Peptide (TFP5/TP5), derived from Cdk5 activator P35, provides neuroprotection in the MPTP model of Parkinson's disease.

Binukumar BK¹, Varsha Shukla¹, Niranjana D. Amin¹, Philip Grant¹, Bhaskar M¹, Susan Kuntz¹, Joseph Steiner¹, and Harish C. Pant¹.

1. National Institute of Neurological Disorders and Strokes, National Institutes of Health, Bethesda, MD, United States 20892,

Abstract

Parkinson’s disease (PD) is a chronic neurodegenerative disorder characterized by the loss of dopamine neurons in the substantia nigra, decreased striatal dopamine levels, and consequent extrapyramidal motor dysfunction. Recent evidence indicates that cyclin-dependent kinase 5 (Cdk5) is inappropriately activated in several neurodegenerative conditions including PD. To date, strategies to specifically inhibit Cdk5 hyperactivity have not been successful without affecting normal Cdk5 activity. Previously, we reported that TFP5 peptide has neuroprotective effects in animal models of Alzheimer’s disease (AD). Here, we show that TFP5/TP5 selective inhibition of Cdk5/p25 hyperactivation in vivo and in vitro, rescues nigrostriatal dopaminergic neurodegeneration induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP/MPP+), in a mouse model of PD. TP5 peptide treatment also blocked dopamine depletion in the striatum and improved gait dysfunction after MPTP administration. The neuroprotective effect of TFP5/TP5 peptide is also associated with marked reduction in neuroinflammation and apoptosis. Here, we show selective inhibition of Cdk5/p25 hyperactivation by TFP5/TP5 peptide, which identifies the kinase as a potential therapeutic target to reduce neurodegeneration in Parkinson's disease.

Introduction

Parkinson’s disease is a neurodegenerative disorder characterized by disabling motor abnormalities, including tremor, muscle rigidity, paucity of voluntary movements, and postural instability (Du et al., 2001). In several mammalian species MPTP produces most of the biochemical and pathological alterations seen in PD, including the loss of dopaminergic neurons of the substantia nigra pars compacta (SNc) (Smith et al., 2003). Current treatment strategies for PD consist primarily of dopamine replacement therapy with levodopa or dopamine agonists (Du

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et al., 2001). Although effective in the early stages of the disease, chronic dopamine replacement therapy can cause debilitating side effects. Accordingly, concerted research efforts have been focused on developing neuroprotective strategies that will halt or slow the progression of PD.

Cdk5 kinase activity was originally identified as a tau kinase implicated in AD progression and independently as a unique cell cycle kinase homolog exhibiting CDK1-like substrate specificity in brain (Zhang et al., 2012). The active kinase was found to be a heterodimer of Cdk5 bound to p25, a truncated fragment derived by calpain cleavage from p35, a larger activator. Whereas Cdk5/p35 is essential for normal development, synaptogenesis and synaptic activity of the mammalian brain, Cdk5/p25 hyperactivity induces cell death and is associated with neuropathology. Stress-induced proteolytic conversion of p35 to p25 and Cdk5/p25 hyperactivity is associated with human AD (Nguyen et al., 2001, Cruz et al., 2003) as well as with animal models of AD, PD, amyotrophic lateral sclerosis, and other neurodegenerative disorders (Nguyen et al., 2002, Qu et al., 2007, Slevin and Krupinski, 2009, Zhang et al., 2012). Significantly, Cdk5 exhibits a critical role in MPTP-mediated neuronal toxicity, in one of the best characterized PD models (Smith et al., 2006, Qu et al., 2007). MPTP, a neurotoxin that leads to selective degeneration of the substantia nigra neurons (Levy et al., 2009), increases Cdk5 activity and triggers neuronal loss through inactivation of survival factor MEF2 (myocyte enhancer factor 2), antioxidant enzyme Prx2 (peroxiredoxin 2) and DNA damage repair enzyme Ape1 (apurinic/apyrimidinic endonuclease 1) (Qu et al., 2007, Huang et al., 2010)

Because of its involvement in PD, Cdk5/p25 has been identified as a prime therapeutic target for PD. Accordingly, compounds like roscovitine, an inhibitor targeting the ATP binding site in Cdk5 and other cell cycle kinases, have been studied as potential therapeutic agents, but they lack specificity (Helal et al., 2004, Helal et al., 2009). Our approach to this problem, based on structure and kinetics of the Cdk5/p25 complex, resulted in the production of several small truncated peptides of p35, which competed with p25 binding and inhibited Cdk5 hyperactivation in vitro (Amin et al, 2002). A small peptide, P5, comprising 24 aa, specifically inhibited Cdk5/p25 activity in cultured cortical neurons, reduced hyperphosphorylated tau and apoptosis, without affecting the normal endogenous Cdk5/p35 activity, nor the activity of several cell cycle kinases (Zheng et al., 2005, Zheng et al., 2010a). The P5 peptide was modified as TFP5 with a transactivator of transcription (Tat) peptide conjugated at the C terminus to facilitate passage
through the blood-brain barrier, and fluoresceinisothiocyanate (FITC; a green fluorescent tag) attached at the N terminus as a marker. When injected intraperitoneally into 5XFAD AD model mice, significantly reduced Cdk5/p25 hyperactivity, hyperphosphorylated tau and rescued behavior deficits of spatial working memory and motor deficits (Shukla et al., 2013). Moreover, TFP5 also reduced toxicity in cortical neurons exposed to high glucose (Binukumar et al., 2014). We now report that intraperitoneal injection of TP5 (TFP5 without FITC) into MPTP-induced mice effectively blocks degeneration of dopamine neurons in the SNpc, and almost completely prevents the loss of striatal dopamine and its metabolites. The peptide treatment also ameliorates the MPTP-induced behavioral deficits, inhibits neuroinflammation \textit{in vivo} and protects MPP+ neurotoxicity \textit{in vitro}. These results suggest that TFP5/TP5 may be effective in the treatment of Parkinson’s disease.

**Results**

**TFP5 inhibits hyperactive Cdk5/p25 in MPP+- induced mesencephalic primary cultures, a dose dependent study.**

We first determined whether 24 hour MPP+ incubation induces Cdk5 hyperactivation in mesencephalic primary cultures in the presence or absence of TFP5 pretreatment and co-incubation. Seven DIC mesencephalic neuron-enriched primary cultures pretreated with TFP5 were coincubated with different um concentrations of MPP+ and TFP5 followed by Cdk5 immunoprecipitation and kinase assay. From 0.01um to 10.0 uM a concentration dependent increase in the activity was observed in the presence of MPP+ treatment (Fig.1 A); the decline at 100uM is probably due to cell toxicity. Pretreatment and coincubation with 0.5uM TFP5, however, was sufficient to significantly reduce the elevated activity (30%) at each concentration (Fig. 1A). In Western blots of the same lysates as in Fig. 1A we see a decline of p35 expression from 0.01 uM to 10 uM with modest expression of p25 (Fig. 1B). The kinase activities however are reduced at all concentrations (20-30%) by TFP5 treatment. Since 10uM MPP+ induced maximum activity, we selected this as the measure of hyperactivity in vitro for further study. It is noteworthy that scrambled peptide did not show any inhibitory effect (Fig1A).

Because the Cdk5/p25 inhibitor, TFP5 was effective at blocking deregulated kinase activity, we further asked whether the effect can be replicated in primary dopaminergic neurons from mesencephalic cultures. Mesencephalic tissues from E14 mouse embryos were cultured and
grown on polylysine coated cover slips. The neuronal cultures were pretreated with/wo TFP5 (500nM) or scrambled peptide for 12 hr then co-incubated in the presence of 10μM concentration of MPP+ and TFP5 for 24hr. TH-positive neurons in the control cells displayed a normal cell body and neurite integration, as shown in Fig. 1C. After exposure to 10μM MPP+ for 48h, dopaminergic neurons with swollen cellbodies and fragmented neurites were observed across the field. The number of TH-positive neurons and the length of TH-positive neurites were significantly reduced (Fig1D). In contrast, treatment with TFP5 (500nM) effectively attenuated MPP+-induced injury of dopaminergic neurons. Scrambled peptide control at 500 nm, however, had no protective effect on these cells. Taken together, these results demonstrate that TFP5 has a neuroprotective effect in cell culture models of dopaminergic neurodegeneration.

TFP5 treatment inhibits MPP+ -induced inflammation and apoptosis in mesencephalic primary cultures

To investigate anti-inflammatory effects of TFP5 against PD-related neurodegeneration, primary neuron-glia cultures from mouse midbrains (approximately 50% glia, 50% neurons), were pretreated with/wo TFP5 (500nM) or scrambled peptide for 12 hr, then co-incubated in the presence of 10μM concentration of MPP+ and TFP5 for 24hr. We measured the expression levels of F4/F8 (microglia marker) and GFAP (astrocytes marker) (Fig 2A,B and 2C,D). It is evident that treatment with 10μM MPP+ increased the expression of both F4/F8 and GFAP (164% and 173%) compared with the control, whereas treatment with TFP5 ameliorates these effects. Scrambled peptide had no effects compared to MPP+ treatment group. Next, we examined the effect of TFP5 on MPP+ induced apoptosis by measuring caspase-3 activation and cytochrome C release in the mixed cultures. Caspase-3 is an important signaling protein located down-stream in the apoptosis signaling pathway. Cytochrome C release and Caspase-3 activation are accepted measures of cellular apoptosis (Gentil et al., 2003). Treatment of mesencephalic primary cultures with MPP+ for 24h resulted in robust activation of caspase-3 and cytochrome C release, Fig.2 (E,F, and I, J). Pretreatment and co-incubation with TFP5 significantly inhibited the increased levels of caspase-3 and cytochromes C. To further confirm the anti-apoptotic function of TFP5, the expression levels of Bcl-2 (an anti-apoptotic factor) was determined by Western blot. As shown in Fig. 2G,H, MPP+ treatment for 24h significantly decreased the protein level of Bcl-2. This result is in line with previous reports (Liu et al., 2010). Treatments
with TFP5 significantly increased Bcl-2 expression (Fig. 2G and H). The above results indicate that TFP5 almost completely blocked the up-regulation of cytochrome C release, caspase-3 activation and decreased Bcl-2 expression. In all cases, scrambled peptide treatment had no effect. Together, these results revealed that TFP5 treatment has anti-apoptotic effects in mesencephalic cells in primary culture.

**TFP5 inhibits MPTP-induced Cdk5/p25 hyperactivity in vivo.**

As a more effective test of the efficacy of TFP5 as a therapy for PD, an in vivo model system should be used. To determine the effect of TFP5 treatment in vivo, we adopted the four dose MPTP mouse model of PD (Jackson-Lewis and Przedborski, 2007) (for details see the material and methods). For the initial sets of experiments, animals were injected intraperitoneally (ip) with a single TFP5 injection, 40 mg/kg (selection of dose based on our previous study) every day for 9 days. Day 2 TFP5 treated animals received four doses of MPTP (15mg/kgX4, total of 60 mg/kg/day). On the 10th day animals were sacrificed and Cdk5 kinase activity was measured in lysates of the SN. We found that the dose of TFP5 was inadequate; TFP5 treated animals did not show significant inhibition of Cdk5/p25 deregulated activity compared to MPTP group (Supplementary Fig. 1). Accordingly, we increased the dose to a single, 80mg/kg ip injection every day for 9 days. We used the peptide without the FITC tag, (TP5) for this higher protocol since TFP5 aggregates at higher doses (unpublished data). In this case we show that TP5 pre-treatment produced significant inhibition of Cdk5/p25 kinase activity (Fig. 3A). Hence, this in vivo protocol, identified as the “standard protocol” was selected for more extensive study. We assessed Cdk5 expression in the SN of mice treated with MPTP and TP5 and noted that Cdk5 expression increased almost 3-fold after MPTP treatment (lanes 3,4 Fig. 3B,C) but over expression was unaffected by TP5 (lanes 5-6, Fig. 3B,C). This observation is consistent with the suggestion that TP5 only inhibits Cdk5 activity without affecting expression. Note that Fig. 3B is a representative sample Western while Fig. 3C quantifies the results of assays of six animals per group. In Fig. 3D, 2 representative blots of the same lysates with p35 antibody (C19), which detects both p35 and p25, show MPTP-induced p25 up-regulation (compare control lanes 1 and 5 with MPTP lanes 2 and 6 respectively). These results are consistent with the data in Fig. 3A; MPTP-induced p25 expression, hyperactivates Cdk5 activity in the SN which is down regulated after TP5 treatment. In all cases scrambled peptide, the negative control had no effect on activity.
Dopaminergic neurons of the SN express Cdk5/p35 and are protected from cell death by TP5 after MPTP induction

To identify the cells of the SN that express Cdk5, reciprocal immunoprecipitations with tyrosine hydroxylase and Cdk5 antibodies were carried out with lysates of the substantia nigra. The results in Fig. 4A show that TH co-immunoprecipitates Cdk5 and p35 while Cdk5 co-immunoprecipitates the expected p35 together with TH. Colocalization of CDk5/p35 with TH was also confirmed in cells of the SN in immunohistological assays of ventral brain sections (Fig. 4B).

To investigate the neuroprotective effects of TP5 on MPTP-induced neuronal death in vivo, we treated C57BL6 mice with TP5 (80 mg/kg /ip) daily for 9 days together with appropriate controls. On day 2, mice were administered MPTP (4 × 15 mg/kg, i.p). Eight days later the brains were analyzed by immunohistochemistry to quantify TH positive neurons in the SNpc. MPTP treatment reduced the number of TH-positive neurons by 77% compared with saline-treated controls (P < 0.001) (Figs. 4C and D). Mice that received daily treatments of TP5 at 80 mg/kg, showed an increase of TH-positive neurons in the SNpc, to 52% of control (P < 0.01 and P < 0.001, respectively) (Fig.4D). The neuroprotective effect of TP5 was dose-dependent as a 40 mg/kg dose of TP5 failed to protect dopamine neurons from MPTP toxicity (data not shown). Moreover, scrambled peptide did not show any dopaminergic neuroprotection when compared to MPTP group. MPTP injections also caused significant decreases (p < 0.01) in the level of dopamine and its metabolites in the striatal region of MPTP-injected mice; dopaminergic cell loss is coupled to reduced dopamine levels (Figs. 4 E,F). MPTP-induced dopamine and HVA depletion of more than 50% was attenuated, almost to control levels in mice treated with TP5 for 9 days as compared to the MPTP-injected mice and/or the scrambled peptide controls. Taken together, these results suggest that TP5 can improve neurochemical deficits in the MPTP mouse model of PD.

TP5 suppresses MPTP-induced astroglial and microglial activation in the SN in vivo.

Microglial activation has been implicated in the propagation of SNpc neurotoxicity in multiple animal models of PD. Post-mortem analysis of idiopathic PD patients has revealed strong immunoreactivity for CD68, a marker of phagocytic microglia (Croisier et al., 2005, Vroon et al., 2007). Furthermore, administration of MPTP has been reliably shown to induce this phagocytic microglia phenotype in the SNpc of mice (Vroon et al., 2007, Chung et al., 2010,
Chung et al., 2011). Previous studies additionally report the presence of reactive microglia in MPTP-treated SN exhibiting nigral DA neuronal degeneration (Wu et al., 2003, Block et al., 2007). Accordingly, we investigated whether a TP5 injection regimen can inhibit MPTP-induced glial activation in the SN in vivo. Nine days after the final MPTP treatment, with or without TP5, brain tissues were processed for immunostaining using an antibody against CD11b and GFAP to detect microglial and astrocyte activation respectively (Fig. 5). Consistent with earlier reports (Wu et al., 2003), numerous GFAP-positive reactive astrocytes (Fig. 5A, B) and CD11b-positive (activated) microglia (Fig 5C) were observed in MPTP-treated SN compared to saline and scrambled peptide controls. TP5 treatment mitigated these effects of MPTP, dramatically decreasing the number of Mac-1- and GFAP-positive reactive astrocytes cells in the MPTP-treated SN. Scrambled peptide had no effects on glial activation when compared to MPTP group (Fig. 5D and H). Other signs of an MPTP inflammatory response were investigated. Post-mortem analysis of human PD tissue has shown that microglia are immunoreactive for multiple pro-inflammatory cytokines including TNFα and IL-1β (McGeer and McGeer, 2004). Further, mice that are genetically altered to inhibit cytokine production or are deficient in receptors for these cytokines provide neuroprotection in the SNpc following MPTP exposure (Klevenyi et al., 1999, Sriram et al., 2002). Moreover, it has been shown that transgenic mice lacking the iNOS gene are resistant to MPTP-induced neurotoxicity (Liberatore et al., 1999). Thus, we determined whether MPTP-induced expression of IL-1β, and TNF-α in the SN was affected by TP5. Nine days after the final MPTP injection, midbrain tissues lysates were analyzed by ELISA (Fig. 5D,E). The results showed that the levels of TNF-α protein (5D) and IL-1 β (5E) were significantly increased in the midbrain of MPTP-treated mice compared to saline controls. Treatment with TP5 inhibited these MPTP-induced effects, reducing levels of TNF-α (P < 0.001; IL-1β (P < 0.001) approximately 50%. Here, too, scrambled peptide had no effects.

**TP5 Improved Locomotor Functions in MPTP-treated Mice**

We examined whether TFP5 protects against neurobehavioral deficits caused by MPTP. Using the same treatment protocol as described above, at 16hr and 48hr after the MPTP injections on day 2, mice from each treated group were tested for locomotor activity in an open field test (Fig. 6). Representative maps using Versaplot software (Accuscan, OH) depict the locomotor activity pattern of mice over a 10-min period (Fig. 6A). We observed a marked
decrease in total distance traveled after MPTP treatment (85%) which was restored approximately 30% after TP5 treatment (p<0.01). Quantification of individual components of the running pattern showed different levels of recovery after TP5 treatment (Fig. 6B); horizontal activity restored 30%, (p< 0.001), vertical activity (60% p<0.0001), whereas increase in rest time was only 10% (p<0.01). We see that TP5 significantly improved MPTP-induced hypolocomotion.

Similar patterns of locomotor behavior were obtained in tests at 48 hr (Supplementary figure 2).

**TP5 protects from chronic MPTP-induced apoptosis**

The loss of nigral dopaminergic neurons in PD results from extensive apoptosis marked by up-regulation of several apoptotic proteins (Levy et al., 2009). To determine whether TP5 inhibits neuronal apoptosis induced by MPTP, groups of mice were subjected to the standard protocol and at day 10, SN tissue was dissected, lysed and prepared for Western blots with antibodies specific for cleaved caspase-3, Bax and cytochrome C as an assay for apoptosis. The Western blot analysis showed that MPTP-evoked PD apoptotic phenotypes were significantly 1.5 -2 fold greater than saline and SCP control injections (Fig. 7A-C). These changes, however, were significantly attenuated by TP5 treatment, in some cases almost to baseline levels (P < 0.05). Moreover, scrambled peptide treated mice did not show any significant alterations. Our results are consistent with our hypothesis concerning the efficacy of TP5 treatment; prolonged treatment of TP5 markedly reduces the expression of apoptotic markers in PD mice.

**Discussion**

For many years, MPTP-induced toxicity in mice has served as a useful model for PD (Przedborski and Jackson-Lewis, 1998; Franco-Iborra et al2015 (Mizuno et al., 1998, Schapira et al., 1998). MPTP, a mitochondrial toxin, when injected into mice, is first converted to MPP+ by monoamine oxidase B-and then taken up selectively into nigral dopaminergic neurons by the dopamine transporter. Here it specifically inhibits mitochondrial complex I activity which disrupts the electron transport chain (Przedborski and Jackson-Lewis, 1998). Defective mitochondria result in ROS over expression, mitochondrial fusions, DNA mutations and alterations, and endoplasmic reticulum interactions resulting in the release of mitochondrial cytochrome C into the cytoplasm where it can complex with apoptosis activating factor 1 (Apaf-1) and caspase-9, caspase 3, the downstream apoptosis executioner (Li et al., 1998). This results
in the morphological changes associated with apoptosis, including DNA fragmentation and cytoskeletal disruption (Stennicke and Salvesen, 2000).

There are additional pathways to PD, however, leading to the death of dopamine neurons (Venderova and Park, 2012) including MPTP-induced hyperactivity of Cdk5 (Smith et al, 2003; 200(Qu et al., 2007), Wen et al, 2014). In the latter study, for example, Cdk5 activation phosphorylates RKIP (Raf kinase inhibitor protein) which undergoes autophagy, releasing the Erk/MAPkinase cascade to drive dopamine neurons into the S phase and cell death. Cdk5 hyperactivation has been implicated as an event associated with neurodegenerative disorders in humans and model rodents such as AD and ALS (Patrick et al., 1999, Ahlijanian et al., 2000, Cruz et al., 2003, Lau and Ahlijanian, 2003, Nguyen and Julien, 2003, Noble et al., 2003, Cheung and Ip, 2004). In fact, upregulated Cdk5 has also been reported in dopamine neurons in postmortem studies of PD patients (Brion and Couck, 1995; Nakamura et al, 1997). Hence, Cdk5 has been identified as a therapeutic target in neurodegenerative disorders (Tsai et al., 2004). Several studies have indicated that hyperactive Cdk5 results from stress-induced cleavage of p35 to p25, by calpains, the calcium-dependent proteases (Lee et al., 2000, Crews et al., 2011). P25 forms a stable hyperactive complex with Cdk5; hence a principal therapeutic target in neurodegeneration is Cdk5/p25 and not the normal Cdk5/p35. In our laboratory we have identified a specific fragment of p35, the Cdk5 inhibitory peptide (CIP), which displayed a specific inhibitory effect on Cdk5/p25 activity in vitro, without affecting “normal” Cdk5/p35 activity, nor other Cdns (Amin et al., 2002, Zheng et al., 2002, Zheng et al., 2005, Shukla et al., 2013). We also showed that the selective inhibition of Cdk5/p25 hyperactivation in vivo, through over expression of CIP in p25 transgenic mice, rescues the neurodegenerative AD pathologies caused by Cdk5/p25 hyperactivation without affecting neurodevelopment afforded by normal Cdk5/p35 activity (Shukla et al., 2013, Sundaram et al., 2013). A further fine-tuning of these studies has identified a smaller 24 aa peptide derived by serial truncation of CIP. This 24 aa peptide (termed, P5) has been shown to inhibit Cdk5/p25 activity in transfected HEK cells and primary neurons without affecting Cdk5/p35 nor other cdkns (Zheng et al., 2010b). P5, modified as TFP5, so as to penetrate the blood-brain barrier after intraperitoneal injections in AD model mice inhibited abnormal Cdk5/p25 hyperactivity and significantly rescued AD pathology in 5XFAD mice (Shukla et al., 2013).
The question arises as to whether hyperactive Cdk5/p25 contributes to the MPTP pathology, and if so, does treatment of these PD mice with TFP5/TP5 decrease or eliminate the iconic pathology? Indeed, our data demonstrate that TFP5/TP5 can effectively protect midbrain dopamine neurons from the toxic effects of MPTP/MPP+ in vivo and in vitro. Moreover, TP5 treatment results in a marked protective effect on the toxic depletion of dopamine and its metabolites in the striatum. Both in vivo and in vitro data show that TP5/TFP5 treatment inhibits MPTP/MPP+-induced hyperactivation of Cdk5/p25 thereby protecting the apoptotic loss of dopamine neurons. This finding, coupled with previous studies, clearly demonstrates the importance of Cdk5/p25 in models of dopaminergic neuron loss (Smith et al., 2003, Qu et al., 2007). The downstream events leading to cell death are uncertain, however. Cdk5 probably acts through multiple targets and kinase crosstalk important for neuronal survival. There are numerous proteins and substrates with which Cdk5/p35 and or Cdk5/p25 interact (Lim et al, 2003). Some targets have been suggested in MPTP toxicity; i.e., an antioxidant enzyme Prx2 (Qu et al., 2007) and/or transcription factor MEF2 (Gong et al., 2003), apurinic/apyrimidinic endonuclease 1 (Ape1), and a protein crucial for base excision repair (BER) following DNA damage (Huang et al., 2010). The coordinated targeting of multiple Cdk5/p25 substrates may contribute to stress-induced neuronal death. Mitochondrial dysfunction triggered by MPP+ can evoke a sustained elevation of cytoplasmic calcium levels (Chen et al., 1995), which, in turn, activate calpains that may cleave p35 to p25 to form hyperactive Cdk5/p25.

The standard TP5 peptide treatment successfully induces some level of behavioral recovery. The most prominent biochemical changes in the striatum of PD patients and MPTP-treated mice are decreased levels of dopamine (Savitt et al., 2006, Jackson-Lewis and Przedborski, 2007). Such deficits in striatal dopamine in MPTP-treated mice led to a decreased latency to fall on an accelerating rotarod apparatus, reflecting diminished coordination and balance (Chung et al., 2010). TP5 treatment was found to increase striatal dopamine levels and ameliorate motor deficits in MPTP-treated mice. Accordingly, mice treated with TP5 also had significantly more TH immunoreactivity in the striatum compared to both MPTP and MPTP plus scrambled peptide treated mice. Furthermore, the depletion of TH positive cells in the SN in response to MPTP was ameliorated by treatment with TP5. Future studies will be needed to analyze the pharmacokinetic and pharmacodynamics of TP5, by tissue selectivity, blood persistence, body clearance, potential brain localization, distribution and also need check the
possibility that the scrambled peptide and the TP5 peptide could have different proteolytic stability in vivo.

The aberrant activation of microglia and astrocytes have been shown to increase the pathophysiology associated with PD; microglia-derived proinflammatory cytokines may be another pathway in nigrostriatal DA neuronal death. Several lines of evidence point to the presence of activated glial cells expressing the proinflammatory cytokines IL-1β and TNF-α in the SN of PD patients (Nagatsu et al., 2000) and MPTP-treated mice (Zhao et al., 2007). TNF-α and IL-1β, originating from activated glia, may trigger intracellular death-related signaling pathways or participate in the induction of iNos expression in the MPTP model (Teismann et al., 2003a, Teismann et al., 2003b). Our current data showed that TP5/TFP5 attenuates the production of both IL-1β and TNF-α in the SN of MPTP-injected mice; it also reduced microglial and astrocyte expression of CD11b and GFAP. These data suggest that treatment with TFP5/TP5 was efficacious in reducing the histological and molecular pro-inflammatory phenotype. This is in line with recent studies showing that selective inhibition of Cdk5/p25 hyperactivation in vivo, through overexpression or ip injection of the Cdk5 inhibitory peptide (CIP)/TFP5 rescues the neuroinflamatory pathologies caused by Cdk5/p25 hyperactivation in AD model mice (Shukla et al., 2013, Sundaram et al., 2013).

Previous studies have shown that mitochondrial fission is a very early invariant event which contributes to cytochrome c release and neuronal apoptosis. Using a small molecule Cdk5 inhibitor, as well as a dominant-negative Cdk5 mutant and RNAi knockdown experiments, (Meuer et al., 2007) identified Cdk5 as an upstream signaling kinase that regulates mitochondrial fission during apoptosis of neurons. They also showed that mitochondrial fission is a modulator contributing to Cdk5-mediated neurotoxicity, thereby intergrating Cdk5 into established neuronal apoptosis pathways. In our study, MPTP was found to elicit cytochrome c release, activation of caspase-3, and Bax in the SN of wild-type mice and these events were found to be attenuated in the TP5 treatment. Studies in primary mesencephalic cultures revealed that this toxicity appears to involve, sequentially, cytochrome c release, caspase-3, and decreased levels of anti apoptotic factor Bcl-2. TFP5/TP5 treatment ameliorates all these changes. We found that TP5 treatment in vivo resulted in significant attenuation of dopaminergic SN cell loss and striatal dopamine/HVA levels after MPTP administration. The findings reported here showing selective inhibition of
Cdk5/p25 hyperactivation by TFP5/TP5 peptide, identify the kinase as a potential therapeutic target to reduce neurodegeneration in Parkinson's disease.

**EXPERIMENTAL PROCEDURES**

**Materials**

P35 (C-19) polyclonal antibody, Cdk5 (C-8) poly-clonal antibody, TH polyclonal antibody and Cdk5 (J-3) monoclonal antibody were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), all used at 1:300–500 dilutions. Antibodies to caspase-3 and cleaved caspase-3 were obtained from Cell Signaling Technologies (Beverly, MA). Cdk5 inhibitor roscovitine was obtained from Biomol Research Laboratories, Inc. (Plymouth, PA). TFP5, p5 conjugated with TAT peptide and FITC, TP5, p5 conjugated with TAT peptide and scrambled TFP5 peptide (Scb) were synthesized by peptide 2.0 (VA, USA). Sequences used were as follows:

TFP5, FITCGGGKEAFWDRCLSVINLMSSKMLQINAYARAAR

TP5, KEAFWDRCLSVINLMSSKMLQINAYARAAR

Scp peptide, FITCGGGGGGFWRCLSNGKGMSSKGGGINAYARAAR

**Mesencephalic cell culture and treatment**

The mesencephalic neuron-glia cultures were prepared from C57BL6/J mice using a modified method reported by (Ossola et al., 2011). In brief, mesencephalic tissues were dissected from embryos at 15–16 days, stripped of the meninges and blood vessels, and minced. The tissues were dissociated by 0.1% trypsin digestion for 15 min at 37°C and gentle trituration. The cells were suspended in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 U/mL penicillin/streptomycin, and plated at equal density of 0.6 million cells per well on 12-mm coverslips precoated with 0.1 mg/ml poly-D-lysine. Before seeding, culture vessels, consisting of 6 cm dishes were coated with poly-D-lysine (PLL, 50µg/ml) at room temperature overnight. It was reported that the composition of these neuron-glial cultures was approximately 11% microglia, 48% astroglia, and 41% neurons of which 2.8–3.8% of the cells were TH-positive DA.
neurons (Gao et al., 2002). The neuron-enriched cultures were prepared by adding 10 mM Ara-C to mesencephalic neuron-glia cultures for 48 h at 72 h after seeding. The standard treatment protocol: cultures were pretreated with/woTFP5 (500nM) or scrambled peptide for 12 hr then co-incubated in the presence of different concentrations of MPP+ and TFP5 or scrambled peptide for 24hr.

**Immunocytochemistry:**

The primary mesencephalic neurons were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and processed for immunocytochemical staining. First, nonspecific sites were blocked with 2% bovine serum albumin, 0.5% Triton X-100, and 0.05% Tween 20 in PBS for 30 min at room temperature. Cells were then incubated with primary antibody such as anti-TH (1:500, mouse monoclonal) at 4 °C overnight. Appropriate secondary antibodies (Alexa Fluor 488 or 594, Invitrogen) were used followed by incubation with 10µg/ml DAPI (Invitrogen) for 5 min at room temperature to stain the nucleus. Cover slips containing stained cells were washed twice with PBS and mounted on poly-D lysine-coated slides (Sigma). Cells were viewed under a NIKON inverted fluorescence microscope, and images were captured with a SPOT digital camera.

**Immunoprecipitation and kinase assays**

Kinase assays were performed as described previously with modification (Binukumar et al., 2014). Briefly, 7 DIC mesencephalic neuron-glia cultures or neuron-enriched cultures, were pretreated with/woTFP5 (500nM) or scrambled peptide for 12 hr then co-incubated in the presence of different concentration of MPP+ and TFP5 or scrambled peptide for 24hr, followed by lysis in T-PER tissue protein extraction reagent (Thermo-Scientific). Cdk5 was immunoprecipitated with the polyclonal C8 antibody for 2 h at 4°C, and immunoglobulin was isolated using Protein A-Sepharose beads for 2 h at 4°C. Immunoprecipitates were washed three times with lysis buffer and then once with 1X kinase buffer containing 20 mMTris-Cl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10 mM MgCl2, 10 mM sodium fluoride, and 1 mM sodium orthovanadate. The samples were added to the reaction mix containing kinase buffer, 50µM ATP, 20 g of histone H1, and 0.5 Ci of 32 P-ATP and incubated at 30°C for one hour. Reactions were halted by the addition of loading buffer, and samples were then electrophoresed on 12%
SDS-PAGE gels. Histone bands were visualized by Coomassie blue staining, and gels were dried, then autoradiographs were scanned on a Phosphor imager. Radioactive band density was analyzed using Image J software, and statistical analysis was performed. In pad assays, 25 µl aliquots of the incubation mixture were placed on a Whatman p81 paper square, air-dried, and washed five times for 15 min each in 75 mM phosphoric acid and once in 95% ethanol. After air drying, squares were transferred to vials containing Bio-Safe II scintillation fluid for counting.

**Animals and MPTP Treatment:**

Eight-ten-week-old male C57BL/6 male mice weighing 24–28 g were housed under standard conditions: constant temperature (22 ±1 °C), humidity (relative, 30%), and a 12-h light/dark cycle. Mice were allowed free access to food and water. Use of the animals and protocol procedures were approved and supervised by the Institutional Animal Care. The following groups of animals were used in most experiments:

(A) *Sham/saline group:* Twenty C57BL/6 mice were injected intraperitoneally (ip) with a single saline (0.9%) injection every 2 hours for a total of 4 injections over the course of 1 day.

(B) *MPTP/saline group:* Twenty C57BL/6 mice were injected ip with a single MPTP injection (15 mg/kg calculated to the freebase) every 2 hours for a total of 4 injections over the course of 1 day (total of 60 mg/kg/day).

(C) *MPTP(15mg/kg) plus TFP5 (40mg/kg) group:* Ten C57BL/6 mice were injected ip with a single TFP5 injection (40 mg/kg, injection) every day for a total of 9 injections over the course of 9 days. At day 2 these mice also received four injections with MPTP (15mg/kg, total of 60 mg/kg/day);

(D) *MPTP (15mg/kg) plus TP5 (80mg/kg) group:* Ten C57BL/6 mice were injected ip with a single TP5 (80 mg/kg,MW) every day for a total of 9 injections over the course of 9 days. At day 2, these mice also received four injections with MPTP (15mg/kg, total of 60 mg/kg/day).

(E) *MPTP (15mg/kg) plus Scrambled peptide (80mg/kg) group:* Ten mice were injected ip with a single Scrambled peptide (80 mg/kg) every day for a total of 9 injections over the course of 9 days. At day 2 MPTP injection (day 2) mice also received four injections with MPTP (15mg/kg, total of 60 mg/kg/day) as similar to MPTP group.
**Western blot analysis**
Western blot analysis was performed as described previously (Binukumar et al., 2014). In brief, cells or brain tissues were lysed or homogenized in extraction buffer, respectively, centrifuged at 10000g for 5 min and supernatants were collected and protein determined. Equal amounts of total protein (50 µg/lane) were resolved on a 4–20% SDS-polyacrylamide gel and blotted onto a PVDF membrane. This membrane was incubated in blocking buffer 5% dry milk powder (w/v) for 1 h at room temperature followed by incubation overnight at 4°C in primary antibodies. Primary antibodies used were as follows: Cdk5 (1:500), p35 (1:1000), TH (1:1000), Cytochrome C (1:250), Bcl2 (1:200), Bax (1:200), all (Biovision), β-actin (1:10,000; Sigma), Caspase 3(1:250), Tubulin (1:10,000; Sigma). The membranes were then washed four times in TTBS (5 min/each), followed by incubation in respective secondary antibody (goat anti-mouse or goat anti-rabbit IgG (L)-HRP conjugate at a dilution of 1:3,000) for 2 h at room temperature. Western blots were analyzed using the Amersham Biosciences ECL kit following the manufacturer’s instructions (GE Healthcare).

**Immunohistochemistry**
Ten micron cryostat sections of mid brain and substantia nigra were collected on slides and prepared for immunohistochemistry which was performed according to standard protocol for single or double immunostaining (Wu et al., 2002). Primary antibodies were p35 (1:100), Cdk5 (1:250), TH (1:500), GFAP (1:300; Santa Cruz Biotechnology), and Iba1 (1:1,000; Wako Chemicals Inc.). Immunostaining was visualized by fluorescein and Texas red secondary antibodies (Vector Laboratories) and was examined by transmitted or confocal microscopy. TH immunostaining was carried out on striatal and midbrain sections and the TH- and DAPI-stained SNpc neurons were counted. The striatal density of TH immunoreactivity was determined as described (Bifsha et al., 2014). To quantify dopamine neuron degeneration, cell counts were performed using ImageJ (National Institutes of Health). For cell counts of degenerating neurons, TH-stained coronal sections were loaded on ImageJ; the sections spanned regular intervals (30 or 100 mm) across the rostrocaudal extent of midbrains of 3 mice each in the different groups. For each section, total numbers of TH+ cells were separately counted for SNc in both hemispheres. Values are reported as means ±2S.D. Statistical significance was calculated using ANOVAs (Bifsha et al., 2014).
Behavioral Measurements
We performed the open-field experiment to test locomotor function of mice after MPTP and TP5/scrambled peptide treatments (Fredriksson et al., 1999, Ghosh et al., 2013). An automated device (AccuScan, model RXYZCM-16, Columbus, OH) was used to measure the spontaneous activity of mice. The activity chamber was 40x40x30.5 cm made of clear Plexiglas and covered with a Plexiglas lid with holes for ventilation. The infrared monitoring sensors were located every 2.54 cm along the perimeter (16 infrared beams along each side) and 2.5 cm above the floor. Two additional sets of 16 sensors were located 8.0 cm above the floor on opposite sides. Data were collected and analyzed by a VersaMax Analyzer (AccuScan, model CDA-8, Columbus, OH). Before any treatment, mice were placed inside the infrared monitor for 10 min daily for three consecutive days to train them. Sixteen and 48 hr after the last MPTP injection, open-field experiments were conducted. In the open-field experiment, mice were monitored for horizontal activity, vertical activity, total distance traveled (cm), total movement time (s), total rest time (s), and rearing activity over a 10-min test session. Using Versaplot and Versadat software we analyzed the data among the four groups.

ELISA dopamine and HVA
Total brain or mesencephalon was collected and fresh 50 µl (1 µg/ml) brain lysates were detected with 50 µl primary antibody (1 h) and 100 ml anti-rabbit secondary antibody (30 min) at RT according to the manufacturer’s protocols (Abnova,) for DA and (Eagle Biosciences,) for HVA.

Cytokine assays: The concentration of TNF-α and IL-1β, and IL-6 in the mixed culture medium/brain lysates was measured with ELISA kit (Thermoscientific), according to the manufacturer’s instructions. Absolute concentrations were derived by comparison with a standard curve.

Statistical Analysis
Data were analyzed with Prism 3.0 software (GraphPad Software, San Diego, CA). Bonferroni and Dunnett multiple comparison testing was used. Differences with \( p < 0.05 \) were considered significant

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Fig 1. TFP5 inhibits hyperactive Cdk5/p25 in MPP+- induced mesencephalic primary cultures, a dose dependent study.

(A). Ventral mesencephalic neuronal-enriched cultures were pretreated with TFP5 (500nM) or scrambled peptide for 12 hr then co-incubated with different concentrations of MPP+ and TFP5 for 24 hr. Cdk5 was immunoprecipitated from equal amounts of lysates using C-8 antibody.
Immunoprecipitates were then subjected to \textit{in vitro} kinase pad assays with histone H1 as substrate. Activity, as counts/min, was quantified from three separate experiments and summarized in the bar graphs (***p< 0.001; **p<0.01; *p<0.05). (B) Ventral mesencephalic neuronal-enriched cultures were treated as in (A) after which SDS-PAGE and Western blots were prepared with the Cdk5 and p35 antibodies. Note p25 expression in all MPP+ lanes. The results are expressed as mean ± s.e.m. of three independent experiments, (***p<0.001). (C). Mesencephalic tissues from E14 mouse embryos were cultured and grown on poly lysine coated cover slips. The neuronal cultures were pretreated with TFP5 (500nM) or scrambled peptide for 12 hr then co-incubated in the presence of 10μM concentration of MPP+ and TFP5 for 24hr. The cells were fixed and stained, the numbers of TH-IR neurons and representative images of experiments are shown; Scale bar=100 μm.
Fig 2. TFP5 treatment inhibits MPP+ -induced inflammation and apoptosis in mesencephalic primary cultures

Ventral mesencephalic cultures were pretreated with TFP5 (500nM) or scrambled peptide for 12 hr then co-incubated in the presence of 10μM MPP+ and TFP5 (500nM) or scrambled peptide for 24hr. The cell lysates were subjected to SDS-PAGE and Western blots were prepared with
antibodies to F4/F8 (A and B), GFAP (C and D), Cytochrome C, cytosol fraction (E and F),
and BCl2 (G and H) and caspase 3 (I and J). Bar graphs represent quantification of respective
mean densities from three separate experiments in each case (**p<0.01; *P<0.05).
Fig 3. TFP5 inhibits MPTP-induced Cdk5/p25 hyperactivity in vivo.

(A). To determine the effect of TFP5 treatment in vivo, we adopted the four-dose MPTP mouse model of PD. Four groups of mice, 6 animals per group, were treated according to the standard protocol as described for animal groups in Experimental Procedures: Group A, controls injected with vehicle, group B MPTP day 2, 4×15mg/kg injections, group D, 9 day pretreatment with TP5 plus MPTP on day 2, group E, 9 day pre treat with Scrambled peptide (Scp) plus MPTP on day 2, All sacrificed on day 10. (A) Ventral midbrains were dissected and homogenized in lysis
buffer, centrifuged and supernatants were collected. Protein concentrations were determined and equal amounts of lysate protein (350µg) were taken for immunoprecipitation with Cdk5 antibody (C-8). Immunoprecipitates were then subjected to *in vitro* kinase assays with histone H1 as substrate. Activity, as counts/min, was quantified from 6 animals per group and summarized in a bar graph (**p< 0.001). (B, C). Lysate samples from each group were subjected to SDS-PAGE and Western immunoblotting. Membranes were probed with Cdk5 antibody and reprobed with an antibody for tubulin as a loading control. A sample blot is shown in (B) and quantification from 6 different animals in bar graph (C). Note that Cdk5 /tubulin ratio, though elevated by MPTP treatment is unaffected by TP5. Quantification of mean density of Cdk5 from three separate experiments (**p<0.001). (D).Western blots were also prepared from these lysates to assess the expression of p35 and p25. A representative sample of experiments are shown with actin as loading control.
Fig. 4. Dopaminergic neurons of the SN express Cdk5/p35 and are protected from cell death by TP5 after MPTP induction

(A). Cdk5 physically interacts and colocalizes with TH and p35. Reciprocal co-immunoprecipitation (pull-down) experiments were carried out with mouse brain lysates, comparing input (10%) and Cdk5 IPs with TH IPs using antibodies to Cdk5 and TH respectively. Both p35 and TH were pulled down with Cdk5 antibody while the reciprocal pull down with TH
show both Cdk5 and p35. This indicates that dopamine neurons are expressing Cdk5/p35. (B). This association was confirmed in the immunohistochemical assay of mouse brain sections showing co-localization of Cdk5/p35 and TH with antibodies to Cdk5, TH, and p35.

(C). Dopamine neurons and processes were identified by TH immunostaining of representative midbrain sections 10 days after MPTP treatment with or without TP5/Scp (80 mg/kg daily, see Materials and Methods for details). Control group A; MPTP-treated group, B MPTP plus TP5 group D and MPTP +SCP, group E. Note the marked reduction in TH-positive cell bodies and processes after MPTP administration (compare a and b) and the protection by TP5. Photomicrographs are from a representative experiment repeated three times with similar results. (D). Quantification of TH positive neurons***P<0.001). (E, F) TP5 prevents striatal loss of dopamine and dopamine metabolites after MPTP administration. Striatal tissues from brains of mouse groups treated as above, were assayed for dopamine and HVA. The levels of dopamine(4E) and HVA(4F) were determined with ELISA and expressed as ng/100 mg of wet tissue. (n=8, ***P<0.001, *P<0.05).
Fig 5. TP5 suppresses MPTP-induced astroglial and microglial activation and inflammation in the SN in vivo.

(A). Sections of SN tissues obtained from the same animals as used in Fig. 3 were immunostained with GFAP Ab for astrocyte and Cdl1b (C) for microglia. The heightened expression of GFAP and Cdl1b were observed in the MPTP group as compared to control group, while the MPTP group treated with TP5 reveals a moderate staining of Cdl1b and GFAP. The control group, however, has shown almost negligible staining. Scale bars: A–F, 200 mm; (B). Western blots of tissue lysates from each group show a marked reduction in the expression of MPTP-induced
GFAP by TP5. In addition, sandwich ELISA assays of lysates from each group also show a TP5 rescue of over expressed TNF-a (D), and IL1b (E) in MPTP-induced inflammation.
Fig. 6. TP5 Improved Locomotor Functions in MPTP-treated Mice

(A). Sixteen hours after standard protocol treatment, the four groups of mice were assessed for spontaneous motor activity in a novel environment (open field) for a 10 min period, as described in Materials and Methods. Compared to controls, MPTP treated mice initiated less spontaneous locomotor activity compared to saline control. After TP5 treatment mice, displayed a significant
improvement in motor activity. (B). Individual movement components were analyzed as shown in the bar graphs. TP5 treated animals showed only a modest improvement in distance run and horizontal activity (*P<0.05), whereas vertical activity was robustly improved (**P<0.01). Resting time also was affected by TP5 treatment (***P<0.001). Data are means ± SEM for eight to ten mice per group. Statistical significance was assessed by one-way repeated measures ANOVA with Bonferroni post hoc test.
Fig 7. TP5 protects from chronic MPTP-induced apoptosis

(A). Striatal tissue lysates from the brains of four groups of mice (as above) were prepared as Western blots to assay for the expression of key apoptotic marker molecules. A blot for cytochrome C quantified as (B) below (ratio cytC to actin) shows a significant increase over controls after MPTP induction that is completely reduced by TP5 treatment. Likewise in (C, D) we see that the expression levels of striatal Cleaved Caspase 3 and Bax (E,F) were also significantly increased in MPTP group mice as compared to controls. Here, too, treatment with TP5 significantly decreased the expression levels. Values are expressed as mean ± SEM (n = 3) (**P<0.01, *P<0.05)