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Ligand for translocator protein reverses pathology in a mouse model of Alzheimer's disease

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Abstract

Ligands of the translocator protein (TSPO) elicit pleiotropic neuroprotective effects that represent emerging treatment strategies for several neurodegenerative conditions. To investigate the potential of TSPO as a therapeutic target for Alzheimer's disease (AD), the current study assessed the effects of the TSPO ligand Ro5-4864 on the development of neuropathology in 3xTgAD mice. The effects of the TSPO ligand on neurosteroidogenesis and AD-related neuropathology including β-amyloid accumulation, gliosis and behavioral impairment were examined under both early intervention (7 month-old young-adult male mice with low pathology) and treatment (24 month-old, aged male mice with advanced neuropathology) conditions. Ro5-4864 treatment not only effectively attenuated development of neuropathology and behavioral impairment in young adult mice, but also reversed these indices in aged 3xTgAD mice. Reduced levels of soluble β-amyloid were also observed by the combination of TSPO ligands Ro5-4864 and PK11195 in non-transgenic mice. These findings suggest TSPO is a promising target for the development of pleiotropic treatment strategies for the management of AD.

Keywords

β-amyloid; gliosis; neuroinflammation; progesterone; Ro5-4864; testosterone

Introduction

Ligands of translocator protein (TSPO), a mitochondrial molecule involved in the control of steroidogenesis (Papadopoulos et al., 2006b), elicit pleiotropic neuroprotective effects, promoting nerve regeneration while reducing oxidative damage, apoptosis and gliosis (Rupprecht et al., 2010). Increased neurosteroid synthesis is perhaps the best characterized effect of TSPO ligands and most likely contributes at least in part to the plethora of established neuroprotective actions and anxiolytic and antidepressant behavioral effects (Rupprecht et al., 2010). Consequently, TSPO ligands are under investigation for use in the

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treatment of a range of neurological disorders including traumatic brain injury (Papadopoulos and Lecanu, 2009), peripheral neuropathies (Giatti et al., 2009), inflammatory conditions (Veiga et al., 2005), chronic pain (Torres et al., 2000), depression (Gavioli et al., 2003) and anxiety (Rupprecht et al., 2009). Although potential therapeutic applications of TSPO ligands to AD has been suggested (Veenman and Gavish, 2000; Papadopoulos et al., 2006a), to date AD-related investigations of TSPO ligands have assessed only their utility in detecting gliosis via *in vivo* PET imaging in AD patients and mouse models of AD (Edison et al., 2008; Ji et al., 2008; Yasuno et al., 2008).

Although the mechanisms of AD pathogenesis remain to be fully resolved, the leading hypothesis posits that the disease is initiated and driven by prolonged elevation in levels of β-amyloid protein (Aβ) (Walsh and Selkoe, 2007). Despite enormous effort to develop therapies that specifically reduce Aβ accumulation, thus far these strategies have proven unsuccessful clinically. TSPO ligands may offer an alternative to specific Aβ-regulating strategies in that they simultaneously target multiple facets of the neurodegenerative cascade, perhaps including not only Aβ accumulation but also aspects of neuroinflammation, oxidative stress, mitochondrial dysfunction and neuronal loss (Rupprecht et al., 2010).

To investigate the potential of TSPO as a therapeutic target for AD, the current study assessed the effects of TSPO ligand Ro5-4864 in the 3xTgAD mouse model of AD (Oddo et al., 2003). The effects of Ro5-4864 on neurosteroidogenesis and AD-related neuropathology including Aβ accumulation, gliosis, and behavioral impairment were assessed in both young-adult 3xTgAD mice with mild pathology and aged 3xTgAD mice with advanced neuropathology, allowing insight into protective actions under early versus late intervention scenarios. Because TSPO ligands can differentially modulate TSPO activities, we also compared the independent and combined effects of Ro5-4864 with PK-11195, a non-benzodiazepine TSPO ligand, on regulation of Aβ levels in non-transgenic mice.

Methods

Animals & treatments

Male homozygous 3xTgAD and non-transgenic (nonTg) C57BL6 mice (Jackson Laboratory, Bar Harbor, ME) were maintained at the University of Southern California (USC) vivarium facilities with food and water available *ad libitum*. In experiments with young-adult mice, males (age 4 months) were gonadectomized (GDX) to deplete gonadal sources of sex hormones and preclude TSPO-ligand induced upregulation of steroidogenesis in the testes. In studies with young adult 3xTgAD mice, GDX and sham-GDX mice were divided into 3 groups (N=7–10/group): Sham + vehicle, GDX + vehicle, and GDX + Ro5-4864; and administered Ro5-4864 (3 mg/kg) or vehicle (1% DMSO in canola oil) by injection (i.p.) once weekly for 3 months. This dose of Ro5-4864 has been widely used to yield neuroprotective effects *in vivo* (Veiga et al., 2005; Giatti et al., 2009). In experiments with aged 3xTgAD mice, males (age 23 months, N=6/group) were similarly injected with Ro5-4864 or vehicle for four weeks. In studies with nonTg C57BL6 mice, males were GDX or sham-GDX at age 3 months then assigned to one of the following groups (N=6–7/group): Sham + Vehicle, GDX + vehicle, GDX + Ro5-4864, GDX + PK-11195, GDX + PK-11195 + Ro5-4864. Treatments of either vehicle, Ro5-4864 (3 mg/kg) or PK-11195 (3 mg/kg) were administered via once weekly injection (i.p.) beginning two weeks following surgery and continuing for four weeks. Mice were anesthetized (80 mg/kg ketamine/5 mg/kg xylazine; i.p.) and perfused with ice-cold saline 48 h after the final injection. Brains were bisected, with one hemisphere immersion fixed in 4% paraformaldehyde/0.1 M PBS for 48 h and the limbic regions of the other hemisphere frozen on dry ice for neurosteroid assessment. All experiments were approved by the USC Institutional Animal Care and Use Committee.

Immunohistochemistry

Fixed hemibrains were sectioned exhaustively in the horizontal plane at 40 μm. Some sections were labeled using antibodies directed against Aβ (#71-58000, 1:300 dilution, Zymed, San Francisco, CA) or hyperphosphorylated tau (AT8, 1:1000 dilution, Peirce, Rockford, IL) using a standard avidin: biotinylated enzyme complex immunoperoxidase method as previously described (Rosario, 2006). Aβ immunoreactive load was calculated as percentage of total pixel area positively labeled in binary images created by thresholding digitally captured grayscale images of high magnification fields (420×330 μm) as previously described (Cummings et al., 2002). Mean load values were averaged from two to three non-overlapping fields from each brain region in five sections per animal. AT8 immunoreactivity was quantified by counting cells strongly immunoreactive over the entire cell surface, as previously described (Carroll et al., 2010).

Other sections were labelled using antibodies directed against ionized calcium binding adaptor molecule-1 (IBA-1, Wako Chemicals, Neuss, Germany) as a marker of microglial activation (Ito et al., 1998) or glial fibrillary acidic protein (GFAP, clone GA5; Sigma-Aldrich, Tres Cantos, Spain) as a marker of astrocyte reactivity (Middeldorp and Hol, 2011), as previously described (Barreto et al., 2009). IBA-1 and GFAP immunoreactivities were quantified by volume density morphometric analysis (Weibel, 1979). Experimenters were blinded to treatment conditions during quantification.

Behavioral testing procedures

Anxiety and exploratory activity were assessed in the elevated plus maze (EPM), and spontaneous alternation behavior (SAB) was assessed in the Y-maze, as previously described (Carroll et al., 2010). Mice were tested in the EPM 24 hrs following the 4^{th} injection. The total number of arm entries was scored as a marker of exploratory activity and duration spent exploring the open arm was scored as an index of anxiety-related behavior. After a 1 h interval, SAB was assessed. Arm choices were recorded and the SAB score was calculated as the number of alternations divided by the total number of alternation opportunities. One outlier was excluded from analysis from the 7 month-old Sham + vehicle group.

LC-MS/MS analysis of neuroactive steroids

Samples were homogenized and purified by organic phase extraction for LC-MS/MS analysis as previously described (Caruso et al., 2008). The LC-MS/MS system consisted of a linear ion trap-mass spectrometer (LTQ, ThermoElectron Co., San Jose, CA, USA) equipped with a Surveyor liquid chromatography (LC) Pump Plus and a Surveyor Autosampler Plus (ThermoElectron Co., San Jose, CA, USA). Chromatographic separation was performed on a Inertsil ODS-2 RP-C18 column (5 μm, 150 mm × 4.6 mm i.d.; GL Sciences Inc., Japan) and the MS was operated with atmospheric pressure chemical ionization (APCI) in the positive ion mode. LC-MS/MS peaks were evaluated using Excalibur® release 2.0 SR2 (ThermoFisher Co, San Jose, CA, USA). Calibration curves were prepared and analyzed using deuterated internal standards. Limits of quantification, precision and accuracy have been previously reported (Caruso et al., 2008). One outlier was excluded from analysis of progesterone levels from the GDX + vehicle group.

Aβ ELISA

Homogenates of hemibrains from nonTg mice were prepared as previously described (Ramsden et al., 2003) and used to quantify levels of soluble Aβx-40 and Aβx-42 using commercially available ELISA kits according to manufacturer's instructions (Wako, Osaka, Japan).

Data analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS: version 11.5; SPSS Inc., Chicago, Illinois, USA). The effect of GDX and Ro5-4864 administration in the young-adult 3xTgAD mice was assessed by one-way ANOVA followed by least-square difference tests. The effect of Ro5-4864 treatment in the aged-senescent 3xTgAD mice was determined by *t*-tests. The effect of treatment on Aβ levels in C57BL6 mice was assessed by linear mixed model followed by least-square difference tests. Significance was set at a threshold of $p<0.05$. Data is presented as mean ± SEM.

Results

Ro5-4864 attenuates hippocampal Aβ accumulation in 3xTgAD mice

In young-adult 3xTgAD mice, a significant effect of treatment was observed on Aβ immunoreactive load in the hippocampal CA1 region ($F=4.53$, $p=0.02$). GDX accelerated Aβ accumulation in hippocampus CA1, increasing Aβ immunoreactive load of vehicle-treated GDX mice compared to sham mice (Fig. 1A-B, K). Ro5-4864 treatment attenuated Aβ accumulation in the CA1 region of GDX mice, with Aβ load almost halved in Ro5-4864 compared to vehicle-treated GDX mice (Fig.1 B-C, K). Aβ load in the subiculum was not significantly affected by either GDX (Fig. 1 F-G, L) or Ro5-4864 administration (Fig. 1 G-H, L; $F=2.86$, $p=0.08$). In aged 3xTgAD mice, Ro5-4864 treatment also decreased Aβ load in the hippocampus CA1 by more than 50% ($t=2.08$, $p=0.03$; Fig. D-E, K). A trend towards decreased subiculum Aβ load was noted in aged mice administered Ro5-4864, however this did not reach significance ($t=1.70$, $p=0.06$; Fig. I-J, L).

As an indicator of tau neuropathology, the number of cells immunoreactive with the phospho-specific tau antibody, AT8, was assessed in aged 3xTgAD mice. No significant difference in the number of AT8 immunoreactive neurons between vehicle (58.3 ±15.3) and Ro5-4864 (40.8 ± 17.3) treated groups was observed ($t=0.76$, $p=0.23$).

Ro5-4864 decreases gliosis in 3xTgAD mice

In young-adult 3xTgAD mice, significant effects of treatment were observed on both GFAP ($F=29.88$, $p<0.001$) and IBA-1 ($F=39.60$, $p<0.001$) hippocampal immunoreactivities. GDX was associated with modest but significant increases in hippocampal GFAP (Fig. 2A-B, K) and IBA-1 immunoreactivities (Fig. 2 F-G, L). Ro5-4864 administration in GDX mice more than halved GFAP (Fig. 1 B-C, K) and IBA-1 (Fig. 2 G-H, L) immunoreactivities. Similarly, Ro5-4864 treatment strongly lowered GFAP ($t=6.76$, $p<0.001$; Fig. 2 D-E, K) and IBA-1 ($t=6.00$, $p<0.001$; Fig. 2 I- J, L) immunoreactivities in the aged 3xTgAD mice.

Ro5-4864 alters brain testosterone and progesterone levels in 3xTgAD mice

Because TSPO regulates synthesis of neuroactive steroids, we measured brain levels of several steroids. In young-adult 3xTgAD mice, a significant effect of treatment was observed on testosterone ($F=5.43$ $p=0.02$) and progesterone ($F=6.25$, $p=0.008$) levels. GDX depleted brain levels of testosterone (Fig. 3A) and progesterone (Fig. 3B), while Ro5-4864 administration in GDX mice increased brain testosterone and progesterone in comparison to vehicle-treated GDX mice (Fig. 3A, B). Brain levels of pregnenolone ($p=0.54$), iso-pregnanolone ($p=0.25$), dihydroprogesterone ($p=0.17$), tetrahydroprogesterone ($p=0.60$), 17β-estradiol ($p=0.15$) and dihydrotestosterone ($p=0.97$) were not significantly affected by Ro5-4864 treatment in GDX mice (data not shown).

Ro5-4864 administration did not significantly alter brain levels of testosterone ($t=-1.51$, $p=0.19$; Fig. 3A) in aged 3xTgAD mice, however brain levels of progesterone ($t=4.15$, $p<0.01$; Fig. 3B) and its metabolites were decreased: dihydroprogesterone (Veh =39.8±3.44;

Ro5-4864 = 20.0±3.51; t=3.96, p<0.01), and tetrahydroprogesterone (Veh =1.7±0.50; Ro5-4864 = 0.2±0.09; t=2.54, p<0.05). Brain pregnenolone (p=0.27), iso-pregnanolone (p=0.18), E2 (p=0.14) and dihydrotestosterone (p=0.34) levels were not significantly changed following Ro5-4864 treatment in the aged mice (data not shown).

Ro5-4864 improves behavioral measures in 3xTgAD mice

In young-adult 3xTgAD mice, anxiety-related behavior in the EPM was significantly affected by treatment (F=6.86, p=0.006). GDX increased anxiety-related behavior, with GDX mice spending significantly less time exploring the open arms of the EPM compared to sham-GDX mice (Fig. 3C). A trend towards decreased anxiety-related behavior was observed in Ro5-4864 treated mice, with a non-significant increase in duration spent exploring the open arm compared to vehicle-treated GDX mice (p=0.054). In the aged 3xTgAD mice, Ro5-4864 treatment was associated with decreased anxiety, with Ro5-4864 treated mice spending more time exploring the open arms of the EPM (t=2.13, p=0.03). No significant effect of treatment was observed on the number of arm entries in either the young-adult or aged mice, suggesting no effect of treatment on exploratory activity.

Spontaneous alternation behavior (SAB) in the Y-maze, a hippocampal-dependent measure of working memory and attention (Lalonde, 2002), was also significantly affected by treatment in the young-adult mice (F=8.69, p=0.002). The GDX group exhibited significantly impaired SAB compared to sham GDX mice (Fig. 3D). Significantly improved SAB performance was observed in GDX mice treated with Ro5-4864 compared to vehicle treated GDX mice. Ro5-4864 treatment also significantly improved SAB performance in aged 3xTgAD mice compared to vehicle-treated controls (t=2.08, p=0.03).

Combined Ro5-4864 and PK-11195 treatment reduces Aβ in non-transgenic mice

There are several known TSPO ligands that differentially activate TSPO. To begin investigating how other TSPO ligands affect AD-related pathologies, we compared Ro5-4864 with PK-11195, a non-benzodiazepine TSPO ligand, for Aβ-lowering actions in non-transgenic male mice. Aβ-40 levels were significantly affected by TSPO ligand treatments in nonTg mice (F=4.33, p=0.003; Figure 4A). Ro5-4864 reduced Aβ-40 levels ~15% compared to GDX mice (p<0.02). Although mean Aβ-40 levels were ~10% lower in PK-11195 treated compared to GDX mice, this was not significantly different compared to either vehicle or Ro5-4864 treated groups. Co-administration of Ro5-4864 and PK-11195 had an additive effect, decreasing Aβ-40 levels by >25% compared to vehicle treated GDX mice (p<0.001). Aβ-40 levels in Ro5-4864 + PK-11195 treated mice were significantly reduced compared to even sham-operated mice (p<0.02). A similar but nonsignificant trend was observed for TSPO ligand treatments on Aβ-42 levels (F=2.33, p=0.061; Figure 4B).

Discussion

TSPO ligands have been found to be neuroprotective following a wide range of insults including peripheral nerve injury (e.g. Giatti et al., 2009), traumatic brain injury (reviewed Papadopoulos and Lecanu, 2009), excitotoxic lesion (e.g. Veiga et al., 2005) and inflammatory insult (e.g. Torres et al., 2000). Our findings suggest TSPO may also be a promising target for the development of therapeutics for the treatment of AD. We observe that TSPO ligands not only reduce the development of AD-related neuropathology in young adult nonTg and 3xTgAD mice, but also reverse pathology and improve cognition in aged 3xTgAD mice.

Increased neurosteroidogenesis is an established mechanism of action of TSPO ligands. Prior observations that neurosteroid levels are altered in both normal aging and AD in

humans (Yue et al., 2005; Rosario et al., 2011), 3xTg-AD mice (Caruso et al., 2013), and non-transgenic rodents (Vallee et al., 1997) suggest that regulation of neurosteroids may contribute to the observed protection. Consistent with this possibility, prior studies have shown that testosterone (Rosario et al., 2010), dihydrotestosterone (Rosario, 2006), progesterone (Carroll et al., 2010) and the progesterone metabolite allopregnanolone (Chen et al., 2011) attenuate Aβ accumulation and improve memory deficits in 3xTgAD mice. In the current study, Ro5-4864 increased both testosterone and progesterone in young adult 3xTgAD mice. However, the absence of similar regulation of neurosteroids by Ro5-4864 in aged 3xTgAD mice suggests that either neurosteroid regulation is not essential to the Aβ-lowering actions of TSPO ligands or that the primary protective mechanism(s) of TSPO ligands differs in young versus aged mice. Elevated levels of additional neurosteroids, including pregnenolone and DHT, have also been observed in response to Ro5-4864 in other rodent paradigms (Giatti et al., 2009). While it is unclear why different patterns of neurosteroid regulation are observed across studies, the spectrum of neurosteroids induced by TSPO ligands and the magnitude of these effects likely depends upon a variety of experimental parameters including brain region, duration of TSPO ligand treatment, and latency between treatment and tissue collection.

Another important mechanism contributing to neuroprotective actions of TSPO ligands is reduction of gliosis and its associated pro-inflammatory effects. We observed strong morphological evidence of decreased astrocytic and microglial activation in young and aged 3xTgAD mice treated with Ro5-4864. While the marked reduction in gliosis may have been a downstream result of reduced Aβ accumulation, previous studies have demonstrated that TSPO ligands act directly on glia to reduce indices of activation and proliferation (Choi et al., 2002). Chronic neuroinflammation is a significant degenerative factor in AD and anti-inflammatory drugs have shown some therapeutic promise for AD (Szekely et al., 2004). While TSPO ligands are under development for *in vivo* imaging of reactive gliosis in AD, our findings suggest that TSPO ligands may represent not only a marker of neuroinflammation, but also a potential anti-inflammatory treatment strategy for AD.

The most important outcome of the Ro5-4864 treatment is arguably the marked improvements in functional outcomes, increased working memory performance and reduced anxiety behaviors in the 3xTgAD mice. Few studies have addressed the effects of TSPO ligands on learning and memory in rodents, although some have reported impaired (Holmes and Drugan, 1991) and others improved (Cunha et al., 1991) performance in a passive avoidance task. In both male and female 3xTgAD mice, we have reliably observed a strong correlation between Aβ neuropathology and SAB impairment (Rosario, 2006; Carroll et al., 2010). Therefore, the improved working memory performance observed in the current study is likely related to reduced neuropathology. Consistent with our observations in the 3xTgAD mice, TSPO ligands are potent anxiolytics in both rodents and humans (Rupprecht et al., 2009). Anxiety may in fact be an early indicator of AD in people with mild cognitive impairment (Perry and Hodges, 1999). Thus, TSPO ligands may simultaneously target multiple clinical features of AD, including memory function and anxiety behaviors.

Many new generation, safe TSPO ligands have been developed for *in vivo* imaging in humans (Chauveau et al., 2008), however these ligands may also have a therapeutic potential for the treatment of AD. Since TSPO expression is upregulated in the most severely affected regions of the AD brain, TSPO ligands may selectively target regions most affected by the disease. Together, these findings indicate that TSPO may be a promising target for the development of pleiotropic treatment strategies for the management of AD.

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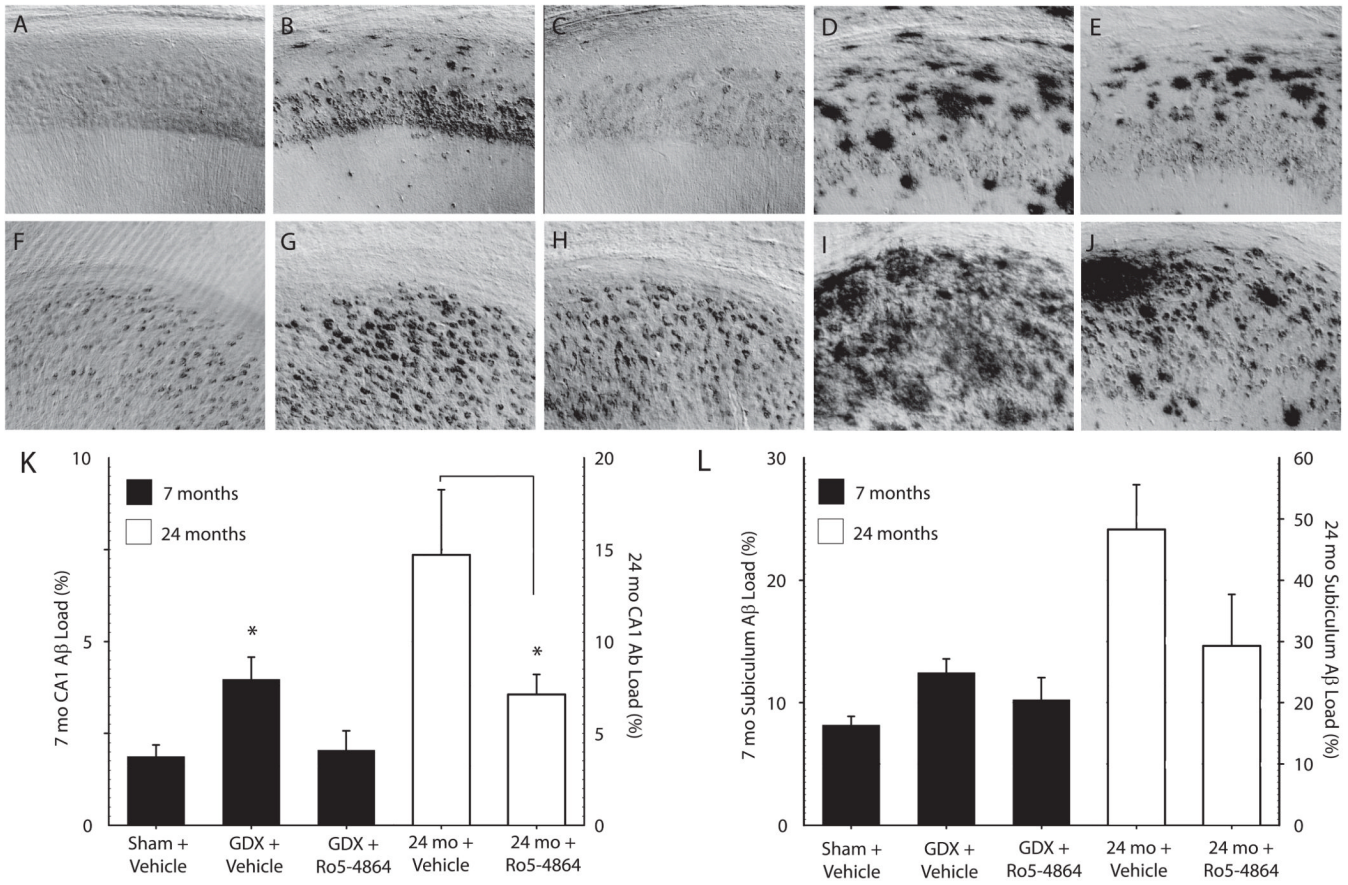

Figure 1. Ro5-4864 reduces Aβ accumulation in the hippocampus of 3xTgAD mice

A-J, Representative photomicrographs show Aβ immunoreactivity in hippocampus CA1 (A-E) and subiculum (F-J) regions of 7 mo-old 3xTgAD mice in the Sham+vehicle (A, F), GDX+vehicle (B, G) and GDX+Ro5-4864 (C, H) conditions; and 24 mo-old 3xTgAD mice administered vehicle (D, I) and Ro5-4864 (E,J). K, Hippocampus CA1 Aβ immunoreactivity load values. L, Subiculum Aβ immunoreactivity load values. *p<0.05 compared to all other groups.

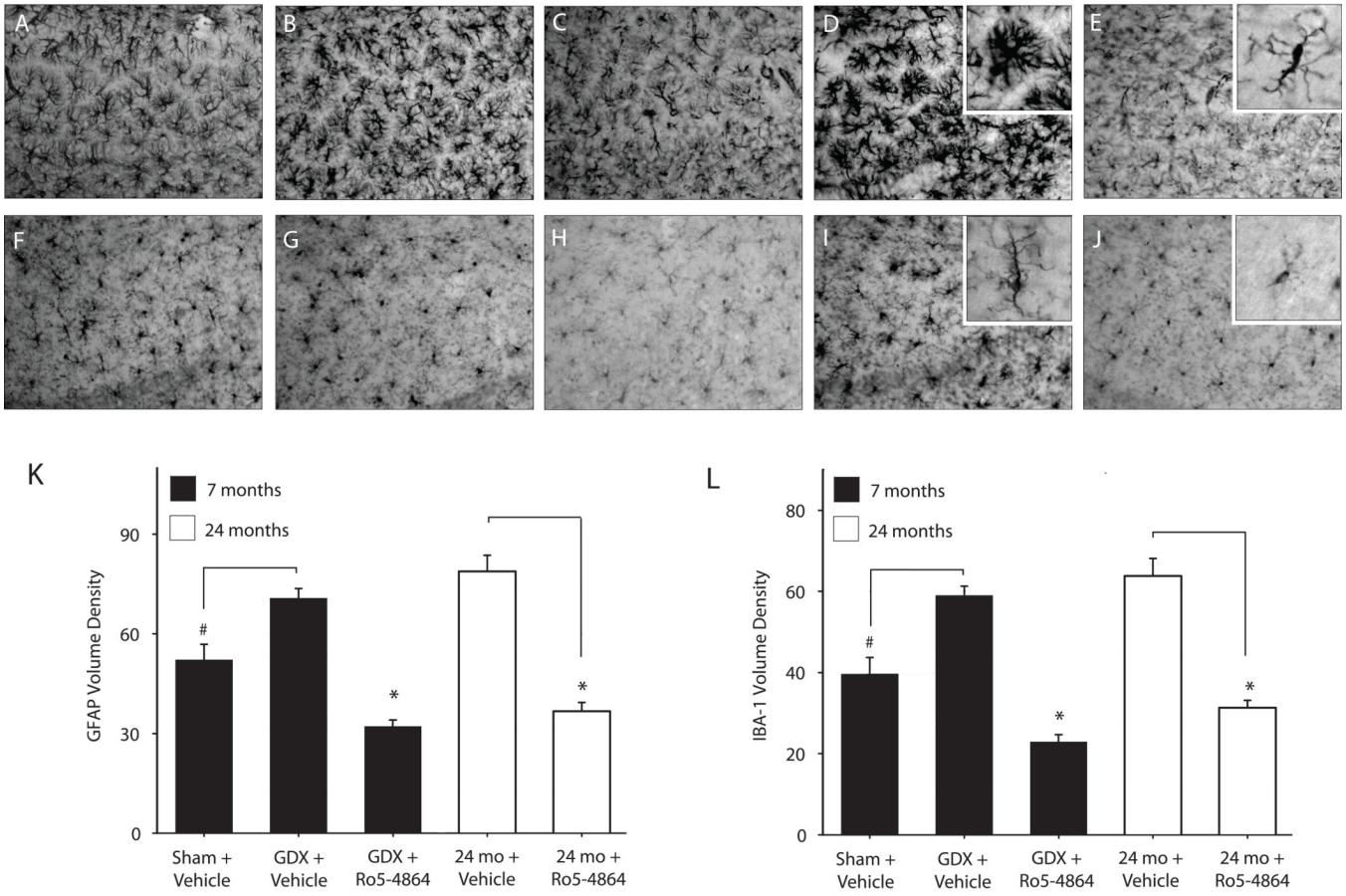

Figure 2. Ro5-4864 reduces hippocampal gliosis in 3xTgAD mice

A-J, Representative photomicrographs show GFAP (A-E) and IBA-1 (F-J) immunoreactivities in the hippocampus CA1 region of 7 mo-old 3xTgAD mice in the Sham +vehicle (A, F), GDX+vehicle (B, G) and GDX+Ro5-4864 (C, H) conditions; and 24 mo old 3xTgAD mice administered vehicle (D, I) and Ro5-4864 (E,J). K, GFAP immunoreactivity volume density values in the hippocampus CA1 region. L, IBA-1 immunoreactivity volume density values in the hippocampus CA1 region. # $p<0.001$ compared to GDX+vehicle. * $p<0.001$ compared to all other groups.

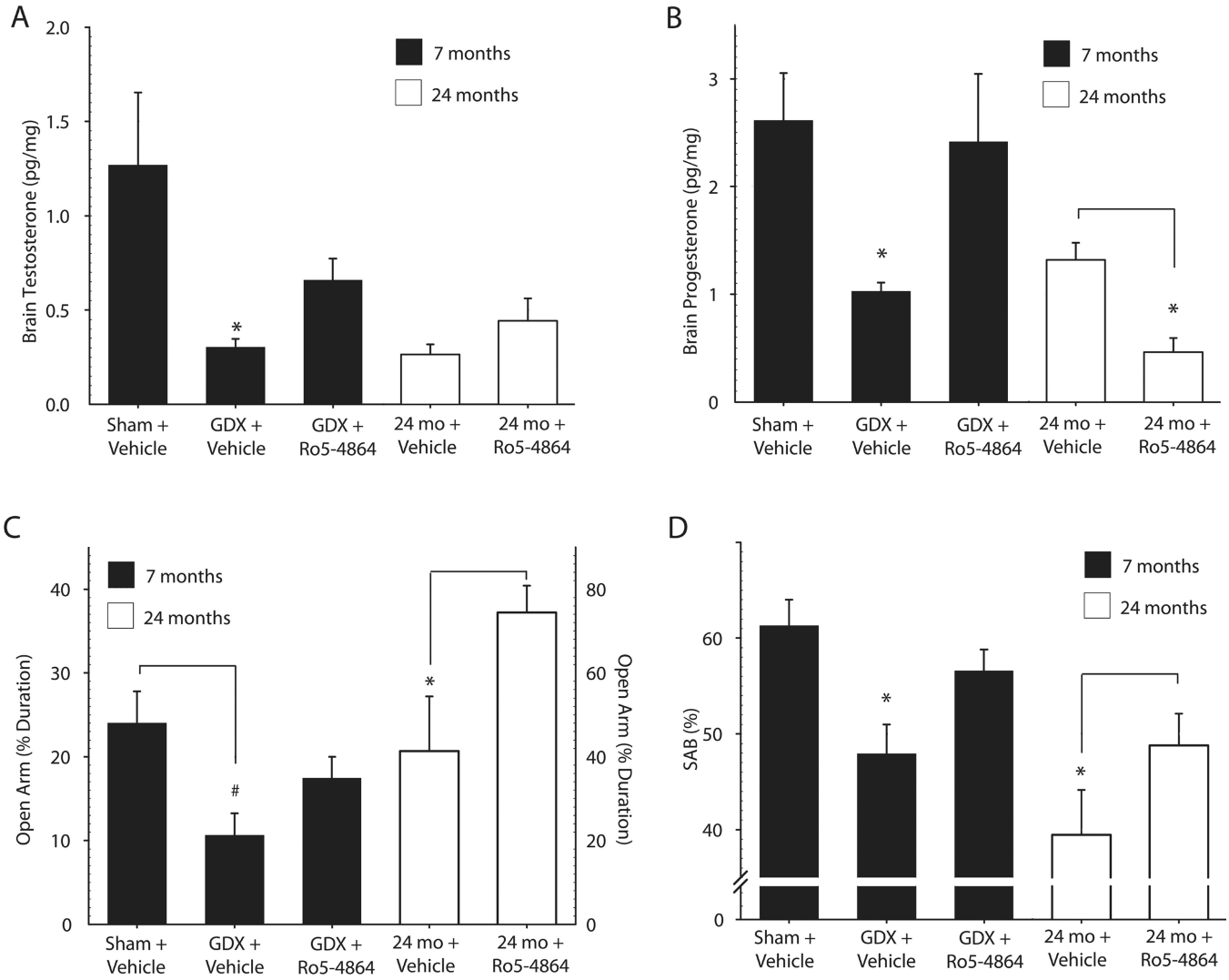

Figure 3. Ro5-4864 regulates brain testosterone and progesterone levels and improves behavioral deficits in 3xTgAD mice

A, Brain testosterone levels were quantified in limbic structures. Ro5-4864 treatment attenuated GDX-induced testosterone depletion in young adult 7 mo-old mice. B, Brain progesterone levels were quantified in limbic structures. Ro5-4864 treatment increased PROG over two-fold in GDX 7 mo-old mice. C, Percent duration spent exploring the open arm of the elevated plus maze, with increased exploration of the open arm indicative of reduced anxiety. D, SAB, represented as percentage alternation, both 7 mo-old GDX and 24 mo-old 3xTgAD mice administered Ro5-4864 exhibited significantly improved SAB performance. # $p<0.05$ compared to Sham+vehicle. *$p<0.05$ compared to all other groups.

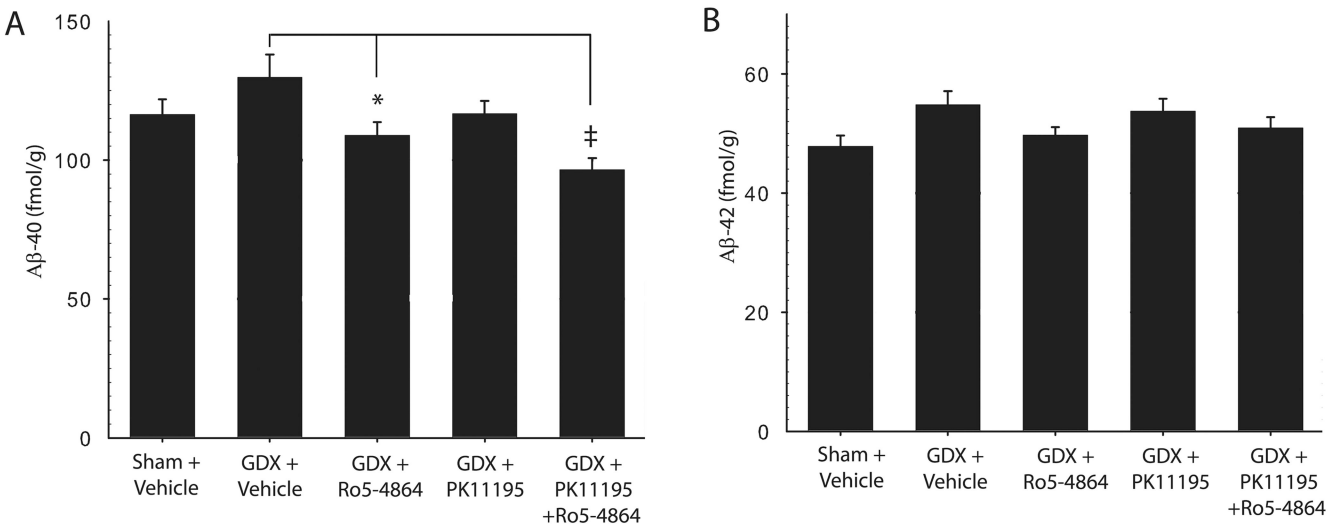

Figure 4. Ro5-4864 alone or in combination with PK-11195 reduces brain Aβ levels in nonTg mice

Brain levels of soluble AβX-40 (A) and AβX-42 (B) from hemibrain homogenates were assessed by ELISA in young adult nonTg mice treated for four weeks with vehicle, Ro5-4864, PK11195, or Ro5-4864 + PK11195. Data show mean values (+SEM). *p<0.02, ‡p<0.001 compared to GDX+vehicle.