

THE ROLE OF IMATINIB METHANESULFONATE SALT-MEDIATED  
REDUCTION OF HIPPOCAMPAL AMYLOID-BETA IN THE  
HYPERPHOSPHORYLATION OF TAU

By

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## ABSTRACT

For years, the prevailing hypothesis in Alzheimer's Disease (AD) has proposed a unified mechanism where deposition of amyloid-beta in the brain induces stress responses that lead to tau-pathologies and ultimately cognitive decline. However, despite extensive research on the disease, the exact mechanism of its onset remains unknown. Previous research in our lab has shown that imatinib-methanesulfonate (IM) lowers the amount of central A $\beta$  and rescues cognitive function following a bout of inflammation. The present study aims to expand our understanding of how IM affects AD pathology and further examine the link between A $\beta$  and tau. We hypothesized that prior and co-administration of IM with lipopolysaccharide (LPS) would eliminate elevations in hyperphosphorylated tau (ptau) by reducing production of A $\beta$ . Our results showed that, despite its poor ability to penetrate the blood brain barrier, peripheral treatment with IM reduces levels of central ptau even in the presence of LPS-induced inflammation. Furthermore, total tau was significantly decreased following fourteen consecutive days of IM administration. These results lead us to propose that the expression of tau following LPS administration may be a protective measure by hippocampal neurons to compensate for the loss of the microtubule-stabilizing protein due to phosphorylation.

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## INTRODUCTION

Over 5 million Americans suffer from Alzheimer's Disease (AD), a neurodegenerative disorder that results in severe cognitive and physical decline and costs upwards of \$214 billion dollars each year to treat (Alzheimer's Fact Sheet, 2014). AD is primarily characterized by the deposition and aggregation of fibrillary amyloid beta ( $A\beta$ ) into plaques and hyperphosphorylated tau (ptau) into neurofibrillary tangles (NFTs). These pathologies are typically followed by cognitive decline resulting from synapse loss and eventually neuronal cell death (Terry et al., 1991, Mattson, 2004). Traditionally,  $A\beta$  plaques have been considered the primary cause of learning and memory impairment; however, recent studies have shown soluble  $A\beta$  oligomers may be the primary neurotoxic species (Klein et al., 2001, De Felice et al., 2007). Among the pathologies correlated with high levels of soluble  $A\beta$  oligomers is an increase in the overall load of NFT's (McLean et al., 1999). These findings present a strong case for the increasingly popular hypothesis that increases in  $A\beta$  triggers a cascade of events that leads to the hyperphosphorylation of tau, linking the primary markers of AD in a singular pathway.

Alzheimer's Disease is also a neurodegenerative tauopathy, a class of diseases characterized by the aggregation of filamentous tau in the brain (Lee et al., 2001). Tau is a microtubule-stabilizing protein that helps regulate axonal transport of molecules and organelles necessary for cell function. Recently, additional roles for tau in DNA stabilization and synaptic function have been proposed (Noble et al., 2013) implicating tau as a critical component in neuronal function. When tau is hyperphosphorylated it detaches from microtubules. As a result, microtubules cannot function properly within the cell (Alonso et al., 1994). These monomeric ptau pieces aggregate to form soluble

oligomers that can be further phosphorylated and ultimately produce paired helical filaments, which are the primary component of NFTs (Wischik et al., 2015). Even in the oligomer stage, ptau is capable of causing toxic effects within the central nervous system (CNS) including synaptic dysfunction, disruptions in calcium homeostasis, and even neuronal death (Yoshiyama et al., 2007, Kopeikina et al., 2012). While NFTs have traditionally been viewed as damaging to the brain (Stancu et al., 2014), recent findings have pointed to the possibility that they may serve a protective function in sequestering the aforementioned tau oligomers (for review, see Spires-Jones et al., 2011). The correlation between NFT's and the progression of tauopathies may be a result of their protective capacity being surpassed, rendering the brain incapable of dealing with the increasing volume of ptau and allowing subsequent neurodegeneration (Kopeikina et al., 2012). Together, these findings implicate tau as a potential target for the treatment of AD and other tauopathies.

There has been considerable evidence supporting the connection of A $\beta$  and ptau in neurodegenerative pathology (Stancu et al., 2014). When directly applied to neurons, both fibrillar and oligomeric A $\beta$  result in a marked increase in tau phosphorylation (Busciglio et al., 1995, De Felice et al., 2008). When those neurons were subsequently treated with phosphatases, tau's ability to bind to microtubules was restored, further supporting the hypothesis that A $\beta$  contributes to the dysregulation of tau (Busciglio et al., 1995). Additionally, A $\beta$  is known to activate multiple kinases linked with the regulation of tau phosphorylation, including cyclin-dependent kinase 5 (cdk5) (Cancino et al., 2011, Noble et al., 2013). Given these findings, it is possible that A $\beta$  may act as the initiator of AD pathology by binding to neuronal receptors and activating a signaling cascade that

ultimately results in the hyperphosphorylation of tau and the ensuing cognitive decline (De Felice et al., 2008).

One way to investigate the role A $\beta$  plays in tau pathology is through inflammation, which is believed to be critical in the progression of AD (Lee et al., 2008). Previous work from our laboratory, and the work of others, has shown that when inflammation is induced via the peripheral administration of lipopolysaccharide (LPS), there is a significant increase in the levels of A $\beta$  and ptau alongside deficits in learning and memory (Lee et al., 2008, Kitazawa et al., 2005, Kahn et al., 2012). In an effort to further explore how inflammation-induced A $\beta$  is responsible for cognitive defects, subsequent studies targeted  $\gamma$ -secretase as a method to disrupt A $\beta$  production. Imatinib methanesulfonate salt (IM), the active component of the anti-cancer drug Gleevec, is known to interfere with the interaction between  $\gamma$ -secretase and  $\gamma$ -secretase activating protein (GSAP), which leads to decreased production of A $\beta$  (He et al., 2010, Sutcliffe et al., 2011). We have previously demonstrated that treatment with IM blocks LPS-induced increases in A $\beta$  within the hippocampus and leads to restored cognition in a hippocampus-dependent task (Weintraub et al., 2013). IM primarily acts as an Abl-specific tyrosine kinase inhibitor. Abl-kinase has been previously shown to be activated by A $\beta$  and can participate in tau pathology via the activation of cdk5 (Cancino et al., 2011), a known tau kinase at multiple AD-associated sites (Wang et al., 2009). Furthermore, activation of the cdk5 pathway is believed to be responsible for LPS-induced hyperphosphorylation of tau (Kitazawa et al., 2005).

In the present study, we tested the hypothesis that a reduction in A $\beta$  following peripheral IM co-administered with LPS would result in a decrease in levels of ptau

within the hippocampus. Due to IM's poor ability to penetrate the BBB, a decrease in ptau levels would lend strong support for a unified pathway whereby peripherally produced A $\beta$  initiates a signaling cascade that leads to elevated levels of ptau and ultimately, increased neurodegeneration in the AD brain.

## METHODS

### *Subjects*

Male C57BL/6J mice between the ages of 4 and 6 months, bred in the Texas Christian University vivarium from a stock obtained from The Jackson Laboratory (Bar Harbor, ME) were used in all experiments. All animals were treated in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of TCU and in accordance with the guidelines described by the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010). Animals were housed in 12.5cm x 15cm x 25cm polycarbonate cages. All experimental groups were subjected to the same 12-hour light/dark cycle with lights on at 06:00 and off at 18:00. Food and water were available continuously.

### *Experimental Design*

Injections were administered intraperitoneally (i.p.) over a two-week period. Subjects were assigned either twice-daily injections of 200  $\mu$ L saline or 20mg/kg IM throughout the 14-day period. The IM dose was chosen based on previous studies (Sutcliffe et al., 2011) and has been successfully used without inducing toxic effects in previously published work from our lab (Weintraub et al. 2012). On day 8 an additional daily injection of either saline or LPS (*Escherichia coli* serotype: 055:B5; Sigma-

Aldrich, St. Louis, MO) at a dose of 250  $\mu\text{g}/\text{kg}$  was administered for the remaining 7 days. There were a total of four treatment groups: saline/saline, IM/saline, saline/LPS, and IM/LPS (Figure 1).

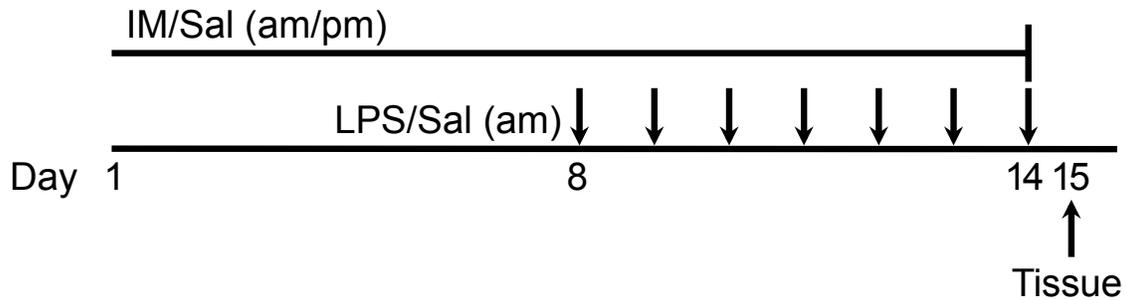


Figure 1. Experimental Timeline. Animals were injected with either i.p. Saline or IM twice daily (am/pm) for 14 days. On day 8 an additional injection of either LPS or Saline was given once daily for the remaining seven days. On day 15 hippocampal tissue was collected.

### *Tissue Preparation*

24 hours after the final injection, subjects were euthanized via  $\text{CO}_2$  inhalation. Hippocampal tissue was collected and homogenized with cell lysis buffer (PRO-PREP Boca Scientific, Boca Raton, FL), containing protease inhibitors. Samples were stored at  $-80^\circ\text{C}$  until processing. Samples were centrifuged at 13,000 rpm for 30 minutes, and the purified lysate was removed. Protein concentrations were quantified using a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

### *Western Blotting*

Samples were diluted to a concentration 0.5 $\mu\text{g}/\mu\text{l}$  and boiled at  $100^\circ\text{C}$  for 5 minutes. 15  $\mu\text{l}$  of each lysate was electrophoresed across a 10% sodium dodecyl sulfate polyacrylamide and transferred onto a polyvinylidene fluoride membrane (Millipore,

Millerica, MA). Membranes were blocked for 2 hours at room temperature with 5% BSA in 1% TBST. Primary antibody was added and membranes were incubated overnight at 4°C. Immunoblots were probed with three antibodies: anti-Tau (C-17), anti-pTau (Ser235), and loading control anti-β-actin polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were rinsed with TBST and incubated in species-specific peroxidase-conjugated AffiniPure secondary antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) for two hours followed by an additional wash with TBST. Detection reagent (SuperSignal West Pico Chemiluminescent substrate, Thermo Scientific, Waltham, MA) was applied and blots were imaged using a Syngene G:Box (Syngene, Frederick, MD). Images were quantitatively analyzed using the Syngene GeneTools software version 4.03.05.0 and reported as arbitrary optical Densitometry Units (DU).

### *Statistical Analyses*

In all experiments, the band intensities (DU) were analyzed using analysis of variance (ANOVA) procedures (Statview 5.0, SAS, Cary, NC), in which Group (IM/SAL) and Treatment (LPS/SAL) were independent variables. The alpha level that was used for all statistical analyses will be 0.05. Any significant main effects and interactions were further analyzed by Fisher's PLSD post-hoc comparisons.

## RESULTS

### *IM treatment does not block LPS-induced weight loss*

We used a 2x2 analysis of variance (ANOVA) to ensure that IM treatment does not block LPS-induced weight loss. As expected, there was no interaction effect between

treatment (LPS or Saline) and condition (IM or Saline)  $F(1, 16): 1.39, ns$ . Furthermore, during the week of LPS administration there was a main effect for treatment whereby animals that received LPS lost significantly more weight than animals that received saline independent of previous IM or saline exposure ( $F(1, 16): 48.08, p < 0.01$ ; Figure 2). No main effect for condition (IM or Saline) was observed ( $F(1, 16): 2.20, ns$ ). Overall, this indicates that IM treatment had no effect on weight loss, but administration of LPS does.

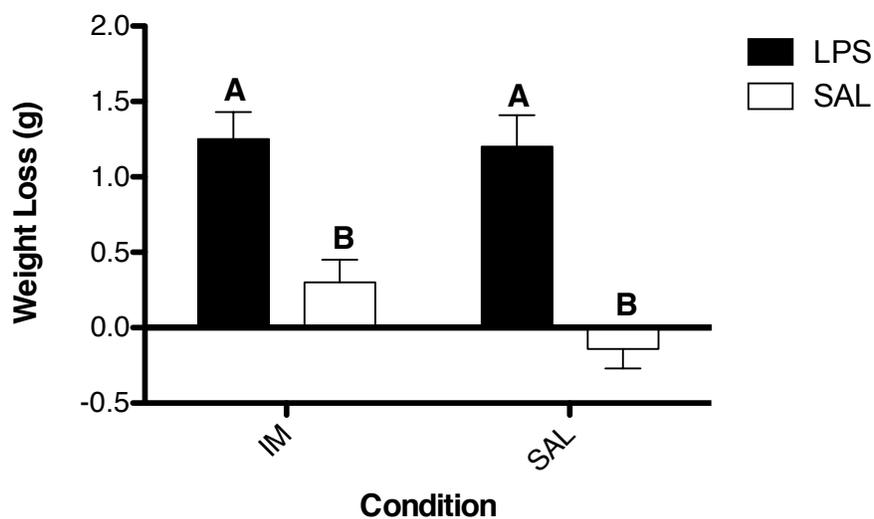


Figure 2. Max weight loss during the 7 days of LPS and saline injections indicate that IM does not block the effects of LPS. Different letters (A,B) represent significant differences ( $p < 0.05$ ) in weight loss. Bars represent mean  $\pm$  SEM.

#### *IM treatment reduces central tau phosphorylation following LPS exposure*

In order to assess if IM affects tau pathology, hippocampal lysates were probed to measure concentrations of ptau. As expected, IM-administration significantly lowered levels of ptau in both LPS and saline treated mice (Figure 3A). An interaction effect was observed between treatment (LPS or Saline) and condition (IM or Saline) ( $F(1,16): 92.03, p < 0.01$ ). Further analysis of pairwise comparisons revealed that ptau levels were

significantly higher in animals that were administered Saline and then LPS versus animals that received only saline ( $p < 0.01$ ). Fisher's PLSD post-hoc analysis demonstrated that animals pre-treated with IM had significantly lower levels of ptau ( $p < 0.01$ ) compared to animals pre-treated with saline across both treatment (LPS or Saline) groups (Figure 3B). These results indicate that IM effectively reduces levels of both LPS-induced and baseline hyperphosphorylation of tau.

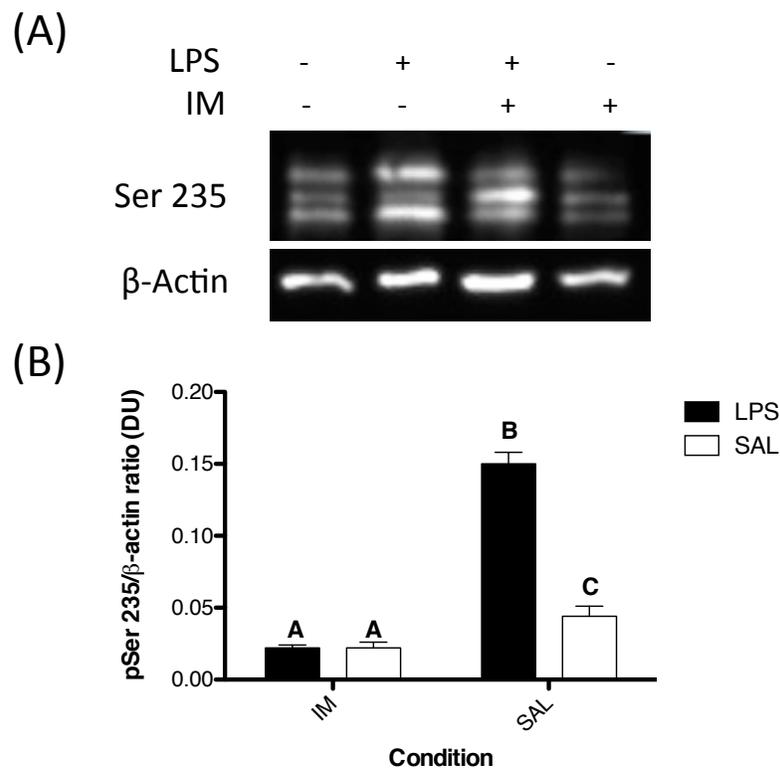


Figure 3. Co-administration of IM prevents LPS-induced tau hyperphosphorylation. (A) Western blot analysis of hippocampal lysates, antibodies to pSer 235 and  $\beta$ -actin were used. (B) Baseline and LPS-induced expression of central ptau are blocked with prior (7 days) and co-administration (7 days) of IM. Different letters represent significant differences ( $p < 0.05$ ) in weight loss. Bars represent mean  $\pm$  SEM. DU = densitometry units.

*IM treatment reduces levels of total tau in the brain following LPS exposure*

We assessed levels of total tau in the brain to observe whether LPS and IM had any affect on overall expression levels of this protein. An interaction effect was observed between treatment (LPS or Saline) and condition (IM or Saline) ( $F(1,16): 64.75, p < 0.01$ ). Unexpectedly, we found that in animals pre-treated with saline, the administration of 7 consecutive days of LPS led to significantly elevated central total tau ( $p < 0.01$ ) (Figure 4A). Post-hoc analysis demonstrated that IM significantly lowered levels of total tau in both the LPS ( $p < 0.01$ ) and the saline ( $p < 0.01$ ) treated animals. On the other hand, within the IM condition, no significant difference in total tau levels were observed between LPS and saline treatment groups (Figure 4B). These results suggest that administration of IM reduces baseline levels of tau and attenuates LPS-induced elevation of the protein.

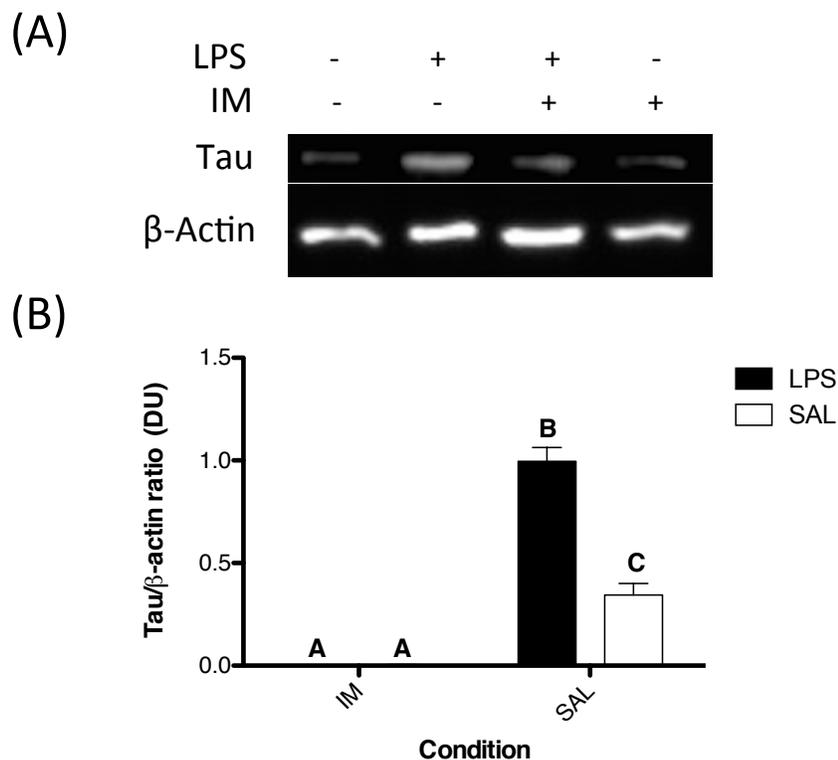


Figure 4. IM reduces overall levels of tau. (A) Western blot analysis of hippocampal lysates probed with total tau (C17) and  $\beta$ -actin antibodies. (B) The graph indicates that tau is significantly elevated following 7 days of LPS treatment ( $p < 0.01$ ). IM blocks LPS-induced and baseline expression of tau. Different letters represent significant differences ( $p < 0.05$ ) in weight loss. Bars represent mean  $\pm$  SEM. DU = densitometry units.

## DISCUSSION

Currently, a popular theory within the field of Alzheimer's research proposes that deposition of  $A\beta$  in the brain initiates a cascade of events that eventually lead to onset of the disease (Karran et al., 2011). Increasingly, evidence suggests that while  $A\beta$  may be the initiator of the disease, it is tau that is responsible for neurotoxicity (For review see Stancu et al., 2014). For example, a reduction in the amount of endogenous tau has been shown to be protective against  $A\beta$ -dependent deficits in cognition (Roberson et al., 2007). Collectively, these findings indicate that  $A\beta$  and tau interact as critical components in the onset of AD pathology.

Previous results from our lab have shown that inflammation caused by seven consecutive days of peripheral LPS administration causes significant increases of both  $A\beta$  (Kahn et al., 2012) and ptau (unpublished data). More recently, we have shown that peripheral IM treatment blocked  $A\beta$  elevation and rescued cognition following a brief bout of inflammation (Weintraub et al., 2013). In order to assess if IM would also have an inhibitory effect on the development of tau pathology, we replicated the methodology of the aforementioned study, but, instead of examining the affects on  $A\beta$ , we observed the compound's effects on ptau accumulation. Our results demonstrate that IM does block LPS-induced elevations in ptau along with lowering the baseline levels of the protein. Due to the compounds poor ability to penetrate the BBB, we argue these results

suggest that IM does not directly affect the phosphorylation of tau within the CNS, but acts indirectly through the inhibition of APP cleavage in the periphery, reducing the amount of A $\beta$  available to initiate central tau pathologies. Alternatively, studies have shown that repeated injections of LPS can compromise the BBB (Xaio et al., 2001), which would then allow IM to act directly within the CNS to decrease tau phosphorylation through Abl-specific tyrosine kinase inhibition. While this mechanism may have the same net result, we do not find that it adequately describes what occurred in the present study. Previous research has shown that when IM is co-administered with LPS, it leads to the inhibited production of peripheral A $\beta$  suggesting that it is the decreased A $\beta$  that is mediating the relationship between IM and ptau (Weintraub et al., 2013).

In addition to inhibiting the buildup of ptau, IM also lowered overall total tau levels in the brain. We observed elevations of total tau in the animals that were pretreated with saline and then administered LPS versus the animals treated with IM and LPS. These findings are consistent with previous research in which absolute levels of tau were elevated after fibrillar A $\beta$  was applied to cultured hippocampal neurons. (Busciglio et al., 1995). We propose that the A $\beta$ -induced phosphorylation of tau seen here can actually trigger an increased expression of the protein. A $\beta$  accumulation is known to be a causative factor in cellular stress responses that initiate tau pathology (Yan et al., 2000). As ptau accumulates, decreasing the availability of healthy tau capable of stabilizing microtubule function, it is possible that neurons increase the expression of the *MAPT* gene responsible for producing functional tau as a protective response. This compensatory mechanism in response to aberrant protein folding has also been found in

relation to LPS and HSP70. Specifically, pilot studies from our lab revealed seven days of consecutive LPS administration significantly increased the expression of HSP70, a protein that is implicated in the correction and destruction of ptau. In some studies, A $\beta$  has been shown to depress the levels of un-phosphorylated tau (Cancino et al., 2011). However, it should be noted that the cells utilized in this experiment were lysed after only 60 minutes of exposure to A $\beta$ , likely resulting in insufficient time to initiate increased gene expression.

In our study, A $\beta$  buildup occurred over the course of seven days, possibly allowing cells sufficient time to respond to the tauopathy by making new proteins in what we propose is a long-term response to the hyperphosphorylation of tau. If the downstream effect of centrally accumulated A $\beta$  is an increase in the expression of tau, as seen in this experiment, then it would make sense that treatment with IM, which reduces A $\beta$  levels in the brain, would also result in lower absolute levels of tau as it would reduce the need for the cell to compensate by up-regulating the amount of healthy tau produced.

Overall, these results, along with our previously reported data (Weintraub et al., 2013), support the link between the parallel increases in A $\beta$  and ptau following LPS-induced inflammation. Our observations demonstrate treatment with IM, known to inhibit the cleavage of APP into pathogenic A $\beta$  peptides, blocks tau pathology in the brain. They also suggest that neurons may increase expression of tau as a protective response to A $\beta$ , indicating a need for future studies to delineate the exact mechanism by which this may work.

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