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Antisense Reduction of Tau in Adult Mice Protects against Seizures

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Introduction

As a member of the microtubule-associated protein family (Weingarten et al., 1975), the protein tau is enriched in axons of mature and growing neurons (Kempf et al., 1998). However, under certain conditions, tau can become hyperphosphorylated and accumulate into oligomeric species and neurofibrillary tangles, resulting in a group of disorders known collectively as tauopathies (Billingsley and Kinsaid, 1997; Lovestone and Reynolds, 1997; Bux and Delacourte, 1999) with the most common being Alzheimer's disease (AD).

Although the role of tau in proteinaceous aggregates has long been studied (Iqbal et al., 1975; Brion et al., 1985), a new role has emerged that implicates tau as a regulator of neuronal hyperexcitability. Tau knock-out (tau −/−) mice demonstrate substantially reduced seizure severity in models of chemically induced seizures (Roberson et al., 2007, 2011; Ittner et al., 2010) and genetic models of severe epilepsy (Holth et al., 2015). These data suggest that tau plays a role in neuronal hyperexcitability and provide evidence that a tau-reducing therapy may be beneficial for those with seizure disorders. In addition, amyloid precursor protein overexpression/amyloid-β depositing mouse lines show increased baseline neuronal hyperexcitability and spontaneous seizures. When placed onto a tau−/− background, these AD mouse models show both decreased seizure frequency and improved learning and memory (Roberson et al., 2007, 2011; Ittner et al., 2010), suggesting that tau-linked neuronal hyperexcitability may be an important component of AD pathophysiology.

However, whether reducing tau levels in an adult animal will modulate neuronal hyperexcitability similar to genetic deletion remains unknown. For example, developmental compensation could contribute to the protective effect of tau−/−, such as the reported increase in microtubule-associated protein 1A (Harada et al., 1994). Here, we tested directly the effect of reducing tau in adult nontransgenic mice by using endogenous tau knock-out mouse models and subsequently analyzing the effects on baseline behavior and induced seizure severity. We reduced murine endogenous tau levels using antisense oligonucleotides (ASOs) delivered directly to the CSF (DeVos and Miller, 2013a). Recent data
demonstrating safety of CSF-delivered ASOs in humans (Miller et al., 2013) suggests that the strategy used here may be translated into therapy for seizures and possibly other neurodegenerative disorders.

Materials and Methods

Animals: All ASO-treated mice were C57BL/6j nontransgenic mice ordered directly from The Jackson Laboratory. Tau−/− mice containing a GFP-molding eDNA integrated into exon 1 of MAPT gene (Tucker et al., 2001) were obtained from The Jackson Laboratory and maintained on a C57BL/6j background. Characterization and behavioral experiments were performed using gender-balanced groups age 2–4 months (Figs. 1,2,3,4,5). Seizure experiments were performed using males age 3–5 months (Figs. 6,7,8). Mice had access to food and water ad libitum and were housed on a 12 h light/dark cycle. All animal protocols were approved by the institutional animal care and use committee at Washington University.

ASOs: The ASOs had the following modifications: 5 nucleotides on the 5′- and 3′-termini containing 2′-O-methoxymethyl modifications and 10 unmodified central oligonucleotides (DeVos and Miller, 2013b) to support RNAase H activity and a phosphorothioate backbone to improve nucleic resistance and promote cellular uptake (Bennett and Swazey, 2010). ASOs were synthesized as described previously (McKay et al., 1999; Chorvatová et al., 2005) and solidified in 0.9% sterile saline immediately before use. ASO sequences were as follows: Tau-s100-5′-GGAGGATCTTCTTAGTCTG-3′; Tau-s200-5′-AAGGGCTTATAGGTACATT-3′; Tau-s400-5′-ATACCTGATTTGAGTGCCC-3′; Scrambled ASO: 5′-CCTGTCCTGACGTGAGGTC-3′.

Surgical placement of intracerebroventricular pumps and tissue collection: As described previously (Smith et al., 2006; DeVos and Miller, 2013a), mice were anesthetized with isoflurane and 28 g osmic intracerebroventricular (ICV) pumps (ALZET) with ASOs implanted in a subcutaneous pocket that was formed on the back of the mouse. The catheter was placed in the right lateral ventricle using the following coordinates based on bregma: −0.5 mm posterior, −1.1 mm lateral (right), −2.5 mm ventral. For CSF collection, mice were placed on a heating pad and anesthetized with isoflurane. CSF was drawn through the catheter magna as described previously and immediately frozen on dry ice (Garten et al., 2011). For tissue collection, mice were anesthetized with isoflurane and perfused using chilled PBS–heparin. Brain and spinal cords were rapidly removed and then snap-frozen in liquid nitrogen and stored at −80°C or postfixed in 4% paraformaldehyde at 4°C and transferred to 30% sucrose 24 h later.

Quantitative real-time PCR: RNA analyses were performed using qRT-PCR. Total RNA was extracted from brain tissue using a Qiagen RNeasy Kit. For total RNA analyses, RNA was reverse transcribed and amplified using the EXPRESS One-Step Superscript qRT-PCR Universal Kit (Invitrogen). For Nsnm RNA levels, RNA was reverse transcribed and amplified using the Power SYBR Green RT-to-Ct 1-Step Kit (Invitrogen). The qRT-PCR were run and analyzed on the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). Total tau and Nsfn mRNA expression levels were normalized to GAPDH mRNA levels and analyzed using the ΔΔCT method for relative expression analysis. The primary probe sequences were as follows: total tau: forward 5′-GAA CGA CCA AAA TCC GGA GTG CTC GCT C3HBFQ+5′-Nsmf: forward 5′-GCATGATTCACTCCTCTTGAACAAGGAGC-3′ reverse 5′-GAACTATACTCTAATGAGGCCCTG-3′; GAPDH: forward 5′-GTC CCC CAT GT TGT GAT G-3′; reverse 5′-TGT GTG CAT GTG CCC CTC C-3′; and Probe 5′/56-FAM-AAT GCA TCG TGC ACC AAC TGC TTT 3′/HBFQ+5′ (IDT).

Tau protein analysis: Tissues were weighed and homogenized in 10X volume RABuffer containing the following (in mm): 100 MES, 1 EDTA, 0.5 MgSO4, 570 NaCl, 20 NaF, and 1 Na2VO4 supplemented with protease inhibitor (Complete Protease Inhibitor; Roche) and phosphatase inhibitor (Sigma). Homogenate was spun at 20,000 x g on a tabletop centrifuge for 10 min at 4°C. Supernatant was collected and protein concentration measured using a Pierce BCA Protein Assay Kit (Thermo Scientific). For tau protein quantification in brain, intestinal fluid (ISF), and CSF, tau concentrations were analyzed using the published Tau-BT2 sandwich ELISA (Yamada et al., 2011). Briefly, 96-well plates (Nunc) were coated with the Tau-5 antibody (Millipore) overnight at 4°C. Plates were blocked with 4% BSA for 60 min at 37°C, brain homogenate, ISF, or CSF diluted in standard buffer (0.25% BSA, 390 mM Tris, 0.65% azide, and 1X protease inhibitor in PBS) was added and incubated overnight at 4°C. For the standard curve, the longest mouse tau isoform recombinant protein was used (mTau60). The detection antibody biotinylated IF-2 (Pierce) was added the next day, followed by streptavidin-peroxydase conjugate-40 ( Fitzgerald). plates. Plates were developed using Super Slow ELISA TMB (Sigma) and read on an Epoch Microplate Spectrophotometer (BioTek).

Immunofluorescence: Brains postfixed in 4% paraformaldehyde were sliced at 50 μm on a freezing microtome. Brain slices were treated with Cita Plus antigen retrieval (BioGenex) before antibody application. Brains were incubated with the primary antibodies Tau-4 (Cell Signaling Technology) and Pan-ASO (1:1000; Invitrogen) in 1% bovine serum overnight at 4°C, followed by a 1 h incubation at room temperature with fluorescent-conjugated secondary antibodies (1:1000, DLight; Thermo Scientific). Fluorescent images were captured using the Olympus Nanozoomer 2.0-HT (Hamamatsu) and processed using the NDP viewer software (Hamamatsu).

In vivo microdialysis for ISF collection: In vivo microdialysis experiments to assess brain ISF tau levels from awake and freely moving mice were developed with modifications of our previously described method (Yamada et al., 2011). A guide cannula (Eisco Microdialysis) was stereotactically implanted in the left hippocampus under isoflurane anesthesia and cemented. After implantation of the cannula and dummy probes (Eisco Microdialysis), mice were habituated to microdialysis cages for one more day. After this recovery period, a 2 mm 1000 kDa cutoff AtmosLM microdialysis probe (Eisco Microdialysis) was inserted through the guide cannula. A probe was connected to a microdialysis peristaltic pump with two channels (MARZ6/2ScPro), which was operated in a push pull mode. The perfusion buffer, 25% human albumin solution (Gemini Bio), was diluted to 4% with rCSF containing the following (in mm): 1.3 CaCl2, 1.2 MgSO4, 3 KCl, 0.4 KH2PO4, 23 NaHCO3, and 122 NaCl, pH 7.35, on the day of use and filtered through a 0.45 μm membrane. ISF was collected at 1 μl/min in a refrigerated fraction collector (ScPro).

Catecholamine: Lactate concentrations in ISF was determined by YSI2700 biochemistry analyzer (YSI Life Sciences).

Sensorimotor battery and 1 h locomotor activity: All mice were evaluated on a battery of sensorimotor tests designed to assess balance (ledge and platform), strength (inverted screen), coordination (pole and inclined screens), and initiation of movement (walking initiation), as described previously (Wozniak et al., 2004; Grady et al., 2006). Locomotor activity was evaluated in all mice over a 1 h period using computerized photobeam instrumentation as described previously (Wozniak et al., 2004, 2007). General activity variables (total ambulations, vertical rearing), along with indices of emotionality, including time spent, distance traveled, and entries made in a 33 × 11 cm central zone, were analyzed.

Elevated plus maze. As described previously (Schafer et al., 2000), the elevated plus maze (EPM) apparatus is a four-arm maze shaped like a plus sign. One set of the opposing arms have walls (closed arms) and the other set is not enclosed (open arms). The number of entries made, time spent, and distance traveled in each set of arms were quantified using a computerized, high-resolution photoamuse (Hamilton-Kinder). These three variables were also analyzed after normalizing the values to reflect percentages calculated out of the totals measured in both sets of arms.

Morris water maze. Spatial learning and memory were evaluated in the Morris water maze using a computerized tracking system (ANY-maze, Stoelting) and procedures that were similar to previously described methods (Wozniak et al., 2004, 2007). The protocol included cued, place, and probe tests and all trials were performed in a 120-cm-diameter pool.
filled with opaque water. Cued trials were performed to identify nonassociative dysfunctions that might affect performance. This involved conducting four trials (60 s maximum) per day for two successive days with two very distinct cues being present and with the platform location being moved for each trial to limit spatial learning. Three days later, place trials were initiated to assess spatial learning; mice were required to learn the single location of a submerged (nonvisible) platform in the presence of several salient distal cues. The place trials were conducted for 5 successive days, each day consisting of 2 blocks of 2 trials (60 s maximum) separated by 2 s. Escape path length, latency, and swimming speeds were calculated for the cued and place trials. A probe trial lasting 60 s was conducted 1 h after the last trial of the place condition. This involved removing the platform and quantifying the time spent in the pool quadrant that had contained the platform (target) and each of the other quadrants and the number of crossings a mouse made over the exact location of where the platform had been (platform crossings). Spatial bias for the target quadrant was analyzed by comparing the time spent in it versus the time spent in each of the other quadrants.

Poreless seizures and EEG recording. In vivo microdialysis experiments from awake and freely moving mice were described. To record EEG activity, bipolar recording electrodes (Teflon-coated, stainless steel wire, 0.0055 inch coated OD, A-M Systems) were attached to the outside of the microdialysis guide cannula via Elmer’s Super-Fast Epoxy Resin. The electrodes extended ~1 mm beyond the tip of guide such that the tips of the electrodes would fall in the center of the 2 mm microdialysis probe membrane once inserted. The guide cannula with attached electrodes (BR-style Bioworld Analytical Systems) was stereotaxically implanted in the left hippocampus under isoflurane anesthesia and cemented. Microdialysis probes (2 mm, BR-2, 30 kDa MWCO membrane, Bioworld Analytical Systems) were inserted into the hippocampus. The perfusion buffer comprised ASF with 0.1% bovine serum albumin filtered through 0.45 μm membrane on the day of use. Once the microdialysis guide cannula and probe were placed into the left hippocampus, 12 h of basal EEG activity was measured. Using a PSI 11K A.C. preamplifier (Grass Instruments), digitized with a DigiData 1222A data acquisition system (Molecular Devices), and recorded digitally with pClamp 9.2 (Molecular Devices). PTX (Sigma-Aldrich) was diluted to the indicated concentrations in 0.15% BSA-ASF perfusion buffer and delivered at a flow rate of 1.9 μl/min, with the lowest dose given first. Each increasing dose was delivered for 60 min and EEG was measured continuously throughout drug delivery. EEG spike frequency was assessed for the last 60 min of each PTX dose and normalized to basal EEG of each mouse.

Experimental penylentetrazole (PTZ; Sigma) was dissolved in sterile PBS at a concentration of 5 mg/ml. A dose of 50 or 80 mg/kg was delivered intraperitoneally for the experiments shown in Figures 7 and 8, respectively. A quiet, isolated room was used for all seizures to minimize noise and/or visual distractions. Immediately after PTZ administration, each mouse was videotaped and 15 min later, the mouse was killed and the brain was snap-frozen for biochemical analyses. Seizures recorded on videotapes were scored in a blinded fashion for severity according to published scales (Lösch and Nolting, 1991; Racine, 1972). The seizure severity score was used as follows: 0 = normal behavior; 1 = immobility; 2 = spasm; tremor, or twich; 3 = tail extension; 4 = forelimb clonus; 5 = generalized tonic activity; 6 = jumping or running seizures; 7 = full tonic extension; and 8 = death.

Statistics. The data were analyzed for statistical significance using GraphPad Prism 5 software. Two-tailed Student’s tests were used for total tau mRNA/protein levels in multiple different brain regions. One-way ANOVA with Bonferroni post hoc analyses were used for total tau mRNA and protein expression when more than one comparison was needed and for the inverted screen task. Two-way ANOVA with Bonferroni post hoc analyses were used for the duration of action study and for ISP tau levels in multiple fractions. Two-way repeated-measures ANOVA (rmANOVA) with Bonferroni post hoc analyses were used to analyze Morris water maze, EPM trials, and PTX dose response. Specifically for the Morris water maze and EPM analyses, the Huynh-Feldt adjustment of α levels was used for all within-subjects effects containing more than two levels to protect against violations of sphericity/compound symmetry assumptions underlying rmANOVA models. The Kruskal-Wallis with Dunn’s post hoc analyses was used for the PTZ seizure severity analysis due to the categorical nature of the seizure severity scale. Linear regression analysis was used to analyze the CSF and brain tau correlations. Linear regression was used to generate the “best-fit” and 95% confidence interval lines on the graphs in Figures 6, 7, and 8, and the Spearman correlation was used to generate r and p-values for all total tau and EEG/seizure comparisons. Error bars in the figures represent SEM.
Results

Tau ASOs reduce endogenous tau mRNA and protein expression.

To determine the functional effect of reducing tau mRNA and protein in vivo, we developed an ASO that reduces endogenous tau mRNA and protein in adult mice. After screening 80 ASOs for their ability to reduce tau mRNA in murine B16-F10 cells, we selected the three most potent ASOs to screen in vivo. We infused 50 μg of each tau ASO into the right hippocampus of adult nontransgenic (NT) mice using saline and a scrambled ASO as controls. One week after ASO infusion, the hippocampus surrounding the injection site was analyzed for total tau mRNA levels (Fig. 1A). All ASOs screened in the hippocampus provided >75% reduction of tau mRNA (ANOVA, \( F_{(4,20)} = 151.4, p < 0.0001 \)). The ASO Tau\(^{ASO3} \) was then tested in a 1 month ICV Alzeto osmotic pump infusion at 100 μg/d. Compared with the saline and scrambled ASO controls, those mice treated with Tau\(^{ASO3} \) had substantially less total tau mRNA (ANOVA, \( F_{(2,16)} = 291.8, p < 0.0001 \)) and protein levels (ANOVA, \( F_{(2,13)} = 7.578, p = 0.007 \); Fig. 1B-C). To test for general off-target ASO effects, we normalized tau mRNA to total RNA input and found no

Figure 3. Total tau protein in the brain ISF is decreased. A Experimental paradigm. NT mice (n = 4-6) were treated with saline or 25 μg/d Tau\(^{ASO3} \) via intracerebroventricular infusion for 1 month and the catheters were removed. After 14 d, a guide cannula and microdialysis probe were placed in the left hippocampus. ISF was collected for 48 h. Each ISF fraction comprised a 90 min collection time. B Total brain tau protein levels in the left hippocampus were measured with R1109, confirming that brain tau was reduced. Two-tailed t test was used. C Total tau protein levels were measured in several ISF fractions. Two-way ANOVA and Bonferroni post hoc analysis was used. D) Fractions from 18-34 h were pooled for each animal to measure total tau protein levels and lactate as a control for probe function. The concentrations were calculated for the 1 μl/min flow rate. There was no significant decrease in lactate levels, demonstrating adequate probe function. Two-tailed t test was used. *p<0.05; **p<0.01; ***p<0.001. Error bars represent SEM.
The immunofluorescence results showed a qualitative decrease in tau protein levels. To quantify more precisely the amount of tau mRNA and protein reduction in the CNS, we measured total tau mRNA and protein levels in multiple CNS regions 8 weeks after implantation of 1 month ICV pumps with 25 μg/d Tau ASO-3 (Fig. 2B). In both the mRNA and protein analyses, total tau levels were decreased in the left and right brain hemispheres and the spinal cord, confirming the widespread reduction of tau expression in the adult mouse CNS (Fig. 2C,D).

**Tau protein levels are reduced in the brain ISF and CSF**

To better understand the full effects of Tau ASO-3, we examined tau protein levels in two additional CNS compartments: the brain ISF and CSF. Recently, reports have placed tau in the extracellular space under physiological conditions (Yamada et al., 2011; Pooler et al., 2013). To determine whether tau ASOs can decrease tau that is secreted into the ISF, we treated a cohort of mice with a 75 μg/d concentration of Tau ASO-3 or saline for 1 month with ICIpumps. At the end of ASO infusion, catheters and pumps were removed and microdialysis probes implanted into the left hippocampus 2 weeks later. We collected ISF for 48 h, with a new fraction being collected every 90 min (Fig. 3A). Immediately after ISF collection, the left hippocampus was dissected out and total tau levels were measured to confirm that brain tau protein was indeed reduced in the Tau ASO-3 cohort (two-tailed t test, t10 = 8.462, p < 0.0001; Fig. 3B).

Total ISF tau protein levels were steady across time in both the saline and Tau ASO-3 groups and substantially reduced in the Tau ASO-3-treated cohort (ANOV A F1,10 = 48.22, p < 0.0001; Fig. 3C), allowing multiple fractions to be combined from the same mouse. These same eight fractions were pooled for each animal and total tau protein levels were again measured. In the Tau ASO-3-treated group, ISF total tau protein levels were greatly reduced compared with the saline control (two-tailed t test, t0 = 9.283, p = 0.003). ISF lactate levels were not significantly different between the saline and Tau ASO-3 groups (two-tailed t test, t0 = 0.6169, p = 0.555). No difference in ISF lactate levels, which usually increase in response to synaptic transmission, suggests that a reduction in endogenous tau does not influence baseline neuronal activity (Bero et al., 2011).
As predicted, brain tau protein levels were decreased at both the first time point and the second (ANOVA: F(1,10) = 20.96, p < 0.0001; Fig. 4A). Interestingly, the CSF total tau was not reduced to the same extent as brain tau at the 4 week collection time, although by the 8 week time point, CSF tau was lower in the Tau ASO-3-treated mice (ANOVA: F(2,28) = 9.720, p = 0.0008; Fig. 4B). Although the reason for this lag in CSF tau reduction is unknown, it may be in part due to a slow turnover of intracellular tau protein to the CSF pool, resulting in a continued decrease in CSF tau while brain tau levels begin to increase. Total brain and CSF tau protein levels were significantly correlated, both in the brain adjacent to the catheter (linear regression R² = 0.8867, F(1,6) = 39.11, p = 0.0008; Fig. 4C) and the contralateral frontal cortex (linear regression R² = 0.657, F(1,6) = 11.49, p = 0.0147; Fig. 4D). These data, in addition to showing a reduction of extracellular tau, suggest that CSF tau levels may be an excellent predictor of brain tau levels in a Tau ASO-3 treatment paradigm.

Reducing tau mRNA and protein does not affect baseline behavior

Before assessing whether tau reduction can provide protection in experimental behavioral paradigms, we first analyzed the mice for any gross motor or cognitive behavioral abnormalities. Tau−/− mice appear normal on learning/memory tasks for up to 1 year (Roberson et al., 2007, 2011; Dawson et al., 2010; Ittner et al., 2010), with some minor parkinsonism motor phenotypes developing at 12 months of age (Lei et al., 2012; Morris et al., 2013). These largely normal behavior phenotypes, however, could be in part due to developmental compensation. We treated a cohort of NT mice with saline, 25 μg/d scrambled control ASO, or 25 μg/d Tau ASO-3 for 1 month and conducted behavioral assessments for 1.5 months after pump removal. Total tau mRNA (ANOVA: F(1,10) = 101.3, p < 0.0001) and protein (ANOVA: F(1,10) = 13.85, p = 0.0002) levels were confirmed to be reduced only in mice treated with Tau ASO-3 (Fig. 5A, B). The mice with decreased tau levels performed similarly to both the saline and scrambled ASO control groups on all seven measures of the sensorimotor battery, including the inverted screen test (ANOVA: F(1,10) = 0.116, p = 0.71; Fig. 5C), suggesting that the Tau ASO-3 mice did not have any gross sensorimotor dysfunctions. Except for a possible hyperactivity in the Tau ASO-3 group, Tau ASO-3 mice also displayed similar behavior during the 1 h locomotor activity test. The Tau ASO-3 mice did not exhibit any significant performance deficits on the place navigation test (ANOVA: F(1,10) = 0.094, p = 0.994) or probe (ANOVA: F(1,10) = 0.0001, p = 1.000) trials in the water maze (Fig. 5D, E), thus providing evidence that their spatial learning and memory were intact. Analysis of the EPM data also showed that the Tau ASO-3 did not differ in levels of anxiety-related behaviors compared with the saline and scrambled ASO control groups (percentage of open arm entries: ANOVA: F(1,10) = 0.284, p = 0.756; percentage of open arm time: ANOVA: F(1,10) = 0.134, p = 0.756; percentage of open arm distance: ANOVA: F(1,10) = 0.211, p = 0.754; Fig. 5F-H). There was also no difference between groups in regard to the total distance traveled in the EPM (ANOVA: F(1,10) = 0.639, p = 0.539). Recognizing that the sample size tested was relatively small, the data suggest that, at least in the short term, reducing tau mRNA and protein in the adult mouse does not appear to result in behavioral impairments.

Reducing tau mRNA and protein protects against chemically induced seizures

To determine whether tau reduction is protective in an induced focal seizure model, we used reverse microdialysis to deliver the noncompetitive GABAA receptor antagonist PTX (Olsen, 2006) locally into the left hippocampus of NT male mice treated with saline, 25 μg/d scrambled ASO, or 12–25 μg/d Tau ASO-3 and simultaneously recorded the EEG activity at the site of PTX delivery. Because this treatment paradigm had not been used previously to study the protective effects of tau reduction, we included a cohort of untreated NT and tau−/− mice to serve as controls. Twelve hours of baseline EEG activity were recorded, followed by continuous infusion of PTX into the left hippocampus with a stepwise increase in concentration every 90 min (4, 20, 100, and 500 μg). Untreated NT and saline-treated mice were com-
both the saline and scrambled ASO control groups (Kruskal-Wallis statistic = 16.26, \( p = 0.0003 \); Fig. 7B). Total tau mRNA (ANOVA \( F_{(3,62)} = 281.5 \), \( p < 0.0001 \)) and protein (ANOVA \( F_{(3,62)} = 45.73 \), \( p < 0.0001 \)) levels were confirmed to be reduced specifically in the Tau\(^{ASO-3}\)-treated group (Fig. 7CD). As further confirmation that the effect of Tau\(^{ASO-3}\) on seizures was secondary to tau reduction and was not an unknown effect of the ASO, we correlated the level of tau protein with seizure severity for individual animals. Indeed, seizure severity and tau protein levels correlated well in all tested mice (Spearman correlations, saline \( r_{(24)} = 0.5889 \), \( p = 0.0016 \); scrambled \( r_{(29)} = 0.6795 \), \( p = 0.0007 \); Tau\(^{ASO-3}\) \( r_{(18)} = 0.504 \), \( p = 0.0236 \); Fig. 7E-G), providing evidence in a second inducible seizure model that a reduction in tau protein is protective against seizures.

**Intrinsic variability in tau protein levels predicts susceptibility to chemically induced seizures**

Due to the variability that has been seen with PTZ seizures (Mandhane et al., 2007), we were surprised that the correlation between seizure severity and tau levels persisted even in NT mice treated with only saline (Fig. 7B). This correlation suggests that, among the NT mouse population, normal endogenous tau levels predict susceptibility to neuronal hyperexcitability. It may be, however, that a combined due to no significant difference between the groups. The tau\(^{−/−}\) mice showed a reduction in normalized spike frequency compared with the NT/saline group at the 500 \( \mu \)M PTX concentration, confirming the protective effect in tau-null mice in this new excitation paradigm (rmANOVA \( F_{(2,22)} = 8.634, p = 0.0009 \); Fig. 6A,B). The Tau\(^{ASO-3}\)-treated group also showed a strong protective effect compared with both the NT/saline and scrambled cohorts (Fig. 6A,B). Furthermore, total tau protein levels in the left hippocampus of pump–treated mice were highly correlated with normalized spike frequency at 500 \( \mu \)M PTX (Spearman correlation, \( r_{(13)} = 0.670, p = 0.0087 \); Fig. 6C). These PTX studies in adult mice support a direct correlation between lower tau protein levels and reduced neuronal hyperexcitability.

In addition to the focal increase in EEG activity, we tested the effects of tau reduction in a widely used seizure paradigm—intraperitoneal PTZ injections. PTZ seizures are considered a gold standard when testing the efficacy of anticonvulsant drugs in the early stages of development in vivo (Löschner, 2011). Three-month-old NT male mice were treated with saline, 25 \( \mu \)g/d scrambled ASO, or 25 \( \mu \)g/d Tau\(^{ASO-3}\) for 1 month and then the pumps were removed. Three weeks later, 55 mg/kg of the GABA antagonist PTZ (Macdonald and Barker, 1977) was administered to the mice by intraperitoneal injection. The mice were video-recorded for 15 min and then immediately collected for total tau analyses (Fig. 7A). Any mouse that had >50% total tau mRNA levels was eliminated from the analysis of the Tau\(^{ASO-3}\) group. Those mice treated with Tau\(^{ASO-3}\) had less severe seizures than more severe seizure results in an acute increase in brain tau protein expression. To test this possibility, we induced severe seizures in a separate cohort of untreated NT mice using a high dose of PTZ and analyzed total tau protein levels immediately afterward. There was no difference in tau protein levels in brain homogenate between those mice that underwent severe seizures secondary to PTZ injection compared with those mice that received a saline injection and did not have seizures (two-tailed \( t \) test \( t_{(13)} = 0.354, p = 0.730 \); Fig. 8A). Interestingly, in the PTZ-injected group, there was an inverse correlation between the time it took to reach a stage 8 seizure and the amount of endogenous tau protein measured. Those mice that had higher levels of endogenous tau protein progressed to severe seizures more quickly than those mice with lower tau (Spearman correlation \( r_{(27)} = -0.887, p = 0.003 \); Fig. 8B). These PTZ data show that the seizure itself does not increase tau protein acutely in brain tissues during the period of the seizure and, together with the Tau\(^{ASO-3}\) PTX and PTZ seizure data, strongly suggest that those mice with higher levels of endogenous tau are inherently more susceptible to neuronal hyperexcitability.

**Discussion**

Using ASO technology directed against endogenous murine tau, total tau mRNA and protein levels were decreased throughout the brain and spinal cord of adult NT mice (Figs. 1,2). In addition, extracellular tau in the brain ISF (Fig. 3) and CSF (Fig. 4) was also
reduced after infusion of Tau\textsuperscript{ASO-3} ASO. After tau was reduced in the adult mice, no significant deviations from baseline were observed in a battery of motor and learning/memory behavior tasks (Fig. 5), demonstrating that short-term tau knock-down is well tolerated in vivo. In the setting of chemically induced seizures, tau reduction protected against seizure severity (Figs. 6, 7), consistent with what has been reported with the genetic tau\textsuperscript{−/−} mouse model. Further, we noted a significant correlation between total tau protein levels in the brain and seizure severity both in treated mice (Figs. 6, 7) and in untreated mice (Fig. 8). These data strengthen the link between total tau expression levels and neuronal hyperexcitability regulation in vivo and demonstrate that the tau\textsuperscript{−/−} effect on neuronal hyperexcitability is likely a tau-mediated event and not a developmental phenomenon.

The tau\textsuperscript{−/−} genotype has been shown in numerous studies to be protective against excitotoxic insults (Roberson et al., 2007, 2011; Ittner et al., 2010), implicating tau in the physiological regulation of aberrant neuronal excitability. In addition, both a complete reduction and haplosufficiency of tau significantly reduced seizures and extended survival in a well-established genetic mouse model of epilepsy, Kv1.1\textsuperscript{−/−} (Glasscock et al., 2010, 2012; Holth et al., 2013). These reports, in conjunction with previous in vivo data using tau knock-down ASOs to protect cells from glutamate-induced excitotoxicity (Pizzii et al., 1999) and our own in vivo tau knock-down data in two different seizure models, support the application of a tau-lowering therapy to regulate hyperexcitability in human patients. Compounds that provide protection against PTZ-induced seizures in vivo have generally been successful in subsequent human clinical trials (Rogawski, 2006). Although many epilepsy patients respond to one or two anticonvulsants, 20–40% of patients remain untreated (Devinsky, 1999; Brodie et al., 2012). Therefore, a tau reduction approach may be an alternative therapy for this refractory population. Given the previous tau\textsuperscript{−/−} protective findings in multiple seizure paradigms (Roberson et al., 2007, 2011; Holth et al., 2013), we predict that our findings of tau knock-down using two different GABA antagonists will also apply broadly to epilepsy in vivo models and to human epilepsy.

The finding that physiological endogenous tau levels in adult mice can affect susceptibility to hyperexcitability not only lends support to the idea that reducing tau may help lower seizure severity, but also implies that endogenous tau levels in humans may influence the risk of developing seizures. Although the exact reason for variability in tau protein expression between mice is unknown, other groups have shown similar variability in marine total tau levels (Holth et al., 2013). Further, tau mRNA and protein levels in human brains can vary by 2–3-fold (Lu et al., 2004; Kattwe et al., 2008; Trabzuni et al., 2012) and CSF total tau levels in control human subjects can differ substantially (Clifford et al., 2009; Fagan et al., 2009; Oka et al., 2013), perhaps due to variability in neuronal expression rates of tau and different baseline tau levels in the brain. Higher levels of tau protein at baseline may not be detrimental, but, upon insult, increased tau expression may
predispose human patients to injury-induced seizures. It is well documented that seizures increases after different types of brain injury, including ischemic stroke (Camilo and Goldstein, 2004; Kwan, 2010) and traumatic brain injury (Angeles et al., 1998; Vesper et al., 2010). If human patients with higher baseline tau are more prone to developing seizures after an injury, being able to identify such patients through genetic studies of tau polymorphisms (Meyers et al., 2007; Kauwe et al., 2008) or tau CSF levels (Palme et al., 2009; Crucaga et al., 2015) may help to risk stratify those patients and aid in determining who would benefit from a preventative antiepileptic therapy.

Tau knock-down has also been studied extensively in the presence of Aβ-deposition and proven to be protective against a growing number of Aβ-induced insults, including cognition (Roberson et al., 2007; Andrews-Zwilling et al., 2010; Ittner et al., 2010; LeRoy et al., 2012), hyperexcitability (Roberson et al., 2007, 2011; Ittner et al., 2010; Suberbielle et al., 2013), decreased survival (Roberson et al., 2007, 2011; Ittner et al., 2010), axonal transport deficits (Vossel et al., 2010), cell cycle reentry (Severd et al., 2013), and double-stranded breaks in DNA (Suberbielle et al., 2013). Several human amyloid precursor protein mouse lines and an ApoE4 mouse line have now been generated that display abnormal EEG and increased seizure frequency (Roberson et al., 2007; Ittner et al., 2010; Vogt et al., 2011; Hunter et al., 2012). Similarly, those with familial AD mutations, ApoE4 genotype, and sporadic late-onset AD also experience an increased incidence in seizures (Takao et al., 2001; Mendez and Lim, 2003; Harden, 2004; Velez-Pardo et al., 2004; Amatniek et al., 2006; Kaufman et al., 2010). This AD-associated excitotoxicity has been implicated in the pathogenesis of the disease (Olney et al., 1997; Mattson, 2004) and has recently been linked to an earlier onset of cognitive decline in AD patients (Vossel et al., 2013).

Because of the limitations in detecting abnormal EEG activity in large populations of AD patients, we currently rely heavily on animal models for predictions regarding hyperexcitability in the context of Aβ. In the human amyloid precursor protein 120 Aβ-depositing line, treatment with the anticonvulsant levetiracetam returned the baseline aberrant excitability back to NT levels and restored cognition (Sanchez et al., 2012), similar to what was seen with the tau+/- genetic cross (Roberson et al., 2007, 2011). This rescue in cognition by means of an anticonvulsant suggests that lowering the abnormal neuronal activity alone may have a positive impact on learning and memory. A similar pilot study was performed in human mildly cognitively impaired (MCI) patients, where patients who were given either placebo or levetiracetam and then recall memory was tested using functional magnetic resonance imaging methods. Levetiracetam treatment significantly improved the recall performance of the MCI patients, again providing evidence that reducing the aberrant excitability in MCI and AD patients may help to restore cognition (Baldner et al., 2012). To test the hypothesis that decreasing aberrant hyperexcitability by means of tau reduction can in turn rescue cognitive decline, we have initiated tau-lowering ASC therapy in an Aβ-depositing mouse model of AD. We propose that tau may be involved in both AD-associated hyperexcitability and neuronal cell loss by means of tau aggregation and neurofibillary tangle formation, making tau knock-down a strong therapeutic target for AD.

The human analog of the mouse tau ASC used here may be readily applicable to human patients, ASOs against superoxide dismutase 1 that extended survival in a rat model of amyotrophic lateral sclerosis (Smith et al., 2006) recently finished a phase 1 clinical trial in human patients. The CSF-delivered ASOs demonstrated excellent safety (Miller et al., 2013). Further, ASOs against survival motor neuron protein that also rescued rodent spinal muscular atrophy models (Hua et al., 2010; Passini et al., 2011; Porensky et al., 2012) are currently being used in phase II studies for children with spinal muscular atrophy. These studies and the growing success of ASOs in preclinical models (Kumar et al., 2006; Yokota et al., 2009; Lamford et al., 2010; DeVos and Miller, 2013b) suggest that the tau reduction strategy outlined here has real potential to be translated into the clinical setting for patients with epilepsy and perhaps tauopathies such as AD, progressive supranuclear palsy, and frontotemporal dementia.

References


