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Research report

NNZ-2591, a novel diketopiperazine, prevented scopolamine-induced acute memory impairment in the adult rat

Jian Guan*, Rong Zhang, Linden Dale-Gandar, Steve Hodgkinson, Mark H. Vickers

Liggins Institute, The University of Auckland, Auckland, New Zealand

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ABSTRACT

In rats, cyclo-L-glycyl-L-2-allylproline (NNZ-2591), a diketopiperazine, is neuroprotective after ischemic brain injury and also improves motor function in a rat model of Parkinson's disease. Given nootropic actions of diketopiperazines, we investigated the effects of and potential role for acetylcholine neurotransmission in NNZ-2591 on spatial memory after scopolamine-induced amnesia in rats.

Adult male Wistar rats were assigned to four groups: saline/water; saline/NNZ-2591; scopolamine/water and scopolamine/NNZ-2591. Morris Water Maze (MWM) tasks were used to determine spatial learning and memory. Thirty minutes prior to each of four daily acquisition trials, rats were intraperitoneally injected with either scopolamine (0.5 mg/kg) or saline. Either NNZ-2591 (30 mg/kg) or water was administered orally (gavages) 10 min after the injection. Immediately after completion of the day 4 acquisition trial a spatial probe trial was performed. The brains were then collected for immunohistochemical analysis.

Scopolamine impaired spatial learning and memory compared to saline treated group, particularly in the day 1 acquisition trial. NNZ-2591 did not reverse this deficit, however it significantly improved memory retention by showing more time spent in the correct quadrant. NNZ-2591 also counteracted the scopolamine-induced up-regulation of choline-acetyltransferase positive neurons in the striatum and similarly counteracted the increased synaptophysin density in the hippocampus. Furthermore, a scopolamine-independent antagonistic effect on muscarinic M2 acetylcholine receptors was found after NNZ-2591 treatment, supporting its modulation of acetylcholine neurotransmission. The data suggest that NNZ-2591 prevents scopolamine-induced acute impairment in memory and modulation of acetylcholine neurotransmission may be the mode of action underlying the memory improvement.

1. Introduction

Diketopiperazines (DKPs), small cyclic peptides, can be derived from the cyclisation of endogenous neuropeptides and their effects on memory have been well documented over the last few decades. For example cyclo-glycyl-proline (cGP), which has been isolated from brain tissues, improves cognitive function in rodents [1,2]. To date, the most studied DKPs are cyclic histidine-proline (cHP), a metabolite of thyrotropin-releasing hormone (TRH), and its derivatives [3–5], which have been shown to be neuroprotective and to improve memory in various rodent models of brain injury. Cyclo-L-glycyl-L-2-allylproline (NNZ-2591) is a modified analogue of cGP and has been shown to be neuroprotective following hypoxic-ischemic brain injury in rats [6]; NNZ-2591 also improves motor function in a rat model of Parkinson's disease [7] although mode of action and effects on spatial learning and memory were not examined.

The hippocampus, a brain region involved in processing memory, is an early target for aging related structural and biological impairments [8] which contribute to the loss of spatial memory [9]. Cholinergic neurotransmission in the hippocampus, striatum and medial septum of the basal forebrain are critical for spatial performance, which can be examined in rodents using the Morris Water Maze (MWM) [10]. The cholinergic effects on GABAergic, glutamatergic and dopaminergic neurotransmission are also essential neuronal circuits for learning and memory [11].

Amnesic agents, such as the muscarinic acetylcholine receptor (MAChR) antagonist scopolamine, have been used for decades in experimental animals to induce impairment in the performance of a variety of tasks requiring intact working and reference memory [12,13]. Scopolamine is a non-selective MAChR antagonist mainly targeting M1 and M2AChRs. It has been utilised in clinical studies for more than 30 years [14]. Those early studies indicated that blockade of central muscarinic receptors could induce patterns of

* Corresponding author at: Liggins Institute, The University of Auckland, 2-6 Park Avenue, Grafton 1023, Auckland, New Zealand. Tel.: +64 9 3737599x6134; DDI: 64 9 9236134; fax: +64 9 3738763.
E-mail address: j.guan@auckland.ac.nz (J. Guan).

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cognitive impairment in young subjects, changes reminiscent of those observed in the aged or in individuals with Alzheimer's disease [15]. In order to tease out the neuroprotective effects that result in the symptomatic cognitive benefits seen in animals, from the possibility of an acute nootropic effect, it is essential to study the drugs in an acute model of non-degenerative impairment. Therefore, using a pharmacological approach, the current study examined the potential effects and the mode of action of NNZ-2591 after scopolamine-induced acute memory impairment in rats.

2. Methods

2.1. Animal experimental procedures

The animal experimental procedures were approved by The Animal Ethics Committee at the University of Auckland and all efforts were made to minimize animal discomfort and reduce the number of animals used. Young adult Wistar rats (male, 4 months of age) were purchased from Harlan, the Netherlands. Animals were housed under standard conditions with a 12:12 light–dark cycle, a temperature and humidity controlled environment and ad-libitum access to food (Harlan-Teklad diet 2018) and water.

Rats were acclimatised to the experimental environments and handling by the experimenters for 2 weeks prior to the start of the study. Rats were then randomly allocated into four treatment groups (n = 12–16 per group) as (1) saline/water; (2) saline/NNZ-2591; (3) scopolamine/water and (4) scopolamine/NNZ-2591. The acute effects of NNZ-2591 were assessed in two groups of animals with or without scopolamine-induced memory impairment to determine any direct pharmacological effect on memory processing. The dose of scopolamine used is well cited in the rodent behavioural literature [16]. The effects of scopolamine and NNZ-2591 on spatial memory were examined using the MWM test for 4 days of acquisition testing. Thirty minutes prior to the first trial of each testing day, either scopolamine (Sigma–Aldrich, 0.5 mg/kg in sterile saline) or saline only was administered by intraperitoneal (i.p.) injection. Either NNZ-2591 (30 mg/kg) or water only was then administered orally (gavage) 10 min after the i.p. administration. The dose of NNZ-2591 was determined based on the effective dose derived from previous rat models [6,7]. The acquisition testing commenced 20 min following the oral administration. The spatial probe trial was tested immediately after the last trial of day 4 acquisition testing. Rats were deeply anaesthetised with sodium pentobarbital (125 mg/kg, i.p.) followed by transcardial perfusion with normal saline followed by 4% paraformaldehyde. Brains were collected for immunohistochemistry within 2 h of completion of the spatial probe.

2.2. Morris Water Maze tests (MWM)

Apparatus: The MWM tests were carried out in a room with dimmed lighting, the equipment surrounded by multiple distal cues (e.g. a white door, a bright yellow bucket and a colorful picture on the wall). A black circular pool (0.6 m × 1.3 m in diameter) was filled with water (consistently maintained at 20–22 °C); a black platform (10 cm × 10 cm) was submerged 2 cm below the water surface and was located in the northeast (NE) quadrant 20 cm away from the wall.

Acquisition testing: During the acquisition testing phase, each rat was given four trials on each testing day for 4 days, with 10 min intervals between trials. The starting position was randomized over each testing day but remained the same for all the rats in each trial. The platform remained in the northeast (NE) quadrant throughout the tests. During each trial, the rat was placed in the water with its head pointed towards the side wall and allowed 90 s to search for the hidden platform. The latency, distance travelled and speed taken to mount the platform was recorded and analyzed with automated tracking software (ANY-maze, v4.2, Stoelting, USA). The rat remained on the platform for 15 s before being removed. Rats that were unable to locate the platform within 90 s were guided to the platform.

Spatial probe trial: A spatial probe trial was used for examining the retention of spatial reference memory immediately after the completion of acquisition testing on day 4. In the spatial probe, the platform was removed from the pool and the rat was allowed to spend 30 s to search for the absent platform before being removed from the pool. The time spent and the swimming speed in the NE quadrant were recorded and analyzed for the retention of spatial reference memory.

2.3. Histology and immunohistochemistry

The methods utilised for brain tissue preparation have been described previously [17]. The brains were removed and placed in the same fixative for a minimum of 48 h before being processed and paraffin embedded. Sequential coronal sections (8 μm) containing the striatum and hippocampus were cut and mounted on chrome-alum coated slides for immunohistochemical staining. Each slide contained three sections with intervals of 40 sections (e.g. 1, 41, 81; 2, 42, 82) and a total of nine sections (or three slides) were used for stereological analysis. The sequential sections from AP + 10.6 to AP + 8.6 mm and from AP + 6.2 to AP + 4.3 mm from the interaural zero planes [18] were used for the parameters examined in the striatum/medial septum and the parameters examined in the hippocampus, respectively. The same sequential sections were used for each parameter staining. The immunostaining was performed simultaneously across treatment groups.

Primary antibodies against muscarinic acetylcholine receptor 2 (M2AChR), choline-acetyltransferase (ChAT), synaptophysin, glutamic acid decarboxylase (GAD), tyrosine hydroxylase (TH) and glutamate receptor-1 (GluR-1) were used to mark neuronal phenotypes, terminals and receptors in the striatum, hippocampus and medial septum. The sections were deparaffinized in xylene, dehydrated in a series of ethanol concentrations and incubated in 0.1 M phosphate-buffered saline (PBS). For antigen unmasking, sections were microwave boiled in 10 mM sodium citrate buffer (pH 6.0) for 1 min. All sections were pretreated with 1% H_2O_2 in 50% methanol for 30 min to quench endogenous peroxidase activity, then incubated with 1.5% normal horse serum/PBS at room temperature to block non-specific staining. The sections were then incubated with the following primary antibodies: rabbit anti-GAD antibody (Sigma, 1:1000), goat anti-ChAT antibody (Chemicon, 1:100), rabbit anti-TH antibody (Protos Biotech Corparation, 1:500), mouse anti-synaptophysin (Sigma, 1:200), rabbit anti-GluR-1 (Chemicon, 1:10) and rabbit anti-AChR M2 (Sigma, 1:150) at 4 °C for 48 h. Sections were incubated with either biotinylated horse anti-mouse or goat anti-rabbit or rabbit anti-goat secondary antibodies accordingly (1:200, Sigma) at 4 °C overnight. ExtrAvidin (Sigma, 1:200) was applied for 3 h at room temperature, and then 0.05% 3,3-diaminobenzidine (DAB) was added for a brown reaction product.

Using light microscopy (×4, Nikon 800, Tokyo, Japan) and an image analyzer (SigmaScan Pro 5.0, SPSS, Chicago, USA) the average density of GluR-1 and synaptophysin in the CA1-2, CA3 and CA4 sub-regions of the hippocampus and TH and GAD terminal staining in the striatum were measured in both hemispheres. The average density was calculated from nine sequential sections. Similarly the number of M2AChR- and GAD-positive neurons in the hippocampus and ChAT-positive neurons in the striatum and the medial septum were counted in both hemispheres of each section, the area of each section used for cell counting measured using the image analyzer and the total number of positive cells/mm^3 from nine sequential sections calculated using the formula:

$$\frac{N}{\mathrm{mm}^3} = \sum \left(\frac{N_1}{A_1} \times I + \frac{N_2}{A_2} \times I + \ldots + \frac{N_9}{A_9} \times I \right)$$

Σ, sum; N_{1-9}, the number of neurons from each of nine sections; A_{1-9}, the area (mm^2) used for assessing the number of neurons; I, the interval between sections is 0.328 mm.

2.4. Data analysis

The effects of scopolamine and NNZ-2591 on spatial reference learning were analyzed using two-way ANOVA repeated measures with training days treated as a dependent factor. The effects of scopolamine and NNZ-2591 on the retention of spatial memory and the immunoreactivity of the neuronal phenotypes, receptors and terminals were analyzed using two-way ANOVA followed by Bonferroni post-hoc testing for specific scopolamine-dependent or -independent effects of NNZ-2591. The data are presented as means ± SEM.

3. Results

3.1. Acquisition of MWM

Latency to platform was significantly different between the treatment groups ($p < 0.0001$, $f = 21.70$ [150,3], n = 12–16, Fig. 1A) and across days of acquisition tests ($p < 0.001$, $f = 133.78$ [150,3] with significant interaction between treatment group and training day ($p < 0.001$, $f = 5.04$ [150,9]), suggesting that the differences between groups was dependent on the day of testing. On day 1 of acquisition testing, both scopolamine treated groups (scopolamine/water and scopolamine/NNZ-2591) showed a significant increase in latency to the platform compared to respective saline controls ($p < 0.01$). While the differences remained significant ($p < 0.01$) between scopolamine/water (35.5 ± 4.7) and saline/water (20.4 ± 3.5) groups on day 2, the latency of the scopolamine/NNZ-2591 group (24.1 ± 2.3) became similar to the saline/control group (saline/NNZ-2591, 23.2 ± 3.1). Post-hoc testing failed to show any statistically significant effects of NNZ-2591 on the latency to the platform compared to water treated controls.

Distance travelled: Two-way ANOVA showed significant differences between the treatment groups ($p = 0.001$, $f = 17.79$ [127,7], n = 9–11, Fig. 1B) and across training days ($p < 0.0001$, $f = 71.26$ [127,3]), with significant interaction between them ($p = 0.005$, $f = 3.6$ [127,9]). The post-hoc test showed that the two groups