LRP1 and p75 Neurotrophin Receptor Collaborate to Trigger Pro-inflammatory Cell-signaling in Response to Extracellular Tau

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Abstract

In Alzheimer's Disease (AD) and other neurodegenerative diseases, microtubule-associated protein Tau forms abnormal intracellular aggregates. Mechanisms by which Tau may promote AD progression remain incompletely understood. Low Density Lipoprotein Receptor-related Protein-1 (LRP1) mediates the uptake of Tau, which is released into the extracellular spaces in the brain and may thereby promote seeding of Tau aggregates in new cells. Herein, we demonstrate that in macrophages, microglia, and astrocytes, extracellular Tau induces an LRP1-dependent pro-inflammatory response, characterized by NFκB activation and expression of diverse pro-inflammatory cytokines. Unlike other LRP1 ligands that elicit LRP1-dependent cell-signaling events, the response to Tau occurs independently of the NMDA Receptor. Instead, Tau-activated cell-signaling requires the low affinity Neurotrophin Receptor ($p75^{NTR}$). The role of $p75^{NTR}$ in Tau-elicited cell-signaling was demonstrated by gene-silencing and/or with TAT-Pep5, in macrophages, astrocytes, and PC12 cells. Because RhoA is activated downstream of $p75^{NTR}$, we studied two Rho kinase pharmacologic inhibitors, Y-27632 and Fasudil Hydrochloride, and demonstrated that both reagents block NFκB activation and cytokine expression in response to Tau. These results define Tau and its receptor assembly, which includes LRP1 and $p75^{NTR}$, as a novel biochemical system that may regulate neuro-inflammation in AD and other neurodegenerative diseases. The ability of Rho kinase inhibitors to antagonize the Tau-LRP1/$p75^{NTR}$ pathway may represent a novel mechanism by which these agents demonstrate efficacy in Alzheimer's Disease.

Key words: Tau, LRP1, p75 neurotrophin receptor, neuro-inflammation, Rho kinase

46 ## Significance Statement

47 In Alzheimer's Disease and other neurodegenerative diseases, microtubule-associated protein
48 Tau forms abnormal intracellular aggregates that contribute to disease progression. When Tau
49 is released by cells, it binds to the transmembrane receptor, LRP1, which is expressed by diverse
50 cells in the CNS. LRP1 has a unique ability to couple endocytosis with activation of cell-signaling.
51 We demonstrated that Tau-binding to LRP1 activates pro-inflammatory responses in
52 macrophages, microglia, and astrocytes. p75 Neurotrophin Receptor served as an essential co-
53 receptor. Targeting Rho kinase, downstream of $p75^{NTR}$, blocked Tau-initiated pro-inflammatory
54 responses. These results define a novel pathway by which Tau may regulate neuro-inflammation
55 in Alzheimer's Disease and other neurodegenerative diseases.

Introduction

Microtubule-associated protein Tau is an intracellular protein that forms abnormal oligomers and fibrils in Alzheimer's Disease (AD) and in neurodegenerative diseases called Tauopathies (Lee et al., 2001; Congdon and Sigurdsson, 2018; Gao et al., 2018). Tau aggregation is regulated by gene polymorphisms, alternative splicing, phosphorylation, and other post-translational modifications. The relationship between Tau aggregation and the pathological changes that occur in AD remains incompletely understood; Tau may contribute to AD progression in the absence of detectable aggregates (Congdon and Sigurdsson, 2018; Gao et al., 2018).

Tau is released by neurons and other cells in the CNS, in extracellular vesicles (EVs) and independently of EVs, allowing for transfer of Tau between cells (Saman et al., 2012; Pooler et al., 2013; Asai et al., 2015; Evans et al., 2018; Merezhko et al., 2018; Brunello et al., 2020). Because Tau transfer between cells in the CNS may play a role in AD progression, identification of Low Density Lipoprotein Receptor-related Protein-1 (LRP1) as an endocytic receptor for extracellular Tau is highly significant (Rauch et al., 2020; Cooper et al., 2021).

LRP1 is a 600-kDa, type 1 transmembrane receptor for over 100 structurally diverse ligands, including apolipoprotein E, α_2-macroglobulin (α_2M), shed non-pathogenic cellular prion protein (S-PrP^C), and proteins released from injured and dying cells (Strickland et al., 2002; Fernandez-Castaneda et al., 2013; Gonias and Campana, 2014; Mantuano et al., 2020). LRP1 ligands are internalized in clathrin-coated pits and usually transferred to lysosomes for degradation. A subset of LRP1 ligands trigger cell-signaling via mechanisms that require co-receptors, such as the NMDA Receptor (NMDA-R), Trk, p75^{NTR}, and/or PrP^C (Backsai et al., 2000; May et al., 2004; Martin et al., 2008; Shi et al., 2009; Mantuano et al., 2013; Stiles et al., 2013; Gonias and Campana, 2014; Krogh et al., 2014; Mattei et al., 2020). Assembly of different LRP1 co-receptors in response to different ligands may explain why the cell-signaling responses elicited by various LRP1 ligands are non-uniform (Gonias and Campana, 2014). LRP1 is expressed by neurons,

81 astrocytes, oligodendrocytes, and microglia in the CNS and by macrophages throughout the body
82 (Gonias and Campana, 2014; Auderset et al., 2016).

83 Binding of Tau to LRP1 is mediated by the Tau microtubule-binding domain (Rauch et al.,
84 2020; Cooper et al., 2021). The Tau alternative splicing variant with four microtubule-binding
85 repeats (2N4R) binds to LRP1 with higher affinity than the variant with three. Tau phosphorylation
86 events, which exacerbate Tau aggregation (Alonso et al., 2001; Augustinack et al., 2002),
87 apparently decrease LRP1-binding affinity (Cooper et al., 2021). Thus, LRP1-mediated uptake
88 of extracellular Tau may be a physiologic process; however, Tau in extracts from Alzheimer's
89 Disease brains partially escapes lysosomal degradation, seeding new intracellular aggregates
90 (Rauch et al., 2020; Cooper et al., 2021).

91 Specific LRP1 ligands, including tissue-type plasminogen activator (tPA), α_2M, and S-PrP^C
92 activate anti-inflammatory cell-signaling responses in macrophages by a pathway that requires
93 the NMDA-R (Mantuano et al., 2017, 2022a). Determining whether Tau initiates LRP1-activated
94 cell-signaling is an important question. There is considerable evidence that Tau accumulation in
95 the CNS is pro-inflammatory (Yoshiyama et al., 2007; Nilson et al., 2017). In P301S-Tau
96 transgenic mice, which express a mutated form of human Tau that forms intracellular filamentous
97 lesions, microglial activation and neuro-inflammation precede formation of detectable Tau
98 aggregates (Yoshiyama et al., 2007). Anti-inflammatory treatment blocks disease progression in
99 P301S-Tau transgenic mice. Although activated microglia demonstrate some activities that are
100 beneficial in AD, excessive microglial activation and chronic neuro-inflammation may promote AD
101 progression (Akiyama et al., 2000; Heneka et al., 2015; Hong et al., 2016; Ransohoff, 2016).

102 In this investigation, we studied the 2N4R variant of Tau. Our results show that extracellular
103 Tau elicits pro-inflammatory responses in macrophages, microglia, and astrocytes. These
104 responses are entirely dependent on LRP1. Unlike anti-inflammatory LRP1 ligands, the cell-
105 signaling pathway triggered by Tau did not require the NMDA-R. Instead, p75 Neurotrophin
106 Receptor (p75^{NTR}) functioned in concert with LRP1 to induce expression of pro-inflammatory

107 mediators in Tau-treated cells. Because Rho is activated downstream of $p75^{NTR}$ (Yamashita et
108 al., 1999), we tested Rho-associated kinase (ROCK) inhibitors and showed that these reagents
109 inhibit Tau-mediated release of pro-inflammatory cytokines. We conclude that Tau, LRP1, and
110 $p75^{NTR}$ constitute a novel system for regulating neuro-inflammation in AD and other
111 neurodegenerative diseases.

112 ## Materials and Methods

113 *Proteins and reagents*

114 Recombinant Human 2N4R Tau was from R&D Systems. Lipopolysaccharide (LPS) serotype
115 055:B5 from *E. coli* was from Sigma-Aldrich. The NMDA-R inhibitors, dizocilpine (MK-801) and
116 dextromethorphan hydrobromide (DXM), were from Cayman Chemicals and Abcam, respectively.
117 Y-27632 was from Cell Signaling Technology. Fasudil Hydrochloride was from Sigma-Aldrich.
118 The $p75^{NTR}$-specific cell-signaling inhibitor, TAT-Pep5, was from Sigma-Aldrich. α_2M was purified
119 from human plasma and activated for binding to LRP1 by reaction with methylamine, as previously
120 described (Imber and Pizzo, 1981). Recombinant S-PrP^C (residues 23-231 from mouse PrP^C)
121 was expressed and purified as previously described (Mantuano et al., 2020) and kindly provided
122 by Dr. Christina Sigurdson (University of California San Diego, La Jolla, CA, USA). All purified
123 protein preparations were cycled through high-capacity endotoxin removal columns and
124 subjected to experiments, as described by Brifault et al. (2017), to confirm that endotoxin was not
125 present.

126

127 *Experimental mice*

128 Wild-type (WT) C57BL/6J mice were obtained from Jackson Laboratory. Mice in which
129 macrophages are LRP1-deficient were obtained by breeding *Lrp1*$^{flox/flox}$ mice with mice express
130 Cre recombinase under the control of the lysozyme-M (LysM-Cre) promoter, in the C57BL/6J
131 background, as previously described (Overton et al., 2007; Staudt et al., 2013; Mantuano et al.,
132 2016). To generate mice in which macrophages are deficient in the essential NMDA-R GluN1
133 subunit, *Grin1*$^{flox/flox}$ mice were bred with mice that express Cre recombinase under the control of
134 the LysM-Cre promoter in the C57BL/6J background (Mantuano et al., 2023). All research
135 protocols involving mice were approved by the University of California San Diego Institutional
136 Animal Care and Use Committee.

137

138 *Cell culture model systems*

139 Bone-marrow-derived macrophages (BMDMs) were harvested from 16-week-old mice, as
140 previously described (Mantuano et al., 2016). Briefly, bone marrow cells were flushed from
141 mouse femurs, plated in non-tissue culture-treated dishes, and cultured in DMEM/F-12 medium
142 containing 10% FBS and 20 ng/mL mouse macrophage colony-stimulating factor (BioLegend) for
143 7 days. Non-adherent cells were removed by washing. Adherent cells included >95% BMDMs,
144 as determined by F4/80 and CD11b immunoreactivity.

145 Microglia were isolated from C57BL/6J mouse pups, as previously described (Ni and
146 Aschner, 2010; Pontecorvi et al., 2019). In brief, brains were harvested from postnatal day 1–6
147 mice. The cortices were dissected from the forebrain, and the surrounding meninges were
148 removed. Intact cortices were mechanically and enzymatically dissociated using the Neural
149 Tissue Dissociation Kit (Miltenyi Biotec). Mixed glial cell cultures were established in DMEM/F-
150 12 supplemented with GlutaMAX, 10% FBS, and 1× Gibco Antibiotic-Antimycotic (Thermo Fisher
151 Scientific). After culturing for 10-14 days, microglia were dissociated by shaking the mixed
152 cultures at 200 rpm for 30 min at 37°C. The floating cells were collected by centrifugation (5 min,
153 600×*g*) and re-plated at 3×10^5 cells/well. Oligodendrocytes were removed by an additional 6 h of
154 shaking. The remaining cells, which included mainly astrocytes, were collected by trypsinization
155 and re-plated at 4×10^5 cells/well. Experiments were performed within 24 h of completing the
156 isolation procedure for microglia and within 48 h of completing the isolation procedure for
157 astrocytes. Microglial cell culture purity was >95% homogeneous as determined by immuno-
158 fluorescence (IF) microscopy for Iba1 (positive), glial fibrillary acidic protein (GFAP) (negative),
159 and OLIG1 (negative). Astrocyte cultures were >95% GFAP-immunopositive.

160 Rat PC12 cells were from the ATCC (CRL-1721) and subjected to quality control tests by the
161 ATCC. PC12 cells were cultured in DMEM, high glucose containing 10% heat-inactivated FBS,
162 5% heat-inactivated horse serum (Thermo Fisher Scientific), in plates that were pre-coated with

163 0.01 mg/mL type IV collagen (Sigma-Aldrich). PC12 cells were passaged no more than eight
164 times.
165
166 *Gene silencing*
167 Microglia were transfected with 10 nm Silencer Select Predesigned *Lrp1* (siRNA ID:s69313), or
168 Silencer Select Negative Control No. 1 siRNA (Ambion), using Lipofectamine RNAiMAX
169 (Invitrogen, Thermo Fisher Scientific). Experiments were performed 24 h after transfection. Gene
170 silencing was confirmed through immunoblot analysis using an anti-LRP1 specific primary
171 antibody (Abcam, catalog #ab92544).
172 PC12 cells were transfected with 300 nM siRNA by electroporation using the Cell Line
173 Nucleofector Kit V (Lonza), following the manufacturer's instructions. Rat-specific ON-
174 TARGETplus SMARTpool siRNA, targeting *Ngfr*, and pooled non-targeting control (NTC) siRNA
175 were from Horizon Discovery. Experiments were performed 48 h after transfection. Gene
176 silencing was confirmed through immunoblot analysis using an anti-NGFR specific primary
177 antibody (Invitrogen, Thermo Fisher Scientific, catalog #MA5-31968).
178
179 *mRNA expression studies*
180 BMDMs, microglia, and astrocytes were transferred to serum-free medium (SFM) for 1 h. Some
181 cultures were pre-treated with MK-801 (1 μM), DXM (10 μM), Y-27632 (10 μM), Fasudil (10 μM)
182 or TAT-Pep5 (0.5 μM) for the final 30 min of the 1 h pre-incubation. The cells then were treated
183 for 6 h with Tau, at the indicated concentrations, LPS (0.1 μg/ml), or vehicle (20 mM sodium
184 phosphate, 150 mM NaCl, pH 7.4, PBS) in the presence of inhibitors, as indicated. RNA was
185 isolated using the NucleoSpin RNA kit (Macherey-Nagel) and reverse-transcribed using the
186 iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using TaqMan gene expression
187 products (Thermo Fisher Scientific). Primer-probe sets were as follows: *Gapdh*
188 (Mm99999915_g1); *Tnf* (Mm00443258_m1); *Il6* (Mm00446190_m1); *Ccl3* (Mm00441259_g1);

189 and *Cxcl2* (Mm00436450_m1). The relative change in mRNA expression was calculated using
190 the $2^{\Delta\Delta Ct}$ method with *Gapdh* mRNA as a normalizer.
191
192 *Cell signaling/immunoblotting experiments*
193 BMDMs, microglia, and astrocytes were transferred to SFM for 1 h and then treated for 1 h with
194 Tau or vehicle as noted. Some cultures were pre-treated with MK-801 (1 μM), DXM (10 μM), Y-
195 27632 (10 μM), Fasudil (10 μM) or TAT-Pep5 (0.5 μM) for the final 30 min of the 1 h pre-
196 incubation, as noted. PC12 cells were cultured in serum-containing medium until ~70%
197 confluent. The cells were then transferred into SFM for 2 h before treatment with various
198 reagents.
199 Extracts of BMDMs, microglia, astrocytes, and PC12 cells were prepared in RIPA buffer
200 (Sigma-Aldrich) supplemented with protease inhibitors and phosphatase inhibitors (Thermo
201 Fisher Scientific). Equal amounts of cellular protein were subjected to SDS-PAGE and electro-
202 transferred to PVDF membranes. The membranes were blocked with 5% nonfat dried milk and
203 then incubated with primary antibodies from Cell Signaling Technology that recognize: phospho-
204 ERK1/2 (catalog #9102); total ERK1/2 (catalog #4370); phospho-IκBα (catalog #2859); total IκBα
205 (catalog #9242); and β-actin (catalog #3700). The membranes were washed and incubated with
206 horseradish peroxidase-conjugated secondary antibody from Jackson ImmunoResearch (anti-
207 rabbit: catalog #111-035-003; anti-mouse: catalog #115-035-003). Immunoblots were developed
208 using Thermo Scientific SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo
209 Fisher Scientific) and imaged using the Azure Biosystems c300 digital system. Image
210 processing/densitometry was performed using Adobe Photoshop 24.7. When immunoblots were
211 re-probed, phospho-IκBα was detected first, followed by total IκBα, then p-ERK, total ERK1/2,
212 and finally, β-actin. Presented results are representative of at least three independent
213 experiments.
214

215 *Proteome profiler mouse cytokine arrays*

216 BMDMs and microglia were transferred to SFM for 30 min and then treated with Tau or vehicle
217 for 6 h, as indicated. Conditioned medium (CM) was collected and clarified by centrifugation. An
218 equivalent amount of CM (1.0 ml for each condition) was incubated with each nitrocellulose
219 membrane provided in the Proteome Profiler Mouse XL Cytokine Array Kit (R&D Systems).
220 Membranes were developed following the instructions of the manufacturer.

221

222 *Statistical analysis*

223 Statistical analysis was performed using GraphPad Prism 9.5.1. All results are expressed as the
224 mean ± SEM. Each replicate was performed using a different BMDM, microglia, astrocyte, or
225 PC12 cell preparation. Comparisons between two groups were performed using two-tailed
226 unpaired t-tests. To compare more than two groups, we performed a one-way ANOVA followed
227 by post-hoc Dunnett's multiple comparison test. When evaluating the effects of two independent
228 factors, we performed a two-way ANOVA followed by Šidák's multiple comparisons test. *p*-values
229 of *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ were considered statistically significant.

230 ## Results

231 **Tau elicits an LRP1-dependent pro-inflammatory response in macrophages**

232 BMDMs were treated with increasing concentrations of Tau for 6 h. RNA was harvested and RT-
233 qPCR was performed to examine mRNA levels for the genes encoding various cytokines. Tau,
234 at concentrations greater than or equal to 5 nM, significantly increased expression of the mRNAs
235 encoding TNFα (*Tnf*), interleukin-6/IL-6 (*Il6*), CCL3/MIP-1α (*Ccl3*), and CXCL2/ MIP-2α (*Cxcl2*)
236 (Fig. 1*A*).

237 IκBα phosphorylation is a key step in the activation of NFκB. Phosphorylated IκBα is
238 ubiquitinated and degraded, allowing NFκB to translocate to the nucleus and bind DNA (Karin
239 and Ben-Neriah, 2000; Viatour et al., 2005). NFκB activation promotes expression of pro-
240 inflammatory cytokines, such as TNFα and IL-6. When BMDMs were treated for 1 h with Tau, at
241 concentrations of 5 nM or greater, IκBα was phosphorylated and a decrease in the total
242 abundance of IκBα was observed (Fig 1*B*). ERK1/2 also was phosphorylated in Tau-treated
243 BMDMs. ERK1/2 phosphorylation is a commonly observed process in LRP1-initiated cell-
244 signaling responses (Gonias and Campana, 2014).

245 To test whether LRP1 is necessary for Tau-induced cytokine expression, we harvested
246 BMDMs from mice in which *Lrp1* is deleted under the control of the LysM promoter (Overton et
247 al., 2007; Staudt et al., 2013; Mantuano et al., 2016). LRP1 protein is decreased by greater than
248 95% in these cells (Staudt et al., 2013). In LRP1-expressing control cells, which carry two floxed
249 *Lrp1* genes but not LysM-Cre, 20 nM Tau significantly increased expression of the mRNAs
250 encoding TNFα, IL-6, CCL3, and CXCL2, as anticipated (Fig. 2A). In LRP1-deficient BMDMs, 20
251 nM Tau failed to induce expression of these cytokines.

252 To examine cytokine expression at the protein level, in an unbiased manner, we treated
253 LRP1-expressing and -deficient BMDMs with 20 nM Tau for 6 h. Conditioned medium (CM) was
254 harvested and subjected to array analysis to detect secreted cytokines. In CM from Tau-treated

255 LRP1-expressing cells, we observed TNFα and IL-6, as anticipated based on our RT-qPCR
256 results (Fig. 2*B*). Other cytokines that were increased in CM from Tau-treated LRP1-expressing
257 cells included but were not limited to CXCL2/MIP2, CXCL1/KC, CXCL10/ IP10, CCL3/CCL4
258 (MIP1α/β), and IL-12. By contrast, CM from Tau-treated LRP1-deficient BMDMs was entirely
259 lacking in the Tau-elicited cytokines detected by this array. In separate studies, Tau failed to
260 induce phosphorylation of IκBα in LRP1-deficient BMDMs, even when the Tau concentration was
261 increased to 20 nM (Fig. 2*C*). Collectively, these results demonstrate that LRP1 is necessary for
262 Tau-induced pro-inflammatory responses in BMDMs.
263

264 **The NMDA-R is not required for macrophages to respond to Tau**

265 tPA, α_2M, and S-PrP^C are examples of LRP1 ligands that activate cell-signaling via pathways that
266 require the NMDA-R (Backsai et al., 2000; Martin et L., 2008; Mantuano et al., 2017; Mantuano
267 et al., 2020). To test whether the NMDA-R is necessary for Tau-elicited responses in BMDMs,
268 first we treated cells with two pharmacologic inhibitors of the NMDA-R. Neither MK-801 nor DXM
269 inhibited the effects of Tau on expression of the mRNAs encoding TNFα or IL-6. (Fig. 3*A*).
270 Similarly, Tau-induced IκBα phosphorylation was unchanged in the presence of MK-801 or DXM
271 (Fig. 3*B*).

272 Next, we isolated NMDA-R-deficient BMDMs from mice in which the gene encoding the
273 essential GluN1 subunit (*Grin1*) is deleted under the control of the LysM promoter (Mantuano et
274 al., 2023). BMDMs from these mice fail to respond to S-PrP^C (Mantuano et al., 2023). However,
275 as shown in Fig. 3*C*, 20 nM Tau significantly increased expression of the mRNAs encoding TNFα,
276 IL-6, CCL3, and CXCL2 in NMDA-R-deficient BMDMs. Furthermore, Tau (10 nM and 20 nM)
277 caused IκBα phosphorylation in NMDA-R-deficient BMDMs, together with the concomitant
278 decrease in total abundance of IκBα (Fig. 3*D*). Taken together, these results demonstrate that
279 Tau-activated cell-signaling in BMDMs occurs independently of the NMDA-R.
280

281 **Tau induces LRP1-dependent pro-inflammatory responses in microglia and astrocytes**

282 Microglia isolated from mouse pups were treated with 20 nM Tau or vehicle for 6 h. CM was
283 harvested and subjected to cytokine array analysis (Fig. 4*A*). Multiple cytokines were detected,
284 in a pattern that overlapped with those detected in Tau-treated BMDMs, including TNFα, IL-6,
285 CCL3/CCL4, and CXCL2. To validate the results of the microarray analysis and test the role of
286 the NMDA-R in Tau-elicited cytokine expression in microglia, we performed RT-qPCR studies.
287 Tau (20 nM) increased expression of the mRNAs encoding TNFα, IL-6, CCL3, and CXCL2 in
288 microglia (Fig. 4*B*). The increases in mRNA expression were not altered by the NMDA-R
289 inhibitors, MK-801 and DXM.

290 ERK1/2 was activated in microglia treated with 20 nM Tau for 1 h (Fig. 4*C*). In the same
291 cells, IκBα was phosphorylated and this event was accompanied by a decrease in the total
292 abundance of IκBα. These cell-signaling events were not inhibited by MK-801 and DXM,
293 confirming that the NMDA-R does is not required for the response to Tau in microglia.

294 To test the role of LRP1 as a mediator of the Tau response in microglia, we silenced
295 expression of *Lrp1* using siRNA. The level of LRP1 was substantially decreased at the protein
296 level, compared with cells transfected with NTC siRNA (Fig. 4*D*). Tau (20 nM) failed to activate
297 ERK1/2 in microglia in which *Lrp1* was silenced.

298 In cultured mouse astrocytes, Tau (20 nM) induced expression of the mRNAs encoding
299 TNFα, IL-6, CCL3, and CXCL2 (Fig. 5*A*). The response to Tau in astrocytes was not inhibited by
300 MK-801 or DXM. Tau also caused IκBα phosphorylation in astrocytes (Fig. 5*B*). This response
301 was not inhibited by MK-801.

302

303 **$p75^{NTR}$ is essential for Tau-activated LRP1 cell-signaling**

304 In neuron-like cell lines, $p75^{NTR}$ serves as an essential LRP1 co-receptor and alternative to the
305 NMDA-R, in mediating cell-signaling in response to the LRP1 ligand, myelin-associated
306 glycoprotein (MAG) (Stiles et al., 2013). When LRP1-initiated cell-signaling involves $p75^{NTR}$, as

307 opposed to the NMDA-R, the cell-signaling factors activated are distinct and include RhoA and
308 Rho-associated kinase-1/2 (Yamashita et al., 1999; Mantuano et al., 2013; Stiles et al., 2013).
309 To test whether $p75^{NTR}$ serves as an LRP1 co-receptor for pro-inflammatory Tau-signaling, first
310 we studied PC12 cells. As a control, we studied the anti-inflammatory LRP1 ligand, $S\text{-}PrP^{C}$
311 (Mantuano et al., 2020).

312 Tau (20 nM) and $S\text{-}PrP^{C}$ (40 nM) both activated ERK1/2 in PC12 cells (Fig. 6*A*). The
313 response to $S\text{-}PrP^{C}$ was inhibited by MK-801 and DXM as anticipated because the NMDA-R
314 serves as an essential co-receptor for LRP1-dependent cell-signaling triggered by $S\text{-}PrP^{C}$
315 (Mantuano et al., 2020). MK-801 and DXM had no effect on Tau-induced ERK1/2 phosphorylation
316 in PC12 cells.

317 Next, we silenced the gene encoding $p75^{NTR}$ (*Ngfr*) in PC12 cells, using siRNA. Gene
318 silencing decreased $p75^{NTR}$ protein to nearly undetectable levels, as determined by immunoblot
319 analysis (Fig. 6*B*). In PC12 cells in which *Ngfr* was silenced, 20 nM Tau failed to activate ERK1/2.
320 By contrast, the NMDA-R-dependent activator of LRP1 signaling, α_2M, retained its ability to
321 activate ERK1/2.

322 Because $p75^{NTR}$ has been identified in macrophages (Barouch et al., 2001; Caroleo et al.,
323 2001; Samah et al., 2008; Meeker et al., 2015; Yu et al., 2022), we tested whether the $p75^{NTR}$-
324 specific inhibitor, TAT-Pep5 (Yamashita and Tohyama, 2003), inhibits Tau-elicited responses in
325 BMDMs. Fig. 6*C* shows that 0.5 μM TAT-Pep5 blocked IκBα phosphorylation in response to Tau
326 in BMDMs. Next, BMDMs were pre-treated with Tau in the presence or absence of TAT-Pep5
327 (0.5 μM) for 6 h. Fig. 6*D* shows that Tau induced expression of the mRNAs encoding TNFα, IL-
328 6, CCL3, and CXCL2 and the response to Tau was inhibited by TAT-Pep5. TAT-Pep5 also
329 blocked expression of the mRNAs encoding TNFα, IL-6, CCL3, and CXCL2 in astrocytes (Fig.
330 6*E*).

331

332 **ROCK inhibitors block the pro-inflammatory effects of Tau**

333 In preclinical animal model systems, the ROCK inhibitor, Fasudil, demonstrates efficacy in
334 inhibiting or reversing many pathological processes associated with AD (Ballard et al., 2020).
335 Amongst the most important activities of ROCK inhibitors is the capacity to antagonize inflam-
336 matory responses in a variety of contexts (Ding et al., 2011; McGown et al., 2011; Zhang et al.,
337 2013; Liu et al., 2014; Guo et al., 2020). Because RhoA is a well described effector of $p75^{NTR}$-
338 activated cell-signaling (Yamashita et al., 1999), we tested whether the ROCK inhibitors, Fasudil
339 and Y-27632, regulate pro-inflammatory responses elicited by Tau in BMDMs.

340 First, BMDMs were pre-treated with Y-27632 (10 μM) or Fasudil (10 μM) and then with 20
341 nM Tau for 1 h. Both ROCK inhibitors blocked ERK1/2 phosphorylation and IκBα phosphorylation
342 in response to Tau (Fig. 7*A*). In BMDMs treated with 20 nM Tau for 6 h, Y-27632 and Fasudil
343 significantly inhibited Tau-induced increases in the mRNAs encoding TNFα, CCL3, and CXCL2
344 (Fig. 7*B*). Fasudil also blocked IκBα phosphorylation in microglia (Fig. 7*C*) and incompletely but
345 significantly inhibited Tau-induced TNFα expression in microglia (Fig. 7*D*). In astrocytes, IκBα
346 phosphorylation in response to Tau and Tau-induced expression of the mRNAs encoding TNFα,
347 IL-6, CCL3, and CXCL2 was entirely inhibited by Fasudil (Fig. 7*E-D*). Taken together, these
348 results suggest that ROCK functions as a targetable, downstream effector of the Tau-
349 LRP1/$p75^{NTR}$ pathway.

350 ## Discussion

351 LRP1 functions in the clearance of proteins from extracellular spaces, in lipoprotein uptake, as a
352 regulator of the plasma membrane proteome, in myelin phagocytosis, in efferocytosis, and as a
353 direct activator of cell-signaling (Strickland et al., 2002; Gonias and Campana, 2014). In AD,
354 LRP1 may regulate disease progression by functioning as a receptor for β-amyloid peptide (Aβ)
355 (Kanekiyo et al., 2013). LRP1 binds secreted forms of amyloid precursor protein (APP) and may
356 regulate processing of cellular APP so that the amount of Aβ generated and secreted by cells is
357 altered (Kounnas et al., 1995; Ulery et al., 2000). Isoforms of apolipoprotein E bind to LRP1 (Liu
358 et al., 2007). Furthermore, LRP1 has been implicated in transport of Aβ across the blood-brain
359 barrier (Deane et al., 2009).

360 The identification of LRP1 as a Tau receptor raises new questions regarding the activity of
361 LRP1 in AD and in Tauopathies (Rauch et al., 2020; Cooper et al., 2021). Because binding of
362 Tau to LRP1 does not require post-translational modification events such as phosphorylation or
363 aggregation (Cooper et al., 2021), LRP1 may play an important physiologic role, removing low
364 levels of Tau form the extracellular spaces in the CNS and delivering the internalized Tau to
365 lysosomes for degradation. However, because Tau may partially escape endosomal transport
366 and enter the cytoplasm, when the amount of Tau in the extracellular spaces in the CNS
367 increases, LRP1 may be involved in propagation of Tau lesions in new cells (Rauch et al., 2020;
368 Cooper et al., 2021).

369 Because LRP1 is known to couple ligand internalization with activation of cell-signaling
370 (Gonias and Campana, 2014), our goal was to test whether the Tau-LRP1 interaction affects cell
371 physiology in a manner that may play a role in progression of AD and other forms of
372 neurodegeneration. We have shown that, in diverse cells, including macrophages, microglia, and
373 astrocytes, the interaction of 2N4R Tau with LRP1 induces robustly pro-inflammatory responses.
374 The responses occur independently of the NMDA-R, which has been implicated in other LRP1

375 cell-signaling events (Backsai et al., 2000; Martin et L., 2008; Mantuano et al., 2017; Mantuano
376 et al., 2020) and instead, appears to be dependent on $p75^{NTR}$. The dependency on $p75^{NTR}$ is
377 reminiscent of the LRP1 cell-signaling pathway activated by myelin-associated inhibitory proteins,
378 including MAG, Nogo-A, OMgp (Mantuano et al., 2013; Stiles et al., 2013). In our previous studies
379 on MAG, Nogo-A, OMgp, we did not examine the capacity of these proteins to stimulate pro-
380 inflammatory responses.

381 RhoA and its effectors, ROCK1 and ROCK2, are activated downstream of $p75^{NTR}$ (Yamashita
382 et al., 1999). We therefore studied two distinct pharmacologic ROCK inhibitors and demonstrated
383 that inhibiting ROCK antagonizes pro-inflammatory cytokine expression in response to Tau.
384 ROCK inhibitors are previously reported to block NFκB activation and cytokine expression in
385 response to pro-inflammatory stimuli other than Tau in macrophages, microglia, and astrocytes
386 (McGown et al., 2011; Ding et al., 2011; Liu et al., 2014; Uchida et al., 2017; Zhang et al., 2022;
387 Guo et al., 2020 Glotfelty et al., 2023). The ROCK inhibitor, Fasudil, has demonstrated efficacy
388 in a number of pre-clinical studies in animal models of neurodegeneration and cognitive
389 impairment (Ballard et al., 2020). In one study in which $A\beta_{1-42}$ was administered by
390 intracerebroventricular injection in rats, increased NFκB activation and production of TNFα and
391 IL-1β were observed in the brain tissue. Fasudil decreased neuro-inflammation and protected
392 against hippocampal neurodegeneration (Song et al., 2013). The ability of ROCK inhibitors to
393 antagonize the pro-inflammatory activity of Tau may have contributed to the previously reported
394 efficacy of these drugs as inhibitors of neuro-inflammation.

395 We hypothesize that under non-pathogenic conditions, mild activation of pro-inflammatory
396 cell-signaling via the LRP1/$p75^{NTR}$ pathway is probably offset by anti-inflammatory cell-signaling.
397 The anti-inflammatory pathways may include LRP1-dependent pathways, such as that activated
398 by binding of S-PrP^{C} to the LRP1/NMDA-R complex (Mantuano et al., 2022b). In future studies,
399 it will be important to understand how various modifications of Tau, which regulate intracellular
400 aggregation, affect the cell-signaling response initiated by LRP1. Given the known variability in

401 cell-signaling elicited by different LRP1 ligands, it should not be assumed that the qualitative
402 nature and magnitude of the signaling responses elicited by different Tau variants will be a simple
403 function of receptor affinity.

404 Stiles et al. (2013) showed that, in neurons, the LRP1/p75^{NTR} receptor assembly functions as
405 an inhibitor of neurite outgrowth and may be involved in the mechanism by which specific proteins
406 inhibit CNS neuronal regeneration following injury. We did not study neurons; however, in neuron-
407 like PC12 cells, p75^{NTR}-dependent cell-signaling was activated by Tau. Understanding the effects
408 of Tau on LRP1-signaling in neurons and the capacity of these cells to differentiate represent
409 important future goals.

410 In response to LPS or interferon-γ, LRP1 is cleaved by transmembrane proteases in the
411 ADAM family, releasing a soluble product that includes the entire α-chain and a fragment of the
412 β-chain ectodomain (Quinn et al., 1999; von Arnim et al., 2005; Gorovoy et al., 2010). Shed LRP1
413 is found in plasma and its level is increased in patients with rheumatoid arthritis or systemic lupus
414 erythematosus (Gorovoy et al., 2010). Shed LRP1 that is purified from human plasma is
415 biologically active and independently pro-inflammatory against macrophages and microglia
416 (Gorovoy et al., 2010; Brifault et al., 2017). It is possible that LRP1 shedding is involved in the
417 pathway by which Tau induces pro-inflammatory responses in macrophages, microglia, and
418 astrocytes. If future studies prove this is true, then there are at least two non-mutually exclusive
419 models to reconcile the activity of p75^{NTR}. In the first model, p75^{NTR} functions with membrane-
420 anchored LRP1 to transduce the initial cell-signaling response to Tau. In the second model,
421 p75^{NTR} functions as a component of the cellular receptor system that mediates the response to
422 shed LRP1.

423 The bimodal activity of LRP1 as a ligand-dependent inhibitor or activator of inflammation is
424 important to consider when reviewing complex animal model studies in which LRP1 may be
425 exposed to diverse ligands. For example, LRP1 deficiency in cultured microglia is mildly pro-
426 inflammatory in the absence of inflammation-regulating mediators (Yang et al., 2016; Brifault et

427 al., 2017). LRP1-deficient microglia in culture demonstrate more robust pro-inflammatory
428 responses to $A\beta_{1-42}$ (He et al., 2020). In these cases, the effects of LRP1 deficiency may be
429 explained by loss of NMDA-R-dependent anti-inflammatory cell-signaling pathways. By contrast,
430 in an *in vivo* model of peripheral nerve injury, microglial LRP1 exacerbates inflammation in the
431 spinal cord, in association with central sensitization of pain processing (Brifault et al., 2019). In
432 this case, the available LRP1 ligands *in vivo* may activate the LRP1/$p75^{NTR}$ pathway and/or induce
433 LRP1 shedding.

434 In summary, the studies presented here identify a novel LRP1-depedent mechanism by which
435 Tau may regulate inflammation, neuro-inflammation, and AD progression. Identification of ROCK
436 as a downstream mediator of the Tau-activated LRP1/$p75^{NTR}$ receptor assembly offers
437 opportunities to target this pro-inflammatory pathway with available drugs that have already been
438 tested using *in vivo* model systems.

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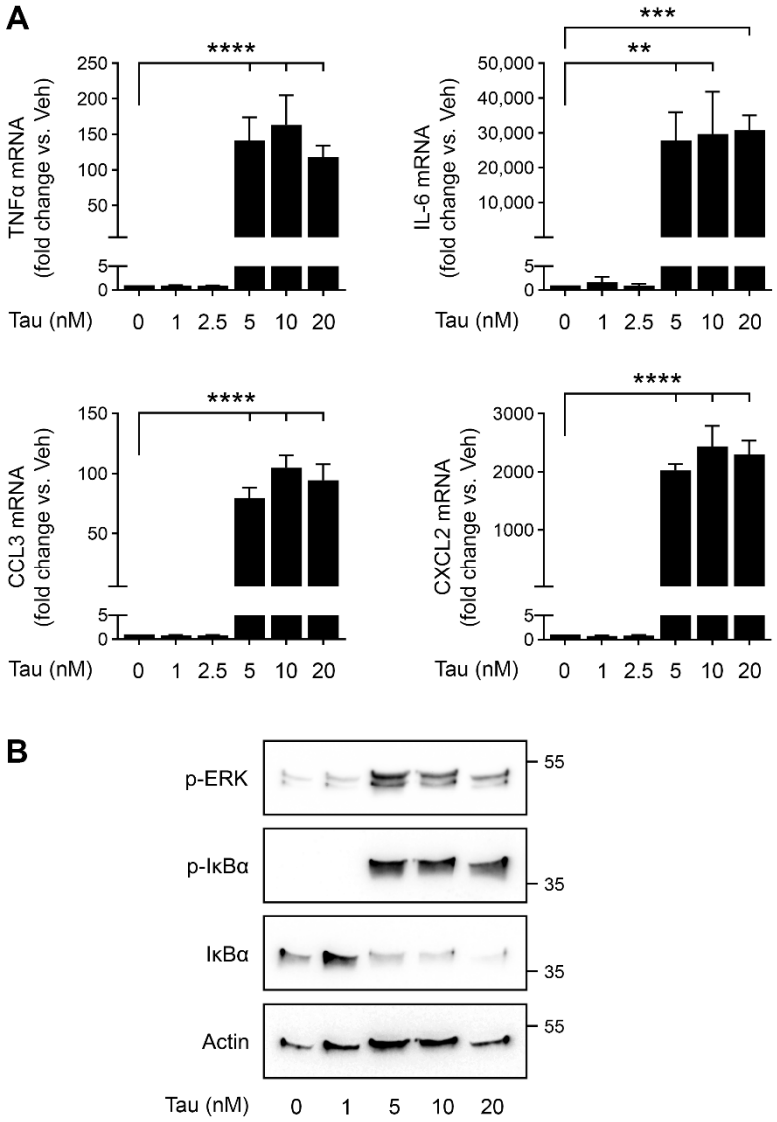
635 ## Figures and Figure Legends



636

637 **Figure 1.** Tau induces an inflammatory response in macrophages. ***A***, BMDMs from wild-type mice
638 were treated for 6 h with increasing concentrations of Tau, as indicated. RT-qPCR was performed
639 to determine mRNA levels for TNFα, IL-6, CCL3, and CXCL2. Data are represented as mean ±
640 SEM (n = 3-8; one-way ANOVA: ***p*<0.01; ****p*<0.001; *****p*<0.0001). ***B***, BMDMs from wild-type
641 mice were treated for 1 h with increasing concentrations of Tau, as indicated. Immunoblot analysis
642 was performed to detect expression of p-ERK, p-IκBα, IκBα, and β-actin.

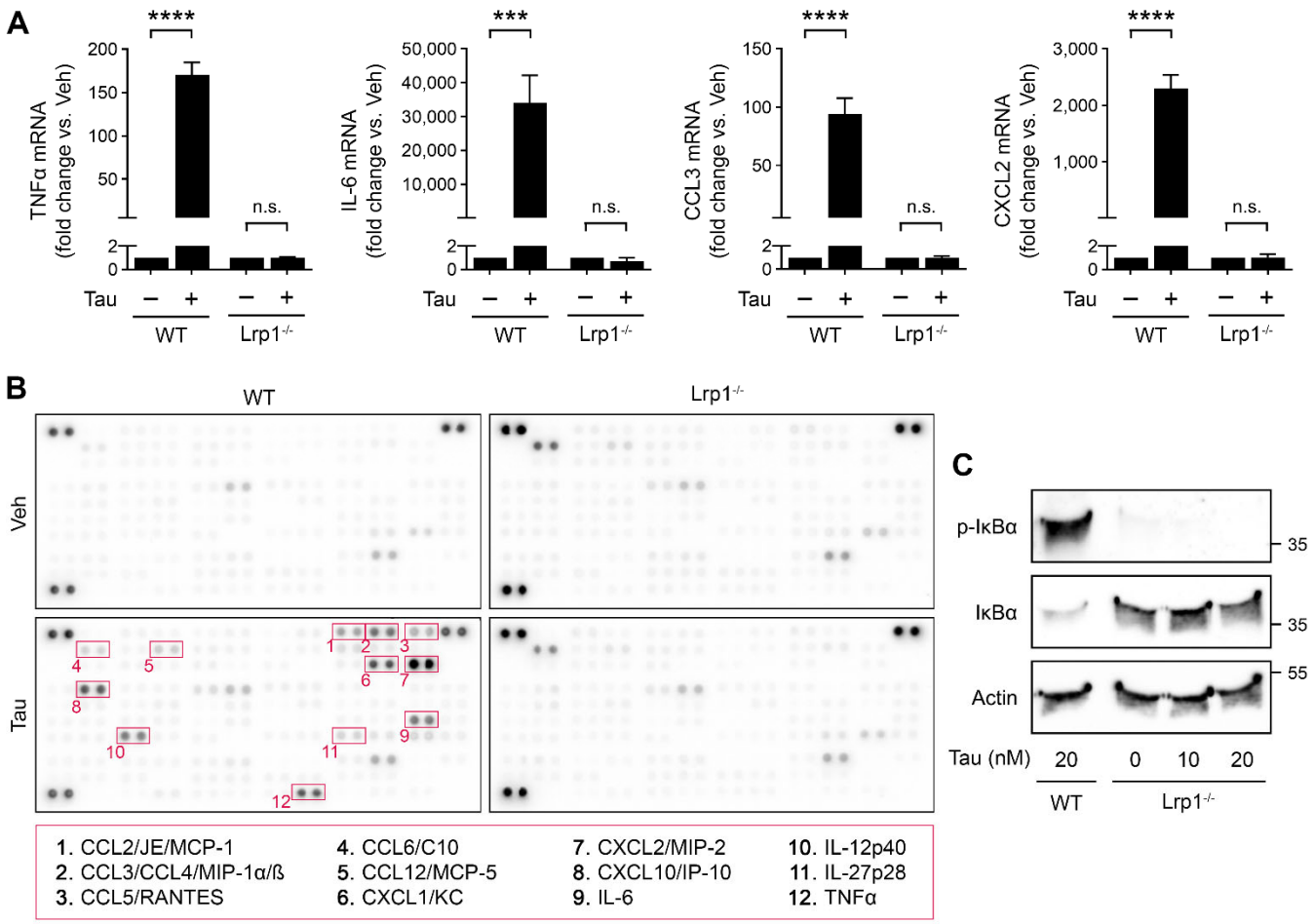
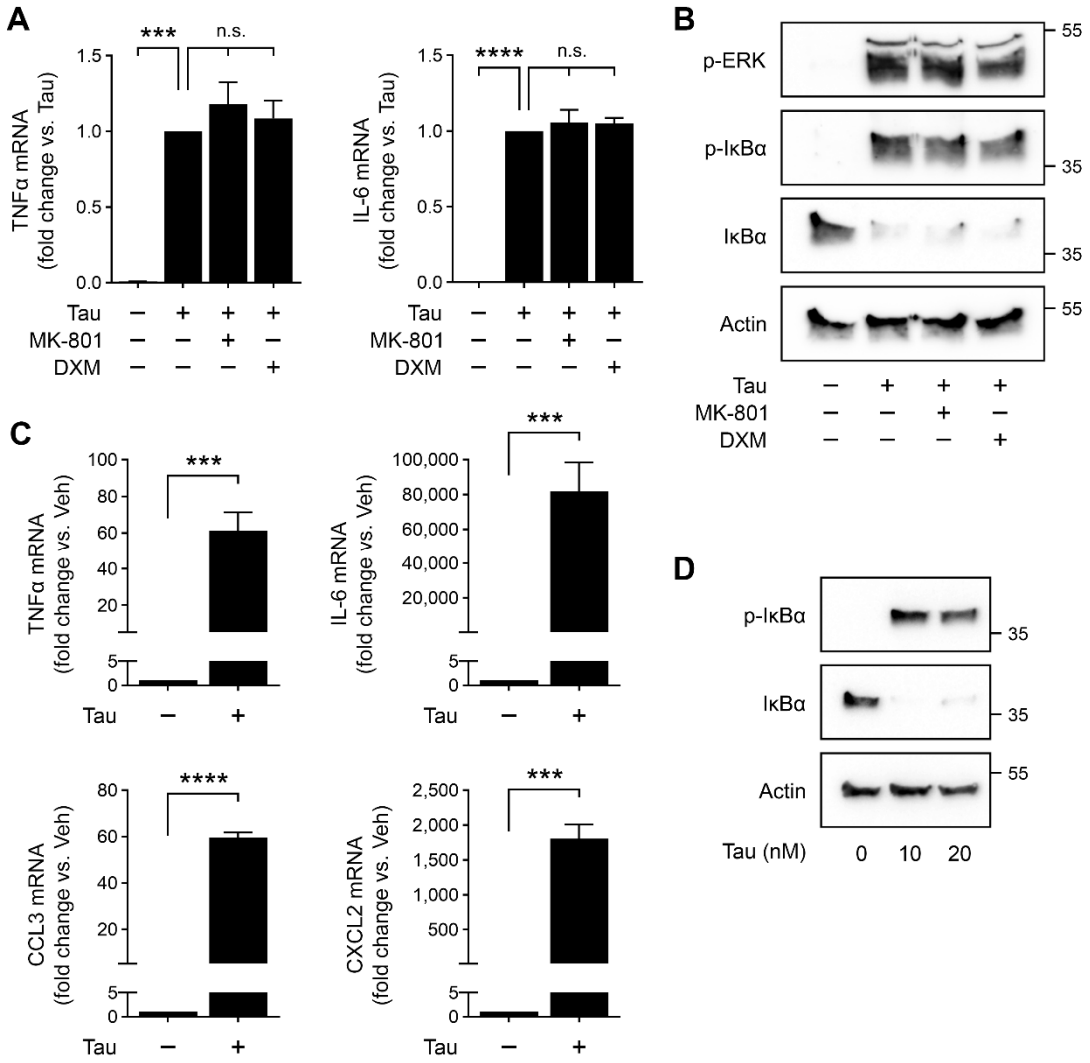


Figure 2. LRP1 mediates Tau-induced inflammation in macrophages. ***A***, BMDMs from wild-type (WT) and *Lrp1*$^{fl/fl}$-LysM-Cre-positive (*Lrp1*$^{-/-}$) mice were treated for 6 h with Tau (20 nM) or vehicle. mRNA levels for TNFα, IL-6, CCL3, and CXCL2 were determined by RT-qPCR. Data are represented as mean ± SEM (n = 3-5; two-way ANOVA: ***$p < 0.001$; ****$p < 0.0001$; n.s., not significant). ***B***, WT and *Lrp1*$^{-/-}$ BMDMs were treated for 6 h with Tau (20 nM) or vehicle. Conditioned medium (CM) was collected and analyzed using the Proteome Profiler Mouse XL Cytokine Array. Representative cytokines that were increased in Tau-treated *Lrp1*$^{-/-}$ BMDMs compared with WT cells are highlighted in red boxes and numbered. ***C***, WT and *Lrp1*$^{-/-}$ BMDMs were treated for 1 h with Tau, at the indicated concentrations. Protein extracts were analyzed by immunoblotting to detect p-IκBα, total IκBα, and β-actin.



654

655 **Figure 3.** The Tau response in macrophages is independent of the NMDA-R. ***A***, BMDMs from
656 wild-type (WT) mice were treated for 6 h with Tau (20 nM), MK-801 (1 μM), DXM (10 μM), or
657 vehicle as indicated. RT-qPCR was performed to determine mRNA levels for TNFα and IL-6
658 (mean ± SEM; n=3-4; one-way ANOVA: ***p<0.001; ****p<0.0001; n.s., not significant). ***B***, WT
659 BMDMs were treated for 1 h with Tau (20 nM), MK-801 (1 μM), DXM (10 μM), or vehicle as
660 indicated. Cell extracts were subjected to immunoblot analysis to detect p-ERK, p-IκBα, total IκBα,
661 and β-actin. ***C***, BMDMs from *Grin1*$^{fl/fl}$-LysM-Cre-positive (*Grin1*$^{-/-}$) mice were treated for 6 h with
662 Tau (20 nM) or vehicle. mRNA levels for TNFα, IL-6, CCL3, and CXCL2 were determined by RT-

663 qPCR (mean ± SEM; n = 5-7; one-way ANOVA: ***p<0.001; ****p<0.0001). ***D***, *Grin1*$^{-/-}$ BMDMs

664 were treated for 1 h with increasing concentrations of Tau, as indicated. Immunoblot analysis was

665 performed to detect p-IκBα, IκBα, and β-actin.

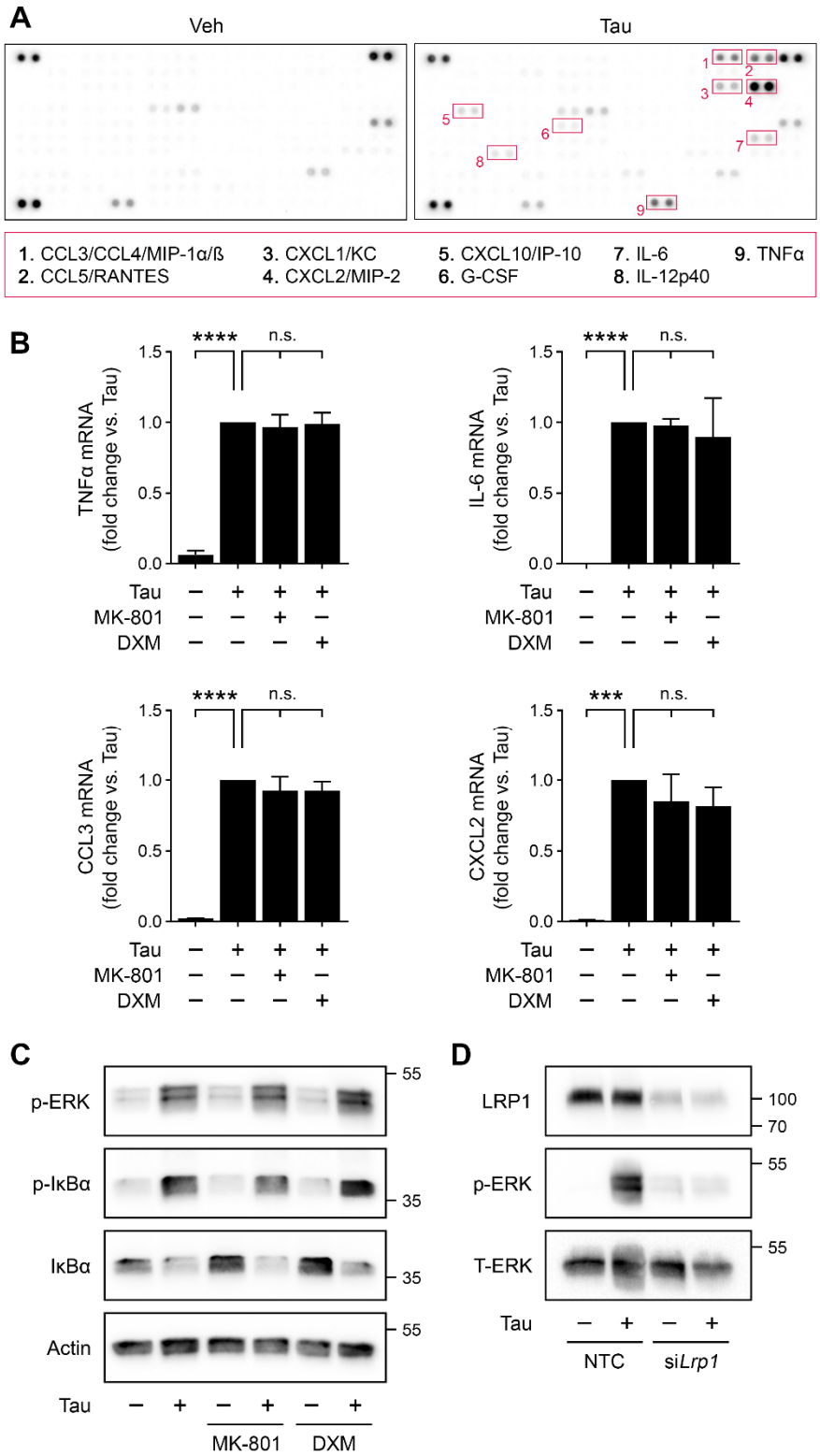
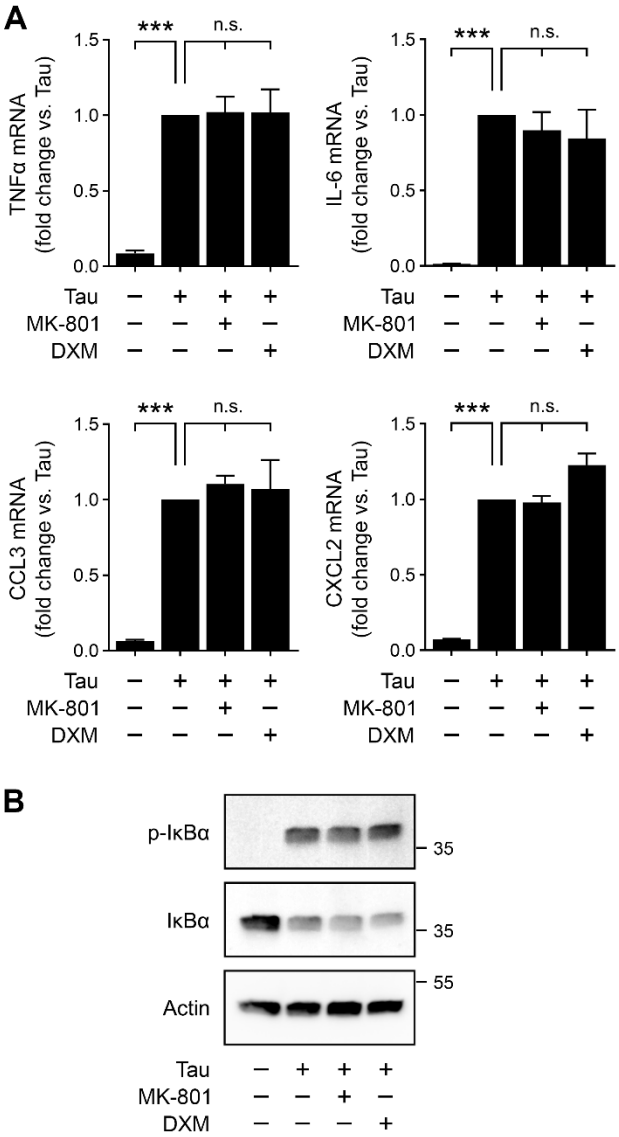


Figure 4. Tau induces a pro-inflammatory response in microglia that requires LRP1, but not the NMDA-R. ***A***, Microglia from mouse pups were treated with Tau (20 nM) or vehicle for 6 h.

669 Conditioned medium (CM) was collected and analyzed using the Proteome Profiler Mouse XL
670 Cytokine Array. Representative cytokines that were increased compared with the control are
671 highlighted in red boxes and numbered. ***B***, Microglia from mouse pups were treated for 6 h with
672 Tau (20 nM), MK-801 (1 μM), DXM (10 μM), or vehicle as indicated. RT-qPCR was performed to
673 determine mRNA levels for TNFα, IL-6, CCL3, and CXCL2 (mean ± SEM; n = 3-5; one-way
674 ANOVA: ***p<0.001; ****p<0.0001; n.s., not significant). ***C***, Microglia from mouse pups were
675 treated for 1 h with Tau (20 nM), MK-801 (1 μM), DXM (10 μM), or vehicle as indicated. Cell
676 extracts were subjected to immunoblot analysis to detect p-ERK, p-IκBα, IκBα, and β-actin. ***D,***
677 Microglia from mouse pups were transfected with *Lrp1*-specific (siL*rp1*) or non-targeting control
678 (NTC) siRNA for 48 h. Cells were then treated with Tau (20 nM) or vehicle for 1 h and then
679 analyzed by immunoblotting to detect LRP1, p-ERK, and T-ERK.



680

681 **Figure 5.** Tau induces a pro-inflammatory response in astrocytes that does not require the NMDA-
682 R. ***A***, Astrocytes from mouse pups were treated for 6 h with Tau (20 nM), MK-801 (1 μM), DXM
683 (10 μM), or vehicle as indicated. RT-qPCR was performed to determine mRNA levels for TNFα,
684 IL-6, CCL3, and CXCL2 (mean ± SEM; n = 3; one-way ANOVA: ***$p < 0.001$; n.s., not significant).
685 ***B***, Astrocytes from mouse pups were treated for 1 h with Tau (20 nM), MK-801 (1 μM), DXM (10
686 μM), or vehicle as indicated. Protein extracts were analyzed by immunoblot to detect levels of p-
687 IκBα, IκBα, and β-actin.

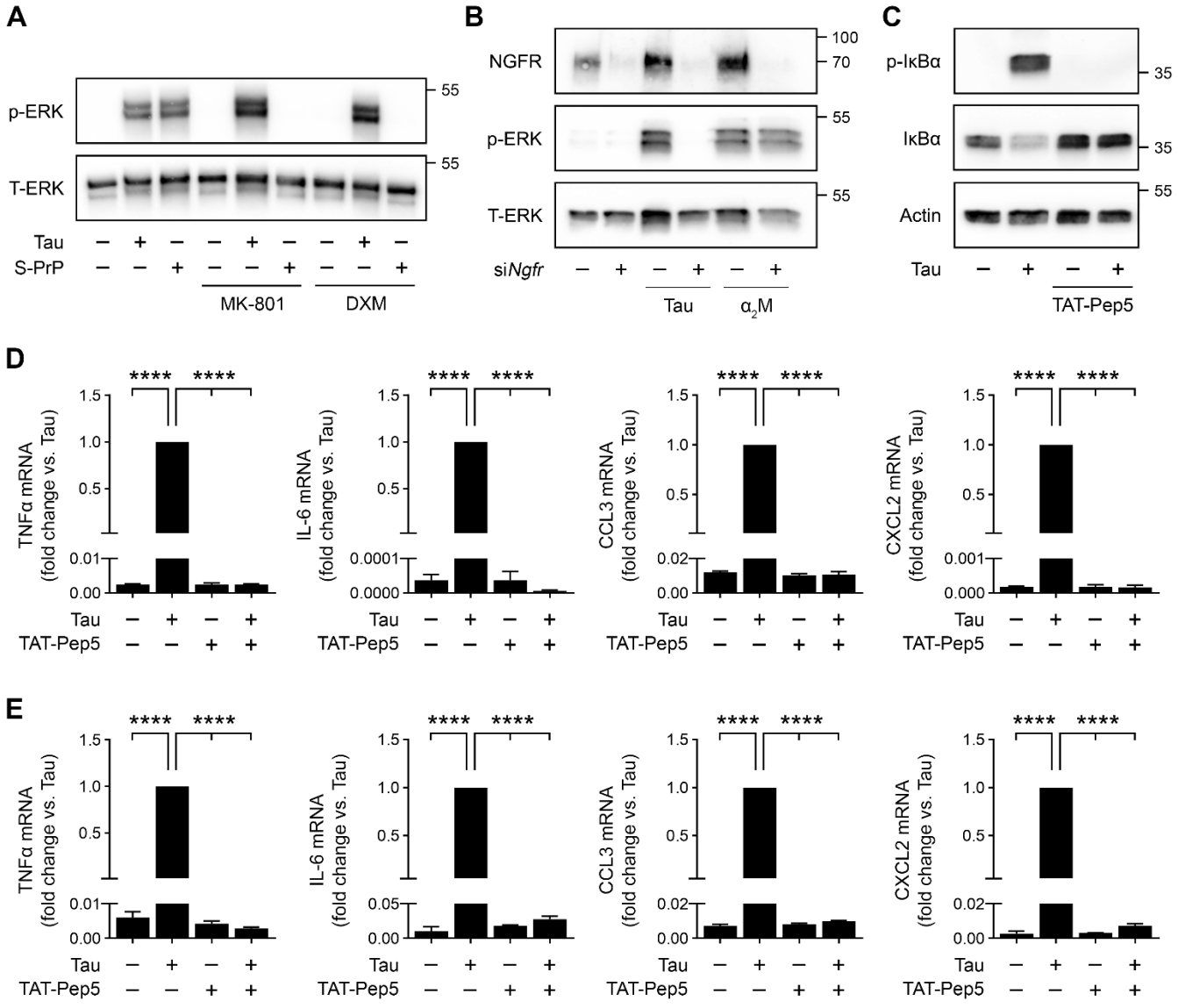


Figure 6. p75^{NTR} is required for Tau-induced cell-signaling. ***A***, PC12 cells were pre-treated with vehicle, MK-801 (1 μM) or DXM (10 μM) for 30 min, and then incubated with Tau (20 nM), S-PrP^{C} (40 nM), or vehicle for 10 min, as indicated. Cell extracts were subjected to immunoblot analysis to detect levels of p-ERK and T-ERK. ***B***, PC12 cells were transfected with *Ngfr*-specific (si*Ngfr*) or non-targeting control (NTC) siRNA for 24 hours. Cells were then treated with Tau (20 nM), α_2M (10 nM), or vehicle for 10 min, then analyzed through immunoblotting to detect levels of NGFR, p-ERK, and T-ERK. ***C***, BMDMs from wild-type mice were pre-treated with TAT-Pep5 (0.5 μM) or vehicle for 30 min and then with Tau (20 nM) for 1 h. Immunoblot analysis was performed to

697 detect expression of p-IκBα, IκBα, and ß-actin. ***D-E***, BMDMs (***D***) and astrocytes (***E***) were pre-
698 treated with TAT-Pep5 (0.5 μM) or vehicle for 30 min and then with Tau (20 nM) for 6 h. RT-
699 qPCR was performed to determine mRNA levels for TNFα, IL-6, CCL3, and CXCL2 (mean ± SEM;
700 n = 3; one-way ANOVA; ****p<0.0001).

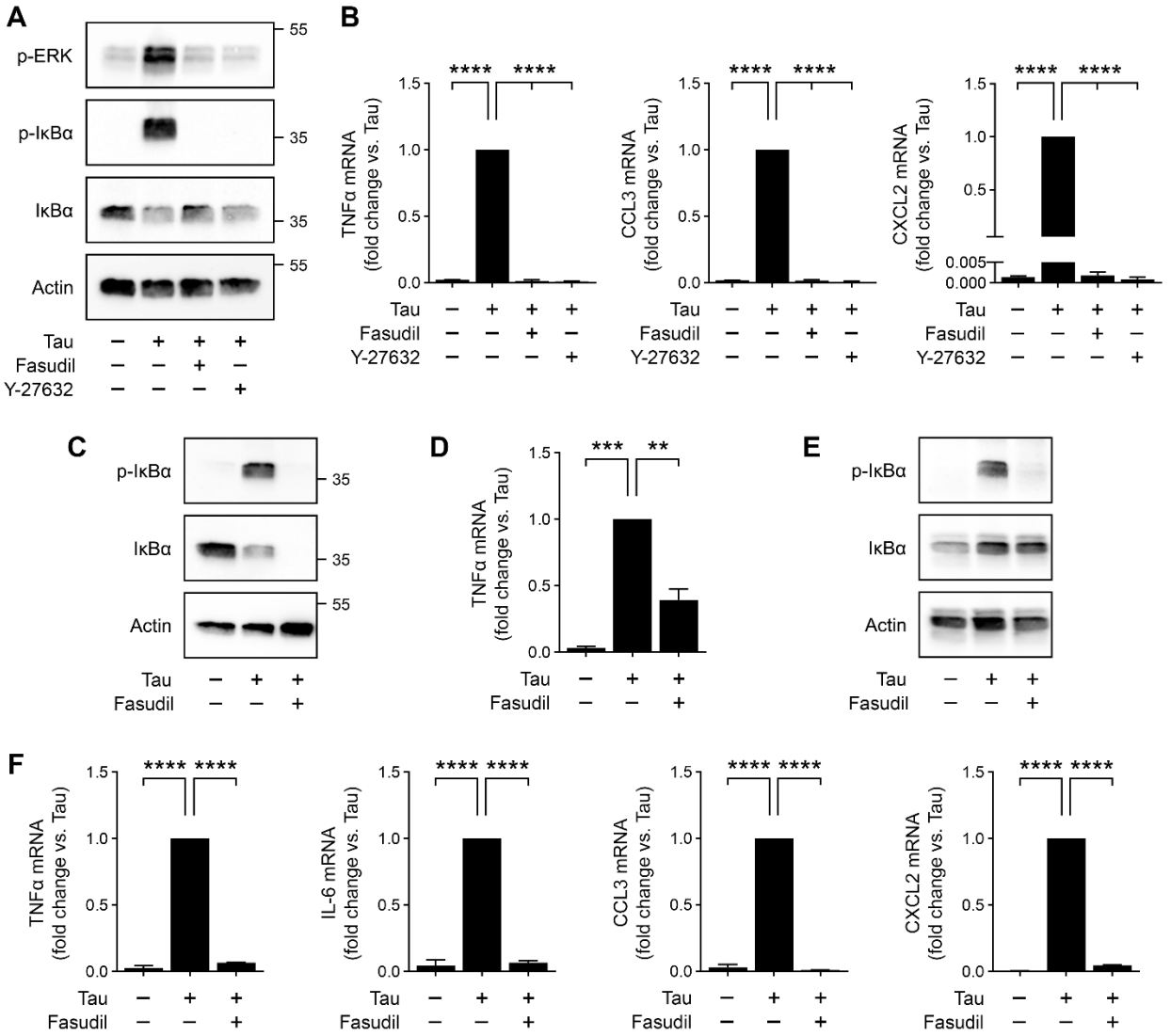


Figure 7. Tau-induced pro-inflammatory responses are neutralized by ROCK inhibitors. ***A***, BMDMs from wild-type mice were pre-treated with Fasudil (10 μM) or Y-27632 (10 μM) for 30 min and then with Tau (20 nM) for 1 h. Cell extracts were subjected to immunoblot analysis to detect p-ERK, p-IκBα, IκBα, and β-actin. **B**, BMDMs from wild-type mice were treated for 6 h with Tau (20 nM) in presence or absence of Fasudil (10 μM) or Y-27632 (10 μM), as indicated. mRNA levels for TNFα, CCL3, and CXCL2 were determined by RT-qPCR (mean ± SEM; n = 3; one-way ANOVA: ****$p < 0.0001$). ***C***, Microglia from mouse pups were treated with Tau (20 nM) in presence or absence of Fasudil (10 μM) for 1 h. Cell extracts were subjected to immunoblot analysis to

710 detect p-IκBα, IκBα, and β-actin. ***D***, Microglia were treated with Tau (20 nM) in presence or
711 absence of Fasudil (10 μM) for 6 h and then analyzed to detect changes of TNFα mRNA by RT-
712 qPCR (mean ± SEM; n = 3; one-way ANOVA: ****p*<0.01; *****p*<0.001). ***E***, Astrocytes were treated
713 for 1 h with Tau (20 nM), in presence or absence of Fasudil (10 μM). Cell extracts were subjected
714 to immunoblot analysis to detect p-IκBα, IκBα, and β-actin. ***F***, Astrocytes were treated for 6 h with
715 Tau (20 nM), in presence or absence of Fasudil (10 μM). RT-qPCR was performed to determine
716 mRNA levels for TNFα, IL-6, CCL3, and CXCL2 (mean ± SEM; n = 3; one-way ANOVA; ******p* <
717 0.0001).