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# Neuroprotective Effect of PACAP on Translational Control Alteration and Cognitive Decline in MPTP Parkinsonian Mice

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**Abstract** Parkinson's disease (PD) is characterized by a triade of motor symptoms due to the degeneration of nigrostriatal pathway. In addition to these motor impairments, cognitive disturbances have been reported to occur in PD patients in the early stage of the disease. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin widely used to produce experimental models of PD. In a previous work, we showed that MPTP altered the expression of proteins involved in mTOR antiapoptotic and PKR apoptotic pathways of translational control (TC) in neuroblastoma cells. In the present study, the results indicated that a subchronic MPTP intoxication in mice decreased the dopaminergic neuron number, produced an activation of PKR way and an inhibition of mTOR way of TC especially in striatum and frontal cortex associated with a great activation of PKR in hippocampus. Moreover, in parallel to biochemical analysis, the mnesic disturbances induced by MPTP were characterized in C57Bl/6 mice, by testing their performance in three versions of the Morris Water Maze task. Behavioral results showed that the MPTP lesion altered mice learning of a spatial working memory, of a cued version and of a spatial reference memory task in the water maze. Furthermore, we previously demonstrated that the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) could counteract the MPTP toxicity on TC factors in neuroblastoma cells. Thus, the second objective of our study was to assess the PACAP effect on MPTP-induced TC impairment and cognitive deficit in mice. The pretreatment with PACAP27 by intravenous injections partially protected TH-positive neuron loss induced by MPTP, prevented the MPTP-induced protein synthesis control dysregulation and mnesic impairment of mice. Therefore, our results could indicate that PACAP may be a promising therapeutic agent in Parkinson's disease.

**Keywords** Parkinson · MPTP · PACAP27 · Translational control · Memory task · Neuroprotection · Mice

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**Abbreviations**

| | |
|---|---|
| 4E-BP1 | 4E-Binding protein 1 |
| eIF2$\alpha$ | Eukaryotic initiation factor 2$\alpha$ |
| eIF4E | Eukaryotic initiation factor 4E |
| FBS | Fetal bovine serum |
| mTOR | Mammalian target of rapamycin |
| PACAP | Pituitary adenylate cyclase activating polypeptide |
| PFC | Prefrontal cortex |
| PKR | Double-stranded RNA-protein dependent kinase |
| p70S6K | Ribosomal p70S6 kinase |
| $MPP^+$ | 1-Methyl-4-phenylpyridinium ion |
| MPTP | 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| PD | Parkinson's disease |
| SN | Substantia nigra |
| TH | Tyrosine hydroxylase |

## Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive and selective loss of

dopaminergic neurons in the substantia nigra (SN) pars compacta (Schultz 1984). This dopaminergic depletion in the striatum leads to the development of clinical and biochemical abnormalities resulting in resting tremor, rigidity and bradykinesia.

Research into the pathogenesis of PD has been rapidly advanced by the development of animal models which also permit the investigation of new treatments. In particular, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a toxin that causes parkinsonism in men and represents the best investigated model of PD (Gerlach and Riederer 1996; Schober 2004). The mechanism for the neurotoxicity of MPTP involves complex I of the mitochondrial respiratory chain inhibition, energy depletion and dopaminergic neurons loss in the SN (Gerlach et al. 2000; Kostrzewa and Segura-Aguilar 2002).

Although the exact cause of the neuronal cell loss is still unknown, human postmortem studies showed the involvement of apoptosis in this disease (Mochizuki et al. 1996; Hartmann et al. 2000). Therefore, it is important to define cellular actors of this cell death presumably critical for the nigral degeneration. Our research group suspected anomalies in protein synthesis regulation in neurodegenerative impairment such as Alzheimer's (Lafay-Chebassier et al. 2005; Paccalin et al. 2005) or Parkinson's diseases (Deguil et al. 2007). Protein synthesis and especially translation initiation is regulated by the control of the availability of two main factors: the eukaryotic initiation factor 2 (eIF2), responsible for the binding of initiator Met-tRNA to the 40S ribosomal subunit, and the cap binding protein eIF4E, which is required for the association of mRNA with the [40S.met-tRNAi] complex (Proud 1992; Clemens 2001).

On the one hand, the activity of eIF2 is controlled by the phosphorylation of its α subunit on Serine 51 by kinases such as PKR (RNA-dependent protein kinase). Several studies have established that activation of PKR can either induce apoptotic cell death or at least enhance this process when apoptosis is initiated by other agents (Der et al. 1997; Balachandran et al. 1998). On the other hand, the activity of the factor eIF4E depends on its reversible sequestration by a family of small binding protein, the 4E-BPs (eIF4E-binding proteins), directly correlated with the phosphorylation level (Gingras et al. 2001). The kinase responsible for these phosphorylations is mTOR (mammalian target of rapamycin) (Raught et al. 2001), which can also phosphorylate the kinase p70S6K that stimulates protein synthesis (Dufner and Thomas 1999). mTOR is a highly conserved serine/threonine protein kinase that has roles in cell metabolism, cell growth and cell survival. Therefore, this initiation step of translational control appears to be regulated by a cascade of phosphorylation affecting proteins of the pro-survival way controlled by mTOR and the pro-apoptotic way controlled by PKR. Recently, a study indicated an increase of RTP801 protein expression leading to repression of mTOR activity in SN of MPTP mice and in postmortem PD patients brain (Malagelada et al. 2006). Another study showed an increase of PKR phosphorylation in hippocampus of PD patients (Bando et al. 2005). Moreover, $MPP^+$ induced in PC12 cells a marked increase in eIF2α phosphorylation which is expected to contribute at least to inhibit the protein synthesis (Ryu et al. 2002). Furthermore, in a previous study, our findings showed that $MPP^+$ induced a phosphorylation of eIF2α and significantly reduced the expression of phosphorylated mTOR, p70S6K, eIF4E and 4E-BP1 in neuroblastoma cells, suggesting its toxicity in the control of protein synthesis (Deguil et al. 2007). Nevertheless, no study investigated the simultaneously regulation of both pathways in various brain regions in a parkinsonian model.

Many works have shown that in addition to well known motor deficits, PD patients displayed cognitive impairments appearing in the early phase of the disease when motor symptoms are barely present (Lees and Smith 1983; Levin et al. 1989). Moreover, several behavioral works reported that MPTP could also produce cognitive impairments in various animal species. In particular, there is a broad consensus in the literature about spatial working memory and habit of learning deficits observed in a rat model of PD induced by intranigral administration of MPTP (Miyoshi et al. 2002; Perry et al. 2004). By contrast, alterations of spatial reference memory dependent on hippocampal integrity are more discussed (Da Cunha et al. 2002; Mura and Feldon 2003). Thus, the assessment of these different memory systems in a mice PD model induced by systemic injection of MPTP could be more relevant to determine cognitive decline which occurs in Parkinson disease.

Pituitary adenylate cyclase activating polypeptide (PACAP) is a pleiotropic peptide which exhibited multiple biological effects in the central and peripheral nervous systems (Arimura et al. 1994; Vaudry et al. 2000b). In vivo, PACAP was shown to exert anti-apoptotic and neuroprotective effects in models of brain injury (Reglodi et al. 2000, 2002; Farkas et al. 2004) and neurodegeneration (Tamas et al. 2006). Indeed, a protective effect of PACAP has been investigated in experimental PD models. Thus, this peptide protects dopaminergic neurons against rotenone, 6-OHDA or $MPP^+$-induced toxicity in cultures (Takei et al. 1998; Chung et al. 2005; Wang et al. 2005) and is also effective in vivo in unilateral 6-OHDA-induced lesion of the *SN* (Reglodi et al. 2004a, b). Moreover, we showed in a previous work that PACAP is a potent neuroprotective agent against $MPP^+$-induced translational control alterations in neuroblastoma cells (Deguil et al. 2007).

In the present work, using mice C57BL/6 as experimental model, we investigated the neurotoxic effect of subchronic administrations of MPTP on two distinct parameters: a biochemical parameter corresponding to the expression in various brain regions of proteins involved in translational control, and a behavioral parameter through the evaluation of animals learning and memory performances in three versions of the water maze, each depending on the integrity of the frontal cortex (Frick et al. 1995; Kesner 2000), the striatum (Packard and McGaugh 1992) and the hippocampus (Morris et al. 1982). At the same time, we evaluated the protective effect of PACAP on these two original parameters.

## Materials and Methods

### Chemicals

Sodium dodecyl sulfate, Triton X-100, sodium fluoride (NaF), phenylmethylsulfonyl fluoride (PMSF), protease and phosphatase inhibitors, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) hydrochloride and PACAP27 were obtained from Sigma (St Quentin Fallavier, France). All reagent-grade chemicals for buffers were obtained from VWR International (Strasbourg, France). Most of the primary antibodies and secondary anti-rabbit IgG antibody conjugated with Horseradish Peroxydase were purchased from Cell Signaling (St Quentin Yvelines, France) except for anti-ß-tubulin antibody from Sigma and peroxidase-conjugated anti-mouse IgG from Amersham Biosciences (Orsay, France).

### Animals and Treatments

Studies were performed on 8-week-old male C57Bl/6 mice purchased from Charles River (France). Animals were treated and kept following the guidelines of the European Union Council. Animals were housed one per cage under controlled temperature and lighting conditions (23 ± 1°C, light on 8 a.m. to 8 p.m.). They had access to both food and water ad libitum. For the chronic paradigm of MPTP administration (Tatton and Kish 1997), mice received one intraperitoneal injection (i.p.) per day of MPTP-HCl (30 mg/kg/day of free base) for five consecutive days (D1–D5). Control mice (sham) received saline injections only. Animals were killed 4 days after the last MPTP injection (D9).

### Intravenous Injection of PACAP

Pharmacokinetic studies demonstrated that after systemic injection, PACAP27 due to its relative low molecular weight associated with its greater lipophilicity, smaller volume of distribution, and longer half-time disappearance from blood, cross the blood–brain barrier and is delivered to brain tissue through a nonsaturable mechanism (Banks et al. 1996; Dogrukol-Ak et al. 2004). For neuroprotective studies, "PACAP + MPTP" animals received an intravenous injection (i.v.) of 15.7 µg/kg/day of PACAP in tail vein for six consecutive days followed 1 h later by an i.p. injection of MPTP for D1–D5. Moreover, the "PACAP" group received an i.v. injection per day of PACAP from D1–D6 and an i.p. injection of saline instead of MPTP. In this set of experiments, animals were killed four days after the last MPTP injection (D9).

### Immunofluorescence

Four brains from each animal group were used for immunochemical assay. Animals were deeply anaesthetized with pentobarbital (40 mg/kg, i.p.) and received an intracardial perfusion of cold PBS (154 mM NaCl, 1.543 mM $KH_2PO_4$, 2.7 mM $Na_2HPO_4.7H_2O$, pH 7.2) followed by an intracardial perfusion with 4% (w/v) paraformaldehyde (PFA) in PBS. Brains were removed and placed in 4% PFA overnight at 4°C. Then, they were dehydrated and embedded in paraffin for sagittal sectioning (4 µm in thickness). Sections were dewaxed 3 × 10 min with Histosol (Shandon, Cergy Pontoise, France) and rehydrated by 5-min incubations each in 100, 95 and 75% ethanol. Sections were rinsed twice in ultra-high quality of water ($H_2O_{UHQ}$) and washed twice in PBS (2 × 5 min). To unmask antigens, the slides were immersed in citrate buffer (10 mM sodium citrate, pH 6) and microwaved 2 × 10 min (with 650 W). Slides were allowed to come to room temperature (RT) before two 5-min incubations in $H_2O_{UHQ}$ and were blocked 60 min at room temperature in TBS/0.05%Tween (50 mM Tris–HCl, 150 mM NaCl, pH 7.6) containing 5% of bovine serum albumin (BSA).

Tyrosine hydroxylase (TH) is a rate-limiting enzyme in dopamine synthesis, thus dopaminergic neurons could easily be detected in the SN using TH antibody. For optical immunofluorescent staining, six sections located in the middle of the rostrocaudal axis of the SN were retained for each mouse. Sections were incubated overnight at 4°C with rabbit polyclonal primary antibodies anti-TH (Ozyme, France) at dilution 1/100 in 1% BSA/TBS/0.05%Tween. After washing with TBST, TH staining sections were incubated with swine polyclonal secondary antibody anti-rabbit immunoglobulins conjugated to TRITC (Dako-Cytomation, Trappes, France) at dilution 1/20 in TBS containing 1% of BSA for 1 h at RT. Then, sections were rinsed three times with TBS for 5 min and twice with water before mounting. Microscopic visualization was performed using a Olympus DP70 digital camera and Olympus

DP-soft (Olympus S.A., Rungis, France). For quantification, after being defined the boundary of the substantia nigra at low magnification (10× objective), TH-positive neurons were counted in five observation fields across the center of mesencephalon at higher magnification (40×). All neurons of the SN were counted three times for each hemisphere by one examiner blind with regard to the experimental condition.

### Mice Brain Preparation for Western Blot

Mice were sacrificed by cervical dislocation and brain regions (striatum, midbrain containing the substantia nigra, hippocampus, frontal cortex) were rapidly dissected ($n = 12$ for sham and MPTP groups, $n = 6$ for PACAP group and $n = 11$ for PACAP + MPTP mice group). Brain regions were weighed and homogenized in 20 volumes of ice cold lysis buffer containing 50 mM Tris–HCl pH 6.8, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM PMSF, 5% (v/w) protease and 1% (v/v) phosphatase inhibitors cocktail.

After centrifugation (15,000*g*, 15 min, 4°C), protein concentrations in the supernatants were determined using protein assay kit (Biorad, France) and 20 μg of proteins per lane were used for Western blot analysis.

### Immunoblottings

Proteins were separated on a 7 or 12% Tris-glycine polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA). Immunoblots were blocked for 2 h in Tris-buffered saline Tween-20 (TBST, 20 mM Tris–HCl, 150 mM NaCl, pH 7.5, 0.05% Tween 20) containing 5% nonfat milk and 0.21% NaF. The blots were incubated with primary antibody in blocking buffer at 4°C overnight. The rabbit primary antibodies used were anti-eIF2α, anti-phosphorylated-p70S6K (Thr 389), (1:1000 dilution); anti-phosphorylated-PKR (Thr 446), anti-phosphorylated-mTOR (Ser 2448), anti-mTOR, anti-p70S6K (1:500 dilution); anti-PKR, anti-phosphorylated eIF2α (Ser 51) (1:250 dilution) and mouse anti-βtubulin ($1{:}10^5$ dilution). Membranes were washed 2 times with TBST and then incubated with the secondary antibody, peroxidase-conjugated anti-rabbit-IgG or peroxidase-conjugated anti-mouse-IgG (1:1000 dilution) during 1 h at 25°C followed by the ECL visualization of the bands after two washes. The optic density (OD) value of each band was evaluated using an automatic image analysis system with the analyser system (gel documentation system, Biorad, France). After two washes in TBST, membranes were probed with antibody against tubulin for 2 h at 25°C. Then, they were washed with TBST, incubated with peroxidase-conjugated secondary antibody for 1 h and analyzed. The protein expressions were adjusted to tubulin expression. The protein levels were expressed as percentage of internal control or of sham animals.

### Memory Tasks

Four days after the first saline or MPTP injection and for 5 days (D4–D9), mice were submitted to one of the three versions of the water maze task used in this work, the spatial working memory version, the cued version of this task and the spatial reference memory version. These tests were conducted in a round tank, 140 cm diameter and 70 cm deep, filled with water (22°C). Several visual cues were placed around the water maze. During experiments, the tank was videotaped and the scores for latency to reach the escape platform and the swimming speed were later computed with an image analyser (Ethovision system by Noldus Information Technology, Wageninger, The Netherlands).

#### *Spatial Working Memory*

In the spatial working memory version and cued version of the water maze, the animals were submitted to four consecutive trials per day over five training days ($n = 10$ for sham, MPTP and PACAP + MPTP groups, $n = 5$ for PACAP group). For the working memory test, mice were left in the tank facing the wall and allowed to swim freely to a transparent acrylic escape platform (11 × 14 cm). The platform was submersed 1 cm under the water surface and placed in the center of one of the four imaginary quadrants of the tank (North-East, South-East, North-West, South-West). The platform remained in the same place during the trials conducted on the same day, but was moved to the center of another quadrant of the tank every day in a pseudo-random manner. If the animal did not find the platform during a period of 60 s it was gently guided to it. The animal was allowed to remain on the platform for 20 s, was then moved to another starting position without leaving the tank, and removed just after completing the fourth trial. Therefore, the animal was forced to use only its short-term memory of the trials conducted on the same day to locate the platform.

#### *Cued Version of the Water Maze*

The cued version of the water maze was similar to the previous version, but the platform was cued by a 7-cm diameter white ball attached on the top, protrubing above the water. In addition, the platform position varied among trials in a pseudo-random manner. As done in the spatial working memory version, after the animal escaped to the

platform, it was allowed to remain on it for 20 s and was then removed from the tank for 10 min before being placed in the next random initial position. Therefore, mice could find the platform only by associating the visible cue with the position of the hidden platform. This protocol of the memory task was proposed by Packard and McGaugh (1992) as a model of habit learning.

*Reference Spatial Memory*

The spatial version of the water maze consisted of four training days, four consecutive trials per day ($n = 18$ sham, $n = 15$ MPTP, $n = 7$ PACAP and $n = 13$ PACAP + MPTP). During trials, the animals were left in the tank facing the wall and allowed to swim freely to the platform visible the first day and submerged 1 cm under the water surface for the three other days. The platform was placed on the center of the quadrant North-East of the tank and maintained in this position throughout the four training days. The initial position in which the animal was left in the tank varied among trials in a pseudo-random way. If the animal did not find the platform during a period of 60 s it was gently guided to it. Then, it was allowed to remain on the platform for 10 s and removed from the tank before being placed in the next initial starting position in the tank. On the 5th day, the animals were submitted to a probe test which consisted of allowing the animals to freely swim for 1 min in the tank without the escape platform. The time spent in the quadrant where the platform was placed (North-East) was analyzed.

Statistical Analysis

Results are expressed as means with SEM. Differences between groups in protein expression were analyzed by ANOVA and the Newman–Keuls test. Escape latencies for the individual trials were averaged by trial (spatial working memory version) or by day (two other versions of the water maze task). Latencies were analyzed over trials or over days using the nonparametric Friedman repeated measures test (Fr values). Comparisons between groups were analyzed using Dunn's test after a Kruskal–Wallis ANOVA according to the statistical program GraphPad Instat. The level of significance was $P < 0.05$.

## Results

Effect of PACAP27 on the MPTP-Induced TH Inhibition

The four animal groups sham, MPTP, PACAP and PACAP + MPTP were sacrificed 4 days after the last MPTP injection (D9). Then, brains were used for the immunofluorescence protocol in order to calculate the number of TH-immunoreactive neurons (TH-ir) reflecting dopaminergic neurons.

Results showed that a massive loss of dopaminergic neurons was observed in the SN of MPTP mice compared to sham animals (Fig. 1a, photos 1 and 2). The intraperitoneal injections of MPTP caused a marked decrease of the TH-ir neuron number in the SN of mice compared to sham mice (47.7%, $P < 0.0005$; Fig. 1b). Furthermore, intravenous injections of PACAP in sham mice did not affect the number of TH-ir neurons in SN (Fig. 1a, photos 1, 3, and 1b). And, for MPTP animals, the intravenous injections of PACAP significantly partially prevented the loss of TH-ir neurons observed in the SN of MPTP mice (63.6%, $P < 0.005$ vs. sham mice, Fig. 1a, photos 4 and 1b). Indeed, the TH-ir neuron number calculated in SN of PACAP + MPTP mice was significantly higher than in MPTP mice (+33%, $P < 0.001$, Fig. 1b).

Effect of PACAP27 on the Translational Control Protein Expression in Sham- and MPTP-Mice

Animals were killed 4 days after the last MPTP injection (D9) since the results of the time course of translational control protein expression conducted from D6 to D12 indicated maximal alterations at this time point (data not shown). Then, brain was dissected and cerebral regions, midbrain containing the SN (Fig. 2a), striatum (Fig. 2b), frontal cortex (Fig. 2c) and hippocampus (Fig. 2d) were used to analyze by Western blot proteins involved in PKR and mTOR pathways of translational control.

*Midbrain Containing the Substantia Nigra*

Figure 2a shows any alteration of the translational control proteins activity calculated by the ratio phospho-protein/total protein which were identical between the four animal groups, sham, MPTP, PACAP and PACAP + MPTP.

*Striatum*

In the striatum of mice intoxicated with MPTP, an increase of the ratio phospho-PKR/total PKR was observed at D9 (137%, $P < 0.05$) compared to sham mice (Fig. 2b) associated with an increase of the phosphorylation of eIF2α compared to control animals (157%, $P < 0.01$, Fig. 2b). In parallel, the analysis of Western blots revealed a significant decrease of the ratio phospho-mTOR/total mTOR and phospho-p70S6K/total p70S6K at D9 (72%, $P < 0.01$ and 69%, $O < 0.05$ respectively, Fig. 2b). These decreases of mTOR and p70S6K phosphorylations were completely prevented by the pretreatment with PACAP (Fig. 2b). In

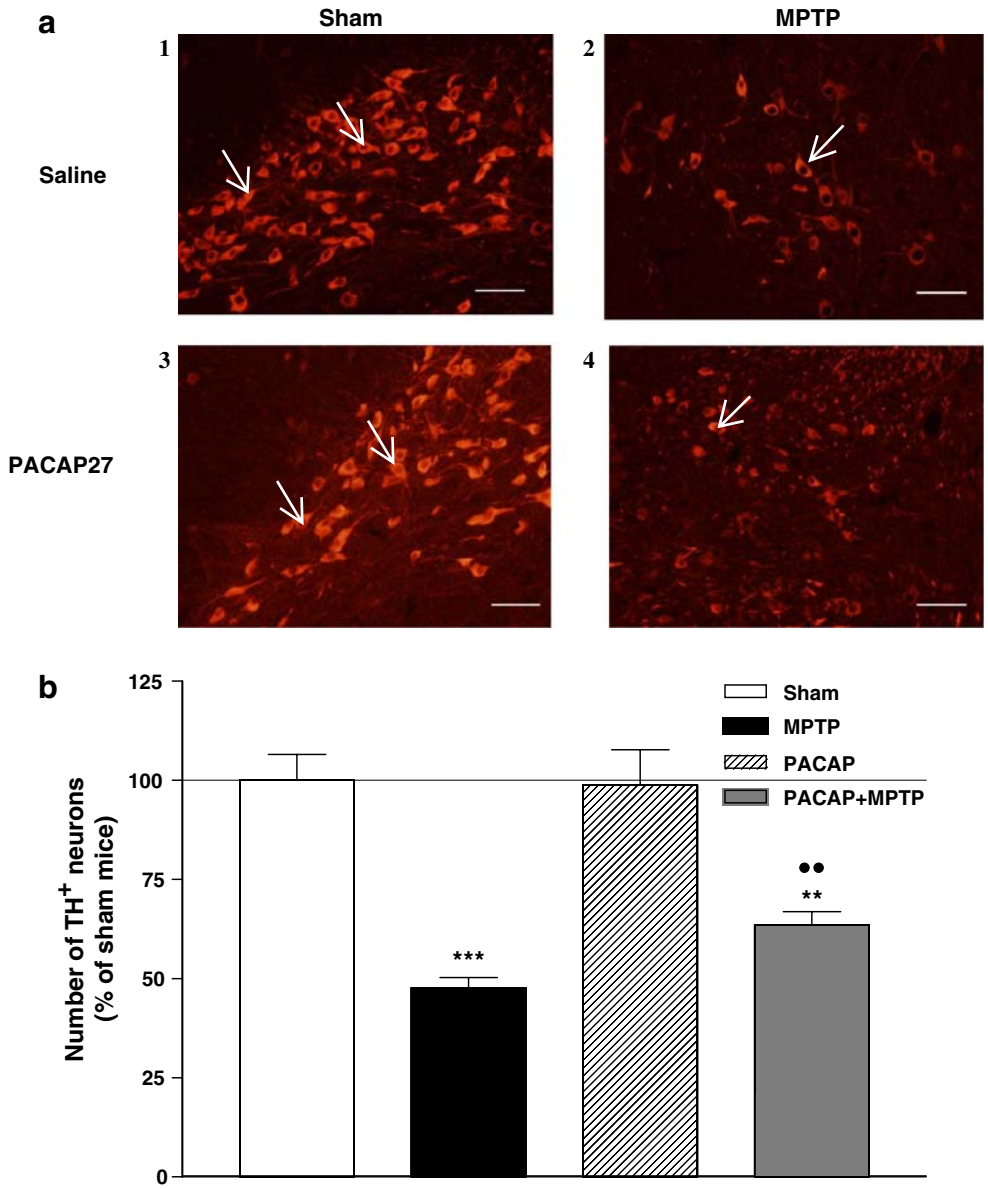


**Fig. 1** Effect of PACAP27 on TH expression in substantia nigra of sham- and MPTP-mice. **a** Immunohistochemical stainings of paraffin-embedded mice brains showing decrease of TH immunopositive neurons (*arrows*) in the SN of MPTP mice (photo 2) related to sham animals (photo 1). The intravenous injection of PACAP did not modify the intense staining of TH immunopositive neurons observed in SN of mice (photo 3) and partially prevented the massive loss of TH-positive neurons observed in the SN of MPTP mice (photos 4 vs. 2). *Horizontal bars*: 20 μm. **b** Effect of PACAP intravenous injections on neuronal density of TH immunoreactive neurons in SN of sham- and MPTP-mice. The count obtained for each animal group is expressed as percentage of TH-positive neurons observed in sham mice (mean ± SEM; $N = 4$ animals per group). $^{**}P < 0.01$, $^{***}P < 0.005$ vs. sham group; $^{\bullet\bullet}P < 0.01$ vs. MPTP group (ANOVA followed by Newman–Keuls test)

addition, we can note that the PACAP + MPTP mice presented a significant increased activity of mTOR in the striatum compared to sham mice (46%, $P < 0.01$) and that a pretreatment of PACAP lightly decreased the phosphorylation of p70S6K related to control animals (−19%, $P < 0.05$). In parallel, the increase of the phosphorylation of PKR and eIF2α observed in the striatum of MPTP mice compared to sham animals was significantly prevented by the pretreatment with PACAP shown in PACAP + MPTP mice ($P < 0.05$ and $P < 0.005$ respectively, Fig. 2b).

### *Frontal Cortex*

Figure 2c shows that the ratio phospho-eIF2α/total eIF2α was significantly increased in the frontal cortex of MPTP mice at D9 (130%, $P < 0.05$) compared to control group.

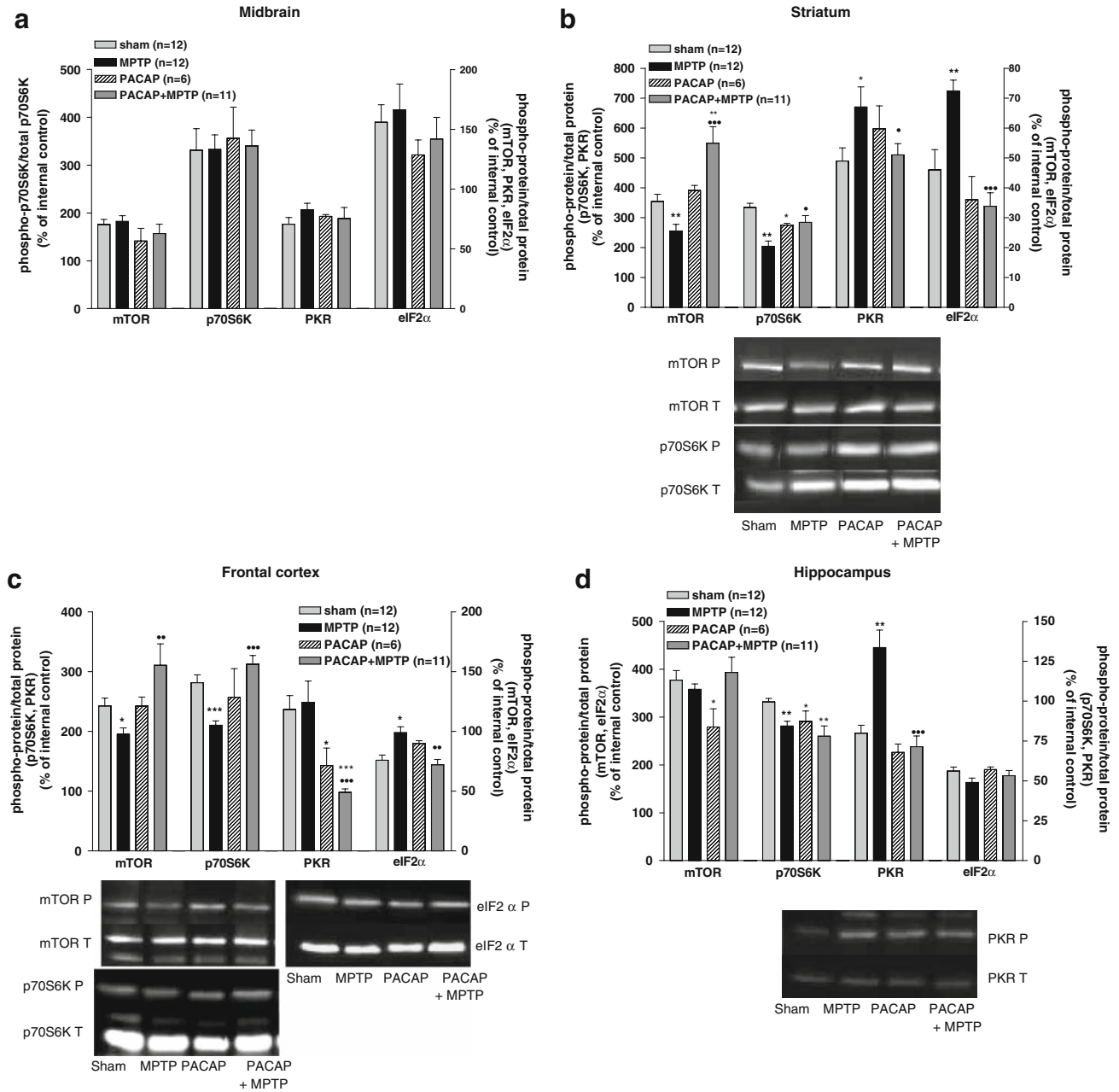


**Fig. 2** Effect of PACAP27 on translational control pathways in several brain regions of mice treated with MPTP. Mice were pretreated or not with PACAP27 and intoxicated with MPTP for 5 days. Then, 4 days after the last MPTP injection (D9), mice were sacrificed and different brain regions, midbrain containing the SN (**a**), striatum (**b**), frontal cortex (**c**) and hippocampus (**d**) were isolated and used for Western blot analysis. Activation of each protein calculated by the ratio between the phosphorylated form and the total form of the protein is expressed as percentage of the internal control and is mean ± SEM ($n = 12$ for sham and MPTP groups, $n = 6$ for PACAP group and $n = 11$ for PACAP + MPTP group). Representative immunoblots show both the immunoreactivity toward the phosphorylated and total forms of mTOR and p70S6K in striatum (**b**), of mTOR, p70S6K and eIF2α in frontal cortex (**c**), and of PKR in hippocampus (**d**) of mice treated or not with MPTP and PACAP. Significant difference is indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. sham group and $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$, and $^{\bullet\bullet\bullet}P < 0.005$ vs. MPTP group (ANOVA followed by Newman–Keuls test)

In addition, Fig. 2c shows similar profiles of the expression of mTOR and p70S6K since the activity of mTOR and p70S6K proteins significantly decreased at D9 (80%, $P < 0.05$ and 75%, $P < 0.005$ respectively) in cortex of animals intoxicated with MPTP related to control mice (Fig. 2c).

The analysis of Western blot revealed that alterations of translational control observed in frontal cortex of MPTP

mice compared to sham mice were totally prevented by the pretreatment with PACAP as we can see in Fig. 2c for the "PACAP + MPTP" group. In addition, a pretreatment by PACAP before saline injection did not modify the protein expression except for PKR marked by an important decrease of the ratio phospho-PKR/PKR in PACAP mice compared to sham animals (−56%, $P < 0.05$). This result was also observed in PACAP + MPTP mice where the phosphorylation of PKR was greatly decreased (−60% vs. sham, $P < 0.005$, Fig. 2c).

*Hippocampus*

Western blots of the hippocampus region revealed an important activation of the PKR protein at D9 in MPTP mice compared to sham animals (167%, $P < 0.01$, Fig. 2d) without modification of eIF2α activity. For the mTOR protein, any modification of the ratio phospho-mTOR/total mTOR was found in MPTP animals, whereas the activity of p70S6K significantly decreased in the hippocampus of these animals group related to sham mice (85%, $P < 0.05$, Fig. 2d). In this brain region, the pretreatment by PACAP before saline injections significantly decreased the mTOR and p70S6K activity compared to sham animals (−26% and −12% respectively, $P < 0.05$, Fig. 2d). Furthermore, the decrease of the phosphorylation of p70S6K observed in MPTP animals compared to sham mice was not counteract by the pretreatment with PACAP. In fact, the ratio phospho-p70S6K/p70S6K calculated in the hippocampus of PACAP + MPTP mice was significantly decreased related to control mice (−22%, $P < 0.01$), and was not different to the ratio calculated for MPTP animals. However, the i.v. injections of PACAP before MPTP intoxication allowed to prevent the modification of the phosphorylation state of PKR observed in MPTP mice (−47% vs. MPTP, $P < 0.005$, Fig. 2d).

## Effect of PACAP27 on the Mnesic Performances in Sham- and MPTP-Mice

Behavioral procedures were performed on days D4–D9 with the four mice groups: sham, MPTP, PACAP and PACAP + MPTP. When submitted to the memory tests, the animals swam normally and no motor disturbances were observed in the water maze. In fact, the mean swimming speed of the animals in the water maze did not differ significantly between groups (mean speed, sham = 0.15 ± 0.05 m/s; MPTP = 0.14 ± 0.04 m/s; PACAP = 0.14 ± 0.04 m/s; PACAP + MPTP = 0.16 ± 0.06 m/s) as it was observed for sham and MPTP mice in previous experiments (Da Cunha et al. 2001; Gevaerd et al. 2001).

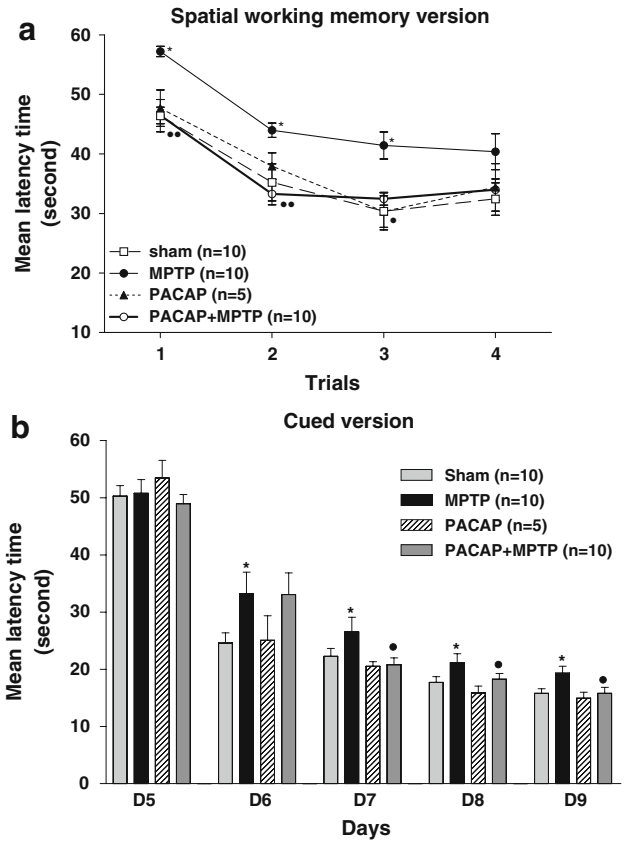


**Fig. 3** Effect of the MPTP intoxication and pretreatment with PACAP in mice on spatial working memory and cued versions of the water maze task. **a** Animals were submitted to four daily trials to find a submersed platform for five training days. The platform remained in the same place during the trials conducted on the same day, but was moved to the center of another quadrant of the tank each day in a pseudo-random manner. Data are averaged by trial and are expressed as mean ± SEM latency to find the platform ($n = 10$ for sham, MPTP and PACAP + MPTP groups, $n = 5$ for PACAP group). $^{*}P < 0.05$ vs. sham group and $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.01$ vs. MPTP group (Dunn's test). **b** Animals were submitted to four daily trials to find a platform cued by a white ball attached to it for five training days. The position of the platform varied among trials in a pseudorandom manner. Data are averaged by day and are expressed as mean ± SEM latency to find the platform ($n = 10$ for sham, MPTP and PACAP + MPTP groups, $n = 5$ for PACAP group). $^{*}P < 0.05$ vs. sham group and $^{\bullet}P < 0.05$ vs. MPTP group (Dunn's test)

*Spatial Working Memory*

In the working memory version (Fig. 3a), the animals were submitted to four consecutive trials per day over five training days (D5–D9). For each mice group, the latencies to find the platform decreased over the course of acquisition training [$Fr_{(3,46)} = 57.41$ for sham group; $Fr_{(3,46)} = 43.07$ for MPTP group; $Fr_{(3,46)} = 73.30$ for PACAP + MPTP group, $P < 0.0001$] and [$Fr_{(3,21)} = 51.08$ for PACAP group, $P < 0.001$]. For sham mice, between trials, there was a significant diminution of latencies between trial

1 and trials 3 ($P < 0.001$) and 4 ($P < 0.01$). Furthermore, for MPTP and PACAP + MPTP groups, between trials, there was a significant diminution of latencies between trial 1 and trials 2, 3 and 4 ($P < 0.01$). And, for PACAP mice, between trials, there was a significant diminution of latencies between trials 1 and 4 ($P < 0.05$). The data presented in Fig. 3a show that the MPTP group scored worse than sham mice in the working memory version of the water maze for the trials 1 ($P < 0.01$), 2 and 3 ($P < 0.05$). Furthermore, a pretreatment with i.v. injections of PACAP did not alter memory performances of mice (Fig. 3a). However, a pretreatment with PACAP before MPTP intoxication significantly prevented the disturbances of working memory observed in MPTP mice for the three first trials of this task ($P < 0.01$ for trials 1 and 2; $P < 0.05$ for trial 2, Fig. 3a).

*Cued Version of the Water Maze*

In the cued version of the water maze (Fig. 3b), the animals were submitted to four trials per day over five training days (D5–D9). We can note that all mice groups were able to learn the cued version of the water maze since their mean escape latency improved throughout the training days. Indeed, for all mice groups, the latencies to find the platform decreased over the course of acquisition training [$Fr_{(4,35)} = 74.44$ for sham group; $Fr_{(4,35)} = 83.88$ for MPTP mice; $Fr_{(4,15)} = 42.30$ for PACAP group and $Fr_{(4,35)} = 67.80$ for PACAP + MPTP group, $P < 0.0001$]. For sham and PACAP mice groups, between days, there was a significant diminution of latencies between day 5 and days 8 and 9 ($P < 0.05$). Furthermore, for MPTP and PACAP + MPTP groups, between days, there was a significant diminution of latencies between day 5 and days 7 ($P < 0.05$), 8 ($P < 0.01$) and 9 ($P < 0.01$). The MPTP group scored worse than the sham group in the cued version of the water maze for the days D6–D9 ($P < 0.05$) but not for the day D5 (Fig. 3b). Furthermore, the PACAP pretreatment did not modify the mnesic performances of sham animals and allowed to prevent at days D7, D8 and D9 ($P < 0.05$) the memory disturbances observed in MPTP mice.

*Reference Spatial Memory*

The spatial version of the water maze consisted of four training days (D5–D8), four consecutive trials per day following by a probe test at D9. The time spent by mice in the tank before finding the platform was not significantly different between the four mice groups throughout the four training days (Fig. 4a). However, it can be noted that only sham- and PACAP-groups were able to learn the spatial reference version of the water maze. Indeed, the latencies

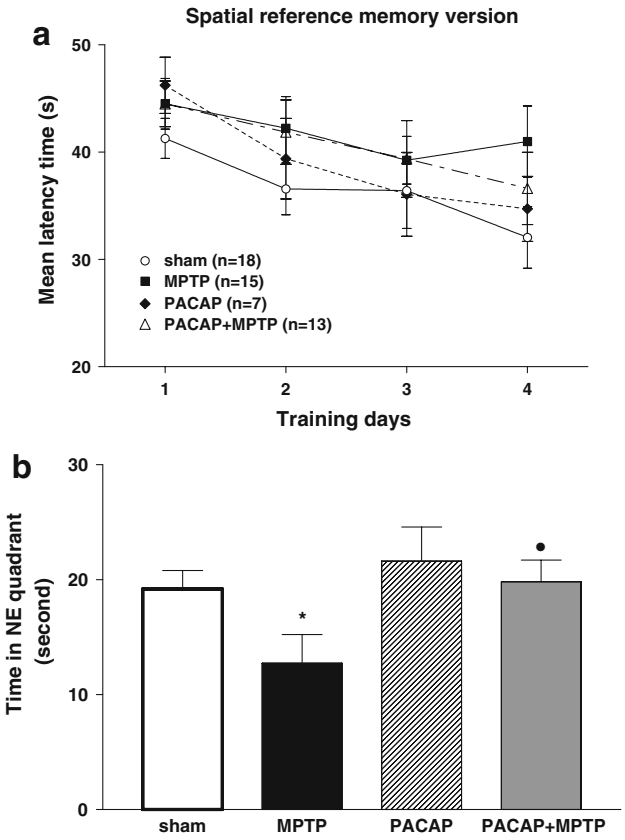


**Fig. 4** Effect of MPTP and pretreatment with PACAP in mice on the spatial reference memory version of the water maze task and during a probe test in which animals were allowed to swim in the water maze without the platform. **a** Spatial learning performance of mice in the water maze reference memory training. Results are expressed in terms of mean latency(s) to find the submerged platform for each of the 4 days of training. The platform remained on the same position over the 4 days and the mice received four trials a day. Data are averaged by day and are expressed as mean ± SEM latency to find the platform ($n = 18$ sham, $n = 15$ MPTP, $n = 7$ PACAP and $n = 13$ PACAP + MPTP). **b** Spatial memory retention performance of mice in the probe test conducted 24 h after training in the water maze reference memory task. Results are expressed in terms of mean time(s) spent in the NE target quadrant (platform removed) during the 60 s probe trial. $^{*}P < 0.05$ vs. sham group and $^{\bullet}P < 0.05$ vs. MPTP group (Dunn's test)

to find the platform decreased over the course of acquisition training [$Fr_{(3,68)} = 140.5$ for sham mice and $Fr_{(3,24)} = 31.23$ for PACAP mice, $P < 0.0001$]. For both animal groups, between trials, there was a significant diminution of latencies between trials 1 and 4 ($P < 0.05$). Indeed, for MPTP- and PACAP + MPTP mice groups, animals spent the same time in the tank before to reach the platform for all the four learning days. The effect of MPTP on memory of mice was also observed in the analysis of probe test scores as shown in Fig. 4b. The mice spent less time in the target quadrant (NE) after intoxication with MPTP than sham mice (−34%, $P < 0.05$). An injection of PACAP in PACAP mice presented no effect on the time

spent in the NE quadrant (Fig. 4b). However, the injection of PACAP 1 h before the MPTP intoxication totally prevented the decrease of time spent in the NE quadrant observed in the MPTP group ($P < 0.05$, Fig. 4b).

## Discussion

The aim of this study was to determine the toxicity of MPTP and the neuroprotective effect of PACAP on translational control.

In our study, we used the mouse experimental model of PD based on an intraperitoneal subchronic administration of MPTP, developed by Tatton and Kish (1997), characterized by a massive dopaminergic degeneration following an apoptotic way. It is based on an intraperitoneal subchronic administration of MPTP which induces dopaminergic cell death and depletes striatal dopamine by about 50%. Furthermore, the number of TH-positive neurons decreases by 60% in the SN of animals and apoptotic markers are commonly observed (Tatton and Kish 1997; Vila et al. 2001). Indeed, our results showed that intraperitoneal injections of MPTP caused a marked decrease of the TH-ir neuron number in the SN of mice of about 50% compared to sham mice.

Furthermore, our data showed an alteration of both mTOR and PKR pathways in several cerebral regions of MPTP mice, especially in striatum and frontal cortex. This translational control dysregulation was characterized by an enhancement of the phosphorylated state of PKR and its substrate eIF2α associated to a reduction of the phosphorylated state of mTOR and its downstream target p70S6K in striatal and frontal cortical regions. These modifications should result in attenuation of global protein synthesis and could influence the demise of neurons survival. The significant depletion of striatal and cortical dopamine level observed in this PD model of MPTP treated mice (Tanila et al. 1998) supports the fact that the translational control disturbance shown in our study could result from a decrease of synaptic dopaminergic neurotransmission in these brain areas. Furthermore, since cell bodies of dopamine neurons innervating the striatum and the frontal cortex respectively arise from SN and ventral tegmental area, our results seem to attest that MPTP induced toxicity on the mesocortical pathway in addition to the nigrostriatal way in our experimental animal model of PD. Moreover, in our model, the significant translational control alteration observed in the striatum at D9 coincided with the peak of activation of the pro-apoptotic protein Bax shown in SN nigra of MPTP mice (Vila et al. 2001). Surprisingly, no modification of the expression of proteins involved in translational control was observed in the midbrain region containing the SN of our MPTP mice. This discrepant finding could be explained by the poor proportion of dopaminergic neuron population present in the whole midbrain region dissected by our experimental procedures that did not allow us to detect molecular variations. In hippocampus, the substantial PKR activation that occurred 4 days after the last MPTP injection (D9) was consistent with a study where an increase of PKR phosphorylation was observed in hippocampus of PD patients (Bando et al. 2005). Although not directly affected by the MPTP, the hippocampal region could be injured by the expansion of pro-inflammatory cytokines expression observed following MPTP treatment (Shen et al. 2005). This result suggests that MPTP could induce, as in human PD, an extrastriatal molecular alteration and a potential neurodegeneration.

In vivo studies have demonstrated that PACAP is neuroprotective and neurotrophic in rat and mouse models of global and focal cerebral ischemia, traumatic brain injury (Reglodi et al. 2000, 2002; Farkas et al. 2004) and neurodegenerative disease such as Huntington chorea, a striatal degeneration model (Tamas et al. 2006). Moreover, recent studies have reported that injection of PACAP into the SN in 6-OHDA rat model of PD can effectively reduce nigral cell death and ameliorate behavioral abnormalities (Reglodi et al. 2004a, b). More recently, in a study where PACAP was administered for 1 week via intravenous injections in MPTP mice, a curative effect was observed marked by a recovery of the TH-positive neurons level and dopamine neurotransmission (Wang et al. 2008). In the present study, we showed that intravenous injections of PACAP significantly partially prevented the loss of TH-ir neurons induced by MPTP intoxication. Indeed, the TH-ir neuron number calculated in SN of PACAP + MPTP mice was significantly higher by about 33% than in MPTP mice, indicating a protective effect of PACAP against MPTP toxicity.

Moreover, we demonstrated that i.v. administrations of PACAP for 6 days significantly prevented the phosphorylation of PKR kinase and its downstream substrate eIF2α in striatal and frontal regions of MPTP mice. At the same time, PACAP counteracted the mTOR pathway inhibition maintaining the basal phosphorylation state of mTOR and p70S6K kinases. Our results also showed that PACAP significantly prevented the great activation of PKR observed in the hippocampus region of MPTP mice. These data are in accordance with our previous study showing that PACAP prevented the $MPP^+$-induced PKR pathway activation and blocked the $MPP^+$ toxicity on molecular factors of the mTOR pathway in neuroblastoma cells (Deguil et al. 2007). Moreover, the protection by PACAP against the death process induced by $MPP^+$ in Neuro2a cells (Deguil et al. 2007) was confirmed in vivo by the significant attenuation of TH-positive neurons loss observed in our MPTP mice model of PD, suggesting a

reduction of cell death. The mechanisms underlying these effects could be multiple. On the one hand, several studies have demonstrated that activation of ERK and PI3K/Akt pathways could mediate some actions of PACAP (Vaudry et al. 2000a; Stumm et al. 2007). Furthermore, both signalling pathways could influence translational control and especially the mTOR signalling. On the other hand, activation of the adenylyl cyclase and phospholipase C pathways leading to inhibition of caspase-3 and apoptotic process also contribute to the neurotrophic effect of PACAP (Vaudry et al. 2000b, 2003; Onoue et al. 2002).

In a second part of this study, we assessed the neuroprotective effects of PACAP on different mnesic performances in mice intoxicated with MPTP. Therefore, we tested mice in three versions of the water maze, each depending on the integrity of the striatum, the frontal cortex and the hippocampus. Indeed, these brain regions were shown to be involved in specific cognitive processes including respectively habit learning, working memory and spatial reference learning and memory that have been shown to be altered in experimental models of PD (Da Cunha et al. 2002, 2003; Miyoshi et al. 2002; Mura and Feldon 2003) and in PD patients (Dubois and Pillon 1997; Lewis et al. 2003). In our MPTP mice model, animals presented learning and memory deficits but no motor impairments, thus modeling the early phase of PD when cognitive impairments are observed but the motor symptoms of the disease are barely present (Lees and Smith 1983; Levin et al. 1989).

First, a learning and memory deficit was observed in MPTP-lesioned animals tested in the spatial working memory version of the water maze. Indeed, MPTP-treated mice displayed a performance level significantly worse than nontreated mice. However, the MPTP mice were able to ameliorate their performances during the four trials of the same day. This finding is consistent with the many reports of spatial working memory deficits observed in PD patients (Owen et al. 1997) and in PD experimental models (Tanila et al. 1998; Miyoshi et al. 2002; Ferro et al. 2005). Working memory is assimilated to a short-term memory. It is a cognitive process which depends on the cortical release of dopamine mesocortical neurons regulating the activity of prefrontal cortical (PFC) neurons (Brozoski et al. 1979). Impairments in delayed alternation, another working memory task, reported in rats following local DA depletion in PFC (Bubser and Schmidt 1990) and the study of Sawaguchi showing that the dopaminergic D1 receptor antagonist SCH23390 impaired working memory-guided movements mediated by frontal cortical areas, confirm the involvement of cortical DA in working memory mechanism (Sawaguchi 2000). Thus, the impairment of our MPTP mice in performing the working memory task could be explained by the depletion of dopamine in cortical region reported in MPTP model (Tanila et al. 1998). Secondly, in the present investigation, MPTP has also moderate deleterious effect on striatal-dependent learning. Indeed, MPTP-treated mice performed significantly worse than sham animals for the four last training days. This cued version of the water maze task was inspired by Packard and McGaugh (1992) and thought to be dependent on the integrity of the dorsolateral striatal area (Miyoshi et al. 2002). This learning process has been shown to be altered in various PD experimental models providing evidence that this type of learning depends more specifically on the integrity of the nigrostriatal dopaminergic system which is the target area of the MPTP toxicity (Miyoshi et al. 2002; Perry et al. 2004). This finding was confirmed by pharmacological studies reporting that dopaminergic D2 receptor agonist and antagonist respectively improved and impaired performance in cued version of water maze (Packard and McGaugh 1992; Setlow and McGaugh 2000). However, in our study, the dopaminergic deficit induced by MPTP seems to be more effectively compensated in striatum than in frontal cortex because the cognitive decline related of this cerebral area appears to be less severe.

Thirdly, in the present study, results showed that the toxic MPTP partially altered the learning capacity in spatial reference version of the water maze. Indeed, MPTP mice were not able to ameliorate their performances throughout the four training days related to sham animals. In addition, the probe test scores analysis revealed a significant deficit in long-term memory in MPTP-treated mice. It has been well established that the integrity of hippocampal formation is essential for spatial learning and memory (Morris et al. 1982). For instance, inactivation of the hippocampus with lidocaine caused an expressive and significant increase in the time spent to find the submerged platform in the spatial task (Da Cunha et al. 2003). Conversely, in PD model, based on intranigral infusion of MPTP, the reference hippocampal-dependent spatial learning seems to be preserved (Packard and McGaugh 1992; Miyoshi et al. 2002). However, the protocol of MPTP intoxication used in these studies selectively damaged the nigrostriatal pathway projecting in dorsal striatum, sparing dopaminergic projections to the ventral striatum (nucleus accumbens), hippocampus, and other limbic brain regions (Gevaerd et al. 2001; Miyoshi et al. 2002). Thus, we might suppose that in our protocol of PD mice model, the systemic administration of MPTP-induced impairments in several cerebral regions including the hippocampus area which could explain the partial mnesic decline of mice in the spatial reference version of the water maze. This hypothesis was confirmed by our results of translational control alteration marked by the great activation of the stress kinase PKR observed in hippocampus of MPTP mice and in accordance with hippocampal atrophy observed in patients with early

stage of PD (Bruck et al. 2004). The impairment in spatial memory version of the water maze also suggested that in this experimental paradigm, other neurotransmission system could be likely affected such as the cholinergic system. Indeed, in human PD as well as in experimental models of PD, dopa-therapy did not reverse all memory impairments, suggesting that dopaminergic transmission changes are not responsible for all cognitive deficits observed in this disease (Cooper et al. 1992; Lange et al. 1992).

Finally, we observed that the pretreatment of MPTP intoxicated mice with PACAP improved learning and memory impairments in the three different paradigms of cognitive tests. Indeed, our results showed that PACAP improved learning and memory performances of mice in spatial working memory and in the cued version of the water maze probably by a neuroprotective effect on dopaminergic neurotransmission. This hypothesis is supported by several studies underlying the role of PACAP on dopamine neurotransmission via increase of DA synthesis, DA uptake and regulation of the expression and activity of the TH enzyme (Takei et al. 1998; Bobrovskaya et al. 2007). Moreover, it has also been reported that injection of PACAP into SN of a 6-OHDA lesioned rat can effectively reduce dopaminergic neurodegeneration in the SN and ventral tegmental area and improve behavioral symptoms (Reglodi et al. 2004a, b). Furthermore, we observed that the pretreatment of MPTP intoxicated mice with PACAP improved memory impairment in the test session of spatial reference version of the water maze. This effect of PACAP on MPTP-induced spatial memory impairments could result from its stimulating effect on cholinergic transmission in the septohippocampal area (Masuo et al. 1993) which could compensate a possible dysfunction of the subcortical cholinergic system induced by MPTP. Furthermore, a recent study indicated that intravenous injection of PACAP offers neuroprotective effects in the MPTP-induced PD mouse model, not only in dopaminergic neurotransmission but also in cholinergic neurotransmission in order to maintain a balance between DA-ACh systems in basal ganglia neuronal pathway (Wang et al. 2008). All these results lead us to consider a potential link between our biochemical and cognitive reports. Indeed, a study raised the intriguing idea that memory formation is regulated via the translational control (Costa-Mattioli et al. 2005). For instance, a study showed that in transgenic mice, in which eIF2$\alpha$ phosphorylation is reduced, spatial learning and memory were enhanced (Costa-Mattioli et al. 2007). Moreover, the administration of rapamycin, a selective mTOR inhibitor, prevented long-term potentiation (LTP) formation in rat hippocampus (Tang et al. 2002). And it was shown that L-LTP inducing stimulation results in a mTOR-dependent increase in active S6K as measured by the phosphorylation of threonine 389 on S6K1 (Cammalleri et al. 2003). Based on these findings, we could suppose that modulation of translational control in specific brain regions could have a role on related learning and memory performances. These findings raise the interesting possibility that regulators of translation could serve as therapeutic targets for the improvement of memory, for instance in PD which is associated with memory loss.

As a conclusion, we observed that the pretreatment of MPTP-intoxicated mice with PACAP partially protected the dopaminergic cell loss, prevented translational control dysregulation in related brains regions and improved learning and memory impairments. Thus, our results could indicate that PACAP may be a promising therapeutic agent in PD.

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