Neuroprotection with Glycine-2-Methylproline-Glutamate (G-2MePE) after hypoxic-ischemic brain injury in adult rats

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Abstract

Background and Purpose: Hypoxic-ischemic brain injury, due to reduced supply of oxygen to brain, is a major cause of death and disability. There is no exclusive treatment available so far. Glycine-2-Methylproline-Glutamate (G-2MePE, NNZ 2556), an analogue of Glycine-Proline-Glutamate reduces neuronal injury after focal ischemia in adult rats. The current study investigated into the neuroprotective effects of G-2MePE after global hypoxic-ischemic brain injury in adult rats. Methods: Adult male rats received a single sub cutaneous injection of G-2MePE (1.2mg/kg) 3h post hypoxic-ischemic brain injury or the same volume of normal saline. Brains were extracted 5 days after the treatment. Tissue damage in the cortex, hippocampus and striatum was assessed. Neuronal survival, glial reactions, caspase-3 activity and TNF-α cytokine activity were also assessed. Results: The treatment with G-2MePE was associated with a significant reduction of tissue damage, improvement in neuronal survival, reduction in reactive microglia, TNF-α positive cells and caspase-3 positive cells in hippocampus and cortex but an elevation of astrocytosis. Conclusions: Neuroprotection with G-2MePE after hypoxic-ischemic brain injury in adult rats is associated with reduced neuronal necrosis, apoptosis, modulated inflammatory responses and augmented astrocytosis.

Introduction

Stroke is a leading cause of death and disability in developed countries affecting 1 in 250 persons. Ischemic stroke is the most common type which occurs when blood supply to the brain is reduced, causing neurons starving for oxygen, resulting in neuronal death or dysfunction, culminating in corresponding loss of neurologic function (Dimagli et al., 1999, Chan, 2001, Hayashi and Abe, 2004). A range of promising drugs have been tested in vitro and in vivo in the past; but the majority have failed to translate to the clinic (Gleichmann et al., 2000, Kawano et al., 2001, Domanska-Janik et al., 2004, Mohammad et al., 2004, Wahlgren and Ahmed, 2004, Kilic et al., 2006, Switzer and Hess, 2006). One obvious reason is the lack of animal models that represent the pathogenesis and pathology of human stroke. Thus testing the potential compound using multiple animal models may provide a more objective view for their preclinical development. The only therapy with proven benefits for acute ischemic stroke patients is the intravenous thrombolytic therapy with recombinant tissue-type plasminogen activator (rt-PA) within 3h of the onset of stroke (Fisher and Schaeibitz, 2000, Acampa et al., 2014), the window which has been recognised as critical for preventing neuronal death after stroke.

N-terminal glycine-proline-glutamate (GPE) is a neurobioactive tri peptide, naturally cleaved from IGF-1 by an acid protease (Sara et al., 1989). GPE crosses the blood brain barrier (BBB) and can prevent brain injury after embolic-stroke in aged rats (Guan et al., 2004, Shapira et al., 2009) and after hypoxic-ischemic (HI) injury in developing adult brain (Sizonenko et al., 2001). GPE is small and does not activate the IGF-1 receptor (Alonso De Diego et al., 2006). GPE has a short half-life (<2min) in plasma due to a rapid peptidase-mediated breakdown (Batchelor et al., 2003).

G-2MePE is a structural analogue of GPE designed to improve protease resistance by alpha methylation of the proline moiety (Harris and Brimble, 2006). It is known that G-2MePE can reduce injury after focal brain injury and trauma (Bickerdike et al., 2009) and HI injury in neonatal rat (Sedin et al., 2007). Reduced inflammation has been reported among the protective effects of G-2MePE (Sedin et al., 2007, Lu et al., 2009, Wei et al., 2009, Cartagena et al., 2013). There are various rodent models of stroke designed for testing the efficacy of neuroprotectants for clinical use with some of them reproducing the pathological changes and others mimicking the pathophysiological events of human stroke. Focal injury models are often severe with limited windows of opportunity to intervene and short outcome evaluation at 24h (Zhou et al., 2013, Gheibi et al., 2014). Therefore testing G-2MePE in different animal models of focal brain injury is desirable. HI induces focal brain damage through a global hypoxic insult with local reduction in blood flow. This approach allows progressive brain damage and provides the opportunity to evaluate longer term outcome. Thus the aim of the study was to examine the treatment effects of G-2MePE after HI brain injury in adult rats.

Material and Methods

Animals

All experimental protocols in this study were approved by the Auckland University Ethics committee. Adult male Wistar rats (280-310g) were
The procedure has been previously described (Guan et al., 2004). Animals were anesthetised with 3% isoflurane/oxygen and the right common carotid artery was permanently ligated with 0/3 silk thread. Rats were left at room temperature for 2h and then placed in an incubator at 90±5% relative humidity and temperature 31±0.5°C for 30 min. Hypoxia was induced by quickly reducing the oxygen level to 6% by introducing nitrogen gas and then maintained at 6% ± 0.2% level by adjusting the flow of nitrogen and air. Hypoxia continued for 15 min under close monitoring.

**Drug preparation and administration**

G-2MePE (Neuren Pharmaceuticals, Auckland) was dissolved in 0.9% saline solution. The experimenter was masked to the treatment groups throughout the experiments. Rats were randomly divided into two treatment groups for receiving either vehicle (0.3 ml saline, n=20) or an equal volume of G-2MePE dissolved in saline (1.2mg/kg, n=18) subcutaneously (SC), 3h after the HI. After the SC injection, animals were housed in groups of 3 in the holding room and weighed daily to ensure their well-being.

**Brain histological preparation**

Five days after the experiments rats were euthanized by an intraperitoneal injection of pentobarbital (125mg/kg) and the brains were transcardially perfused with normal saline followed by fixation with 10% neutral buffered formalin, by gravity feed. The brains were left in formalin solution for 2 days for continued fixation, coronally sliced to 3mm blocks, processed and paraffin embedded. 8μm thick coronal sections were cut at 3 levels i.e., at the level of striatum, at the level of cerebral cortex & dorsal hippocampus and at the level of cortex & ventral hom of the hippocampus (Guan et al., 2000, Shapira et al., 2009) and mounted on albumen-chrome alum pre-coated slides.

**Brain damage assessment**

The degree of tissue damage was assessed using a scoring system after thionin-acid fuchsine staining. Briefly, deparaffinised and hydrated sections were stained with aqueous solutions of thionin and acid fuchsine. Three coronal sections from each brain region were used for scoring in the ipsilateral (ischemic) hemisphere. Tissue damage was assessed at three levels anterior from inter-aural reference zero plane, (A–P 6.45 and 4.2mm) for the lateral cortex; at two levels (A-P 4.5 and 4.2mm) for the hippocampus & the dentate gyrus and at one level (A–P 8mm) for the striatum respectively (Paxinos and Watson, 1982). A 4 point scoring system was used to assess the histopathological damage (0 = no damage; 1 = few cells damaged; 2 = half of brain tissue damaged; 3 = > half of the tissue damaged and 4 = almost all neurons died and no tissue survived) (Rochester et al., 2005). Brain tissue with selective neuronal death, pan-necrosis and brain retraction was treated as damaged (Guan et al., 2000). Neuronal death was confirmed by the presence of acidophilic (pink) cytoplasm and contracted nuclei (Brown and Brierley, 1972, Auer et al., 2005). Brain tissue with selective neuronal death, pan-necrosis and brain retraction was treated as damaged (Guan et al., 2000).

**Immunohistochemical labelling**

The extent of neuronal survival in different parts of the brain, caspase-3 expression and inflammatory responses in the cortex & hippocampus were evaluated by immunohistochemical staining in brain paraffin preparations from experiment 1. 10 sections each from the experimental groups were taken for each antibody labelling. Primary and secondary antibodies used and their dilutions were as follows, (i) monoclonal mouse anti-NeuN for labelling surviving neurons (Chemicon International, Temecula, CA, USA, 1:200) (ii) goat polyclonal anti-ionised calcium-binding adaptor molecule-1(ba-1) for reactive microglia (Abcam, via Sapphire Bioscience, Hamilton, New Zealand , 1:200) (iii) polyclonal rabbit anti-gial fibrillary acidic protein (GFAP) for reactive astrocytes (Chemicon International, Temecula, CA, USA, 1:500), (iv) monoclonal mouse anti-TNF-α for pro-inflammatory cytokine TNF-α (AbD Serotec, Auckland, 1:200) and (v) rabbit polyclonal anti-cleaved caspase-3 ASP 175 for cells undergoing caspase-3 mediated apoptosis (Cell Signalling Technology, U.K; 1:200) (vi) biotin conjugated anti-mouse, anti-goat and anti-rabbit secondary body (Vector Laboratories, Burlingame, CA, 1:200).

Xylene-deparaffinised slides were stained in decreasing concentrations of ethanol and washed with 0.1m/L phosphate buffered saline except in alcohol dehydrated sections were then cleared in xylene and permanently mounted with DPX. Control sections were processed in the same way except that primary antibody was excluded from the incubating solution. Caspase-3 immunolabelled slides were counterstained with weak thionin solution to distinguish the area for counting. Alcohol dehydrated sections were then cleared in xylene and permanently mounted with DPX.

All immunohistochemical assessments were done in a masked fashion in both ipsilateral and contralateral hemispheres. Neuronal survival was assessed by estimating NeuN positive cells in the lateral cortex, CA1-2, CA3, CA4 sub regions of the hippocampus, dentate gyrus and the striatum. ba-1, TNF-α, GFAP and Caspase-3 positive cells were estimated in cortex and hippocampus. Immunopositive cell counts were done from the incubating solution. Caspase-3 immunolabelled slides were counterstained with weak thionin solution to distinguish the area for counting. Alcohol dehydrated sections were then cleared in xylene and permanently mounted with DPX.

The treatment effects of G-2MePE on the tissue damage scores (thionin/acid fuchsine), neuronal survival (NeuN), immunomodulation (ba-1, GFAP, TNF-α) and apoptosis (Caspase-3) on the ipsilateral and contralateral sides of the brain were analysed using two way ANOVA followed by Bonferroni post-hoc tests, with brain regions and treatments as dependant factors. Data are presented as mean ± SEM. Statistical significance was assumed at P < 0.05.

**Results**

G-2MePE treatment attenuated brain tissue damage after HI injury
HI induced unilateral damage in the middle cerebral artery territory ipsilateral to the ligated carotid arteries including the lateral cortex (Figure 1A), hippocampus (Figure 1 C, E G) and striatum (Figure 1I). The insult was associated with selective neuronal loss, acidophilic staining in cytoplasm, tissue pan-necrosis, cellular reactions and tissue infarction.
Figure 1.
Photomicrographs showing hypoxic-ischemic injury in different areas of rat brain after vehicle or G-2MePE treatment. Brains tissues extracted 5 days post HI, formalin fixed, coronally cut at 8µm thickness and stained with thionin and acid fuchsin. Left panel shows sections from vehicle treated brains (A, C, E, G, I) and right panel shows brain sections from G-2MePE (1.2µg/kg) sub-cutaneously treated rats 3h after HI injury (B, D, F, H, J). Note the lesser number of dead or damaged neurons in the G-2MePE treated group. Arrowheads represent areas of pan-necrosis. Neurons undergoing ischemic cell changes were characterised by pink acidophilic cytoplasm and contracted nuclei as shown by arrows. CA, cornu ammonis of hippocampus; DG, dentate gyrus. Scale bar=40µm

Sub cutaneous treatment with 1.2mg/kg G-2MePE reduced overall tissue damage scores compared to that of vehicle treated animals (F [1,156] =15.85, p=0.05, Figure 2A). However, post-hoc tests did not show specific protective effects of G-2MePE in the individual brain regions.

Figure 2.
Hypoxic ischemic Brain damage scores after treatment with vehicle or G-2MePE on brain regions. Sub-cutaneous treatment with G-2MePE (1.2mg/kg) 3h post hypoxic-ischemic brain injury caused an overall reduction in brain damage scores Data are presented as mean ±SEM *p <0.05; **p

G-2mPE treatment improved neuronal survival
Photomicrographs show the distribution and morphological changes of neuronal survival in experiment 1(Figure 3A-J). Morphologically, the surviving neurons in the injured areas were smaller in size, with fewer processes and dendrites. Compared to the vehicle treated group, G-2MePE treated neurons showed almost normal neuronal morphology in all brain regions. There was no difference in number of neurons in the vehicle treated and G-2MePE treated experimental groups in the contralateral cortex, CA1-2, CA3, CA4, dentate gyrus or striatum (Figure 4A-F). HI injury caused significant loss of NeuN positive neurons in the ipsilateral cortex, sub regions of hippocampus and the striatum, with more severe loss in the cortex (p<0.001), CA3, CA4 areas of the hippocampus (p<0.001) and striatum (p<0.001). Sub cutaneous treatment with G-2MePE significantly improved the neuronal density (cells/mm²) associated with HI injury on the ipsilateral cortex (598.75±93.45 vs 882.52 ±60.52; p<0.05), CA1-2 (975.89±245.40 vs 1733.36 ±112.77; p<0.01), CA3 (371.55±78.62 vs 1082.64 ±134.36; p<0.001), CA4 (229.38±39.34 vs 414.57±17.67; p<0.001) areas of the hippocampus, the dentate gyrus (1229.52±309.56 vs 2897.94 ±155.04; p<0.001) and striatum (544.22±109.23 vs 1023.74 ±50.23; p<0.001) to a level similar to the counts in contra lateral counterpart area (Figure 4A-F).
Figure 3.

Representative photomicrographs showing morphology of NeuN immunopositive neurons in HI brain regions after vehicle or G-
2MePE treatment. HI injury caused apparent reduction in the neuronal population and many cells appeared pycnotic (arrow) whereas subcutaneous treatment with G-2MePE (1.2mg/kg) was associated with improved population in cortex (A, B), CA1-2 (C, D), CA3 (G, H), CA4 areas of the hippocampus, the dentate gyrus (G, H) and the striatum (I, J) and the cells appeared morphologically normal. Scale bar=40um.

Figure 4.
Neuronal counts (cells/mm²) in HI brain regions after vehicle or G-2MePE treatment. HI injury caused a remarkable decrease in the neuronal density in the ipsilateral side of the brain in the cortex (A), CA1-2 (B), CA3 (C), CA4 areas of the hippocampus, the dentate gyrus (E) and striatum (F). Sub-cutaneous treatment with G-2MEPE (1.2mg/kg) 3h post HI was associated with a...
significant improvement in the neuronal outcome in all these brain areas analysed. Data are presented as mean ± SEM. ##p

**G-2MePE SC treatment augmented astrocytosis**

Astrocytes were present throughout hippocampus and cortex. HI injury was associated with induction of hypertrophic astrocytes with thicker cell bodies (Figure 5A, B). GFAP positive cell counts (cells/mm$^2$) in the hippocampus and cortex were similar between the two hemispheres of the vehicle treated group hippocampus and cortex. Treatment with G-2MePE was associated with a significant increase in reactive astrocytes in the ipsilateral hippocampus (184.52±29.19 vs 302.79±47.73, p<0.05) and cortex (144.04±23.78 vs 291.18±42; p<0.001; (Figure 6A, B).

**Figure 5.**

Photomicrographs showing astrocytes, microglia, TNFα positive cells, and cleaved caspase-3 positive cells in HI cortex and hippocampus after vehicle or G-2MePE treatment. HI injury was associated with remarkable induction of astrocytes in the vehicle ipsilateral cortex (A) while G-2MePE treatment was associated with a further increase of hypertrophic astrocytes in the
treated ipsilateral cortex (B). Also, G-2MePE treatment was associated with marked attenuation of inflammatory responses as evidenced by reduced number of activated microglia (D), TNFα positive cells (F) & reduced number of caspase-3 positive cells in the ipsilateral treated cortex (H). Corner inserts are the images of cells (arrow marked) magnified. Scale bar A-F= 40μm; GH=20μm.
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**Discussion**

The modified analogue of GPE, G-2MePE, has a much longer half-life in plasma (49 min) compared to GPE (2 min) due to improved enzymatic resistance (Bickerdike et al., 2009). Although ANOVA indicated an overall reduction in tissue damage scores after single dose treatment the efficacy in individual brain regions was not significant. The treatment effects of G-2MePE were further also not evaluated by measuring the number of survival neurons using NeuN immunolabelling. We found the significant improvements of neuronal survival in all brain regions examined. While the tissue damage scores reflected the changes that associated with necrotic neuronal death the evaluation of surviving neurons appeared to be a more sensitive measure for the treatment effects of G-2MePE. In addition, HI injury also induced obvious morphological changes in the neurons by showing the deformed and shrinking cell bodies. The treatment of G-2MePE also prevented HI-induced morphological changes in neurons.

The protective effects of G-2MePE were also demonstrated by showing reduced apoptotic neuronal death. It is known that the activation of caspase-3 is involved in neuronal death after hypoxic-ischemia particularly in brain regions with more progressive neuronal loss (Guan et al., 2013). There were 50% more caspase-3 positive cells in the ipsilateral hippocampus than on the ipsilateral cortex, suggesting neuronal death may be more progressive in the hippocampus whereas necrotic death may be the major factor involved in cortical neuronal loss. Thus the protective effects of G-2MePE may be mediated through necrotic and apoptotic pathways.

Microglia plays an important role in the inflammatory response in the brain and produce pro-inflammatory cytokines like TNF-α or IL-1β. We observed inhibition of reactive microglia and expression of TNF-α after G-2MePE treatment. This is consistent with evidence that anti-inflammation contributes to the neuroprotection of G-2MePE as the improved neuronal outcome was associated with reduction of pro-inflammatory cytokine IL-6 after HI injury in neonatal rats (Svedin et al., 2007). The effects of TNF-α are mediated through two membrane receptors, TNF-α-receptor 1 & 2, and activation of one of these receptors lead to cell death (Aggarwal and Natarajan, 1996, Nakazawa et al., 2006). The SC treatment with G2-mPE also reduced the density of microglia in the ipsilateral hippocampus and cortex (711.4±174.80 vs 105.65±20.24; p<0.001; Figure 6C, D).

**G-2MePE treatment inhibited TNF-α expression**

Hypoxic-ischemic injury was associated with induction of pro-inflammatory cytokines. TNF-α positive cells were diffusely distributed in all sub-regions of the hippocampus and cortex. Morphologically there was a gradient of caspase-3 labelled cells; most recently cleaved caspase being densely stained (Figure 5G, H). G-2MePE treatment significantly reduced the apoptotic cell counts (cells/mm²) in the ipsilateral hippocampus (693.5±104 vs 297.7±104.5 p<0.001) and cortex (88.97±12.54 vs 53.60±8.02; p<0.05; Figure 6G, H).

**G-2MePE treatment reduced caspase-3 mediated apoptosis**

Hypoxic-ischemic injury was associated with induction of pro-inflammatory cytokines. TNF-α positive cells were diffusely distributed in all sub-regions of the hippocampus and cortex. Morphologically there was a gradient of caspase-3 labelled cells; most recently cleaved caspase being densely stained (Figure 5G, H). G-2MePE treatment significantly reduced the apoptotic cell counts (cells/mm²) in the ipsilateral hippocampus (132.63±17.72 vs 68.62±11.22; p<0.01) and cortex (86.97±12.54 vs 53.60±8.02; p<0.05; Figure 6G, H).

**Morphologically** there was a gradient of caspase-3 labelled cells; most recently cleaved caspase being densely stained (Figure 5G, H). G-2MePE treatment prevented HI-induced morphological changes in neurons.

Astrocytes, microglia, TNFα positive and caspase-3 positive cell counts in HI brain hippocampus and cortex after vehicle or G-2MePE treatment. On the contrary sub-cutaneous treatment with G-2MePE was associated with a marked decrease in reactive microglia(C, D), TNF-α positive cells (E, F) and caspase-3 positive cells (G, H) in the ipsilateral hippocampus and cortex. HP= Hippocampus, CTX= cortex. Data are means±SEM. #p

**G-2MePE treatment inhibited microglial activation**

HI injury was associated with marked increase in reactive microglia with amoeboid morphology, throughout the ipsilateral hemisphere. There was intense infiltration in the necrotic areas (Figure 5C). Compared to the contralateral hemisphere there was approximately a 9 fold increase in reactive microglia population in the ipsilateral hippocampus and 8.5 fold increase in the lateral cortex. Treatment with G-2MePE significantly reduced the density of microglia (cells/mm²) in the ipsilateral hippocampus (867.08±195.49 vs 201.37±88.98; p<0.001) and cortex (711.4±174.80 vs 105.65±20.24; p<0.001; Figure 6C, D).

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Summary:

G-2MePG is neuroprotective in adult rats when administered 3 h after HI injury subcutaneously. The mechanisms of neuroprotection may include blocking the necrotic or apoptotic pathways of cell death as well as modulating the immune response and augmenting astrocytosis.

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