Tau pathology involves protein phosphatase 2A in Parkinsonism-dementia of Guam

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Contributed by Ralph M. Garruto, December 9, 2013 (sent for review September 13, 2013)

Parkinsonism-dementia (PD) of Guam is a neurodegenerative disease with parkinsonism and early-onset Alzheimer-like dementia associated with neurofibrillary tangles composed of hyperphosphorylated microtubule-associated protein, tau. β-*N*-methylamino-L-alanine (BMAA) has been suspected of being involved in the etiology of PD, but the mechanism by which BMAA leads to tau hyperphosphorylation is not known. We found a decrease in protein phosphatase 2A (PP2A) activity associated with an increase in inhibitory phosphorylation of its catalytic subunit PP2Ac at Tyr^{307} and abnormal hyperphosphorylation of tau in brains of patients who had Guam PD. To test the possible involvement of BMAA in the etiopathogenesis of PD, we studied the effect of this environmental neurotoxin on PP2A activity and tau hyperphosphorylation in mouse primary neuronal cultures and metabolically active rat brain slices. BMAA treatment significantly decreased PP2A activity, with a concomitant increase in tau kinase activity resulting in elevated tau hyperphosphorylation at PP2A favorable sites. Moreover, we found an increase in the phosphorylation of PP2Ac at Tyr^{307} in BMAA-treated rat brains. Pretreatment with metabotropic glutamate receptor 5 (mGluR5) and Src antagonists blocked the BMAA-induced inhibition of PP2A and the abnormal hyperphosphorylation of tau, indicating the involvement of an Src-dependent PP2A pathway. Coimmunoprecipitation experiments showed that BMAA treatment dissociated PP2Ac from mGluR5, making it available for phosphorylation at Tyr^{307}. These findings suggest a scenario in which BMAA can lead to tau pathology by inhibiting PP2A through the activation of mGluR5, the consequent release of PP2Ac from the mGluR5–PP2A complex, and its phosphorylation at Tyr^{307} by Src.

Alzheimer's disease | amyotrophic lateral sclerosis | tauopathies | tau phosphorylation | cycad

Indigenous residents and immigrants of the Pacific Island of Guam suffer from a high incidence of a progressive and fatal neurodegenerative tauopathy called "parkinsonism-dementia" (PD). This is a long-latency disease with phenotypic characteristics of idiopathic parkinsonism with an early-onset dementia. The disorder is found together with a second high-incidence disease, amyotrophic lateral sclerosis (ALS). The incidence of both diseases has declined dramatically over the past half century, suggesting an environmental etiology (1). The neuropathology of PD is hallmarked by neurofibrillary tangles (NFTs) (2) of paired helical filaments (PHFs) composed of abnormally hyperphosphorylated forms of the microtubule-associated protein tau. The NFTs found in PD brains are ultra-structurally and biochemically similar to those in Alzheimer's disease (AD) (3). In contrast to AD pathology, however, studies using postmortem brains confirmed the absence of amyloid β plaques in most PD cases (2). Many studies have demonstrated that abnormal hyperphosphorylation and aggregation of tau are crucial to neurodegeneration in AD and tauopathies (4). Although the mechanism leading to the formation of NFTs is still obscure, it has been well recognized that an imbalanced regulation in protein kinases and protein phosphatases can directly cause AD-like tau hyperphosphorylation (4). Among the various kinases, glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinase 5, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), MAP kinase kinase (MEK 1/2), ERK 1/2, protein kinase A, casein kinase 1, and p70 S6 kinase have been most often implicated in tau phosphorylation (4). Among the phosphatases, protein phosphatase 2A (PP2A) accounts for ∼70% of tau phosphatase activity in the human brain (5). PP2A is responsible for dephosphorylating most of the hyperphosphorylated sites of tau, and its activity is compromised in the AD brain (6). Given the increasing evidence that PD histopathologically and genetically should be classified as a tauopathy (7, 8), identifying the protein kinases/phosphatases and the associated signaling cascades that regulate tau phosphorylation is of crucial importance.

Several etiological factors, especially environmental factors, genetic susceptibility, and metabolic alterations, have been proposed to contribute to PD. The nonprotein amino acid β-*N*-methylamino-L-alanine (BMAA) has been implicated as a potential environmental factor in PD, ALS, AD, and other neurodegenerative disorders (9–11). Although the neurotoxic effects of BMAA are not conclusive, BMAA concentrations as low as 30 μM can cause selective death of motor neurons (12), and a 10-μM concentration can potentiate neuronal injury induced by exposure to amyloid-β, 1-methyl-4-phenylpyridinium, or methylmercury in mixed cortical cultures (13). The mechanism of BMAA toxicity is complex and several fold: (*i*) BMAA displays agonistic properties for NMDA (14), AMPA/kainate (12), and metabotropic glutamate receptor 5 (mGluR5) (14, 15) and for mGluR1 receptors (16) in a cell-specific manner, and (*ii*) it increases intracellular calcium levels (17) and oxidative stress (15). BMAA was reported to induce learning and memory deficits accompanied by neuronal cell death in rats (18), although the underlying molecular mechanism by which BMAA affects

Significance

Parkinsonism-dementia (PD) of Guam is a classical tauopathy in which abnormal hyperphosphorylation of tau leads to neurodegeneration and dementia. A key unresolved question is the mechanism of abnormal hyperphosphorylation of tau in this disease. This study reports the involvement of a signaling pathway in which the inhibition of protein phosphatase 2A (PP2A) through phosphorylation of its catalytic subunit PP2Ac at Tyr^{307} induced by the activation of metabotropic glutamate receptor 5 (mGluR5) leads to hyperphosphorylation of tau. These findings suggest that the mGluR5–PP2A axis has a central role in neurofibrillary degeneration in Guam PD and thus may be a therapeutic target for the treatment of this disease and related tauopathies.

Author contributions: I.G.-I. and K.I. designed research; M.A., S.F.K., and K.I. performed research; R.M.G. contributed new reagents/analytical tools; M.A. and K.I. analyzed data; and M.A. and K.I. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1322614111/-/DCSupplemental.

tau phosphorylation and eventually toxicity has not been established.

In the present study, we report the involvement of PP2A signaling in PD and show that BMAA can produce similar changes by activating the mGluR5 receptor, leading to the dissociation of PP2A from the receptor, followed by its phosphorylation at Tyr^{307} by Src, a nonreceptor tyrosine kinase. Phosphorylation of PP2A at Tyr^{307} inhibits its activity and leads to hyperphosphorylation of tau. Together these data reveal an etiopathogenic mechanism of neurofibrillary pathology in PD, AD, and ALS involving mGluR5-dependent inactivation of PP2A.

Results

PP2A Activity Is Inhibited by Phosphorylation of the PP2A Catalytic Subunit at Tyr^{307}, and Tau Is Abnormally Hyperphosphorylated in PD Brains. PP2A is the major protein phosphatase that regulates tau phosphorylation in vivo and accounts for ~70% of the total tau phosphatase activity in the mammalian brain (5). In AD brain PP2A activity is compromised, most likely because of the up-regulation of its inhibitors I_1^{PP2A} and I_2^{PP2A} (19) and demethylation of its catalytic subunit, PP2Ac (20). To examine whether PP2A inactivation is involved in abnormal hyperphosphorylation of tau in PD cases, we assayed the PP2A activity in autopsied brains from patients with Guam PD and age-matched controls from Guam by phosphatase ELISA (21). We found a significant ($P < 0.05$) decrease in PP2A activity in PD compared with nonneurological controls from Guam (Fig. 1*A*). PP2A is known to be down-regulated by phosphorylation at Tyr^{307} (22), demethylation at Leu^{309} (23), and endogenous inhibitors I_1^{PP2A} and I_2^{PP2A} (19, 24). We looked for the possible involvement of all PP2A regulatory factors in PD. We observed a significant increase ($P < 0.05$) in PP2A phosphorylation at Tyr^{307} in PD brains as detected by quantitative Western blot analysis (Fig. 1 *B* and *C*). However, we did not detect any significant changes in the levels of demethylated PP2Ac or I_1^{PP2A} and I_2^{PP2A} (Fig. 1 *B* and *C*). Western blots developed with phosphorylation-dependent/site-specific tau antibodies revealed that tau phosphorylation increased at all phosphorylation sites examined in PD cases as compared with the controls (Fig. 1 *D* and *E*). We also observed similar results in four cases of Guam PD and five age-matched nonneurological control cases from the United States mainland (Fig. S1). Interestingly, we found that the total tau level was markedly higher in PD cases than in the controls and displayed an upward gel mobility shift with higher molecular weight smears (Fig. 1*D* and Fig. S1*D*) that resemble those previously reported in AD (25). These data suggested the involvement of Tyr^{307} phosphorylation of PP2Ac in PD brain.

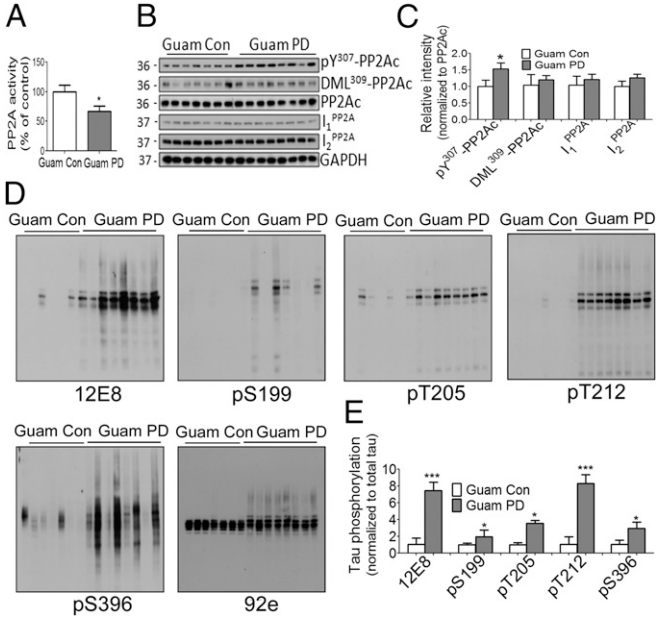

Fig. 1. PP2A activity is decreased because of the increase in phosphorylation of PP2Ac at Tyr^{307}, and tau is hyperphosphorylated in Guam PD brains. (*A*) PP2A activity assayed by phosphatase ELISA in Guam PD and Guam nonneurological control frontal cortices. (*B*) Western blots of PP2A and its inhibitors. (*C*) Quantitative analysis of blots in *B*. (*D*) Western blots of tau phosphorylated at different sites. (*E*) Quantitative analysis of blots in *D*. Data are expressed as mean ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

BMAA Inhibits PP2A, Increases Tau Hyperphosphorylation, and Causes Death of Hippocampal Neurons. Because BMAA has been widely suspected as a possible environmental toxin in the etiopathogenesis of PD (9–11), we investigated whether BMAA can inhibit PP2A activity, induce abnormal hyperphosphorylation of tau, and cause neuronal death. We determined the PP2A activity toward phosphorylated tau as a substrate after treatment with BMAA in mouse mixed primary neuronal cultures. We found that BMAA concentrations of 100 μM to 1 mM significantly decreased PP2A activity for 3–48 h without any detectable effect on its protein expression (Fig. 2 *A* and *B* and Fig. S2). Quantitative analysis of Western blots developed with site-specific phospho-tau antibodies revealed that hyperphosphorylation of tau was significantly increased at $Ser^{262/356}$ (12E8 site; $P < 0.01$), Ser^{199} ($P < 0.05$), Thr^{205} ($P < 0.01$), and Thr^{212} ($P < 0.05$) but not at Ser^{396} in BMAA-treated cells as compared with controls (Fig. 2 *C* and *D*). We found, no significant change in the level of total tau (R134d) between control and BMAA-treated neurons (Fig. 2*D*). Furthermore, immunohistofluorescent analysis revealed that BMAA increased the expression of tau phosphorylated at $Ser^{262/356}$ (Fig. 2 *E* and *F*). Using a lactate dehydrogenase (LDH) release assay, we also observed that 1 mM BMAA caused cell death in mixed primary neuronal culture (Fig. 2*G*), as is consistent with previous reports (12–14). Collectively these results suggest that BMAA can cause cell death which is associated with the inhibition of PP2A and the increase in hyperphosphorylation of tau in primary neurons.

BMAA-Induced Hyperphosphorylation of Tau Is Associated with an Increase in PP2A Phosphorylation at Tyr^{307} in Metabolically Active Brain Slices. To investigate (*i*) whether BMAA has an effect on tau hyperphosphorylation in brain similar to that seen in primary neuronal cultures and (*ii*) whether the PP2A signaling involved in this effect is similar to that found in PD brains, we treated metabolically active brain slices from adult Wistar rats with 1 mM BMAA for 2–3 h, determined the level of tau phosphorylation, and studied the involvement of PP2A and tau kinases regulated by it. We found that BMAA increased phosphorylation of tau at $Ser^{262/356}$ (3.4-fold), Ser^{199} (2.6-fold), Thr^{205} (1.8-fold), and Thr^{212} (3.7-fold) (Fig. S3 *A* and *B*). As in primary hippocampal neurons, we did not find any significant change in the phosphorylation of tau at Ser^{396} (Fig. S3*B*). Next, we assayed PP2A activity and found a significant inhibition of PP2A activity in brain slices incubated with BMAA for 2 h in artificial cerebrospinal fluid (Fig. 3*A*). To understand the mechanism underlying BMAA-induced down-regulation of PP2A, we measured phosphorylation of PP2A at Tyr^{307}, demethylation at Leu^{309}, and the levels of the PP2A inhibitors I_1^{PP2A} and I_2^{PP2A}. We found a dramatic (3.7-fold) increase in the phosphorylation of PP2Ac at Tyr^{307} in BMAA-treated slices, but we detected no significant changes in the level of Leu^{309} demethylated PP2A, I_1^{PP2A}, or I_2^{PP2A} (Fig. 3 *B* and *C*).

It is known that Src family kinases such as c-Src and Lck phosphorylate PP2Ac at Tyr^{307} and inhibit its activity (22). We therefore examined whether Src could play a role in BMAA-induced PP2A inactivation. We found that pretreatment with PP2, a broad-spectrum Src kinase inhibitor, blocked the BMAA-induced tau hyperphosphorylation in brain slices (Fig. 3 *D* and *E*).

PP2A can regulate phosphorylation of tau both directly and through the activation of several tau protein kinases that are

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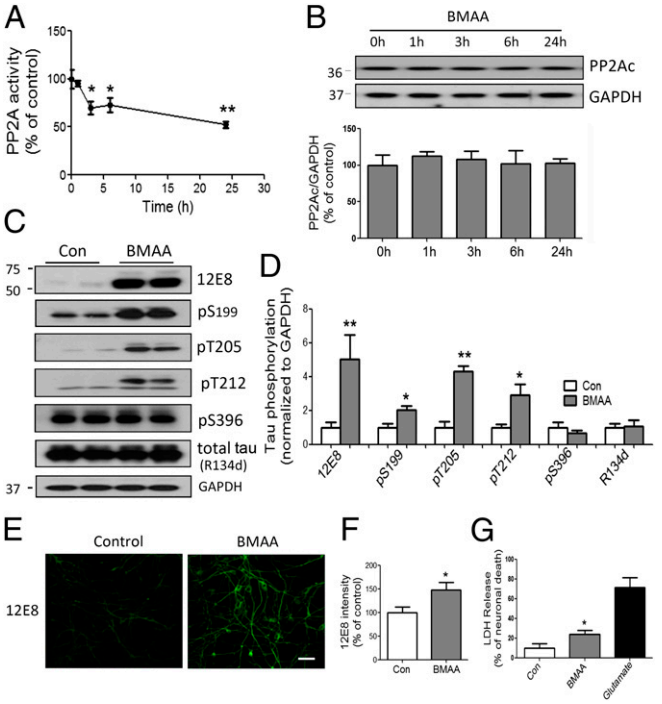

Fig. 2. BMAA inhibits PP2A activity and increases tau hyperphosphorylation in mouse hippocampal primary neurons. (*A* and *B*) PP2A activity (*A*) and PP2Ac protein level (*B*) were measured in mixed mouse primary hippocampal neurons at 0, 1, 3, 6, and 24 h after treatment with BMAA (1 mM). (*C*) Representative Western blots showing hyperphosphorylation of tau. (*D*) Quantification of hyperphosphorylation of tau at different sites and R134d (total tau) shown after normalization with GAPDH. (*E* and *F*) Representative photomicrographs (*E*) and the corresponding quantitation of phospho-tau (12E8) staining (*F*) in control and BMAA-treated cultured primary neurons. (Scale bar, 100 μm.) (Magnification: 40×.) (*G*) LDH release was measured 24 h after treatment with BMAA (1 mM) in cultured neurons; 2 mM glutamate was used as a positive control. Data are expressed as mean ± SEM for three separate experiments. $*P < 0.05$; $**P < 0.01$.

regulated by it (4). We therefore measured levels of total and activated/inactivated forms of several PP2A-regulated protein kinases. The levels of phosphorylated/activated CaMKII, ERK1/2, and MEK1/2 increased noticeably after treatment with BMAA (Fig. 3 *F* and *G*). However, we detected no significant changes in the levels of phosphorylated GSK-3β, JNK, or p70 S6 kinase in BMAA-treated brain slices. We further observed that the inhibition of PP2A activity by okadaic acid up-regulates ERK1/2 phosphorylation, similar to our findings following BMAA treatment (Fig. S4). However, we cannot rule out the involvement of any nonspecific cytotoxic effect of BMAA in increasing the phosphorylation of ERK1/2. Collectively these results suggest that (*i*) inhibition of PP2A plays an important role in BMAA-induced tau hyperphosphorylation, (*ii*) the effect of BMAA is modulated both directly by PP2A and indirectly by the tau kinases that are regulated by PP2A, and (*iii*) the effect of BMAA on PP2A signaling in brain slices is similar to the effect we observed in PD brains.

BMAA Induces an Increase in Phosphorylation of PP2Ac at pTyr^{307} and Tau Hyperphosphorylation in Vivo in Rat Brain. Our observations in primary neuronal culture and brain slices prompted us to study the effect of BMAA on tau hyperphosphorylation in vivo in rat brain. Because of poor blood–brain barrier permeability of BMAA in the adult as compared with the neonatal brain (26), we selected newborn rats for these studies. We administered BMAA intracerebroventricularly (i.c.v.) in neonatal rats and then at various time points analyzed their brains for changes in PP2A and hyperphosphorylation of tau. We found that after BMAA treatment the level of phosphorylation of PP2Ac at Tyr^{307} in rat brains increased markedly for 24–48 h and then decreased to a normal level by 72–96 h (Fig. 4 *A* and *B*). Correspondingly, PP2A activity decreased, and this change was most significant at 48 h after BMAA treatment (Fig. 4*C*). In BMAA-treated rats the hyperphosphorylation of tau at Ser$^{262/356}$ and Thr^{212} peaked at 24–48 h, whereas phosphorylation at Ser^{199}, Thr^{205}, and Ser^{396} peaked at 72–96 h (Fig. 4*D*); in vehicle-treated rats the hyperphosphorylation of tau at all the sites studied also reached the highest levels by 72–96 h. Together these findings revealed that the effect of BMAA on PP2A signaling in vivo in rat brain was similar to the changes in PP2A we observed in PD brains and that hyperphosphorylation of tau at Ser$^{262/356}$ and Thr^{212} precedes that at Ser^{199}, Thr^{205}, and Ser^{396} in BMAA-treated rats.

PP2A Reverses BMAA-Induced Tau Hyperphosphorylation. To confirm the role of PP2A in BMAA-induced tau hyperphosphorylation, we treated the metabolically active rat brain slices with D-Erythro-S (DES), a PP2A activator (27), or expressed wild-type DsRed-tagged PP2Ac in primary neurons before BMAA treatment. We found that either activation of PP2A (Fig. 5 *A* and *B*) or overexpression of PP2Ac (Fig. 5 *C* and *D* and Fig. S5) blocked the BMAA-induced tau hyperphosphorylation.

To evaluate whether BMAA has any direct effect on PP2A, we carried out an in vitro PP2A activity assay using bovine purified PP2A holoenzyme in the presence or absence of BMAA (Fig. 5*E*). We found no effect of BMAA on PP2A activity. Collectively these findings suggest that BMAA does not promote PP2A inactivation directly but most probably affects its signaling.

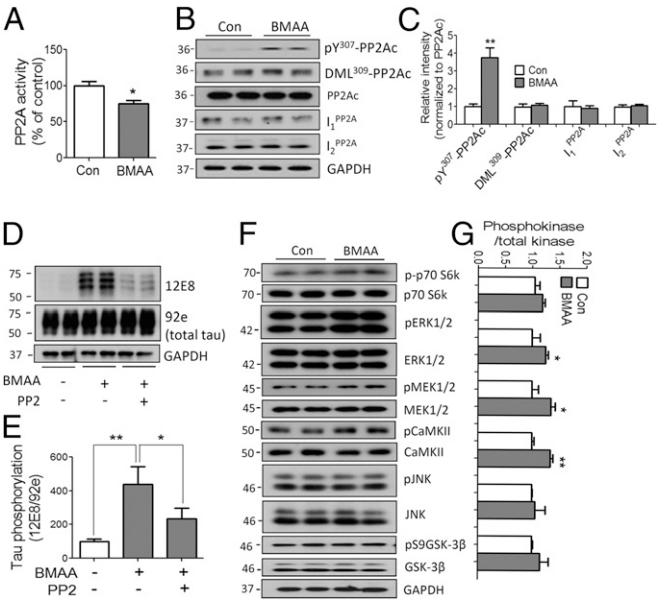

Fig. 3. BMAA induces an increase in pTyr^{307} PP2Ac, inhibits the phosphatase activity, and activates several tau protein kinases in rat brain slices. Rat hippocampal brain slices were treated with BMAA (1 mM) for 2 h, and the tissue homogenate was used for Western blots and to measure PP2A activity. (*A*) PP2A activity. (*B*) Western blots. (*C*) The quantitative analysis of pTyr^{307}-PP2Ac, DML^{309}-PP2Ac, I_1^{PP2A}, and I_2^{PP2A} of Western blots in *B* after normalization with total PP2Ac. (*D*) Western blots of brain slices treated with PP2, an Src kinase inhibitor (10 μM), for 30 min before treatment with BMAA. (*E*) Quantitative analysis of blots in *D*. (*F* and *G*) The Western blot pattern of total and phosphorylated kinases (*F*) and quantitation of phosphorylated kinases normalized with the level of corresponding kinase (*G*). Data are expressed as mean ± SEM for three separate experiments. $*P < 0.05$; $**P < 0.01$.

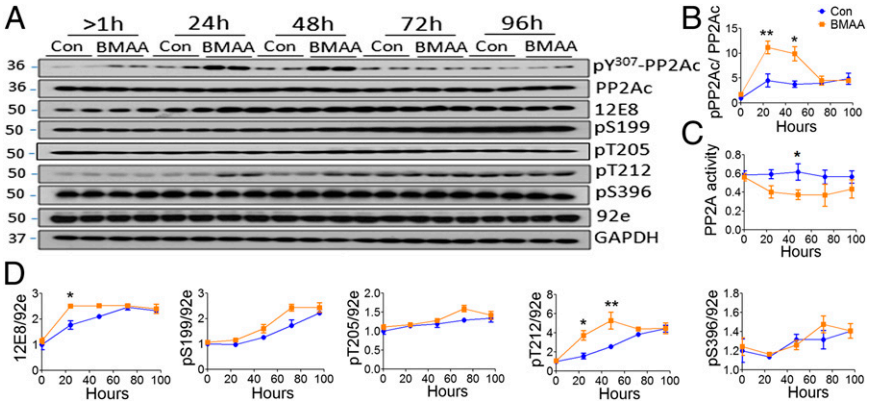

Fig. 4. The i.c.v. infusion of BMAA induces an increase in pTyr^{307}-PP2Ac, inhibition of PP2A activity, and hyperphosphorylation of tau in vivo in rat pups. Rat postnatal day 2 hippocampus was collected at the indicated time points after i.c.v. injection of BMAA (1.3 μmol) and was analyzed by Western blots to detect tau phosphorylation at individual sites and PP2A phosphorylation at Tyr^{307}. (*A*) Representative Western blots. (*B*) pTyr^{307}-PP2A level normalized with total PP2A level. (*C* and *D*) PP2A activity (*C*) and quantification of hyperphosphorylation of tau normalized with total tau (92e) (*D*). Data are expressed as mean ± SEM for two separate experiments (n = 4–5). pPP2Ac, pY^{307}-PP2Ac. *P < 0.05; **P < 0.01.

BMAA-Induced PP2A Inactivation Is Dependent on mGluR5 Activation. To identify the up-stream molecular mechanism involved in BMAA-induced PP2A (Tyr^{307}) phosphorylation, we incubated rat brain slices with and without 2-methyl-6-(phenylethynyl) pyridine (MPEP, an antagonist of mGluR5), MK-801 (an antagonist of NMDA), or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, an antagonist of AMPA) for 30 min before the addition of BMAA. We found that preincubation of the brain slices with 50 μM MPEP significantly blocked tau hyperphosphorylation at the 12E8 site induced by BMAA, but no significant change was observed when the slices were treated with either MK-801 (10 μM) or CNQX (20 μM) alone (Fig. 6 *A* and *B*). The BMAA-induced decrease in PP2A activity was blocked completely by pretreatment with MPEP but not with MK-801 or CNQX (Fig. 6*C*). Interestingly, cotreatment with MPEP and MK-801 entirely prevented the BMAA-induced tau hyperphosphorylation and PP2A inhibition. These data suggest that the activation of the mGluR5 receptor might be primarily involved in the inactivation of PP2A and the resultant hyperphosphorylation of tau in BMAA-treated brain slices. PP2A is known to be associated with mGluR5, and activation of mGluR5 leads to the dissociation of PP2A from this receptor and consequently to PP2A inactivation (28). To confirm whether mGluR5 activation is the underlying cause of PP2A inactivation through dissociation, we carried out coimmunoprecipitation experiments and analyzed the amount of PP2Ac associated with mGluR5 in primary neurons in the presence or absence of 1 mM BMAA. We found that the treatment of the primary neuronal cultures with BMAA led to the dissociation of PP2Ac from mGluR5, and cotreatment with MPEP completely blocked the dissociation of PP2A from the receptor (Fig. 6 *D* and *E*). These data suggest that BMAA-induced mGluR5 activation probably leads to the dissociation and consequently to the inactivation of PP2A by its phosphorylation at Tyr^{307}.

Discussion

Neurofibrillary pathology made up of abnormally hyperphosphorylated tau is a hallmark of several neurodegenerative diseases which, in addition to PD, include AD, frontotemporal dementia-tau, Pick disease, cortico-basal degeneration, progressive supranuclear palsy, and adults with Down syndrome, suggesting that several different etiopathogenic mechanisms probably lead to this lesion. Thus, elucidation of these various mechanisms is critical for the development of rational therapeutic drugs for these diseases and is a major goal in the field. Off and on, over the last five decades, BMAA has been considered a candidate neurotoxin in Guam ALS and PD, but experimental evidence to support its role has been lacking. The present study identifies the association of tau pathology with a decrease in PP2A activity and increased phosphorylation of PP2Ac at Tyr^{307} as a cause of this deficit in the phosphatase activity in PD. Furthermore, we show that all the changes in PD brains that we described above can be replicated by treatment with BMAA in rat brain. We found that treatment with BMAA (*i*) can produce an increase in phospho-Tyr^{307}-PP2Ac, (*ii*) can inhibit PP2A activity, and (*iii*) can produce abnormal hyperphosphorylation of tau in hippocampal primary neurons, in metabolically active brain slices, and in vivo in rats. Finally, using primary neurons and brain slices, we show that BMAA produces the increase in phospho-Tyr^{307}-PP2Ac and consequent hyperphosphorylation of tau by activating mGluR5, leading to the dissociation of PP2Ac from the receptor and then its phosphorylation at Tyr^{307} by Src. Although we did not measure Src activity in our studies, the report that Src activity is up-regulated mainly through Tyr^{416} phosphorylation (29) certainly rules out the direct possible involvement of BMAA, because it is not a kinase. This molecular mechanism (Fig. 7) is different from that involving cerebral ischemia and hypoxia, which inhibit PP2A by the activation of asparaginyl endopeptidase and the consequent cleavage and translocation of I_2^{PP2A} in AD and ALS (6, 19, 21, 30).

BMAA exposure is not localized to Guam but rather is ubiquitous throughout the world (31). BMAA can enter the food chain from cyanobacteria, can be incorporated into plant and animal proteins, and can be bio-concentrated in the ecosystem

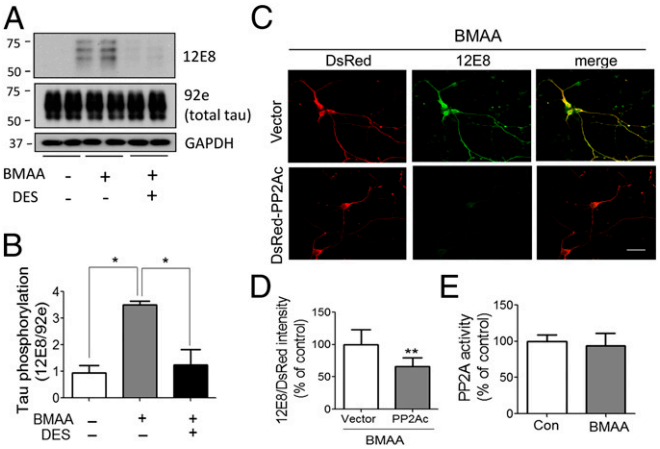

Fig. 5. Up-regulation of PP2A by DES or PP2A (DsRed) reverses BMAA-induced tau hyperphosphorylation in rat brain slices and mouse hippocampal primary neurons. (*A*) Western blots of brain slices treated with BMAA (1 mM) for 2 h with or without pretreatment with the PP2A activator DES (15 nM). (*B*) Quantitation of the blots in *A*. (*C*) The primary hippocampal neurons were transfected with DsRed-vector or DsRed-PP2Ac at 6 days in vitro (DIV) and then were treated with BMAA (1 mM) for 3 h at 8 DIV. The neurons were fixed and costained with DsRed (red) and 12E8 (green). (Scale bar, 50 μm.) (Magnification: 40×). (*D*) Quantitation of 12E8 staining normalized with DsRed. (*E*) In vitro PP2A activity was measured using purified PP2A holoenzyme in the presence or absence of BMAA. Data are expressed as mean ± SEM for three separate experiments. *P < 0.05; **P < 0.01.

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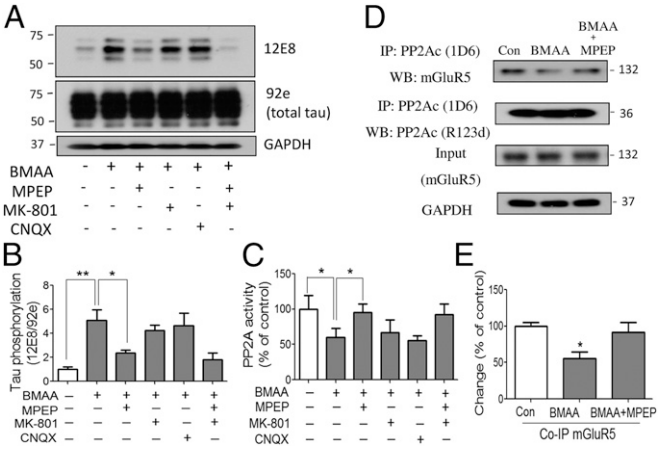

Fig. 6. BMAA induces tau hyperphosphorylation by activating mGluR5. Rat Hippocampal brain slices were incubated with BMAA (1 mM) for 2 h with or without the 30-min pretreatment with MPEP (50 μM), MK-801 (10 μM), or CNQX (20 μM). (*A*) Representative Western blots. (*B*) Quantitation of blots from *A*. (*C*) PP2A activity. (*D*) PP2Ac immunoprecipitates blotted for coimmunoprecipitating mGluR5 after BMAA treatment in primary neuronal cultures. (*E*) Quantitation of data from *D*. Data are expressed as mean ± SEM for three separate experiments. $*P < 0.05$; $**P < 0.01$.

(32, 33). The bound form of BMAA may function as an endogenous neurotoxic reservoir, releasing free BMAA and causing neurodegeneration (34). BMAA promotes neuronal death both in vitro and in vivo (15, 18, 35). In the present study, we found that 1 mM BMAA caused cell death in mixed primary neuronal cultures. We used 1 mM BMAA for most of the experiments, considering that the reported average brain concentrations of the compound in Guam PD and North American AD cases were 627 μg/g, 5 mM and 95 μg/g, 0.8 mM, respectively (9, 36). Therefore, the 1-mM BMAA concentration used in our experimental paradigms for modeling PD-related pathology is highly relevant. In the AD brain phospho-Tyr^{307}-PP2Ac is increased (37), and phosphatase activity is decreased (6). Thus, taken together, the results in the present study support the idea that tau phosphorylation might result from an environmental as well as from an endogenous toxin such as BMAA and that this mechanism could be common to AD, PD, and ALS.

In the present study, we found that the inhibition of PP2A activity could be accounted for by the phosphorylation of PP2Ac at Tyr^{307}. We obtained similar results in the rat brain slices and in vivo in rat brain by treatment with BMAA. Preincubation of brain slices with PP2, an inhibitor of Src responsible for PP2A (Tyr^{307}) phosphorylation, significantly attenuated BMAA-induced tau phosphorylation, suggesting that BMAA may inhibit PP2A through Tyr^{307} phosphorylation. The critical role of PP2A in BMAA-induced tau hyperphosphorylation was confirmed further by both pharmacological and genetic approaches; the activation of PP2A by DES in brain slices and the overexpression of PP2Ac in primary neuronal cultures entirely prevented the hyperphosphorylation of tau when treated with BMAA. The increase we observed in the activation of CaMKII, ERK1/2, and MEK1/2 by BMAA in rat brain slices probably is secondary to the inhibition of PP2A, which is known to regulate these kinases (4).

We found a marked increase in the abnormal hyperphosphorylation of tau in PD brains at Ser^{199}, Thr^{205}, Thr^{212}, and $Ser^{262/356}$. All these phosphorylation sites are known to be involved in promoting the assembly of tau into filaments (38). We observed similar patterns of hyperphosphorylation of tau in BMAA-treated primary neurons and brain slices. Among all the sites we investigated, Ser^{262} and Ser^{356} reside within the microtubule-binding domains of tau, and phosphorylation of Ser^{262} is most implicated in inhibition of tau's interaction with microtubules (39, 40). Although Ser^{199}, Thr^{205}, and Thr^{212} are not within the microtubule-binding domains, the phosphorylation of these sites primes the phosphorylation at other sites as well as increasing tau's microtubule inhibitory activity and self-aggregation into filaments (41). In addition to the phosphorylation of tau at different sites, we observed some interesting time-dependent sequential changes of tau-site–specific phosphorylation in BMAA-infused neonatal rat brain. Phosphorylation of $Ser^{262/356}$ and Thr^{212} preceded the appearance of phosphorylation at Ser^{199} and Thr^{205}. The earlier phosphorylation of Ser^{262} is in agreement with the observation that this phosphorylation detaches tau from microtubules and thereby makes it available for the formation of PHFs. In line with our findings, Augustinack et al. (42) reported that the preneurofibrillary tangles stain positively primarily for $pSer^{262/356}$, whereas both intra- and extracellular NFTs stain positively most prominently for $pSer^{199}$/$pSer^{202}$/$pThr^{205}$, $pThr^{212}$/$pSer^{214}$, and $pSer^{396}$/$pSer^{404}$. In contrast, we observed that $pSer^{396}$ expression was unchanged in BMAA-treated mouse hippocampal primary neuronal cultures, hippocampal brain slices, and even in in vivo rat brain. The apparent discrepancies may be caused by the genomic and cellular complexity of human vs. mouse/rat tissues. Another possible explanation could be that Ser^{396} is the least favorable site for dephosphorylation by PP2A (5, 43) in response to BMAA treatment. Based on the data in the present study, it is reasonable to delineate an association between disease progression and abnormal hyperphosphorylation of tau at specific sites in PD, similar to those reported previously in AD (42, 44).

The present study suggests the direct involvement of a PP2A-dependent disease mechanism. PP2A has been shown to form a complex with NMDA (45) and mGluR5 (28) receptors, and dissociation of PP2A from these receptors reduces PP2A activity. Moreover, BMAA has been reported to affect all the main glutamate receptors, i.e., NMDA, AMPA/kainate, and metabotropic receptors (35). This evidence suggests that the effect of BMAA on either NMDA or mGluR5 can facilitate PP2A inactivation through dissociation from the receptor. In the present study, however, we found that activation of mGluR5, but not of the NMDA receptor, was responsible for BMAA-induced tau hyperphosphorylation in brain slices. The exact reason underlying the selective action of BMAA on mGluR5 is not known. Possible explanations include the predominant expression of mGluR5 in hippocampal neurons and the particular localization of mGluRs on the dendritic membrane (46) making the receptor selectively accessible to BMAA. In the present study, coimmunoprecipitation

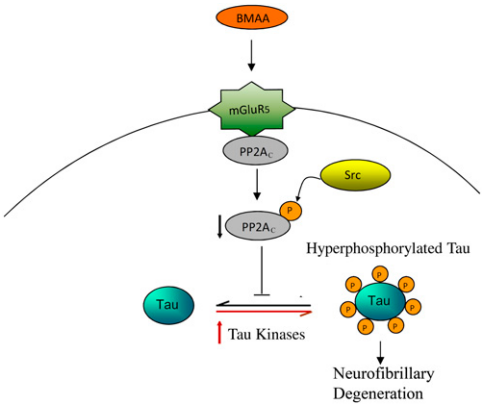

Fig. 7. A proposed schematic representation of the possible mechanism leading to neurofibrillary degeneration in PD. BMAA activates the mGluR5 receptor and dissociates PP2Ac from the receptor. Src kinase then acts on free and available PP2Ac and phosphorylates at Tyr^{307}, inhibiting its activity and shifting the balance toward tau kinases, thus leading to the hyperphosphorylation of tau as evident in Guam PD brain.

experiments further confirmed that the activation of mGluR5 dissociates PP2A from the mGluR5 receptor. Taken together, the results of the present study suggest that activation of mGluR5 facilitates a series of biochemical events, including dissociation of PP2A from mGluR5, Tyr^{307} phosphorylation of PP2Ac, reduction of PP2A activity, and tau hyperphosphorylation.

In summary, based on the findings in the present study, we propose the involvement of a mechanistic pathway underlying the neurofibrillary degeneration in PD, so that an environmental toxin, such as BMAA, down-regulates PP2A activity and induces tau hyperphosphorylation by activating mGluR5; the activation of mGluR5 results in the dissociation of PP2Ac from the receptor, followed by its phosphorylation at Tyr^{307} by Src.

Materials and Methods

Two sets of frozen autopsied tissue samples of frontal cortices from histopathologically confirmed Guam PD cases and age-matched controls were used. The first set consists of eight Guam PD cases and seven nonneurological controls from Guam (Fig. 1 and Table S1); the second set comprises four Guam PD cases and five nonneurological controls from the United States mainland (Fig. S1 and Table S2). For detailed information on these cases, mouse embryonic day 18 hippocampal primary neuronal cultures and transfections, preparation of rat brain slices, BMAA treatment, i.c.v. infusion of rat pups with BMAA, coimmunoprecipitation, PP2A activity assays, SDS/PAGE, Western blots, densitometry, immunofluorescent staining, and statistical analyses, see *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Jian-Zhi Wang (Tongji Medical College) for providing the DsRed-wtPP2Ac vector, Dr. Maria del Carmen Cardenas-Aguayo (New York State Institute for Basic Research and Developmental Disabilities) for assisting in primary neuronal culture, Ms. Janet Murphy for secretarial assistance, and Ms. Kaitlin Drouin for preparation of Guam brain tissues. This work was supported in part by the New York State Office of People with Developmental Disabilities and National Institutes of Health Grant AG019158.

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