Regulation of nuclear TDP-43 by NR2A-containing NMDA receptors and PTEN

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Summary
The dysfunction of TAR DNA-binding protein-43 (TDP-43) is implicated in neurodegenerative diseases. However, the function of TDP-43 is not fully elucidated. Here we show that the protein level of endogenous TDP-43 in the nucleus is increased in mouse cortical neurons in the early stages, but return to basal level in the later stages after glutamate accumulation-induced injury. The elevation of TDP-43 results from a downregulation of phosphatase and tensin homolog (PTEN). We further demonstrate that activation of NR2A-containing NMDA receptors (NR2ARs) leads to PTEN downregulation and subsequent reduction of PTEN import from the cytoplasm to the nucleus after glutamate accumulation. The decrease of PTEN in the nucleus contributes to its reduced association with TDP-43, and thereby mediates the elevation of nuclear TDP-43. We provide evidence that the elevation of nuclear TDP-43, mediated by NR2AR activation and PTEN downregulation, confers protection against cortical neuronal death in the late stages after glutamate accumulation. Thus, this study reveals a NR2AR–PTEN–TDP-43 signaling pathway by which nuclear TDP-43 promotes neuronal survival. These results suggest that upregulation of nuclear TDP-43 represents a self-protection mechanism to delay neurodegeneration in the early stages after glutamate accumulation and that prolonging the upregulation process of nuclear TDP-43 might have therapeutic significance.

Key words: TAR DNA-binding protein-43, NR2A-containing NMDA receptor, PTEN, Glutamate neurotoxicity, Neuroprotection, Neurodegeneration

Introduction
The TAR DNA-binding protein-43 (TDP-43) is a highly conserved, heterogeneous ribonucleoprotein. TDP-43 has both nuclear export and import signals, but its distribution is primarily nuclear (Ayala et al., 2008; Winton et al., 2008). It has been shown that TDP-43 regulates gene transcription, exon splicing and exon inclusion (Sreedharan et al., 2008). However, the function of TDP-43 and its underlying mechanisms are not fully understood. Evidence from in vivo models of murine TDP-43 deletion indicates that TDP-43 is required for embryogenesis and is essential for viability (Kraemer et al., 2010; Sephton et al., 2010; Wu et al., 2010). Recently, the major proteins of the pathological inclusions in amyotrophic lateral sclerosis (ALS) have been identified as TDP-43 and its C-terminal fragments of ~20–25 kDa (Arai et al., 2006; Igaz et al., 2008; Mackenzie et al., 2007; Neumann et al., 2006). TDP-43 has also been identified as a component in the inclusions of frontotemporal lobar degeneration (FTLD) and other neurodegenerative diseases (Arai et al., 2009; Hasegawa et al., 2007; Neumann et al., 2006).

Glutamate accumulation-mediated neurotoxicity is known to play a crucial role in traumatic and ischemic brain injuries, as well as in neurodegenerative diseases including ALS (Culcas et al., 1994; Fiszman et al., 2010; Grosskreutz et al., 2010; Lafon-Cazal et al., 1993; Perry et al., 1987; Plaikakis and Caroscio, 1987). The elevation of glutamate concentration causes neurotoxicity through overactivation of ionotropic glutamate receptors (Arundine and Tymianski, 2004; Hanson et al., 2010; Hardingham et al., 2002; Lee et al., 1999; Rothstein et al., 1990; Sarraf-Yazdi et al., 1998). NMDA receptors are the major subtypes of ionotropic glutamate receptors to mediate glutamate neurotoxicity-induced neuronal death or neurodegeneration (Annis and Vaughn, 1998; Arundine and Tymianski, 2004; Brunet et al., 2009; Hanson et al., 2010; Hardingham et al., 2002; Lee et al., 1999; Sarraf-Yazdi et al., 1998; Sen et al., 2005). However, how NMDA receptors induce neuronal death or neurodegeneration remains unclear. The NMDA receptors containing NR2A and NR2B subunits (NR2ARs and NR2BRs) are the major subtypes of NMDA receptors expressed in the central nervous system (CNS) (Dingledine et al., 1999). Studies demonstrate that NR2ARs and NR2BRs play opposing role in regulating neuronal survival or death (Chen et al., 2008; DeRidder et al., 2006; Hardingham et al., 2002; Liu et al., 2007; Ning et al., 2004; Vanhoutte and Bading, 2003). This might explain why use of NMDA receptor antagonists as neuroprotective agents has been disappointing in clinical trials (Gredal et al., 1997; Traynor et al., 2006). Thus, investigating the specific effects differentially mediated by NR2AR- and NR2BR-dependent intracellular signaling would provide molecular evidence for the development of selective neuroprotection therapies.

Studies by others and us have revealed that suppression of PTEN (phosphatase and tensin homolog) protects against neuronal death (Chang et al., 2007; Ning et al., 2004). Although it functions in the cytoplasm, PTEN can enter the nucleus to regulate transcription, alternative splicing and mRNA stability (Planchnon et al., 2008). Under normal conditions, PTEN shuttles between the cytoplasm and nucleus (Gil et al., 2006; Planchnon et al., 2008). In the nucleus, PTEN has been shown to...
cause downregulation of extracellular signal-regulated kinase (ERK), leading to a decrease in cyclin D1 levels and G0–G1 arrest (Planchon et al., 2008). By interacting with CENP-C, PTEN enhances centromere stability and overall genomic stability (Planchon et al., 2008). Similar to its role in the cytoplasm, nuclear PTEN also induces apoptosis (Planchon et al., 2008).

To understand the cellular and molecular mechanisms that mediate the role of TDP-43 in both physiological and neurodegenerative conditions, we investigated the effect of NMDA receptors and PTEN on TDP-43 expression in an in vitro glutamate accumulation-induced neurodegeneration model using cultured mouse cortical neurons. We show that the protein level of endogenous TDP-43 in the nucleus is increased in the early stages after glutamate accumulation. The increase of TDP-43 is mediated through a reduced association of PTEN with TDP-43 in the nucleus, which results from the activation of NR2ARs and subsequent downregulation of nuclear PTEN. We also provide evidence that the upregulation of nuclear TDP-43 by NR2AR activation and PTEN downregulation in the early stages after glutamate accumulation is neuroprotective. Thus, the NR2AR–PTEN–TDP-43 signaling pathway might represent a general mechanism against neuronal death in the early stages of glutamate neurotoxicity-induced neuronal injury and neurodegeneration.

Results

The protein level of nuclear TDP-43 is increased in cortical neurons in the early stages after endogenous glutamate accumulation

To investigate the functional consequences of TDP-43 in the CNS, we established an in vitro glutamate-induced neurotoxicity model in cultured mouse cortical neurons. DL-threo-beta-hydroxyaspartate (THA), an inhibitor of glutamate transporters, was used to induce neuronal injury. By promoting extracellular glutamate accumulation, THA treatment causes glutamate neurotoxicity and has been used to induce neuronal injury in vitro (Corse et al., 1999; Kidd and Isaac, 2000; Matyja et al., 2006; Nagaiška et al., 2010; Tolosa et al., 2008; Van Westerlaak et al., 2001). To characterize this model, we first performed a lactate dehydrogenase (LDH) release assay to measure THA-induced neuronal damage in the cortical cultures. We showed that the rate of neuronal death was not significantly altered during the first 3 days after treatment with 100 μM THA, but increased at 6 days after treatment (Fig. 1A). We then performed western blot assays to measure the protein level of TDP-43 in the specific injury paradigm. We found that the level of TDP-43 was remarkably increased in cortical neurons during the first 3 days after THA treatment (Fig. 1B). However, the increased expression of TDP-43 returned to basal levels at 6 days after THA insult (Fig. 1B). Our data also showed that there were no C-terminal fragments of TDP-43 in THA-treated groups or in the respective controls (Fig. 1C).

Because TDP-43 is mainly expressed in the nucleus, we next tested whether the distribution of TDP-43 was altered in the nucleus of cortical neurons after glutamate accumulation. Immunocytochemical staining was performed to determine TDP-43 expression with the use of propidium iodide (PI) to label the nucleus in cultured cortical neurons (Fig. 1D). Although we confirmed that TDP-43 expression was increased at 3 days after THA treatment (Fig. 1D), we found that the increased TDP-43 was confined to the nucleus (Fig. 1D). We did not observe significant expression of TDP-43 in the cytoplasm in either control or THA-treated neurons (Fig. 1D). Taken together, these results indicate that TDP-43 expression is increased only in the nucleus in the early stages after glutamate accumulation.

Downregulation of PTEN contributes to the upregulation of TDP-43 after glutamate accumulation

Given that the phosphatase PTEN is involved in regulating neuronal survival and exerts its effects in both cytoplasm and nucleus, we tested the effect of PTEN on TDP-43. We first measured the level of PTEN protein in cortical neurons at 3 days after THA treatment. We showed that, in contrast to the increase of TDP-43, PTEN was decreased at 3 days after THA treatment (Fig. 2A). To determine whether PTEN downregulation contributed to TDP-43 upregulation, we tested the effect of a selective PTEN inhibitor bisperoxovanadium [bpV(pic)] on the protein expression of TDP-43 in the cortical neurons (Schmid et al., 2004). We found that PTEN inhibition by bpV(pic) not only enhanced TDP-43 expression in control cortical neurons (Fig. 2B), but also increased TDP-43 expression at 6 days after THA treatment (Fig. 2C). However, bpV(pic) treatment had no significant effect on TDP-43 expression at 3 days after THA treatment (Fig. 2D). To provide further evidence for the regulation of TDP-43 by PTEN, the cultured neurons were transfected with PTEN siRNAs (siRNApten) or PTEN cDNAs (Fig. 2E–H). Our data showed that suppression of overexpression of PTEN increased and inhibited TDP-43 expression, respectively (Fig. 2E–H). These results suggest that TDP-43 is negatively regulated by endogenous PTEN, and that the upregulation of TDP-43 expression is mediated by PTEN downregulation in the early stages after glutamate accumulation.

To determine how TDP-43 was regulated by PTEN, two different dominant-negative forms of phosphatase-inactivated PTEN mutants, C124A and G129E, were used to inhibit endogenous lipid and protein phosphatase activities of PTEN (Myers et al., 1998; Tamura et al., 1998; Weng et al., 2001). The C124A mutation causes a loss of both lipid and protein phosphatase activities, and the mutant G129E results only in loss of lipid phosphatase activity with retention of protein phosphatase activity. Our results demonstrated that the level of TDP-43 was increased in neurons transfected with both C124A and G129E (Fig. 3A,B), suggesting that the downregulation of lipid phosphatase activity of PTEN contributes to upregulation of TDP-43.

We also performed cycloheximide chase experiments to determine the effect of PTEN on TDP-43 stability. The neuronal cultures were first treated with vehicle or THA (100 μM) for 3 days and then the cultures were treated with cycloheximide (20 μg/ml) for 1, 3, 6 and 12 hours (Turgeon et al., 2001). We found that the protein level of TDP-43 was significantly decreased in control neurons at 6 hours after cycloheximide treatment (Fig. 3C). By contrast, in THA-treated neurons, the protein level of TDP-43 remained unchanged following treatment with cycloheximide for 12 hours (Fig. 3D). Moreover, our data showed that bpV(pic) treatment stabilized the level of TDP-43 after 12 hours of treatment with cycloheximide (Fig. 3E). These results indicate that downregulation of PTEN leads to the enhancement of TDP-43 protein stability, which might contribute to THA-induced increase of TDP-43 expression in the early stages after glutamate accumulation.
NR2AR activation leads to PTEN downregulation and subsequent TDP-43 upregulation after glutamate accumulation

To further reveal the upstream signaling to regulate TDP-43, we tested the effects of NMDA receptors on the protein expression of both PTEN and TDP-43 after glutamate accumulation. We showed that the NR2AR antagonist NVP-AAM077 (0.4 μM) but not NR2BR antagonist Ro25-6981 (0.5 μM) attenuated THA-induced PTEN downregulation after glutamate accumulation (Fig. 4A). Although NVP-AAM077 has small degree of cross-inhibition on NR2BRs (Liu et al., 2007), this cross-effect can be ignored because our data indicated that NR2BR inhibition had
no effect on TDP-43 expression (Fig. 4A). Thus, PTEN downregulation is in part mediated by NR2AR activation after glutamate accumulation.

As our data indicated that TDP-43 was a downstream effector of PTEN (Fig. 2), we reasoned that NR2AR activation would lead to TDP-43 upregulation through PTEN downregulation after glutamate accumulation. To verify this possibility, the effects of NVP-AAM077 (0.4 μM) and Ro25-6981 (0.5 μM) on TDP-43 expression were tested in cortical neurons treated with THA (100 μM). We showed that the inhibition of NR2ARs but not NR2BRs blocked the upregulation of TDP-43 expression at 3 days after THA treatment (Fig. 4B). We also showed that...
inhibiting NR2AR activity prevented THA-induced upregulation of TDP-43, and this effect was blocked by treatment of PTEN inhibitor bpV(pic) (Fig. 4C). These data suggest that NR2AR activation leads to PTEN downregulation, which in turn results in TDP-43 upregulation in the early stages after glutamate accumulation.

**Decreased association of PTEN with TDP-43 in the nucleus mediates TDP-43 upregulation after glutamate accumulation**

Because the downregulation of PTEN might occur in the nucleus to regulate TDP-43 expression, we performed subcellular fractionation assays to measure the PTEN levels in both the cytoplasm and nucleus. Our data showed that the protein expression of PTEN was decreased not only in the cytosolic fraction but also in the nuclear fraction at 3 days after THA treatment (Fig. 5A), suggesting that a reduced import of PTEN from cytoplasm into the nucleus leads to the decrease of PTEN in the nucleus.

To understand how reduced nuclear PTEN led to the upregulation of TDP-43 in the nucleus, we performed a co-immunoprecipitation assay to test whether PTEN could physically associate with TDP-43 to regulate TDP-43 expression. Indeed, our data indicated that TDP-43 was co-immunoprecipitated with PTEN by an anti-PTEN antibody in the homogenates of cultured cortical neurons (Fig. 5B). Conversely, immunoprecipitation with an anti-TDP-43 antibody co-precipitated PTEN (Fig. 5C). These data indicate that PTEN forms a protein complex with TDP-43 in the nucleus to negatively regulate TDP-43 expression. To further determine whether this was also true in cortical neurons subjected to glutamate insult, we measured the association levels of PTEN with TDP-43 in cortical neurons in both control and THA-treated conditions. Our data showed that both the level of co-precipitated TDP-43 by anti-PTEN antibody and the level of co-precipitated
PTEN by anti-TDP-43 antibody were significantly lower at 3 days after THA treatment compared with those in control cortical neurons (Fig. 5D,E), and that the NR2AR antagonist NVP-AAM077 (0.4 μM) but not the NR2BR antagonist Ro25-6981 (0.5 μM) reduces THA-induced PTEN downregulation at 3 days after 100 μM THA treatment (n=7 for each group; *P<0.05 vs control; **P<0.05 vs 3d/THA; data are normalized to control). (B) Representative immunoblots (left) and summarized data (right) show that NVP-AAM077 (0.4 μM) but not Ro25-6981 (0.5 μM) prevented upregulation of TDP-43 expression at 3 days after 100 μM THA treatment (n=6 for each group; *P<0.05 vs control; **P<0.05 vs 3d/THA; data are normalized to control). (C) Sample immunoblots (left) and summarized data (right) show that bpV(pic) (100 nM) prevented NR2AR-inhibition-induced blockade of TDP-43 upregulation in cortical neurons at 3 days after 100 μM THA treatment (n=6 for each group; *P<0.05 vs control; **P<0.05 vs 3d/THA; **P<0.05 vs NVP-AAM077+3d/THA; data are normalized to control). All bar graphs show means ± s.e.m.

To determine whether PTEN regulated TDP-43 through direct binding, we performed an in vitro binding assay (Ning et al., 2004). Our data showed that PTEN was not directly associated with TDP-43 (Fig. 5F). To determine whether PTEN-dependent dephosphorylation was involved in the regulation of TDP-43 expression after glutamate insult, we measured the levels of serine and tyrosine phosphorylation of TDP-43 in neurons treated with THA or PTEN inhibitor (Wan et al., 1997a). TDP-43 was immunoprecipitated by antibody against TDP-43, and then anti-phosphoserine/phosphotyrosine antibodies were used to detect the phosphorylation level of precipitated TDP-43 proteins. We found that both serine and tyrosine phosphorylation of TDP-43 were not altered at 3 days after THA treatment and that PTEN inhibitor bpV(pic) had no significant effects on serine and tyrosine phosphorylation of TDP-43 (Fig. 5G,H). These data indicate that regulation of TDP-43 by PTEN is not mediated through a dephosphorylation process.

Upregulation of nuclear TDP-43 confers protection against glutamate accumulation-induced neuronal death

Because NR2AR activation and PTEN downregulation are believed to promote neuronal survival (Chang et al., 2007; Chen et al., 2008; Liu et al., 2007; Ning et al., 2004), it is possible...
Fig. 5. The association of PTEN with TDP-43 is reduced at the early stage after glutamate accumulation. (A) Sample immunoblots (left) and summarized data (right) from subcellular fractionation assays show that the protein expression of PTEN is decreased in both cytosolic and nuclear fractions at 3 days after 100 μM THA treatment (n=6 for each group; *P<0.05 vs cytoplasm or nucleus control; data were normalized to cytoplasm control). Tubulin, a marker of cytoplasmic fraction; p85, a marker of nucleus fraction. (B) Representative immunoblots from co-immunoprecipitation assays show that TDP-43 is co-immunoprecipitated by an antibody against PTEN. No Ab, no antibody added to the assay. (C) Sample immunoblots from co-immunoprecipitation assays show that PTEN is co-immunoprecipitated by an antibody against TDP-43. (D) Representative immunoblots (left) and summarized data (right) from co-immunoprecipitation assays show that the level of co-precipitated TDP-43 by anti-PTEN antibody is significantly reduced at 3 days after 100 μM THA treatment compared with that in control cortical neurons, and that the NR2AR antagonist NVP-AAM077 (0.4 μM) blocks the reduction of coprecipitated TDP-43 induced by THA treatment (n=6 for each group; *P<0.05 vs control; #P<0.05 vs 3d/THA; data are normalized to control). (E) Representative immunoblots (left) and summarized data (right) from co-immunoprecipitation assays show that the level of coprecipitated PTEN by anti-TDP-43 antibody is significantly decreased at 3 days after 100 μM THA treatment compared with that in control cortical neurons, and that the NR2AR antagonist NVP-AAM077 (0.4 μM) blocks the reduction of coprecipitated PTEN induced by THA treatment (n=6 for each group; *P<0.05 vs control; #P<0.05 vs 3d/THA; data are normalized to control). (F) In vitro binding assay showing the direct binding of [35S]PTEN to GST–NR1-1aCT but not GST–TDP-43. (G) Representative immunoblot (left) and summarized data (right) show that THA (treatment for 3 days) and bpV(pic) have no significant effect on the serine phosphorylation of TDP-43 (n=3 for each group). (H) Representative immunoblot (left) and summarized data (right) show that THA (treatment for 3 days) and bpV(pic) have no significant effect on the tyrosine phosphorylation of TDP-43 (n=3 for each group). All bar graphs show means ± s.e.m.
that the effect delivered by nuclear TDP-43 upregulation in the early stages after glutamate accumulation represents a neuroprotective response. To test this possibility, we used siRNA to knock down TDP-43 (siRNAtdp43) in the nucleus of cultured cortical neurons (Fig. 6A). We showed that the cell death in cortical neurons transfected with siRNAtdp43 was enhanced at 3 days after THA treatment compared with the control neurons transfected with non-targeting control siRNA (NsiRNA) (Fig. 6B). These data indicate that the endogenous TDP-43 in the nucleus is a pro-survival signal in nature. To determine whether NR2AR and PTEN were upstream of TDP-43 to regulate neuronal survival, we first measured the effect of the NR2AR antagonist NVP-AAM077 on neuronal death in cortical neurons subjected to glutamate insult. We found that NR2AR inhibition by NVP-AAM077 (0.4 μM) remarkably increased cell death in cortical neurons at 3 days after THA treatment (Fig. 7A). However, the transient overexpression of TDP-43 (Fig. 7B) or inhibition of PTEN by bpV(pic) attenuated the NR2AR inhibition-induced increase of neuronal death after

Fig. 6. Suppressing nuclear TDP-43 increases the death of cortical neurons at early stages after THA treatment. (A) Sample images (left) and summarized data (right) indicate that TDP-43 siRNA (siRNAtdp43) but not the non-targeting control siRNA (NsiRNA) inhibits TDP-43 expression in the nucleus of normal cortical neurons (means ± s.e.m.; for each group, n=35 cells from three independent experiments; *P<0.05 vs NsiRNAtdp43; data are normalized to NsiRNA). (B) Representative images (left) and summarized data (right) show that knockdown of TDP-43 with siRNAtdp43 promotes cortical neuronal death at 3 days after 100 μM THA treatment compared with the control neurons transfected with NsiRNA (means ± s.e.m.; for each group, n=50 cells from three independent experiments; *P<0.05 vs GFP; data are normalized to GFP). The neurons were transfected with siRNAs at 1 day after THA treatment.
glutamate insult (Fig. 7A). Thus, the upregulation of TDP-43 by NR2AR activation and PTEN downregulation plays a neuroprotective role in the early stages after glutamate neurotoxicity-induced injury.

**Discussion**

By knockdown or deletion of TDP-43 in vitro and in vivo, recent studies suggest that TDP-43 is a pro-survival protein in the nucleus and that the loss of function of nuclear TDP-43 promotes neurodegeneration (Fiesel et al., 2010; Iguchi et al., 2009). However, there is no direct evidence showing the neuroprotective effect of endogenous TDP-43 in neuronal injury and neurodegeneration. Our study provides the first evidence that nuclear TDP-43 expression is increased at the early stages in an in vitro glutamate accumulation-induced neurodegeneration model (Corse et al., 1999; Kidd and Isaac 2000; Matyja et al., 2006; Nagaiška et al., 2010; Tolosa et al., 2008; Van Westerlaak et al., 2001). We demonstrate that glutamate accumulation results in an
upregulation of nuclear TDP-43, and that the TDP-43 upregulation confers a neuroprotective effect. These results reveal a self-protection mechanism mediated by TDP-43 upregulation at the early stage of neurodegeneration. It would be interesting in the future to test whether TDP-43 also protects from other forms of neuronal death or cytotoxicity in different cell types.

Aberrant regulation of TDP-43 has been found to play complex roles in both the nucleus and cytoplasm in the pathogenesis of ALS (Barmada et al., 2010; Winton et al., 2008). In ALS patients, TDP-43 expression is remarkably reduced in the nucleus in the affected neurons with cytoplasmic inclusions (Cairns et al., 2007; Hasegawa et al., 2007; Neumann et al., 2006). In transgenic mice with TDP-43 overexpression, TDP-43 accumulates in the nucleus and aggregates in the cytoplasm (Wils et al., 2010). These findings indicate that a translocation of TDP-43 from the nucleus to cytoplasm might contribute to the accumulation of TDP-43 in the cytoplasmic inclusions. In the in vitro neurodegeneration model, we demonstrate that in response to glutamate accumulation, endogenous TDP-43 is only increased in the nucleus and does not translocate to cytoplasm. Thus, although TDP-43 behaves as a pro-survival signaling protein in the nucleus, the upregulation of endogenous TDP-43 in the nucleus would not lead to its translocation to the cytoplasmic compartment.

To understand the cellular and molecular mechanism by which TDP-43 plays a neuroprotective role during the neurodegeneration process, we investigated the effect of NMDA receptors on TDP-43 expression in an in vitro neurodegeneration model. We demonstrate for the first time that the upregulation of TDP-43 is mediated by the activation of NR2AR after glutamate accumulation. Recent evidence shows that NR2AR activation promotes neuronal survival in acute CNS injury such as cerebral ischemia and traumatic spinal injury (Allain and Goshgarian, 2008; Liu et al., 2007; Terasaki et al., 2010). However, the underlying mechanisms remain largely unknown. In the present study, we identify TDP-43 as a novel downstream signaling protein of NR2ARs in a glutamate neurotoxicity-induced neurodegeneration model. Our results suggest that upregulation of TDP-43 by NR2AR activation counteracts neurodegeneration in the early stages after glutamate insult. By contrast, NR2BR is found to have no effect on TDP-43 expression in our experimental model, although previous studies, including ours, indicate that NR2BR overactivation increases neuronal death by suppression of CREB-, ERK- and PINK1-dependent survival signaling pathways (Hardingham et al., 2002; Shan et al., 2009; Wang et al., 2004). Because blocking NMDA receptors would suppress NR2AR-mediated neuroprotection while inhibiting NR2BR-mediated neuronal death, simply inhibiting NMDA receptors would not be a proper strategy to prevent neurodegeneration. This might explain why the use of glutamate antagonists as neuroprotective agents has been disappointing in clinical trials (Gredal et al., 2006; Wan et al., 1997b). Thus, investigating cellular and molecular mechanisms mediating the differential role of NR2AR and NR2BR in neuronal survival or death is crucial for us to develop a selective neuroprotection strategy for the treatment of ischemic or traumatic CNS injury and neurodegenerative diseases.

Our results also show that PTEN downregulation mediates NR2AR-dependent elevation of nuclear TDP-43 following glutamate accumulation. Interestingly, we provide evidence that the downregulation of PTEN leads to a reduced association of PTEN with TDP-43 in the nucleus, which contributes to the increased nuclear expression of TDP-43 after glutamate accumulation. It is unclear how the decreased association of PTEN and TDP-43 affects TDP-43 levels. However, based on our evidence that: (1) TDP-43 expression is negatively regulated by PTEN (Fig. 2); (2) PTEN does not directly interact with TDP-43 (Fig. 5F); (3) PTEN has no effect on serine/tyrosine phosphorylation of TDP-43 (Fig. 5G,H); and (4) PTEN downregulation enhances the stability of TDP-43, we reason that the decrease in the PTEN–TDP-43 association might indirectly lead to a relief of TDP-43 inhibition by PTEN in a phosphorylation-independent manner, which enhances the stability of TDP-43 and thus increases TDP-43 expression. Future studies are required to further address how this process occurs.

In summary, the present study provides the first evidence that upregulation of TDP-43, mediated by NR2AR activation and PTEN downregulation, confers neuroprotection in the early stages after glutamate accumulation. Given that NMDA receptors, PTEN and TDP-43 are involved in various CNS disorders, the NR2AR–PTEN–TDP-43 signaling might represent novel therapeutic targets for the development of a neuroprotective strategy.

**Materials and Methods**

**Culture of mouse cortical motor neurons and THA treatment**

The cortical neuronal cultures were prepared from C57BL/6 mice at gestational day 17 using a modified protocol (Shan et al., 2009). After removing meninges, cortices were placed in ice-cold plating medium (Neurobasal medium, 2% B-27 supplement, 0.5% FBS, 0.5 mM L-glutamine and 25 mM glutamic acid). Dissociated motor neurons were prepared from precentral gyrus that was carefully dissected from the cortices. The neurons were suspended in plating medium and plated on Petri dishes coated with poly-D-lysine. After 3 days in culture, half of the plating medium was removed and replaced with maintenance medium (Neurobasal medium, 2% B-27 supplement, and 0.5 mM L-glutamine). Thereafter, maintenance medium was changed in the same manner every 3 days.

To induce chronic glutamate neurotoxicity, the cultures were exposed to THA (100 μM) at 8 days after plating. The cultured neurons were collected for experiments at 1, 3 and 6 days after THA treatment.

**Western blotting, co-immunoprecipitation and in vitro binding assays**

Western blotting, co-immunoprecipitation and in vitro binding assays were performed as reported previously (Ning et al., 2004). For western blotting, total proteins were extracted with lysis buffer. Equal amounts of proteins were separated by 8–10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrophoretically transferred to polyvinylidene difluoride membrane and incubated with a blocking buffer for 1 hour at room temperature. The membranes were incubated with primary antibodies overnight at 4°C, and then incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. The anti-TDP-43 antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA) and the anti-PTEN antibody was purchased from Chemicon (Temecula, CA). The protein bands were imaged using ECL (Amersham Pharmacia Biotech). The membranes were re-probed with anti-GAPDH antibody as a loading control. For co-immunoprecipitation experiments, the cell lysates were pre-absorbed with 20 μl protein A/G agarose beads at 4°C for 30 minutes, spun at 10,000 r.p.m for 10 seconds, and the supernatant was incubated with specific primary antibody at 4°C overnight. After incubation with 20 μl protein A/G agarose beads for 1.5 hours at 4°C, the immunocomplexes were collected by centrifugation and washed three times with ice-cold washing buffer. The final products were boiled for 5 minutes and resolved with SDS-PAGE, and immunoblotted with specific antibodies. Images were analyzed using ImageJ software (Version 1.34). For in vitro binding assay, GST–NR1–1aG, GST–TDP-43 or GST (10 μg) alone was incubated with [32P]SIPETN probe for 4 hours at room temperature. The beads were washed six times with tissue homogenizing buffer and eluted with 10 mM glutathione. Eluates were separated by SDS-PAGE and visualized by autoradiography (Ning et al., 2004).

**Immunocytochemical staining**

Immunocytochemical staining was performed as described previously (Liu et al., 2006; Wan et al., 1997b). Briefly, the transfected cells were fixed with 4% paraformaldehyde and blocked in 5% normal goat serum. The specimen was incubated with a blocking buffer for 1 hour at room temperature. The anti-TDP-43 antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA) and the anti-PTEN antibody was purchased from Chemicon (Temecula, CA). The protein bands were imaged using ECL (Amersham Pharmacia Biotech). The membranes were re-probed with anti-GAPDH antibody as a loading control. For co-immunoprecipitation experiments, the cell lysates were pre-absorbed with 20 μl protein A/G agarose beads at 4°C for 30 minutes, spun at 10,000 r.p.m for 10 seconds, and the supernatant was incubated with specific primary antibody at 4°C overnight. After incubation with 20 μl protein A/G agarose beads for 1.5 hours at 4°C, the immunocomplexes were collected by centrifugation and washed three times with ice-cold washing buffer. The final products were boiled for 5 minutes and resolved with SDS-PAGE, and immunoblotted with specific antibodies. Images were analyzed using ImageJ software (Version 1.34). For in vitro binding assay, GST–NR1–1aG, GST–TDP-43 or GST (10 μg) alone was incubated with [32P]SIPETN probe for 4 hours at room temperature. The beads were washed six times with tissue homogenizing buffer and eluted with 10 mM glutathione. Eluates were separated by SDS-PAGE and visualized by autoradiography (Ning et al., 2004).
incubated with primary antibody overnight at 4°C and then incubated with fluorochrome-conjugated secondary antibody for 1 hour at room temperature. The primary anti-TDP-43 antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA). Secondary antibodies, Alexa Fluor 594 (red fluorescence) and Alexa Fluor 488 (green fluorescence), were purchased from Invitrogen (Carlsbad, CA). DAPI or propidium iodide (PI) was used to label the nuclei. Fluorescent-labeled proteins were imaged using a 63× or 40× objective in a Zeiss LSM 510 META confocal microscope (Oberkochen, Germany) as described previously (Liu et al., 2006; Wan et al., 1997b). Images were acquired using a Zeiss AxioCam digital camera in the linear range with constant settings and were analyzed using ImageJ software. Each image was a z-series of 6–13 images, taken at 0.75-μm depth intervals. The resultant stack was flattened into a single image using maximum projection. For individual experiments, all images in all experiments were analyzed using identical acquisition parameters. During data acquisition and analysis, the investigator was blind to the treatment group.

Transfection
The small interfering RNAs specific to mouse TDP-43 and PTEN (siRNA Tdp43 and siRNA PTEN) and non-targeting control siRNA (NsRNA) were purchased from Santa Cruz Biotech (Santa Cruz, CA). The cortical neurons were transfected with using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol (Ning et al., 2004). cDNA encoding green fluorescent protein (GFP) was used as a marker of successful neuronal transfection. The cells were fixed for immunocytochemical labeling at 48 hours after transfection.

Subcellular fractionation assay
The assay was performed as described (Ayala et al., 2008). Briefly, cultured neurons were harvested and washed in PBS by repeated centrifugation. The neurons were harvested and washed in PBS by repeated centrifugation. The culture medium of cells co-transfected with GFP and protein (GFP) was used as a marker of successful neuronal transfection. The cells were fixed for immunocytochemical labeling at 48 hours after transfection.

Neuronal death assays
After incubation for 5 minutes, nuclei were pelleted and gently resuspended in 1 ml of buffer N plus 0.6% NP-40. After incubation for 5 minutes, nuclei were pelleted and gently resuspended in 1 ml of buffer N. The nuclei were again pelleted and lysed using an equal volume of solution 2 (20 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EGTA, 0.1 mM DTT, 0.5 mM PMSE, 5% glycerol, 0.4 M NaCl). The nuclear fraction was cleared by centrifugation following incubation for 30 minutes. Nuclear and cytoplasmic fractions were quantified and visualized by western blotting.

Statistics
The cell count was blinded to the experimental treatment.

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