● INVITED REVIEW

TFP5/TP5 peptide provides neuroprotection in the MPTP model of Parkinson's disease

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Abstract

Cyclin-dependent kinase 5 (Cdk5) is a member of the serine-threonine kinase family of cyclin-dependent kinases. Cdk5 is critical to normal mammalian nervous system development and plays important regulatory roles in multiple cellular functions. Recent evidence indicates that Cdk5 is inappropriately activated in several neurodegenerative conditions, including Parkinson's disease (PD). 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. During neurotoxicity, p35 is cleaved to form p25. Binding of p25 with Cdk5 leads deregulation of Cdk5 resulting in number of neurodegenerative pathologies. To date, strategies to specifically inhibit Cdk5 hyperactivity have not been successful without affecting normal Cdk5 activity. Here we show that inhibition of p25/Cdk5 hyperactivation through TFP5/TP5, truncated 24-aa peptide derived from the Cdk5 activator p35 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 inhibition of Cdk5/p25-hyperactivation by TFP5/TP5 peptide, which identifies Cdk5/p25 as a potential therapeutic target to reduce neurodegeneration in PD.

Key Words: cyclin-dependent kinase 5; Parkinson's disease; neurodegeneration; therapeutic target; TP5 TFP5/TP5 peptide; MPTP

Introduction

Cyclin dependent kinase-5 (Cdk5), a family member of the cyclin dependent kinases, plays a pivotal role in the nervous system development and function. Cdk5 was first purified as one (TPKII) of two tau protein kinases, namely TPKI and TPKII, from a bovine brain microtubule fraction (Ishiguro et al., 1992), as neuronal cdc2-like kinase from bovine brain extracts (Lew et al., 1992), and as a Lys-Ser-Pro (KSP) sequence phosphorylating kinase from rat spinal cord (Shetty et al., 1993). We had also identified kinase and phosphatase players regulating neurofilament and tau phosphorylation in mammalian and squid neurons (Floyd et al., 1991; Veeranna et al., 1998; Veeranna et al., 2000; Grant and Pant, 2004), identified Cdk5 as a principal kinase phosphorylating KSP sites in neurofilament subunit (NFH) tail domains (Sharma et al., 1998), and in a collaborative study of a Cdk5 knockout, demonstrated its key role in neuronal development, function and survival (Ohshima et al., 1996). Initially, its activity was found to be restricted to neurons in the central nervous system due to the expression of neuronal specific activators p35 and p39. However, in the last decade p35 distribution and Cdk5 activity have been found in a growing number of tissues such as the testis, pancreas, cornea and most recently in glial cells of the brain (Kesavapany et al., 2007).

The tight regulation of Cdk5 activity is achieved through the specific cellular localization of its major activator p35. With an N-terminal myristoylation sequence of the activator, which anchor the membranes, Cdk5 activity is therefore restricted and localized to this region of the cell. When a neurotoxic insult involving amyloid-β peptides, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), reactive oxygen species (ROS) or glutamate is initiated, there is an increase in intracellular calcium, which then activates the calcium-dependent protease calpain. Calpain cleaves p35 into a p10 product that is still anchored to the membrane and a p25 moiety that is a potent hyperactivator of Cdk5. p25 is now free to move around the neuronal compartments and the net effect is that Cdk5 is hyperactivated by p25 leading to hyper phosphorylation of the substrates. To confound the misery of the neuron, p25 has a very strong affinity to Cdk5 but also has a longer half-life than its parent p35 protein, which leads to an aberrant hyperactivation of Cdk5 (Kesavapany et al., 2007). The result of this is an abnormal phosphorylation of cytoskeletal proteins, such as the microtubule-associated protein tau and neurofilaments (NFs) and the hyperphosphorylation of these cytoskeletal proteins are found in pathological hallmarks of a number of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease

(PD) and amyotrophic lateral sclerosis (ALS). One type of such lesion in AD is the neurofibrillary tangles (NFTs) where the epitopes found to be phosphorylated in these inclusions are readily hyperphosphorylated by Cdk5/p25. Lewy bodies, the inclusions found in PD brain, contain deposits of α-synuclein and phosphor NF-H (Hill et al., 1991; Spillantini et al., 1997) and Cdk5 (Brion et al., 1995; Nakamura et al., 1997; Smith et al., 2003), while inclusions in the spinal cord contain hyperphosphorylated aggregated NF-H (Bajaj et al., 1998, 1999). In PD, early involvement of Cdk5 in the disease pointed to its presence in Lewy bodies (Nakamura et al., 1997). Since then, experiments using MPTP to induce dopaminergic neuronal loss showed that the downstream effector of this is Cdk5 (Smith et al., 2003; Alvira et al., 2006). Upon MPTP treatment, calpains produces p25 and hyperactive Cdk5 (Cdk5/p25) phosphorylates the transcription factor myocyte enhancer factor 2 (MEF2) to inactivate it. The inactivation of MEF2 causes neuronal loss (Smith et al., 2006). In a recent report, Cdk5 has been shown to interact with and phosphorylate Parkin, a protein that contains E3-ubiquitin ligase activity. Mutations in Parkin are responsible for a large percent of autosomal recessive juvenile parkinsonism cases. Cdk5 phosphorylation of Parkin decreased its auto-ubiquitination activity and a phospho-deficient mutant of Parkin displayed increased ubiquitination towards itself as well as its substrates synphilin and α-synuclein, both constituents of Lewy body inclusions in PD. The results showed that Cdk5 activity is important in regulating inclusion formation and accumulations of toxic Parkin substrates in a PD paradigm (Avraham et al., 2007).

Evolution of CIP, P5 and TFP5/TP5

Current strategies to produce inhibitors of kinase activity target ATP binding regions of the kinases. However, since all kinases depend on these regions as a mode of action, the specificity of this approach has often been called into question. 'Normal' p35-mediated Cdk5 activity, as mentioned before, is required for the proper formation and function of the nervous system and thus, only aberrant p25-mediated Cdk5 hyperactivity must be targeted and ATP analogues would not be useful in this paradigm. Indeed, known inhibitors such as butyralactone, olomoucine and roscovitine all inhibit normal as well as aberrant Cdk5 activity. While attempting to fully characterize the behavior of p25 and its subsequent hyperactivation of Cdk5, we identified CIP (126a.a. residues), a truncated fragment of the Cdk5 regulator, p35, which specifically inhibited hyperactive Cdk5/p25 (Amin et al., 2002; Sundaram et al., 2013), presumed to contribute to the pathology of AD (Patrick et al., 1999; Smith and Tsai, 2002; Tsai et al., 2004).

A further fine-tuning of these studies has identified a smaller, 24-aa peptide (termed P5) derived by serial truncation of CIP. This 24-aa peptide (termed P5) has been shown to inhibit Cdk5/p25 activity in transfected human embryonic kidney 293 (HEK) cells and primary neurons

without affecting normal Cdk5/p35 or other Cdks (Zheng et al., 2002, 2005, 2007, 2010; Kesavapany et al., 2007). Subsequently, 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 AD model mice (Shukla et al., 2013). Moreover, TFP5 also reduced toxicity in cortical neurons exposed to high glucose (Binukumar et al., 2014).

TFP5 Treatment Inhibits MPP[+]-induced Inflammation and Apoptosis in Mesencephalic Primary Cultures

Encouraged by our previous results and Cdk5 involvement in PD, Cdk5/p25 has been identified as a prime therapeutic target for PD. In a recent study the efficacy of the TFP5 peptide was tested in a PD model (Binukumr et al., 2015). Consistent with previous studies, we observed 24 hours of MPP[+] incubation induces Cdk5 hyperactivation in mesencephalic primary cultures. Pretreatment and coincubation with TFP5, however, was sufficient to significantly reduce the elevated activity. Because the Cdk5/p25 inhibitor TFP5 was effective at blocking deregulated kinase activity, we further tested whether the effect can be replicated in primary dopaminergic neurons from mesencephalic cultures. The number of tyrosine hydroxylase (TH) positive neurons and the length of TH-positive neurites were significantly reduced after MPP[+] treatment. In contrast, treatment with TFP5 effectively attenuated MPP[+] induced toxicity in dopaminergic neurons. Scrambled peptide control however, had no protective effect on these cells. Taken together, those results demonstrate that TFP5 has a neuroprotective effect in cell culture models of dopaminergic neurodegeneration. We also investigated the anti inflammatory action of TFP5, primary neuron-glia cultures from mouse midbrains with TFP5 or scrambled peptide. It is evident that treatment with MPP[+] increased the expression of both F4/F8 and GFAP, astocytic marker compared with the control, whereas treatment with TFP5 ameliorates these effects. Scrambled peptide had no effect compared with MPP[+] treatment. Further we examined the effect of TFP5 on MPP[+] induced apoptosis by measuring caspase-3 activation and cytochrome c release in the mixed cultures. Treatment of mesencephalic primary cultures with MPP[+] for 24 hours resulted in robust activation of caspase-3 and cytochrome c release. TFP5 significantly inhibited the increased levels of caspase-3 and cytochrome c. To confirm further the antiapoptotic function of TFP5, we determined the expression levels of Bcl-2. MPP[+] treatment 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. Together, these results showed that TFP5 treatment has antiapoptotic effects in mesencephalic primary culture.

Dopaminergic Neurons are Protected from Cell Death by TP5 after MPTP Induction

As a more effective test of the efficacy of TFP5 as a therapy for PD, we used an *in vivo* model system. For the initial sets of experiments, animals were injected intraperitoneally (i.p.) with a single TFP5 injection, 40 mg/kg every day for 9 days. On day 2, TFP5-treated animals received four doses of MPTP. We found that the dose of TFP5 was inadequate; TFP5-treated animals did not show significant inhibition of Cdk5/p25-deregulated activity compared with the MPTP group accordingly, we increased the dose to a single, 80 mg/kg i.p. injection every day for 9 days. We used the peptide without the FITC tag (TP5) since TFP5 aggregates at higher concentration. In this case, TP5 pretreatment produced significant inhibition of Cdk5/p25 kinase activity. MPTP treatment also reduced the number of TH-positive neurons by 77% compared with saline-treated controls. Mice that received daily treatments of TP5 at 80 mg/kg showed an increase of TH-positive neurons in the SNpc. The neuroprotective effect of TP5 was dose dependent, as a 40 mg/kg dose of TP5 failed to protect dopamine neurons due to MPTP toxicity. Moreover, scrambled peptide did not show any dopaminergic neuroprotection compared with the MPTP group. MPTP injections also caused significant decreases in the level of dopamine and its metabolites in the striatal region of MPTP-injected mice. MPTP-induced dopamine and homovanillic acid (HVA) depletion was attenuated almost to control levels in mice treated with TP5. 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 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 revealed strong immunoreactivity for CD68, a marker of phagocytic microglia (Croisier et al., 2005; Vroon et al., 2007). 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, 2011). Previous studies reported in addition 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 SNpc *in vivo*. Consistent with earlier reports (Wu et al., 2003), numerous GFAP-positive reactive astrocytes and CD11b-positive (activated) microglia were observed in MPTP-treated SNpc compared with saline and scrambled peptide controls. TP5 treatment mitigated these effects of MPTP. Scrambled peptide had no effects on glial activation compared with the MPTP group. Post-mortem analysis of human PD tissue showed that microglia are immunoreactive for multiple proinflammatory cytokines, including tumor necrosis factor α (TNF-α) and interleukin-1β (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 after MPTP exposure (Klevenyi et al., 1999; Sriram et al., 2002). Thus we examined whether MPTP-induced expression of IL-1β and TNF-α in the SN was affected by TP5. The results showed that the levels of TNF-α protein and IL-1β were significantly increased in the midbrain of MPTP-treated mice compared with saline controls. Treatment with TP5 inhibited these MPTP-induced effects, reducing levels of TNF-α and IL-1β ~50%. Here, too, scrambled peptide had no effects. Further we examined whether TFP5 protects against neurobehavioral deficits caused by MPTP. We observed a marked decrease in total distance traveled after MPTP treatment (85%), which was restored ~30% after TP5 treatment. We see that TP5 significantly improved MPTP-induced hypolocomotion.

Our recent data for the first time identify that intraperitoneal injection of peptide, TP5 into MPTP-induced mice effectively blocks degeneration of dopamine neurons in the SNpc and prevents the loss of striatal dopamine and its metabolites. The peptide treatment also ameliorates the MPTP-induced behavioral deficits, inhibits neuroinflammation *in vivo*, and protects against MPP$^+$ neurotoxicity *in vitro*. These results suggest that TFP5/ TP5 may be effective in the treatment of Parkinson's disease.

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