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Neuroprotective effects of the N-terminal tripeptide of insulin-like growth factor-1, glycine-proline-glutamate (GPE) following intravenous infusion in hypoxic–ischemic adult rats

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Abstract

The N-terminal tripeptide of insulin-like growth factor-1, GPE is neuroprotective when given intracerebroventricularly 2 h after hypoxic–ischemic (HI) brain injury in rats. We have now examined whether GPE can cross the blood–brain barrier and exert neuroprotective actions following intravenous administration.

Following a single bolus intravenous injection, GPE was rapidly metabolized and cleared from the circulation. The short half-life (<2 min) in blood was subsequently associated with modest and inconsistent neuroprotection. In contrast, potent neuroprotection of GPE was consistently observed in all brain regions examined following 4 h intravenous infusion (12 mg/kg). The neuroprotective effects of GPE after infusion showed a broad effective dose range (1.2–120 mg/kg) and an extended window of treatment to 7–11 h after injury. The central penetration of GPE after intravenous infusion was injury-dependent. GPE also improved long-term somatofunction with a comparable neuronal outcome. GPE reduced both caspase-3-dependent and -independent apoptosis in the hippocampus. Treatment with GPE also inhibited microglial proliferation and prevented the injury-induced loss of astrocytes.

In conclusion, the neuroprotective actions of GPE infusion were global, robust and displayed a broad effective dose range and treatment window. GPE's activity included the prevention of neuronal apoptosis, promotion of astrocyte survival and inhibition of microglial proliferation. With injury specific central penetration, GPE has considerable promise as a systemic neuroprotective treatment after acute encephalopathies.

Keywords: Glycine-proline-glutamate; Neuroprotection; Intravenous administration; Blood–brain barrier; Hypoxia–ischemia; Adult rat

1. Introduction

Acute ischemic brain injury is one of the major causes of death and long-term disability in adult life. Currently, it can be treated by thrombolytics to enhance brain reperfusion if patients can be registered to the clinic within 3 h of the onset of stroke. Neuroprotection has been considered to be another critical mechanism for treating acute ischemic brain injuries (Lutsep and Clark, 1999). It has been well documented that the majority of neurons die several hours, even days following ischemic injuries, such as stroke or neurological complications associated with open heart surgery (Coimbra et al., 1996; Beilharz et al., 1995; Gallyas et al., 1992; Hsu et al., 1994). This evolution of cell loss is progressive due to the initiation of programmed cell death pathways, which offers a window of opportunity for treatment intervention.

Insulin-like growth factor-1 (IGF-1) is a naturally occurring peptide in the central nervous system (CNS) (Baskin et al., 1988), and plays an important role in the CNS development and acts as a survival and differen-

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Table 1
Experimental groups

Experiments	Treatment groups				
	Non-injured		Injured		
			Vehicle	GPE doses and time of treatment	
Half-life in plasma				n= 10	15 mg/kg 2 h post-injury
Central penetration	Vehicle $n = 6$	GPE $n = 9$	$n = 6$	$n = 9$	12 mg/kg 1–5 h post-injury
Single bolus			$n = 14$	$n = 14$	15 mg/kg 2 h post-injury
Single bolus + i.v. infusion			$n = 16$	$n = 16$	3 mg/kg + 12 mg/kg 1–5 h post-injury
Dose dependency			$n = 11$	$n = 9$	1.2 mg/kg 1–5 h post-injury
			$n = 16$	$n = 16$	12 mg/kg 1–5 h post-injury
			$n = 10$	$n = 12$	120 mg/kg 1–5 h post-injury
Window of treatment			$n = 13$	$n = 12$	12 mg/kg 3–7 h post-injury
			$n = 16$	$n = 16$	12 mg/kg 7–11 h post-injury
			$n = 16$	$n = 15$	12 mg/kg 24–48 h post-injury
Long-term effects	Normal $n = 6$	Sham $n = 6$	$n = 6$	$n = 6$	12 mg/kg 1–5 h post-injury

tiation factor for both neuronal and glial cells (Aberg et al., 2000; O'Donnell et al., 2002). It has been well documented that treatment with IGF-1 can prevent neuronal death from several forms of ischemic injury in both the mature (Gluckman et al., 1992; Guan et al., 1993, 1996) and developing brain (Johnston et al., 1996). An anti-apoptotic role for IGF-1 has also been demonstrated in vitro (Yin et al., 1994; Galli et al., 1995). However, clinical application of IGF-1 can be problematic due to the limited capability for IGF-1 to cross the blood–brain barrier (BBB) and the potential for mitogenic and metabolic effects.

Sara et al. (1989) first suggested that IGF-1 can be naturally cleaved into des-N (1-3)-IGF-1 (des-IGF-1) and the N-terminal tripeptide, glycine-proline-glutamate (GPE); a process mediated by an acid protease (Yamamoto and Murphy, 1994, 1995; Sara et al., 1989). Without interacting with IGF-1 receptors, GPE has been demonstrated to stimulate dopamine and acetylcholine release in vitro (Nilsson-Håkansson et al., 1993). We have previously demonstrated that intracerebroventricular administration of GPE can protect neurons after hypoxic–ischemic (HI) brain injury, particularly in the cerebral cortex and the CA1-2 subregions of the hippocampus (Guan et al., 1999). With its small molecular size, GPE may have the potential to cross the compromised BBB after ischemic injury. In the current study, we have investigated the pharmacokinetics and neuroprotective effects of GPE following intravenous (i.v.) administration in HI injured rats.

2. Methods

2.1. Animals and surgery

These studies were approved by the Animal Ethics Committee of the University of Auckland. Every effort was made to minimize animal suffering and to reduce the number of animals used.

Adult male Wistar rats (280–310 g) were obtained from the Animal Resources Unit colony, University of Auckland. Acute brain injury was induced using the modified Levine preparation and has been described previously (Guan et al., 1993). Briefly, the unilateral brain injury was induced by ligation of the right carotid artery followed by inhalation hypoxia. The right carotid artery was double ligated under general anaesthesia (3% halothane/oxygen). After 1 h recovery from the anaesthesia, the rats were placed in an incubator where the humidity ($90 \pm 5\%$) and temperature ($31 \pm 0.5\ ^{\circ}C$) were controlled for a further 1 h. The rats were then exposed to 15 min hypoxia ($6 \pm 0.2\%$ oxygen). The animals were maintained in the incubator for a further 30 min after the hypoxia before being removed to a holding room.

To allow treatments to be administered by continuous intravenous (i.v.) infusion, rats in some protocols were chronically catheterized 3 days prior to the experiment as described previously (Thomas et al., 1997). Rats were surgically fitted with an indwelling jugular venous catheter and housed individually in metabolic cages. The surgery was conducted under general anaesthesia with 3% halothane/oxygen, where the right jugular vein was exposed and a polyethylene catheter inserted. The catheter was exteriorized and passed out of the cage via a protective stainless steel spring and connected with a fluid-tight swivel joint. This was to allow the animal free movement within the cage. After a 3 day post-surgery recovery period, the catheter was connected to a peristaltic infusion pump to facilitate the infusion of GPE.

2.2. Experimental groups

Experimental groups are shown in Table 1.

2.2.1. Pharmacokinetic studies

Ten HI injured rats were used to determine the half-life of GPE after a single bolus i.v. injection given 2 h after HI injury. Blood samples were collected into heparinised tubes on ice containing protease inhibitor cocktail (Sigma-Aldrich, Sydney, Australia) at 10, and 0 min before, and 1, 2, 4, 8, 16 and 32 min after the i.v. bolus injection of 3 mg/kg GPE (Bachem AG, Basal, Switzerland). The plasma was stored at −80 °C for GPE radioimmunoassay.

In two additional experiments, the central penetration of GPE was determined in both normal ($n = 15$) and HI injured rats ($n = 15$). Nine rats from each group of animals were given a continuous 4 h infusion of 12 mg/kg GPE. The remaining six rats from each group received a control infusion of 10 mM succinate buffer, pH 6.0 (GPE-vehicle). In HI injured rats, GPE treatment was administered 1–5 h after injury. At the end of the 4 h infusion period, the animals were anaesthetized and cerebral spinal fluid (CSF) was collected into the tubes containing protease inhibitors from the cisterna magna using a 29-G ultra-fine needle and syringe. The animals were then killed using an overdose of pentobarbital. The CSF samples were stored at −80 °C for GPE assay.

2.2.2. Treatment studies

In experiment 1, two groups of 14 HI injured rats received either a single i.v. injection of 15 mg/kg GPE administered 2 h after HI injury or an injection of GPE-vehicle. After 4 days, the rats were killed and the brains collected for histological analysis.

In experiment 2, 16 rats were given a single 3 mg/kg i.v. bolus injection of GPE 1 h after HI injury and immediately followed by a continuous 4 h i.v. infusion (12 mg/kg) of GPE. The control HI group ($n = 16$) received GPE-vehicle alone. The rats were killed and the brains collected for histological and immunohistochemical analysis 4 days after the HI injury.

In experiment 3, groups of HI injured rats ($n = 9$–16) received a continuous 4 h i.v. infusion of either 1.2, 12 and 120 mg/kg GPE or their vehicle beginning 1 h after HI injury. The rats were killed and the brains collected for histological analysis 4 days after the HI injury.

In experiment 4, groups of HI injured rats ($n = 12$–16) received a continuous 4 h i.v. infusion of 12 mg/kg GPE beginning either 3, 7 or 24 h after HI injury. Control HI injured rats received a 4 h infusion of GPE-vehicle alone corresponding to the relevant treatment window of the experiment (e.g. 3–7, 7–11 or 24–28 h after HI injury). The rats were killed and the brains collected for histological analysis 4 days after HI injury.

In experiment 5, 24 rats were used to examine the long-term effects of GPE on both neuronal outcome and functional recovery after HI injury. Rats were randomly divided into four groups of normal controls, sham operated, HI injured treated with either GPE or its vehicle. All rats were habituated with the bilateral tactile tests for 3 sequential days prior to HI injury. Either GPE (12 mg/kg) or the vehicle was infused 1–5 h following HI injury. The procedure of the bilateral tactile tests has been described previously (Guan et al., 2001). All HI injured rats were tested at 3, 5, 10, and 20 days post-insult and the normal controls and sham operated rats were tested in parallel. Each rat had four trials on each day of testing. In each trial, an adhesive label (11 mm^2) was applied on the distal radial portion of the left and right forelimbs. The rat was put into a clear perspex observation chamber (190 mm wide× 210 mm high × 330 mm long). The time taken for the rat to contact each patch was measured (in minutes). The time between trials for each rat on each day of testing was between 5 and 10 min, and the order of patch placement was pseudo-random, with the left and right patch each being placed first on two of the four trials. The trial was ended at 5 min if the rat failed to contact to the patch. The time of contact to the patch was considered an indication of somatosensory-motor function of damaged (right) and control (left) hemispheres in the rats. L/R ratio of time taken to contact to the patch was used to quantify the asymmetry between performance on the contralateral limb (left, with potential deficit) and the ipsilateral limb (right, without potential deficit) to the damaged hemispheres. The mean of the ratio for each rat on each day of testing was calculated. Experimenters were blinded to the treatment groups. Rats were killed 21 days after the HI injury for histological analysis.

2.3. Histology

The histological procedures have been described previously (Guan et al., 1993, 1996). Briefly, 4 days after HI injury and GPE treatment, the rats were perfused transcardially under deep anaesthesia with normal saline followed by 10% formalin. The brains were removed and placed in the same fixative for 2 days before being processed using a paraffin procedure. Three coronal (6 μm) sections were cut from the striatum, cerebral cortex and hippocampus, mounted on glass slides and stained with thionine and acid fuchsin.

Dead neurons were identified as those with acidophilic (red) cytoplasm and contracted nuclei (Auer et al., 1985; Brown and Brierley, 1972). Brain tissues with selective neuronal death, cellular reaction and/or pan-necrosis were considered to be damaged (Guan et al., 2000; Markgraf et al., 1993). In additional to the above described pathology, the tissue damage score

also included the tissue atrophy and cavitation in the group used for long-term histological examination 21 days after the HI injury. The severity of brain damage in the lateral cortex was assessed using three levels, the dentate gyrus and the CA1-2, 3 and 4 sub-regions of the hippocampus using two levels, and the striatum using one level as following: $0 =$ no damage; $1 \leq 5\%$ tissue damaged; $2 \leq 50\%$ tissue damaged; $3 \geq 50\%$ tissue damaged and $4 \geq 95\%$ damaged (Guan et al., 2000; Lundgren et al., 1992). The average tissue damage scores in different brain regions were used for data analysis (Guan et al., 2000). Any animals that died before the termination of experiments were rejected from the histological analysis. The histology was analyzed by an individual blinded to the treatment groups.

2.4. Immunohistochemistry

Primary antibodies against glial fibrillary acidic protein (GFAP), isolectin B4, caspase-3, and proliferating cell nuclear antigen (PCNA) were used to mark reactive glial cells and cells undergoing apoptosis and proliferation, respectively.

Immunohistochemical staining was performed in both control and GPE treated HI rats (experiment 2) on paraffin tissues, along with four normal control rats. Coronal sections (6 μm) containing the level of the hippocampus were cut and mounted on chrome-alum coated slides for staining. The sections were deparaffinized in xylene, dehydrated in a series of ethanol and incubated in 0.1 M phosphate buffered saline (PBS). For antigen unmasking (caspase-3 and PCNA staining), sections were heated in 10 mM sodium citrate buffer (pH 6.0) for 1 min at high power. All sections were pretreated with 1% H_2O_2 in 50% methanol for 30 min to quench the endogenous peroxidase activity. Then, either 1.5% normal horse serum or 2.5% normal sheep serum in PBS was applied for 1 h at room temperature to block non-specific background staining. The sections were then incubated with following primary antibodies: monoclonal mouse anti-GFAP antibody (Sigma, St. Louis, MO, USA diluted 1:500); polyclonal rabbit anti-caspase-3 p17 antibody (Cleaved Caspase-3 Antibody, detects only the large 17–20 kDa fragment of activated caspase-3, Cell Signaling Technology, USA, diluted 1:1000); mouse anti-PCNA antibody (DAKA, A/S, Denmark, diluted 1:100). After incubation with primary antibodies at 4 °C for 2 days (except for PCNA staining which was incubated overnight), the sections were incubated with biotinylated horse anti-mouse or goat anti-rabbit secondary antibody (1:200, Sigma) at 4 °C overnight. The ExtrAvidin (Sigma, 1:200), which had been prepared 1 h before use, was applied for 3 h at room temperature, and then reacted in 0.05% 3,3-diaminobenzidine (DAB) and PBS to produce a brown reaction product. Sections were dehydrated in a series of alcohols to xylene and coverslipped with mounting medium. Control sections were processed in the same way except the primary antibody was omitted from the incubation solution.

For specific visualization of microglia, isolectin B4 from *Griffonia simplicifolia* seeds (Sigma, St. Louis, MO, USA) was used as a marker. The sections were pretreated with 1% H_2O_2 in 50% methanol for 30 min to quench the endogenous peroxidase activity after being deparaffinized. The sections were then incubated overnight at 4 °C with the isolectin primary antibody, diluted (1:4) in Tris buffered saline before being developed in DAB.

For TdT-mediated dATP nick end labeling (TUNEL) staining, the sections were pretreated for 15 min with Proteinase K (40 μg/ml; Sigma Chemical, St. Louis, MO), washed in PBS, then kept for 10 min with methanol containing 1% H_2O_2 to block non-specific peroxidase activity. Sections were then washed again in PBS and incubated for 5 min with TdT buffer (GIBCO-BRL, Life Technologies, Gaithersburg, MD). DNA fragments were labeled with TdT and biotin-14-dATP (Gibco-BRL) for 1 h at 37 °C. Subsequently, sections were washed in SSC buffer and incubated for 2 h with ABC reagent (Vector Laboratories). After washing, the sections were developed with DAB substrate. Sections were dehydrated in graded alcohols and mounted using DPX. A section that was pretreated with DNase 1 (Sigma Biosciences) to nick all DNA served as a positive control. A negative control slide was obtained with the omission of TdT from the incubation solution.

The number of caspase–3, TUNEL, GFAP, PCNA and isolectin B4 positive cells were counted within the pyramidal layer of the CA1-2, CA3 and CA4 subregions on both sides of the hippocampus.

2.5. Radioimmunoassay

The concentration of GPE in plasma and CSF was measured by a novel and specific double antibody radioimmunoassay (Batchelor et al., 2003). Prior to assay, the GPE samples, standards and tracer were derivatised with Bolton and Hunter reagent (Sigma-Aldrich, Sydney, Australia) to standardize the antibody binding configuration and maximize antibody recognition. The derivatised GPE radioimmunoassay shows complete parallelism with rat plasma and a recovery of unlabelled GPE added before assay of 83% ($n = 6$ experiments). The ED-50 was 195 pg/tube, and the limit of detection was 2 pg/ml. The intra-assay CV was $<10\%$ over the range 0.5–25 ng/ml. Any samples reading off the standard curve were further diluted before being re-assayed.

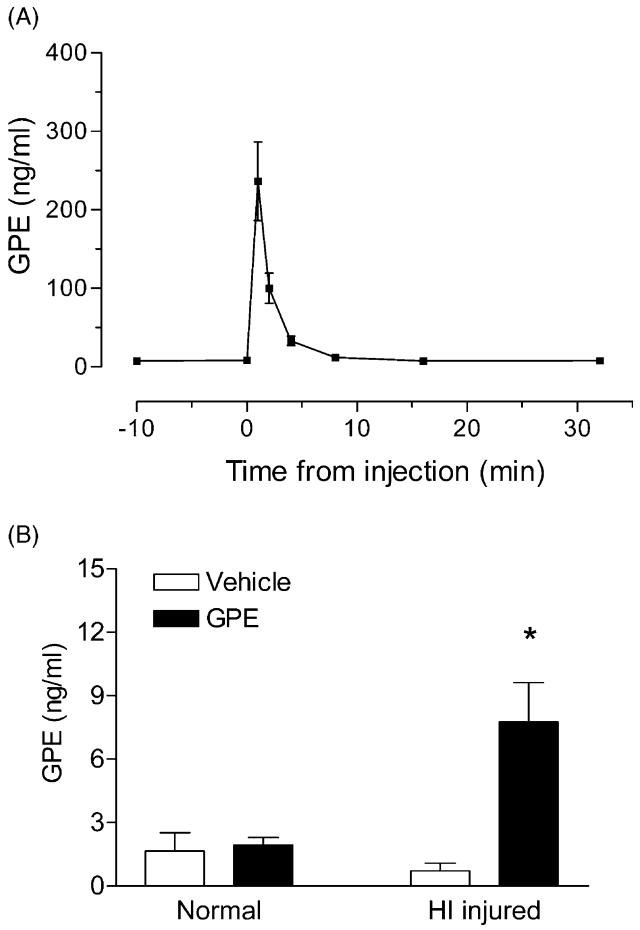

Fig. 1. Plasma concentration of GPE following a single i.v. bolus injection of 3 mg/kg GPE given 2 h after HI injury in adult rats. $n = 10$ animals (A). The levels of GPE in the CSF after a 4 h i.v. infusion of 12 mg/kg GPE in normal and HI injured rats. HI injured rats were treated 1–5 h after injury. Data are presented as mean ± SEM. $n = 6$–9 animals per group. $^{*}P < 0.05$ compared with the HI injured vehicle control group (B).

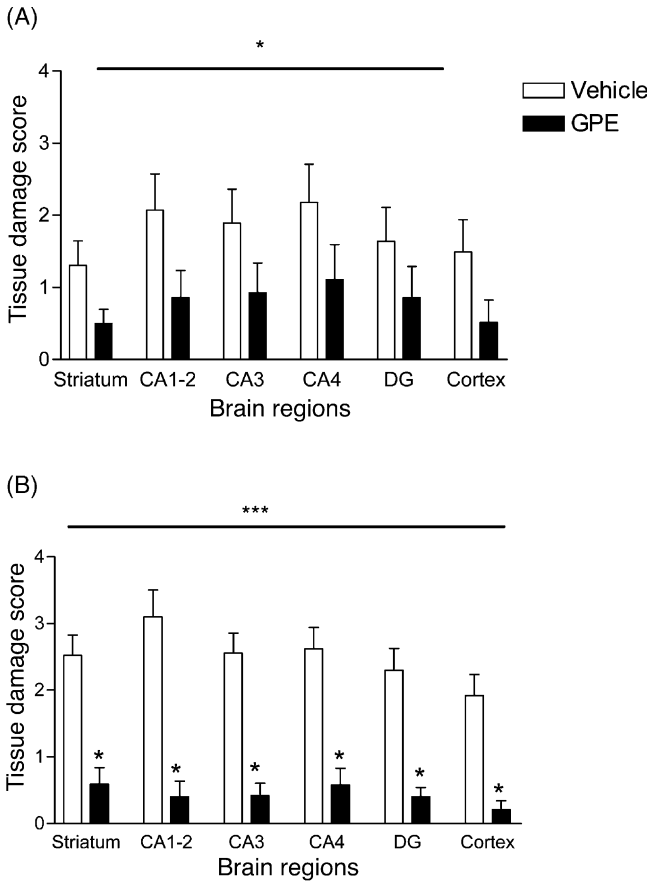

Fig. 2. Comparison between a single i.v. bolus injection of 15 mg/kg GPE given 2 h after injury (A), and a single i.v. bolus injection of 3 mg/kg followed by a continuous 4 h i.v. infusion of 12 mg/kg GPE (total dose 15 mg/kg) given 1–5 h after injury (B). Animals were killed after 4 days. Data are presented as mean ± SEM. $n = 14$–16 animals per group. $^{*}P < 0.05$, $^{***}P < 0.0001$ compared with the vehicle control groups. DG = dentate gyrus.

2.6. Statistical analysis

Histological and immunohistochemical data were analyzed using two-way ANOVA followed by Bonferroni post hoc tests for multiple comparisons, with brain regions treated as dependent factors. The levels of GPE in the CSF and plasma were analyzed using a one-way ANOVA. Data are presented as mean ± SEM.

3. Results

3.1. GPE Pharmacokinetics

Following a single 3 mg/kg i.v. injection of GPE in HI injured rats, plasma concentrations of GPE immediately increased from the baseline (8.1 ± 4.1) to 237.6 ± 50.0 ng/ml (Fig. 1A). The levels of GPE then rapidly declined back to baseline values (9.8 ± 1.33 ng/ml) within the next 8 min. The half-life of GPE in plasma was estimated to be less than 2 min.

There was a significant increase ($P < 0.05$) in GPE levels in the CSF following a continuous 4 h i.v. infusion of GPE (12 mg/kg) in HI injured rats (7.8 ± 1.9 ng/ml) compared with the vehicle treated HI control group (0.72 ± 0.36 ng/ml, Fig. 1B). There was no difference in GPE levels in the CSF between GPE (1.94 ± 0.36 ng/ml) and the vehicle (1.55 ± 0.88 ng/ml) treated groups in non-HI injured rats.

3.2. GPE treatment studies

HI brain injury resulted in severe neuronal injury in the ligated right hemisphere 4 days after HI injury. Massive neuronal loss was seen in all sub-regions of the hippocampus. A mixture of selective neuronal loss, tissue pan-necrosis and cellular reaction were found in the cerebral cortex, all sub-regions of the hippocampus,

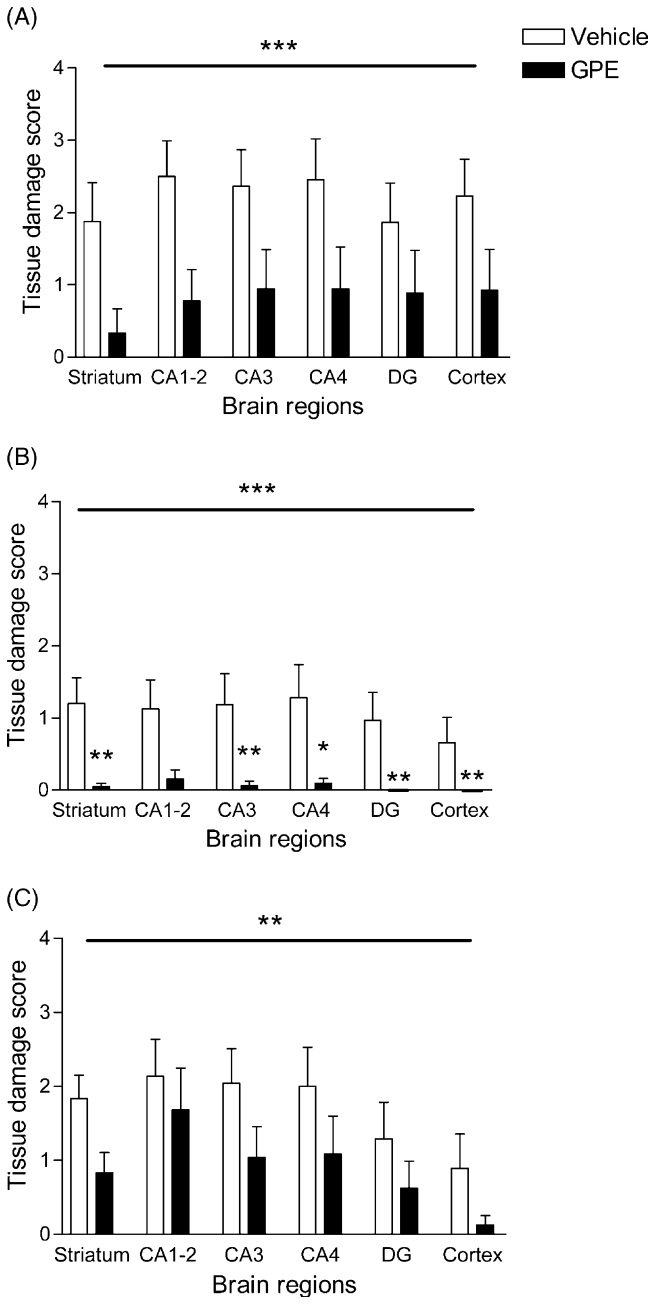

Fig. 3. Dose response of GPE following HI brain injury. Three doses of GPE (1.2 mg/kg, A; 12 mg/kg, B and 120 mg/kg, C) and their vehicle were administered 1–5 h after HI injury and the animals killed 4 days later. Data are presented as mean ± SEM. $n = 9$–16 animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the vehicle control groups. DG = dentate gyrus.

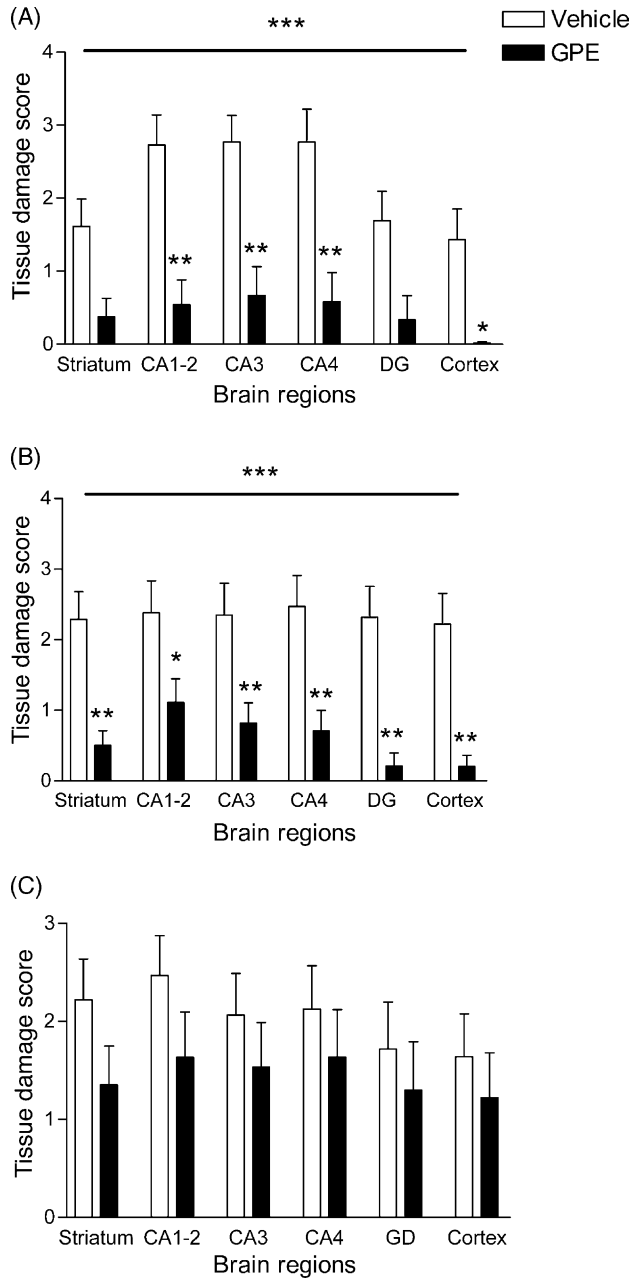

Fig. 4. Treatment window for GPE administration following HI brain injury. Four hour infusion of GPE (12 mg/kg, i.v.) or vehicle was initiated at 3 h (A), 7 h (B) and 24 h (C) after HI injury and the animals killed 4 days later. Data are presented as mean ± SEM. $n =$ 12–16 animals per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the vehicle control groups.

the dentate gyrus and the striatum. There was no neuronal loss in the left hemisphere.

3.2.1. Experiments 1–2

In animals injected with single i.v. bolus dose of 15 mg/kg GPE 2 h after the HI injury there was a modest reduction (overall $P = 0.047$) in tissue damage scores compared with the vehicle treated group, with no difference between the groups in the individual brain regions (Fig. 2A). In contrast, in animals injected with a 3 mg/kg bolus followed by a continuous 12 mg/kg i.v. infusion of GPE 1–5 h after HI injury (total dose 15 mg/kg), there was a highly significant reduction (overall $P < 0.0001$) in the tissue damage scores when compared with the vehicle treated group (Fig. 2B). Post hoc analysis showed that GPE treatment significantly

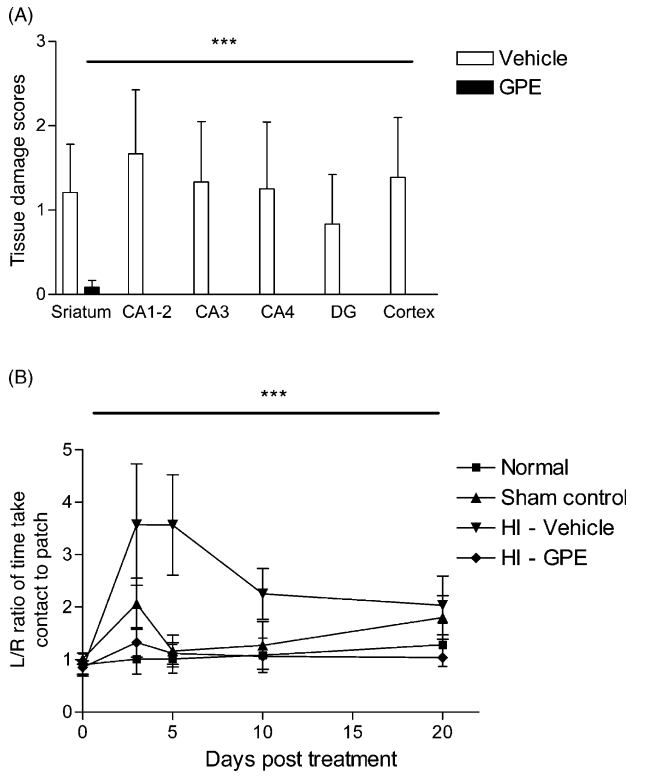

Fig. 5. Effects of GPE on long-term neuronal survival (A) and somatofunctional recovery (B). Animals were infused with 12 mg/kg GPE ($n = 6$) or vehicle ($n = 6$) 1–5 h after HI brain injury. The long-term histological and behavioral outcome examined 21 days after HI injury. Data presented as mean ± SEM, $^{***}P < 0.001$ compared to the vehicle control group.

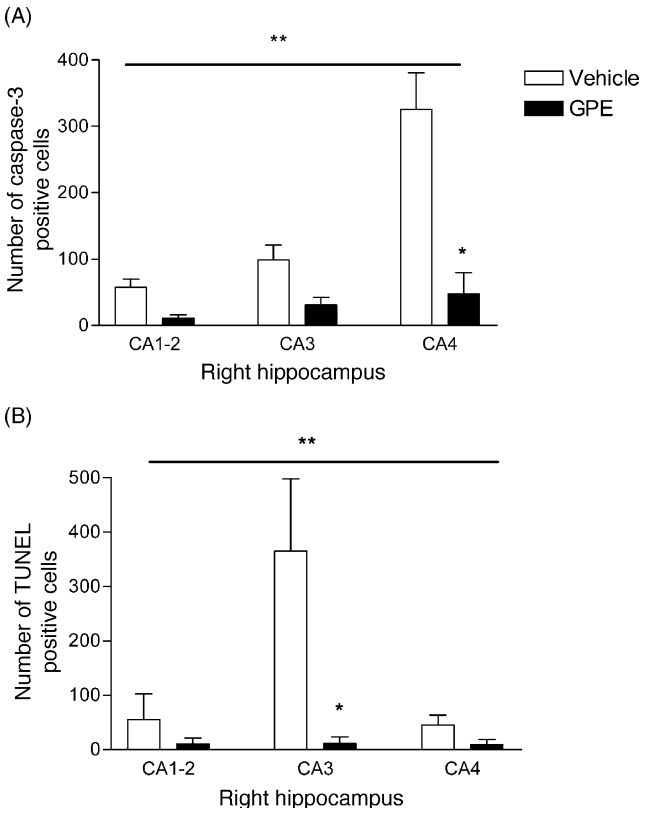

Fig. 6. Effects of GPE on apoptotic cells in the injured right hippocampus detected with an antibody to caspase-3 (A), or with TUNEL staining (B). Data are presented as mean ± SEM. $n = 11$–16 animals per group. Animals were infused with 12 mg/kg GPE or vehicle 1–5 h after HI brain injury and killed 4 days later. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the vehicle control group.

reduced ($P < 0.01$) the tissue damage in all the brain regions examined.

3.2.2. Experiments 3–4

GPE exhibited a broad effective dose range between 1.2 and 120 mg/kg) (Fig. 3A–C) when the 4 h i.v. infusion initiated at 1 h post-injury without initial bolus i.v. injection. GPE also reduced the injury when the treatment was delayed to either 3–7 or 7–11 h after the injury (Fig. 4A and B). No effect was observed when GPE was administered 24–28 h after the injury (Fig. 4C).

3.2.3. Experiment 5

In the vehicle treated group, the tissue cavitations and atrophy were found within the ipsilateral hemisphere in the rats with severe brain damage 21 days after HI injury. The average tissue damage scores were 1.28 ± 0.11 ($n = 6$). Treatment with GPE (12 mg/kg 1–5 h post-HI, without bolus) significantly reduced the tissue damage scores, with mild striatal damage observed in one of six rats treated with GPE (0.01 ± 0.01, $n = 6$, $P < 0.0001$, Fig. 5A).

HI injury significantly increased the L/R ratio of the time taken to contact the patch in (overall 2.45 ± 0.51, $P < 0.0001$, Fig. 5B) when compared to the normal control group (1.05 ± 0.06). Similar to our previous report (Guan et al., 2001), the behavioral deficit was developed and maximized at day 3 followed by a spontaneous recovery at day 10 in the vehicle treated group. Treatment with GPE, (12 mg/kg 1–5 h post-HI significantly reduced the L/R ratio of the time contact to the patch (1.08 ± 0.07) compared to the vehicle treated group (2.45 ± 0.51, $P < 0.01$, Fig. 5B).

3.3. Immunohistochemical analysis

There were few caspase-3 positive cells observed in the control (left) side of the hippocampus (average 18.9 ± 3.9 cells, data not shown). HI brain injury resulted in an increase in caspase-3 positive cells in all subregions of ipsilateral (right) hippocampus (160.5 ± 83.4 vs. 18.9 ± 3.9 cells, Fig. 6A). This increase in caspase-3 positive cells was more pronounced in the CA4 subregion (325.5 ± 55.2 cells). Treatment with GPE significantly reduced the number of caspase-3 positive cells in

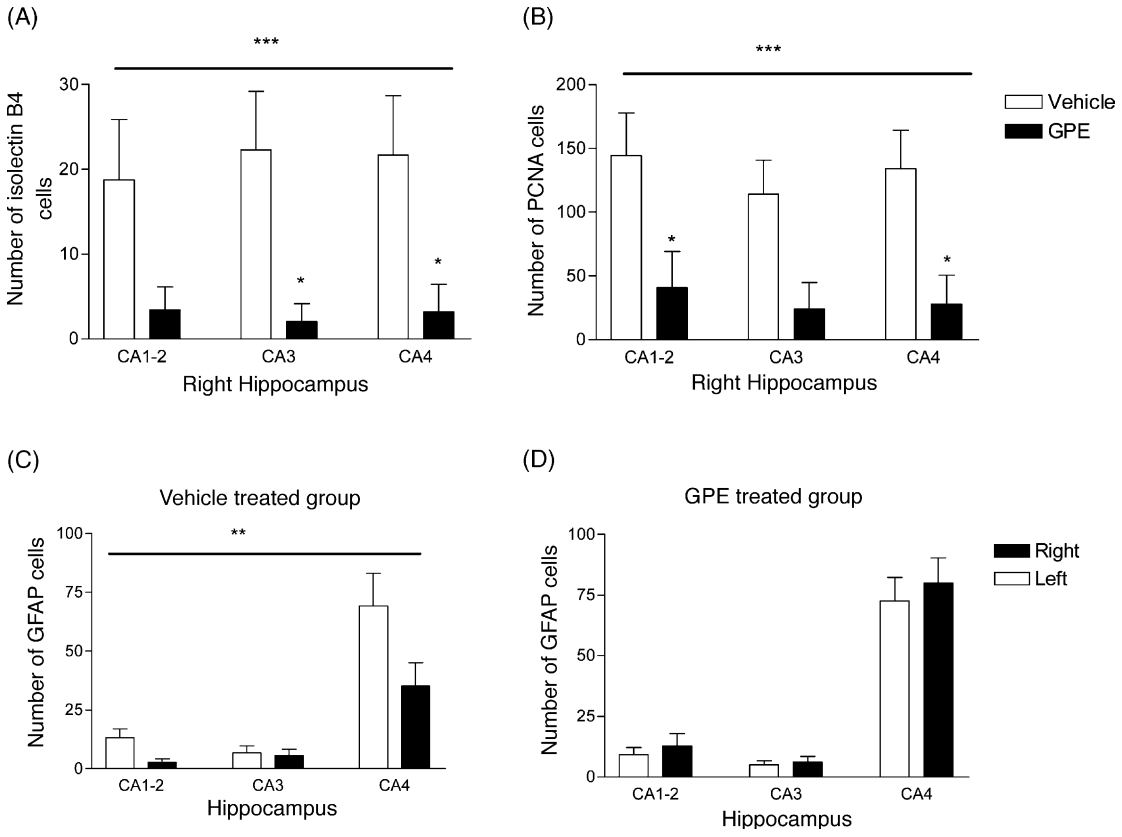

Fig. 7. Effects of GPE on isolectin B4 positive microglia (A), PCNA positive cells (B), and GFAP positive astrocytes (C, D) in the hippocampus. Data are presented as mean ± SEM. $n = 13$–14 animals per group. Animals were infused with 12 mg/kg GPE or vehicle 1–5 h after HI brain injury and killed 4 days later. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with vehicle control group.

the hippocampus (29.9 ± 10.6 cells, overall $P< 0.01$), particularly in the CA4 sub-region of the hippocampus (47.7 ± 31.8 cells, $P< 0.01$) compared with the vehicle treated group (Fig. 6A).

The TUNEL positive cells were only detected in ipsilateral hippocampus (155.5 ± 105.0 cells), particularly in the CA3 sub-region (365.5 ± 132.6 cells, Fig. 6B). GPE treatment significantly reduced ($P < 0.01$) the number of TUNEL positive cells in the right hippocampus (10.6 ± 0.7 cells), particularly in the CA3 sub-region of the hippocampus (11.7 ± 11.7 cells) when compared with the vehicle treated group (Fig. 6B). There were no TUNEL positive cells observed in the control side of the hippocampus.

In response to HI injury, there was an increase in the number of isolectin B4 positive microglia in all the sub-regions of injured hippocampus (20.9 ± 1.18 cells) when compared to the control side, where isolectin B4 positive cells were absent. Treatment with GPE significantly reduced (2.9 ± 0.4 cells, overall $P < 0.001$) the number of isolectin B4 positive cells, particularly in the CA3 and CA4 sub-regions ($P < 0.05$) when compared with the vehicle treated group (Fig. 7A).

The number of PCNA positive cells was increased in the ipsilateral hippocampus (130.9 ± 8.9 cells) compared to the control side (1.6 ± 0.7 cells, data not shown). Treatment with GPE significantly reduced ($P < 0.05$) the number of PCNA positive cells in the ipsilateral hippocampus (30.9 ± 5.1 cells, $P < 0.001$), particularly in the CA1-2 and CA4 sub-regions ($P < 0.05$) when compared to the vehicle treated group (Fig. 7B).

In vehicle treated animals, HI injury significantly reduced ($P< 0.05$) the number of GFAP positive astrocytes in the ipsilateral hippocampus, particularly in the CA4 sub-region, when compared with the uninjured control hippocampus (Fig. 7C and D). In contrast, there was no difference in the number of GFAP positive cells between the ipsilateral and contralateral hippocampus in the GPE treated group (Fig. 7C and D).

4. Discussion

This present study demonstrates that GPE exhibits robust and potent neuroprotective effects following continuous 4 h i.v. infusion in adult rats after HI brain injury. The treatment effects of GPE were global, with a broad effective dose range from 1.2 to 120 mg/kg and extended treatment window of 7–11 h after HI

injury. GPE treatment also achieved long-term neuroprotection, with improved long-term somatosensory-motor function. The neuroprotective effects of GPE in the hippocampus were associated with the inhibition of both caspase-3-dependent and -independent neuronal apoptosis. There was also evidence that GPE promoted the survival of astrocytes and suppressed the proliferation of microglial following ischemic injury.

Although several forms of growth factors, including IGF-1 have been reported to be neuroprotective after various forms of ischemic brain injuries, their potential mitogenic effects and difficulties in crossing the BBB are well-recognized limitations for their clinical development. Given that a small peptide is likely to be more accessible to the CNS (Pardridge, 2002), drug development has now focused on small molecules.

Our data clearly demonstrated an injury-dependent central penetration of GPE, since a significant elevation of GPE in the CSF following treatment was only found in HI injured rats. Ischemic injury-induced disruption of the BBB can be as early as 2–4 h after the injury due to the loss of tight junctions of the endothelium and disrupt the endothelial basal lamina by activation of matrix metalloproteinases (Fujimura et al., 1999; Planas et al., 2001). Although GPE is a small molecule, the hydrophilic nature can be problematic for its central penetration. Therefore, the disruption of basal lamina after ischemic injury (Fujimura et al., 1999) may be more associated to the enhanced central uptake of GPE. MK-801, a non-competitive NMDA receptor antagonist is a small molecule with well-demonstrated neuroprotection in ischemic animal models. In contrast to this injury elevated central penetration, ischemic brain injury reduces MK-801 tissue binding after administered peripherally (Wallace et al., 1992). Compared to the substances with non-specific access to the CNS, the injury-mediated central penetration of GPE could provide more specific targeting to the injured brain regions and minimize unwanted interactions to the regions un-affected by ischemic damage.

The degree of neuroprotection of GPE following a single bolus injection was limited and variable. This variability was most probably due to the extremely short half-life of GPE in plasma, which was estimated to be less than 2 min (Batchelor et al., 2003). A rapid break down of GPE into its major metabolites has also recently been reported in the normal rats (Batchelor et al., 2003). Therefore, given the need to maintain efficacious blood levels in order to maintain a stable central uptake of GPE, continuous infusion appeared to be a reliable and effective route of administration for GPE treatment. Intravenous infusion of GPE consistently achieved robust neuroprotection in all the brain regions examined, with a broad effective dose range. Tissue damage in the dentate gyrus and the cerebral cortex was completely prevented following the treatment with the most effective dose of GPE (3 mg/kg/h for 4 h). Although the fast clearance in plasma of GPE suggested a limited application for chronic neurological conditions, this could be a favorable aspect for treating acute neurological conditions since drug accumulation associated adverse effects may be minimized.

Another key practical issue for drug discovery is that recruitment of patients in a timely manner is a formidable practical problem. For example, despite the proven efficacy of thrombolytics after acute ischemic stroke, the great majority of patients are not enrolled within the 3 h treatment window (Famularo et al., 1998; Fisher and Schaebitz, 2000). The majority of compounds demonstrated to be neuroprotective in experimental models have a rather short window of opportunity (Fisher and Schaebitz, 2000). Few compounds can be administered no later than 6 h after injury with declined efficacy compared to early treatment (Mary et al., 2001; Williams et al., 2003). However, a delayed treatment with GPE at either 3 or 7 h after HI injury showed a similar degree of neuroprotection compared to the earlier administration. The treatment was not effective when the 4 h infusion initiated 24 h after HI injury and the treatment window between 11 and 24 h needs to be further explored. With a broad effective dose range, the extended the window of opportunity that GPE provides offers further promise to its clinical development.

In addition to the well-demonstrated secondary phase of neuronal loss occuring during the first week after injury, this delayed cell death can continue in a progressive ''tertiary'' phase over many months (Coimbra et al., 1996; Colbourne et al., 1999; Gallyas et al., 1992; Hsu et al., 1994; Jeon et al., 1995), which has also been previously demonstrated in the current HI injury model (Guan et al., 2001). In reality, most experimental studies report histological endpoints exclusively between 24 h and 1 week after initial injury, however, early protection by some compounds may not be maintained in long-term (Fisher and Schaebitz, 2000; Gladsrone et al., 2002). Therefore, short-term neuronal outcome cannot be seen to reflect a realistic efficacy of treatment and can be misleading in clinical development. Current experiments showed GPE i.v. infusion initiated 1 h after the injury improved long-term neuronal outcome examined 21 days following HI injury. The progressive neuronal damage examined 21 days after the injury has been previously reported in this particular animal model, speculatively due to the biphasic effects of products during endogenous response to brain injury and recovery process (Guan et al., 2001; Sharp et al., 2000).

One of the well-recognized pitfalls for the failure in the transformation of results from animal models to humans is that the pre-clinical development is

evaluated solely depending on histological outcome, while clinical trails are evaluated differently depending upon long-term functional and neurological outcomes (Fisher and Schaebitz, 2000; Gladsrone et al., 2002). Our previous studies have shown that HI injury resulted in unilateral damage within the territory of the middle cerebral artery (Guan et al., 2001; Ginsberg and Busto, 1989), which is largely associated with somatosensory function (Guan et al., 2001). This particular distribution of neuronal damage in the cerebral cortex resulted in significant loss of somatosensory function on the contralateral side to the damaged hemisphere and was most pronounced at the early time points (day 3 and 5). A spontaneous functional recovery was found 10 days after HI injury, probably associated with endogenous production of various growth factors (Yamaguchi et al., 1991; Gasser et al., 1986; Gomez Pinilla et al., 1992; Klempt et al., 1992). There was no significant recovery in somatosensory function over the period examined in the vehicle treated group. On the other hand, rats that showed delayed contact to the patch were also often observed missing the patch during the trial, which may suggest a deficiency of motor co-ordination as the loss of multiple phenotypic neurons in the striatum has been reported after HI injury (Guan et al., 1999). Like its parent peptide IGF-1 (Guan et al., 2001), treatment with GPE also improved the recovery in somatofunction, with a comparable long-term neuronal outcome (Fig. 5A and B). We have previously reported that the functional recovery by IGF-1 is more associated to preventing progressive neuronal loss rather than the reduction of infarct size (Guan et al., 2001). The current study showed that the tissue cavitation and atrophy was only found in the minority rats (2/6 vehicle treated rats), probably due to less degree of damage in current experiment. However, the GPE treated group had a minimum histological damage and no behavioral deficit.

Acute hypoxic–ischemic insult to brains results in neuronal loss with a mixed pathogenesis. Necrosis, a morphology recognized for a more rapid evolution of neuronal death initiated by a rupture of cell membranes, whilst others cells are committed to die via a more progressive process initiated by nuclear condensation (e.g. apoptosis). Both forms of neuronal death do not occur immediately following the injury, which provides a window of opportunity for treatment. TUNEL and caspase-3 positive immunostaining have been broadly used as markers of cells that undergo apoptosis (Snider et al., 1999; Velier et al., 1999). Given that the tissue damage scores currently used assessed a mixture of neuronal necrosis and apoptosis, a similar degree of neuronal damage was found cross the CA1-2, CA3 and CA4 sub-regions of the hippocampus in the vehicle treated group. Interestingly, while an increased TUNEL positive cells were seen mainly in the CA3 sub-region of the hippocampus, the majority of caspase-3 positive cells were located differently in the CA4 sub-regions in the vehicle treated group. Both TUNEL and caspase-3 positive cells were relatively low in the CA1-2 sub-regions. It is thought that, as an execution phase protease, caspase-3 activation (Yakovlev and Faden, 2001) leads to the fragmentation of DNA (Springer et al., 2001), where TUNEL can then be positively labeled. This HI injury resulted spatial difference between caspase-3 activation and TUNEL labeling indicates that caspase-3 pathway may not necessarily lead to positive TUNEL labeling. This disassociation between the TUNEL and caspase-3 immunoreactivity has also been suggested outside of the CNS (Donoghue et al., 1999). Therefore, these spatial differences indicated that both caspase-3-dependent and -independent pathways were involved in neuronal injury in the hippocampus following HI injury. Our data clearly show that GPE treatment 1–5 h after HI injury significantly reduced the tissue damage, as well as TUNEL and caspase-3 positive cells, suggesting inhibition of both neuronal necrosis and apoptosis.

A role for glial cells in neuronal damage and recovery has been controversially documented (Kraig et al., 1995). Our data show that GPE strongly suppressed microglial proliferation, and completely prevented the HI-induced loss of astrocytes. A physiological role for reactive astrocytes has been suggested to be involved in BBB integrity, cell-to-cell communication, intracellular iron-homeostasis, plasticity of neurons, and neurotrophic actions by regulating growth factor metabolism (Kraig et al., 1995). Under physiological conditions, excitatory amino acid release from astrocytes is receptor mediated, whereas injury-induced excitatory amino acid leakage from astrocytes is due to astrocyte swelling (Kraig et al., 1995), which can lead to a damaged homeostasis and contribute to further neuronal injury. The loss of astrocytes following ischemic injury has also been suggested to be an important part of evolution of tissue infarction (Matsui et al., 2002; Tateishi et al., 2002). Therefore, maintaining astrocyte integrity may be critical for the neuroprotection by GPE.

Microglial cells are generally believed to have a role in brain inflammation, autoimmune responses and neuronal degeneration (Kraig et al., 1995). Unlike its parent peptide IGF-1 (Cao et al., 2003), GPE treatment reduced HI injury-induced isolectin B4 positive microglial cells, probably through inhibiting cell proliferation, since PCNA positive cells, a marker of cell proliferation was also reduced by GPE treatment. Several neuroprotective agents have been identified to have anti-inflammatory properties, such as TGFβ-1 (McNeill et al., 1994), which could be involved in neuroprotection of GPE after HI injury. In contrast to GPE, IGF-1 promotes the proliferation of both

astrocytes and microglia after ischemic brain injury (Cao et al., 2003; O'Donnell et al., 2002). This may suggest a different mode of action between GPE and IGF-1 in glial/neuronal interaction.

In conclusion, these studies demonstrate that GPE exerts robust and potent effects in preventing neuronal injury after HI brain injury. In addition to its injury-dependent central penetration and rapid plasma clearance, a broad effective dose range, extended treatment window and long-term functional recovery make GPE a potential candidate to be developed for treating acute ischemic brain injury. Promoting astrocyte survival and inhibiting microglia proliferation may be important for GPE in preventing both neuronal apoptosis and necrosis.

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