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Differential regulation of NMDA receptor function by DJ-1 and PINK1

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Summary

Dysfunction of PTEN-induced kinase 1 (PINK1) or DJ-1 promotes neuronal death and is implicated in the pathogenesis of Parkinson's disease, but the underlying mechanisms remain unclear. Given the roles of *N*-methyl-D-aspartate receptor (NMDAr)-mediated neurotoxicity in various brain disorders including cerebral ischemia and neurodegenerative diseases, we investigated the effects of PINK1 and DJ-1 on NMDAr function. Using protein overexpression and knockdown approaches, we showed that PINK1 increased NMDAr-mediated whole-cell currents by enhancing the function of NR2A-containing NMDAr subtype (NR2ACNR). However, DJ-1 decreased NMDAr-mediated currents, which was mediated through the inhibition of both NR2ACNR and NR2B-containing NMDAr subtype (NR2BCNR). We revealed that the knockdown of DJ-1 enhanced PTEN expression, which not only potentiated NR2BCNR function but also increased PINK1 expression that led to NR2ACNR potentiation. These results indicate that NMDAr function is differentially regulated by DJ-1-dependent signal pathways DJ-1/PTEN/NR2BCNR and DJ-1/PTEN/PINK1/NR2ACNR. Our results further showed that the suppression of DJ-1, while promoted NMDA-induced neuronal death through the overactivation of PTEN/NR2BCNR-dependent cell death pathway, induced a neuroprotective effect to counteract DJ-1 dysfunction-mediated neuronal death signaling through activating PTEN/PINK1/NR2ACNR cell survival–promoting pathway. Thus, PINK1 acts with DJ-1 in a common pathway to regulate NMDAr-mediated neuronal death. This study suggests that the DJ-1/PTEN/NR2BCNR and DJ-1/PTEN/-PINK1/NR2ACNR pathways may represent potential therapeutic targets for the development of neuroprotection strategy in the treatment of brain injuries and neurodegenerative diseases such as Parkinson's disease.

Key words: neurodegeneration; NR2A; NR2B; PARK7; PTEN-induced kinase 1.

Introduction

DJ-1 (also called *PARK 7)* was recently cloned as a putative oncogene (Nagakubo *et al.*, 1997). Study on *DJ-1* gene deletion reveals that DJ-1 is an atypical peroxiredoxin-like peroxidase (Andres-Mateos *et al.*, 2007). Loss-of-function mutations in *DJ-1* were recently identified in patients with early-onset autosomal recessive Parkinsonism (Bonifati *et al.*, 2003), suggesting that dysfunction of DJ-1 may contribute to the dopaminergic neurodegeneration in Parkinson's disease (PD). DJ-1 is widely expressed in the brain and peripheral tissues and normally present in the cytosol, nucleus and mitochondria (Zhang *et al.*, 2005). The oxidative stress promotes DJ-1 to translocate to mitochondria, which correlates with the neuroprotective role of DJ-1 in mitochondria (Canet-Aviles *et al.*, 2004). The knockdown of DJ-1 in *Drosophila* is shown to cause mitochondrial dysfunction (Yang *et al.*, 2005). Although the loss of DJ-1 is insufficient to lead to dopaminergic neurodegeneration, the dopamine neurons with DJ-1 deficiency are sensitive to oxidative stress and neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine (MPTP) (Kim *et al.*, 2005c). Recent study demonstrates that DJ-1 is involved in stroke-induced brain injury (Aleyasin *et al.*, 2007; Yanagisawa *et al.*, 2008), indicating that DJ-1 dysfunction may play a broad role in the CNS damage.

The newly identified *PINK1* (PTEN-induced kinase 1; also called *PARK6*) gene encodes a serine/threonine kinase (Valente *et al.*, 2004). Similar to DJ-1, loss-of-function mutations in the *PINK1* gene have been linked to early onset of PD (Healy *et al.*, 2004; Valente *et al.*, 2004). In the brain, PINK1 is primarily expressed in the neurons of hippocampus, substantia nigra and cerebellar Purkinje cells (Blackinton *et al.*, 2007). PINK1 is located in both the mitochondria and the cytoplasm (Beilina *et al.*, 2005; Silvestri *et al.*, 2005). Recent studies show that PINK1 plays crucial roles in the regulation of mitochondrial dynamics, and the loss

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of PINK1 function causes changes in mitochondrial morphology (Thomas & Cookson, 2009). Inactivation of Drosophila PINK1 results in the progressive loss of dopaminergic neurons (Wang *et al.*, 2006), and that the loss of PINK1 leads to functional defects of mitochondria, causes mitochondrial calcium overload, increased sensitivity to oxidative stress, reduced dopamine release and impaired synaptic plasticity (Kitada *et al.*, 2007; Gautier *et al.*, 2008). PINK1 is neuroprotective in both *in vitro* and *in vivo* experimental models (Haque *et al.*, 2008; Wood-Kaczmar *et al.*, 2008).

It has been well established that *N*-methyl-D-aspartate receptor (NMDAr)-mediated neurotoxicity causes neuronal death in various CNS disorders including cerebral ischemia, traumatic brain injury and neurodegenerative diseases including PD (Lee *et al.*, 1999; Koutsilieri & Riederer, 2007). However, the use of NMDAr antagonists as neuroprotective agents has been disappointing in the clinical trials (Lipton, 2004). One possible explanation is that these antagonists, while suppressing NMDAr-mediated neurotoxicity, block the physiological effects of NMDArs. Thus, development of selective antagonists that can specifically block NMDAr-mediated neurotoxicity would bring hope for the effective treatment of CNS injuries and neurodegenerative diseases. NMDArs are composed of multiple subunits, including NR1, NR2 (NR2A-D) and NR3 (NR3A-B) (Dingledine *et al.*, 1999). The NR2A- and NR2B-containing NMDAr subtypes (NR2ACNRs and NR2BCNRs) are the predominant NMDArs expressed in the adult forebrain. Different NR2 subunits confer distinct electrophysiological and pharmacological properties on the NMDAr subtypes and link them with different intracellular signaling. In the mature neurons of hippocampus and cortex, NR2ACNRs are mainly expressed on postsynaptic membrane and NR2BCNRs are predominantly distributed on the extrasynaptic membrane (Rumbaugh & Vicini, 1999; Tovar & Westbrook, 1999). It has been demonstrated that enhancing extrasynaptic NMDArs triggers cell death while activating synaptic NMDArs promotes neuronal survival (Hardingham *et al.*, 2002; Liu *et al.*, 2007). Recent studies in both *in vitro* and *in vivo* conditions further demonstrate that overactivation of NR2BCNRs induces neuronal death (Hardingham *et al.*, 2002; Liu *et al.*, 2007; Martel *et al.*, 2008) and that activation of NR2ACNRs promotes neuronal survival (DeRidder *et al.*, 2006; Liu *et al.*, 2007; Anastasio *et al.*, 2009). These results suggest that NR2ACNRs and NR2BCNRs may exert opposing effects on neuronal death. However, *in vitro* evidence also suggests that NR2ACNRs and NR2BCNRs may play a neurotoxic and neuroprotective role, respectively, in different developmental stages (von Engelhardt *et al.*, 2007; Martel *et al.*, 2008). These findings suggest that NR2ACNRs and NR2BCNRs may play complex roles in mediating neuronal survival and/or death. Thus, identification of intracellular signaling that contributes to NR2ACNR- or NR2BCNR-mediated neuronal survival or death at synaptic and/or extrasynaptic sites would provide molecular basis for the development of selective and potent therapeutics for CNS injuries and neurodegenerative diseases.

Given that the impaired mitochondrial function is a common death pathway whereby the NMDAr-mediated neurotoxicity and the dysfunction of DJ-1 and PINK1 promote neuronal death (Greenwood & Connolly, 2007; Deas *et al.*, 2009; Thomas & Cookson, 2009), we set up to investigate whether dysfunction of DJ-1 and PINK1 was linked to NMDAr-mediated neurotoxicity to exert their effects on NMDAr-mediated neuronal death in the CNS. Using rat cortical neurons as our experimental model, we have revealed that the DJ-1 suppression, while increases sensibility of cortical neurons to NMDA-induced neurotoxicity through DJ-1/PTEN/NR2BCNR pathway, induces a self-protective signaling through DJ-1/PTEN/PINK1/NR2ACNR pathway. We also provide evidence that knockdown of DJ-1 together with the suppression of PINK1 or NR2ACNRs markedly increases NMDA-induced neuronal death, indicating that PINK1 acts with DJ-1 in a common pathway to regulate NMDAr-mediated neuronal death. Thus, the DJ-1-dependent dual signal pathways DJ-1/PTEN/NR2BCNR and DJ-1/PTEN/PINK1/NR2ACNR may represent potential therapeutic targets for the treatment of brain injuries and neurodegenerative diseases.

Results

DJ-1 negatively regulates NMDAr function

We first examined whether DJ-1 regulated the function of NMDArs. We recorded NMDAr-mediated whole-cell currents in rat cortical neurons at 10–12 days in culture. DJ-1 protein expression was suppressed by transfecting the neurons with DJ-1 siRNA (siRNAdj-1). As illustrated in Fig. 1A,B, siRNAdj-1, but not the scrambled DJ-1 siRNA (SsiRNAdj-1) as control, markedly reduced DJ-1 protein expression in the cultured neurons. Two additional DJ-1 siRNAs with different target sequences were also shown to effectively suppress DJ-1 expression (data not shown). Whole-cell patch-clamp recordings showed that NMDAr-mediated currents were significantly increased in neurons transfected with siRNAdj-1 compared with that of control neurons transfected with SsiRNAdj-1 (Fig. 1C). We also showed that overexpression of DJ-1 in the cortical neurons with transfection of DJ-1 cDNAs remarkably inhibited NMDAr-mediated currents (Fig. 1D,E). These results provide the first evidence that NMDAr function is negatively regulated by DJ-1.

PINK1 positively regulates NMDAr function

We then examined whether PINK1 modulated the function of NMDArs. NMDAr-mediated whole-cell currents were recorded in cultured cortical neurons transfected with PINK1 siRNA (siRNApink1). Two additional PINK1 siRNAs with different target sequences were also shown to effectively suppress PINK1 expression (data not shown). Our data showed that the suppression of PINK1 by siRNApink1 (Fig. 2A), in contrast to DJ-1 suppression, significantly reduced NMDAr-mediated currents (Fig. 2B). This evidence suggests that endogenous PINK1 may play a different role than DJ-1 in regulating NMDAr function. To

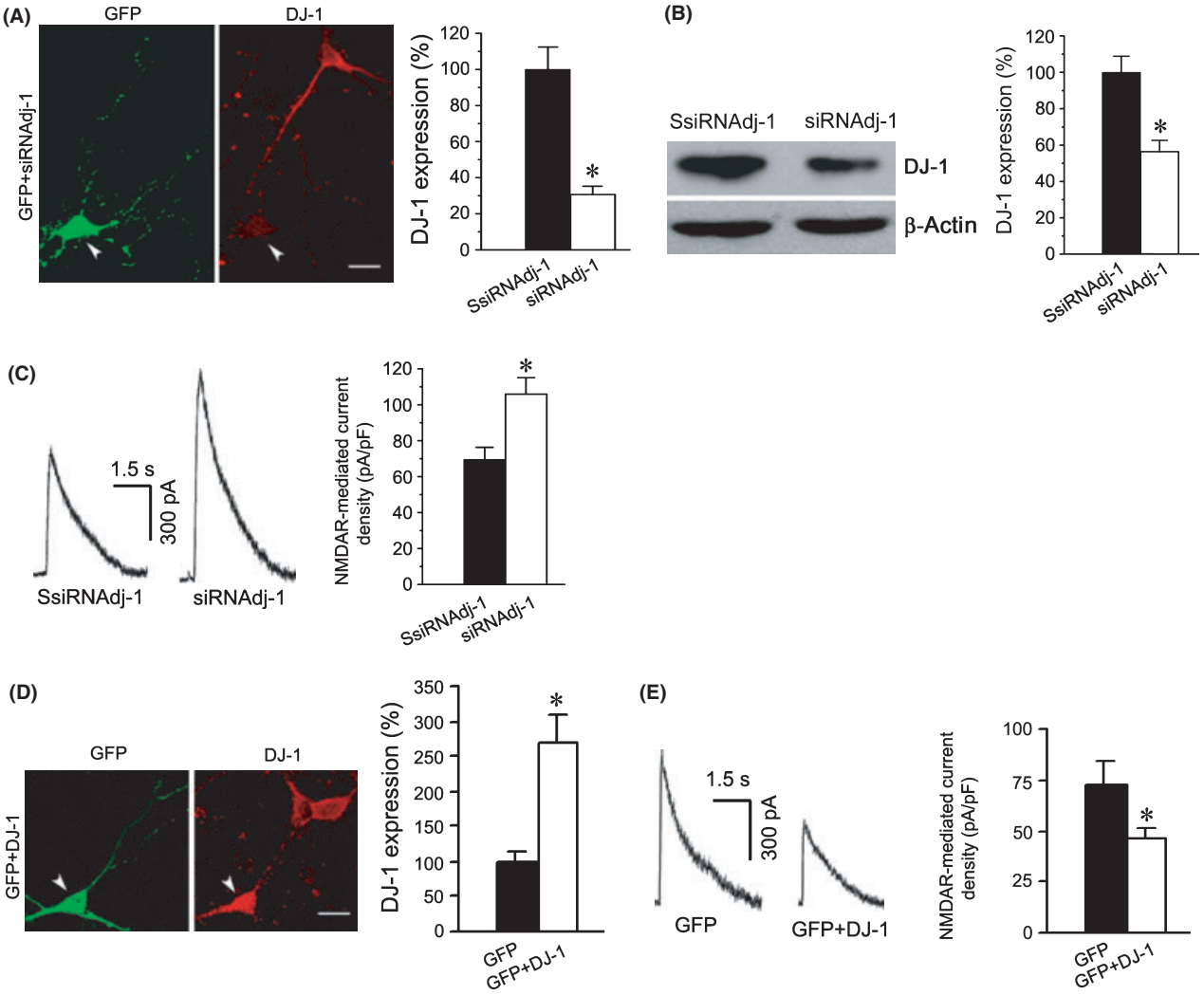

Fig. 1 Effects of suppression or overexpression of DJ-1 on NMDAr-mediated whole-cell currents. (A) Left panel: Knockdown of DJ-1 protein in cultured cortical neurons by transfecting DJ-1 siRNA (siRNAdj-1). The scrambled siRNA (SsiRNAdj-1) was used as controls. Green fluorescent protein was cotransfected with siRNAs to label the transfected neurons. Right panel: Summarized data of DJ-1 protein suppression by siRNAdj-1 in cultured cortical neurons (n = 15 for each group, $^*P < 0.05$). Scale bar = 15 μm. (B) Left panel: sample immunoblots showing that knockdown of DJ-1 protein in cultured cortical neurons by transfecting DJ-1 siRNA (siRNAdj-1). The scrambled siRNA (SsiRNAdj-1) was used as controls. Right panel: Summarized data of DJ-1 protein suppression by siRNAdj-1 in cultured cortical neurons (n = 6 for each group, $^*P < 0.05$). (C) Left panel: Representative traces of NMDAr-mediated whole-cell current in cortical neurons transfected with siRNAdj-1 and SsiRNAdj-1. Right panel: NMDAr-mediated current density is significantly increased in cells transfected with siRNAdj-1 compared to those transfected with SsiRNAdj-1 (SsiRNAdj-1, n = 22; siRNAdj-1, n = 22; $^*P < 0.05$). (D) Left panel: overexpression of DJ-1 protein in cultured cortical neurons by cotransfecting DJ-1 and GFP cDNAs. The neurons transfected with GFP alone were used as controls. Right panel: Summarized data of DJ-1 protein overexpression in cultured cortical neurons (n = 20 for each group, $^*P < 0.05$). Scale bar = 15 μm. (E) Left panel: Representative traces of NMDAr-mediated whole-cell current in cortical neurons transfected with GFP and GFP + DJ-1 cDNAs. Right panel: NMDAr-mediated current density is significantly reduced in cells transfected with GFP + DJ-1 cDNAs compared to those transfected with GFP alone (GFP, n = 26; GFP + DJ-1 cDNAs, n = 28; $^*P < 0.05$). NMDAr, *N*-methyl-D-aspartate receptors.

provide further evidence to support the regulation of NMDArs by PINK1, cortical neurons were transfected with PINK1 cDNAs (Fig. 2C). As expected, the overexpression of PINK1 was shown to increase NMDAr currents (Fig. 2D).

PINK1 regulates NMDAr function through acting on NR2ACNRs

Given that NR2ACNRs and NR2BCNRs may exert opposing effects on neuronal survival/death (Hardingham *et al.*, 2002; Liu *et al.*, 2007), we reasoned that the opposing effects of DJ-1 and PINK1 on NMDAr-mediated whole-cell currents might be attributed to the differential regulation of NR2ACNR and NR2BCNR subtypes by DJ-1 and PINK1, respectively. To test this possibility, we performed whole-cell patch-clamp recordings to measure NR2ACNR- and NR2BCNR-mediated currents in rat cortical neurons with the use of NR2ACNR antagonist NVP-AAM077 and NR2BCNR antagonist Ro 25-6981 (Liu *et al.*, 2007; Fantin *et al.*, 2008; Schotanus & Chergui, 2008). We measured the inhibited peak levels of NMDAr-mediated whole-cell currents by NVP-AAM007 (50 nM) or Ro 25-6981 (1 μM) so that NR2ACNR- and

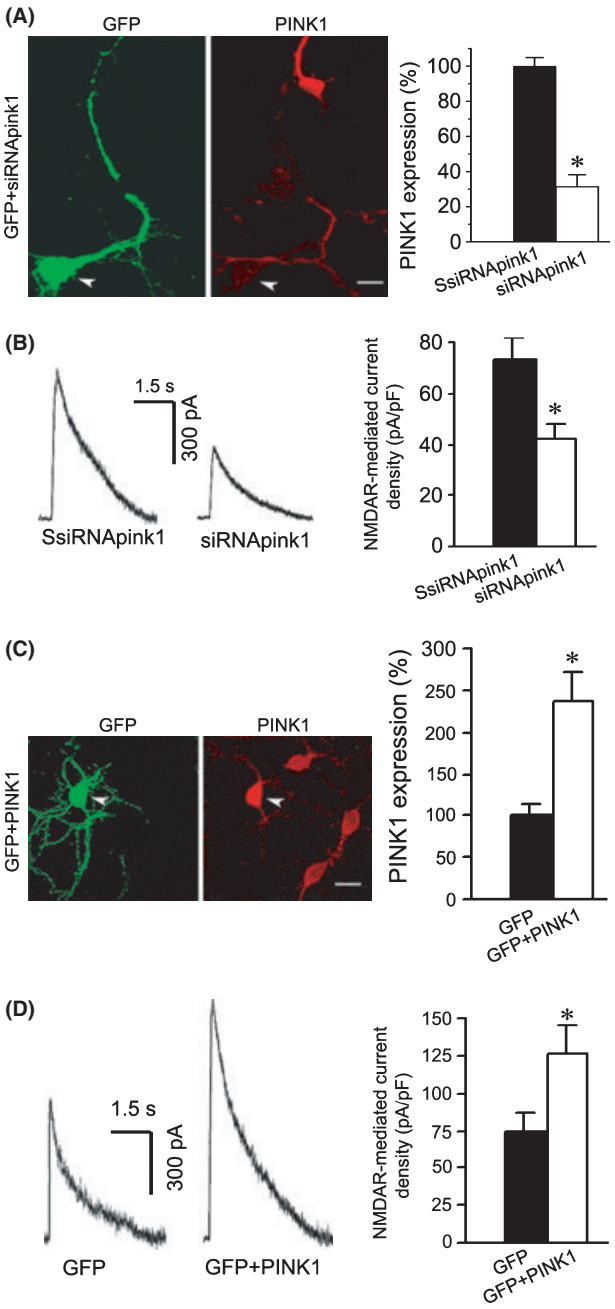

Fig. 2 Effects of suppression or overexpression of PINK1 on NMDAr-mediated whole-cell currents. (A) Left panel: Knockdown of PINK1 protein expression in cultured cortical neurons by PINK1 siRNA (siRNApink1). The scrambled siRNA (SsiRNApink1) was used as controls. Green fluorescent protein (GFP) was cotransfected with siRNAs to label the transfected neurons. Right panel: Summarized data of PINK1 protein suppression by siRNApink1 in cultured cortical neurons (n = 19 for each group, $^*P < 0.05$). Scale bar = 15 μm. (B) Left panel: Representative NMDAr-mediated whole-cell current in cortical neurons transfected with siRNApink1 and SsiRNApink1. Right panel: NMDAr-mediated current density was inhibited in cells transfected with siRNApink1 compared with those transfected with SsiRNApink1 (SsiRNApink1, n = 19; siRNApink1, n = 23; $^*P < 0.05$). (C) Left panel: overexpression of PINK1 protein in cultured cortical neurons by cotransfecting PINK1 and GFP cDNAs. The neurons transfected with GFP alone were used as controls. Right panel: Summarized data of DJ-1 protein overexpression in cultured cortical neurons (n = 22 for each group, $^*P < 0.05$). Scale bar = 15 μm. (D) Left panel: Representative traces of NMDAr-mediated whole-cell current in cortical neurons transfected with GFP and GFP + PINK1 cDNAs. Right panel: NMDAr-mediated current density was significantly increased in cells transfected with GFP + PINK1 cDNAs compared to those transfected with GFP (GFP, n = 23; GFP + PINK1 cDNAs, n = 25; $^*P < 0.05$). NMDAr, *N*-methyl-D-aspartate receptors.

NR2BCNR-mediated components of NMDAr currents could be isolated (Fig. 3A,B,D,E). We also used the NR2BCNR antagonist ifenprodil for the experiments and found that ifenprodil had the same effect as Ro 25-6981 (Fig. 3C,F). In the neurons transfected with PINK1 cDNAs and siRNAs, we found that the overexpression and suppression of PINK1, respectively, increased and inhibited NR2ACNR-mediated currents without effect on NR2BCNR-mediated currents (Fig. 3A–C). These data indicate that the PINK1 regulation of NMDAr function is resulted from altered NR2ACNR activity. As NR2ACNR-dependent signaling is believed to be neuroprotective, these results suggest a possibility that PINK1 dysfunction may promote neuronal death through inhibition of NR2ACNRs.

DJ-1 regulates NMDAr function through acting on both NR2ACNRs and NR2BCNRs

By measuring NR2ACNR- and NR2BCNR-mediated currents using NR2ACNR antagonist NVP-AAM077 (50 nM) and NR2BCNR antagonist Ro25-6981 (1 μM), our results showed that suppressing DJ-1 protein expression in neurons transfected with DJ-1 siRNAs increased both NR2ACNR and NR2BCNR currents (Fig. 3D–F). We also showed that DJ-1 overexpression inhibited NR2BCNR but not NR2ACNR currents (Fig. 3D–F). These results led us to hypothesize that DJ-1 dysfunction, while promoting neuronal death through enhancing NR2BCNR-dependent cell death signaling, might induce self-protective signaling through increasing NR2ACNR function (Fig. 8).

DJ-1 regulates the surface expression of NR2BCNRs

The regulation of NMDAr function by DJ-1 and/or PINK1 could be attributable to the altered expression of NMDArs on the cell surface (Carroll & Zukin, 2002). We found that transfections of siRNApink1 or PINK1 cDNAs had no significant effects on the surface and total protein expression of NR2A subunits in the cultured cortical neurons (Fig. 4A,B). However, siRNAdj-1 transfection resulted in an increased surface expression of NR2B (Fig. 4C) but not NR2A subunits (data not shown). We also showed that DJ-1 cDNA transfection led to a decreased surface expression of NR2BCNRs in cortical neurons (Fig. 4D). Because the total NR2B levels were not altered by DJ-1 knockdown or overexpression (Fig. 4C,D), the altered surface expression of NR2B could be the result of an altered delivery and/or internalization of NMDArs. Thus, the post-transcriptional mechanisms may mediate the DJ-1 regulation of NR2B surface expression. Thus, PINK1 affects NR2ACNR function not by regulating NR2ACNR total protein/surface expression but via other unknown mechanism which is different from DJ-1 effect on NR2BCNRs.

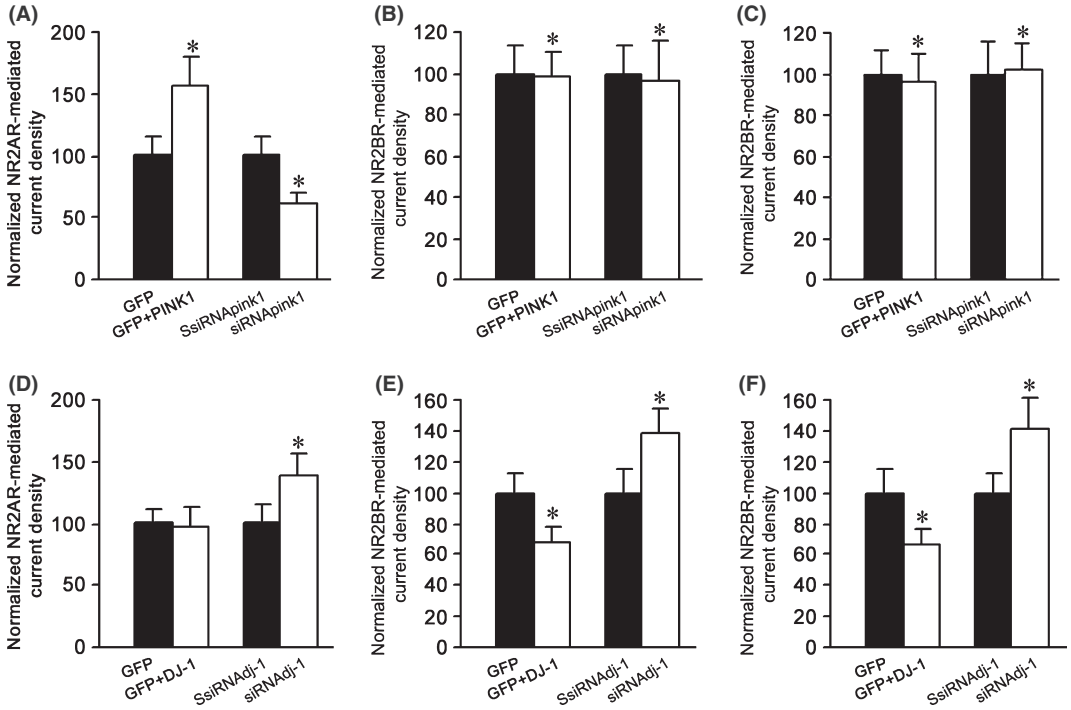

Fig. 3 Differential regulation of NR2ACNR and NR2BCNR function by DJ-1 and PINK1 in cultured cortical neurons. NR2ACNR- and NR2BCNR-mediated currents in rat cortical neurons were recorded with the use of NR2ACNR antagonist NVP-AAM077 (A, D) and NR2BCNR antagonist Ro 25-6981 (B, E). The NR2BCNR antagonist ifenprodil was also used to measure NR2BCNR-mediated currents (C, F). (A) PINK1 overexpression and knockdown, respectively, increases and decreases the function of NR2ACNRs ($n = 10$ for each group, $^*P < 0.05$ vs. GFP or SsiRNApink1). (B, C) Both PINK1 overexpression and knockdown have no effects on the function of NR2BCNRs ($n = 10$ for each group, $^*P > 0.05$). (D) DJ-1 knockdown increases the function of NR2ACNRs, but DJ-1 overexpression has no effect on NR2ACNR function ($n = 11$ for each group, $^*P < 0.05$ vs. SsiRNAdj-1). (E, F) DJ-1 overexpression and knockdown, respectively, inhibits and enhances the function of NR2BCNRs ($n = 11$ for each group, $^*P < 0.05$ vs. GFP or SsiRNAdj-1). GFP, green fluorescent protein.

PTEN mediates DJ-1 regulation of NR2BCNR function

We have previously shown that the phosphatase PTEN positively regulates the function of NR2BCNRs (Ning *et al.*, 2004; Chang *et al.*, 2007). As DJ-1 is a negative regulator of PTEN (Kim *et al.*, 2005b), we hypothesized that PTEN upregulation might contribute to DJ-1 knockdown-induced enhancement of NR2BCNR function. We therefore performed Western blots to test PTEN expression in cultured cortical neurons transfected with DJ-1 siRNAs. Indeed, our data showed that DJ-1 knockdown resulted in an increased protein expression of PTEN in the cultures (Fig. 5A). We then performed whole-cell patch-clamp recording to test whether PTEN upregulation mediated DJ-1 suppression-induced NR2BCNR potentiation. Using a specific PTEN inhibitor bpV(pic) (Schmid *et al.*, 2004), we showed that bpV(pic) (100 nM) significantly reduced DJ-1 suppression-induced increase in NR2BCNR currents but without effect on DJ-1 suppression-induced increase in NR2ACNR currents (Fig. 5B,C). Together with our previous findings that PTEN positively regulates NR2BCNRs and thereby promoting neuronal death (Ning *et al.*, 2004; Chang *et al.*, 2007), we propose that DJ-1 knockdown-induced potentiation of NR2BCNR function is mediated in part by increased PTEN expression, which may promote neuronal death and neurodegeneration.

DJ-1 regulates NR2ACNR function via PTEN/PINK1 signaling

It has been demonstrated that enhancement of PTEN expression induces the expression of PINK1 (Unoki & Nakamura, 2001). As DJ-1 knockdown results in an upregulation of PTEN expression (Fig. 5A), we have hypothesized that PTEN upregulation-induced increase in PINK1 expression could be responsible for DJ-1 knockdown-induced potentiation of NR2ACNR function. We therefore examined both PTEN and PINK1 expression in cultured neurons transfected with DJ-1 siRNAs. As expected, DJ-1 knockdown increased not only the protein expression of PTEN but also the protein expression of PINK1 (Fig. 5A). We then tested whether DJ-1 suppression-induced increase in NR2ACNR currents was resulted from PINK1 upregulation in neurons transfected with DJ-1 siRNAs. We showed that suppressing PINK1 expression in cortical neurons significantly reduced DJ-1 knockdown-induced increase in NR2ACNR currents but had no effect on DJ-1 knockdown-induced increase in NR2BCNR currents (Fig. 5D,E). Together with our finding that PINK1 positively regulates NR2ACNR function (Fig. 3A), these data suggest that the enhanced NR2ACNR function by DJ-1 suppression may be due at least in part to the upregulation of PINK1 expression.

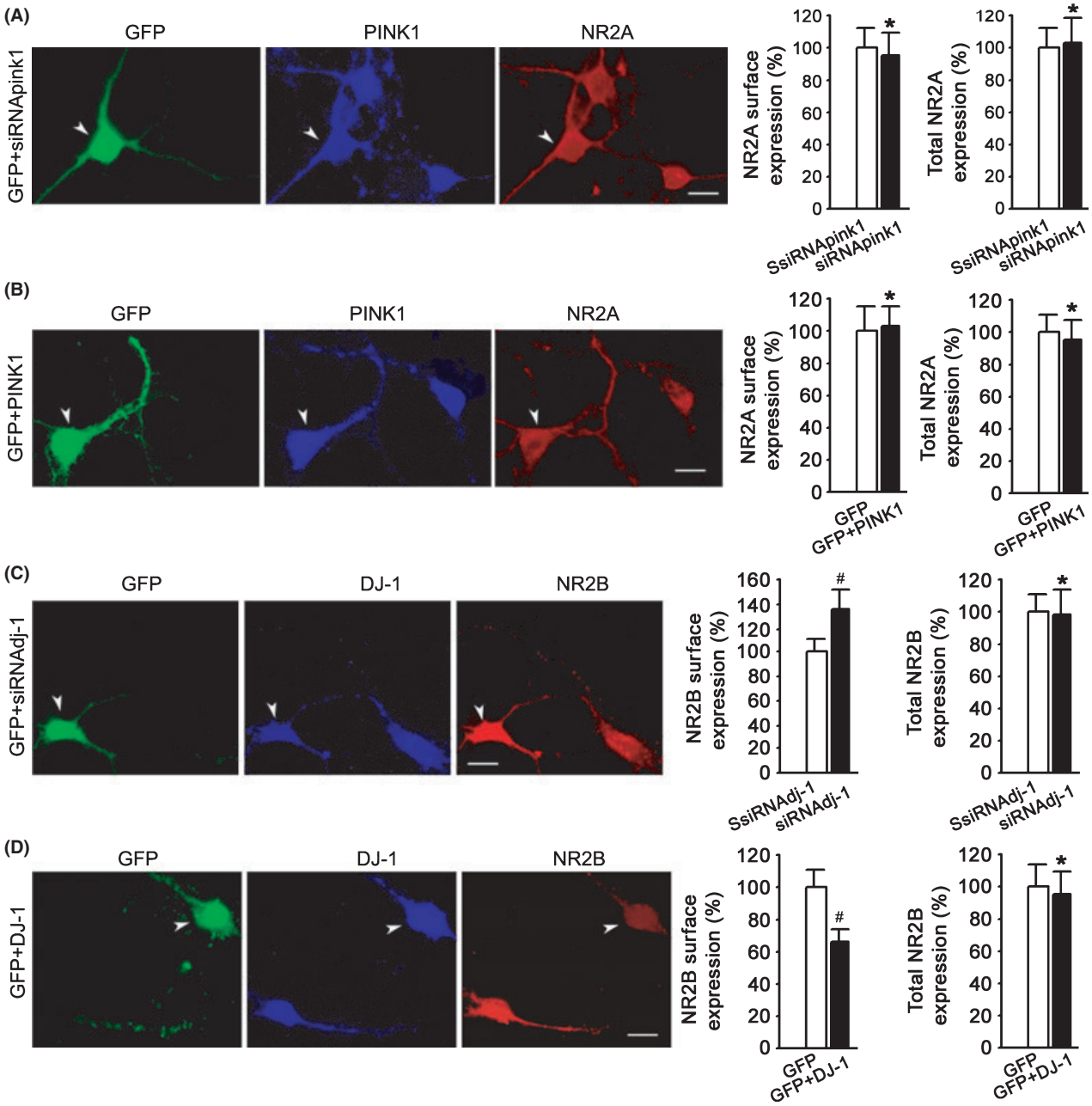

Fig. 4 Regulation of NR2BCNR surface expression by DJ-1. (A) Left, Sample images showing that PINK1 knockdown has no effect on the surface NR2ACNR expression. Scale bar = 15 μm. Right, Summarized data showing that both total and surface NR2ACNR expression are not altered by PINK1 knockdown (n = 30 for each group in both bar graphs, $^{*}P > 0.05$ vs. SsiRNApink1). (B) Left, Sample images showing that PINK1 overexpression has no effect on the surface NR2ACNR expression. Scale bar = 15 μm. Right, Summarized data showing that both total and surface NR2ACNR expression are not altered by PINK1 overexpression (n = 26 for each group in both bar graphs, $^{*}P > 0.05$ vs. GFP). (C) Left, Sample images showing that DJ-1 knockdown increases the surface NR2BCNR expression. Scale bar = 15 μm. Right, Summarized data showing that the surface NR2BCNR expression is increased by DJ-1 knockdown (n = 28 for each group, $^{\#}P < 0.05$ vs. SsiRNApink1), but the total NR2BCNR expression is not altered by DJ-1 knockdown (n = 25 for each group, $^{*}P > 0.05$ vs. SsiRNApink1). We also show that DJ-1 knockdown has no effect on the surface/total NR2ACNR expression (Data not shown). (D) Left, Sample images showing that DJ-1 overexpression decreases the surface NR2BCNR expression. Scale bar = 15 μm. Right, Summarized data showing that the surface NR2BCNR expression is reduced by DJ-1 overexpression (n = 28 for each group, $^{\#}P < 0.05$ vs. GFP), but the total NR2BCNR expression is not altered by DJ-1 overexpression (n = 25 for each group, $^{*}P > 0.05$ vs. GFP). GFP, green fluorescent protein.

To provide direct evidence that the increased PINK1 expression was induced by PTEN upregulation, we performed immunocytochemical staining to examine PINK1 expression in cultured neurons transfected with PTEN cDNA or siRNA. Our results showed that PTEN overexpression markedly enhanced PINK1 expression (Fig. 6A), whereas the suppression of PTEN did not exert significant effect on PINK1 expression (Fig. 6B). Taken together, these data suggest that the enhanced NR2ACNR function by DJ-1 suppression may be due at least in part to the upregulation of PTEN and subsequent increase in PINK1 expres-

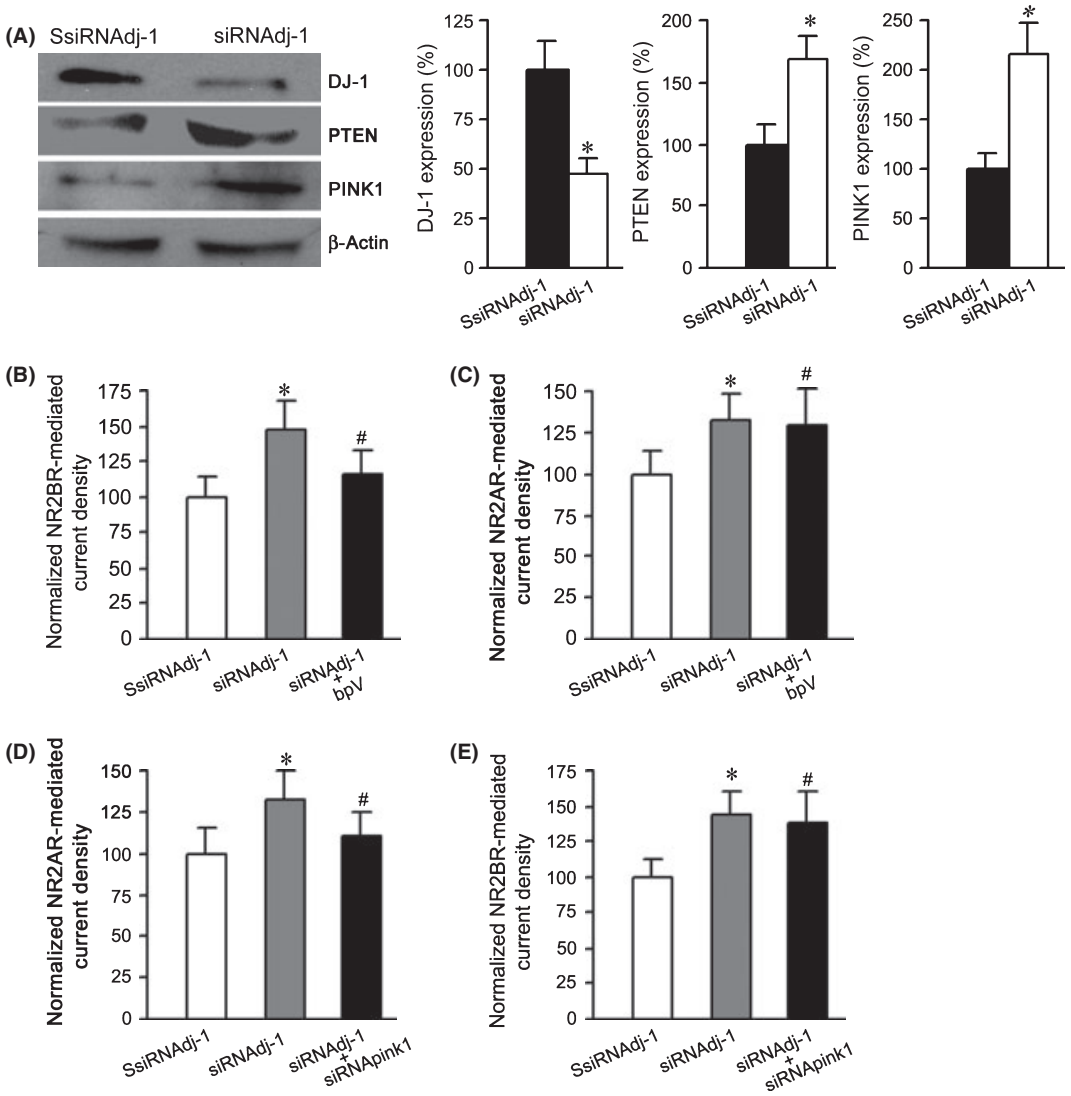

Fig. 5 Roles of PTEN and PINK1 in mediating DJ-1 regulation of NR2ACNRs and NR2BCNRs. (A) Western blots show that PTEN and PINK1 levels are enhanced by DJ-1 knockdown in rat cortical neurons (n = 3, $^*P < 0.05$ vs. SsiRNAdj-1). (B) PTEN inhibitor bpV(pic) (100 nM) significantly blocks DJ-1 knockdown-induced increase in NR2BCNR currents (SsiRNAdj-1, n = 12; siRNAdj-1, n = 12; siRNAdj-1 + bpV(pic), n = 13, $^*P < 0.05$ vs. SsiRNAdj-1; $\#P < 0.05$ vs. siRNAdj-1). (C) PTEN inhibitor bpV(pic) (100 nM) has no effect on DJ-1 knockdown-induced increase in NR2ACNR currents (n = 12 for each group, $^*P < 0.05$ vs. SsiRNAdj-1; $\#P > 0.05$ vs. siRNAdj-1). (D) Cotransfection of PINK1 siRNA (siRNApink1) with siRNAdj-1 significantly reduces DJ-1 knockdown-induced increase in NR2ACNR currents (n = 15 for each group, $^*P < 0.05$ vs. SsiRNAdj-1; $\#P < 0.05$ vs. siRNAdj-1). (E) Cotransfection of siRNApink1 with siRNAdj-1 has no effect on DJ-1 knockdown-induced increase in NR2BCNR currents (n = 15 for each group, $^*P < 0.05$ vs. SsiRNAdj-1; $\#P > 0.05$ vs. siRNAdj-1).

sion. This study indicates that PINK1 acts with DJ-1 in a common pathway (DJ-1/PTEN/PINK1/NR2ACNR) to enhance NR2ACNR function (Fig. 8). As activation of NR2ACNRs is thought to promote neuroprotection (DeRidder *et al.*, 2006; Liu *et al.*, 2007), PINK1 may act on NR2ACNRs to counteract DJ-1 dysfunction-induced neurodegenerative effect mediated by DJ-1/PTEN/NR2BCNR signaling (Fig. 8).

PINK1 confers neuroprotection through activation of NR2ACNRs

To determine the roles of DJ-1 suppression-dependent dual signal pathways DJ-1/PTEN/PINK1/NR2ACNR and DJ-1/PTEN/NR2BCNR in NMDAr-mediated neuronal survival/death, we first tested whether PINK1 conferred neuroprotection through the activation of NR2ACNRs. Neuronal death was induced by incubating the cultured neurons with NMDA (plus NMDAr co-agonist glycine) for 30 min. At 6 h after treatments, the propidium iodide (PI) labeling was performed to measure neuronal death (Ning *et al.*, 2004), and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay that measures the mitochondrial function was performed to measure neuronal viability (Choi *et al.*, 2004; Wang *et al.*, 2007). We showed that the overexpression of PINK1 in cortical neurons significantly decreased NMDA-induced neuronal death and that the NR2ACNR antagonist NVP-AAM077 (50 nM) reduced PINK1-mediated neuroprotective effect (Fig. 7A). We also provided evidence that cortical

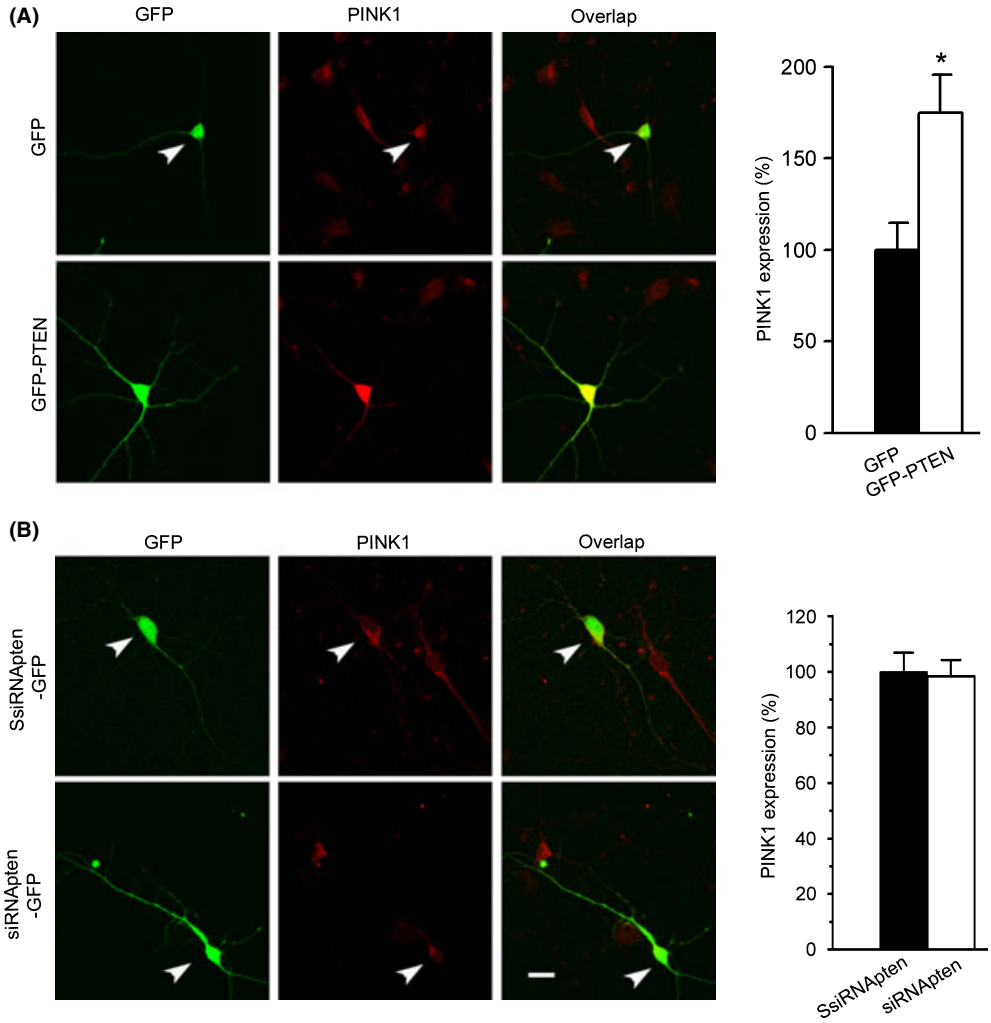

Fig. 6 PTEN overexpression induces increased protein expression of PINK1 in cultured rat cortical neurons. (A) PINK1 protein expression is significantly enhanced by PTEN cDNA transfection (GFP, $n = 68$ cells; PTEN-GFP, $n = 65$ cells, $^*P < 0.05$). (B) Downregulation of PTEN protein by PTEN siRNA transfection does not result in significant change in the protein expression of PINK1. Scale bar = 20 μm. All data were collected from three independent neuronal cultures. GFP, green fluorescent protein.

neurons with PINK1 suppression had increased sensitivity to NMDA-induced neurotoxicity (Fig. 7B,C). Together, these data suggest that PINK1 upregulation of NR2ACNRs plays a neuroprotective role in NMDA-induced neuronal death.

DJ-1 and PINK1 in a common pathway regulate NMDA-induced neuronal death

We next tested whether PINK1 acted with DJ-1 in a common pathway, the DJ-1/PTEN/PINK1/NR2ACNR, to regulate NMDA-induced neuronal death. Our results showed that while the treatment of NMDA (plus glycine for 30 min) significantly increased the death rate in cortical neurons with the knockdown of DJ-1 or PINK1 alone at 6 h after NMDA treatment, the neuronal death was further increased in neurons treated with DJ-1 siRNAs plus either PINK1 siRNAs or NR2ACNR antagonist NVP-AAM077 (50 nM) (Fig. 7B,C). Together with our findings that DJ-1 suppression induces the enhancement of PINK1 expression and subsequent potentiation of NR2ACNR currents (Fig. 5) and that PINK1 exerts its neuroprotective effect through the activation of NR2ACNRs (Fig. 7A), these results suggest that PINK1 may act downstream of DJ-1 to counteract DJ-1 dysfunction-induced neuronal death through the activation of NR2ACNRs (Fig. 8).

PTEN/NR2BCNR signaling mediates DJ-1 suppression-induced neuronal death

To determine the role of DJ-1/PTEN/NR2BCNR pathway in regulating neuronal death, we tested the effects of PTEN inhibitor bpV(pic) and NR2BCNR antagonist Ro25-6981 on DJ-1 knockdown-mediated neuronal death. We showed that both bpV(pic) (100 nM) and Ro25-6981 (1 μM) significantly blocked NMDA-induced death in cortical neurons with DJ-1 knockdown (Fig. 7D,F). We also showed that the treatment of NR2B antagonist Ro25-6961 failed to further reduce neuronal death in DJ-1-

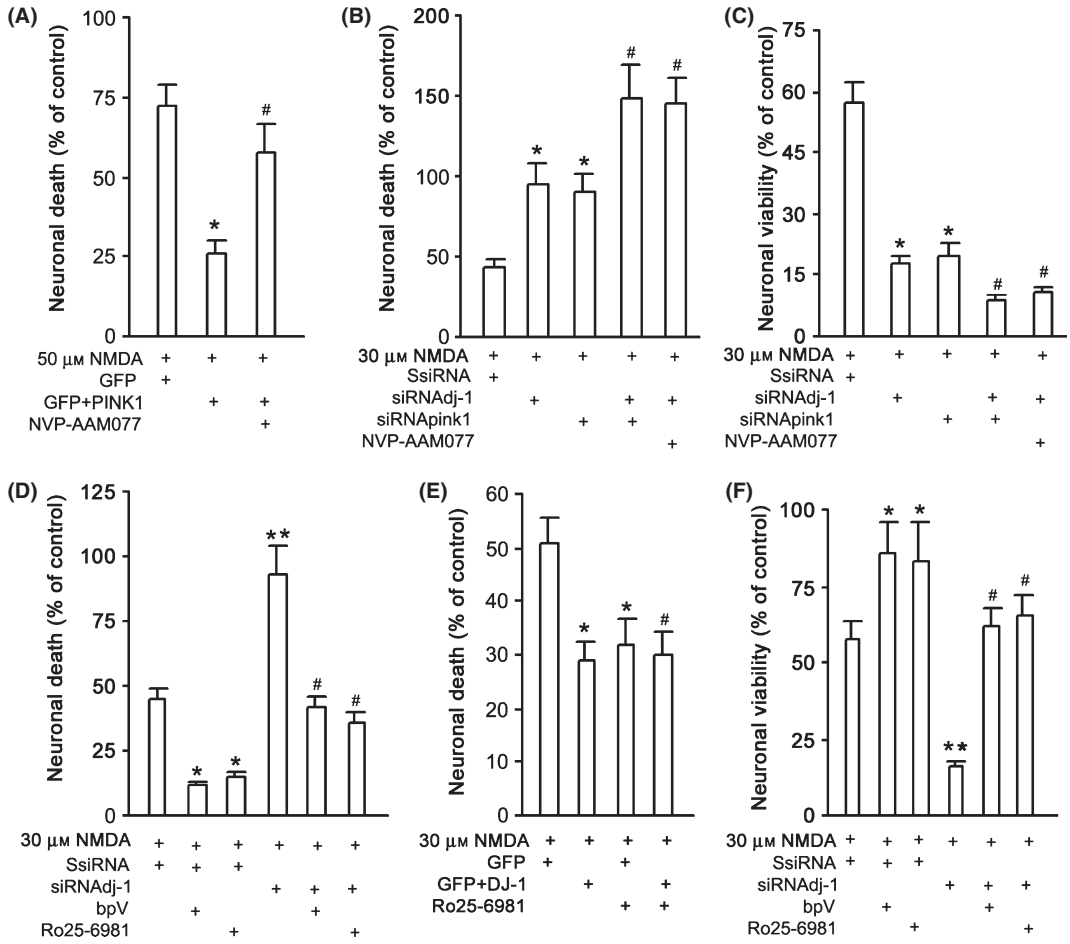

Fig. 7 Effects of DJ-1/PTEN/PINK1/NR2ACNR and DJ-1/PTEN/NR2BCNR pathways on NMDA-induced neuronal death. (A) Summarized data of PI labeling show that overexpression of PINK1 reduces NMDA-induced cortical neuronal death, and this neuroprotection is blocked by NR2ACNR antagonist NVP-AAM077 (50 nM) (n = 200 cells for each group, data were normalized to the NMDA untreated control; $^{*}P < 0.05$ vs. GFP + 50 μM NMDA, $^{\#}P < 0.05$ vs. GFP + PINK1 + 50 μM NMDA). The percentage of dead neurons in NMDA untreated control is 7%. (B) PI labeling assay shows that neuronal death is significantly increased in neurons treated with siRNAdj-1 + siRNApink1 or siRNAdj-1 + NVP-AAM077 (n = 200 cells for each group; data were normalized to the NMDA untreated control; $^{*}P < 0.05$ vs. SsiRNA + 30 μM NMDA, $^{\#}P < 0.05$ vs. siRNAdj-1 + 30 μM NMDA or siRNApink1 + 30 μM NMDA). (C) MTT assay shows that neuronal viability is significantly decreased in neurons treated with siRNAdj-1 + siRNApink1 or siRNAdj-1 + NVP-AAM077 (n = 7 for each group; data were normalized to the NMDA untreated control; $^{*}P < 0.05$ vs. SsiRNA + 30 μM NMDA, $^{\#}P < 0.05$ vs. siRNAdj-1 + 30 μM NMDA or siRNApink1 + 30 μM NMDA). (D) PI labeling assay shows that PTEN inhibitor bpV(pic) (200 nM) and NR2BCNR antagonist Ro25-6981 (1 μM) block DJ-1 suppression-mediated increase in neuronal death by NMDA-induced neurotoxicity (n = 200 cells for each group; data were normalized to the NMDA untreated control; $^{*}P < 0.05$ vs. SsiRNA + 30 μM NMDA, $^{**}P < 0.05$ vs. SsiRNAdj-1 + 30 μM NMDA, $^{\#}P < 0.05$ vs. siRNAdj-1 + 30 μM NMDA). (E) Summarized data of PI labeling show that overexpression of DJ-1 reduces NMDA-induced cortical neuronal death, and this neuroprotection is mediated through the inhibition of NR2BCNRs (n = 200 cells for each group, data were normalized to the NMDA untreated control; $^{*}P < 0.05$ vs. GFP + 30 μM NMDA, $^{\#}P > 0.05$ vs. GFP + DJ-1 + 30 μM NMDA or Ro25-6981 + 30 μM NMDA). (F) MTT assay shows that PTEN inhibitor bpV(pic) (200 nM) and NR2BCNR antagonist Ro25-6981 (1 μM) block DJ-1 suppression-mediated decrease in neuronal viability (n = 7 for each group; data were normalized to the NMDA untreated control; $^{*}P < 0.05$ vs. SsiRNA + 30 μM NMDA, $^{**}P < 0.05$ vs. SsiRNAdj-1 + 30 μM NMDA, $^{\#}P < 0.05$ vs. siRNAdj-1 + 30 μM NMDA). NMDAr, *N*-methyl-D-aspartate receptors.

overexpressed cells (Fig. 7E), suggesting that the overexpression of DJ-1 enhances survival by reducing NR2BR function. These results indicate that DJ-1 dysfunction, through the upregulation of PTEN and subsequent overactivation of NR2BCNRs, promotes neuronal death and neurodegeneration (Fig. 8).

Discussion

Dysfunction of DJ-1 and PINK1 contributes to neurodegeneration and may contribute to the early onset of PD (Bonifati *et al.*, 2003; Healy *et al.*, 2004; Valente *et al.*, 2004 May 21). Recent evidence demonstrates that DJ-1 is involved in ischemia-induced neuronal injury (Aleyasin *et al.*, 2007; Yanagisawa *et al.*, 2008), suggesting a broad role of DJ-1 in the regulation of neuronal death. Because mitochondrial dysfunction plays crucial roles in neuronal death and neurodegeneration that are induced or enhanced by NMDAr-mediated neurotoxicity and the dysfunction of DJ-1 and PINK1 (Greenwood & Connolly, 2007; Plun-Favreau & Hardy, 2008; Thomas & Cookson, 2009), we investigated the effects of DJ-1 and PINK1 on NMDAr-mediated neuronal survival/death. We provide the first evidence suggesting that DJ-1 suppression, while increasing neuronal susceptibil-

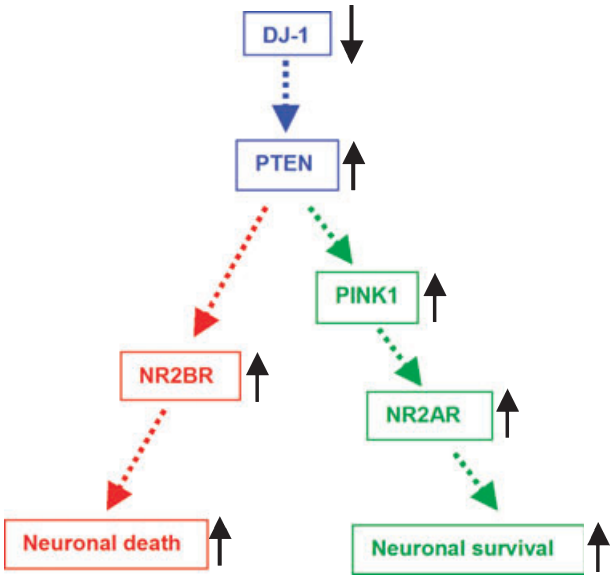

Fig. 8 Model of DJ-1 suppression-dependent dual signal transduction pathways. Suppression of DJ-1 leads to increased PTEN expression, which activates two signal pathways: (1) the increased expression of PTEN results in the overactivation of NR2BCNRs that promote neuronal death and (2) the increased expression of PTEN also induces PINK1 expression, which enhances NR2ACNR function to protect against DJ-1 dysfunction-mediated neurotoxicity.

ity to neurotoxicity by overactivating PTEN/NR2BCNR signaling, may induce a self-protective signaling through activating PTEN/PINK1/NR2ACNR pathway (Fig. 8). These results suggest that as a downstream signal of DJ-1, PINK1 may respond collaboratively to counteract DJ-1 dysfunction-induced neuronal damage (Fig. 8) (Tang *et al.*, 2006), which may delay neuronal death and possibly contributes to the slow neurodegenerative process in PD. To support this notion, our study also provides evidence that suppression of DJ-1 together with PINK1 knockdown, compared with suppression of DJ-1 or PINK1 alone, significantly increases NMDAr-mediated neuronal death (Fig. 7). As the haploinsufficiency of PINK1 and DJ-1 is a possible mechanism of early-onset PD, these data suggest that a concomitant dysfunction of DJ-1 and PINK1 may accelerate neurodegeneration process that may lead to the early onset of PD (Tang *et al.*, 2006).

We have previously reported that suppressing the protein expression or activity of PTEN protects against ischemic neuronal death, which is in part mediated through the inhibition of NR2BCNR-mediated neurotoxicity (Ning *et al.*, 2004). In this study, we provide new evidence that PTEN upregulation mediates DJ-1 suppression-induced increase in NR2BCNR function and neuronal death. This finding suggests that DJ-1 may act upstream of PTEN to regulate NR2BCNR-dependent neuronal death. Our data indicate that the phosphatase PTEN may play bi-directional roles to regulate neuronal survival/death in DJ-1-dependent pathways DJ-1/PTEN/NR2BCNR and DJ-1/PTEN/PINK1/NR2ACNR (Fig. 8). We showed that DJ-1 suppression-induced increase in PTEN expression, while promoting NMDA-mediated neuronal death through increased NR2BCNR activity, induced a neuroprotective effect by enhancing PINK1/NR2ACNR signaling (Figs 7 and 8). However, PINK1/NR2ACNR pathway is not involved in PTEN suppression-induced neuroprotection because PTEN suppression has no effects on PINK1 expression and NR2ACNR function. Thus, unlike DJ-1 suppression, DJ-1 potentiation couples to PTEN/NR2BCNR but not to PTEN/PINK1/NR2ACNR pathway to regulate neuronal death. As Unoki & Nakamura (2001) have shown that *PINK1* gene is transcriptionally transactivated by PTEN overexpression, a transcriptional mechanism may mediate the PTEN upregulation of PINK1 expression in our experimental conditions. However, it is unclear why the suppression of PTEN expression does not inhibit PINK1 expression. Future study is required to investigate the underlying mechanisms. It is interesting to note that the DJ-1 knockdown-induced potentiation of NR2BCNR currents can be partially inhibited by PTEN inhibitor (Fig. 5B). These data suggest that the PTEN-independent mechanisms may mediate DJ-1 knockdown-induced increase in NR2BCNR function. Indeed, this possibility is supported by recent findings that DJ-1 regulates neuronal survival through MEKK1-SEK1-JNK1 signaling pathway and alters the phosphorylation of 5′-AMP-activated protein kinase (AMPK) (Mo *et al.*, 2008; Vasseur *et al.*, 2009). The fact that PTEN inhibitor only affects NR2BCNR-specific component and has no effect on NR2ACNR pathway also suggests that the phosphatase activity of PTEN is important for NR2BCNR effect, whereas other attribute of PTEN is important for NR2ACNR effect (Fig. 5).

Although the results of clinical trials testing NMDAr antagonists as neuroprotectants after stroke and brain trauma have been discouraging (Hoyte *et al.*, 2004; Lipton, 2004), revealing neuronal survival and death signaling that selectively couple to NMDArs is a critical step to provide molecular basis for the development of novel neuroprotection strategy. In our study, we demonstrate that DJ-1 and PINK1, two recently identified intracellular signals associated with neurodegeneration, play differential roles in regulating the function of NR2BCNRs and NR2ACNRs. Importantly, we demonstrate that cosuppression of DJ-1 and PINK1 promotes neuronal death through increasing NR2BCNR-mediated neurotoxicity and inhibiting NR2ACNR-mediated neuronal survival signaling. Thus, selectively suppressing NR2BCNR-dependent neurotoxicity and specifically enhancing NR2ACNR-dependent neuroprotective signaling may be an effective strategy for the treatment of brain injury and neurodegeneration. The NMDAr overactivation induces massive increase in cytosolic calcium, the excitotoxicity that contributes to mitochondrial dysfunction including depolarization, decreased ATP synthesis, structural collapse and potential opening of the permeability transition pore (Greenwood & Connolly, 2007). Because the deficiency of both DJ-1 and PINK1 is involved in mitochondrial dysfunction (Deas *et al.*, 2009; Thomas & Cookson, 2009), the aberrant regulation of NMDArs by DJ-1 and PINK1 may promote NMDAr excitotoxicity-induced mitochondrial dysfunction in the neurodegenerative process of PD and other neurological disorders.

In summary, this study provides the first evidence that DJ-1-dependent pathways DJ-1/PTEN/NR2BCNR and DJ-1/PTEN/PINK1/NR2ACNR differentially regulate the function of NR2BCNRs and NR2ACNRs and that DJ-1 and PINK1 in a common pathway regulate NMDAr-mediated neuronal death. Both DJ-1/PTEN/NR2BCNR and DJ-1/PTEN/PINK1/NR2ACNR pathways function independently, not competitively. These results support the notion that NR2ACNR- and NR2BCNR-dependent signaling may play opposing roles in regulating neuronal death. Thus, our study reveals the mechanism by which DJ-1 and PINK1 regulate neuronal survival/death. Given the interactions between DJ-1, PINK1 and NMDArs in contributing to neuronal survival/death, the signal pathways DJ-1/PTEN/NR2BCNR and DJ-1/PTEN/PINK1/NR2ACNR may be the potential targets for the treatment of neurodegenerative diseases and CNS injury.

Experimental procedures

Rat cortical neuronal culture

The cortical neuronal cultures were prepared from Wistar rats at gestation day 17 using a modified protocol as described in our previous report (Brewer *et al.*, 1993; Shan *et al.*, 2009). Briefly, dissociated neurons were suspended in plating medium (Neurobasal medium, 2% B-27 supplement, 10% FBS, 0.5 μM L-glutamine and 25 μM glutamic acid) and plated on poly-D-lysine-coated Petri dishes. 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 μM L-glutamine). Thereafter, maintenance medium was changed in the same manner every 3 days.

Transfections

Specific siRNAs targeting PINK1 (siRNApink1) and DJ-1 (siRNAdj-1) were designed by IDT international (Kim *et al.*, 2005a). The scrambled siRNA (SsiRNA) was transfected in control neurons. Green fluorescent protein (GFP) vectors were cotransfected as a marker for transfected neurons with the siRNAs in all groups. Transfections of siRNAs and plasmids including DJ-1 and PINK1 cDNAs were performed at 7–9 days in cultures using Lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada) as described previously (Wan *et al.*, 1997; Ning *et al.*, 2004; Kim *et al.*, 2005a). The neurons were used for experiments at 48 h after transfections.

Western blotting

Western blotting and immunocytochemical staining were performed as described previously (Wan *et al.*, 1997; Liu *et al.*, 2000). Monoclonal antibody (mAb) against PTEN (Chemicon, Temecula, CA, USA), polyclonal antibody (pAb) against PTEN (Cell Signaling Technology, Beverly, MA, USA), pAb against PINK1 (Santa Cruz Biotech, Santa Cruz, CA, USA) that is characterized in our recent report (Shan *et al.*, 2009), pAb against DJ-1 (Santa Cruz) and mAb against actin (Chemicon) were used. Primary antibodies were labeled with horseradish peroxidase-conjugated secondary antibodies, and bands were imaged using SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA). The protein levels were quantified using ImageJ (NIH, Bethesda, MD, USA) software as described in our previous study (Ning *et al.*, 2004; Liu *et al.*, 2006, 2010).

Immunocytochemical staining, image acquisition and analysis

The anti-DJ-1 or PINK1 primary antibody (Santa Cruz) and Alexa Fluor 594 (red fluorescence) or Alexa Fluor 350 (blue fluorescence) secondary antibody (Invitrogen, Carlsbad, CA, USA) were used for the labeling of DJ-1 and PINK1. To measure the surface expression of NR2A and NR2B subunits, the cells were not permeabilized and labeled with anti-NR2A or NR2B antibody against the extracellular domain, and Alexa Fluor 594 (red fluorescence) secondary antibody (Invitrogen) were used (Ning *et al.*, 2004; Liu *et al.*, 2006). To measure the whole-cell expression of NR2A and NR2B, neurons were permeabilized with the treatment of 4% paraformaldehyde in PBS for 20 min and then 0.3% Triton X-100 for 10 min (Ning *et al.*, 2004; Liu *et al.*, 2006). Images were observed using a 63 × objective mounted on a Zeiss (Oberkochen, Germany) LSM 510 META confocal microscope as described previously (Liu *et al.*, 2006) and acquired using a Zeiss AxioCam digital camera in the linear range with constant settings. The collected images were analyzed for the expression of NR2A, NR2B, DJ-1 and PINK1 using ImageJ software as described in the following paragraph.

As the transfection efficiency is much lower in primary neurons compared to cell lines, in our experimental conditions, we found that only the transfection efficiency for DJ-1 siRNA, but not for the PINK1 siRNA, DJ-1 cDNA and PINK1 cDNA, is high enough to allow us to measure the knockdown of DJ-1 protein with Western blot assay. Thus, the immunocytochemical staining method had to be used to measure the changes in protein levels after transfections of PINK1 siRNA, DJ-1 cDNA or PINK1 cDNA. We have extensively used this method in our previous studies (Ning *et al.*, 2004; Liu *et al.*, 2006, 2010). To avoid the subjective measurements of knockdown and overexpression of DJ-1 and PINK1 in immunofluorescence labeling assay, we acquired the images in the linear range with constant settings. 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 a maximum projection. For all experiments, we analyzed fluorescent signal in the regions of interest by measuring the average fluorescence intensity per unit area. All images in all experiments were analyzed using identical acquisition parameters. During data acquisition and analysis, the investigator was blind to the treatment group. In each experiment, neurons were selected randomly, and fluorescent images of each neuron acquired from a single plane were transferred for analysis (Ning *et al.*, 2004; Liu *et al.*, 2006, 2010).

Electrophysiology

The neurons transfected with GFP were identified in our patch-clamp setup equipped with fluorescence microscope. The method for the recording of NMDAr-mediated whole-cell currents was described in detail previously (Ning *et al.*, 2004). Recording electrode resistance was 3–5 MΩ when filled with solution containing 140 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES, 4 mM K_2ATP, titrated to pH 7.3 with CsOH, and the osmolality was 280–290 mOsm. Bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES, 33 mM Glucose, titrated to pH 7.4 with osmolarity of 300–320 mOsm. Strychnine (1 μM) was added into bath solution to inhibit glycine receptor-mediated currents. The holding potential was +60 mV to remove the blockade effect of Mg^{2+} on NMDAR channels. *N*-methyl-D-aspartate receptor-mediated whole-cell currents were recorded by pressure application of 300 μM NMDA and 1 μM glycine (150 kPa, 200 ms) from a micropipette with its tip located ~20 μm from the recorded cell. Drugs were delivered at intervals of 1 min. Data were acquired with an Axopatch 200B amplifier and pCLAMP 10 software interfaced to a Digidata 1322A acquisition board (Molecular Devices, Sunnyvale, CA, USA), and signals were filtered at 2 kHz and digitized at 10 kHz. Peak currents were measured and normalized to the cell capacitances to acquire current densities (pA/pF). NR2ACNR antagonist NVP-AAM077 and NR2BCNR antagonist Ro 25-6981 were used to isolate NR2ACNR- and NR2BCNR-mediated currents. NVP-AAM077 or Ro 25-6981 was administered into bath solution after stable recordings of NMDAr currents induced by NMDA and glycine, and the NMDAr currents were continuously recorded until the peak currents were inhibited to stable levels. To measure the current densities of NR2ACNR- or NR2BCNR-mediated currents, the amplitude value of inhibited NMDAr currents by NR2ACNR or NR2BCNR antagonist was divided by the cell capacitance (pA/pF).

Cell death and viability assays

PI labeling was performed to detect neuronal death as described in our previous study (Ning *et al.*, 2004). Briefly, all the experimental cultures were plated with equal amount of cells. As a marker for transfected neurons, GFP was cotransfected with siRNAs or cDNAs. The medium for neuronal cultures with or without transfections was replaced by extracellular solution containing PI (Molecular Probes, Eugene, OR, USA) at a final concentration of 50 μg mL^{-1}. After 20 -min incubation in an ambient gas incubator at 37°C, cultures were washed with extracellular solution and then fixed with 4% paraformaldehyde. Neuronal death (percentage) was determined by calculating the number of PI labeling in neurons expressing GFP over total number of the green neurons (green neurons with PI labeling/green neurons with PI labeling + green neurons without PI labeling). For all the experiments, at least ten independent experiments were performed and randomly selected 20 transfected cells per group/experiment were analyzed by an investigator blind to the transfected constructs.

We also measured the neuronal viability by performing MTT assay that measures the mitochondrial function (Choi *et al.*, 2004; Wang *et al.*, 2007). Briefly, 100 μM MTT (5 mg/10 mL of medium) was added to the cultures, and the plate was incubated for 4 h at 37°C. The MTT solution was removed, 100 μM of dimethyl sulfoxide was added to each well, and the color intensity was assessed using a plate reader at 590 nm. Values from each treatment were expressed as a percentage of the untreated control.

Statistics

All population data were expressed as mean ± SE. The Student's *t*-test or the ANOVA test was used when appropriate to examine the statistical significance of the differences between groups of data. Significance was placed at $P < 0.05$.

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