Crry silencing alleviates Alzheimer’s disease injury by regulating neuroinflammatory cytokines and the complement system

Abstract

Complement component (3b/4b) receptor 1 (CR1) expression is positively related to the abundance of phosphorylated microtubule-associated protein tau (tau), and CR1 expression is associated with susceptibility to Alzheimer’s disease. However, the exact role of CR1 in tau protein-associated neurodegenerative diseases is unknown. In this study, we show that the mouse Cr1-related protein Y (Crry) gene, Crry, is localized to microglia. We also found that Crry protein expression in the hippocampus and cortex was significantly elevated in P301S mice (a mouse model widely used for investigating tau pathology) compared with that in wild-type mice. Tau protein phosphorylation (at serine 202, threonine 205, threonine 231, and serine 262) and expression of the major tau kinases glycogen synthase kinase-3 beta and cyclin-dependent-like kinase 5 were greater in P301S mice than in wild-type mice. Crry silencing by lentivirus-transfected short hairpin RNA led to greatly reduced tau phosphorylation and glycogen synthase kinase-3 beta and cyclin-dependent-like kinase 5 activity. Crry silencing reduced neuronal apoptosis and rescued cognitive impairment of P301S mice. Crry silencing also reduced the levels of the neuroinflammatory factors interleukin-1 beta, tumor necrosis factor alpha, and interleukin-6 and the complement components complement 3 and complement component 3b. Our results suggest that Crry silencing in the P301S mouse model reduces tau protein phosphorylation by reducing the levels of neuroinflammation and complement components, thereby improving cognitive function.

Key Words: Alzheimer’s disease; cognitive function; complement system; CR1; Crry; neurodegeneration; neuroinflammation; tauopathy

Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by cognitive function deficits and progressive memory loss; it was first discovered in 1906 by Alois Alzheimer (Alzheimer, 1906). Two histopathological hallmarks of AD are intraneuronal neurofibrillary tangles, caused by abnormal aggregation of hyperphosphorylated microtubule-associated protein tau (tau), and extracellular amyloid plaques, composed of amyloid beta (Aβ) peptides (Kidd, 1963; Hardy and Higgins, 1992). As a microtubule-associated protein, tau is usually expressed in neuronal axons and is implicated in the dissociation of microtubules by binding to the tubulin dimers that constitute microtubules (Cleveland et al., 1977). Truncation and phosphorylation of tau protein cause tau to aggregate as neurofibrillary tangles in the proximal axoplasm. A study based on the P301S mouse model (widely used for investigating tau pathology) previously indicated that filamentous tau lesions were associated with loss of neurons and atrophy of the hippocampus and entorhinal cortex; microgliosis and impaired hippocampal synaptic function occurred prior to the formation of filamentous tau tangles (Yoshiyama et al., 2007).

Complement cascades are a vital part of the immune system that function to protect the central nervous system (CNS) from infection; dysregulation of the complement system in the CNS results in neurodevelopmental damage and disease (Lee et al., 2019; Lo

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Graphical Abstract

Crry silencing alleviates tau pathology

Truncation and phosphorylation of tau protein cause tau to aggregate as neurofibrillary tangles in the proximal axoplasm. A study based on the P301S mouse model (widely used for investigating tau pathology) previously indicated that filamentous tau lesions were associated with loss of neurons and atrophy of the hippocampus and entorhinal cortex; microgliosis and impaired hippocampal synaptic function occurred prior to the formation of filamentous tau tangles (Yoshiyama et al., 2007).

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Lee, 2021; Yang et al., 2021). Several potential genes and loci, including the complement component (3b/4b) receptor 1 (CR1) gene (CR1), are associated with susceptibility to AD (Corneveaux et al., 2010; Kucukkilic et al., 2018; Kunkle et al., 2019). The CR1 protein affects the deposition of Aβ in brain tissue (Biffi et al., 2012, Thambisetty et al., 2013). Furthermore, Zhu et al. (2017) implicated CR1 in the progression of AD because of its effect on Aβ deposition, clearance, and tau pathology. The recent observation that the role of CR1 in AD-related tautopathy is not clear. Killick et al. (2013) showed that the levels of complement factor H and the phosphorylation of tau protein at serine 235 were greatly decreased in Cr1-related protein Y (Crry)-deficient (−/−) mice in comparison with controls, implicating Crry protein in AD-associated tautopathy. However, these observations illustrate only that Crry affects tau phosphorylation and tauopathy, and it is not clear whether this is related to the mechanisms of action of Crry in AD-related tautopathy. The expression and distribution of CR1 proteins differ between rodents and humans: the murine complement receptor type 2 (CR2) gene (Cr2) encodes both CR1 and CR2 proteins, whereas human CR1 and CR2 proteins are encoded by separate genes (Jacobson and Weis, 2008). Crry, a rodent-specific gene also known as Cr1-like (Cr1l), is more highly expressed in megakaryocytes than astrocytes and is expressed only to the RNA level in mouse neurons (Davoust et al., 1999). The Crry gene in rodents is the closest ortholog of the human CR1 gene, and it is widely accepted that Crry in murine models acts as the equivalent of CR1 in humans (Molina et al., 1992; Jacobson and Weis, 2008; Killick et al., 2013). Therefore, the Crry gene in murine models can be used to investigate the possible role of CR1 in AD-related tautopathy, which may contribute to further understanding the neuropathological processes in AD.

The complement system is a crucial driver of inflammation (Lee et al., 2019). Overactivation of complement signals can result in neuron injury and neuroinflammation in many neurological diseases (Krance et al., 2019; Parker et al., 2019). Synapse loss in AD is mediated by the complement pathway and microglia and leads to cognitive decline (Stevens et al., 2007). Complement C1q levels were elevated in postsynaptic densities of P301S mice and patients with AD, and the accumulation of C1q was correlated with phosphorylated tau levels and synapse loss (Dejanovic et al., 2018). Wu et al. (2019) showed that P301S mice had elevated levels of classical complement components and the central complement component complement 3 (C3), and C3 expression was associated with tau phosphorylation. Litvinchuk et al. (2018) demonstrated positive correlations between cognitive decline and the expression levels of C3 and C3a anaphylatoxin chemotactic receptor in patients with AD.

The tau P301S (PS19) mouse model is one of the most widely used models for investigating AD-related tautopathies (Yoshiyama et al., 2007; Iba et al., 2013). In this study, the role of CR1 protein in AD-related tautopathy was investigated by examining the role of Crry in with our current mice. Our study aimed to discover the role that the CR1 protein plays in the progression of AD and to identify potential therapeutic targets for AD.

Materials and Methods

Animals

We acquired the mice used in our current study from the Model Animal Research Center, Nanjing University. We used heterozygous transgenic mice (Tg[Prnp-MAPTTP301S]PS19Vie/Nju, specific-pathogen-free grade, 3–9 months old, 12–25 g) that had the murine prion protein gene driven on a B6C3F1 background and expressed the human P301S human 1N4R tau isoform on chromosome 3 (Yoshiyama et al., 2007). For WT controls, we selected littermates with matched genetic background and age. We selected only male mice to minimize the interference of estrogen on cognitive function and neuroinflammation (Wei et al., 2014). Mice were housed at 22°C and 50% relative humidity in a 12-hour light-dark cycle. The Medical Ethics Committee of the Wuxi No. 2 People’s Hospital affiliated to Nanjing Medical University, Wuxi, China, approved the study protocol with approval No. 2020-Y-45 on October 21, 2020. All operations were carried out as per the Animal Research: Reporting of experiments (ARRIVE) guidelines (Kilkenny et al., 2010).

The mice were divided into groups randomly. Signals were collected at the endpoint of each cycle extension, and amplification curve data were evaluated. The double immunofluorescence staining protocol on a 7500 Real-Time PCR system (Thermo Fisher Scientific Inc.). Reverse transcription was carried out at 37°C for 15 minutes, 85°C for 5 seconds, and then samples were kept at 4°C. The complementary DNA was then amplified under the following reaction conditions: 95°C for 30 seconds, 55°C for 30 seconds, and 60°C for 34 seconds. The primer sequences used are listed in Additional Table 2. Signals were collected at the endpoint of each cycle extension, and amplification curve data were evaluated. The 2-ΔΔCT method (Livak and Schmittgen, 2001) was used for analyzing expression data.
Cresyl violet staining

Briefly, all tissue samples were paraffin embedded, cut, dewaxed, rehydrated, and stained in cresyl violet (10 mg/mL, MilliporeSigma). The sections were rinsed with water, dehydrated in increasing concentrations of ethanol, and viewed with a light microscope. Neurons in the hippocampus and ventricle of mice using cresyl violet staining and image software (National Institutes of Health, Bethesda, MD, USA) (Schneider et al., 2012). Two independent, experienced experimenters performed the neuron counts.

Morris water maze test

The Morris water maze test was carried out 2 months following lentiviral particle injection as per the methods provided in a previous study (Jiang et al., 2014), with 6 mice in each group. Briefly, for the hidden-platform training, a circular platform with a diameter of 10 cm was submerged 0.5 cm below the water surface in the southeast quadrant of a large, water-filled container. A video camera (SMART Video Tracking System; Panlab, Harvard Apparatus, Barcelona, Spain) was used to track the swimming paths for 60 seconds, and then a computer-controlled system (SMART Video Tracking System; Panlab, Harvard Apparatus) was used to analyze the swimming paths. All mice were trained to complete 4 trials per day for 5 consecutive days. The length of time taken to reach the submerged platform over the four trials was averaged. When a mouse failed to find the platform within 60 seconds, we picked it up and placed it on the platform for 15 seconds. The platform was removed 24 hours after the last trial, and this was followed by a probe test for 60 seconds. In a probe test, the platform was removed, and the swimming path of the mouse was recorded.

Passive avoidance step-through task

The passive avoidance step-through task was carried out 2 months following lentiviral particle injection, as previously described (Kumaran et al., 2008), with six mice in each group. The test cage (TopScan; CleverSys Inc, Reston, VA, USA) had two equally sized compartments, with a guillotine door separating light and dark compartments. For the acquisition trial, mice were kept in the test cage for 5 minutes and had free movement between the light and dark compartments. For the training test, mice were placed in the light compartment with the closed door and administered a 0.5 mA foot shock for 10 seconds. We excluded animals that took more than 100 seconds to cross to the dark compartment. When a mouse did not enter the dark compartment, we recorded the latency as 300 seconds.

Statistical analysis

The sample sizes in this study were not predetermined using statistical methods, but the sample sizes we used are similar to those in previous studies (Ruan et al., 2020; Izy et al., 2021). All experiments were evaluated using blind methods. Statistical analyses were performed with SPSS Statistics for Windows software, version 25.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of the mean (SEM). The Kolmogorov-Smirnov test was employed for analysis of variables with normal distribution. Independent sample t-tests were applied for pairwise group comparisons. For comparisons among multiple groups, we performed one-way analysis of variance followed by Tukey’s post hoc test. For real-time RT-PCR and qRT-PCR, the melt curve of the target gene was used to verify that a single product was amplified. The relative expression was calculated using the 2-ΔΔCt method. The p-values were obtained using the Student’s t-test or one-way analysis of variance followed by Tukey’s post hoc test. A value of P < 0.05 was considered significant.

Results

Cry protein expression increases with age in the context of AD

The localization of Cry in the brain was explored via double immunofluorescence staining in 6-month-old P301S mice. Cry and the microglial marker ionized calcium-binding adapter molecule 1 (Iba-1) (Ohsawa et al., 2004) were colocalized, suggesting Cry is expressed in microglia (Figure 1A). There was relatively weak colocalization between Cry and glial fibrillary acidic protein, an astrocyte marker (Takizawa et al., 2008), suggesting lower expression of Cry in astrocytes (Figure 1B). However, we observed no association between Cry and neurons labeled with neuronal nuclei antigen (NeuN) (Duan et al., 2016) or oligodendrocytes labeled with oligodendrocyte-specific protein (OSP) (Bronstein et al., 1996), suggesting that Cry is not expressed in neurons and oligodendrocytes of P301S mice (Figure 1C and D).

Figure 1 | Double immunofluorescence staining to identify the localization of Cry in the brains of 6-month-old P301S mice (original magnification 400X).

(A) Cry (FITC, green fluorescence) and the microglial marker Iba-1 (rhodamine, red fluorescence) colocalize in the brains. (B) Weak colocalization between Cry and the astrocyte marker GFAP (rhodamine, red fluorescence). (C) No colocalization between the oligodendrocyte marker OSP (rhodamine, red fluorescence) and Cry. Scale bars: 100 μm (upper) and 25 μm (lower). (D) Cry: Cr1-related protein Y; FITC: fluorescein isothiocyanate; GFAP: glial fibrillary acidic protein; Iba-1: ionized calcium binding adaptor molecule 1; NeuN: neuronal nuclei; OSP: oligodendrocyte specific protein.

The change in Cry protein levels in the brain during the aging process in the AD context was revealed by western blot assay. Our results show that Cry protein levels in the hippocampus and cortex were markedly upregulated in 6-month-old and 9-month-old P301S mice in comparison with 3-month-old P301S mice (n = 6, both P < 0.05), but no statistical difference was found between 6-month-old and 9-month-old P301S mice (n = 6, P > 0.05; Figure 2A–D). Moreover, Cry protein levels in the hippocampus and cortex were also markedly increased in 6-month-old and 9-month-old P301S mice compared with those in age-matched WT mice (n = 6, both P < 0.05; Figure 2A–D). Immunofluorescence staining of Cry confirmed that Cry protein levels in the hippocampus and cortex were increased in 6-month-old P301S mice compared with those in age-matched WT mice (Additional Figure 2).

Furthermore, analysis based on the microglia marker Iba-1 demonstrated increasing microglial activation in the hippocampus and cortex of 3-, 6-, and 9-month-old P301S mice compared with that in age-matched WT mice (n = 6, all P < 0.05), with no statistical difference among 3-, 6-, and 9-month-old P301S mice (n = 6, all P > 0.05; Figure 2A, C, E, and F). However, statistically significant differences were observed when Cry levels were normalized to Iba-1 expression in microglia in 6-month-old P301S mice (n = 6, both P < 0.05; Figure 2G and H), which indicates that Cry upregulation is not a result of the increased number of microglia in this AD mouse model.

Cry expression in brain tissues is inhibited by Cry-specific shRNA

We investigated Cry gene and protein expression and the silencing efficiency of Cry shRNA infusion. Cry gene and protein expression in the cortex of 6-month-old mice that received Cry shRNA decreased by 41.0% and 42.6%, respectively, compared with those in 6-month-old mice that received control shRNA (n = 6, both P < 0.05; Figure 3A–C). Similarly, Cry shRNA infusion decreased Cry gene and protein expression in the hippocampus of 6-month-old mice by 37.2% and 35.3%, respectively, compared with those in...
6-month-old mice that received control shRNA (n = 6, both P < 0.05; Figure 3D–F). These results indicate that Crry expression is successfully inhibited by shRNA infusion.

Decrease in hyperphosphorylated tau levels after Crry silencing

Western blot analyses showed changes at four phosphorylation sites (serine 202, threonine 205, threonine 231, and serine 262) in tau protein in 6-month-old WT and P301S mice that received Crry shRNA or control shRNA. AT8/total tau, threonine 231/total tau, and serine 262/total tau were significantly elevated in whole brains of 6-month-old WT and P301S mice compared with those in WT mice (n = 6, all P < 0.05; Figure 4A–D). Following Crry shRNA infusion, AT8/total tau, threonine 231/total tau, and serine 262/total tau decreased in whole brains of 6-month-old WT and P301S mice by 59.0% (n = 6, P < 0.05; Figure 4A and B), 59.85% (n = 6, P < 0.05; Figure 4A and C), and 52.4% (n = 6, P < 0.05; Figure 4A and D), respectively, compared with those in 6-month-old WT and P301S mice that received control shRNA. Total tau levels did not change in any of the mice tested.

Figure 2 | Crry protein expression increases with age in an Alzheimer’s disease context.

(A, C) Crry, Iba-1, and β-actin protein expression in the cortex and hippocampus of P301S mice and age-matched WT mice at different ages, as visualized using western blot. (B, D–F) Quantification of Crry and Iba-1 protein levels in the cortex and hippocampus of P301S mice and age-matched WT mice. (G, H) Crry expression was significantly upregulated in the cortex and hippocampus of 6-month-old P301S mice after normalizing Crry expression to microglial Crry using western blot. (B, D–F) Quantification of Crry and Iba-1 protein levels in the cortex and hippocampus of P301S mice and age-matched WT mice. (G, H) Protein level analysis was followed by Tukey’s multiple comparison test (G, H). † Data are represented as the mean ± SEM (n = 6 for each group) and were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison test (B, D–F) and by independent sample t-test (G, H). †P < 0.05. Crry: Crry-related protein Y; Iba-1: ionized calcium binding adaptor molecule 1; WT: wild type.

Figure 3 | Crry expression in the brains of P301S mice is significantly decreased by Crry shRNA.

(A–C) mRNA and protein expression of Crry in the cortex of 6-month-old WT and P301S mice after treatment with control shRNA or Crry shRNA. (D–F) mRNA and protein expression of Crry in the hippocampus of 6-month-old WT and P301S mice after treatment with control shRNA or Crry shRNA. mRNA and protein expression levels were normalized to β-actin. Data are represented as the mean ± SEM (n = 6 for each group) and were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison test. †P < 0.05. Crry: Crry-related protein Y; shRNA: short hairpin RNA; WT: wild type.
Cry silencing influences the balance of tau kinases and tau phosphorylation
The activation of major tau kinases, including glycogen synthase kinase-3 beta (GSK3β), AMP-activated protein kinase (AMPK), mitogen-activated protein kinase p38 beta (p38 MAPK), c-Jun N-terminal kinase 1 (JNK), cyclin-dependent-like kinase 5 (CDK5), and calcium/calmodulin-dependent protein kinase type II subunit alpha (CaMKKII-α), was studied to determine the effect of Cry silencing on tau kinases (Figure 5A–G). The expression levels of p-GSK3β, p-AMPK, p-JNK, CDK, and p-CaMKII-α were elevated in 6-month-old P301S mice compared with those in WT mice. In addition, GSK3β phosphorylation and CDK5 phosphorylation levels were significantly decreased in P301S mice after treatment with Cry shRNA (all P < 0.05). However, there were no significant differences in phosphorylation for the other tau kinases tested (all P > 0.05). Furthermore, P301S mice had lower levels of protein phosphatase 2A (PP2A) catalytic subunit (PP2Ac) expression than WT mice, and the activity of the main tau phosphatase, PP2A, remained unchanged in P301S mice after treatment with Cry shRNA (P < 0.05; Figure 5H and I). These results indicate that infusion of Cry shRNA decreased hyperphosphorylated tau by repressing the activity of the tau kinases GSK3β and CDK5, without influencing tau phosphatase levels.

Cry silencing reverses neurodegenerative changes in the brains of P301S mice
P301S mice had a lower neuron count in the hippocampus and cortex compared with WT mice. In addition, the neuron count in these two brain regions of P301S mice injected with control shRNA was significantly lower than that of P301S mice injected with Cry shRNA (both P < 0.05; Figure 6A–C). We found higher cleaved caspase-3 levels in the hippocampus and cortex of P301S mice compared with those in WT mice, as measured by western blot. Moreover, cleaved caspase-3 expression was lower in P301S mice that received control shRNA (Figure 6D and E), which suggests that Cry shRNA may have reduced apoptosis. Furthermore, we analyzed hippocampal atrophy and ventricle volume based on cresyl violet staining. We observed hippocampal atrophy in P301S mice, and Cry silencing decreased this change in ventricle volume (P < 0.05; Figure 6F). Ventricle volume was increased in P301S mice, and Cry silencing decreased this change in ventricle volume (P < 0.05; Figure 6G). Synaptophysin expression was lower in P301S mice compared with that in WT mice, and synaptophysin levels in P301S mice were upregulated after Cry shRNA infusion compared with that in P301S mice injected with control shRNA (P < 0.05; Figure 7). These results indicate that there were significant neurodegenerative changes in the brains of P301S mice. When Cry protein expression in the brain was decreased, the neuron count and synaptophysin expression levels increased in the cerebral cortex and hippocampus.

Cry silencing restores the cognitive function of P301S mice
The Morris water maze test was used to determine the influence of Cry shRNA infusion in 6-month-old P301S mice. We found that the difference in swimming speed between individual 6-month-old P301S mice was not significant, which eliminates any potential for interference of motivation effects and sensorimotor input on measurements of cognitive performance (P > 0.05; Figure 8A). The hidden-platform task, which assesses cognitive deficiency, showed that P301S mice had higher latencies compared with WT mice when finding the submerged platform (P < 0.05; Figure 8B). After infusion with Cry shRNA, the average time it took to reach the hidden platform was markedly decreased in P301S mice in comparison with P301S mice infused with control shRNA or WT mice on days 4 and 5. No significant differences were found within the group of P301S mice infused with control shRNA or within the group of WT mice during this test (P > 0.05; Figure 8B).

For the probe test, we removed the platform in the water and performed probe trials to evaluate the effects of infusion with Cry shRNA on 6-month-old P301S mice. As shown in Figure 8C, three groups of mice (6-month-old WT mice with control shRNA, 6-month-old WT mice with Cry shRNA, and 6-month-old P301S mice with Cry shRNA) spent markedly more time in the target quadrant than in the other three quadrants (all P < 0.05), while 6-month-old P301S mice with control shRNA spent similar amounts of time in all quadrants (P > 0.05). Our results indicate that infusion with Cry shRNA significantly alleviated spatial memory impairment in P301S mice. Our results also indicate that infusion with Cry shRNA in 6-month-old P301S mice reduced the number of errors and improved latency times (both P < 0.05; Figures 8D and E).

Cry silencing modulates neuroinflammation and the complement system in the brains of P301S mice
We studied the effects of Cry shRNA treatment on neuroinflammation by analyzing the expression of interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6). Downregulation of Cry significantly suppressed the expression of IL-1β (by 47.3%, n = 6, P < 0.05), TNF-α (by 53.4%, n = 6, P < 0.05), and IL-6 (by 35.4%, n = 6, P < 0.05) compared with those in P301S mice infused with control shRNA (Figure 9A–C). Furthermore, we observed a decrease in Cry levels and proinflammatory cytokines after infusion with Cry shRNA. We also found that the levels of C3 and complement component 3b (C3b) proteins decreased by 48.6% and increased by 51.9%, respectively, after infusion with Cry shRNA compared with those in P301S mice that received control shRNA (both P < 0.05; Figure 10A–C).

**Figure 4** | Hyperphosphorylated tau is decreased after infusion with Cry shRNA in the brains of 6-month-old P301S mice.
(A) Levels of AT8 (which recognizes serine 202 and threonine 205 hyperphosphorylated tau), threonine (thr) 231 hyperphosphorylated tau, serine (ser) 262 hyperphosphorylated tau, and total tau were measured by western blot. (B–D) Quantification of the proportion of AT8/total tau (B), threonine 231 hyperphosphorylated tau/total tau (C), and serine 262 hyperphosphorylated tau/total tau (D). Protein expression levels were normalized to β-actin. Data are presented as the mean ± SEM (n = 6 for each group) and were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison test. *P < 0.05, Cry: Cr1-related protein Y, shRNA: short hairpin RNA; WT: wild type.
Figure 5  |  Effect of Crry shRNA on the tau kinase/phosphatase balance in the brains of 6-month-old P301S mice.
(A) Expression levels of p-GSK3β (serine 9), total GSK3β, phospho-AMPKα (threonine 172), total AMPK, phospho-p38 MAPK (threonine 180 and tyrosine 182), p38 MAPK, phospho-JNK (threonine 183 and tyrosine 185), JNK, CDK5, phospho-CaMKII-α (tyrosine 231), and total CaMKII-α, as detected by western blot. (B–G) Quantification of p-GSK3β/GSK3β, p-p38/p38, p-AMPK/AMPK, p-JNK/JNK, CDK5/β-actin, and p-CaMKII-α/CaMKII-α. (H) PP2Ac levels were detected by western blot. (I) Quantification of PP2Ac levels. Protein expression levels were normalized to the WT + control shRNA group. Data are presented as the mean ± SEM (n = 6 for each group) and were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison test. †P < 0.05. AMPK: AMP-activated protein kinase; CaMKII-α: CaM-dependent protein kinase II-α; CDK5: cyclin-dependent kinase 5; Crry: Cr1-related protein Y; GSK3β: glycogen synthase kinase-3beta; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinases; PP2Ac: protein phosphatase 2A catalytic subunit; shRNA: short hairpin RNA; WT: wild type.

Figure 6  |  Decreasing Crry expression in the brain can delay neuron loss in P301S transgenic mice.
(A) Cresyl violet staining shows the survival of neurons in the cortex and hippocampus. Mice in the WT + control shRNA and WT + Crry shRNA groups have more neurons than P301S mice, and mice in the P301S + control shRNA group have more neurons compared with mice in the P301S + control shRNA group. Arrows indicate the neurons stained by cresyl violet. Scale bar: 100 μm. (B, C) Quantification of surviving neurons stained by cresyl violet in the cortex and hippocampus of mice in each group. (D, E) Cleaved caspase-3 expression in neurons of the cortex and hippocampus of mice in each group visualized by western blot. Protein expression levels were normalized to the WT + control shRNA group. (F, G) Quantitative analysis of hippocampal volume (F) and ventricle volume (G). Data are presented as the mean ± SEM (n = 6 for each group) and were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison test. †P < 0.05. Crry: Cr1-related protein Y; shRNA: short hairpin RNA; WT: wild type.
Cognitive function deficiency is rescued in P301S mice after infusion with Crry shRNA.

Latency (s)

Infusion with Crry shRNA modulates neuroinflammation in P301S+Crry

IL-1β

α.

IL-6: interleukin-6; shRNA: short hairpin RNA; TNF-α: tumour necrosis factor

Reduced glyceraldehyde-phosphate dehydrogenase; IL-1β: interleukin-1beta; IL-6: interleukin-6; shRNA: short hairpin RNA; WT: wild type.

Infusion with Crry shRNA modulates the complement system

IL-1β

α.

IL-6: interleukin-6; shRNA: short hairpin RNA; TNF-α: tumour necrosis factor

Reduced glyceraldehyde-phosphate dehydrogenase; IL-1β: interleukin-1beta; IL-6: interleukin-6; shRNA: short hairpin RNA; WT: wild type.

Infusion with Crry shRNA modulates the complement system

IL-1β

α.

IL-6: interleukin-6; shRNA: short hairpin RNA; TNF-α: tumour necrosis factor

Reduced glyceraldehyde-phosphate dehydrogenase; IL-1β: interleukin-1beta; IL-6: interleukin-6; shRNA: short hairpin RNA; WT: wild type.
Discussion

For this study, we intended to explore the potential role of the CR1 gene in AD-related tauopathy by investigating the rodent-specific Crry gene involved in human P301S tauopathy mouse model. Double immunofluorescence staining showed that Crry was expressed in microglia and had lower expression in astrocytes (weak colocalization with the astrocyte marker glial fibrillary acidic protein) but was not expressed in neurons. In mice, Davoust et al. (1999) were the first to detect mRNA and protein expression of Crry in astrocytes and microglia, and they showed that microglia had higher Crry expression levels than astrocytes. Only Crry mRNA was observed in neurons. Our results are consistent with the findings of Davoust et al. (1999). Microglia-mediated neuroinflammation is a hallmark of a variety of neurodegenerative diseases, including AD (Tang and Le, 2016). CR1, SP1, and CD33 are highly expressed in microglia and contribute to the progression of AD in a non-AB-dependent manner (Villegas-Llerena et al., 2016; Ethymiou and Goate, 2017). Therefore, we speculated that CR1 expressed in microglia might contribute to AD progression by influencing tau pathology.

We detected tau phosphorylation levels and the activity of tau kinases by silencing Crry expression with a Crry shRNA encoding lentiviral vector in P301S mice. P301S mice had significantly elevated tau protein phosphorylation at the AT8, threonine 231, and serine 262 sites, and had increased activity of the major tau kinases GSKβ and CDK5 in comparison to WT mice. Silencing decreased tau protein phosphorylation and the activity of these two tau kinases. GSKβ is a serine/threonine protein kinase that regulates the phosphorylation of many cellular substrates and has a crucial role in the phosphorylation of tau protein (Hernández et al., 2009). GSKβ expression was significantly elevated in P301S mice compared with WT mice, and aged P301S mice had increased levels of hyperphosphorylated tau coupled with increased GSKβ expression (Dumont et al., 2011; Hou et al., 2020). CDK5 is a cyclin-dependent kinase and also a tau kinase that phosphorylates tau at serine/threonine residues (primarily serine 202, serine 235, serine 404, and threonine 205) in serine/threonine-proline sequences (Kimura et al., 2014; Cortés et al., 2019). CDK5 is abnormally activated in AD and its deregulation contributes to various histopathological changes in patients with AD (Liu et al., 2016). Saito et al. (2019) showed that CDK5 could elevate the phosphorylation and accumulation of tau by regulating tau phosphorylation at serine 262. Hyperphosphorylation of tau is one of the main pathological changes in AD. Our results suggest that Crry silencing might partly improve AD by alleviating tauopathy. Killick et al. (2013) found that Crry−/− mice had decreased levels of complement factor H (a serum biomarker for AD progression) and tau phosphorylation at the serine 235 site. To a certain extent, the study of Killick et al. (2013) confirmed our speculation that Crry silencing might improve AD-related tauopathy by interfering with tau pathology. In addition, we found that Crry silencing decreased neuron death and hippocampal atrophy and improved the deficiency in cognitive function in P301S mice, as expected. Therefore, we confirm that Crry silencing is beneficial in the P301S tauopathy model mouse.

The complement system represents a crucial part of the innate immune system. Overactivation of the complement system will directly or indirectly lead to damage of the body’s own tissues (Bajic et al., 2015). Crry is an important regulator of the complement pathway that has the same effect as other complement regulatory proteins, such as complement decay-accelerating factor and membrane cofactor protein. Crry can repress the activity of C3/C5 convertase to block complement activation (Kim et al., 1995). Crry−/− mice are embryo lethal, but they can be rescued by C3 deletion as complement activation in the mother cannot be inhibited when Crry is absent, which results in complement attacks on the placenta (Xu et al., 2000). The double knockout of Crry in kidney causes microglial priming via interaction of iC3b with CR3; microglial priming has been associated with experimental autoimmune encephalomyelitis (Ramaglia et al., 2012). Ramaglia et al. (2012) suggested that elevated local expression of C3 and FB were observed in Crry−/− mice, and both C3 and FB were dependent on and required for microglial priming, indicating microglial priming was caused by uncontrolled complement activation through an alternative pathway. In Crry−/− mice, FB catalyzes the first cleavage of C3b, resulting in iC3b accumulation (Kim et al., 1995), and Ramaglia et al. (2012) suggested that local accumulation of iC3b engaged its receptor CR3 and triggered microglial priming in the Crry−/− mice. However, complement mediates microglial phagocytosis and clearance of fibrillar Aβ by the interaction of iC3b with CR3 on the microglia surface (Fu et al., 2012). Shi et al. (2017) suggested that inhibition of complement and downstream iC3b/CR3 signals could protect synapses against Aβ aggregation-related loss in an APPPS1 mouse model before amyloid plaque aggregation. C3b was also implicated in the clearance of Aβ in peripheral blood by interacting with CR1 on erythrocytes (Rogers et al., 2006). Increasing evidence indicates that Aβ plays roles in the pathways upstream of tau and induces neurotoxicity mediated by tau (Rapport et al., 2002; Roberson et al., 2007; Choi et al., 2014). Therefore, we speculate that C3b or iC3b might contribute to the clearance of Aβ by interacting with the corresponding receptors and decreasing neuronal toxicity. Studies have also suggested that microglia can degrade and clear pathological tau (Luo et al., 2015; Bolós et al., 2016). For example, Luo et al. (2015) found that hyperphosphorylated pathological tau derived from brain tissue from patients with AD was rapidly internalized and degraded by microglia. We speculate that complement might also mediate microglial phagocytosis and clearance of pathological tau by the interaction of iC3b/CR3 with the CR3 receptor on the microglia surface.

Our findings emphasize the complexity of the complement system. Therefore, more work is required to understand the dual nature of the complement system (namely, that it can exacerbate or attenuate neurodegenerative disease), especially for Crry and human CR1 in the CNS. Moreover, more experiments should be carried out to determine the activities of iC3b, CR3, C3a, and the terminal complement complex, as well as their associations with microglial priming and activation. Additionally, studies have reported that Crry is involved in regulation of CD4+ T cell activation and acts as a costimulatory molecule to elevate T cell receptor-dependent activation signals (such as intraextracellular-signaled-regulated kinase and p38 mitogen-activated protein kinase) in peripheral blood (Ramaglia et al., 2012). MAPK and JNK (Fernández-Centeno et al., 2000; Almeida-Salazar et al., 2005). MAPK and JNK were investigated in this study because of their roles in tau phosphorylation. To comprehensively understand the role of Crry, the effects of Crry on immunobiology or other biological processes should also be further investigated.

Our study’s design and methods had some limitations. We performed all experiments in vivo, and no in vitro experiments were designed. We used quantitative real-time polymerase chain reaction for the detection of inflammatory cytokines, but we did not use western blot on tissue lysates or enzyme-linked immunosorbent assays on tissue homogenates to investigate inflammatory cytokines. In conclusion, Crry might play an important role in the P301S tauopathy mouse model, and Crry downregulation might be associated with modulation of microglial activation, neuroinflammation, and the complement system. Our study indicates that complement dysregulation is likely to be important in neurodegenerative diseases. Drugs that modulate complement activation may be repurposed, or brain-targeting complement therapies could be developed for the treatment of neurodegenerative diseases.
Group 1
(To verify the location of CR1 in brains)

P301S mice N=2

Group 2
(To evaluate the change of Crry protein levels in brain during aging process under AD context)

3 months in P301S mice N=6; 3 months in wide-type mice N=6
6 months in P301S mice N=6; 6 months in wide-type mice N=6
9 months in P301S mice N=6; 9 months in wide-type mice N=6

Group 3
(To verify the changes in P301s mice after infusion with shRNA)

6 months in P301s mice + Crry shRNA N=6;
6 months in P301s mice + Control shRNA N=6;

Group 4
(To verify the Cognitive changes in P301s mice after infusion with shRNA)

6 months in P301s mice + Crry shRNA N=6;
6 months in P301s mice + Control shRNA N=6

Additional Figure 1 The detailed experimental protocol.

CR1: Complement component 3b/4b receptor 1; Crry: Cr1-related protein Y; shRNA: short hairpin RNA; WT: wild type.
Additional Figure 2 Immunofluorescence staining to determine the expression of Crry in cortex and hippocampus of 6-month-old WT and P301S mice.

(A) Representative images of immunofluorescence staining (original magnification, 200×) and quantified for Crry immunopositivity in cortex of WT and P301S mice. (B) Representative images of immunofluorescence staining (original magnification, 400×) and quantified for Crry immunopositivity in hippocampus of WT and P301S mice. The arrows indicate the merged fluorescent signal from green and red fluorescent signals. Immunofluorescence staining of Crry in these two brain regions were obviously increased in P301S mice versus to that of age-matched WT mice. Data are presented as the mean ± SEM (n = 6 for each group), and analyzed by independent sample t-test. Crry: Cr1-related protein Y; DAPI: 4′,6-diamidino-2-phenylindole; Iba-1: ionized calcium binding adaptor molecule 1; WT: wild type.
## Additional Table 1 Antibodies used in western blot and double immunofluorescence staining

| Antibody                                      | Concentration | RRID No.     | Species and clonality | Catalog No. | Supplier                          |
|-----------------------------------------------|---------------|--------------|-----------------------|-------------|-----------------------------------|
| Crry                                           | 1:200         | AB_2085152   | Rabbit monoclonal     | sc-30214    | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| Iba-1                                          | 1:500         | AB_2636859   | Rabbit monoclonal     | ab178846    | Abcam, Cambridge, UK              |
| GFAP                                           | 1:500         | AB_305808    | Rabbit polyclonal     | ab7260      | Abcam, Cambridge, UK              |
| NeuN                                           | 1:1000        | AB_2532109   | Rabbit monoclonal     | ab177487    | Abcam, Cambridge, UK              |
| OSP                                            | 1:500         | AB_305935    | Rabbit polyclonal     | ab7474      | Abcam, Cambridge, UK              |
| Hyperphosphorylated tau at Ser\(^{202}:Thr^{205}\) (AT8) | 1:1000        | AB_223647    | Mouse monoclonal      | mn1020      | ThermoFisher Scientific, Sunnyvale, CA, USA |
| Hyperphosphorylated tau at Thr\(^{231}\)        | 1:1000        | AB_2533742   | Rabbit polyclonal     | 44-746G     | ThermoFisher Scientific, Sunnyvale, CA, USA |
| Hyperphosphorylated tau at Ser\(^{262}\)        | 1:1000        | AB_2533743   | Rabbit polyclonal     | 44-750G     | ThermoFisher Scientific, Sunnyvale, CA, USA |
| Total tau                                      | 1:1000        | AB_628327    | Mouse monoclonal      | sc-32274    | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| Phospho-GSK-3\(^\beta\) (Ser\(^9\))            | 1:1000        | AB_2798546   | Rabbit monoclonal     | 5558T       | Cell Signaling Technology, Danvers, MA, USA |
| GSK-3\(^\alpha:\beta\)                         | 1:1000        | AB_2636978   | Rabbit monoclonal     | 12456T      | Cell Signaling Technology, Danvers, MA, USA |
| Antibody                        | Dilution | Catalog Number | Species          | Category         | Source                          |
|--------------------------------|----------|----------------|------------------|------------------|---------------------------------|
| AMPK                           | 1:1000   | AB_1118940     | Mouse monoclonal | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| Phospho-AMPKα (Thr^{172})      | 1:1000   | AB_2799368     | Rabbit monoclonal| Cell Signaling Technology, Danvers, Massachusetts, USA |
| Phospho-p38 MAPK (Thr^{180}Tyr^{182}) | 1:1000   | AB_2139682     | Rabbit monoclonal| Cell Signaling Technology, Danvers, MA, USA |
| p38 MAPK                       | 1:1000   | AB_2533144     | Mouse monoclonal | ThermoFisher Scientific, Sunnyvale, CA, USA |
| JNK                            | 1:1000   | AB_2140722     | Mouse monoclonal | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| Phospho-JNK (Thr^{183}Tyr^{185}) | 1:1000   | AB_2574777     | Mouse monoclonal | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| CDK5                           | 1:1000   | AB_627241      | Mouse monoclonal | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| Synaptophysin                  | 1:1000   | AB_2286949     | Rabbit monoclonal| Abcam, Cambridge, UK |
| PP2Ac                           | 1:1000   | AB_2170107     | Mouse monoclonal | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| C3                             | 1:500    | AB_2066623     | Rat monoclonal   | Abcam, Cambridge, UK |
| C3b                             | 1:2000   | AB_627277      | Mouse monoclonal | Santa Cruz Biotechnology, Santa Cruz, CA, USA |
| Antibody Description                  | Dilution | Catalog Number | Species       | Type          | Supplier Information                                      |
|---------------------------------------|----------|----------------|---------------|---------------|------------------------------------------------------------|
| Phospho-CaMKII-α (Tyr<sup>231</sup>) | 1:1000   | AB_2070310     | Rabbit        | polyclonal    | Cell Signaling Technology, Danvers, MA, USA                |
| CaMKII-α                              | 1:1000   | AB_2721906     | Mouse         | monoclonal    | Cell Signaling Technology, Danvers, MA, USA                |
| Cleaved caspase-3                     | 1:500    | AB_2341188     | Rabbit        | polyclonal    | Cell Signaling Technology, Danvers, MA, USA                |
| β-Actin                               | 1:1000   | AB_2714189     | Mouse         | monoclonal    | Santa Cruz Biotechnology, Santa Cruz, CA, USA              |
| Goat anti-Mouse IgG (H+L) Poly-HRP    | 1:1000   | AB_1965958     | Polyclonal    | 32230         | ThermoFisher Scientific, Sunnyvale, CA, USA                |
| Secondary Antibody                    |          |                |               |               |                                                             |
| Fluorescein-conjugated affinipure Goat anti-Rabbit IgG antibody | 1:200 | AB_2571576 | Polyclonal | ZF-0311 | Zhongshan Goldenbridge Inc., Beijing, China |
| FITC labeled Goat anti-mouse IgG antibody | 1:200 | AB_2716306 | Polyclonal | ZF-0312 | Zhongshan Goldenbridge Inc., Beijing, China |

AMPK: AMP-activated protein kinase; C3: complement 3; C3b: complement component 3b; CaMKII-α: CaM-dependent protein kinase II-α; CDK5: cyclin-dependent kinase 5; Crry: Cr1-related protein Y; GFAP: glial fibrillary acidic protein; GSK: glycogen synthase kinase; Iba-1: ionized calcium binding adaptor molecule 1; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinases; NeuN: neuronal nuclei; OSP: oligodendrocyte specific protein; PP2Ac: protein phosphatase 2A catalytic subunit.
**Additional Table 2 Primer sequences used in this study**

| Name   | Primer forward (5'-3')       | Primer reverse (5'-3')       | GenBank accession number* | Amplicon size (bp) |
|--------|------------------------------|------------------------------|---------------------------|--------------------|
| Crry   | CTTCCCTTCGCATCAGTG           | AAGGATACCCCTCATTGG           | NM_01349                 | 209                |
|        | TTGCA                        | TTCCTC                      |                           |                    |
| TNF-α  | GTCTACTGAACTTCGGGG           | ATGATCTGAGTGAGGTG          | NM_01369                 | 102                |
|        | TGAT                         | CTG                         |                           |                    |
| IL-1β  | GAAGAGCCCATCCTCTGT           | TTCATCTCGGAGCTGTAGT         | NM_00836                 | 96                 |
|        | GA                           | G                           |                           |                    |
| IL-6   | ACAAGCCAGAGTCCTTC            | CATTGGAATTGCGGTAGG          | NM_03116                 | 105                |
|        | AGAG                         | A                           |                           |                    |
| GAPD   | CAAACAGAATCCACCTC            | GGTCCAGGGTTTCTTACTCC        | NM_00808                 | 164                |
| H      | TTC                          | TT                          |                           | 4.3                |

* The GenBank accession numbers were obtained from the NCBI [https://www.ncbi.nlm.nih.gov/](https://www.ncbi.nlm.nih.gov/).

Crry: Cr1-related protein Y; GAPDH: reduced glyceraldehyde-phosphate dehydrogenase; IL-1β: interleukin-1beta; IL-6: interleukin-6; TNF-α: tumour necrosis factor alpha.