N-terminal tripeptide of IGF-1 improves functional deficits after 6-OHDA lesion in rats

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Central administration of N-terminal tripeptide of IGF-1 (GPE) prevents the loss of dopamine neurons. We now examine effects of GPE administered peripherally, on long-term functional recovery after 6-OHDA lesion in rats. GPE treatment (3 mg/kg, i.p.), 3 days after the lesion reduced the number of rotations ($p < 0.005$) and the time over meter ($p < 0.005$) compared to vehicle treatment. Step length and number of adjusting steps were increased in the GPE group ($p < 0.005$), particularly at 12 weeks post lesion. However, GPE treatment did not prevent the loss of tyrosine hydroxylase in the substantia nigra pars compacta and the striatum. The study suggests that peripheral administration of GPE after onset of nigrostriatal dopamine depletion improves long-term Parkinsonian motor deficits, independent of neuronal outcome. *NeuroReport* 15:1601–1604 © 2004 Lippincott Williams & Wilkins.

Key words: GPE; Long-term recovery; 6-OHDA lesion; Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder characterised by severe motor disabilities such as bradykinesia, tremor and rigidity. Current treatments are not effective and often produce unpleasant side effects [1]. Thus the search for effective drugs for the treatment of PD continues.

Central administration of insulin-like growth factor (IGF)-1 has been shown to be neuroprotective after various types of brain injury *in vivo* [2] and neurotrophic on dopaminergic neurons *in vitro* [3]. Although IGF-1 has been reported recently to protect dopaminergic neurons and improve motor function when given peripherally prior to 6-OHDA lesion in rats [4], the limited central penetration [5,6] and potential growth effects of IGF-1, have been recognized as major obstacles for clinical application.

Glycine-proline-glutamate (GPE) is naturally cleaved from IGF-1 in the brain by an acid protease [7]. It has been shown to stimulate dopamine release from the striatum without interacting with the IGF-1 receptor [8] and central administration of GPE has been shown to be neuroprotective after a hydroxydopamine (6-OHDA) lesion in rats [9]. It has also been reported recently that GPE crosses the blood–brain barrier after peripheral administration [10].

The current study was carried out to determine whether delayed peripheral administration of GPE improves long-term functional deficits and neuronal outcome in rats following a unilateral 6-OHDA induced nigrostriatal lesion.

MATERIALS AND METHODS

This study was approved by the Animal Ethics Committee of Auckland University. All efforts were made to minimise animal suffering and to reduce the number of animals used.

Surgery: Wistar rats (male, 280–310 g) were used. The nigrostriatal lesion was induced by infusing 6-OHDA into the medial forebrain bundle (MFB). The surgical procedure has been described previously [9]. Briefly, the head of the rat was fixed on a stereotaxic frame and an incision was made to expose the skull under halothane (3%) anaesthesia. 6-OHDA (8 μg in 2 μl 1% ascorbic acid/saline) was infused into the right MFB (AP + 4.7 mm, R 1.6 mm, V –8 mm), using a 25G needle connected to a 100 μl Hamilton syringe. The infusion was controlled by a microdialysis infusion pump at the infusion rate of 0.5 μl/min. The needle was left in the brain for a further 3 min before being slowly withdrawn. The skin was sutured with 2.0 silk.

Rotation tests: To identify the degree of dopamine lesion, rotation tests were carried out on day 3, prior to drug treatment. The subsequent tests were at 8, 15, 22, 29, 43, 57 and 85 days (representing week 1, 2, 3, 4, 6, 8, and 12 tests) for the groups treated with 3 mg/kg (n=10) and its vehicle (n=10), and at 8, 15, 22 and 29 days (representing week 1, 2, 3 and 4 tests) for the groups treated with 0.3 mg/kg (n=5) and 30 mg/kg (n=8) GPE and their vehicles (n=7, n=8 respectively). Apomorphine (0.1 mg/kg in 0.1% ascorbic acid, Sigma Chemicals) was injected s.c. and the rats were placed in opaque plastic cylinders (30 cm wide, 37 cm high).

The number of contralateral rotations over a minute was counted from the 5th min after the apomorphine injection and then every 5 min for 1 h (12 measurements). Full body contralateral turns were counted and the total number of rotations/h was calculated.

GPE treatment: Rats were then paired into either GPE or vehicle treatment groups depending on the number of rotations at day 3. Rats that did not rotate were eliminated from the study. A single dose of GPE (0.3, 3 and 30 mg/kg in 0.1%BSA/0.1 M PBS, Bachem, USA) or its vehicle was then administered i.p. 3 days after 6-OHDA lesion. The experimenter was blind to the treatment groups.

Stepping tests: Rats were preconditioned to the time over meter, step length and adjusting steps tests for 3 consecutive days before 6-OHDA lesion [11] both time over meter and step length tests were initiated 1 week after the lesion and the adjusting steps test was initiated 2 weeks after the lesion. The subsequent tests were one day before each rotation test at 7, 14, 21, 28, 42, 56 and 84 days, representing weeks 1, 2, 3, 4, 6, 8 and 12 tests in the groups treated with 3 mg/kg (n=10) GPE and its vehicle (n=7, three rats were excluded from stepping tests due to severe lesion in vehicle group). The front contralateral paw of the rat was dipped in Indian ink. A U-shaped wooden platform (1.5 × 0.4 m) base covered with chart paper was used for the stepping tests. A start line was marked on one end of the platform and other end was connected to the home cage. The time taken to walk over one meter from the starting mark to the end of the platform was recorded for time over meter using a millisecond counter. The average length between forepaws on the contralateral side was measured for step length (cm) by dividing the number of paw-prints made on the paper over the meter by 100 cm. The number of adjusting steps made by the contralateral forepaw was measured after the above tests were completed. The animal was held by its hind limbs and the ipsilateral forepaw, and dragged sideways over 90 cm in 5 s. The number of steps made by the contralateral paw was counted in the forehand direction.

Immunocytochemistry: The brains were transcardially perfused with normal saline followed by 10% buffered formalin and a standard paraffin tissue preparation was used for immunohistochemistry [9]. Coronal sections from the striatum and the SNc (8 μm) were deparaffinized, rehydrated and washed in phosphate buffered saline (PBS, 0.1 M). After pre-treatment with 1% H_2O_2, sections were incubated in rabbit polyclonal antisera raised against tyrosine hydroxylase (1:500, Protos Biotech, New York, USA). The sections were then incubated in donkey anti-rabbit biotinylated secondary antibody (1:200, Amersham, Life Science), followed by incubation in Streptavidin linked horseradish peroxidase (1:200, Amersham, Life Science), then reacted in 0.05% 3,3-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2.

The number of TH positive neurons on both sides of the SNc was counted using light microscopy (Nikon 800, × 20) at three representative levels (AP + 4.2 mm, AP + 3.8 mm, AP + 3.4 mm). The average density of TH in the striatum (minus background) was also measured using three adjacent sections using the Sigma Scan image analysis software. The experimenter who accessed the histology was blind to the treatment groups.

Statistical analysis: The effects of GPE on functional recovery were analysed using repeated measures two-way ANOVA. The difference within the groups was analysed using repeated measures one-way ANOVA followed by Dunnett's multiple comparison test (GraphPad Software, San Diego, California, USA). Data from day 3 were taken as pretreatment baseline for rotations, and habituation data were used as the pretreatment baseline for stepping tests. Right/left (R/L) ratio of TH-immunopositive neurons from the three levels of SNc and the TH density from three striatal sections was averaged and compared between the two groups using the t-test (GraphPad Software, San Diego, California, USA). Data are presented as mean ± s.e.m.

RESULTS

Rotation test: The mean of rotations was similar between GPE (109.5 ± 24.9) and vehicle (111.2 ± 35.1) groups before treatments. A single dose of GPE treatment (3 mg/kg) significantly reduced the overall number of rotations compared to the vehicle treatment ($p<0.005$, Fig. 1a); however there was no difference between two groups in individual time points. Compared to pre-treatment, the number of rotations increased significantly ($p<0.001$, Fig. 1a) at all the time points examined in the vehicle treated group. In the GPE-treated group the number of rotations also increased significantly ($p<0.001$) compared to pretreatment at all time points examined except week 8 after the lesion (Fig. 1a). Neither a lower (0.3 mg/kg) nor a higher (30 mg/kg) dose of GPE reduced the number of rotations compared to their own vehicle-treated groups (Table 1).

Stepping tests: There was a significant ($p<0.005$, overall) decrease in time over meter in the GPE-treated group compared to the vehicle-treated group (Fig. 1b). In the vehicle group, time over meter was significantly increased at weeks 3, 4, 6, 8 and 12 (6.9 ± 1.4, 7.2 ± 1.8, 8.1 ± 2.1, 7.1 ± 1.1 and 5.8 ± 0.9, respectively, $p<0.05$) compared to pretreatment (2.7 ± 0.3). In the GPE group, a significant increase in time over meter was only seen at week 4 (6.1 ± 1.6, $p<0.05$) compared to pretreatment (2.7 ± 0.3, Fig. 1b).

GPE treatment also showed a significant ($p<0.005$, overall) increase in average step length compared to vehicle treatment, particularly at 12 weeks post-lesion (16.6 ± 0.6 *vs* 13.9 ± 0.8, $p<0.05$, Fig. 1c). There was no significant difference in step length in either vehicle- or GPE-treated groups compared to pretreatment.

The number of adjusting steps in the contralateral paw was significantly increased ($p<0.005$, overall) in the GPE-treated group compared to the vehicle-treated group, particularly at 2 and 12 weeks ($p<0.05$, Fig. 1d). In the vehicle group, the number of adjusting steps decreased significantly at all the time points examined ($p<0.001$) compared to pretreatment (20.1 ± 0.9). There was a further significant decrease at week 12 (6.1 ± 0.7, $p<0.01$) compared to week 6 (11.7 ± 0.9) in the vehicle-treated group. In GPE-treated animals the decrease in the number of adjusting steps was only seen at weeks 3 (11.1 ± 1.6), 4 (11.5 ± 2.1) and 12 (11.3 ± 1.8) compared to pretreatment (20.1 ± 0.9, Fig. 1d).

Immunocytochemistry: There was no difference in the R/L ratio of the number of TH positive neurons in the substantia nigra between GPE- (20.0 ± 6.2%) and vehicle- (14.3 ± 5.7%) treated groups (Table 2). There was a complete loss of TH staining on the ipsilateral (right) striatum in both groups.

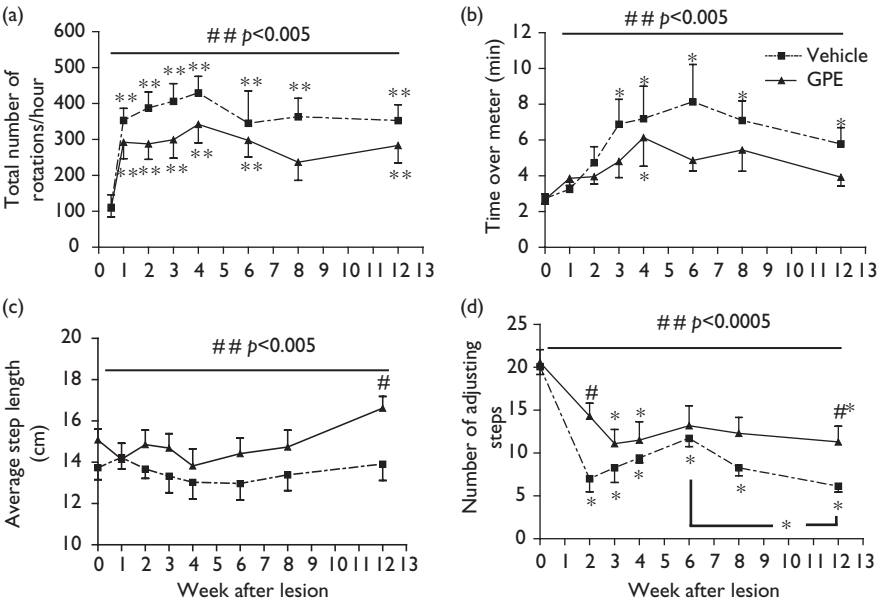

Fig. 1. GPE treatment (3 mg/kg) significantly reduced the number of rotations (**a**; overall, $\#\#p < 0.005$), and the time over meter (**b**; overall $\#\#p < 0.005$) compared to vehicle treatment. Compared to the vehicle-treated group, GPE treatment also increased the average step length (**c**; overall $\#\#p < 0.005$), particularly at 12 weeks post-lesion ($\#p < 0.05$). The number of adjusting steps was also significantly increased (**d**; overall, $\#\#p < 0.005$) in the GPE-treated group compared to the vehicle-treated group, particularly at weeks 2 and 12 after the lesion ($\#p < 0.05$). #indicates difference between two treatment groups; *indicates the difference between each of the individual time points and the pretreatment value for the same experimental group.

Table 1. Dose response of GPE on apomorphine-induced rotations.

	0.3 mg/kg	Vehicle	30 mg/kg	Vehicle
Week 0.5	255.9 ± 29.3	258.8 ± 29.2	134.0 ± 26.6	114.4 ± 22.49
Week 1	322.5 ± 20.8	271.3 ± 60.8	333.0 ± 41.3	278.3 ± 16.7
Week 2	443.8 ± 70.5	421.7 ± 12.7	224.6 ± 27.6	238.8 ± 31.7
Week 4	332.5 ± 56.2	370.6 ± 28.3	339.0 ± 56.3	291.7 ± 27.8

There was no difference in the total number of rotations between the GPE and its vehicle treated groups over the period of testing.

Table 2. Effects of GPE on TH immunoreactivity.

	SNc (R/L ratio of TH neurons)	Striatum (R/L ratio TH density)
Vehicle	14.3 ± 5.7%	0.0%
GPE	20.0 ± 6.2%	0.0%

GPE treatment improved TH immunoreactivity in neither the SNc nor the striatum.

DISCUSSION

The current study suggested that peripheral administration of GPE following the onset of 6-OHDA induced nigrostriatal dopamine depletion improves long-term recovery of Parkinsonian motor deficits, independent of neuronal outcome.

A unilateral 6-OHDA lesion resulted in progressive dopamine depletion, demonstrated by apomorphine-induced rotations (Fig. 1a). These drug-induced rotations occur due to supersensitivity of post-synaptic D2 receptors in denervated striatum following loss of dopaminergic neurons in the SNc, leading to increased dopamine levels on lesioned side. Therefore rotations may likely reflect an imbalanced dopaminergic function due to unilateral dopamine depletion rather than measure Parkinsonian motor deficit.

A single dose of GPE given peripherally 3 days after the lesion reduced the overall rotations in a dose-dependent manner, suggesting a role for GPE in suppressing the progression of nigrostriatal dopamine depletion. Although there was no difference between two treatment groups at individual time points, a transient recovery was seen in week 8 after GPE treatment, followed, however, by a further progression in dopamine depletion at week 12 compared to pretreatment (Fig. 1a). The lack of effect on loss of TH immunoreactivity may be partially associated with this further progression in dopamine depletion at the end of the experiment. It may suggest that a single treatment with GPE may have delayed, but not prevented the progression of nigrostriatal dopamine depletion.

Our previous studies have shown that intracerebroventricular injection of GPE prevented the loss of TH immunoreactivity in the striatum and substantia nigra when given 2 h after a 6-OHDA lesion [9]. Given that apomorphine induced contralateral rotations, detected before the treatment, occur due to ~70–90% loss of nigrostriatal dopamine function [12], our previous study appeared to measure the efficacy of GPE on neurotoxicity, while the current study examined the effects on neuronal degeneration, as GPE treatment was initiated 3 days post-lesion.

The effects of GPE on apomorphine-induced rotation appeared to be dose dependent, as either a lower or a higher dose failed to reduce the number of rotations. A similar bell shaped dose response treatment effect of GPE has been reported recently after hypoxia-ischemia in infant rats [13].

In addition to apomorphine-induced rotations, GPE treatment attenuated long-term Parkinsonian motor deficits. Stepping tests were used in this study as a measure of forelimb akinesia [11]. In contrast to the effects GPE on rotations at the end of experiment, the effects of GPE on step tests either remained (time over meter) or showed further improvement (adjusting steps and average step length) at the end of experiment. This difference in long-term recovery between rotation and step tests further suggests that apomor-

phine-induced rotations may be more indicative of nigrostriatal dopamine depletion while stepping tests likely represent Parkinsonian motor dysfunction. Traditionally, the loss of dopaminergic neurons in the substantia nigra has been recognized as the major pathology reflecting Parkinsonian motor dysfunction [14]. Our current data showed that the Parkinsonian motor deficit shown by stepping tests were associated neither histologically with dopamine depletion nor to apomorphine-induced rotations. These data suggest that GPE treatment associated motor functional recovery was, at least partially independent of the dopamine depletion.

GPE treatment may also have altered the temporal developments of the motor deficits. In the vehicle-treated group the deficit indicated by a reduced walking speed (time over meter) developed earlier at week 3 and persisted during the 12 weeks of tests, whereas a transient deficit, with a delayed onset was seen at week 4 in the GPE-treated group (Fig. 1b). The most pronounced 6-OHDA induced motor deficit was the slowness in initiating movements as indicated here by the number of adjusting steps. Following a partial recovery at 6 weeks after the lesion, the vehicle-treated group showed further progression in this particular motor deficit at week 12 (6 weeks *vs* 12 weeks), which may have contributed to a statistical difference between the groups at end of experiments. In addition to the overall treatment effects in adjusting steps, GPE delayed the onset of the deficits (Fig. 1d). Unlike the adjusting steps and time over meter tests, the deficit in step length was not pronounced in both treatment groups, probably due to immature habituations. However GPE treatment resulted an overall increase in step length, particularly at the end of experiments compared to the vehicle treatment. The patterns of development of Parkinsonian deficits varied in our experiment, which may be due to the unique temporal development of individual motor deficits.

Recent studies in animal models of PD have shown that the glutamatergic pathway may be involved in compensatory mechanisms after dopaminergic denervation resulting in improved motor function [15,16]. Overactivity of the subthalamic nucleus (the indirect pathway) is thought to contribute to the pathology of PD [17]. Several studies have demonstrated improved functional outcomes following manipulation of glutamatergic transmission in the 6-OHDA lesion model [18,19]. Our results show that despite the loss of dopamine neurons and dopamine receptor function, GPE improved the long-tem recovery of Parkinsonian motor deficits. The mechanism of this nigrostriatal dopamine depletion independent functional recovery is not clear, however the improved the function may be due to neuroplasticity outside of the nigrostriatal system, since both NMDA agonist and antagonist effects of GPE have been reported previously [20–22]. With its ability to cross the BBB and provide long-term functional benefits, GPE may have the potential to be developed for clinical application in Parkinson's disease.

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