NMDA receptor mediates tau-induced neurotoxicity by calpain and ERK/MAPK activation

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The altered function and/or structure of tau protein is postulated to cause cell death in tauopathies and Alzheimer's disease. However, the mechanisms by which tau induces neuronal death remain unclear. Here we show that overexpression of human tau and of some of its N-terminal fragments in primary neuronal cultures leads to an *N*-methyl-D-aspartate receptor (NMDAR)-mediated and caspase-independent cell death. Death signaling likely originates from stimulation of extrasynaptic NR2B-subunit-containing NMDARs because it is accompanied by dephosphorylation of cAMP-response-element-binding protein (CREB) and it is inhibited by ifenprodil. Interestingly, activation of NMDAR leads to a crucial, sustained, and delayed phosphorylation of extracellular-regulated kinases 1 and 2, whose inhibition largely prevents tau-induced neuronal death. Moreover, NMDAR involvement causes the fatal activation of calpain, which, in turn, degrades tau protein into a 17-kDa peptide and possibly other highly toxic N-terminal peptides. Some of these peptides are hypothesized, on the basis of our *in vitro* experiments, to initiate a negative loop, ultimately leading to cell death. Thus, inhibition of calpain largely prevents tau degradation and cell death. Our findings unravel a cellular mechanism linking tau toxicity to NMDAR activation and might be relevant to Alzheimer's disease and tauopathies where NMDAR-mediated toxicity is postulated to play a pivotal role.

extracellular-regulated kinase | mitogen-activated protein kinase | neurodegenerative diseases | glutamate receptors | Alzheimer's disease

Abnormalities of the protein tau have been implicated in neuronal death in a variety of tauopathies, such as frontotemporal dementia and Alzheimer's disease (AD) (1).

Tau protein supports the microtubule system responsible for intracellular transport, axonal morphology, and cell physiology. The correct functioning of tau depends upon a balance between the different tau isoforms, its state of phosphorylation, and its structural integrity. A perturbation of these parameters may cause tau dysfunction and neurodegeneration (2).

Different cellular and animal models of tau pathology have been created to discern the molecular and cellular processes underlying tau-mediated toxicity. From these studies it has emerged that tau-induced neuronal dysfunction and neuronal loss may be attributed to the reduced functionality of tau, to the toxic function gained by phosphorylated, mutated, cleaved, or aggregated tau (3–6), and to altered expression levels of normal tau isoforms (7). Although this evidence suggests that alterations of tau may directly cause neuronal degeneration and cell death, little is known about the molecular and cellular mechanisms underlying tau-mediated cellular toxicity and, more importantly, the mode of cell death caused by tau aberrations. Morphological changes consistent with apoptosis, with or without activation of caspases, have been detected "*in vitro* " in neurons overexpressing pseudohyperphosphorylated tau (4) or tau cleaved at residue D421 (8) as well as "*in vivo* " in some neuronal and glial cells of transgenic mice expressing human tau (htau) isoforms (9) and in oligodendrocytes of mutant htau-transgenic mice (10). However, a nonapoptotic neurodegeneration process has been described in transgenic mice overexpressing the mutated forms of htau P301S (11), V337M (12), and P301L (13) and in astrocytes expressing the longest htau isoform (14). Furthermore, other studies have provided evidence that overexpression of htau in transgenic mice caused extensive organelle swelling and cytoplasmic vacuolization more suggestive of necrosis and likely of glutamate-mediated excitotoxicity (9). In this context, it is known that *N*-methyl-D-aspartate receptor (NMDAR)-expressing neurons are vulnerable to AD loss, supporting the hypothesis of excitotoxic NMDAR activity-mediated death in AD (15–17).

To decipher the molecular determinants of tau-induced neurotoxicity we overexpressed full-length htau and a variety of N-terminal tau fragments in primary neuronal cultures. We found that activation of NMDAR accompanied by calpain activation and defects in regulation of extracellular-regulated kinase (ERK) signaling, combined with a reduced activity of phospho-cAMP-response-element-binding protein (CREB), may be a crucial mechanism by which tau and some of its N-terminal fragments may induce toxicity.

Results

Tau Overexpression Is Toxic. In our previous study, we observed that moderate overexpression of full-length htau in cerebellar granule cells (CGCs), although protective against K^+ deprivation-induced cell death, caused a rapid ≈20% cell death, and we surmised that it would be attributed to the high level of tau expression reached in some neurons (18). To test our hypothesis we infected CGCs at 4 days *in vitro* with various multiplicities of infection (MOIs) of tau-(1–441) vector and Lac-Z vector as control and assayed for neuronal death 24 and 48 h later. Indeed, we found that, in this condition, increased cell death was observed in CGCs expressing increased level of htau protein (Fig. 1*A*). At 24 h after infection ≈83%, 76%, 63%, and 60% of neurons were viable after transduction of tau-(1–441) vector at MOIs of 30, 60, 120, and 200. At 48 h, viability was reduced to ≈75%, 70%, 55%, and 53%, respectively. In sharp contrast, neurons infected with control vector at various MOIs remained viable. These data indicate that tau toxicity is dose-dependent and the highest effect was observed when exogenous tau was ≈2.5- 3-fold the endogenous tau level (Fig. 1*B Lower*). The same profile was also observed in primary cortical cultures (Fig. 1 *C* and *D*).

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Abbreviations: AD, Alzheimer's disease; CGCs, cerebellar granule cells; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CREB, cAMP-response-element-binding protein; ERK1/2, extracellular-regulated kinases 1 and 2; htau, human tau; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMDAR, *N*-methyl-D-aspartate receptor; ODN, oligodeoxynucleotide.

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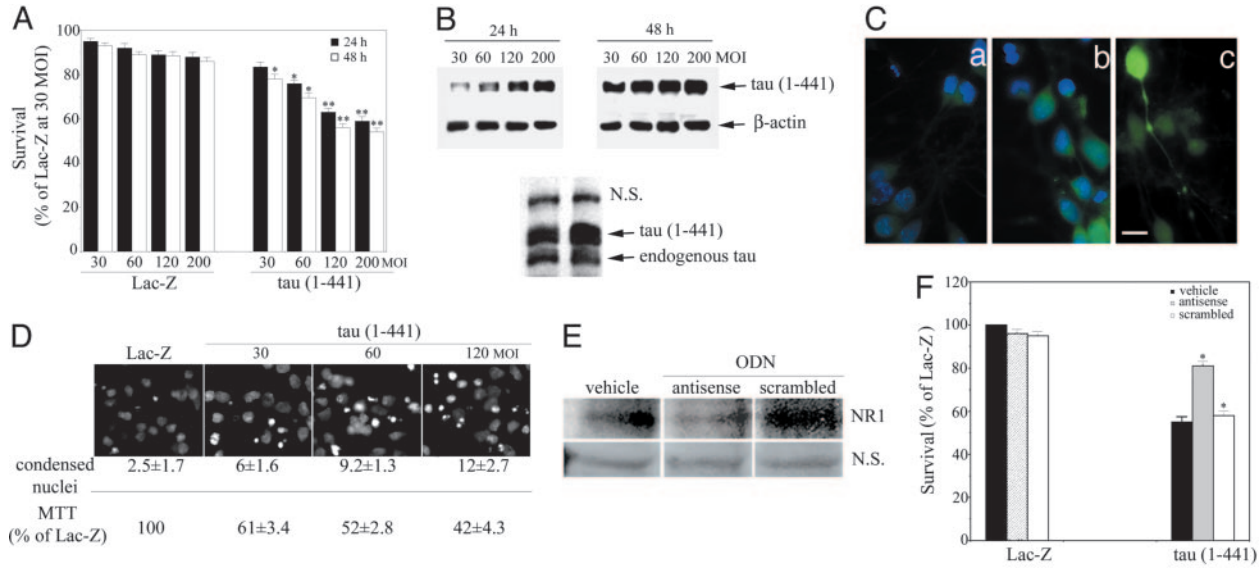

Fig. 1. Reduced viability of tau-(1–441)-infected neurons. (*A*) CGCs were infected at 4 days *in vitro* with either Lac-Z- or tau-expressing adenovirus vectors at the MOIs indicated. Survival was assessed 24 and 48 h later by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Each data point is the mean ± SE of triplicate determinations of three independent experiments and is expressed as percentage of Lac-Z-infected cells, considering the value obtained in these cells as 100% (*, $P < 0.05$; **, $P < 0.01$ compared with Lac-Z-infected neurons). (*B*) Western blot analysis of lysates from tau-(1–441)-infected CGCs performed with mAb 9E10 and normalized with β-actin (*Upper*) and with mAb TAU-1 (*Lower*) and normalized with a nonspecific (N.S.) band. (*C*) Immunofluorescence analysis of tau-(1–441)-infected cortical neurons at MOIs of 30 (*a*) and 120 (*b*), immunostained with mAb 9E10 (green). Forty-eight hours after infection, nuclei were stained with Hoechst 33258 (blue). A cortical neuron expressing tau with a sign of degeneration (varicosity along a neurite) is shown (*c*). (Scale bar, 20 μm.) (*D*) Micrographs of Hoechst 33258-stained cortical neurons after 48 h of infection. Values corresponding to condensed nuclei and to MTT assay are reported below. (*E*) Western blot analysis of the NR1 subunit expression in CGCs treated with the antisense and scrambled ODNs. (*F*) CGCs were incubated with NR1 antisense and scrambled ODNs and then infected with Lac-Z and tau-(1–441) vectors for 48 h, when survival was determined. Data are expressed as in *A*.

Tau Toxicity Is NMDAR Dependent. To determine whether caspases mediate tau-induced cell death, we tested the protective effect of a broad-spectrum caspase inhibitor, Z-Val-Ala-Asp fluoromethyl ketone (z-VAD-fmk) in CGC and cortical neurons infected with tau-(1–441) at an MOI of 120. As shown in Table 1, z-VAD-fmk (100 μM) was unable to alleviate the loss of neurons, suggesting that this mode of death is caspase independent, as confirmed by the inability to detect by Western blot analysis the cleaved, active fragment of caspase-3 (Fig. 5, which is published as supporting information on the PNAS web site). Moreover, overexpression of Ad-Bcl2, a key antiapoptotic regulator of neuronal cell death in a variety of *in vivo* and *in vitro* paradigms, failed to rescue tau-(1–441)-infected neurons from death (Table 1). These data suggest that the mode of tau-mediated cell death, in both cerebellar granule and cortical neurons, is not classic apoptosis as also reported by other authors (9, 13, 14). Moreover, we obtained data suggesting that this observation could also be extended to hippocampal neurons overexpressing tau (data not shown). Tau-(1–441)-infected CGCs acquired a necrotic-like morphology (data not shown) indicative of glutamate-mediated toxicity that is mainly mediated by NMDAR (19). Consistently, three antagonists of NMDAR, MK-801 (10 μM), 2-amino-5-phosphonovaleric acid (APV) (100 μM), and memantine (10 μM), but not 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (40 μM) an antagonist of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor, and GYKY52466 (100 μM), an AMPA receptor antagonist, were able to protect CGCs from tau toxicity (Table 1). To confirm the involvement of NMDAR in tau toxicity we transfected CGCs with an NR1 antisense oligodeoxynucleotide (ODN) proved to be effective and specific in previous study (20). This strategy allowed us to reduce the NR1 expression by ≈50%, as assessed by immunoblotting (Fig. 1*E*).

Neurons treated with NR1 antisense ODN were protected from tau-induced toxicity (survival 83 ± 2.5% at 48 h), whereas neurons treated with scrambled ODN were susceptible to tau toxicity as well as neurons treated with vehicle alone and infected with tau-(1–441) (survival 51.8 ± 3% and 55.5 ± 3% at 48 h, respectively) (Fig. 1*F*).

Table 1. Percent survival at 48 h of tau-(1–441)-infected CGCs and cortical neurons under various treatments

	CGCs		Cortical neurons	
Treatment	Lac-Z	Tau-(1–441)	Lac-Z	Tau-1(1–441)
—	100	51 ± 2.8	100	40 ± 3
z-VAD-fmk	95 ± 3.2	52 ± 4**	97 ± 2	44 ± 2**
Ad-Bcl2	92 ± 2.8	48 ± 4.7**	92 ± 3.2	47 ± 2**
MK-801	98 ± 1.7	92 ± 3.4**	97 ± 2	100 ± 2.2**
APV	93 ± 3.7	87 ± 3.7**	95 ± 2.8	98 ± 2**
Memantine	100 ± 2	98 ± 2.4**	97 ± 3	97 ± 1.8**
CNQX	95 ± 4	50 ± 4.2**	95 ± 2	35 ± 3**
GYKY52466	97 ± 3.7	48 ± 3.2**	93 ± 4	38 ± 4**

z-VAD-fmk, Z-Val-Ala-Asp fluoromethyl ketone (100 μM); MK-801 (10 μM); APV, 2-amino-5-phosphonovaleric acid (100 μM); memantine (10 μM); CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione, (40 μM); GYKY52466 (100 μM). Data are expressed as in Fig. 1*A*. **, $P < 0.01$ compared with untreated tau-expressing neurons.

Two N-Terminal Tau Regions Mediate Different Types of Tau Toxicity. To map the tau protein regions involved in NMDAR-mediated toxicity, various tau fragments were cloned into adenoviral vector (Fig. 2*A*) and expressed in CGCs at various MOIs. All fragments affected, in a dose-dependent manner and at different extent, the viability of CGCs (Fig. 2*B*). The more harmful were tau-(1–44), tau-(26–44), and tau-(26–230), which reached their maximal neurotoxic effect at an MOI of 50 with only ≈20% of cells viable 48 h after infection. By contrast, for tau-(1–156) and

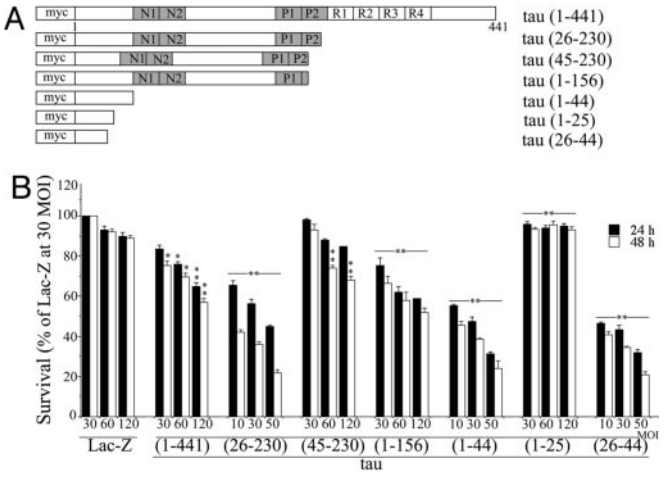

Fig. 2. Impact of various tau fragments on CGC viability. (*A*) Diagram of tau vectors used in this study. All constructs are derived from the longest htau isoform and expressed with 6 Myc epitope tags fused to the N terminus. (*B*) CGCs were infected at various MOIs with the vectors indicated. Viability was determined 24 and 48 h after infection and is expressed as reported for Fig. 1*A*. **, $P < 0.01$; *, $P < 0.05$ compared with Lac-Z-infected CGCs at an MOI of 30.

tau-(45–230) the survival was 52 ± 2.3% and 72 ± 1.8% ($n = 4$), respectively, at an MOI of 120. All fragments, except tau-(45–230), were rapidly toxic also at the lowest MOI. In the case of tau-(45–230) the decrease of survival became significantly detectable only at 48 h, when, at MOIs of both 60 and 120, corresponding to 2- to 3-fold the level of expression of endogenous tau (data not shown), caused ≈30% death, which remained substantially unchanged at 96 h after infection (data not shown). It must be pointed out that vector encoding the first 25 aa of htau resulted in the complete absence of toxicity even when the highest MOIs were used (Fig. 2*B*). We found that tau-(1–44), as previously reported (19), tau-(26–230), tau-(1–156), and tau-(26–44) caused an NMDAR-mediated cell death (Fig. 6, which is published as supporting information on the PNAS web site) because it was inhibited by antagonists of NMDAR. No classical hallmarks of apoptosis were observed, as also reported for full-length tau (Figs. 5 and 6). In contrast, the toxicity exerted by tau-(45–230) did not adequately fulfill either the criteria of classical apoptosis or those of necrosis, suggesting that an alternative mechanism of death is activated by tau-(45–230). In conclusion, we found that a high level of full-length tau exerts its toxic action by activating NMDAR and that the minimal region bearing this property is that encoded by the tau-(26–44) vector.

Tau-Induced Cell Death Is Mediated by NR2B Receptor. NMDARs mediate opposing effects according to their localization. Stimulation of synaptic NMDAR induces prosurvival events, whereas activation of extrasynaptic NMDAR leads to excitotoxic death (21). It has been reported that whereas NR2A receptors are predominantly confined to synapses, NR2B-containing receptors are particularly distributed extrasynaptically (22). To test whether extrasynaptic NMDARs were involved in tau-induced cell death, we treated Lac-Z- and tau-infected neurons with ifenprodil (10 μM), an antagonist of NR2B receptors (23). Fig. 3*A* shows that ifenprodil effectively provided neuroprotection against cell death caused by tau-(1–441) and tau-(1–44), whereas it did not affect the decrease in survival caused by tau-(45–230). This observation further suggests that two modes of cell death are induced by different portion of tau protein. Activation of extrasynaptic NMDAR has been reported to trigger a CREB shut-off signal (24), and we found that tau-(1–441) and tau-(1–44) overexpression induced a clear decrease (≈36% and 77%

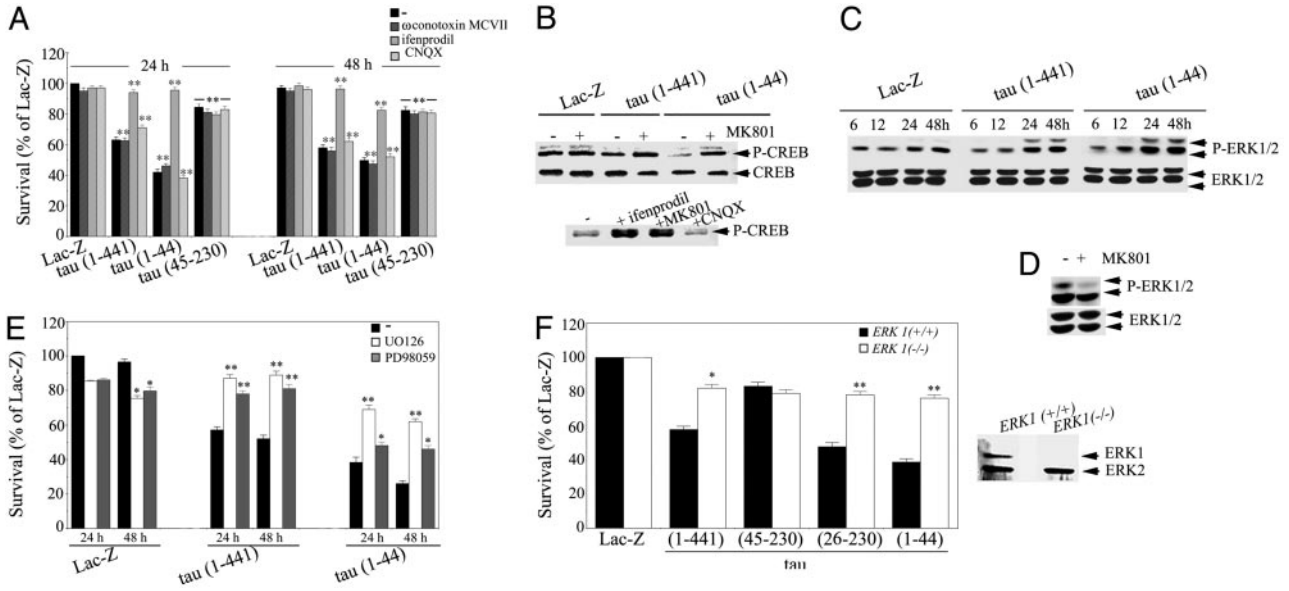

Fig. 3. Tau toxicity involves extrasynaptic NMDAR, CREB dephosphorylation, and ERK1/2 activation. (*A*) Survival of CGCs infected with vectors indicated in the absence or in the presence of ifenprodil (10 μM), CNQX (40 μM), or ω-conotoxin MCVII (1 μM), a blocker of N-, P-, and Q-type calcium channels, reported to be inhibited by ifenprodil. Data are reported as in Fig. 1*A*. (*B*) Lysates from Lac-Z-, tau-(1–441)-, and tau-(1–44)-infected CGCs treated with MK-801 (10 μM), ifenprodil (10 μM), or CNQX (40 μM) for 24 h were probed for phospho-CREB (P-CREB) and total CREB. Representative blots of two independent experiments with similar results are shown. (*C* and *D*) Time course analysis of phospho-ERK1/2 (P-ERK1/2) analyzed by Western blotting from lysates of Lac-Z-, tau-(1–441)-, and tau-(1–44)-infected CGCs in the absence (*C*) or in the presence (*D*) of MK-801. Representative blots of three independent experiments with similar results are shown. (*E*) Survival at 24 and 48 h of tau-infected neurons in the absence or in the presence of UO126 (5 μM) or PD98059 (15 μM). Data are reported as in Fig. 1*A*. *, $P < 0.05$; **, $P < 0.01$ compared with tau-untreated cells). (*F*) Both wild-type (ERK1$^{+/+}$) and ERK1-deficient (ERK1$^{-/-}$) CGCs were prepared from 6-day-old mice and infected with the vectors indicated. Data are reported as in Fig. 1*A*. *, $P < 0.05$ compared with Lac-Z-infected CGCs. (*Upper Right*) Survival was determined 48 h later by the MTT assay. (*Lower Right*) Western blot detecting ERK1/2 in CGC lysates from ERK1$^{+/+}$ and ERK1$^{-/-}$ mice.

respectively, compared with phospho-CREB level in Lac-Z-infected neurons) in CREB phosphorylation at Ser-133. Treatment with NMDAR antagonists, MK-801 and ifenprodil, significantly sustained a robust activation of CREB in tau-infected neurons, whereas CNQX was unable to restore or increase the level of phospho-CREB (Fig. 3*B*).

Tau Induces NMDAR-Dependent Activation of ERK1/2. A role for ERK1/2 signaling pathway has been proposed in NMDAR-mediated neuronal death (25). We investigated whether tau neurotoxic effect could be mediated by these kinases. We found that tau-(1–441) and tau-(1–44) overexpression in CGCs led to ERK1/2 activation (detected by phosphorylated ERK1/2, P-ERK1/2) observed 12 h after transduction and until 48 h (Fig. 3*C*). ERK1/2 activation was more pronounced for tau-(1–44) compared with tau-(1–441). Increased phosphorylation of ERK1/2 was not detected in neurons overexpressing tau-(45–230) (data not shown). To determine whether ERK1/2 activation is NMDAR dependent, we exposed CGCs to MK-801 (10 μM) for 24 and 48 h after transduction, and we found a suppression of ERK1/2 activation (Fig. 3*D*). To test whether ERK1/2 activation contributes to tau-induced neurotoxicity, we evaluated the effect of UO126 (5 μM) and PD98059 (15 μM), two inhibitors of mitogen activated protein kinase (MEK)1/2 on the survival of neurons overexpressing tau-(1–441), tau-(1–44), and tau-(45–230). UO126 and PD98059, although toxic for Lac-Z-infected CGCs with a total loss of ≈35% of neurons at 48 h, markedly prevented tau-(1–441) and tau-(1–44)-induced cell death. For example, expression of tau-(1–44) produced 65 ± 5.7% and 74 ± 4.8% neuronal death (n = 3) measured, respectively, 24 and 48 h later. In the presence of UO126 (5 μM) cell death was reduced to 25 ± 4.5% at 24 h and to 50 ± 3.5% at 48 h. Similar results were obtained with PD98059 (15 μM) (Fig. 3*E*). To provide independent evidence for the proposed role of these kinases on tau-induced cell death, we overexpressed tau vectors in CGCs obtained from ERK1$^{-/-}$ mice (26). ERK1-deficient CGCs exhibited significant protection from tau-(1–441)-, tau-(1–44)-, and tau-(25–230)-induced death in comparison with cell death induced in ERK1$^{+/+}$ CGCs. In contrast, tau-(45–230)-mediated cell death was not affected by ERK1 signaling (Fig. 3*F*). Thus the inhibition of ERK1/2 pathway, by pharmacological treatment as well as by genetic ablation of ERK1, can prevent tau-mediated cell death, pointing out a crucial role for ERK1 in tau-induced neuronal degeneration.

NMDAR-Mediated Tau Toxicity Is Associated with Calpain Activation and Tau Cleavage. Ca^{2+} influx through NMDAR may activate calpain, a major effector protease in glutamate-mediated toxicity by degrading key cytoskeletal proteins (27). To establish whether NMDAR-mediated calpain activation occurred in tau-expressing neurons, we performed Western-blot analysis with an antibody against calpain-1 able to recognize both the latent (80-kDa) and the activated (78- to 76-kDa and 58-kDa) forms (28, 29). We found that in tau-(1–441)- and tau-(1–44)-, but not tau-(45–230)- and Lac-Z-infected neurons, there was an increase both in the latent and activated forms of calpain blocked by MK-801 (10 μM) and by calpeptin (10 μM), a cell-permeant calpain-specific inhibitor (Fig. 4*A*).

We determined whether calpain inhibition could prevent tau toxicity, finding that both calpeptin (10 μM) and Ad-calpastatin overexpression provided robust protection against tau-(1–441) and tau-(1–44) toxicity, survival being 88 ± 4% and 82 ± 3.4%, respectively, for tau-(1–441) and 78 ± 4% and 79 ± 3%, respectively, for tau-(1–44). Again, tau-(45–230)-mediated cell death was not affected by these treatments (Fig. 4*B*).

It has been reported that activation of calpain leads to degradation of tau with the formation of a 17-kDa fragment corresponding to the region encoded by the tau-(45–230) vector

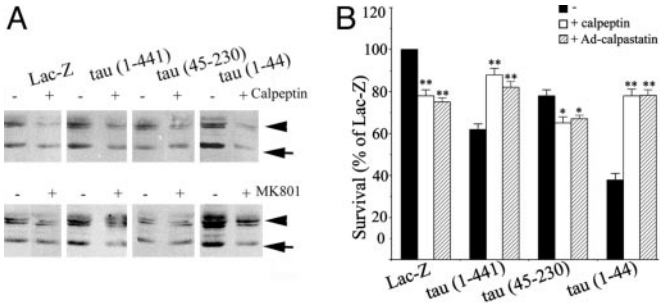

Fig. 4. Tau toxicity is accompanied by NMDAR-dependent activation of calpain. (*A*) Western blot analysis, with antibody against calpain, of lysates from CGCs infected with the vectors indicated, in the absence or in the presence of MK-801 or calpeptin (10 μM) for 24 h (arrowhead: mature calpain; arrow, active calpain). (*B*) Viability of CGCs 24 h after infection with LacZ or tau vectors, in the absence or in the presence of calpeptin (10 μM) or Ad-calpastatin. Data are reported as in Fig. 1*A*. **, $P < 0.01$ by unpaired t test compared with untreated tau-infected cells.

(30, 31). We tested whether tau-mediated calpain activation resulted in the appearance of this fragment. Thus, CGCs were infected with tau-(1–441) vector at MOIs ranging from 30 to 120 for 24 h after infection, and Western blot analysis of lysates prepared from these cells was performed with mAb TAU-1. Fig. 7, which is published as supporting information on the PNAS web site, shows the presence of the 17-kDa tau fragment, whose amount was correlated with the expression of exogenous tau and prevented by MK-801 and calpeptin, indicating that it was due to tau-induced NMDAR-dependent calpain activation.

Discussion

It is a generally accepted notion that tau plays a crucial role in neuronal loss associated with tauopathies and AD.

Nevertheless, how this protein causes cell death remains unclear. The findings reported in this study constitute an attempt to elucidate its possible mode of action.

We found that the extent of tau-induced neuronal loss, both in cultured cortical neurons and in CGCs, is directly related to the level of expression of the transgene. The most potent neurotoxic effect was observed when human tau was 3-fold higher than that normally expressed in rats, and our study confirms other studies reporting that tau pathology is dose dependent (6, 7, 32, 33). The most notable result of our study is that the longest htau isoform and some, but not all, N-terminal fragments cause cell death through an involvement of NMDAR. Thus, pharmacological blockade or inhibition of NR1 expression with antisense ODN renders CGC and cortical neurons immune to tau toxicity.

The conclusion that tau influences NMDAR signaling and induces a necrotic type cell death (24) is demonstrated by (*i*) the rapidity of neurotoxic effect, (*ii*) the lack of classical hallmarks of apoptosis, (*iii*) the neuroprotective role offered by memantine, an NMDAR antagonist approved in the United States for the treatment of moderate to severe AD, and by ifenprodil, an inhibitor of extrasynaptic NR2B receptor, and (*iv*) the dephosphorylation of CREB.

A decreased CREB phosphorylation has been detected in brain of AD patients (34). Recently, it has been reported that β-amyloid may affect neuronal survival through this transcription factor (35). However, in this case CREB dephosphorylation was attributed to the reduced survival-promoting signals derived from synaptic receptors, due to β-amyloid increased NMDAR endocytosis. In this context, the findings reported in this paper add further knowledge to the mechanisms leading to CREB dephosphorylation in AD.

Stimulation of either synaptic or extrasynaptic NMDARs is linked to the activation of ERK/mitogen-activated protein kinases, which therefore appear to play a dual role as prosurvival (36) and prodeath kinases (37). We have found that tau toxicity is accompanied by sustained and delayed activation of ERKs and that this activation is NMDAR dependent and critically involved in cell death. Thus, two MEK inhibitors, UO126 and PD98059, as well as genetic ablation of ERK1, inhibit tau toxicity, in agreement with previous results reporting a critical role for ERKs in oxidative glutamate toxicity in cortical neurons and in other paradigms of neuronal death (38, 39).

ERK1/2 kinase activation has been reported to correlate with an increased phosphorylation of tau at TAU-1 and PHF-1 sites (40, 41). We have found that the expression of htau in primary neuronal cultures is not accompanied by an abnormal tau phosphorylation at these sites, although a consistent increase of phosphorylated (PHF-1, P262, but not AT8) tau with longer infection times is observed. This effect is proportional to total tau and, as previously reported in another paradigm of tauopathy (14), the fraction of phosphorylated tau remains unchanged during the time course of infection (Fig. 8, which is published as supporting information on the PNAS web site). All together, these data suggest that this posttranslational modification of tau is probably not involved in our model of cell death, in agreement with a growing number of studies reporting that tau can induce death independently of its state of phosphorylation (14, 33). Indeed, we have also shown that NMDAR-mediated tau toxicity is also exerted by tau fragments 1–44, 1–156, and 26–44, which do not contain sites recognized by mAbs PHF-1 and TAU-1, suggesting that other posttranslational modifications are involved in tau toxicity.

Many of the used N-terminal peptides of tau have been designed around the putative calpain cleavage sites on tau sequence (30, 31). This protease is activated by calcium influx through NMDAR during excitotoxicity (27). We found that in CGCs the activation of calpain mediated by NMDAR plays an important role in tau toxicity because its inhibition by calpeptin as well as by overexpression of its endogenous inhibitor calpastatin, significantly prevents neuronal death. Calpain is overactivated in AD brains (42, 43) and in several tauopathies (44, 45) in which it has been postulated to play an important role in development of cytoskeleton pathology by directly degrading or inducing the phosphorylation of its components. Among these, we have found that a fraction of tau is cleaved by calpain, with accumulation of a 17-kDa diagnostic fragment corresponding, as previously reported, to the tau region coded by tau-(45–230) vector (30, 31). It is noteworthy that cleavage at this site is expected to produce also the highly toxic tau-(1–44) fragment, although several attempts to detect it by Western blotting were unsuccessful, probably because it is further and rapidly degraded. These observations suggest that overexpression of tau can induce cell death by causing its own cleavage with the production of N-terminal toxic fragments. The N-terminal cleavage of tau could be of relevance to AD, in which it is postulated to play an important role in tangle maturation and in the evolution to a more severe AD pathology (46). Cleavage of the N terminus of tau and accumulation of the diagnostic 17-kDa tau fragment has been reported to occur also *in vitro* during apoptosis of CGCs (32). In a subsequent study, it was reported that also in β-amyloid-induced death of cultured hippocampal neurons, a 17-kDa fragment (residues 45–230) is produced as the result of a calpain-mediated attack, and it was believed to be responsible for β-amyloid-induced death (31). Our studies add further crucial information demonstrating that another N-terminal fragment (residues 1–44) is by far more toxic than the 17-kDa larger peptide and that such toxicity initiates a negative loop whereby calpain not only contributes to the generation of the 1–44 peptide but also is responsible for its toxicity.

How tau overexpression causes NMDAR activation, whether this effect is mediated by synaptically released glutamate, or whether tau, directly or indirectly, affects NR1 receptor function, thereby increasing the sensitivity to the resting level of glutamate, must be clarified.

In conclusion, this paper describes the signaling triggered by tau overexpression as being mediated through a neurotransmitter receptor and lends further support to the increasing evidence of the importance of NMDAR in neurodegenerative diseases.

Materials and Methods

Materials. (+)-MK-801 [dizocilpine hydrogen maleate, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate], GYKI52466 [1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride], and memantine hydrochloride (3,5-dimethylamantadine hydrochloride) were from Sigma-Aldrich. Ifenprodil hemitartrate [2-(4-benzylpiperidino)-1-(4-hydroxyphenyl)-1-propanol hemitartrate], calpeptin (*N*-benzyloxycarbonyl-L-leucylnorleucinal), and CNQX were from Tocris Bioscience (Bristol, U.K.). UO126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthiobutadiene)] and PD98059 (2′-amino-3′-methoxyflavone) were from Calbiochem.

Preparation of Primary Neuronal Cultures. CGCs were obtained from dissociated cerebella of 8-day-old Wistar rats (Charles River, Calco, Italy), or from 6-day-old ERK1$^{-/-}$ mice as previously described (47). Cortical neurons were prepared from embryonic day 17 (E17) embryos from timed pregnant Wistar rats (Charles River) as previously reported (48).

Construction and Purification of Recombinant Adenoviral Vectors. Plasmids encoding full-length or deleted tau cDNA were generated by PCR amplifications using tau40pSG5 vector as template (30). The numbering of tau fragment is according to the longest htau isoform.

The 5′ primer for tau-(1–441), tau-(1–156), tau-(1–44), and tau-(1–25) was 5′-AGCTGAATTCAATGGCTGAGCCCCGCCAG-3′, and the 3′ primers were 5′-GACCGCTCGAGTCACAAACCCTGCTTGGC-3′, 5′-GACCGCTCGAGTCATCCCCGCGGTGTGGCGAT-3′, 5′-GACCGCTCGAGTCACAGGCCAGCGTCCGTGTC-3′, and 5′-GACCGCTCGAGTCAATCTTTCCTGTCCCCCAA-3′, respectively. The 3′ primer for tau-(26–230) and tau-(45–230) was 5′-GACCGCTCGAGTCAACGGACCACTGCCACCTT-3′, and the 5′ primers were 5′-AGCTGAATTCACAGGGGGGCTACACCATG-3′ and 5′-AGCTGAATTCAAAAGAATCTCCCCTGCAG-3′, respectively. The 5′ primer for tau-(26–44) was 5′-AGCTGAATTCACAGGGGGGCTACACCATG-3′, and the 3′ primer was 5′-GACCGCTCGAGTCACAGGCCAGCGTCCGTGTC-3′. PCR primers contained EcoRI (5′) and XhoI (3′) sequences for insertion into EcoRI–XhoI-cut pCS2+MT downstream from the six Myc tags (30).

Replication-deficient Ad5 adenoviral vectors encoding human full-length or truncated tau proteins were prepared as described in ref. 18.

Adenovirus expressing β-galactosidase (Ad-Lac-Z) and Bcl2 (Ad-Bcl2) were provided by Marco Crescenzi (Laboratorio di Tossicologia Comparata ed Ecotossicologia, Istituto Superiore di Sanitá, Rome). Adenovirus expressing a 593-aa rat calpastatin isoform was purchased from the Biomedical Research Service Center, State University of New York at Buffalo.

Infection of Neurons. GCCs and cortical neurons were infected at 4 and 7 days *in vitro*, respectively, as reported in ref. 18.

Assessment of Neuronal Viability. Viable CGCs and cortical neurons were quantified by the MTT tetrazolium salt assay, by

counting the number of intact nuclei (18), or by counting the number of condensed nuclei after Hoechst 33342 staining.

ODNs. The NMDAR1 antisense is an 18-mer phosphodiester ODN (5′-CAG CAG GTG CAT GGT GCT-3′) targeted to the nucleotide sequence 4 through 21 that directly follows the initiation codon of rat NMDAR1. This antisense ODN has been proven effective and specific in a previous study (20). The scrambled ODN (5′-GAG CAG CTG CGT GAT GCT-3′) contains the same C+G nucleotide content as the antisense but in a different sequence. A database search using the BLAST program (National Center for Biotechnology Information, Bethesda, MD) revealed no identity with other rodent sequences. Both ODNs contained three phosphorothioate linkages at 3′ and 5′ ends. The 5′ ends of both ODNs were conjugated to fluorescein to monitor the transfection efficacy. The ODNs were delivered into CGCs at 3 days *in vitro*, using Lipofectamine 2000 (Invitrogen), and the CGCs were infected with tau vector at 4 days *in vitro*. They were lysed for Western blot analysis and assayed for survival 48 h later.

Western Blot Analysis. Total proteins were extracted by scraping the cells in SDS/reducing sample buffer and then by boiling for 5 min and subjected to SDS/PAGE on 10% or 7.5–15% linear gradient polyacrylamide gels. Membranes were prepared by scraping off the plate in a buffer containing 10 mM Tris·HCl, 2 mM EGTA, 1 mM $MgCl_2$ (pH 7.5), plus protease inhibitor mixture (Sigma), and centrifuged for 60 min at 100,000 rpm in an Airfuge to separate membranes from the cytosol fraction. The membranes were then resuspended in buffer containing 0.2% Triton X-100 and 0.2% Tween 20 and sonicated for 5 s on ice with a probe sonicator. Equivalent amounts of membrane proteins were analyzed on an SDS/7.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose paper for 1 h at 4°C. After electroblotting on nitrocellulose membrane (Hybond-C; Amersham Pharmacia), proteins were visualized by using appropriate primary antibodies. All primary antibodies were diluted in 0.5% (wt/vol) nonfat dry milk and incubated with the nitrocellulose blot overnight at 4°C. Incubation with secondary peroxidase-coupled anti-mouse/rabbit was performed by using the ECL system (Amersham Pharmacia).

Statistical Analysis. All experiments were performed in triplicate and repeated at least three times. Data were expressed as means ± SE ($n = 3$). Statistical significance was analyzed by Student's *t* test (STATVIEW 4.01; SAS Institute, Cary, NC). *P* values are *, $P < 0.05$; **, $P < 0.01$.

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