three NF κ B-related messages (Nfkbia, Tank, and Tnfrsf1a). In addition, cytokine signaling may have also been suppressed by upregulation of 2 genes known to be suppressors in this pathway (Socs2 and Socs5), as well as by upregulation in lipin-1 (Lpin1) which has been shown to reduce secretion of inflammatory markers [61].

Several other processes associated with aging or AD included two mRNA species linked to lipid shuttling and A β processing (apolipoprotein E (APOE) and amyloid β A4 precursor protein (apba2)) which were found to be decreased by PIO. We also found PIO-mediated increases in lipin-2 (Lpin2) message, another gene associated with lipid homeostasis. Message for the cardiac ryanodine receptor type 2 (Ryr2), an integral protein mediating Ca^{2+} -induced Ca^{2+} -release (CICR) in excitable cells was significantly reduced by PIO as was 11- β dehydrogenase type 1 (Hsd11b1) message. This later enzyme generates the active form of glucocorticoids and is a sought after pharmacological target since it directly impacts glucocorticoid levels in the brain and downregulation improves learning [62, 63]. PIO-mediated downregulation of genes associated with glutamatergic neurotransmission included the genes for glutamate transporters (Slc1a1 and Slc1a2), metabotropic glutamate receptor 3 (Grm3), AMPA receptor 2 (Gria2), glutamate receptor delta-2 subunit (Grid2), and the zeta-1 subunit of the NMDA receptor (Grin1). Trends for down regulation ($p < 0.1$) were also seen for the glutamate receptor subunits epsilon-3 (Grin2c) and epsilon 1 (Grin2A), as well as for the metabotropic glutamate receptor 1 (Grm1). While several RNA species associated with glutamatergic neurotransmission were also found to be upregulated by PIO treatment, including glutamate receptor delta-1 subunit (Grid1) and glutamate receptor subunit 3A (Grin3A), this does not necessarily detract from a relative loss of glutamatergic function given that Grin3A confers reduced Ca^{2+} permeability through the NMDA receptor [64], and Grid1's function outside of the auditory system is not yet clear [65].

DISCUSSION

Because of potential links between AD and diabetes, we investigated whether the anti-diabetic drug PIO could attenuate cognitive impairment and markers of pathology in an accelerated mouse model of AD. Treatment was initiated at 10 months, a time point when hippocampal A β deposits are well-established and pathology is still ongoing in this model [31]. Following 4 months of treatment, when animals were 14 months of age, we found PIO improved performance in the active avoidance task, increased short- and long-term hippocampal plasticity (synaptic hyperpolarization and LTP, respectively), reduced A β and

tau staining, and altered expression of several known pharmacologically-relevant gene targets (e.g., TNF- α , NF κ B- and interleukin-related genes). In addition, microarray analyses also revealed several novel targets of PIO known to be associated with cognition, including estrogenic and glutamatergic pathways. Interestingly, it appears that PIO also selectively targeted some of the same processes that are dysregulated by the transgenes (e.g., lipid/fatty acid metabolism, mitochondrial energy processes, and synaptic neurotransmission [66, 67]), providing further support for the potential efficacy of PIO in the treatment of AD.

Pioglitazone facilitates learning and improves short-and long-term plasticity

Consistent with prior reports of PPAR- γ agonists in the brain [68, 69], improved LTP maintenance was seen in animals treated with PIO (Fig. 5). PIO also counteracted learning deficits on the active avoidance task (Fig. 3B) suggesting that PIO may have improved learning through actions on LTP expression. Although optimal NMDA- and AMPA-mediated functions are essential for LTP [70], aberrantly elevated glutamatergic neurotransmission has been seen in AD models [71–74]. Consistent with this aberrant glutamatergic activity, memantine, a weak NMDA antagonist used in the treatment of AD [73–75] enhances LTP in models of aging and AD [76–78], restores neuronal encoding in aging (i.e., hippocampal place field plasticity) [79], and slows cognitive decline in the 3 $\times$ Tg-AD model [78]. Together with evidence that PIO is able to reduce NMDA-mediated Ca²⁺ currents and levels [80], the permissive effect of PIO on learning and LTP reported here appears to resemble some aspects of memantine action. Along these lines, it is interesting to note that drugs that target the glutamatergic system can be used to treat mood disorders [81]. Thus, the effects observed here on glutamate signaling may also underlie the purported ability of PIO to reduce symptoms of depression [82] and may hold relevance for the AD population which suffers from a high degree of depression.

Additional electrophysiological analyses identified a PIO-mediated increase in membrane hyperpolarization during repetitive synaptic stimulation (Fig. 4B). The synaptically-driven membrane hyperpolarization is typically larger in younger compared to older neurons [59] and thus, the effect of PIO seen here appears to confer a younger phenotype. This greater hyperpolarization may also have contributed to improved learning in the PIO-treated animals by enhancing signal to noise and facilitating throughput during synaptic communication. These actions also are very similar to those of memantine in AD models [73, 83]. Moreover, the effect of PIO on synaptic hyperpolarization may also provide an underlying mechanism for the reported ability of PIO to reduce seizures in mouse models of epilepsy [84, 85]. Another mechanism possibly underlying the effects of PIO on learning and plasticity may depend in part on changes in hyperpolarization-activated cation channels (HCN). Our microarray analysis showed that PIO significantly downregulated Pex5l, a gene that promotes HCN incorporation into dendritic membranes [86]. Since knockdown of HCN is associated with an improvement in learning and LTP [87], inhibition of Pex5l (and hence HCN) may also underlie the beneficial actions of PIO on learning and LTP seen here.

Another measure of intracellular hyperpolarization, the Ca²⁺-dependent afterhyperpolarization (AHP) (Fig. 4A) was unaffected by PIO. The Ca²⁺-dependent AHP is larger with aging and is considered a major electrophysiological biomarker of brain aging [60, 88]. We also noted that when a similar number of action potentials was used to evoke the AHP, it was markedly smaller in 3 $\times$ Tg-AD mice compared to our prior measures in aged F344 rats [58, 59]. While we have previously shown that PIO reduces the AHP in aged rats [54], perhaps the small amplitude AHPs in the 3 $\times$ Tg model precludes additional reductions by PIO. Smaller AHPs in these animals have previously been noted [89]. Future studies will be needed to resolve differences between animal models of aging and those of AD. Interestingly, however, the reduced AHPs are also consistent with increased excitability and seizures in 3 $\times$ Tg and other models of AD [90–92]. Overall, the results from the

electrophysiology experiments show that PIO induces effects consistent with mechanisms associated with improved learning.

Role of PIO in the periphery

Given the evidence for potentially causal associations between dyslipidemia and cognitive decline and AD [2–4, 93–96], we tested whether the effects of PIO on serum lipid levels (decreased cholesterol, HDL and triglycerides, Table 1) were correlated with improved learning. No significant correlations were detected between any of the lipids measured and learning. We also investigated whether decreased serum lipid levels were associated with brain A β levels [93, 97] and observed a trend toward a positive correlation between serum cholesterol and brain A β ($p = 0.076$, please see Results). It is noteworthy that while serum lipid levels were not correlated with learning, we did observe that A β levels and behavioral performance were inversely correlated (Fig. 3D) as seen in other AD experimental models and in humans [98–101].

Transcriptional effects in hippocampus and associations with cognition

Lipid-related genes/pathways—Microarray results indicate that lipid metabolism was significantly downregulated by PIO in the hippocampus. Contrary to what is typically seen in the periphery, where PIO mediates lipid storage and adipose tissue expansion through upregulation of genes such as lipoprotein lipase, acyl-CoA synthetase, fatty-acid binding protein 4, and adipocyte protein 2 [12–15], in the brain, an alternative process of reduced fatty acid metabolism seems to emerge (Fig. 6, bottom right). Of interest, we identified many mitochondrial/peroxisomal enzymes linked to the processing/synthesis of various fatty acids (Fasn, Mecn, Scd2, Hadh, Fads6, Fa2h, Elovl5, Acsbg1), glycerolipids (Gpam, Gpd2), or myelin (Npc1, Qk, Mbp, Lgi4, Acsbg1), which were all shown to be downregulated by PIO. We also observed a significant downregulation in genes mediating cholesterol transport (Fig. 6). Taken together, it seems that in the brain PIO may target different lipid-related genes and elicit changes opposite to those seen in the periphery.

Glutamatergic neurotransmission genes/pathways—In addition to the effects of PIO on the glutamate-dependent electrophysiological measures highlighted above (i.e., LTP and synaptic hyperpolarization), several categories of genes associated with glutamatergic neurotransmission also were affected by PIO. These include genes for glutamate transporters (Slc1a1 and Slc1a2), metabotropic glutamate receptor 3 (Grm3), AMPA receptor 2 (Gria2), glutamate receptor delta-2 subunit (Grid2), and the zeta-1 subunit of the NMDA receptor (Grin1), which were all downregulated. Thus, consistent with evidence that the 3 $\times$ Tg model has aberrant glutamatergic neurotransmission [71–74] the PIO-mediated downregulation of molecules involved in glutamate signaling may have rescued some aspects of the phenotype resulting in improvements in cognition, perhaps similar to the effect of memantine in AD and related models [78, 80, 102].

Estrogen-related genes/pathways—An important series of genes upregulated by PIO in the hippocampus and associated with cognition, are those linked to the steroid hormone estrogen (Fig. 6). Numerous prior studies have reported on the beneficial impact of estrogen in brain aging [103–110] and in female 3 $\times$ Tg-AD mice as well [109, 111, 112]. Thus, increased estrogen-dependent signaling may also contribute to the improved learning seen with PIO in female 3 $\times$ Tg-AD animals. While estrogen levels in 3 $\times$ Tg-AD mice are unaltered until approximately 12 months of age [111, 112], it remains to be determined whether some effects of PIO on estrogen-related gene targets occur through changes in circulating estrogen levels. It is also unclear whether the estrogen-dependent changes observed here also extend to males. However, it is important to note that TZDs have been shown to have beneficial effects in the brains of male and female mice.

Amyloid processing genes/pathways—Work from several groups has provided evidence for the role of PPAR- γ agonists in reducing A β levels through inhibition of β -secretase 1 (BACE-1) [18, 113], by increasing insulin-degrading enzyme (IDE) [28], Abca1-mediated clearance [24], or microglia mediated phagocytosis [24]. Our microarray analyses on hippocampal tissue did not provide supporting evidence for changes in BACE-1 or IDE messages, but did show a significant decrease in Abca1 message levels with PIO. Further studies are needed to determine whether the duration of treatment, type of TZD used, potential adaptation to the TZD, differences in TZD dose [18, 29], or animal models can account for these discrepancies.

CONCLUSIONS

To our knowledge, this is the first comprehensive microarray analysis on the long-term effects of PIO in the brain of a transgenic mouse model of AD. PIO also exerted beneficial effects on pathological biomarkers of AD, counteracted impaired learning, and enhanced synaptic plasticity in these same animals. Insights from the genome-wide analyses regarding the mechanisms of PIO reveal multiple novel targets related to estrogen, glutamate, and lipid signaling in the brain. Interestingly several processes related to lipid signaling, such as myelinogenesis and cholesterol trafficking, are increased with aging and cognitive decline [38, 39] and here we show that PIO can oppose these actions. Finally, our results also demonstrate the efficacy of PIO treatment, even if initiated when pathological processes are prominent and ongoing, suggesting that PIO could represent a clinically-relevant approach in the management of AD, particularly given its pleiotropic effects.

Supplementary Table 1. This table lists all genes significantly affected by PIO treatment. One of the column headers indicates the presence or absence of the consensus PPAR- γ DNA binding sequence. Other information about direction of change, *p* value, gene symbol and function are also listed. Please see the Microsoft Excel spreadsheet at <http://www.j-alz.com/issues/30/vol30-4.html#supplementarydata07>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Assessment of pioglitazone effects on peripheral tissues. A) Heart (left axis, white bars) and adrenal weights (right axis, black bars) were normalized to body weight (mg/gr) in animals fed either control diet (CTRL) or PIO-enriched diet (PIO). B) Left, representative examples of liver sections stained with oil red O and hematoxylin from a CTRL and PIO-treated animal. Right, data showing percent of sections stained for lipids (% area stained) in both groups. Data represent Mean $\pm$ SEM, $*p < 0.05$, *T*-test.

Fig. 2. Hippocampal A β levels and tau immunoreactivity. A) Top, representative examples of CA1 area photomicrographs taken from animals fed either control diet (CTRL) or PIO-enriched diet (PIO) and stained using WO2 antibody against the A β peptide. Bottom, quantification of WO2 staining shows decreases in integrated A β density, and in the area covered by A β positive cells (percent). B) Results from Sandwich ELISA method to measure soluble (left) and insoluble (right) A β fractions. While not significant, a trend was seen in the soluble fraction decreasing with PIO treatment ($p = 0.07$). C) Photomicrographs of hippocampal sections stained with PHF-1 antibody to monitor the presence of hyperphosphorylated tau. A

significant reduction in tau immunoreactivity was seen (see text). Data represent Mean $\pm$ SEM, *T*-test, $*p < 0.05$.

Fig. 3.

Active avoidance learning. A) Pretraining measures on time spent in each compartment indicate all animals spend more time in the dark rather than the light chamber ($***p < 0.001$, Two-way ANOVA). B) Percentage avoidance in PIO fed mice was significantly greater than in the CTRL group across all training sessions ($*p < 0.05$ Two-way ANOVA). For graphical comparison only, the percentage avoidance from non-transgenic control mice (non-Tg) is also shown (inset). C) Ranked escape latencies across the first 3 sessions show better performance in the PIO fed mice compared to the CTRL animals ($*p < 0.05$ Two way ANOVA). D) Ranked escape latencies (higher numbers indicate poorer performance) correlated well with ranked integrated A β densities (higher numbers indicate greater A β density), as increased A β deposits were seen in animals with poor learning ($r = 0.66$, $p < 0.01$). Data represent Mean $\pm$ SEM.

Fig. 4. Membrane hyperpolarization measures. A) Example of afterhyperpolarization (top) elicited intracellularly with three action potentials and recorded in a 3×Tg-AD mouse fed the control diet (action potentials are truncated). Bar graphs show the amplitude of the medium afterhyperpolarization (mAHP) and slow AHP (sAHP) as well as the duration of the afterhyperpolarization recorded in mice fed either control diet (CTRL) or PIO-enriched diet (PIO) (*T*-test, $p > 0.05$). Note that the AHPs are particularly small in this animal model. B) Top, train of action potentials elicited synaptically with repetitive stimulation for 10 s at 7 Hz. Bottom, PIO animals showed a significant increase in maximal membrane hyperpolarization (measured from baseline – dashed line, ΔmV) at the three frequencies tested. * $p < 0.05$, Two-way ANOVA. Data represent Mean ± SEM.

Fig. 5.

High frequency induced synaptic potentiation. Normalized EPSP slopes measured in CTRL (white circles) and PIO (black circles) fed animals during baseline and following a 1 s 100 Hz stimulation burst. All measures taken after LTP induction were significantly greater in the PIO group. Inserts represent EPSP traces taken before LTP induction (black), and in the last 5 minutes of recording (red). Data represent Mean $\pm$ SEM, ** $p < 0.01$, RM-ANOVA.

Fig. 6. Statistical approach and gene clustering. Inset shows the significant genes (up and down) that were identified from a total list of 1936 (FDR = 0.29). An unbiased DAVID analysis shows overrepresentation categorization into clusters sensitive to PIO in the up (left) and down (right) direction. The length of each bar represents the *p* value associated with this gene. Representing the most populated categories, were genes associated with DNA binding (UP - red) and neurotransmission (DOWN - blue).

Table 1

Blood chemistry panel, animal weights, and food consumption. Blood serum markers in animals were measured following a 4 month treatment with either a control (CTRL) or a PIO-enriched diet (PIO)

	CTRL	PIO
Calcium (mg/dL)	9.3 ± 0.39	8.5 ± 0.42
Glucose (mg/dL)	167.4 ± 19.23	169.5 ± 18.16
BUN (mg/dL)	26.4 ± 4.29	24.1 ± 5.78
ALT (U/L)	93.1 ± 27.95	43.4 ± 4.17
CK (U/L)	799.4 ± 192.73	1207.1 ± 252.65
Uric acid (mg/dL)	1.7 ± 0.24	2.2 ± 0.23
Cholesterol (mg/dL)	113.9 ± 13.40	57.4 ± 5.67 [#]
HDL (mg/dL)	63.6 ± 7.91	33.6 ± 3.29 ^{**}
LDL (mg/dL)	11.3 ± 1.46	7.9 ± 0.96
Triglyceride (mg/dL)	101.8 ± 19.42	55.4 ± 3.51 [*]
Body weight (g)	34.6 ± 2.42	29.3 ± 0.79 ^{***}
Food (g/mouse/day)	2.9 ± 0.42	2.6 ± 0.14

Abbreviations: BUN, blood urea nitrogen; ALT, alanine aminotransferase; CK, creatine kinase; HDL, high density lipoprotein; LDL, low density lipoprotein. Body weights and food intake (Food) are also presented and were averaged across the duration of the study.

^{*}
 $p < 0.05$,

^{**}
 $p < 0.01$,

[#]
 $p < 0.001$

^{***}
 $p < 0.0001$ indicate a significant effect of PIO (*T*-test). Data represent Mean ± SEM.

Table 2

Electrophysiological parameters

	CTRL	PIO
Electrode resistance (MΩ)	117.2 ± 6.50	121.1 ± 7.55
Input resistance (MΩ)	56.4 ± 6.37	66.8 ± 5.19
Holding current (pA)	-361.7 ± 47.04	-276.2 ± 28.27
Action potential height (mV)	68.9 ± 3.40	71.1 ± 3.00
Current injection (pA)	230.3 ± 18.43	214.1 ± 18.73

Data represent Mean ± SEM on measure of electrode resistance, input resistance, holding current necessary to hold the cell at -70 mV, action potential height (measured from -65 mV), and intracellular current injection necessary to elicit 3 action potentials (duration 100 ms). No significant difference was found in any of these parameters across treatment groups (*T*-test, *p* > 0.05).