Withania somnifera reverses Alzheimer's disease pathology by enhancing low-density lipoprotein receptor-related protein in liver

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A 30-d course of oral administration of a semipurified extract of the root of *Withania somnifera* consisting predominantly of withanolides and withanosides reversed behavioral deficits, plaque pathology, accumulation of β-amyloid peptides (Aβ) and oligomers in the brains of middle-aged and old APP/PS1 Alzheimer's disease transgenic mice. It was similarly effective in reversing behavioral deficits and plaque load in APPSwInd mice (line J20). The temporal sequence involved an increase in plasma Aβ and a decrease in brain Aβ monomer after 7 d, indicating increased transport of Aβ from the brain to the periphery. Enhanced expression of low-density lipoprotein receptor-related protein (LRP) in brain microvessels and the Aβ-degrading protease neprilysin (NEP) occurred 14–21 d after a substantial decrease in brain Aβ levels. However, significant increase in liver LRP and NEP occurred much earlier, at 7 d, and were accompanied by a rise in plasma sLRP, a peripheral sink for brain Aβ. In WT mice, the extract induced liver, but not brain, LRP and NEP and decreased plasma and brain Aβ, indicating that increase in liver LRP and sLRP occurring independent of Aβ concentration could result in clearance of Aβ. Selective down-regulation of liver LRP, but not NEP, abrogated the therapeutic effects of the extract. The remarkable therapeutic effect of *W. somnifera* mediated through up-regulation of liver LRP indicates that targeting the periphery offers a unique mechanism for Aβ clearance and reverses the behavioral deficits and pathology seen in Alzheimer's disease models.

herbal extract | dementia | neurodegenerative disease

Alzheimer's disease (AD) is characterized by progressive dysfunction of memory and higher cognitive functions. Pathological hallmarks include senile plaques, neurofibrillary tangles, dystrophic neurites, gliosis, and neuroinflammation. Cholinesterase inhibitors and the NMDA antagonist memantine, the commonly used drugs for AD, provide symptomatic relief but do not alter the course of disease. No curative treatment is available, and research focuses on drugs for slowing disease progression or providing prophylaxis.

The majority of AD cases are sporadic in nature. The small fraction of familial cases are caused primarily by mutations in three genes: amyloid precursor protein (APP), presenilin1 (PS1), and presenilin 2 (PS2). These mutations result in abnormal processing of APP and increased generation of β amyloid peptide 1-42 (Aβ42), which aggregates as β sheets (1). Treatment strategies have focused on reducing β-amyloid load through (*i*) inhibition of γ- or β-secretases or activation of α-secretase; (*ii*) inhibition of Aβ aggregation; (*iii*) activation of proteases, such as neprilysin (NEP); and (*iv*) active and passive immunotherapy (2, 3).

Among other mechanisms, influx and efflux of brain Aβ are regulated by receptor for advanced glycation end products (RAGE) and low-density lipoprotein receptor-related protein (LRP), respectively (4). The soluble form of LRP in plasma (sLRP) is a peripheral sink for Aβ that aids its sequesteration. In AD, plasma sLRP and LRP1 at the blood-brain barrier are reduced, whereas RAGE expression is increased, resulting in accumulation of brain Aβ (5, 6). Thus, enhancing Aβ transport across the blood-brain barrier and Aβ sequestration by sLRP can help reduce AD pathology.

Traditional systems of medicine, such as Ayurveda, offer a knowledge base that can be drawn on to develop novel therapeutic strategies. *Withania somnifera* (WS), also known as Ashwagandha, is a nootropic agent that promotes cognition, including memory (7). Withanolide A and withanoside IV from WS roots help promote neurite outgrowth in cultured neurons and in rodents injected with Aβ 25–35 (8, 9). Here we demonstrate that a WS extract reverses behavioral deficits and plaque pathology and reduces the Aβ burden in middle-aged and old APP/PS1 mice through up-regulation of liver LRP, leading to increased clearance of Aβ. The therapeutic effects of WS were reproducible in APPSwInd J20 mice, another model of AD, in which behavioral deficits were reversed and plaque load decreased significantly.

Results

WS Reverses Behavioral Deficits and Plaque Pathology in APP/PS1 and APPSwInd J20 Mice. A 30-d course of treatment with WS extract (Fig. S1) led to complete reversal of the behavioral deficits seen in the radial maze task in middle-aged (9–10 mo old) male APP/PS1 mice (Fig. 1*A*) and in the Morris water maze in APPSwInd J20 mice (Fig. S2 *A–D*). Old APP/PS1 mice (23–24 mo) showed enhanced performance on the radial arm maze starting on day 21 of treatment and increasing progressively up to day 30 (Fig. 1*B*). Amyloid plaques were eliminated in the cortex (CT) and hippocampus (HP) of middle-aged APP/PS1 mice of both sexes (Fig. 1*C* and Fig. S3*A*) and substantially reduced in old mice as detected by stereologic examination after immunostaining with antibody to Aβ42, ubiquitin, silver, or thioflavin (Fig. 1*C* and Fig. S4 *A* and *B*). In 8- to 10-mo-old APPSwInd J20 mice, plaque density was reduced by 58% in CT and 63% in HP (Fig. S2*E*). Cerebral amyloid angiopathy (CAA), observed as deposition of Aβ in brain microvessels, seemed substantially reduced in old APP/PS1 (Fig. S5 *A–C*) and APPSwInd J20 (Fig. S5*D*) mice.

WS Decreases Aβ Levels and Promotes Disaggregation of Oligomers in APP/PS1 Mice. Aβ42 levels were reduced in middle-aged male APP/PS1 mice by 77% in CT and 78% in HP and in old mice by 49% in CT and 52% in HP (Fig. 1*D*). Brain Aβ40 (Fig. S6*A*) and plasma Aβ42/40 were significantly reduced as well (Fig. 1*E* and Fig. S6*B*). Similar reductions in brain and plasma Aβ42 levels were seen in middle-aged female APP/PS1 mice (Fig. S3*B*). Aβ

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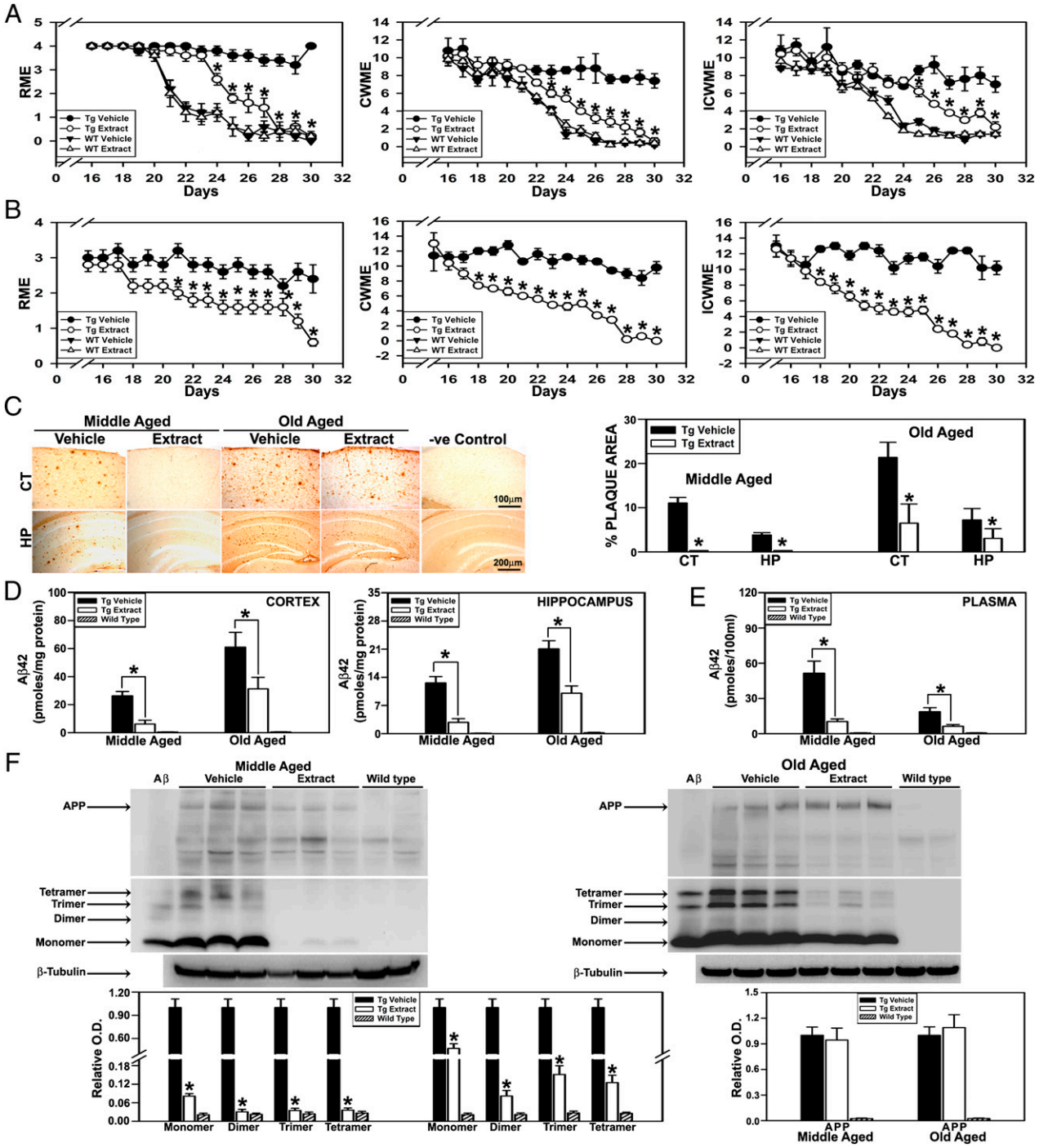


Fig. 1. Behavioral recovery and reduction of amyloid pathology in middle-aged and old APP/PS1 mice after 30 d of WS treatment. (*A* and *B*) Reversal of behavioral deficits in middle-aged (*A*) and old (*B*) APP/PS1 (Tg) mice measured as a significant decrease in reference memory errors (RME), correct working memory errors (CWME), and incorrect working memory errors (ICWME) on an eight-arm radial maze (RAM) task. (*C*) The plaque area was decreased in the CT and HP of middle-aged and old animals. Sections stained by normal IgG were used as negative controls. (*D* and *E*) Aβ42 levels were significantly reduced in the CT, HP, and plasma of middle-aged and old animals treated with WS. Aβ42 levels in WT and middle-aged and old mice were as follows: CT, 0.63 pmol/mg protein; HP, 0.30 pmol/mg protein; plasma, 0.52 pmol/100 mL and CT, 0.78 pmol/mg protein; HP, 0.33 pmol/mg protein; plasma, 0.61 pmol/100 mL, respectively. (*F*) A significant reduction of Aβ42 monomer and lower oligomers was seen in CT. Values are mean ± SEM (*A* and *B*) or mean ± SD (*C*–*F*), $n = 10$ mice. $^*P \leq 0.05$.

oligomers (dimers, trimers, and tetramers), which contribute significantly to AD pathology (10, 11), were significantly decreased in the CT of APP/PS1 mice treated with WS for 30 d (Fig. 1*F*).

APP Processing Is Not Altered by WS. To rule out the possibility that therapeutic effects of WS could be caused by repression of APP expression (both transgene and WT), we quantified APP protein (Fig. S7*A*), and mRNA (Fig. S7*B*) in APP/PS1 mice and found that they were unaffected by WS. We also evaluated CTFs of APP and found no change in levels of α, β, and γ CTFs (Fig. S7*C*). β-secretase (BACE) levels were unaffected as well (Fig. S7*D*), indicating that WS does not influence APP processing.

Temporal Sequence of Therapeutic Action of WS in APP/PS1 Mice. Plaque density decreased significantly after 14 d of treatment (Fig. 2*A*). Activated microglia were observed around the plaques (Fig. S8*A*). The number of activated microglia proximal to the plaques increased significantly from day 7 to day 14 and decreased thereafter (Fig. S8 *A* and *B*). The number of microglia distal to the plaques decreased from day 7 to day 21, as did the total number of Iba-1 positive microglial cells (Fig. S8 *A* and *B*).

Aβ42 monomer decreased after 7 d in CT, followed by decline in the levels of oligomers (dimers, trimers, and tetramers) and total Aβ42/40 after 14 d (Fig. 2 *B* and *C*). Interestingly, plasma Aβ42/40 levels increased significantly between day 7 and day 14, possibly indicating clearance of Aβ from the brain into the circulation (Fig. 2*D*).

Consequently, we examined the status of LRP, which mediates transport of Aβ from the brain into the periphery (12, 13). Expression of LRP mRNA and protein increased significantly in CT after 14–30 d of treatment (Fig. 3*A*). LRP expression increased progressively in endothelial cells, as demonstrated by colocalization of LRP with CD31, a marker of endothelial cells (Fig. 3*C* and Fig. S9*A*). Neuronal LRP remained unchanged (Fig. 3*D*). RAGE and clusterin expression decreased significantly at 14–30 d posttreatment, whereas ApoE was unaffected (Fig. S9*B*). The mRNA, protein, and catalytic activity of NEP increased only after 21 d (Fig. 3*B*).

Because the decrease in brain Aβ42 monomer and increase in plasma Aβ were detected before significant enhancement of brain LRP or NEP, we examined the peripheral effects of WS. Liver NEP (16%) and LRP mRNA (57%) and plasma sLRP (46%) increased after 7 d and increased progressively up to 30 d (by 45%, 239%, and 274% respectively; Fig. 3 *E* and *F*). Significant negative correlations was seen among cortical Aβ42, hepatic LRP and NEP, and plasma sLRP levels (Fig. 3*G*).

WS Enhances LRP and NEP mRNA in WT Mice. To identify Aβ-independent effects, we treated young adult WT mice with WS and observed increased expression of hepatic, but not brain, LRP and NEP (Fig. 4*A*). Plasma sLRP was increased, whereas brain and plasma Aβ were decreased (Fig. 4 *B* and *C*), indicating that the profound effect of WS on liver LRP and NEP leads to a decrease in Aβ load even in WT mice.

Down-Regulation of Hepatic LRP, but Not Neprilysin, Abrogates the Therapeutic Efficacy of WS in APP/PS1 Mice. We down-regulated hepatic LRP selectively in WS-treated APP/PS1 mice by i.p. administration of antisense oligodeoxynucleotides (oligos) for 7 d. Liver LRP mRNA and protein and plasma sLRP decreased

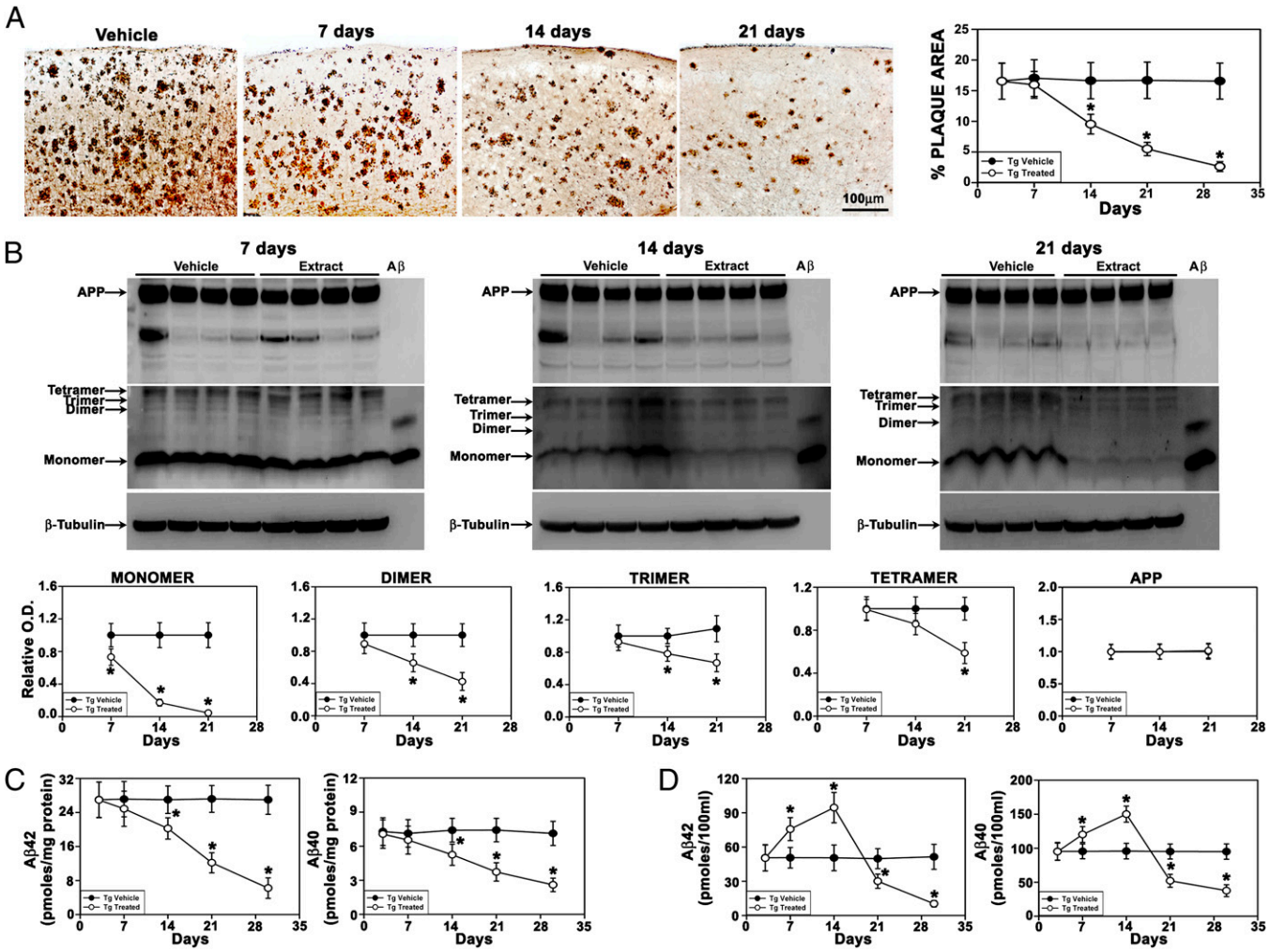
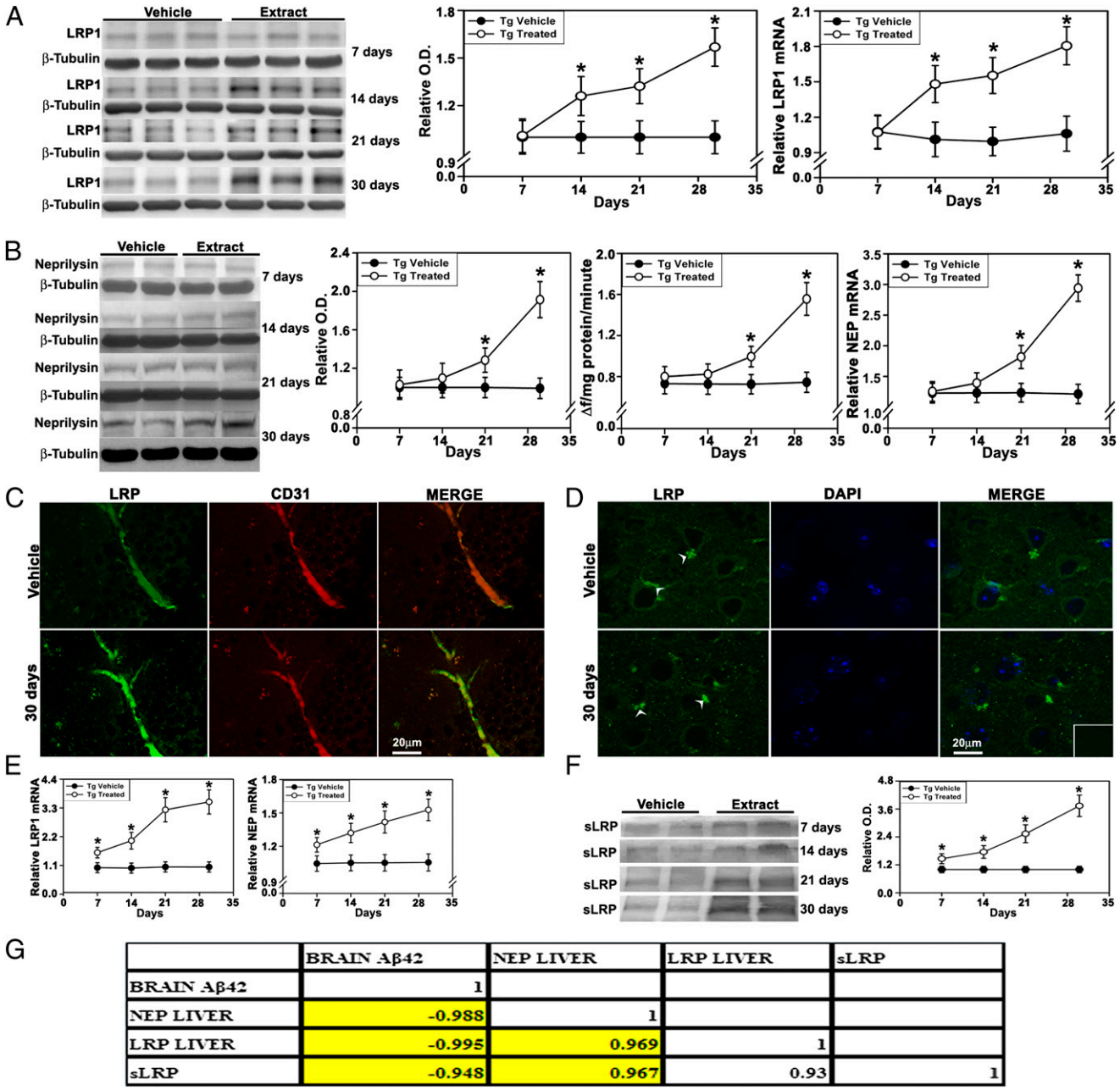


Fig. 2. Time course of clearance of AD pathology by WS in APP/PS1 mice. (*A*) A significant decrease in silver-stained plaques was seen in CT after 14–30 d of treatment. (*B*) Aβ42 monomer and oligomers were decreased significantly after 7 and 14 d, respectively. (*C* and *D*) Cortical Aβ42/40 decreased progressively from day 14 to day 30; however, corresponding plasma levels were significantly elevated from day 7 to day 14 and decreased thereafter up to day 30. Values are mean ± SD (n = 8 mice). *$P \leq 0.05$.



	BRAIN Aβ42	NEP LIVER	LRP LIVER	sLRP
BRAIN Aβ42	1			
NEP LIVER	-0.988	1		
LRP LIVER	-0.995	0.969	1	
sLRP	-0.948	0.967	0.93	1

Fig. 3. WS induces the expression of LRP, NEP, and sLRP in APP/PS1 mice. (*A* and *B*) mRNA and protein levels of LRP (*A*) increased progressively in CT from day 14 onward, whereas NEP mRNA, protein, and activity (*B*) increased after day 21. (*C* and *D*) LRP expression increased in endothelial cells as detected by colocalization with CD31, but not in cortical neurons (arrowheads). (*Inset*) Negative control stained by normal IgG. (*E* and *F*) In contrast, hepatic LRP, NEP (*E*), and plasma sLRP (*F*) increased significantly from day 7 onward. (*G*) Pearson correlation analysis indicates significant negative correlations among brain Aβ42 and liver LRP, NEP, and sLRP. Values are mean ± SD (n = 8 mice). *$P \leq 0.05$.

by 23–38% (Fig. 4 *D* and *E*), which led to increased brain Aβ and decreased plasma Aβ (Fig. 4 *G* and *H*). This treatment paradigm did not affect brain LRP or hepatic NEP (Fig. 4 *D* and *F*). Inhibition of hepatic, but not brain, NEP (Fig. S10*A*) by thiorphan had no effect on Aβ levels in brain or plasma (Fig. S10*C*), indicating that hepatic NEP does not play a critical role in the therapeutic effect of WS. Thiorphan had no effect on hepatic LRP (Fig. S10*B*).

Discussion

The major constituents of the roots of WS are alkaloids (eg, withanine, somniferine) and steroidal lactones, withanolides, and their glycosides, withanosides. We report reversal of AD pathology in APP/PS1 mice by a partially purified WS extract consisting of 75% withanolides and 20% withanosides by increasing the clearance of the toxic Aβ peptide from the brain, promoting its sequestration in plasma and ultimately its degradation in the periphery. The aforementioned beneficial effects are associated with reversal of behavioral deficits and reduced AD pathology in very old AD Tg mice (22–24 mo old), a phenomenon not reported previously. The therapeutic benefits of WS on behavioral deficits and Aβ pathology are also confirmed in APPSwInd J20 mice, another AD mouse model. WS treatment decreased CAA in both models (Fig. S5). However, CAA is scarce even in old (23–24 mo) APP/PS1 mice and detectable mainly in the HP of adult APPSwInd J20 mice, and thus a thorough analysis is

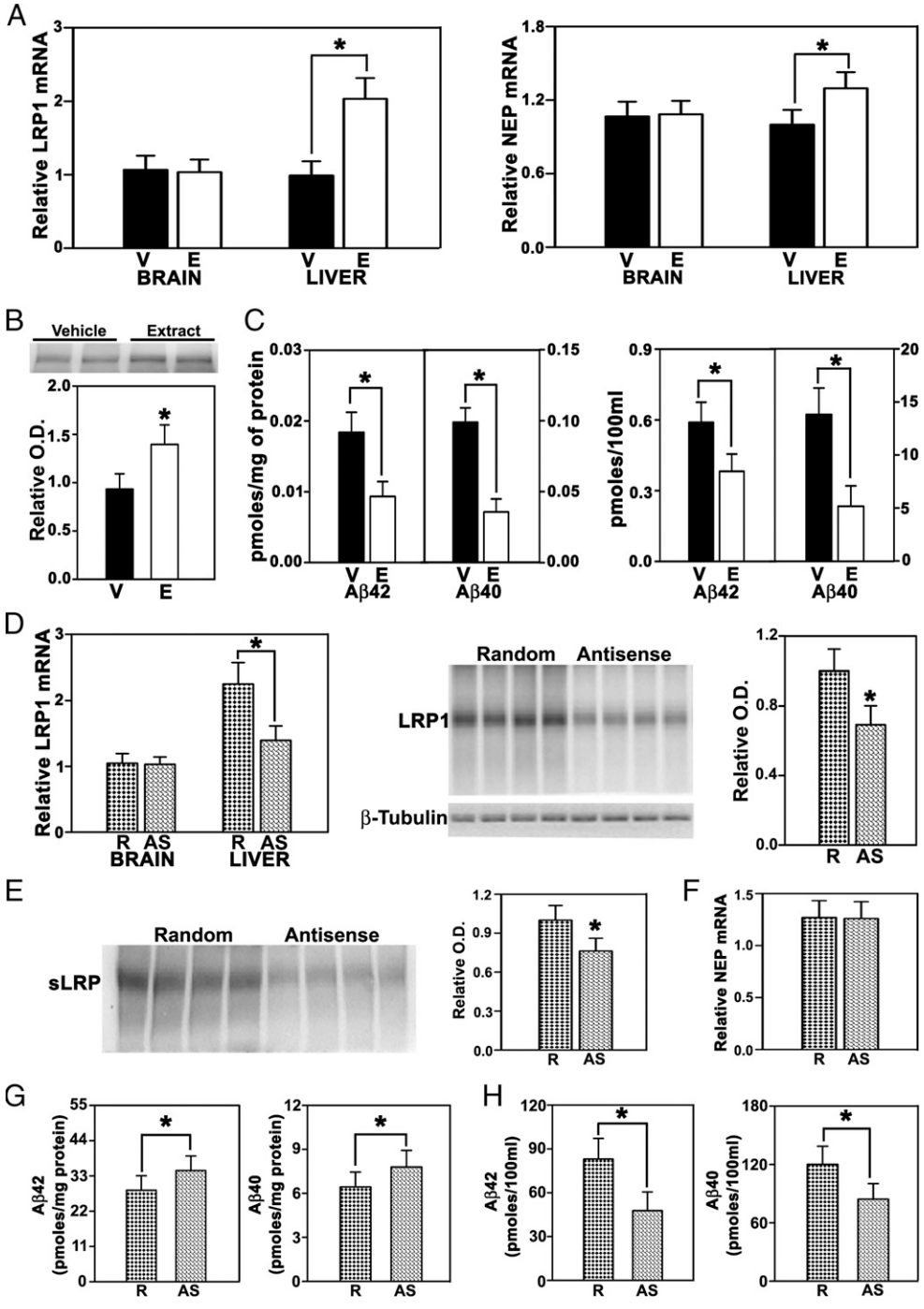


Fig. 4. Down-regulation of hepatic LRP abolishes the therapeutic efficacy of WS. Young adult WT mice treated with WS for 30 d showed significant increases in hepatic LRP, NEP (*A*), and plasma sLRP (*B*), but no change in brain levels. (*C*) Aβ42/40 was decreased in brain and plasma. APP/PS1 mice given WS for 7 d along with antisense oligos to LRP (AS) showed a significant decrease in mRNA and protein of liver LRP (*D*) and plasma sLRP (*E*), leading to an increase in brain Aβ42/40 (*G*) and a decrease in plasma (*H*) Aβ42/40 compared with mice treated with WS and random (R) oligos. (*F*) Antisense oligos to LRP had no effect on liver NEP. Values are mean ± SD (n = 9 mice). *$P \leq 0.05$.

needed with a more suitable AD mouse model with extensive CAA, such as the vasculotropic Dutch/Iowa E693Q/D694N mutation (Tg-SwDI).

One of the first effects seen after 7 d of WS treatment is increased levels of sLRP in plasma and enhanced expression of LRP and NEP in the liver, accompanied by a concomitant increase in plasma Aβ42/40 and decrease in brain Aβ monomer levels, indicating the efflux of Aβ42/40 from the brain into plasma. Following the course of therapeutic action of WS, we see a decrease in the lower oligomers of Aβ after 2–3 wk, indicating that the disaggregation process starts with a decrease in brain Aβ42 monomer.

Cleavage of the N terminus of hepatic LRP releases extracellular domain of LRP into the plasma, where it is present as sLRP (14) and acts as a peripheral sink for Aβ. Intravenous delivery of LRP decreases Aβ42/40 in the brain, with a corresponding increase in the plasma (5). Liver LRP mediates endocytosis of Aβ peptides present in the plasma, which are then cleared through NEP and other proteases present in the liver (15). Indeed, the ability of WS to induce liver LRP may be extremely important, given that cell surface LRP in liver is required for the rapid systemic clearance of the toxic Aβ peptide (16) and subsequent degradation of this peptide by proteases in the liver.

In young adult (3-mo-old) WT mice, WS enhances the expression of liver LRP and NEP but has no effect on the brain. The increase in liver LRP leads to a decrease in Aβ in the brain and plasma in these WT mice, indicating that enhanced expression of liver LRP and thus of sLRP can lead to efflux of Aβ from the brain to the plasma. Selective down-regulation of liver

LRP, but not NEP (Fig. 4*D*), abrogates the therapeutic effect of WS and increases Aβ levels in the brain (Fig. 4 *G* and *H*), indicating that the primary effect of WS on liver LRP and sLRP contributes to the clearance of brain Aβ. In APP/PS1 mice, WS enhances liver LRP by 57% within 7 d and by up to 239% after 30 d. Several proteases, including NEP, are involved in the degradation of Aβ in the liver. Although WS induces hepatic NEP, its inhibition does not have a profound effect on therapeutic efficacy, indicating that other proteases present in the liver can potentially degrade Aβ. The preferential effect of WS on inducing the expression of LRP in the liver but not in the brain could be due to differential penetration of the extract and/or regulation of gene expression.

Sequentially, there were two distinct effects: before and after a decrease in brain Aβ. The early events seen at 7 d, when total brain Aβ level was not significantly decreased, include increases in liver LRP and NEP and plasma sLRP, with no change in the brain other than Aβ monomer loss. However, after 14 d, when brain Aβ levels were decreased, LRP expression increased as measured by quantitative RT-PCR and immunoblot analysis. NEP levels increased after 21 d. The increase in LRP mRNA occurs earlier and is more pronounced in the liver (a 2.5-fold increase after 21–30 d) compared with the brain (a 0.7-fold increase).

Increased production of Aβ peptides is known to depress the expression of critical genes, which is restored when Aβ levels are decreased (4). The delayed effect of WS on the expression of brain LRP and NEP seems to indicate that a similar phenomenon might be operating here as well. This idea is supported by the lack of effect of WS on LRP and NEP expression in the brain of WT animals, unlike in the liver.

Although LRP is the major cell surface receptor for clearance of Aβ from brain interstitial fluid across the blood-brain barrier, it is also involved in the endocytosis of APP and thereby influences Aβ production within neurons (17). In fact, AD transgenic mice crossed with mice overexpressing neuronal LRP exhibit increased soluble Aβ levels and enhanced memory deficits (18). WS did not substantially increase neuronal LRP, although enhanced LRP staining was seen in endothelial cells lining microvessels (Fig. 3 *C* and *D*). The other proteins involved in trafficking of Aβ between the brain and the periphery are RAGE and clusterin. The expression of RAGE is increased in AD. WS decreased the expression of RAGE and clusterin, the physiological and pathological chaperones that influence Aβ aggregation (19).

We have demonstrated the profound effect of the withanosides/ withanolides in reducing amyloid load through their direct peripheral effect on liver LRP and sLRP. The remarkable effect of WS in clearing the amyloid load in familial forms of AD is of significance, given that these deposits are poorly cleared from the brain, resulting in earlier onset of the familial forms. Thus, WS may be of potential use in the treatment of familial AD.

As noted earlier, the extract used in this study is a mixture of several withanolides and withanosides, and a rather high dose was used. Notwithstanding, one of the salient features of this study is that oral administration of WS proved highly effective in reversing the behavioral deficits and pathological features in two mouse models of AD. Purification and identification of the active principles of WS is required such that the effective dosage of administration can be decreased. It is likely that more than one compound will be necessary to bring about the therapeutic action. Because WS is composed of several compounds, it is possible that pathways other than those examined in this study may contribute to the ultimate therapeutic effect seen. Nevertheless, the potent effect of WS in rapidly clearing Aβ is mainly related to its effect in the periphery through increases in the levels of liver LRP and sLRP, indicating that targeting the periphery for Aβ clearance may provide a unique mechanism for rapid elimination of Aβ, eventually leading to reversal of behavioral deficits in AD transgenic mice.

Materials and Methods

Preparation of Plant Extract. The powdered root of *W. somnifera* obtained from an authenticated source (Arya Vaidya Sala) was serially extracted with chloroform-methanol and dried to remove all traces of the solvent. The residual material, comprising 75% withanolides and 20% withanosides, is referred to as WS extract. The LC-MS fingerprint of the extract is shown in Fig. S1.

Animals and Treatment. Transgenic mice B6C3-Tg (APPswe,PSEN1)85Dbo/J were procured from Jackson Laboratory. An additional group of APPSwInd mice (J20 line) was used to confirm data on cognition and amyloidosis. WT and APP/PS1 or APPSwInd J20 mice were divided into two groups for treatment with vehicle or WS extract. WS extract was suspended in ethanol (1 g/mL), and a single daily oral dose of 1 g/kg body weight was administered for 7–30 d, with no animal receiving more than 30 μL of ethanol per day. Control animals received and equal volume of ethanol. Details are provided in *SI Materials and Methods*.

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