The cannabinoid CB$_2$ receptor agonist GW405833 does not ameliorate brain damage induced by hypoxia-ischemia in rats

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HIGHLIGHTS

- The CB$_2$ agonist GW405833 was not neuroprotective in rat hypoxia-ischemia.
- Neither pre nor post injury drug administration was protective.
- Neither single nor multiple administrations were protective.
- Neither histological nor behavioral benefits were observed.
- Brain damage and behavior was measured out to 15 days after surgery.

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ABSTRACT

The cannabinoid CB$_2$ receptor has been under investigation as a potential target for neuroprotection with the suppression of neuroinflammation as the proposed mechanism of action. Several studies have now reported that CB$_2$ agonists are neuroprotective in models of cerebral ischemia. However, these studies have tended to measure brain infarctions in rodents 1–3 days after drug administration and have not assessed behavioral outcomes. As it has been shown that apparent protection soon after injury is not necessarily correlated with improved outcome after several weeks, we tested the CB$_2$ selective agonist GW405833 in a model of cerebral hypoxia-ischemia, and assessed histological and behavioral outcomes 15 days after injury. Many putatively neuroprotective drugs have failed to translate from promising preclinical results to clinical success. We designed our experiments to not only test CB$_2$ mediated neuroprotection, but also to test several drug administration regimens to maximize the chance of detecting any therapeutic effect. However, GW405833 failed to provide neuroprotection in any of our experiments. These results challenge how far the results of earlier studies into CB$_2$ mediated neuroprotection as measured at early time points may be extrapolated to later time points or to other models.

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1. Introduction

Several studies have shown that cannabinoid CB$_2$ receptor agonists are neuroprotective when brain damage is assessed 1–3 days following cerebral ischemia. CB$_2$ receptor activation reduces infarction size in mice 24 h after middle cerebral artery occlusion (MCAO) [21,22] and 48 h after MCAO [13]. Reductions in infarction size and improvements in neurological score in mice 48 h after MCAO have also been observed when CB$_2$ agonists have been administered up to 3 h after injury [20]. However, it has been shown that apparent neuroprotection as measured a short time after injury and drug administration does not necessarily correlate with improved long term outcomes [8,17] and that improvements in histological scores do not always correlate with improved behavioral outcomes [4]. Nor do small focal lesions necessarily model the extensive damage often seen in malignant human stroke [3] where patients are often co-morbid with a variety of cardiovascular disorders. Therefore, in this study we tested whether GW405833 – a well characterized selective CB$_2$ receptor agonist [18] – could improve longer term outcomes (in this study 15 days post-injury for infarction size and up to 13 days for behavior) in rats that had been exposed to hypoxia-ischemia (HI), a model of cerebral ischemia that reliably produces a unilateral lesion in the brain and in which the non-selective cannabinoid CB$_1$/CB$_2$ receptor agonist WIN55,212–2 has previously

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been shown to be neuroprotective following hypoxia-ischemia (HI) in rats [5,6].

2. Methods and materials

2.1. Experimental approach

We tested the effects of GW405833 (Tocris Bioscience, UK) in employing an HI model that we previously developed and described [17]. All procedures were approved by the University of Otago Animal Ethics Committee. Outcome measures were both histological (infarction size scoring and lectin binding) and behavioral (Morris Water Maze). We tested both pre- and post-injury drug administration regimens, as well as single and multiple administration regimens when the drug was given after injury. Activity of the drug was validated by assessing the concentration dependent suppression of activation in RAW 264.7 mouse macrophage cells that were treated with the endotoxin lipopolysaccharide (LPS) (data not shown). For experiments described below the animals were randomly allocated to each group and the surgeon and experimenter were blinded to the treatment groups and any previously recorded data. All rats used in these experiments were male Wistar rats and were provided by the University of Otago Taiieri Animal Facility. They were obtained at postnatal day 19 (P19) and gentled daily prior to the experiments. Animals underwent hypoxia ischemia (HI) at P26. The age was chosen by using myelination, cell migration and behavior as measures to correspond to the target human age of 6 months to 18 years old [15]. Infarction size assessment was as previously described (toluidine staining for experiments 1 and 2, and TTC staining for experiment 3) [17].

2.2. Experimental groups

2.2.1. Post-surgery single administration

Thirty minutes after hypoxia animals were randomly allocated to receive either i.p. 3 mg/kg GW405833 (n = 8) in 25% (w/v) 2-hydroxypropyl-β-cyclodextrin (HPCD) (H107, Sigma–Aldrich®) in 0.1 M PBS or in HPCD vehicle alone (n = 8). The other animals received only the 25% (w/v) HPCD in 0.1 M PBS vehicle. In all experiments the injection volume was 4 ml/kg. Fifteen days after HI the animals were sacrificed and the infarction volume was measured using a toluidine stain (the “Variable HI” model in [17]).

2.2.2. Post-surgery multiple administration

The aim of this experiment was to maintain CB2 receptor activation over the course of 6 days, a period that correlates with the development of damage in the ischemic penumbra [5]. In order to ensure that CB2 activation was prolonged through the day following each administration we increased the dose to 10 mg/kg, the maximum dose at which we considered that CB2 activation could be avoided at the cMax (see below for further details). Thirty minutes after (Variable) HI rats were administered the vehicle (n = 12) or 10 mg/kg GW405833 (n = 12). The insolubility of this amount of GW405833 in HPCD necessitated the use of a different vehicle: 5% DMSO, 5% (v/v) Cremophor-EL (C-EL) (#CS15, Sigma Aldrich®), 5% (v/v) ethanol, 85% (v/v), 0.01 M PBS solution. The animals were re-administered with the same treatment every 24 h for 6 days following HI. At the end of the night cycle on day 7 the Morris water maze (MWM) task was performed (see [11]). On day 15 the rats were sacrificed and the brains were then sectioned and assessed for infarction and lectin staining. For the MWM experiments an additional 8 animals were used that underwent a sham surgery identical to that of the ligation surgery except without occluding the vessel and the animals were not subjected to hypoxia.

2.2.3. Pre-surgery drug administration

Rats received either (s.c.) HPCD vehicle (n = 11) or 3 mg/kg GW405833 (n = 11) immediately before HI surgery was performed. In this experiment the infarction areas were visualized 3 days after HI (the “Fixed HI” model in [17]) using a TTC stain.

2.3. Lectin staining

We adapted the protocol established by Acarin et al. [1]. The sections of the brain immediately adjacent to those that were used to measure infarction size were used for lectin staining. These sections were immersed in 0.1% (v/v) Triton X (#UN3082, BDH Laboratory Supplies®) in 0.01 M PBS for 15 min, and this was followed by a single 3 min wash in 0.01 M PBS. Non-specific binding was then blocked by a 30 min incubation in 1% (w/v) bovine serum albumin (BSA) (#A9418, Sigma–Aldrich®) in 0.01 M PBS. The blocking solution was dabbed off, and the lectin solution was applied at the optimal dilution of 1–200 of original stock solution using 2% (w/v) BSA in 0.01 M PBS. The optimal dilution was established using 7 different lectin concentrations applied to adjacent sections; the concentration that produced the most specific staining and the greatest difference in staining density between the ipsilateral and contralateral hemispheres was a 1–200 dilution; this was then used for all experiments (data not shown). The stock lectin solution consisted of 5 mg/ml of the biotin conjugated, lyophilized powdered lectin extracted from Lycopersicon esculentum (#L0651, Sigma Aldrich®) in 2% (w/v) BSA in 0.01 M PBS. This was allowed to incubate at room temperature (RT) for 4 h. This was then washed with five 5 min immersions in 0.01 M PBS at RT. The sections were then incubated for 30 min at RT with a 0.01 M PBS solution containing horseradish peroxidase (HRP) conjugated-biotin and avidin, at the recommended concentration of the provided kit (#PK6100, VectaStain®). After a washing step, the sections were then developed using a 0.05% 3,3’,-diaminobenzidine tetrahydrochloride e (DAB) 0.01 M PBS solution (#5K-4100 Vector Labs®). The reaction was stopped by immersion in dH2O for 2 min followed by two 0.01 M PBS washes. The slides were then wet mounted using Permaflour (#FTA-030-FM, thermo-Scientific®). The sections were then scanned using a Biorad® GS-710 calibrated imaging densitometer scanner and analyzed for optical density using Image J® software (National Institute of Health, USA). For each section, the whole hemisphere mean optical density was measured, as well as the mean optical density of the striatum and the hippocampus. Percentage increase in staining was calculated using Equation (1). This was calculated for each section across the cortex, and plotted against the location of the section in the cortex. The area under of the curve of this plot was defined as “mean density”.

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\frac{\text{percentage increase in lectin staining of the ipsilateral hemisphere}}{\text{mean optical density of ipsilateral hemisphere}} = \left(\frac{\text{mean optical density of contralateral hemisphere}}{\text{mean optical density of contralateral hemisphere}}\right) - 100
\] (1)

The calculation used to approximate the percentage increase in lectin staining of the ipsilateral hemisphere following HI.

2.3.1. Morris water maze

We used methods adapted from Vorhees and Williams [19]. The tank was 155 cm in diameter with a submerged 15 cm platform. Data were analyzed using Noldus Ethovision® Version 6 software. At 7 days after HI day rats were habituated to the maze room. Training trials took place over days 8–11 after HI. Each rat had four trials per day for the four days; each rat was placed in a randomly assigned position that was the same for each rat (between 1 and 12 o’clock). The rats were allowed to swim for 90 s before...
being guided by hand to the platform. If the rat was guided to the platform, the rat received the maximum time of 90 s. If the rat found the platform prior to the 90 s, it was allowed to rest on the platform for 20 s before removal. Finding of the platform was only considered successful if the rat remained on it for more than 2 s. Once the rat was on the platform, if the rat jumped off it was placed back on the platform until it remained on the platform for 10 s, or 60 s had passed from the time it was successful. The rat was then dried and rested for 30 s before it was either trialed again, or, if it had finished the trials, it was returned to its home cage. Probe trials were performed 12 days after HI day. The platform was removed and the rat allowed to swim for 30 s. The number of passes over the location of the platform, path taken and total distances were recorded along with other outcome measures. Cued trials took place 13 days after HI; a white striped platform was placed in the NE quadrant and the rat was placed in the SW position. The water level was lowered to allow the rat to see the platform. To reduce the distraction of distal cues, the high contrast pictures were removed from view. The time taken to reach the platform was recorded, with a maximum time of 120 s allowed before removal.

### 2.3.2. Statistical analysis

When assessing the difference between means of two groups, independent two-tailed Student t tests were used. For all two factor analyses mixed model two-way ANOVAs were used. Sphericity was assessed using Mauchly’s test for sphericity. If this assumption was violated, the Greenhouse–Geisser correction was made [7]. For all analyses model assumptions were tested and were not significantly violated. Homogeneity of variance was assessed with the Levine’s test and normality was evaluated with the Kolmogorov–Smirnov test. The likelihood ratio Chi squared test was used to determine if the success rate on finding the platform differed between groups. All analyses were performed using IBM SPSS 19v Statistical Software© and graphs drawn using GraphPad Prism (v. 6.02, GraphPad Software Inc.).

### 3. Results

There were no significant differences in infarction sizes between the vehicle groups and the GW405833 treated groups in all three experiments (Fig. 1). Differences between groups were not significant (p > 0.05) with respect to total infarction volume (Fig. 1B, 1C, 1D, 1E, 1F).
D and F). Similarly, no significant differences were detected for either the drug treatment factor, nor for the interaction of drug treatment with position in the brain (Fig. 1A, C and E) using a two-way ANOVA \((p>0.05)\). Furthermore, there was no evidence of any dose-response effect, with 3 mg/kg GW405833 (Fig. 1A) producing a greater (though not statistically significant) apparent reduction in damage than 10 mg/kg GW3405833 (Fig. 1B).

Treatment with multiple administrations of 10 mg/kg GW405833 did not have a significant effect in any of the analyses carried out on the MWM experiment when compared with the vehicle control (Fig. 2). The animals that underwent HI took significantly longer to locate the platform than those that had undergone sham surgery (Fig. 2A) \((p<0.05\) for the HI/vehicle group compared with the sham group; \(p<0.01\) for the HI/GW405833 group compared with the sham group). However, there was no significant difference between vehicle and GW405833 treated groups \((p>0.05)\). There were no significant differences \((p>0.05)\) between any of the groups for the cued trial (Fig. 2B). Animals that had undergone HI traveled significantly greater distances than sham animals; planned comparisons showed significant differences between the sham group and both the HI/vehicle group \((r=0.47, t(23)=-2.57, p=0.017)\) and the HI/GW405833 group \((r=0.59, t(23)=-3.47, p=0.002)\). During the probe trial (Fig. 2D) the sham animals were 9.0 times more likely to enter the platform zone compared to the HI/vehicle group and 4.8 times more likely than the HI/GW405833 group. However, this effect was not statistically significant \((\chi^2(2)=3.42, p=0.18)\).

The lectin stain visualized both activated microglia/macrophages and endothelial cells. As these are morphologically distinct, it was visually determined that the lectin staining was predominantly due to activated microglia/macrophages, and so could be used as a general measure of inflammatory activity.

Fig. 2. The effects of 10 mg/kg GW405833 administered over 6 successive days on performance in the Morris Water Maze following HI, compared with vehicle and sham (“Naive/controls”) controls. (A) Mean latency (s) to the platform over the four trial days. (B) Mean latency (s) to the platform during a cued trial. (C) Mean total distance (m) traveled by each treatment group during the 30 s probe trial. (D) Percentage of animals from each group to enter the platform zone during the probe trial. Error bars are standard error; \(p<0.05\) and * \(p<0.01\) compared with the sham surgery group.

Fig. 3. Representative images of lectin-stained brain sections 15 days after HI. Arrows indicate unilateral staining in the cortex, striatum, and hippocampus. Scale bars: A = 1.2 mm; B = 1.6 mm; C = 1.1 mm.
Fig. 4. The effects of 10 mg/kg GW405833 administered over 6 successive days on lectin staining in different regions of the brain following HI. Mean optical density of lectin staining, expressed as a percentage increase from contralateral side for sequential sections in the ipsilateral cortex (A), striatum (C) and hippocampus (E) with the overall mean lectin staining for each region depicted in (B), (D) and (F), respectively. Error bars are standard errors. * p < 0.05 and ** p < 0.01 compared with the no-treatment group (“HI”). Other conventions are as for Fig. 2C and D.
this approach has been successfully employed by Ramirez and colleagues in the study of cannabinoids and neuroprotection [14]. Lectin staining was consistently unilateral, localized to the infarct and peri-infarct zone (Fig. 3). GW405833 treatment (10 mg/kg for 6 days) did not significantly affect lectin staining in the cortex or striatum (Fig. 4A–D) assessed either by distance along the brain or by area under the curve (AUC). There appeared to be a moderate effect of GW405833 on lectin staining in the hippocampus (Fig. 4E) (r = 0.37), however, this effect was also not statistically significant (t(13) = 1.42, p = 0.17) (Fig. 4E and F).

4. Discussion

GW405833 failed to provide a detectable therapeutic effect with any of the dosing strategies used in our experiments, in terms of histological markers and performance in the MWM. Even when a high dose (10 mg/kg/day) was administered over 6 days, the outcomes for the treated rats were not significantly different from controls when measured 15 days after the initial surgery.

In addition to testing for neuroprotection in general, the HI model allowed us to test whether there was a neuroprotective effect localized to the penumbra. HI is characterized by a clear distinction between an initial phase of damage at the ischemic core and a later period of secondary damage in the penumbra. These processes are partially independent [9] with damage in the penumbra thought to be partly caused by neuroinflammation. In some (but not all) studies which have shown neuroprotective properties for CB2 agonists this has been hypothesized to be due to effects on inflammation [16] as CB2 agonists are anti-inflammatory [16,12]. GW405833 in particular has been found to have anti-inflammatory properties in both in vivo and in vitro models [18]. If GW405833 has anti-inflammatory effects in this HI model in the ischemic penumbra, the distribution of damage in the drug treated brains should be altered such that damage is concentrated more in the ischemic focus than in the penumbra (which should be protected by the drug). In addition, GW405833 would be expected to reduce markers of inflammation in the penumbra; damage should be reduced more by administering the drug during the secondary phase of injury than by administration before surgery; sustained drug delivery should be more effective that acute delivery; and behavioral functions associated with the penumbra should be rescued by the drug. However, none of these predicted effects were observed. For example, the distribution of damage in the brains of treated rats was not significantly different from control rats; the areas more distant from the center of damage in the graphs that show the distribution of damage in the rat brains correspond to the penumbra, but this was no more protected than the ischemic focus (the center) – and delivering the drug during the second phase of injury was no more effective than delivering the drug immediately before or after surgery. Thus not only did we not detect any general neuroprotective effect by GW405833, but our experiments also appear to provide evidence against these more specific anti-inflammatory effects.

The lack of efficacy for GW405833 in our experiments could be explained in several ways. Considering one possible explanation, it may have been that the highest doses used in our experiments may have been insufficient to suppress the inflammatory response or other pathological processes relevant to 

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