British Journal of Pharmacology (2007) 151, 688–700
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

The synthetic cannabinoid HU210 induces spatial memory deficits and suppresses hippocampal firing rate in rats

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Background and purpose: Previous work implied that the hippocampal cannabinoid system was particularly important in some forms of learning, but direct evidence for this hypothesis is scarce. We therefore assessed the effects of the synthetic cannabinoid HU210 on memory and hippocampal activity.
Experimental approach: HU210 (100 μg kg^{-1}) was administered intraperitoneally to rats under three experimental conditions. One group of animals were pre-trained in spatial working memory using a delayed-matching-to-position task and effects of HU210 were assessed in a within-subject design. In another, rats were injected before acquisition learning of a spatial reference memory task with constant platform location. Finally, a separate group of animals was implanted with electrode bundles in CA1 and CA3 and single unit responses were isolated, before and after HU210 treatment.
Key results: HU210 treatment had no effect on working or short-term memory. Relative to its control Tween 80, deficits in acquisition of a reference memory version of the water maze were obtained, along with drug-related effects on anxiety, motor activity and spatial learning. Deficits were not reversed by the CB_1 receptor antagonists SR141716A (3 mg kg^{-1}) or AM281 (1.5 mg kg^{-1}). Single unit recordings from principal neurons in hippocampal CA3 and CA1 confirmed HU210-induced attenuation of the overall firing activity lowering both the number of complex spikes fired and the occurrence of bursts.
Conclusions and implications: These data provide the first direct evidence that the underlying mechanism for the spatial memory deficits induced by HU210 in rats is the accompanying abnormality in hippocampal cell firing.
British Journal of Pharmacology (2007) **151,** 688–700; doi:10.1038/sj.bjp.0707273; published online 14 May 2007

Keywords: cannabinoid; HU210; SR141716A; AM281; spatial learning; water maze; single unit recording; hippocampus; rat

Abbreviations: BLA, basolateral amygdala; DMTP, delayed-matching-to-position; DNMS, delayed-non-match-to-sample; FR, mean frequency; IBI, inter-burst interval; i.p., intraperitoneally; ISI, inter-spike interval; ITI, inter-trial interval; LC, locus coeruleus; *S*, surprise; Δ^9-THC, Δ^9-tetrahydrocannabinol

Introduction

The identification of cannabinoid receptors and the development of synthetic cannabinoids led to significant advances in assessing the effects of marijuana on cognitive processes. It enabled research to develop more specific pharmacological tools for endogenous receptors and confirmed the long-held hypothesis that marijuana intake leads to receptor-mediated specific alterations in mental abilities (Riedel and Davies, 2005). However, a better understanding of these effects on learning and memory function is warranted, since marijuana is the most widely used recreational drug and also because of its potential for therapeutic applications (Pertwee, 2000; Robson, 2001).

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Received 9 January 2007; revised 27 February 2007; accepted 14 March 2007; published online 14 May 2007

Cannabinoid CB_1 receptors are widely distributed throughout the central nervous system (CNS), with a particularly high density in cerebral cortex and hippocampus. This anatomical location correlates well with effects of cannabinoids on memory formation. CB_1 receptor agonists have been investigated on numerous occasions using different behavioural paradigms. Both acute and chronic administration of CB_1 agonists, including the primary psychoactive constituent of marijuana, Δ^9-tetrahydrocannabinol (Δ^9-THC), the endocannabinoid anandamide and the synthetic cannabinoids, WIN-55212,2 and CP55940, induced learning and memory impairments in rats and mice (for review see Davies *et al.*, 2002; Robinson *et al.*, 2004; Robinson and Riedel, 2004). Severe impairments occur in the short-term domain of spatial memory (for reviews see Robinson *et al.*, 2004; Robinson and Riedel, 2004). Despite its wide use in memory research, application of the open-field water maze to cannabinoid research is relatively

recent. Varvel *et al*. (2001), for instance, reported impairments in spatial reference and working memory after Δ^9-THC administration in mice. In the reference memory version, mice overtrained to the platform location were subsequently impaired at doses of Δ^9-THC that also produced gross sensory and motor disturbances ($100\,mg\,kg^{-1}$). By contrast, mice were deficient at a much lower dose ($3–5\,mg\,kg^{-1}$), with less severe side effects in a working memory version, in which the platform was changed to a new position each day. Reference and working memory versions of the water maze, however, access two different memory mechanisms. Overtraining in the spatial reference memory task followed by drug administration tests effects of Δ^9-THC on recall. Such memory retrieval is insensitive to cannabinoid treatment (Da Silva and Takahashi, 2002) and deficits only occur with drug doses severely impeding motor coordination (see Varvel *et al*., 2001).

By contrast, despite extensive training in the working memory task, animals still learn the novel platform location and this encoding of spatial information is CB_1 sensitive (Hampson and Deadwyler, 1998, 1999, 2000, 2003). Our work proved that hippocampal CA3 and CA1 neural firing rates were not only reduced in the presence of Δ^9-THC and WIN 55212-2, but behavioural task-specific firing rate changes were also suppressed in rats performing a spatial delayed-non-match-to-sample (DNMS) task. In those studies, hippocampal principal cell firing increased 10- to 20-fold over background (1–5 Hz) within ± 1.5 s of the Sample (encoding) and Nonmatch (recall) phases of DNMS trials. Despite a 20–40% decrease in the number of correct DNMS trials, Δ^9-THC and WIN 55212-2 suppressed this firing peak only for the Sample and not the Nonmatch phase, suggesting that cannabinoids selectively impaired the encoding of trial-specific working memory but not memory retrieval.

The effect of HU210, a synthetic analogue of Δ^9-THC, has not been investigated in detail in learning paradigms and physiological recordings. HU210 is a classical cannabinoid with high lipophilicity. Classical cannabinoids are tricyclic dibenzopyran derivatives occurring naturally in cannabis (Δ^9-THC) or synthetic analogues of these compounds, as is the case for HU210 (for review see Howlett *et al*., 2002). Efficacy at both $CB_1$and CB_2 receptors is similar to that of other cannabinoids; however, the affinity of HU210 for these receptors is higher (Pertwee, 2001; Howlett *et al*., 2002). This results in HU210 being a potent cannabinoid agonist with long-lasting pharmacological effects *in vivo*. An initial study by Ferrari *et al*. (1999) revealed a drug-induced dose-dependent learning deficit in spatial reference memory tested in the water maze, but there was no effect on a visible platform task. The deficit was interpreted as being due to a spatial learning impairment, presumably due to CB_1 receptors located in hippocampus. Drug effects on parameters of anxiety were also presented, inviting the alternative interpretation that HU210, similar to Δ^9-THC, may have caused place aversion (Cheer *et al*., 2000). A more recent account extended these results and revealed a deficit in the acquisition of a working memory task in the water maze in rats pretreated with HU210 for 15 consecutive days (Hill *et al*., 2004). The working memory deficit was specific for inter-trial interval (ITI) of 5 min but not for 30 s. Since animals were naïve to the task, there was also a deficit in procedural learning, making it difficult to determine the contribution of hippocampus and spatial deficits to the overall acquisition impairment. A more detailed analysis is warranted, which should also explore whether HU210 effects on spatial learning are possibly mediated via the hippocampal cannabinoid system.

Our main aims were first the detailed assessment of the effects of HU210 on spatial learning and memory in rats using the open-field water maze. Experiment 1 employed a working memory task using a delayed-matching-to-position (DMTP) paradigm as described previously (Roloff *et al*., 2002a, b). Performance on a DMTP task in the water maze is sensitive to hippocampal lesions, intra-hippocampal *N*-methyl-D-aspartate (NMDA) receptor blockade (Steele and Morris, 1999), scopolamine treatment, combined β-amyloid and scopolamine exposure (Roloff *et al*., 2002b), and Δ^9-THC administration to stimulate CB_1 receptors (Varvel *et al*., 2001; Da Silva and Takahashi, 2002; Fadda *et al*., 2004). As we did not observe the expected results, experiment 2 repeated the work of Ferrari *et al*. (1999). This yielded the proposed deficit and we then assessed, whether the effect is mediated by CB_1 receptors using co-administration of the two CB_1 antagonists, SR141716A and AM281, with HU210 in some groups.

The second aim of the study was to determine whether the observed behavioural deficits in spatial reference memory could have resulted from alterations in hippocampal neural firing. Recent publications by Pistis and co-workers (Pistis *et al*., 2004; Muntoni *et al*., 2006) provide contrasting results for the effects of cannabinoids on firing of locus coeruleus (LC) and basolateral amygdala (BLA) neurons *in vivo*. WIN-55212-2 and Δ^9-THC increased firing of LC neurons, while WIN-55212-2 and HU210 decreased firing of BLA neurones in anaesthetized animals. Given that we have shown cannabinoid-elicited inhibition of hippocampal neural firing in behaving animals, it is important to confirm these effects with HU210.

Materials and methods

Subjects

Male Lister Hooded rats from commercial sources (Rowett Research Institute, Aberdeen or Harlan, UK), aged 7–8 weeks and weighing 250–300 g at the start of training were used in all experiments (exp. 1, $N=8$; exp. 2, $N=63$; exp. 3, $N=8$). Subjects were group housed (four per cage), with free access to food and water on a 12:12 h day/night cycle (lights on at 0700 am). Animals were housed in a pathogen-free animal facility in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines. Recording took place between the daylight hours of 01000 am and 0400 pm. All experiments were performed under UK Home Office regulations.

Drug treatment and groups

Stock solutions of HU210 ($5\,mg\,ml^{-1}$) (Tocris Cookson, Bristol, UK), AM281 ($10\,mg\,ml^{-1}$) (Tocris Cookson, Bristol,

UK) and SR141716 A (10 mg ml^{-1}) (Research Triangle Inst., Natl. Inst. Drug Abuse, Cary, NC, USA) in ethanol were used. Drugs were prepared fresh each day using these stock solutions in a vehicle of Tween 80, evaporated and further diluted with saline (0.9%) to the final doses of HU210 100 μg kg^{-1}; SR 141716 A 3 mg kg^{-1}; AM281 0.5 mg kg^{-1} and 1.5 mg kg^{-1}. Tween 80 was used as the vehicle in this study. Drug doses were selected based on published efficacy of the respective doses in behavioural terms in Lister Hooded rats and were administered intraperitoneally (i.p.) 1 h before testing at a volume of 5 ml kg^{-1}. The antagonists were administered immediately before HU210. All drugs were administered at the same time on each day of testing.

Behavioural apparatus

A circular white Perspex water maze (150 cm diameter, 50 cm depth) was placed in a room surrounded by various spatial cues. The pool was filled with water (25 $\pm$ 2°C) to a depth of 35 cm. A clear Perspex platform (10 cm diameter) was placed at a predetermined platform position approximately 1 cm below the surface of the water. Trials were recorded by an overhead video camera and tracker with data video taped and stored online for later analysis using a PC-based software (HVS Image, Hampton, UK).

Behavioural training and testing

Experiment 1 – HU210 and DMTP performance. All animals were fully pretrained in the delayed-matching-to-sample task before testing in the presence of drug. This training lasted for at least 2 weeks (four trials per day) and performance in trial 2 of the DMTP task was below 20 s latency to find the platform. The procedure was identical to testing in the presence of drug. Testing comprised of the following protocol: HU210 injection was followed by behavioural testing an hour later. The next day was drug and test-free to allow wash-out and we administered Tween 80 on the following day and performed another test. This was again followed by an injection and a test-free day. A fully counterbalanced within-subject design was employed in which each animal went through all drug and control conditions. Twelve predetermined, randomly located platform positions distributed in all parts of the pool were selected. The platform location remained constant within each session (one session of four trials per day), but was changed between sessions. Subjects were released facing the wall of the pool from one of four cardinal release sites (N, S, E or W) in a semi-random manner (all release sites used on each day). A maximum time allowance of 90 s was set for rats to locate the submerged platform (diameter 10 cm, 1 cm below water surface), where they remained for 30 s before being returned to their cages. These were placed into a heating box to avoid hypothermia. If rats failed to find the platform within 90 s, the experimenter guided them to it.

DMTP testing involved a within-subject design, with all animals receiving both drugs and also performing at both delays in an alternating manner. The ITI between trials 1 and 2 was either 30 s or 1 h; all other ITIs were 30 s. It follows that performance in trial 2 (path length, latency and swim speed) is of particular relevance, as it reflects spatial short-term memory of today's platform location (Steele and Morris, 1999; Roloff *et al*., 2002a, b). Performance in trial 4 represents the floor level that can be achieved under drug/control conditions.

Experiment 2 – HU210 and reference memory. Reference memory was examined by following the protocol of Ferrari *et al*. (1999). The platform location was constant for each animal. It was placed at the centre of one pool quadrant and target quadrants were counterbalanced for all groups. Animals were naïve at the start of training and were given four trials per day on four consecutive days. On each trial, rats were released from one of the four release sites (N, S, E or W) facing the wall of the pool and allowed 90 s to locate the platform, where they remained for a further 30 s. All ITIs were 30 s. During ITIs, animals stayed in the heating box. If rats failed to locate the platform within 90 s, the experimenter guided them to it. A probe trial was administered 24 h following acquisition training, which lasted 60 s, with the platform removed from the pool and animals being released opposite to the target quadrant.

In experiments 1 and 2, path length taken to locate the platform on each trial, time spent in a swim corridor (Whishaw, 1984) directly connecting the release site with the platform location and latency to target area were recorded as indices of spatial memory. The target area was two times the size of the platform (20 cm diameter) and centres over the platform position. Path length was used as a measure of spatial learning and memory, as unlike latency, it also takes into account a difference in swim speed. Path length has been suggested as the most important measure of spatial memory (Lindner, 1997). Swim speed and thigmotaxis (time spent in the outer 10 percent of the pool) were assessed as measures of procedural memory.

Experiment 3 – surgery. Rats were anaesthetized under a constant flow of isoflurane (Abbot laboratories, IL, USA) and positioned in a stereotaxic frame before unilateral implantation with a multi-electrode recording array (Neurolinc, NY, USA) consisting of 16 stainless steel electrodes (40 μm) arranged in two rows (800 μm between rows), such that each row consisted of eight electrodes with 200 μm centre-to-centre spacing. The array was constructed with asymmetric lengths such that CA3 electrode tips would be automatically positioned 1.2 mm latero-ventral to the CA1 electrode tips.

The scalp was incised along the midline, pulled back to the lateral cranial ridges and single holes drilled for insertion of a 100 μm silver ground wire into the parietal cortex. The centre of the array was positioned 3.4 mm posteriorly and $\pm$2.7 mm mediolaterally from Bregma, depending on whether the array targeted the left or right hippocampus. The posterior end of the array was angled laterally such that the longitudinal axis was 30° from midline of the skull. An oval-shaped craniotomy was made approximately 2 mm larger than the array, the dura resected and the long electrode tips placed in contact with the surface of the brain. The electrode was then driven in 50 μm steps to a depth of 2.8 mm for the

CA1 leads and 4.0 mm for the CA3 leads, then lowered to a final depth of approximately 3.0 mm for CA1 and 4.2 mm for CA3, using continuous recording of neural activity to confirm optimum placement of the electrode tips in the respective cell layers. Neural activity was continuously monitored throughout surgery to ensure that the electrode array maintained placement in the appropriate hippocampal subfields. Following a brief settling time (15–20 min) for electrodes to stabilize in place, the electrode depths were adjusted, if necessary, and the craniotomy sealed with dental cement.

Electrophysiological recording

Following stabilization of the electrode array, selected principal cells with firing rate of 0.5–5 Hz were isolated using a Multiunit Acquisition Processor (MAP, Plexon Inc., Dallas, TX, USA). The neuronal ensemble activities of these preselected CA3/CA1 principal cells were tracked and recorded following the aforementioned treatments.

Recording parameters

Single neuron spike trains were analyzed using Neuroexplorer (Nex Technologies, MA, USA) software. The following parameters were computed for each selected principal cell across treatments and animals: (1) mean frequency of firing (FR, Hz) and (2) mean inter-spike intervals (ISIs). In addition, 'bursts' of spikes were characterized using the 'Surprise' method that identified sequences of at least three consecutive spikes with ISIs less than one-half of the mean ISI over all spike occurrences. Bursts were identified by: (1) calculating mean FR and ISI for all spikes, (2) identifying sequences of three or more consecutive spikes with ISIs, in which all ISIs were less than one-half the mean ISI, (3) computing 'Surprise' (S) such that $S=-\log_{10}(P)$ where probability that the same sequence of spikes could occur in a random Poisson distribution with the same mean frequency as FR, (4) maximizing S by either adding consecutive spikes to the end of the burst, or removing consecutive spikes from the beginning of the burst. Bursts with S-values >10 were then characterized according to mean burst duration, mean interburst interval (IBI), mean number of spikes in burst, mean frequency of bursts in the spike train, mean FR within bursts and mean ISI within bursts.

Data analysis

Behavioural data were analyzed 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 trials, delay and drug treatment as factors were employed. Appropriate planned comparisons including further two-way ANOVAs and t-tests were performed with the significance level set to $P<0.05$.

Electrophysiological data were analyzed by Student's t-tests (paired) to compare the means$\pm$s.e.m. between HU210 and Tween 80 across all parameters described above.

Results

General behavioural observations

It was obvious from observations on dry land surfaces that animals injected with HU210 presented with some cataleptic symptoms and, although we quantified this using the bar test as descent latencies, HU210-treated animals were not significantly different from Tween 80 controls ($df=11$; $t=1.276$; $P>0.2$) and descent latencies hardly exceeded 10 s (data not shown). The limb positions during swimming were normal and the typical forelimb inhibition was seen in all rats.

Experiment 1 – HU210 does not affect performance in DMTP in water maze

After 2 weeks of pretraining, all animals were competent in performing the DMTP task. Relative to the Tween 80 treatment, there was no impairment in trial 2 performance in rats under the influence of HU210. This was observed for path length (Figure 1a), corridor analysis (Figure 1b) and latency to target area (Figure 1c). Statistical analysis confirmed reliable effects of trial (F-values >2.7; $P\geqslant0.05$) but not drug and delay (all F-values <1.2) on all these parameters. Similarly, effects of trial (F-values >3.5; $P<0.02$) were obtained for procedural measures (swim speed and thigmotaxis; Figure 1d and e), and the main effect of drug ($F(3,112)=17.1$; $P<0.0001$) was reliable only for swim speed.

Contrary to expectation (Hill *et al*., 2004), data from this experiment indicated that despite differences in swim speed, there was no spatial working/short-term memory deficit in HU210-treated rats. Previous work (Bannerman *et al*., 1995; Cain *et al*., 1997, 2002) using the water maze suggests that many drug treatments lead to procedural deficits in naïve but not pretrained animals. We therefore reasoned that pretraining, as conducted here to familiarize subjects with the task requirements, may have eliminated differences in procedural parameters and also the spatial memory deficit. Pretraining can indeed render these learning and memory processes insensitive to drug actions (Cain *et al*., 1997, 2002) or hypoxic insult (Row *et al*., 2003), and we therefore progressed by testing naïve animals in a spatial reference memory task as previously suggested.

Experiment 2 – acquisition learning of a spatial reference memory task is impaired by HU210

The failure of HU210 to induce spatial short-term/working memory impairment in the DMTP task may be due to the previously reported procedural memory deficit (Ferrari *et al*., 1999; Hill *et al*., 2004) and the pretraining-induced familiarization with the procedural task demands may thus have rendered the task HU210 insensitive. Therefore, re-examination of the effects of HU210 on acquisition learning of a spatial reference memory as reported by Ferrari and coworkers should reveal an overall deficit. At the same time, we examined whether HU210-induced memory deficits are mediated by CB_1 receptors? Figure 2 depicts the effect of HU210 on path length required to localize the platform

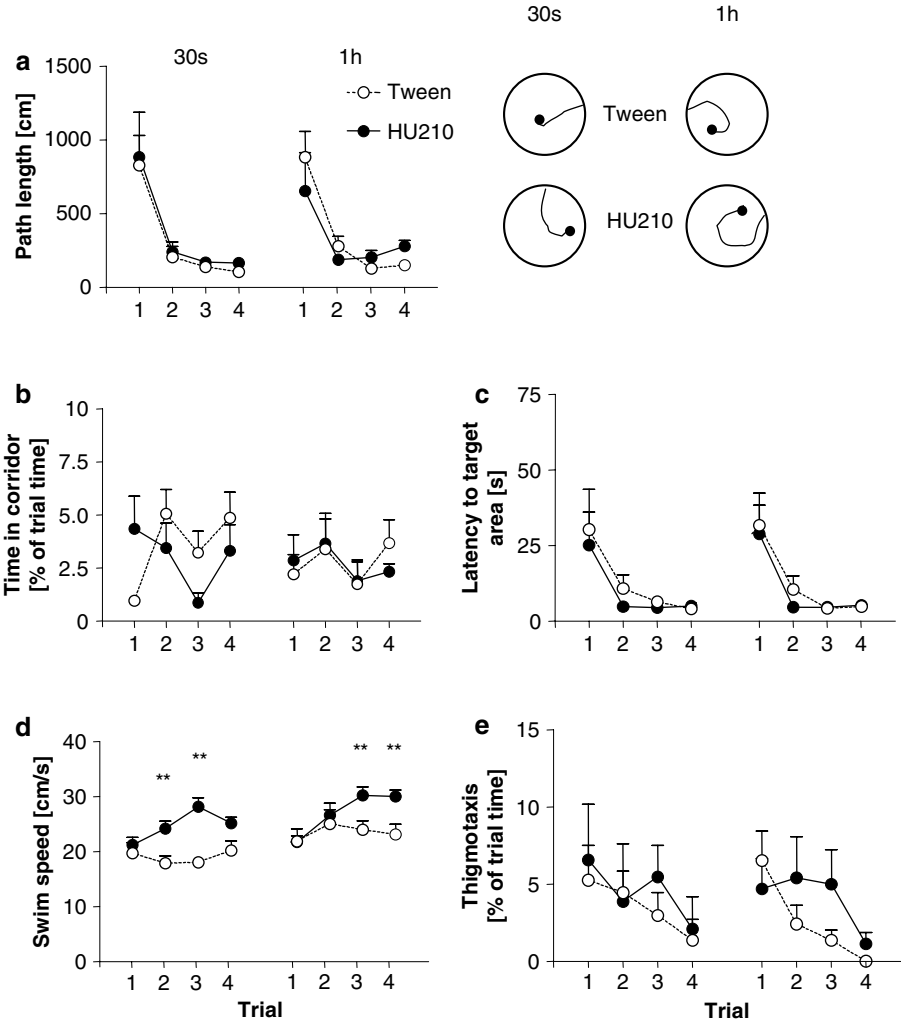

Figure 1 HU210 did not affect short-term memory assessed in a delayed-matching-to-position task in pre-trained animals. Values shown in (**a**–**e**) are means ± s.e.m. HU210 treatment did not lead to an overall increase in path length (**a**), and recorded values did not reveal any spatial (**b, c**) or thigmotaxis-related (**e**) differences between groups at either delay (30 s or 1 h). Swim speed was higher in the HU210 group (**d**; asterisks indicate $P<0.01$.). Performance improved on trial 2 independent of drug treatment. Representative swim traces indicate trial 2 performance for both delays.

during acquisition learning in a spatial reference memory task. Animals treated with HU210 swam longer distances on most trials compared with Tween 80-treated rats. This impression was confirmed statistically with a main effect of drug treatment ($F(1,240)=39.8$; $P<0.0001$), trial ($F(15,240)=22.4$; $P<0.0001$), and an interaction ($F(1,240)=3.2$; $P<0.0001$) suggesting that the deficit did not affect all trials equally. Spacing trials over repeated sessions attests different qualities to trials 1 and 4 of each session. Learning between days refers to long-term memory formation and includes processes of consolidation (Riedel and Micheau, 2001), while within-session learning (from trial 1 to 4 in our case) may be an index of short-term memory (Kesner *et al*., 1993).

Detailed behavioural analysis of all groups, with a focus on trials 1 and 4, was conducted in order to distinguish between these memory processes, and results are summarized in Figure 3. HU210 was very effective and both antagonists, AM281 and SR141716A, did not reverse the deficits. For trial 1, (Figure 3a) this was confirmed by a 7×4 factorial ANOVA, with drug treatment as between- and day as within-subject factors. Overall, there were significant effects of drug treatment ($F(6,192)=10.83$; $P<0.0001$), day ($F(3,192)=71.48$; $P<0.0001$), and an interaction ($F(18,192)=1.93$; $P=0.01$). All drug groups were significantly different to Tween 80 (all F-values >15; $P<0.0003$), apart from the AM281 1.5 mg kg^{-1} group ($F=1.1$). Both AM281 alone groups were found to be significantly different to all other HU210 groups (all F-values >16; $P<0.0001$), yet they were not different from each other ($F<4$; $P>0.05$). All HU210-containing drug treatments also did not differ from each other (all F-values <4; $P>0.05$). It is obvious, however, that all groups irrelevant of drug treatment improved their performance over days (all F-values >7.1; $P<0.001$).

A qualitatively different result was obtained for trial 4 (Figure 3b). Some drug groups were initially impaired on days 1 and 2, but then attained floor levels similar to the Tween 80 group on the fourth day of training. It appeared,

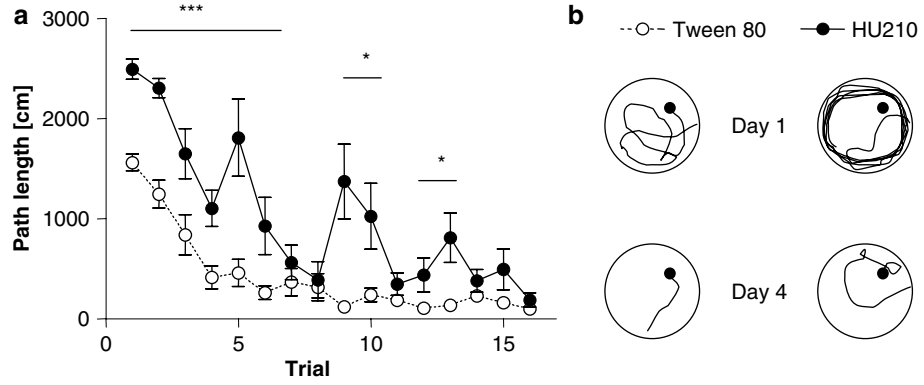

Figure 2 HU210 impaired spatial learning in the water maze. (**a**) Path length across all trials of acquisition for both Tween 80- and HU210 (100 μg kg^{-1})-treated animals. Values shown in (**a**) are means$\pm$s.e.m.. HU210 animals were severely impaired and required significantly longer swim paths to locate the platform. Asterisks indicate reliable differences (*$P<0.05$; ***$P<0.001$) between groups for the time periods indicated by the horizontal bars. In (**b**), representative swim paths indicate performance under the two drugs for the first (day 1) and final day (day 4) of training.

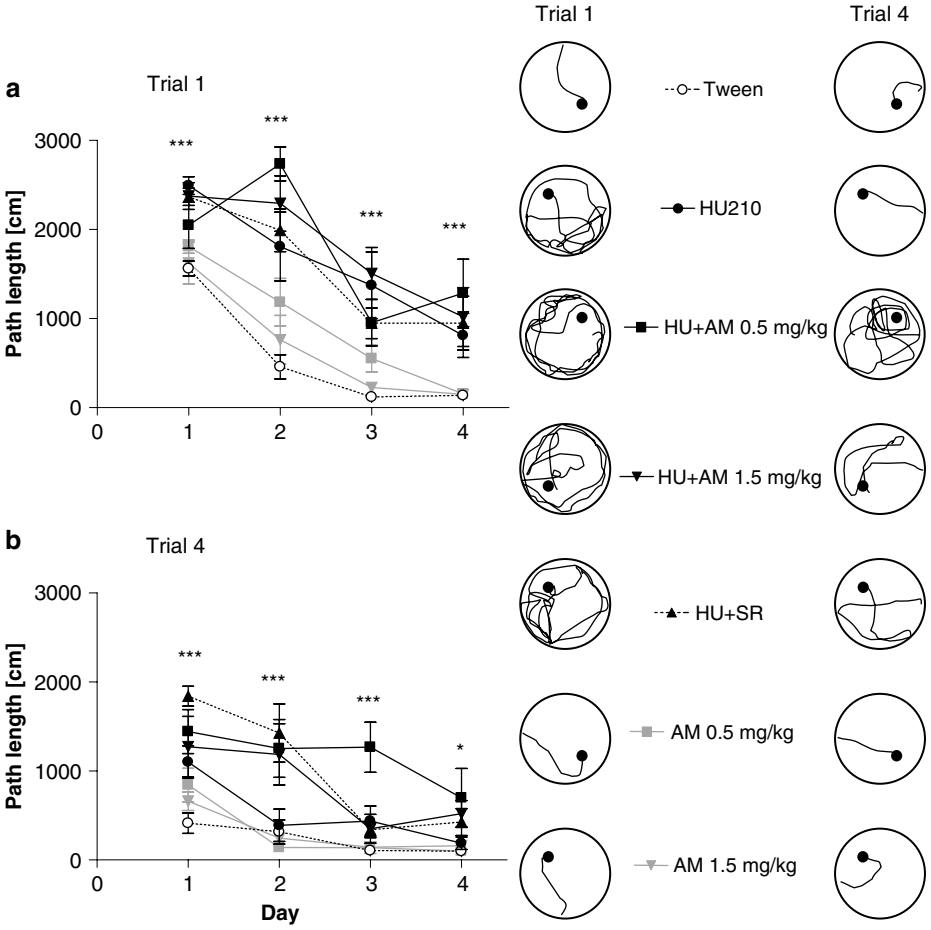

Figure 3 HU210-induced deficits in spatial learning were not reversed by cannabinoid antagonists. Values shown in (**a**) and (**b**) are means$\pm$s.e.m. (**a**) Trial 1 performance (long-term memory between sessions) of all drug groups. There was no reversal of the HU210 deficit with cannabinoid antagonists SR141716A and AM281. (**b**) Trial 4 performance (short-term memory within each session) for all drug groups. HU210 alone induced a minor deficit. Co-administration of HU210 and cannabinoid antagonists led to short-term memory deficits. Swim traces show representative examples of swim paths for all drug groups during trials 1 and 4 on day 4. Asterisks indicate significant differences between HU210-containing groups and Tween 80 controls at the specific time points (*t*-test, *$P<0.05$; ***$P<0.001$).

however, that neither AM281 nor SR141716A could reverse the deficit induced by HU210. Analysis of all treatments yielded a main effect of drug treatment ($F(6,192)=7.58$; $P<0.0001$), day ($F(3,192)=30.30$; $P<0.0001$) and also an interaction ($F(18,192)=2.32$; $P=0.0026$). As with trial 1, all treatment groups containing HU210 were significantly impaired in trial 4 relative to Tween 80 (*F*-values >26; $P<0.0001$), apart from both AM281 alone groups (*F*-values <1.8). Further

comparisons revealed that co-administration of HU210 with antagonists impaired performance compared with HU210 alone (*F*-values >6.7; $P<0.014$), but HU210 + antagonist groups did not differ from each other (*F*-values <1). These data clearly suggest that the performance of animals treated with HU210 (alone or co-administered with AM281 or SR141716A) is compromised. Interestingly, antagonist treatment enhanced the deficit observed for trial 4.

Overall, these data suggest that HU210 treatment impairs both spatial long-term and short-term memory in rats. Analysis of latency to target area (twice the size of the platform) for trial 1 (Figure 4a) confirmed a main effect of drug treatment ($P>(6,192)=3.47$; $P=0.01$), day ($F(3,192)=11.91$; $P<0.0001$) and an interaction (F (18,192) $=2.95$;$P=0.0001$); all groups treated with HU210 differed from Tween 80 controls (F's>4.52; P-values <0.05), apart from both AM281 alone groups (F-values <2.64; $P>0.1$). This strongly suggests that both antagonists (SR141716A and AM281) did not reverse the spatial long-term memory deficits induced by HU210. For trial 4 latency to target area (Figure 4b), analysis confirmed main effects of drug treatment ($F(4,128)=3.14$; $P=0.03$), day ($F2,128)=10.4$;

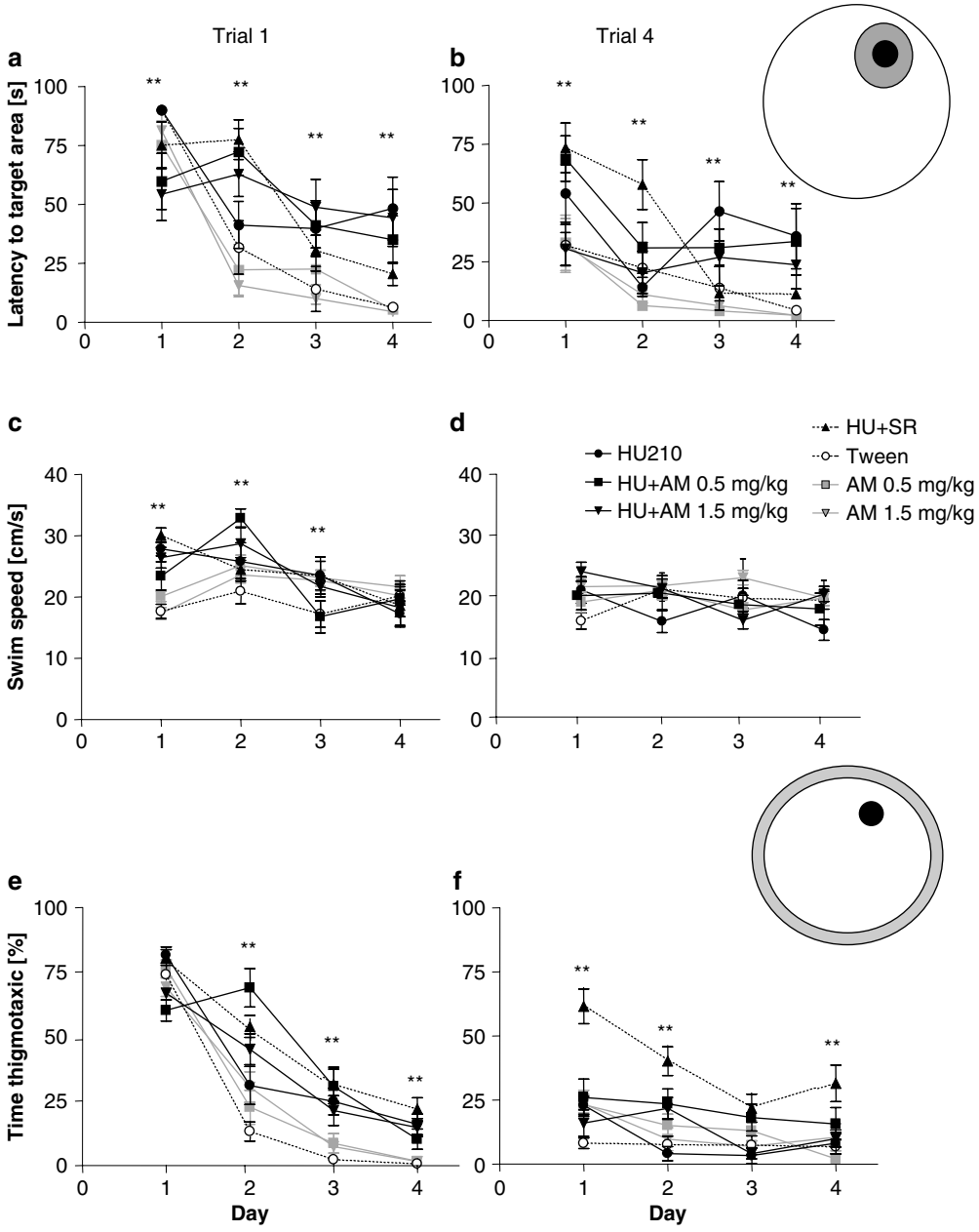

Figure 4 Analysis of spatial and non-spatial parameters monitored during acquisition of the reference memory task. Values shown in (**a–f**) are means ± s.e.m. HU210 treatment impaired performance on the spatial parameter of latency to target area (**a, b**) for trials 1 and 4. In general, co-administration of antagonists increased the deficit caused by HU210. Swim speed was increased in all HU210-treated groups for trial 1 during the initial training days (**c**); however, no difference was observed at trial 4 (**d**). All HU210-treated animals presented with severely increased levels of thigmotaxis during trial 1 (**e**), but HU210 alone had only minor effects on thigmotaxis at trial 4 (**f**). However, combined administration especially with SR141716A caused a dramatic rise in anxiety-related swimming along the edges of the pool. Asterisks indicate reliable differences between HU210-containing groups and Tween 80 controls at the specific time points (*t*-test, $^{**}P<0.01$).

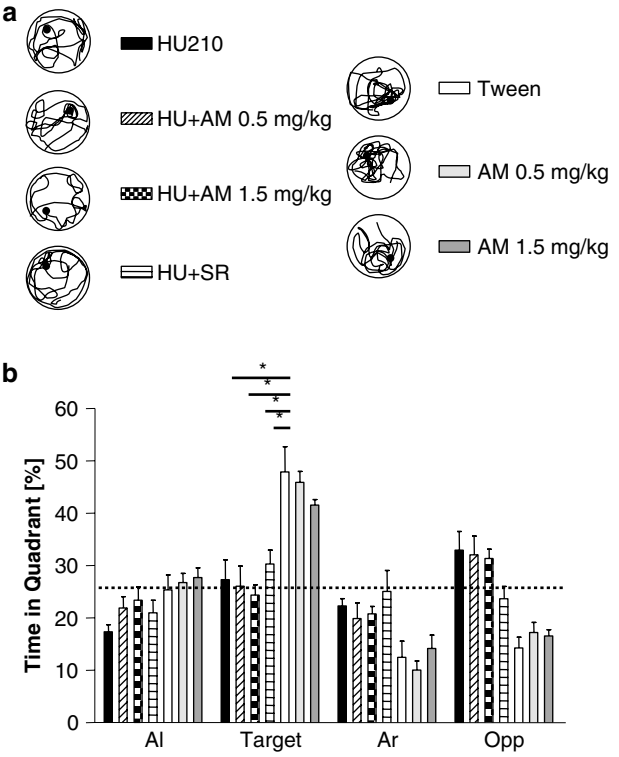

Figure 5 Probe trial conducted 24 h after the last training trial with platform removed. In (**a**), representative swim traces of individuals of the different drug groups obtained during the probe trial. In (**b**), values shown are means ± s.e.m.; horizontal line indicates chance level; Al, adjacent left; Ar, adjacent right; Opp, opposite. A spatial bias for the target quadrant was only found in animals treated with Tween 80 or AM281 alone (0.5 and 1.5 mg kg^{-1}), whereas all other groups displayed no retention. Asterisks indicate reliable difference between Tween 80 controls and HU210-containing groups indicated by the bars (*$P<0.05$).

$P<0.0001$) and an interaction ($F(12,128)=2.65$; $P=0.003$). The HU + AM 1.5 mg kg^{-1} and AM281 alone groups were the only groups that did not differ from Tween 80 (*F*-values <1.67; $P>0.2$), while all other groups were different from Tween animals (*F*-values >8.75; $P\leqslant0.01$). This suggests that the higher dose of AM281 reversed the spatial short-term memory deficit induced by HU210. This was confirmed in that the group of animals treated with (HU210 + AM 1.5 mg kg^{-1}) performed significantly better than all other HU210 groups (*F*-values $\geqslant6.4$; $P<0.02$).

Analysis of non-spatial parameters for trials 1 and 4 of each day are summarized in Figure 4c–f. At trial 1, swim speeds (Figure 4c) were not different between drug treatments ($F(4,128)=1.7$; $P=0.17$), but we obtained effects of day ($F(3,128)=17.3$; $P<0.0001$) and an interaction ($F(12,128)=3.2$; $P=0.0004$). Planned comparison confirmed that all drug groups differed from Tween 80 controls (all *F*-values >6.8; $P<0.01$), but not between each other (all *F*-values <1), suggesting that antagonists did not reverse the HU210-induced increase in swim speed. For trial 4, swim speeds (Figure 4d) were more stable and we did not obtain reliable differences between days, drug groups or an interaction (all *F*-values <2; $P>0.05$).

Data for trial 1 (Figure 4e) indicate substantially higher thigmotaxis in all groups relative to Tween 80. Statistical comparison confirmed this impression with main effects of drug treatment ($F(4,128)=10.5$; $P<0.0001$), day ($F(3,128)=176$; $P<0.0001$) and an interaction ($F(12,128)=6.7$; $P<0.0001$). All drug groups, apart from the higher dose of AM281 (1.5 mg kg^{-1}) ($F=2.7$; $P>0.1$), were significantly different from Tween 80 controls (all *F*-values >40; $P<0.0001$). Furthermore, there were subtle differences between the drug treatments, namely between HU210 and HU210 + SR141716A, as well as HU210 + SR141716A and HU210 + AM281 (1.5 mg kg^{-1}) (all *F*-values >13; $P\leqslant0.001$). There was, however, no reversal of the HU210-induced increase in anxiety. Thigmotaxis measured at trial 4 of each session (Figure 6d) revealed main effects of drug treatment ($F(4,128)=16.4$; $P<0.0001$), day ($F(3,128)=13.1$; $P<0.0001$) and an interaction ($F(12,128)=2.9$; $P=0.002$). Controls showed practically no swimming in the outer zone (apart from release). This was similar for the HU210 and AM281 (1.5 mg kg^{-1}) alone groups (*F*-values <4.32; $P>0.05$), but all drug groups combining HU210 with antagonists were significantly worse than Tween 80 and HU210 treated rats (all *F*-values >3.2, $P<0.05$); the most pronounced deficit was observed for the HU210 + SR141716A combination.

Retention test

Performance of the groups differed greatly in the probe test conducted 24 h after the last training trial (Figure 5). Compared with Tween 80 controls and the AM281 alone groups, all HU210 treatments failed to show a spatial bias for the target quadrant providing evidence for the lack of spatial memory. This was confirmed statistically in a 5 (drug treatment as between-subject factors) × 4 (quadrant as within-subject factor) factorial ANOVA. We obtained a highly significant interaction between these parameters ($F(12, 160)=7.5$; $P<0.0001$), and a *post hoc* pairwise comparison confirmed interactions for all HU210-treated drug groups relative to Tween 80 (all *F*-values >9.5; $P<0.0001$), whereas AM281 groups did not differ from controls (*F*-values <1.6; $P>0.2$). This confirms that despite some learning during each session (as indicated in trial 4 performances) and small overall improvements in performance over days, HU210-treated animals did not form a spatial memory of the location of the hidden platform.

HU210 attenuates spontaneous firing activity of principle neurons in hippocampus

Extracellular action potentials were recorded from 45 principal cells ($N=8$ animals) in hippocampal CA3 and CA1 subfields. Average firing frequency and short duration 'bursting' characteristics of the spike train were analyzed. Exposure to HU210 produced significant suppression of average firing frequency ($t=7.941$, $P<0.001$) throughout the spike trains (Figure 6). As a result, mean ISIs were significantly increased ($t=3.871$, $P<0.001$) following HU210 treatment, confirming that the decrease in spontaneous neural activity was coupled to a prolongation of the interval between any two spike occurrences in the spike train.

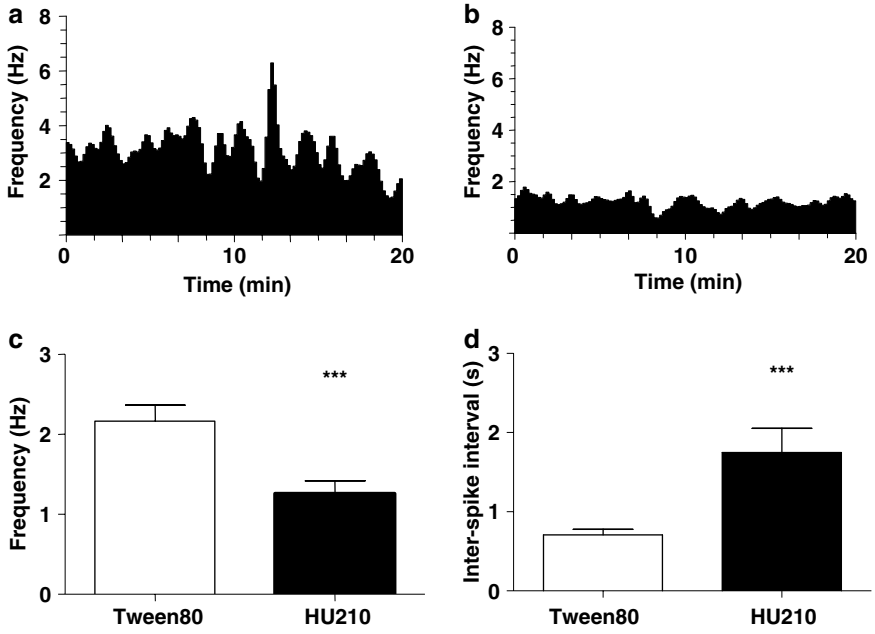

Figure 6 Spike firing properties of hippocampal cells were attenuated after HU210 treatment. The rate histograms (mean frequency/10 s bins) depict firing activity of a preselected principal cell during a 20 min recording epoch following Tween 80 (**a**) and HU210 (**b**) treatments. Values shown in (**c**) and (**d**) are means±s.e.m. of frequency (**c**) and ISIs (**d**) pooled across 1 h electrophysiological recordings calculated from 45 hippocampal principal cells located in the dorsal CA3 and CA1 subfields. Asterisks indicate $P<0.001$.

Moreover, HU210 treatment revealed a marked reduction ($t=6.057$, $P<0.001$) in the average number of bursts (Figure 7a) and number of spikes per bursts ($t=2.274$, $P<0.05$; Figure 7f) in comparison to controls (Tween 80) across all preselected principal cells. HU210 significantly attenuated the average burst duration ($t=2.409$, $P<0.05$; Figure 7b) and IBI ($t=3.848$, $P<001$; Figure 7c) in comparison to controls, suggesting that not only were spontaneous burst occurrence, spikes per burst and burst duration reduced, but the interval between successive bursts was increased as a consequence of HU210 treatment. In addition, HU210 noticeably reduced the average frequency within bursts ($t=3.816$, $P<0.001$; Figure 7d) and increased mean ISI within bursts ($t=4.220$, $P<0.001$; Figure 7e). These findings suggest that the overall changes in spike train firing characteristics were replicated in burst activity following HU210 treatment.

Finally, we also assessed the effects of AM281 against HU210-induced suppression of firing activity. In line with the behavioural results, AM281 at either 0.5 or 1.5 mg kg^{-1}, injected 1 h post-HU210 treatment, did not reverse the reduction in firing frequency or bursting (all $P>0.05$). Relative to Tween 80, depression of activity persisted (all $P<0.05$) (data not shown).

Discussion

We here report, that HU210 induced a spatial deficit in the water maze in learning a reference memory task in numerous parameters (Table 1 for summary) together with alterations in hippocampal firing patterns of single principal neurons. Pretraining in a working memory task, however, prevented these deficits.

HU210 induces spatial and non-spatial deficits

Plant-derived and synthetic cannabinoids impair working or short-term memory, with more severe effects when animals are exposed to long delays between trials (for review see Deadwyler *et al*., 1995; Robinson and Riedel, 2004), which is consistent with the involvement of the hippocampus in longer but not shorter delays (Hampson *et al*., 1999). Deficits in spatial learning after Δ^9-THC administration have been reported for both rats (Stiglick and Kalant, 1982; Nakamura *et al*., 1991; Lichtman *et al*., 1995; Molina-Holgado *et al*., 1995; Lichtman and Martin, 1996; Ferrari *et al*., 1999; Hernandez-Tristan *et al*., 2000; Mishima *et al*., 2001; Fadda *et al*., 2004) and mice (Varvel *et al*., 2001, 2005; Da Silva and Takahashi, 2002) tested in radial or water maze. Although usually injected i.p., a few studies confirm that cannabinoids also act via direct intrahippocampal administration (Lichtman *et al*., 1995; Egashira *et al*., 2002). This suggests that the hippocampal CB_1 receptor population is crucial for spatial learning.

HU210 is a Δ^9-THC analogue, with higher efficacy and much more potent and long-lasting effects on CB_1 receptors than Δ^9-THC (Pertwee, 1999). Its behavioural effects have not been explored in great detail. HU210-induced effects on spatial learning in the water maze have been reported in naïve rats (Ferrari *et al*., 1999; Hill *et al*., 2004). Pretraining animals in a DMTP version of the water maze (Roloff *et al*., 2002a, b), however, enables rats to learn and consolidate the procedural elements of the task (Saucier *et al*., 1996; Cain

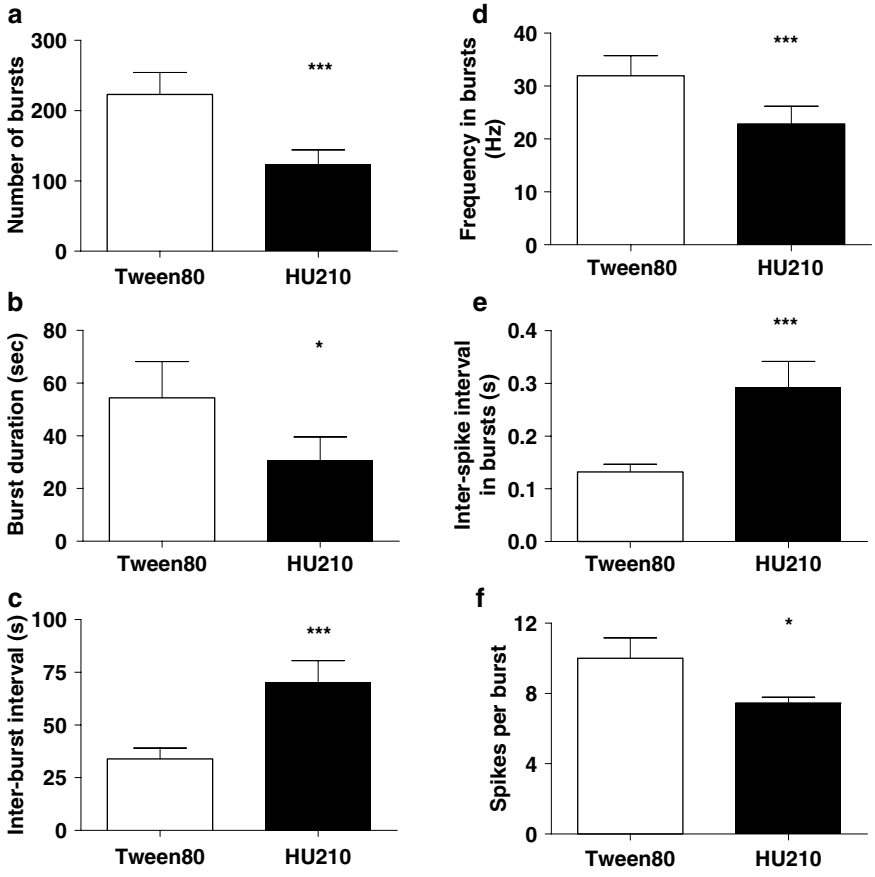

Figure 7 Overall burst analysis of recordings from hippocampal cells. Values shown in (**a**–**f**) are means ± s.e.m. of number of bursts (**a**); burst duration (**b**); IBI (**c**); frequency in bursts (**d**); ISI in bursts (**e**) and spikes per burst (**f**) across 1 h electrophysiological recordings calculated from the same 45 dorsal hippocampal principal cells located in the CA3 and CA1 subfields, following Tween80 and HU210 treatment. Relative to Tween 80, HU210 induced a strong reduction in excitability, as evidenced by changes in burst characteristics. Asterisks indicate $*P<0.05$; $***P<0.001$.

Table 1 Summary of experimental results of HU210 on DMTP and reference memory and retention in the water maze, as well as electrophysiological responses of single hippocampal neurons

	DMTP		*Reference memory*		*Retention*	*Electrophysiology*
	30 s	*1 h*	*Trial 1*	*Trial 4*		
Path length	NS	NS	↑ * No reversal	↑ * No reversal	* Impairment	Frequency
Latency to target area	NS	NS	↑ * No reversal	↑ * Reversal with AM281 1.5 mg kg^{-1}	No reversal with AM281 or SR	↑ * of bursts
Swim speed	↑ *	↑ *	↑ * No reversal	NS		↑ * of spikes
Thigmotaxis	NS	NS	↑ * No reversal	HU210 + AM281 or SR impaired		No reversal with AM281

Abbreviations: DMTP, delayed-matching-to-position; NS, not significant.
Arrows indicate an increase or decrease in respective measures, together with asterisks denoting their significance $P<0.05$.

et al., 1997, 2002) and to form a gross spatial map of the environment (O'Keefe and Nadel, 1978). Daily movement of the platform would require the animal to memorize the novel location of the platform and spatial deficits could be recorded uncontaminated of any drug effects on the learning procedure. However, we obtained no memory deficit in the HU210 group despite implementation of a delay of up to 1 h between trials 1 and 2 (exp. 1). This finding is contrary to work by Hill *et al*. (2004), who found HU210-induced deficits in acquisition of a DMTP task in the water maze at ITIs of 5 min but not 30 s following exposure of rats to HU210 (100 μg kg^{-1}) daily for 15 consecutive days.

The absence of impairment with HU210 in short-term/working memory is also in contrast to previous studies that

have used Δ-^{9}THC and other CB_1 agonists (see Fadda *et al*., 2004, 2006). Therefore replicating the work of Ferrari *et al*. (1999) aimed at gaining a deeper insight into psychopharmacological mechanisms underlying the actions of HU210 and also to determine if there was indeed a differential effect on spatial working and reference memory as spatial reference memory protocols may activate different brain circuits and/or different cellular mechanisms compared to short-term or working memory paradigms (Izquierdo *et al*., 1999).

In the reference memory task, we found a learning deficit similar to the one reported by Ferrari and colleagues, and further analysis revealed that both spatial and non-spatial parameters were affected by HU210. There was a clear increase in swim speed observed in the reference memory task. However, this could not explain the deficit obtained in spatial learning since a similar increase in swim speed was obtained in pre-trained animals in the working memory paradigm (exp.1), yet there was no learning or memory impairment. We take this as evidence for a dissociation of cannabinoid effects on motor activity and on spatial learning. They are also independent of previous experience with the drug, as faster swim speeds were executed on all drug days (Figures 1d and 4c). Δ^9-THC had similar effects in humans, where enhanced speed of performance correlated with increases in errors (Gibson's maze, double digit cancellation; Curran *et al*., 2002), indicating a trade-off between speed and accuracy. Riskier driving at high speed under the influence of cannabis (Ashton, 2001) may indeed be related to changes in performance speed. We obtained contrasting results in our animal study with accuracy being independent of swim speed.

HU210-induced thigmotaxis was high in the reference memory paradigm, but not different from controls in the working memory protocol suggesting that anxiety-related parameters may account for the observed learning deficit. Close inspection of Figure 4f, however, indicates that HU210-treated animals reach floor level in their wall hugging, while spatial task parameters (path length (Figure 3b) and latency to target area (Figure 4b)) remain increased. This suggests that the initial increase in anxiety is overcome during daily training and cannot explain the remaining spatial within-session learning deficit. The simplest explanation thus is that the deficit seen in trial 4 after HU210 treatment arises from deficits in spatial processing, most likely due to reduced firing of principal neurons in hippocampus.

Memory deficits are not reversed by cannabinoid antagonists

Co-administration of HU210 and AM281 or SR141716A aimed at reversing the reference memory deficits. SR141716A is a well studied highly potent CB_1 receptor antagonist that has previously been shown to reverse the CB_1 agonist-mediated impairments in cognition (Mallet and Beninger, 1998; Nava *et al*., 2000; Mishima *et al*., 2001; Da Silva and Takahashi, 2002; Hampson and Deadwyler, 1998, 1999, 2000, 2003). AM281 is a structural analogue of SR141716A.

Co-administration of cannabinoid agonists and antagonists is a common way of assessing the specificity of a drug for the CB_1 receptor and has, in the case of Δ^9-THC and WIN55,212-2, yielded convincing evidence for CB_1 receptor-mediated actions (Lichtman and Martin, 1996; Hampson and Deadwyler, 1998, 1999, 2000; Mishima *et al*., 2001; Varvel *et al*., 2001; Da Silva and Takahashi, 2002). We expected a similar result in our protocol, but were unable to reverse deficits with either AM281 or SR141716A. This not only pertained to the spatial parameters recorded during spatial learning but also to procedural memory and motor activity. Rather, HU210-induced thigmotaxis reflecting heightened anxiety was exacerbated by the antagonists (exp. 2), but was reversed by pretraining in the DMTP task (exp.1). Anxiety and procedural memory-related deficits thus seem not to be mediated via CB_1 receptors, although the doses of AM281 or SR141716A used here were effective in work reported by others (Hampson and Deadwyler, 1999; Cosenza *et al*., 2000; Varvel *et al*., 2001). Together with the exacerbation in thigmotaxis, this strongly implies that doses of AM281 and SR141716A were effective. A more speculative explanation may even assume that while HU210 may have acted on non-CB_1 receptors (for review see Howlett *et al*., 2002; Wilson and Nicoll, 2002) or via a non-receptor mechanism, both antagonists may have acted as inverse agonists (Landsman *et al*., 1997; Cosenza *et al*., 2000), thereby enhancing some but reversing the other behavioural deficits induced by HU210.

HU210 acts via hippocampal mechanisms

Electrophysiological recordings from hippocampal principal cells have revealed that cannabinoids affect task-related firing (Hampson and Deadwyler, 2000; Hampson *et al*., 2003). In rats trained to perform a DNMS task, hippocampal CA3 and CA1 neurons increase firing in response to one or more behaviourally relevant events, such as Sample (encoding) or Non-match (recall) lever press responses. WIN 55212-2 and Δ^9-THC administered before starting the DNMS task suppress firing during the Sample phase but not the Nonmatch phase. The Sample phase neural activity was shown to be necessary for correct performance of the DNMS task. Here, we recorded baseline firing of CA3/CA1 neurons to determine the effects of HU210, given that hippocampal neural activity is essential for performance in the water maze (Hollup *et al*., 2001). Figures 6 and 7 reveal that HU210 suppresses spontaneous firing rates of CA3 and CA1 of principal cells, consistent with our previous observations of cannabinoid effects in freely moving animals (Hampson *et al*., 2003). In addition, the alteration of spike train burst firing characteristics (Figure 7) are likewise in agreement with the reduction of peak firing rates in the DNMS task (Hampson and Deadwyler, 2000). Cannabinoid-induced reductions in baseline spike firing have also been observed by others (Pistis *et al*., 2004) in BLA.

Exposure to HU210 did not result in a complete silencing of hippocampal neurons; rather, frequency and duration of burst firing, as well as firing within bursts, were reduced by approximately 50%. Since hippocampal cells have previously been shown to encode information pertinent to memory formation by brief episodes (0.5 s) of increased firing rate, the overall diminution of burst firing by HU210 would likewise

prevent such episode in the water maze. Thus, reduced baseline activity and attenuated burst firing would block neural encoding episodes and thereby account for the failure of HU210-treated animals to acquire spatial elements relevant to learning the platform location in the water maze.

Conclusions

In summary, our results corroborate previous work suggesting a role of cannabinoids in learning and memory. In the spatial reference memory task conducted in the open-field water maze, HU210 impaired memory formation, but this concerned both spatial and non-spatial elements of the task. While exposure to the water maze progressively reduced non-spatial parameters, a spatial deficit still persisted, even in the presence of cannabinoid antagonists. This spatial impairment may be due to HU210 causing a reduction of hippocampal neural excitation.

Acknowledgements

Supported by a Component grant from the MRC to GR and RGP, and NIH grant DA08549 to REH.

Conflict of interest

The authors state no conflict of interest.

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