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Research report

WIN55,212-2 induced deficits in spatial learning are mediated by cholinergic hypofunction

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ABSTRACT

Cannabinoids acting on CB_1 receptors induce learning and memory impairments. However, the identification of novel non-CB_1 receptors which are insensitive to the psychoactive ingredient of marijuana, Δ^9-tetrahydrocannabinol (Δ^9-THC) but sensitive to synthetic cannabinoids such as WIN55,212-2 (WIN-2) or endocannabinoids like anandamide lead us to question whether WIN-2 induced learning and memory deficits are indeed mediated by CB_1 receptor activation. Given the relative paucity of receptor subtype specific antagonists, a way forward would be to determine the transmitter systems, which are modulated by the respective cannabinoids. This study set out to evaluate this proposition by determination of the effects of WIN-2 on acquisition of spatial reference memory using the water maze in rats. Particular weight was given to performance in trial 1 of each daily session as an index of between-session long-term memory, and in trial 4 as an index of within-session short-term memory. Intraperitoneal (i.p.) administration of WIN-2 (1 mg/kg and 3 mg/kg) prior to training impaired long-term, but not short-term memory. This deficit was not reversed by the CB_1 antagonists/inverse agonists Rimonabant (3 mg/kg i.p.) and AM281 (0.5 mg/kg i.p.), but recovered in the presence of the cholinesterase inhibitor rivastigmine (1 mg/kg). Reversal by rivastigmine was specific to WIN-2, as it failed to reverse MK801 (0.08 mg/kg) induced learning impairments.

Collectively, these data suggest that in this spatial reference memory task WIN-2 causes a reduction in cholinergic activation, possibly through a non-CB_1-like mechanism, which affects long-term but not short-term spatial memory.

1. Introduction

Delta-9-tetrahydrocannabinol (Δ^9-THC), the main constituent of the hemp plant *Cannabis sativa* has long been used as a recreational drug, but understanding its functions in the central nervous system has been greatly advanced by the discovery of selective cannabinoid receptors (CB_1 [34]; CB_2 [38]). In vivo, cannabinoid receptors are activated by endogenous ligands such as arachidonoyl ethanolamide (anandamide), 2-arachydonoyl glycerol, or arachidonyl glyceryl ether (noladin ether) [44]. Synthetic compounds that selectively stimulate or antagonise CB_1 or CB_2 receptors have been developed to assess the effects of marijuana on cognitive processes and potential therapeutic applications [53]. In humans these drugs are psychoactive and may lead to euphoria, enhancement of sensory perception and, above all, severe memory impairments [35,15].

Cognitive effects of cannabinoids have been investigated in numerous learning paradigms in rodents with the overall result that both acute and repeated administration of Δ^9-tetrahydrocannabinol (Δ^9 THC), HU210, WIN55,212-2 (WIN-2), CP-55,940, or anandamide impaired learning and memory [9,49,50,46]. From this work, it appears that cannabinoid receptors are particularly important for encoding of task-specific information required to perform working/short-term memory paradigms [22–24]. Co-administration or pre-treatment with CB_1 antagonists including Rimonabant and AM281 reversed the impairments in learning and memory processes induced by the cannabinoid agonists [33,30,32,5,22–24,36,8]. These data suggest that CB_1 receptor activation is responsible for the cognitive decline.

These seemingly consistent findings of CB_1 receptor stimulation-induced memory deficits are complicated by the discovery of novel cannabinoid receptors. A G protein-coupled, non-CB_1, Δ^9 THC-insensitive receptor has been identified in the brain of CB_1 knock-out mice [12,21,4]. It shows high expression

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levels in hippocampus, is activated by WIN-2 and anandamide, does not affect GABA release but inhibits glutamatergic neurotransmission [4,21]. In addition there is recent evidence to support that the orphan G protein coupled receptor GPR55 is a cannabinoid receptor. GPR55 is differentially sensitive to various plant, synthetic and endogenous cannabinoids including AM251 (an antagonist/inverse agonist at CB_1 receptors), Δ^9-THC, HU210, CP55,940 and anandamide, and are blocked by the non-psychoactive phytocannabinoid cannabidiol [55]. However, WIN-2 and AM281 neither activate nor antagonise the receptor [56]. The discovery and cloning of these novel cannabinoid-sensitive receptors offers an alternative interpretation of previous data making it possible that some effects of exogenously administered synthetic cannabinoids are due to non-CB_1 receptor mediated actions. One way of distinction thus may be the identification of the transmitter system that is affected by the cannabinoid and attempt reversal by re-activation through transmission enhancers. This study attempts to highlight the functional connection between cannabinoids and the cholinergic system.

Cannabinoids are known to regulate the release of excitatory and inhibitory transmitters, and CB_1 receptors in particular have been linked with cholinergic, glutamatergic, GABAergic and dopaminergic neurotransmission [1,49]. While initial studies indicated an indirect link between cannabinoid receptors and cholinergic activation (reviewed in [50]), there is also evidence for the expression of pre-synaptic CB_1 receptors on cholinergic nerve terminals suggesting a direct inhibition of acetylcholine release [20]. Thus, in vivo effects of cannabinoids could be due to cholinergic hypofunction and not due to the curtailing actions on GABA-ergic release [45,27]. However, this is difficult to dissociate with cannabinoid receptor antagonists since high doses of Rimonabant (and presumably AM281) increase hippocampal acetylcholine release [16,17] suggesting that CB_1 receptors are constitutively or tonically active. Nevertheless, cholinergic hypofunction may be countered by cholinesterase inhibitors to re-establish normal cholinergic tone; the reduction in GABAergic inhibition, however, would be difficult to counteract by systemic administration of drugs.

Indeed, cholinesterase inhibitors differentially affect cannabinoid induced working memory deficits: impairments induced by CP,55-940 and Δ^9-THC were reversed by eptastigmine [3] as well as physostigmine and tetrahydroaminoacridine [37]. In light of the selectivity of Δ^9-THC for CB_1, these data suggest a direct link between cannabinoid and cholinergic system. However, this observation is not consistent and [30] have failed to reverse working memory deficits with physostigmine. We thus re-examined this issue using WIN-2, an exogenous cannabinoid agonist with mixed receptor properties together with the anti-Alzheimer drug rivastigmine (Exolon©). We report a novel spatial long-term memory deficit induced by WIN-2, which is reversed by rivastigmine.

2. Material and methods

2.1. Subjects

Male Lister Hooded rats obtained from a commercial source (Rowett Research Institute, Aberdeen, UK) weighing 250–350 g at the start of testing were used in all of the experiments. Animals were group housed (4 per cage) in climatised holding rooms with free access to food and water and maintained on a 12 h day/night cycle (lights on at 7 a.m.). All experiments were performed between 9 a.m. and 5 p.m. and in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines as well as the UK Animals (Scientific Procedures) Act 1986.

2.2. Drug treatments and groups

Animals were assigned to 1 of 11 possible drug conditions with $n = 9$ in each group: (1) Tween 80; (2) WIN-2 (1 mg/kg); (3) WIN-2 (3 mg/kg); (4) WIN-3 (1 mg/kg); (5) WIN-2 (1 mg/kg) + AM281 (0.5 mg/kg); (6) WIN-2 (1 mg/kg) + Rimonabant (3 mg/kg); (7) WIN-2 (1 mg/kg) + rivastigmine (1 mg/kg); (8) WIN-2 (1 mg/kg) + scopolamine hydrobromide (0.2 mg/kg); (9) saline; (10) MK801 maleate (0.08 mg/kg); (11) MK801 maleate (0.08 mg/kg) + rivastigmine (1 mg/kg). Doses were chosen based on previous studies from our group and others [47,48,51,13,11,60,31]. Animals were run in a series of replications with control groups (saline, Tween 80, or WIN 55,212-3 (WIN-3), the inactive isomer to WIN-2) present in each replication. They were in turn for presentation purposes re-organised into a logic sequence of four experiments. Rimonabant was supplied by Sanofi (Montpellier, France). All other drugs were purchased from commerical sources; WIN-3 and Scopolamine, (Sigma–Aldrich, Poole, UK), WIN-2, MK801 and AM281 (Tocris Cookson,Bristol, UK) and Rivastigmine (Novartis, West Sussex, UK). All cannabinoids were freshly prepared each day by combining the cannabinoid/ethanol stock solutions with a vehicle of Tween 80. The solutions were evaporated using a rotary evaporator and further diluted with saline (0.9%) in order to produce the required final doses. Tween 80 was prepared in a similar manner but with the drug omitted. Scopolamine, rivastigmine and MK801 were mixed in saline. All drugs were sonicated before being injected intraperitoneally (i.p.) 30 min prior to each daily training session at an injection volume of 5 ml/kg body weight. Injection times and doses were in line with our previous studies [11,13,47,51,52]. In case of multiple drugs, antagonists were administered first followed 2–3 min later by the cannabinoid; in case of reversal by rivastigmine, it was always administered last.

2.2.1. Behavioural testing

2.2.1.1. Apparatus. A circular white Perspex water maze (150 cm diameter, 50 cm depth) was positioned in a room surrounded by numerous extra-maze cues. It was filled with water ($25 \pm 2\,^{\circ}C$) to a depth of 35 cm and a clear Perspex platform (10 cm diameter) was submerged 1–2 cm below the surface at pre-determined locations as the only means of escape. The swim paths of the animals were recorded by an overhead video camera and tracker for online storage and analysis using the PC based software (Ethovision 3.1, Noldus, NL).

2.2.1.2. Training. A standard reference memory task was employed with the platform location remaining constant for each given animal throughout training [47,51,52]. The platform was positioned in the centre of one pool quadrant (NE, NW, SE, SW) and these were counterbalanced for all treatment groups. Animals were naïve at the start of training and given four trials per day for four consecutive days. They were released from four cardinal release sites (N, S, E, W) in a pseudo-random manner facing the wall of the pool and given a maximum time allowance of 90 s to find the platform and climb onto it. Animals that failed to locate the platform within this allotted time period were guided to the platform by the experimenter. All rats remained on the platform for 30 s before being removed, towel dried and returned to their home cage for an inter-trial interval of 30 s before the next trial.

2.2.1.3. Data analysis. The path length taken to locate the hidden platform was recorded for each trial during acquisition. Particular weight was given to trial 1 each day as an index of between-session learning reflecting long-term memory (24 h), while trial 4 may be an index for within-session learning reflecting short-term memory [51]. Behavioural analysis of the drug treatments with a focus on trial 1 and trial 4 was performed in order to distinguish between these two memory processes. Other behavioural parameters analysed included swim speed and thigmotaxis (time spent swimming in the outer 10% close to the pool walls). Data are expressed as group means ± SEM and were analysed using the computer based statistics package Graphpad Prism (Version 4.01 for Windows, Graphpad software, San Diego, CA, USA). Repeated measures two-way analysis of variance (ANOVA) with treatment and days as factors were employed, followed by further planned comparisons and post hoc tests to determine the sources of reliability. Significance levels were set to $p < 0.05$ and only reliable terms are given in the text.

3. Results

3.1. Experiment 1: acquisition of a spatial reference memory is impaired by WIN-2

Since the majority of previous work has focused on working memory paradigms using the radial arm maze or delayed-match/non-match-to-position tasks our first objective was to establish whether systemic administration of WIN-2 would affect spatial reference memory in the water maze. Results are summarised in Fig. 1 with trial 1 and trial 4 of each day presented as long-term and short-term memory measures, respectively [51]. For trial 1 (Fig. 1A) there was an impairment in both WIN-2 groups (1 and 3 mg/kg) compared with Tween 80 or the inactive isomer WIN-3 (1 mg/kg). This was supported by statistical analysis which yielded a main effect of day ($F(3,96) = 49.5$; $p < 0.0001$) indicat-