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Loss of tau and Fyn reduces compensatory effects of MAP2 for tau and reveals a Fyn-independent effect of tau on glutamate-induced Ca²⁺ response

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Summary Statement

The downstream effect of the interaction between microtubule-associated protein tau and Src family non-receptor tyrosine kinase Fyn was investigated with a tau/Fyn double KO mouse. We demonstrate that tau has a Fyn-independent role in glutamate-induced calcium response and that MAP2 can compensate for tau in interacting with Fyn in dendrites.

Abstract

Microtubule-associated protein tau associates with Src family tyrosine kinase Fyn. A tau-Fyn double knockout (DKO) mouse was generated to investigate the role of the complex. DKO mice resembled Fyn KO in cognitive tasks and resembled tau KO mice in motor tasks and protection from pentylenetetrazole-induced seizures. In Ca^{2+} response, Fyn KO was decreased relative to WT and DKO had a greater reduction relative to Fyn KO, suggesting that tau may have a Fyn-independent role. Since tau KO resembled WT in its Ca^{2+} response, we investigated whether MAP2 served to compensate for tau, since its level was increased in tau KO but decreased in DKO mice. We found that like tau, MAP2 increased Fyn activity. Moreover, tau KO neurons had increased density of dendritic MAP2-Fyn complexes relative to WT neurons. Therefore, we hypothesize that in the tau KO, the absence of tau would be compensated by MAP2, especially in the dendrites, where tau-Fyn complexes are of critical importance. In the DKO, decreased levels of MAP2 made compensation more difficult, thus revealing the effect of tau in the Ca^{2+} response.

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Introduction

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Tau was initially discovered as a microtubule-associated protein enriched in axons (Binder et al., 1986; Weingarten et al., 1975). Interest in tau dramatically increased after hyperphosphorylated tau was found in neurofibrillary tangles (NFT), a major hallmark of Alzheimer's disease (AD) (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Nukina and Ihara, 1986; Wood et al., 1986), and after mutations in the tau gene were found to cause frontotemporal dementia with Parkinsonism linked to chromosome 17 (Clark et al., 1998; Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). Subsequent studies have revealed new functions for tau, such as the regulation of motor-driven axonal transport (Dixit et al., 2008; Trinczek et al., 1999), formation and trafficking of stress granules (Vanderweyde et al., 2016), and postsynaptic scaffolding (Ittner et al., 2010).

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However, despite the multiple roles played by tau, initial studies of five lines of independently generated tau KO mice did not suggest any gross deficits or loss of viability (Dawson et al., 2001; Fujio et al., 2007; Harada et al., 1994; Tan et al., 2018; Tucker et al., 2001). Further testing of tau KO mice has yielded controversial results, as some studies reported minor memory deficits (Ahmed et al., 2014; Ikegami et al., 2000; Lei et al., 2014; Ma et al., 2014) while a larger number of studies reported no cognitive deficits (Dawson et al., 2010; Dawson et al., 2001; Ittner et al., 2010; Kimura et al., 2014; Lei et al., 2012; Li et al., 2014; Morris et al., 2013; Regan et al., 2015; Roberson et al., 2007; Tan et al., 2018; van Hummel et al., 2016). Morphological studies of tau KO mice reported alterations in small caliber axons as the only abnormality (Harada et al., 1994) while low density neuronal cultures from another line of tau KO mouse indicated slowed axonal development, although brain development appeared normal (Dawson et al., 2001). The lack of more pronounced behavioral and morphological phenotypes has largely been attributed to functional compensation by other microtubule-binding proteins such as microtubule associated protein 1A/1B (MAP1A/1B) and MAP2 (Harada et al., 1994; Ma et al., 2014). While it is thought that compensating for a loss of microtubule stabilizing activity is important, given the additional functions for tau, other compensatory mechanisms may be needed if these functions are critical for mouse viability and depend on tau.

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Following our finding that tau could associate with the cytoplasmic face of the plasma membrane (Brandt et al., 1995), we found that the proline-rich domain of tau interacts with the SH3 domain of Src family non-receptor tyrosine kinases (SFK) such as Fyn and Src (Lee et al., 1998) and that Fyn phosphorylated tau on Tyr18 (Lee et al., 2004). In addition, we showed that tau increased the auto-phosphorylation of Fyn as well as the enzymatic activity of Fyn (Sharma et al., 2007). Also, tau was critical for nerve growth factor (NGF)-induced mitogen activated protein kinase (MAPK) activation, suggesting a

98 role for tau in signal transduction (Leugers et al., 2013; Leugers and Lee, 2010). Interest in the tau-Fyn
99 interaction has been increased by the presence of phospho-tyr18-tau in NFTs(Bhaskar et al., 2010; Lee et
100 al., 2004), reported alterations of Fyn expression in AD brain(Ho et al., 2005; Shirazi and Wood, 1993), and
101 the neuroprotective effects of Fyn depletion in AD models(Chin et al., 2004; Lambert et al., 1998).
102 Furthermore, Fyn is highly expressed in the central nervous system(Umemori et al., 1992) and
103 phosphorylates the N-methyl-D-aspartate receptor (NR) subunit 2B (NR2B) at Y1472 residue(Nakazawa et
104 al., 2001; Rong et al., 2001), which facilitates the interaction between the NR and the postsynaptic density
105 95 protein (PSD-95)(Tezuka et al., 1999) and regulates long-term potentiation (LTP) and memory
106 formation(Kojima et al., 1997; Stein et al., 1992). Tau has been implicated in targeting Fyn to the
107 postsynaptic space, as tau KO mice showed decreased levels of Fyn and pY1472 NR2B in synaptosome
108 preparations(Ittner et al., 2010). As a result, the PSD-NR interaction was disrupted, leading to protection
109 from both pentylenetetrazole (PTZ) induced seizures and amyloid- β induced excitotoxicity (Ittner et al.,
110 2010; Roberson et al., 2007). However, electrophysiological studies of tau KO resembled WT (Ittner et al.,
111 2010; Kimura et al., 2014; Roberson et al., 2011), further underlying the need to elucidate the function of
112 the tau-Fyn interaction and its significance in neuronal cell function.

113 To further understand the impact of the combined action of tau and Fyn, we generated a tau-Fyn
114 double knockout (τ^{-}/Fyn^{-}) mouse, characterized its behavioral, biochemical, and neurophysiological
115 properties, and found a role for tau in the neuron's calcium response in a Fyn-independent manner.
116 Additionally, we identified the location of tau-Fyn complexes in neurons and determined that in dendrites,
117 the loss of such complexes led to compensation mediated by MAP2.

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Results

120 Generation of tau-Fyn DKO mice

121 In order to investigate the downstream effects of the tau-Fyn interaction, we generated τ^{-}/Fyn^{-}
122 double knockout mice (DKO) by breeding tau KO (C57BL/6) mice(Dawson et al., 2001) with Fyn KO
123 (C57BL/6-S129) mice(Stein et al., 1992) (Fig. S1). Polymerase chain reaction (Fig. 1A) was used for
124 genotyping and western blotting (Fig. 1B) was used to confirm the loss of tau and Fyn.

125 During breeding, DKO animals exhibited a propensity to develop non-obstructive hydrocephalus
126 postpartum, consistent with the observation that having the homozygous Fyn KO trait on a C57BL/6
127 background increased the occurrence of hydrocephalus (Goto et al., 2008). While mice with severe
128 hydrocephalus could be identified within 6 weeks of age by their domed heads and hunched backs, less
129 severe levels of hydrocephalus escaped visual detection. Therefore, to identify mice with moderate
130 hydrocephalus, MRI was used to image the lateral ventricle. Quantification of the images allowed us to

131 classify mice as being normal or having moderate or severe hydrocephalus (Fig. 1C). Behavioral tests
132 showed that both Fyn KO and DKO mice with mild and severe hydrocephalus had increased total
133 movements in the open field test (Fig. 1D) and decreased total freezing in contextual fear conditioning (Fig.
134 1E) when compared to normal mice of the same genotype. This suggested that hydrocephalus caused the
135 animal to be hyperactive. Thus, subsequent behavioral and biochemical tests were performed using only
136 non-hydrocephalic mice with normal brain structure that had been screened by MRI. Beginning at eight
137 weeks of age, the mice underwent a series of motor and behavioral tests.

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139 **DKO mice recapitulate specific behaviors of Fyn KO and tau KO mice**

140 Using the open field apparatus, mice of the four genotypes had similar total movements and percent
141 movement in the center (Fig. S2A, B). This indicated an absence of motor deficits or anxiety phenotype at
142 young age. However, when mice were aged to 12 months and underwent the pole test, tau KO and DKO
143 mice required more time to descend the pole relative to WT and Fyn KO mice (Fig. S2C).

144 In novel object recognition, mice from the four genotypes displayed equal preferences for two
145 identical objects on training day (Fig. 2A, left). However, on testing day, both Fyn KO and DKO mice
146 performed significantly worse than WT and tau KO mice in interacting with the new object (Fig. 2A, right).
147 There were no differences between WT and tau KO mice ($p=0.9012$), and between Fyn KO and DKO mice
148 ($p=0.1301$). The absence of anxiety as monitored by the open field test suggested that Fyn KO and DKO
149 had a cognitive deficit.

150 In contextual fear conditioning, Fyn KO and DKO mice spent significantly less time freezing as
151 compared to WT and tau KO mice on training day (Fig. 2B, left). On testing day, Fyn KO and DKO mice
152 also spent less time freezing than WT and tau KO mice as measured using either minute by minute
153 measurements (Fig. 2B right panel) or total time freezing (Fig. 2C). There was no statistically significant
154 difference between Fyn KO and DKO mice ($p=0.8442$) and between WT and tau KO mice ($p=0.7076$) (Fig.
155 2C). To assess learning versus memory, the amount of freezing during the first minute of the testing day
156 was divided by the amount of freezing during the last minute of the training day. There was no significant
157 difference between the ratios of the four genotypes (Fig. 2D), indicating that the mice did not differ with
158 respect to memory and that the deficits exhibited by Fyn KO and DKO mice on day 2 were due to a
159 learning deficit.

160 Previous reports have also shown that tau ablation protected against pentylentetrazole (PTZ)
161 induced seizures (Roberson et al., 2007). This property has been attributed to tau depletion reducing levels
162 of Fyn at the post-synaptic region leading to disrupted NR stability within the post-synaptic density (Ittner et
163 al., 2010). In terms of both latency to develop seizures and maximum seizure stages reached, DKO and tau
164 KO mice were equally and dramatically protected against PTZ whereas Fyn KO mice were only moderately

165 protected relative to WT mice (Fig. 2E, F). There was no significant difference between tau KO and DKO
166 mice ($p=0.9752$; Fig. 2F). To summarize, in motor tasks and PTZ induced seizures, DKO mice resembled
167 tau KO mice while in cognitive and memory tasks, DKO mice resembled Fyn KO mice.

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169 **Localization of Tau- SFK complexes in WT primary hippocampal culture**

170 To further investigate the functions of the tau/Fyn complex, proximity ligation assays (PLA) were
171 used to localize tau-Fyn complexes in WT primary hippocampal cultures (Fig. 3A). PLAs are able to detect
172 endogenous protein-protein interactions *in situ*; signals are recovered when the two PLA probes lie within
173 40 nm of each other, using rolling circle amplification to yield a marker for the interaction (Gullberg et al.,
174 2004) (Soderberg et al., 2006). As a control, tau KO cultures were similarly probed (Fig. S3B). Tau-Fyn
175 complexes were found in all cellular compartments of WT hippocampal neurons (axons (Fig. 3B), dendrites
176 (Fig. 3C), and cell bodies (Fig. 3D)). For quantitation, PLA dots were counted in both dendrites and axons.
177 Dot intensity was not used because the intensity of a PLA signal reflected the rolling circle step, making the
178 intensity level an artificial characteristic. Upon quantitation, the density of tau-Fyn complexes in dendrites
179 (number of PLA puncta/process length) was 2.54 fold higher than that in axons (Fig. 3E). As expected,
180 there were no tau-Fyn complexes in tau KO neurons (Fig. S3A). Since we have previously shown that tau
181 increased Fyn auto-phosphorylation (Sharma et al., 2007), complexes between tau and activated SFK
182 (pSFK) were also examined (Fig. 3F-J). Similar to the tau-Fyn complexes, the density of tau-pSFK
183 complexes in dendrites was 2.04 fold higher than that in axons (Fig. 3J). However, since the pSFK antibody
184 cannot distinguish between activated Fyn and activated Src, tau-Src complexes were also examined as a
185 control. The density of tau-Src complexes in dendrites was increased by 2.78 fold relative to that in the
186 axon (Fig. 3O).

187 Since tau is known to be enriched in axons, we found it surprising that the density of tau-SFK
188 complexes was higher in the dendrites relative to axons. However, we noted that axons were significantly
189 longer and more numerous than dendrites and that the density of PLA puncta was relatively high in the
190 proximal axon but dramatically decreased in the distal axon whereas the density of PLA puncta in the
191 shorter dendrites was uniformly high. In fact, if only the proximal axon was used to calculate density, the
192 density of tau-Fyn complexes in axons would match that of the dendrites (Fig. S3). Therefore, when the
193 total axon length was used, the calculated density in axons was lower than that in dendrites.

194

195 **Fyn KO and DKO hippocampal insoluble PSD fractions have decreased phospho-Y1472 NR2B and** 196 **phospho-SFK levels**

197 Because the density of tau-Fyn complexes in dendrites exceeded that in axons and because tau has
198 been shown to target Fyn to the post-synaptic density to affect NR2B phosphorylation (Ittner et al., 2010),

199 we wanted to determine if NR2B phosphorylation was altered in the DKO mice. Crude hippocampal
200 synaptosomes were fractionated into “soluble non-PSD” and “insoluble PSD” fractions using Triton X-100
201 (Fig. 4A) (Ittner et al., 2010; Lopes et al., 2016b; Milnerwood et al., 2010). The presence of synaptophysin
202 in the “soluble non-PSD” and the presence of PSD-95 in the “insoluble PSD” fractions (Fig. 4B)
203 demonstrated the separation of PSD and non-PSD membranes from crude WT synaptosomes; tau and Fyn
204 were present in both fractions (Fig. 4B). In examining the “insoluble PSD” fraction from 9-12 month old
205 hippocampus, PSD-95 and total NR2B levels were not significantly different between the four genotypes
206 (One-way ANOVA $p=0.6462$, $p=0.3439$, respectively; Fig. 4C, D). However, relative to WT, pY1472-
207 NR2B levels were decreased by 54.6 % in Fyn KO and 64.0 % in DKO insoluble PSD fractions, with Fyn
208 KO and DKO not being significantly different from each other ($p=0.8871$; Fig. 4C, D). WT and tau KO
209 were also not significantly different from each other ($p=0.8284$; Fig. 4C, D). We then examined whether
210 Src might be compensating for Fyn. In the PSD fraction, although the Src level was unchanged in the four
211 genotypes ($p=0.7699$; Fig. 4C, D), the activated SFK (pSFK) level in Fyn KO and DKO was decreased by
212 73.2% and 84.0% with respect to that of WT. There was no significant difference between tau KO and WT
213 ($p=0.9946$) and between Fyn KO and DKO ($p=0.8512$; Fig. 4C, D). While the pSFK antibody cannot
214 distinguish between activated Fyn and activated Src, all four genotypes had similar Src levels in the PSD
215 but only Fyn KO and DKO had decreased pSFK levels. Since the absence of Fyn did not alter Src levels but
216 reduced pSFK levels, we concluded that most of the activated tyrosine kinase in WT and tau KO PSD
217 fractions was Fyn that was responsible for phosphorylating Y1472-NR2B.

218 219 **Decreased glutamate-induced Ca^{2+} response in Fyn KO and DKO primary hippocampal neurons**

220 Since phosphorylation of NR2B has been shown to be involved in facilitating Ca^{2+} response upon
221 NR activation (Nakazawa et al., 2001; Rong et al., 2001; Tezuka et al., 1999), we were interested in
222 determining the glutamate-induced Ca^{2+} influx of DKO neurons. In addition, depleting tau with shRNA in
223 WT neurons had already been shown to affect glutamate-induced, NMDA receptor-dependent Ca^{2+} influx
224 (Miyamoto et al., 2017), so we were also interested in the response of tau KO neurons. Sample tracings of
225 $[Ca^{2+}]_i$ from five individual cells of each genotype are shown in Fig. 5A. At baseline, unstimulated
226 intracellular $[Ca^{2+}]_i$ from the four genotypes were similar ($p=0.2808$; Fig. 5B). Upon stimulation, Fyn KO
227 neurons had a 29.9% reduction and DKO neurons had a 53.1% reduction relative to WT neurons; tau KO
228 neurons were not significantly different from WT neurons ($p=0.7264$). However, Ca^{2+} response in DKO
229 neurons was significantly different from Fyn KO neurons (Fig. 5C).

230 The fact that DKO neurons had an even greater reduction relative to Fyn KO neurons suggests that
231 tau had a Fyn-independent role in regulating Ca^{2+} response that was unmasked in the absence of Fyn. In
232 addition, the absence of any changes in the Ca^{2+} response of tau KO neurons suggested that another protein

233 could compensate for tau in the tau KO. However, this compensatory effect was apparently lost in the
234 DKO, leading to an exacerbated decrease in Ca^{2+} influx.

235

236 **Microtubule-associated protein 2 (MAP2) was altered in KOs and like tau, could increase Fyn**
237 **activity**

238 Our finding that tau KO had no memory deficits, no changes in pY1472-NR2B, and no changes in
239 glutamate-induced Ca^{2+} response suggested that either Fyn did not require tau to mediate these functions or
240 that a different protein was compensating for the loss of tau with respect to its Fyn interaction. Interestingly,
241 another microtubule-associated protein, MAP2, has been found to be upregulated in tau KO mice (Ma et al.,
242 2014) and in agreement with the previous data, we also found MAP2 to be increased by 48.9% in the tau
243 KO mice ($p=0.021$, Fig. 6A). However, while it has been shown that MAP2 compensated for the loss of tau
244 by maintaining microtubule stability, as measured by tubulin acetylation (Ma et al., 2014), the possibility of
245 other compensating mechanisms remained unexplored.

246 Similar to tau, MAP2 has a PXXP domain that interacts with the SH3 domain of Fyn (Zamora-
247 Leon et al., 2001). While we have shown that for tau, this interaction potentiated Fyn and Src activities
248 (Sharma et al., 2007), the ability of MAP2 to exhibit a similar property has not been demonstrated. To
249 investigate, MAP2c, the shortest isoform in the MAP2 family was tested for its ability to increase auto-
250 phosphorylation of Fyn and to increase the enzymatic activity of Fyn. To examine auto-phosphorylation of
251 Fyn, as detected by antibodies against activated SFK, 3T3 cells were transfected with Fyn alone or with
252 MAP2c (Fig. 6B). After Fyn was immunoprecipitated (IPed) and probed for activated SFK, we found that
253 the phosphorylation of Fyn expressed with MAP2c was increased by 61.9% relative to Fyn expressed alone
254 (Fig. 6C). This indicated that the presence of MAP2c increased the auto-phosphorylation of Fyn. To ensure
255 that the anti-Fyn antibody was specific for Fyn and not other SFKs, the IPed Fyn was also probed for Src.
256 Src was not detected (Fig. 6C), indicating that the IP was indeed specific for Fyn. To investigate the ability
257 of MAP2c to increase the enzymatic activity of Fyn, *in vitro* kinase assays were conducted using tubulin, a
258 known substrate for Fyn (Marie-Cardine et al., 1995). Because MAP2c can bind tubulin, an N-terminal
259 fragment of MAP2c (N-MAP2c) containing the Fyn binding motif but lacking the microtubule binding
260 domain was used. Fyn and N-MAP2c were separately expressed in 3T3 cells and IP'ed for use in the kinase
261 reaction. Tubulin was incubated either alone, with Fyn, or with pre-incubated Fyn and N-MAP2c. The
262 presence of N-MAP2c increased tyrosine phosphorylation of tubulin by 59.4% relative to tubulin incubated
263 with Fyn alone ($p=0.049$; Fig. 6D). Our data provide evidence that similar to tau, MAP2 can increase Fyn
264 activity. As an additional indication that MAP2 resembled tau with regards to potentiating SFK activity, we
265 examined the ability of MAP2c to sustain SFK activation in stimulated 3T3 cells. In this system, SFK
266 activation was indirectly assessed and we had previously shown that tau was able to prolong SFK activation

267 following stimulation. When MAP2 was compared to tau in this assay, we found that MAP2 resembled tau,
268 prolonging SFK activation in cells (Fig. S5).

269 Interestingly, when MAP2 levels were assessed in Fyn KO and DKO mice, MAP2 was decreased
270 by 23.6% and 33.2 %, respectively, relative to WT mice ($p=0.044$ and $p=0.0045$; Fig. 6A), with was no
271 significant difference between the two ($p=0.438$; Fig. 6A). The labeling of MAP2 in 12-month-old
272 hippocampal sections from the four genotypes confirmed the biochemical result (Fig. S4). Compared to
273 WT, hippocampal sections of Fyn KO and DKO mice appeared to display shorter MAP2 fibers and weaker
274 MAP2 intensity while tau KO mice appeared to show stronger MAP2 intensity (Fig. S4). Therefore, in
275 regulating MAP2 expression levels, Fyn appeared to act downstream of tau, since the DKO resembled the
276 Fyn KO. In the DKO, the decrease in MAP2 most likely eliminated the ability of MAP2 to compensate for
277 the loss of tau. Thus, when DKO neurons experienced a further decrease in calcium influx, relative to the
278 Fyn KO, we were able to identify a Fyn-independent effect that tau had on glutamate-induced calcium
279 influx. In the tau KO, it was likely that this effect was not detected because of the increased MAP2 level.

280

281 **Tau KO neurons had more dendritic MAP2-Fyn complexes than WT neurons**

282 To determine whether MAP2-Fyn complexes might compensate for the loss of tau-Fyn complexes
283 in the tau KO, MAP2-Fyn and MAP2-pSFK complexes were visualized in WT and tau KO primary
284 hippocampal cultures using PLA (Fig. 7). Relative to WT dendrites, tau KO dendrites had a 32.5% increase
285 in MAP2-Fyn density (Fig. 7A, B, G) and a 39.9% increase in MAP2-pSFK density (Fig. 7C, D, H). As
286 expected, we did not find any MAP2-containing complexes in axons, identified as MAP2 negative and
287 tubulin positive processes (Fig. 7, open arrowheads). In tau KO neurons, MAP2-Src complex density was
288 increased 36.8% relative to WT neurons (Fig. 7 E, F, I). Together, these data support the notion that MAP2
289 compensates for tau in terms of interacting with SFKs in dendrites.

290

291 **Discussion**

292 In the present study, we generated tau^{-/-}/Fyn^{-/-} DKO mice to investigate the combined effect of tau
293 and Fyn and found that they resembled tau KO mice in motor tasks and protection from PTZ while
294 resembling Fyn KO mice in cognitive tasks. DKO and Fyn KO mice also had decreased Y1472-NR2B
295 phosphorylation levels in hippocampal insoluble PSD fractions. These changes were accompanied by a
296 reduction in glutamate-induced Ca²⁺ response in Fyn KO and DKO primary hippocampal neurons, with
297 DKO neurons having a more severe reduction than Fyn KO neurons, indicating that tau may have a Fyn-
298 independent effect on regulating Ca²⁺ influx. Together with our findings that MAP2 potentiated Fyn activity
299 and that dendritic MAP2-Fyn complexes were increased in tau KO neurons relative to WT neurons, our

300 data suggest that MAP2 may compensate for the loss of tau by increasing the levels of MAP2-Fyn and
301 MAP2-pSFK complexes in dendrites.

302 In motor tasks, our aged tau KO and DKO mice had deficits in the pole test, which was consistent
303 with results obtained by other laboratories (Ikegami et al., 2000; Lei et al., 2014; Lopes et al., 2016a; Morris
304 et al., 2013). Since aged Fyn KO mice did not exhibit such deficits, we concluded that tau, not Fyn, was
305 involved in the motor pathway. Another known property for tau KO mice is their protection from PTZ-
306 induced seizures and A β induced excitotoxicity (Roberson et al., 2007), traits attributed to reduced Fyn
307 levels at the post-synaptic region and disrupted NR stability within the post-synaptic density (Ittner et al.,
308 2010). However, in PTZ-induced seizures, our finding that tau KO mice had greater protection than Fyn
309 KO mice suggested that tau may participate in Fyn independent pathways to regulate seizure susceptibility.
310 If tau acted only with Fyn, Fyn KO mice would have shown protection against PTZ equal to that of the tau
311 KO. The fact that in response to PTZ, DKO and tau KO mice had similar seizure thresholds argued that
312 Fyn's protective effect against seizure was encompassed by tau's protective effect and did not represent a
313 separate pathway since the DKO mice did not exhibit an additional level of protection relative to tau KO
314 mice. Besides interacting with Fyn and affecting NR2B-PSD95 association, tau has been reported to affect
315 two other pathways that regulate seizure susceptibility. One pathway involves SynGAP1, a site-specific
316 inhibitor of Ras, where it has been found that reducing SynGAP1 in tau KO mice increased susceptibility to
317 PTZ-induced seizure (Bi et al., 2017). A second pathway has been reported where the loss of tau prevented
318 the depletion of Kv4.2, a dendritic potassium channel, and mitigated hyperexcitability in an AD mouse
319 model (Hall et al., 2015). These data indicate that tau is involved in other mechanisms that modulate seizure
320 susceptibility and excitotoxicity in dendrites. We speculate that one or both of these mechanisms will be
321 Fyn-independent.

322 Our data also provide evidence that Fyn plays a role in regulating PTZ seizure severity, as our non-
323 hydrocephalic Fyn KO mice had decreased PTZ-induced seizure susceptibility. Our results disagreed with
324 previous reports that Fyn KO mice had either increased seizure susceptibility (Miyakawa et al., 1996) or
325 similar susceptibility (Kojima et al., 1998) relative to WT mice. However, these previous studies likely
326 included mice with undetected mild/moderate hydrocephalus. By using MRI to identify Fyn KO mice with
327 mild/moderate hydrocephalus, we had found that mice with hydrocephalus had increased seizure
328 susceptibility compared to WT (Fig. S6). In addition, when a cohort of superficially normal Fyn KO mice,
329 with and without hydrocephalus, was analyzed as a group, the seizure threshold resembled that of WT (Fig.
330 S6). Therefore, because prior studies were not aware that the Fyn KO mice might have had mild/moderate
331 hydrocephalus (Kojima et al., 1998; Miyakawa et al., 1996), their data did not reflect the sole contribution
332 of the Fyn KO trait. Our data suggest that the Fyn KO trait conferred mild protection against seizures.

333 In agreement with prior studies where mice lacking Fyn, but not other members of Src family non-
334 receptor tyrosine kinases, had deficits in LTP and performance in the Morris water maze (Grant et al.,
335 1992), we found Fyn, not tau, to be critically involved in cognitive tasks (Dawson et al., 2010; Dawson et
336 al., 2001; Ittner et al., 2010; Kimura et al., 2014; Lei et al., 2012; Li et al., 2014; Morris et al., 2013;
337 Roberson et al., 2007; van Hummel et al., 2016). In contextual fear conditioning, both Fyn KO and DKO
338 mice already showed deficits on training day, where the mice differed from WT at the 5th (DKO) or 6th (Fyn
339 KO) minute (Fig. 2B). Thus, it is possible that Fyn depletion could have changed the way animals perceived
340 pain, leading to a decrease in the saliency of the shock and weakening the association between shock and
341 the chamber, with learning and memory being normal. Another possibility is that the expression of freezing
342 behavior itself was impaired. Future studies using cue induced freezing or predator odor induced freezing
343 would determine whether Fyn and/or tau depletion affected freezing behavior. In Fyn KO and DKO mice,
344 the cognitive deficits were accompanied by decreases in Y1472-NR2B phosphorylation levels in the
345 hippocampal insoluble PSD fractions while WT and tau KO mice had no memory deficits and no decreases
346 in pY1472-NR2B. The similarity in the levels of pY1472-NR2B in our WT and tau KO mice agree with
347 other findings using the same tau KO line used here (Lopes et al., 2016b). However, another report using a
348 different tau KO line found reduced levels of pY1472-NR2B (Ittner et al., 2010). The fact that different
349 lines of tau KO mice exhibited different phenotypes underlines the complexity of the analysis of tau KO
350 mice (Ke et al., 2012; Lei et al., 2014).

351 In the brain, tyrosine phosphorylation of NR2B is important in regulating receptor trafficking,
352 gating kinetics, and Ca²⁺ permeability (reviewed by (Groverman et al., 2012; Kalia et al., 2004; Trepanier et
353 al., 2012)). Since Fyn KO and DKO mice had reduced levels of pY1472-NR2B, we examined calcium
354 influx using hippocampal neurons of each genotype. As expected, Fyn KO neurons had reduced glutamate-
355 induced Ca²⁺ response relative to WT neurons. Interestingly, DKO neurons had an even greater reduction
356 relative to Fyn KO neurons while tau KO neurons had a response similar to WT neurons, suggesting that
357 tau had a Fyn-independent role in regulating Ca²⁺ response that was unmasked in the absence of Fyn.
358 Possible mechanisms include interactions of tau with other Ca²⁺ permeable channels such as voltage gated
359 Ca²⁺ channels, regulators of NMDARs, or other components of the PSD, with tau modulating signaling
360 pathways that increase intracellular Ca²⁺ levels. Additional studies are needed to explore these nonexclusive
361 possibilities. Our Ca²⁺ response data from the tau KO neurons agree with a report showing that brain slices
362 from tau KO mice had no change in glutamate-induced Ca²⁺ response profiles (Bi et al., 2017) and is
363 consistent with the overall picture of the tau KO having no changes in excitatory post-synaptic currents
364 (EPSCs), AMPA/NMDA current ratios, and LTP (Ittner et al., 2010; Kimura et al., 2014; Roberson et al.,
365 2011). However, when tau was acutely depleted by shRNA in WT neurons, a decrease in Ca²⁺ response
366 after glutamate stimulation was revealed (Miyamoto et al., 2017). This suggested that extended tau

367 depletion, achieved through genetic ablation in a tau KO mouse, may be fundamentally different from acute
368 tau depletion achieved through the use of tau shRNA. Indeed, although no permanent deficits in axonal
369 development were reported in neurons from tau KO mice (Dawson et al., 2001; Harada et al., 1994), WT
370 neurons treated with tau antisense oligonucleotides or shRNA exhibited dramatic deficits in axon formation
371 defective migration properties, abnormalities in mitochondria transport, and developmental deficits
372 (Caceres and Kosik, 1990); (Sapir et al., 2012). These findings indicate that comparing neurons from a
373 knockout animal to those created by treating WT neurons with shRNA may be less straightforward than
374 expected. Successful creation of a KO animal may be required to select genetic alterations that allow pups
375 to be born and animals to be bred. Moreover, the extent of compensation in the tau KO mouse has been
376 analyzed by microarray analysis and it was found that in the brain, several signal transduction genes such as
377 fosB and c-fos were up-regulated 5-24 fold (Oyama et al., 2004). Based on these findings, we demonstrated
378 that tau was required for NGF-induced AP-1 activation (Leugers and Lee, 2010). The fact that the loss of
379 tau required up-regulation of both fosB and c-fos for compensation suggested that AP-1 activation was a
380 critical tau function required by a mouse to survive.

381 Our finding that tau KO had no memory deficits, no changes in pY1472-NR2B, and no changes in
382 glutamate induced Ca^{2+} response suggested that either Fyn did not require tau to mediate these functions or
383 that another protein was compensating for the loss of tau with respect to its Fyn interaction. Interestingly,
384 other microtubule-associated proteins (MAPs), MAP1A/1B and MAP2, are upregulated in tau KO mice
385 (Harada et al., 1994; Ma et al., 2014) and in agreement with previous data reported for our tau KO mouse
386 line (Ma et al., 2014), we found MAP2 to be increased in our tau KO mice (Fig. 6A). In contrast, MAP2
387 was significantly decreased in Fyn KO mice, correlating with the reduction and shortening of MAP2
388 positive dendrites described previously (Kojima et al., 1997). In DKO mice, MAP2 was decreased,
389 indicating that the Fyn effect superseded the tau effect on MAP2 levels. The mechanisms by which Fyn
390 depletion down-regulated or tau depletion up-regulated MAP2 are not known. However, since Fyn has been
391 shown to affect protein translation (White et al., 2008) and tau, fos activation (Leugers and Lee, 2010), the
392 effects of Fyn may be downstream of those of tau. Therefore, in the DKO, the Fyn KO trait resulted in the
393 loss of MAP2-mediated compensation for the loss of tau whereas in the tau KO, up-regulated MAP2 levels
394 would be capable of compensating for the loss of tau. In examining the glutamate induced Ca^{2+} response,
395 the response in tau KO resembled those in WT due to compensation by MAP2. In contrast, with the
396 reduction of MAP2 in both the Fyn KO and DKO and the attenuation of Ca^{2+} influx in the DKO relative to
397 the Fyn KO, we were able to uncover a role for tau in the Ca^{2+} response that was independent of Fyn (Fig.
398 5C).

399 In the tau KO mice, if the tau-Fyn interaction was of critical importance, one would predict that
400 compensation for the loss of the tau-Fyn interaction would occur. Our finding that tau KO neurons had a

401 significantly higher density of MAP2-Fyn complexes in the dendrites relative to WT neurons suggested that
402 MAP2 would have an increased role in interacting with Fyn in the absence of dendritic tau. We also showed
403 that similar to tau, MAP2 also potentiated Fyn activity (Fig. 6B, C), leading us to hypothesize that MAP2
404 would have an increased role in interacting with Fyn, affecting NR2B phosphorylation and NR function in
405 the absence of dendritic tau. Future studies using gain-of-function and loss-of-function experiments would
406 further test this hypothesis.

407 In summary, we generated a tau^{-/-}/Fyn^{-/-} DKO mouse and found that tau was capable of regulating
408 Ca²⁺ influx in a Fyn-independent manner, a property that we propose to be mimicked by MAP2 in the
409 absence of tau. As dysregulation of Ca²⁺ influx is a known mechanism leading to excitotoxicity in AD, our
410 findings identify tau as a new therapeutic target in the regulation of neuronal Ca²⁺. Most importantly,
411 increased levels of dendritic MAP2-Fyn complexes in the tau KO highlight the importance of the tau-Fyn
412 interaction and indicate the need to compensate if tau is lost. Our findings suggest that the association and
413 activation of Fyn by tau in dendrites are critical neuronal functions.

414

415

Methods and Materials

416 Mice

417 Tau KO (C57BL/6) mice (Dawson et al., 2001) and Fyn KO (C57BL/6/S129) mice (Stein et al.,
418 1992) were obtained from Jackson Laboratories and crossed to generate heterozygotes. The heterozygotes
419 were then bred to generate tau^{-/-}/Fyn^{-/-} and tau^{+/-}/Fyn^{+/-} mice (Fig. S1). Mice were genotyped as described
420 by Jackson Laboratories. All procedures were approved by the University of Iowa Institutional Animal Care
421 and Use Committee and in strict accordance with the NIH Guide for the Care and Use of Laboratory Mice.

422 When the homozygous Fyn KO trait was bred into a C57BL/6 background, the mice exhibited a
423 50% chance of developing non-obstructive hydrocephalus postpartum (Goto et al., 2008). The DKO mice
424 also developed hydrocephalus. Magnetic resonance imaging (MRI) was used to differentiate normal from
425 hydrocephalic mice. Only normal mice screened by MRI were used for behavioral and biochemical tests.

426

427 Magnetic resonance imaging

428 Varian Unity/Inova 4.7 T small-bore MRI system (Varian, Inc., Palo Alto, CA; Small Animal
429 Imaging Facility, University of Iowa) with an in-plane resolution of 0.13 x 0.25 mm² and 0.6 mm slice
430 thickness was used. Coronal images were collected and lateral ventricle volume sizes were analyzed with
431 ImageJ (Carter et al., 2012). A lateral ventricle volume ≤0.2 units was normal, ≥0.2 and ≤ 2 units was
432 moderate, and ≥ 2 units was severe hydrocephalic.

433

434 **Open field test**

435 The open field test was done at 8 weeks as described(Coryell et al., 2007) with a dimension of 40.6
436 \times 40.6 \times 36.8 cm open-field chamber (San Diego Instruments, San Diego, CA), 55 lux, for 20 min. Total
437 activity was measured by total beam breaks and central activity was measured by beam breaks in center
438 (15.2 \times 15.2 cm). 14 WT (14 male), 20 Tau KO (9 male, 11 female), 18 Fyn KO (15 male, 3 female), 13
439 (10 male, 3 female) DKO mice were tested. Since there were no statistically significant differences between
440 males and females, their results were combined.

441

442 **Pole test**

443 The pole test was done at 12 months of age as described(Lei et al., 2014). Trials were excluded if
444 the mouse jumped or slid down the pole. Between tests, the pole was cleaned using 70% ethanol. 9 WT, 13
445 tau KO, 11 Fyn KO, 18 DKO male mice were tested.

446

447 **Novel object recognition**

448 The novel object recognition test was done at 10 weeks as described(Tang et al., 2001). The time
449 each animal spent exploring objects (defined as nose within an inch of the object) was manually
450 determined. Discrimination index [DI = $(X_1 - X_2) / (X_1 + X_2)$] was calculated. 17 WT (12 male, 5 female), 17
451 tau KO (12 male, 5 female), 28 Fyn KO (16 male, 12 female), 18 DKO (14 male, 4 female) mice were used.
452 Since there were no statistically significant differences between males and females, their results were
453 combined.

454

455 **Contextual fear conditioning**

456 Contextual fear conditioning was done at 11 weeks as described(Sowers et al., 2013), using a near-
457 infrared video-equipped fear conditioning chamber (Med Associates, Inc., St. Albans, VT). Freezing was
458 scored with VideoFreeze software (Med Associates, Inc.). 32 WT (27 male, 5 female), 29 tau KO (21 male,
459 8 female), 21 Fyn KO (14 male, 7 female), 20 DKO (14 male, 6 female) mice were tested. Since there were
460 no statistically significant differences between males and females, their results were combined.

461

462 **Pentylentetrazole-induced seizures**

463 Pentylentetrazole (PTZ)-induced seizure was done at 12 weeks as described(Roberson et al.,
464 2007). A modified Racine seizure scale was used: 0: normal behavior; 1: immobility; 2: tail extension; 3:
465 forelimb clonus; 4: generalized clonic activity; 5: bouncing; 6: tonic extension; 7: death(Loscher et al.,
466 1991; Racine, 1972). 25 WT (14 male, 11 female), 21 tau KO (9 male, 12 female), 36 (25 male, 11 female)

467 Fyn KO, and 26 DKO (19 male, 7 female) mice were used. Since there were no statistically significant
468 differences between males and females, their results were combined.

469

470 **Tissue preparation and subcellular fractionation**

471 PSD preps were isolated from hippocampi of 9-12 months old mice (Ittner et al., 2010; Lopes et al.,
472 2016b; Milnerwood et al., 2010). As described, crude synaptosomal membranes were resuspended with 1%
473 Triton X-100 containing buffer and after centrifugation, the supernatant containing non-PSD membranes
474 was retained as the “soluble non-PSD” fraction. The pellet was resuspended in 1% Triton X-100, 1%
475 deoxycholic acid, 1% SDS containing buffer and after centrifugation, the supernatant was retained as the
476 triton “insoluble PSD” fraction.

477

478 **Primary hippocampal neuron culture**

479 Primary hippocampal neuronal culture was performed as described (Beaudoin et al., 2012).
480 Hippocampi from P0 pups were isolated and digested in 1 mg/ml trypsin (Sigma-T7409) and sequentially
481 triturated with Pasteur pipettes. Cells were plated with MEM media (Gibco) containing 10% horse serum
482 and 1 μ M insulin (Sigma-I5500) and maintained with Neurobasal-A Plus and 2% B27 Plus (Gibco) at 37 °C
483 and 5% CO₂ until harvest. 50% of media was changed every 3 days.

484

485 **In situ proximity ligation assay (PLA) and immunofluorescence**

486 PLA components were purchased from Sigma (Duolink® *in situ*) and the assay was performed per
487 manufacturer’s instructions. Cells were imaged using confocal microscopy (Leica SP8 STED) and exported
488 to ImageJ for analysis.

489 Primary antibodies used were: DA9 (1:50, mAb, generous gift of Dr. Peter Davies), Fyn3 (sc-16,
490 1:250, Santa Cruz), MAP2 (1:1000, chicken polyclonal, Thermo Fisher, Cat # PA1-10005), MAP2 (HM-2,
491 1:250, Sigma M4403), Src (36D10, 1:250, rabbit polyclonal, Cell Signaling, Cat #2109), phospho-Src
492 (1:250, rabbit polyclonal, Tyr418; Thermo Fisher, Cat #44660G), and tubulin (1:50, YL1/2; Accu-Spec).
493 Secondary antibodies used were anti-mouse Alexa 488 (1:250, Molecular Probes), anti-chicken Alexa 633
494 (1:1000, Thermo Fisher), and anti-rat Alexa 647 (1:250 Jackson Immunoresearch).

495 To quantify the density of PLA puncta, ten random areas on each coverslip were photographed.
496 Axons were identified by either tau or tubulin positive staining and MAP2 negative staining; dendrites were
497 identified by MAP2 positive staining. The lengths of all visible processes were measured using ImageJ and
498 all visible PLA puncta were manually counted. For each of the 10 areas, the densities of dendritic or axonal
499 PLA puncta were calculated by dividing the number of PLA puncta located on dendrites or axons by the
500 total dendrite or axon length, respectively. The experiment was performed 3 times. In Fig. 3, the density of

501 PLA in dendrites was expressed relative to the density in axons, where the average value was set as “1”. In
502 Fig. 7, the density of PLA in tau KO mice was expressed relative to the density in WT mice, where the
503 average value was set as “1”. In Fig. S3, for each neuron, the proximal axon length was defined as the
504 average length of the dendrites from that neuron.

505

506 **Ca²⁺ imaging**

507 Ca²⁺ imaging was performed on primary hippocampal neurons (DIV 11-12) as described (Kim et al.,
508 2009). Hippocampal neurons were loaded with 2 μM Fura-2/AM (Invitrogen) and then mounted on an
509 inverted microscope (Olympus IX-71). The cells were perfused with HEPES buffered Hank's salt solution
510 (HH buffer) to establish baseline and then stimulated with 100 μM Glutamate, 10 μM Glycine, and 0.2 μM
511 Tetrodotoxin (TTX) in HH buffer. [Ca²⁺]_i changes were continuously recorded by alternately exciting
512 fluorescence at 340 nm (12-nm bandpass) and 380 nm (12-nm bandpass) using a Polychrome V
513 monochromator (TILL Photonics, Munich, Germany). Fluorescence was recorded at 530 nm (50-nm
514 bandpass) using an IMAGO charge-coupled device camera (640 × 480 pixels; TILL Photonics). A 20x
515 objective (numerical aperture = 0.75, Olympus, Japan) and a 2×2 binning set at room temperature was used.
516 [Ca²⁺]_i was derived using the formula $[Ca^{2+}]_i = K_d \beta (R - R_{min}) / (R_{max} - R)$, with $K_d = 275$ nM (Shuttleworth and
517 Thompson, 1991), $R = F_{340nm} / F_{380nm}$, $R_{min} = 0.21$, $R_{max} = 3.45$, and $\beta = 6.97$. Data were analyzed using
518 TILLvisION version 4.0.12 software (TILL Photonics).

519

520 **Immunoprecipitation**

521 3T3 (NIH) cells were grown in α-MEM supplemented with 10% fetal bovine serum (Hyclone) and
522 transfected with either Fyn alone or with Fyn and MAP2c together. Cells were harvested with RIPA lysis
523 buffer. Fyn was IPed with 1 μg Fyn antibody (mouse monoclonal antibody [mAb], sc-434, SantaCruz) and
524 10 μL Protein G plus-agarose (CalBiochem). Washed Protein G bead pellets were resuspended in 2X
525 Laemmli buffer and subjected to Western blot analysis. 3T3 cells have not been reported as
526 contaminated or misidentified (International Cell Line Authentication Committee, October 14,
527 2018).

528

529 **Plasmids**

530 pRc/CMV expressing the N-terminal fragment of human MAP-2c was synthesized by site-directed
531 mutagenesis using the pRc/CMV MAP2c plasmid (Albala et al., 1993) as template. Residue 320 on MAP-
532 2c was mutated to a stop codon. The construct was sequenced to verify the MAP2c sequence and the
533 mutation site.

534

535 ***In vitro* phosphorylation of tubulin**

536 *In vitro* phosphorylation of tubulin was performed as described (Sharma et al., 2007). Fyn or
537 truncated MAP2c (human N-MAP2c residue 1-319) were expressed in 3T3 cells and IPed using 1 µg anti-
538 Fyn (mAb, sc-434, SantaCruz) or 4 µg anti-MAP2 (HM-2, mAb, Sigma, Cat # M4403) and Protein G beads
539 (CalBiochem). The beads containing Fyn were resuspended in 100 µL buffer (0.1% Triton, 50mM Tris
540 pH7.5, 150 mM NaCl, 1 mM EDTA, 1mM AEBSF) and equally divided into two tubes. N-MAP2c beads
541 were added to one of these tubes and nutated at 4°C for 1 hour, centrifuged, and supernatant discarded.
542 Control tube was similarly treated, where Protein G beads were added to beads containing Fyn. 10 µg taxol-
543 stabilized tubulin and kinase reaction buffer was added to both tubes and incubated for 5 min at 37°C. A
544 third tube containing only tubulin, kinase reaction buffer, and 1µg mouse non-specific IgG was used as
545 control. Samples were subjected to western blot analysis.

546

547 **Western blot analysis**

548 Western blotting was performed as described(Lee et al., 1998). Primary antibodies used were:
549 Tau5-HRP (1:10,000, (Leugers and Lee, 2010)); Fyn3 (sc-16, 1:1000, rabbit polyclonal, Santa Cruz);
550 GAPDH (1:25,000, mAb, Chemicon, Cat # MAB374); synaptophysin (SP15, 1:1000, mAb, Millipore, Cat
551 # MAB329-C); PSD-95 (EP2652Y, 1:1000, rabbit monoclonal, Millipore, Cat# 04-1066); NR2B (1:1000,
552 mAb, One World Labs, StressMarq, Cat # SMC-33D); phospho-Y1472-NR2B (1:1000, rabbit polyclonal,
553 PhosphoSolutions, Cat # p1516-1472); Src (GD-11, 1:1000, mAb, Milipore, Cat # 05-184); phospho-SFK
554 (1:1000, phospho-Src Tyr 418, rabbit polyclonal, Cell Signaling, Cat # 2101); MAP2 (HM-2, mAb, 1:1000,
555 Sigma, Cat # M4403); generic phospho-tyrosine (4G10, 1:1000, mAb, Millipore, Cat # 05-321); and tubulin
556 (1:1000, YL1/2; Accu-Spec). Quantification was done by densitometry using ImageJ. Protein levels from
557 the PSD fraction were normalized to β-actin (Fig. 4) and protein levels from crude lysates were normalized
558 to GAPDH (Fig. 6A), with values subsequently normalized to WT levels, which were represented as “1”. In
559 Fig. 6C, pSFK was normalized to total Fyn and values from Fyn+MAP2c were normalized to Fyn alone
560 condition with the latter represented as “1”. In Fig. 6D, 4G10 was normalized to both tubulin and Fyn and
561 the Fyn+N-MAP2c condition was normalized to the Fyn alone condition with the latter represented as “1”.

562

563 **Statistical Analysis**

564 Statistical analysis was carried out with either Microsoft Excel or GraphPad Prism 7.0 using
565 ordinary one way ANOVA with Tukey’s post hoc multiple comparison or unpaired parametric t-test when
566 appropriate. For Fig. 2B and 2E, 2-way ANOVA with Tukey’s post hoc multiple comparison was used.

567 $p \leq 0.05$ was considered as statistically significant, with $p \leq 0.05$ denoted as *, $p \leq 0.01$ denoted as **, $p \leq 0.001$
568 denoted as ***, and $p < 0.0001$ denoted as ****.

569

570

571 **Declarations**

572

573 **Animals**

574 Animal care was in compliance with National Institutes of Health guidelines for the care and use of
575 laboratory animals and all animal procedures were approved by the University of Iowa Institutional
576 Animal Care and Use Committee.

577 **Availability of data and material**

578 The datasets analysed in the current study are available from the corresponding author on
579 reasonable request.

580 **Competing interests**

581 No competing interests declared.

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586 **Authors' contributions**

587 G Liu designed the experiments, performed the PCR, behavioral, biochemical, calcium imaging,
588 and PLA experiments, analyzed data, and wrote the manuscript. R Thangavel and E Adams
589 performed IHC and analysis, MB Francis generated the double knockout mice, Y Kim assisted with
590 PLA experiments, J Rysted and Z Lin assisted with calcium imaging, RJ Taugher assisted with
591 behavioral work, and JA Wemmie and YM Usachev provided valuable discussions and data
592 analysis. G Lee designed experiments, analyzed data, and revised the manuscript.

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600

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821

822

Figure Legends

822

823

824 **Fig. 1: Confirmed by MRI, tau^{-/-}/Fyn^{-/-} double knockout (DKO) mice with hydrocephalus exhibited**
825 **behavioral abnormalities.**

826 A) Polymerase chain reaction (PCR) products from heterozygous (Het), WT, and DKO mice are
827 shown.

828 B) Western blot of crude brain lysate showed that DKO mice did not express tau or Fyn.

829 C) Examples of T2 weighted MRI of coronal mouse brains showing normal, moderate, or severe
830 hydrocephalus.

831 D) In the open field test, Fyn KO and DKO mice with hydrocephalus (moderate or severe) had
832 increased total movements relative to mice with no hydrocephalus. n=18 Fyn KO, 13 DKO, 41 Fyn KO
833 hydro, 19 DKO hydro. ****p≤ 0.0001.

834 E) In contextual fear conditioning, Fyn KO and DKO mice with moderate or severe hydrocephalus
835 had decreased freezing relative to mice with no hydrocephalus. n=21 Fyn KO, 20 DKO, 33 Fyn KO hydro,
836 23 DKO hydro. **p=0.0014, ****p≤ 0.0001.

837 Mean ± S.E.M. are shown. Ordinary one-way ANOVA with Tukey's post-hoc multiple comparison was
838 used for both panels D and E.

839

840 **Fig. 2: In memory tasks, DKO recapitulated the behavior of Fyn KO mice whereas in PTZ induced**
841 **seizures, DKO mimicked the tau KO mice.**

842 A) In novel object recognition, there was no difference in object preference on training day (One-way
843 ANOVA p= 0.3221; left panel) while on testing day, Fyn KO and DKO mice had significantly reduced
844 interaction with the novel object (right panel; One-way ANOVA: p<0.0001; ***p=0.0004, ****p≤
845 0.0001). n= 17 WT, 17 tau KO, 28 Fyn KO, 18 DKO.

846 B) In contextual fear conditioning, using minute by minute measurements, WT and tau KO mice spent
847 more time freezing compared to Fyn KO and DKO mice on both training day (left) and testing day (right).
848 n= 32 WT, 29 tau KO, 21 Fyn KO, 20 DKO.

849 C) Fyn KO and DKO mice spent less time freezing than WT (p=0.0002, p<0.0001, respectively) and
850 tau KO mice (p=0.0075, p=0.0004, respectively). One-way ANOVA: p<0.0001; **p= 0.0075, ***p=
851 0.0002 or 0.0004, ****p≤ 0.0001.

852 D) Ratio of time freezing in first minute of day 2 to time freezing in last minute of day 1 show an
853 average of 1 for all genotypes, indicating a learning rather than memory deficit for Fyn KO and DKO mice.
854 One-way ANOVA: p=0.0509

855 E) In PTZ induced seizures, tau KO and DKO mice were equally protected while Fyn KO mice were
856 only moderately protected as measured in latency to reach each seizure stage. n= 25 WT, 21 tau KO, 36 Fyn
857 KO, and 26 DKO.

858 F) For maximum seizure stage reached, tau KO and DKO mice were similarly protected relative to
859 WT and Fyn KO mice; Fyn KO mice were only slightly protected relative to WT mice. One-way ANOVA:
860 $p < 0.0001$; $**p = 0.0011$, $***p = 0.0003$, $****p \leq 0.0001$. n= 25 WT, 21 tau KO, 36 Fyn KO, and 26 DKO.
861 Mean \pm S.E.M. are shown. Ordinary one-way ANOVA with Tukey's post-hoc multiple comparisons were
862 used for A, B, C, D, F. Ordinary two-way ANOVA with Tukey's post-hoc multiple comparisons were used
863 for B and E.

864

865 **Fig. 3: Tau-Fyn, tau-pSFK, and tau-Src complexes are present in neurons.**

866 A,F,K) Using WT hippocampal neurons, tau-Fyn (A), tau-pSFK (F), and tau-Src (K) complexes were
867 identified using proximity ligation assay as described in Materials and Methods. Scale bar: 25 μ m.

868 B-D, G-I, L-N) Tau-Fyn, tau-pSFK, and tau-Src complexes were found in axons (B, G, L), dendrites (C, H,
869 M), and cell bodies (D, I, N), respectively. Scale bar: 10 μ m (B, C, G, H, L, M); 20 μ m (D, I, N).

870 E, J, O) PLA complex density in WT neurons was quantitated as described in Materials and Methods.

871 Mean \pm S.E.M. are shown. n=30 areas from each condition were analyzed. $****p \leq 0.0001$ as determined by
872 unpaired parametric 2 tailed t-test.

873

874 **Fig. 4: Fyn KO and DKO hippocampal PSD fraction had decreased phospho-Y1472 NR2B and**
875 **phospho-SFK levels**

876 A) Schematic for separating insoluble PSD and soluble non-PSD fractions from crude synaptosomes.

877 B) Soluble non-PSD fraction isolated from crude hippocampal synaptosomes of WT mice showed
878 synaptophysin, a pre-synaptic marker, while insoluble PSD fraction showed PSD95, a post-synaptic marker.
879 Fyn and tau were found in both fractions.

880 C) Western blotting of hippocampal insoluble PSD fractions prepared from WT, tau KO, Fyn KO, and
881 DKO.

882 D) Blots were quantitated by densitometry and normalized. n=9 animals for each genotype. For
883 pY1472-NR2B, relative to WT, levels were decreased in Fyn KO and in DKO ($***p = 0.0008$,
884 $****p < 0.0001$, respectively); relative to tau KO, levels were also significantly decreased in Fyn KO and in
885 DKO ($****p < 0.0001$, $****p < 0.0001$, respectively). For pSFK, relative to WT, levels were decreased in Fyn
886 KO and in DKO ($****p < 0.0001$, $****p < 0.0001$, respectively); relative to tau KO, levels were also
887 decreased in Fyn KO and in DKO ($****p < 0.0004$, $****p < 0.0004$, respectively).

888 Mean \pm S.E.M. are shown. Unpaired two-tailed parametric t-test was used for Fyn and ordinary one-way
889 ANOVA with Tukey's post-hoc multiple comparisons was used for others.

890

891 **Fig. 5: Glutamate induced Ca^{2+} response was impaired in Fyn KO and DKO hippocampal neurons**

892 A) Ca^{2+} response traces from five representative neurons from each genotype are shown; traces were
893 obtained in response to glutamate and glycine as described in Materials and Methods.

894 B) No difference in baseline intracellular $[\text{Ca}^{2+}]_i$ was found among the hippocampal neurons from the
895 different genotypes. n= 105 WT, 115 tau KO, 114 Fyn KO, and 181 DKO cells were analyzed. p=0.2808.

896 C) Upon stimulation, Fyn KO neurons had a 29.9% reduction (****p<0.0001) and DKO neurons had a
897 53.1% reduction (****p<0.0001) in Ca^{2+} response relative to WT neurons. The reduction of the DKO
898 neurons was significantly different from that of Fyn KO neurons (***p=0.0001).

899 Mean \pm S.E.M. are shown. Ordinary one-way ANOVA with Tukey's post-hoc multiple comparisons was
900 used.

901

902 **Fig. 6: Microtubule-associated protein 2 (MAP2) is increased in tau KO and its association with Fyn**
903 **increases Fyn activity**

904 A) Crude mouse brain lysates from four genotypes were probed for MAP2, with GAPDH as control.
905 Relative to WT mice, MAP2 was increased in tau KO mice (* p=0.021), decreased in Fyn KO mice
906 (*p=0.044) as well as in DKO mice (**p=0.0045). Mean \pm S.E.M. are shown 7 WT, 8 tau KO, 8 Fyn KO,
907 and 8 DKO mice were used.

908 B) Lysates from 3T3 cells confirmed transfection with Fyn alone or Fyn plus MAP2c.

909 C) Fyn IP from cells co-transfected with Fyn plus MAP2c showed increased pSFK levels relative to
910 Fyn IP from cells transfected with Fyn alone. No Src was detected (C). n=4 independent experiments were
911 conducted. *p=0.0254.

912 D) Tubulin was incubated either alone, with Fyn, or with Fyn plus N-MAP2c, then probed with anti-
913 phospho-tyrosine (4G10). n=6 independent experiments.

914 Unpaired two-tailed parametric t-test was used for A, C, D. Mean \pm S.E.M. are shown.

915

916 **Fig. 7: Tau KO neurons have more MAP2-Fyn, MAP2-pSFK, and MAP2-Src complexes relative to**
917 **WT neurons.**

918 MAP2-Fyn (A, B), MAP2-pSFK (C, D), and MAP2-Src complexes (E, F) were detected in WT neurons
919 (A,C,E) or tau KO neurons (B, D, F) using proximity ligation assay. No MAP2-SFK complexes were
920 visualized in axons (open arrow heads). Scale bar: 25 μm . MAP2-Fyn (G), MAP2-pSFK (H), and MAP2-
921 Src (I) complex densities were quantitated in both WT and tau KO neurons. Mean \pm S.E.M. are shown, 88

922 images for MAP2-Fyn, 30 images for MAP2-pSFK, and 30 images for MAP2-Src were analyzed. Unpaired
923 parametric two-tailed t-test was used. For G) and I), **** $p \leq 0.0001$ and for H), ** $p=0.0033$.

924

925

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927

Figure 1

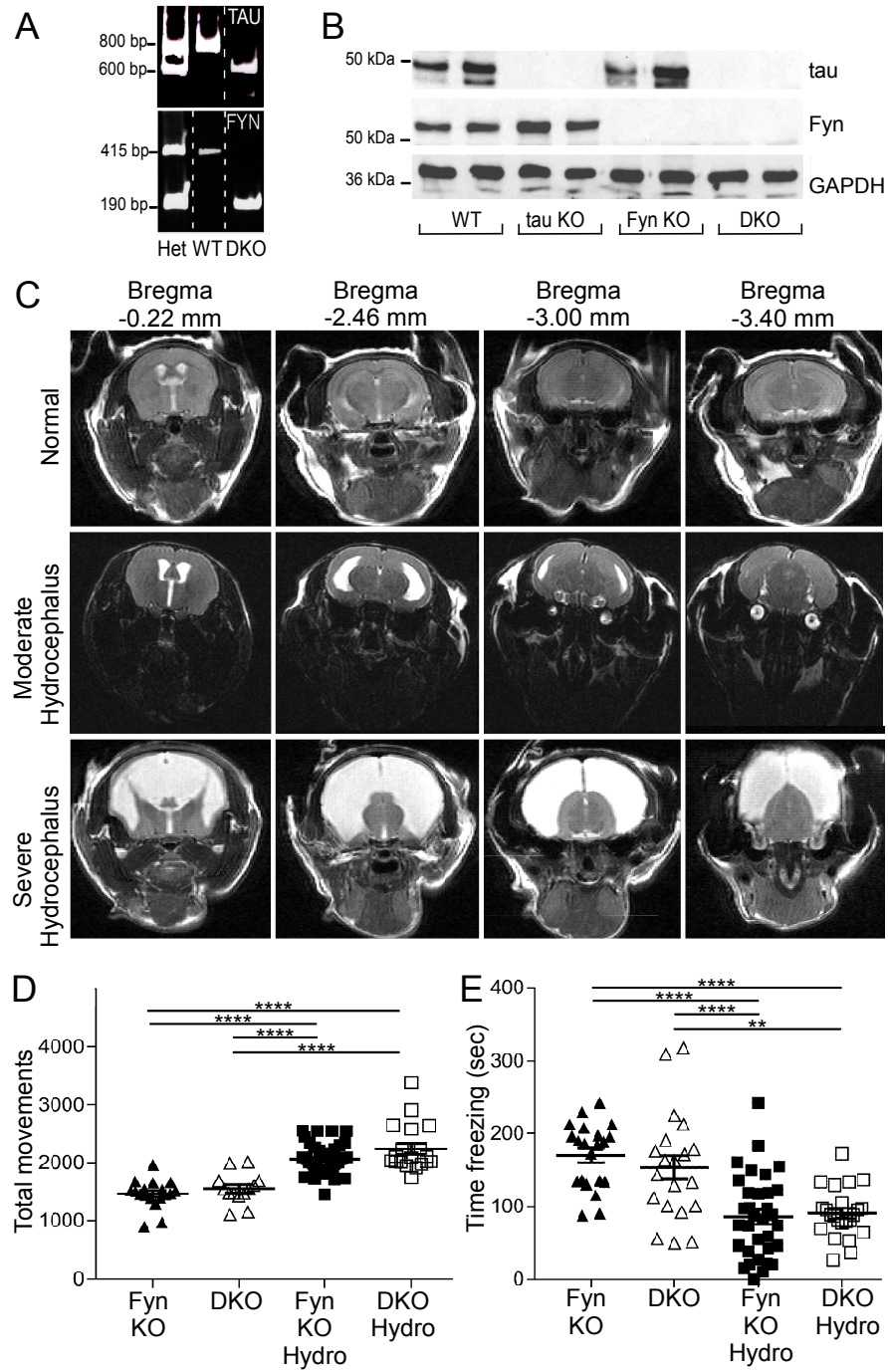


Figure 2

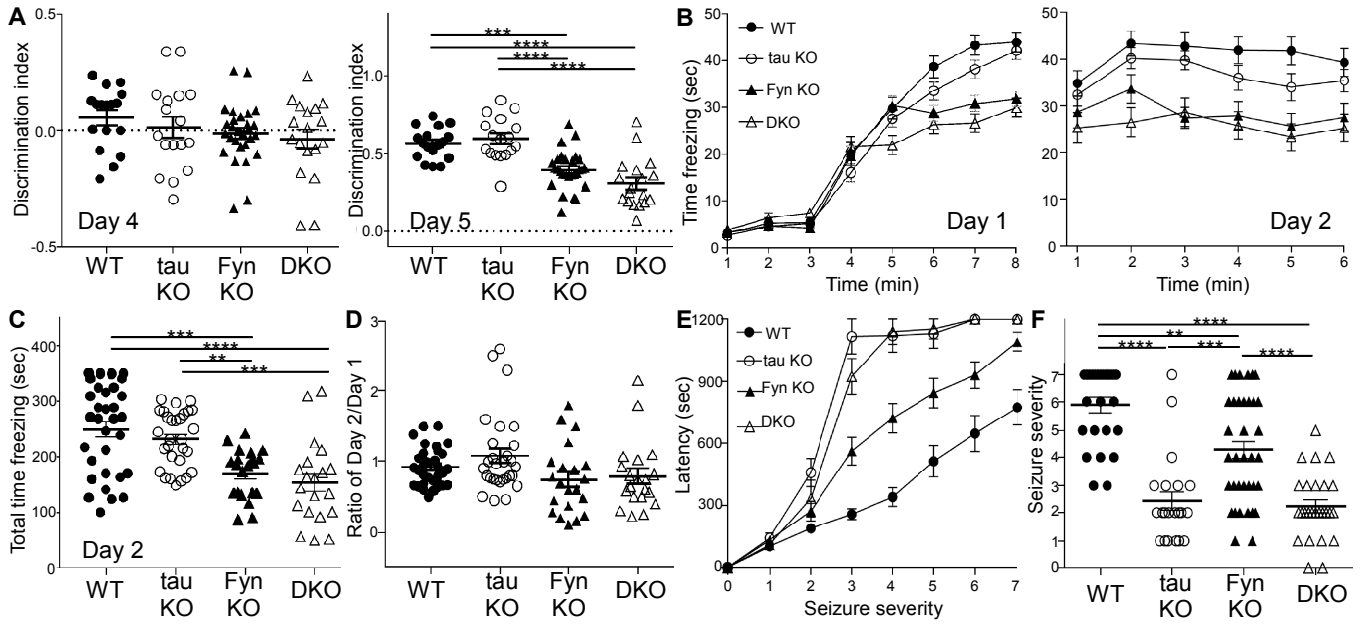


Figure 3

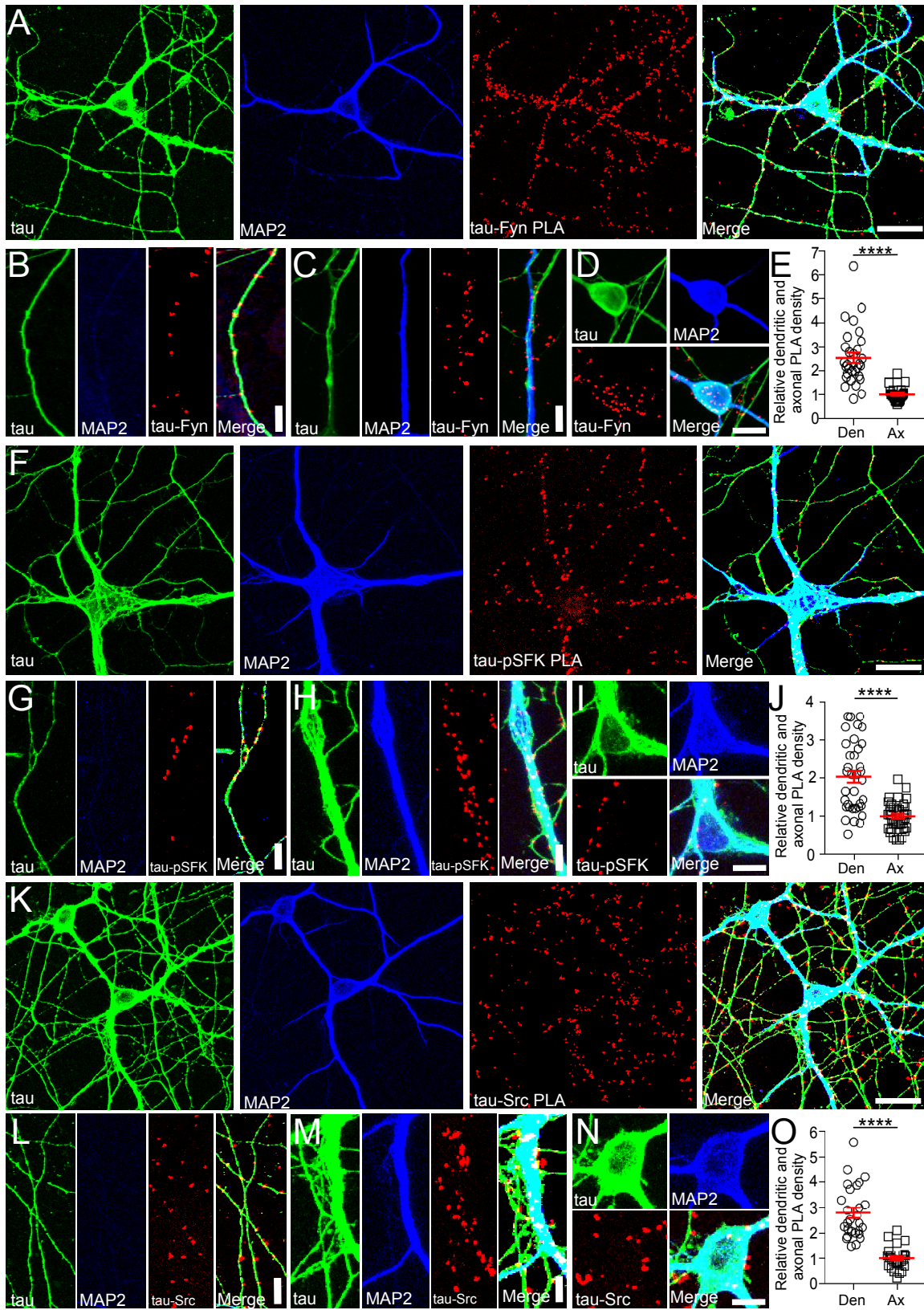


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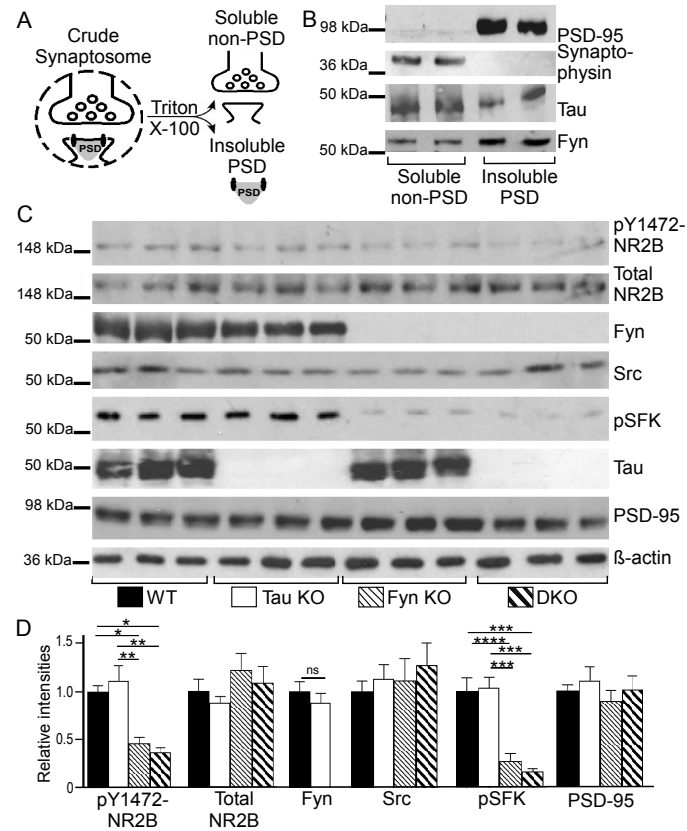


Figure 5

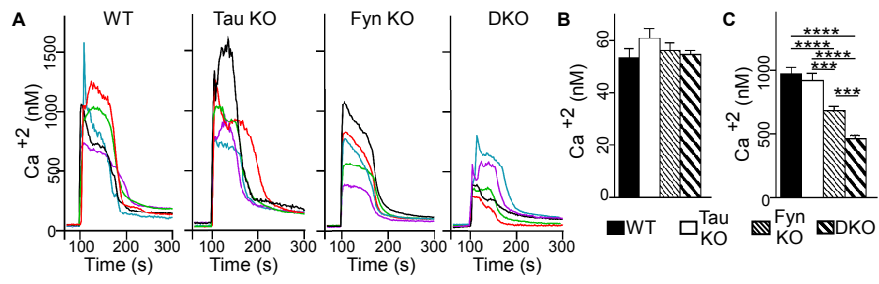


Figure 6

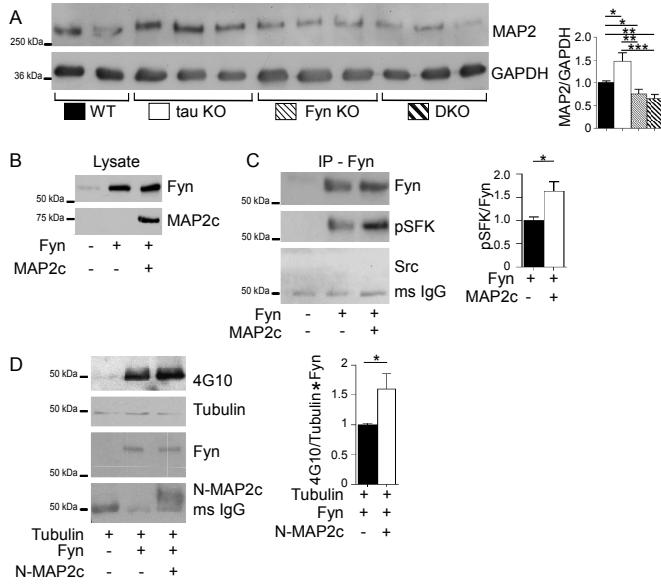


Figure 7

