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Pharmacological elevation of anandamide impairs short-term memory by altering the neurophysiology in the hippocampus

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Abstract

In rodents, many exogenous cannabinoid agonists including Δ^9 -THC and WIN 55,212-2 (WIN-2) have been shown to impair short-term memory (STM) by inhibition of hippocampal neuronal assemblies. However, the mechanisms by which endocannabinoids such as anandamide and 2-arachidonylglycerol (2-AG) modulate STM processes are not well understood. Here the effects of anandamide on performance of a Delayed Non-Match to Sample (DNMS) task (i.e., STM task) and concomitant hippocampal ensemble activity were assessed following administration of either URB597 (0.3, 3.0mg/kg), an inhibitor of the Fatty Acid Amide Hydrolase (FAAH), AM404 (1.5, 10.0mg/kg), a putative anandamide uptake/FAAH inhibitor, or *R*-methanandamide (3.0, 10.0mg/kg), a stable analogue of anandamide. Principal cells from hippocampal CA3/CA1 were recorded extracellularly by multi-electrode arrays in Long-Evans rats during DNMS task (1–30s delays) performance and tracked throughout drug administration and recovery. Both *R*-methanandamide and URB597 caused dose- and delay-dependent deficits in DNMS performance with suppression of hippocampal ensemble activity during the encoding (sample) phase. *R*-methanandamide induced effects were not reversed by capsaicine excluding a contribution of TRPV-1 receptors. AM404 produced subtle deficits at longer delay intervals but did not alter hippocampal neuronal activity during task-specific events. Collectively, these data indicate that endocannabinoid levels affect performance in a STM task and their pharmacological elevation beyond normal concentrations is detrimental also for the underlying physiological responses. They also highlight a specific window of memory processing, i.e. encoding, which is sensitive to cannabinoid modulation.

Keywords

delayed non-match to sample; electrophysiology; endocannabinoid; hippocampus; short-term memory; single-unit recording; ensemble code

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1. Introduction

Systemic administration of phytocannabinoids or synthetic receptor agonists such as Δ^9 -Tetrahydrocannabinol (Δ^9 -THC), WIN55,212-2 and HU-210 impair working memory (WM) and short-term memory (STM) in rodents (Heyser et al., 1993; Lichtman et al., 1995; Ferrari et al., 1999; Hampson and Deadwyler, 2000; Mishima et al., 2001; Varvel and Lichtman, 2002; Hampson et al., 2003; Goonawardena et al., 2010a, 2010b; Robinson et al., 2010). Unexplored, however, remains the role(s) played by endogenous cannabinoids such as arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG) on these different memory processes. Their presence in memory-relevant areas of the rodent brain (Di Marzo et al., 2001) together with the localization of cannabinoid (CB1) receptors on different cell types (Marsicano and Lutz, 1999; Domenici et al., 2006) in hippocampus and pre-frontal cortex (Herkenham et al., 1991) suggests that the endocannabinoid system could play a pivotal role in modulating both WM and STM processes. Typically, endocannabinoid function has been probed by administration of SR141716A, a well-known CB1 receptor antagonist. When given alone SR141716A enhanced STM in a radial-arm maze at long, but not short delays (Lichtman, 2000; Wolff & Leander, 2003), short-term social recognition memory (Terranova et al., 1996) and passive avoidance retention (Mazzola et al., 2003). More recently, we provided evidence for an intriguing prolongation of long-term spatial memory in rats (Robinson et al., 2008) suggesting that the time course of memory is normally curtailed by the activation of CB1 receptors. This is in line with evidence that SR141716A impairs extinction learning in the Morris water maze (Varvel et al., 2005) and extinction of conditioned freezing either to a tone or context (Marsicano et al., 2002; Suzuki et al., 2004) suggesting that an endocannabinoid tone is crucial for various forms of learning and memory.

Anandamide, the first endocannabinoid to be isolated (Devane et al., 1992) produces WM deficits in rats trained to perform a non-match-to-position operant conditioning task (Mallet and Beninger, 1998). Around the same time, Murillo-Rodriguez and co-workers (1998) confirmed that intra-cerebroventricular (icv) administration of anandamide weakens memory consolidation. Such a direct role of the hippocampal endocannabinoid system was proven in long-term memory since intra-hippocampal infusions of anandamide produced anterograde amnesia in rats trained to perform a one trial, step-down inhibitory avoidance task (Barros et al., 2004), and chronic infusions of rimonabant (CB1 receptor antagonist/inverse agonist) via minipumps enhanced acquisition learning and prolonged spatial memory possibly by preventing extinction learning (Robinson et al., 2008).

Since anandamide is rapidly metabolized and/or recycled into the cell, other studies have used *R*-methanandamide, a metabolically stable analogue of anandamide, to better understand how this particular endocannabinoid modulates learning and memory. *R*-methanandamide dose-dependently disrupted operant conditioning through a CB1 receptor mediated mechanism (Brodkin and Moerschbaecher, 1997), impaired recognition memory in rats (Kosiorsek et al., 2003), and disrupted WM in the water maze in mice (Varvel and Lichtman, 2002; Varvel et al., 2006). These data strongly implicate endogenous cannabinoids in several forms of memory and warrant its use in this study. However, *R*-methanandamide may also activate transient receptor potential vanilloid type-1 (TRPV-1) receptors in the hippocampus (Chavez et al., 2010; Bhaskaran and Smith, 2010) and any drug-related action may not be specific to CB1 receptor mediated functions. Consequently, *R*-methanandamide was also assessed in the presence of capsazepine in an attempt to define a putative involvement of the TRPV-1 receptor in this STM task.

An alternative approach to modulate endocannabinoid levels is by interference with the metabolism of anandamide. It is metabolized by Fatty Acid Amide Hydrolase (FAAH), an

integral membrane protein widely distributed in the rodent brain including the hippocampus (Freund et al., 2003). Various FAAH inhibitors have been developed including the well characterized URB597, known to deactivate FAAH and increase anandamide levels in the rodent brain at a dose of 0.3mg/kg (Fegley et al., 2004). The same dose caused WM deficits in a delayed alternation task in a T-maze (Seillier et al., 2010) without producing side effects (e.g. hypomotility, hypothermia, etc.) that often accompany exogenously administered CB1 receptor agonists. Such a pharmacological approach is thus preferred over synthetic cannabinoids.

A third possibility for pharmacological elevation of brain levels of anandamide is via the inhibition of its putative transporter protein. AM404 is an inhibitor of this transporter and lacks any direct action on cannabinoid receptors (Kelley and Thayer, 2004; Fegley et al., 2004; Fowler et al., 2004). However, AM404 may also act as a FAAH inhibitor (Glaser et al., 2003; Abush and Akirav, 2010) thereby inhibiting two possible mechanisms that physiologically can curtail the actions of anandamide. Systemic injections of AM404 (5 mg/kg) impaired spatial learning in the water maze (Abush and Akirav, 2010), but it dose-dependently (2 – 10mg/kg) enhanced extinction (i.e. block reconsolidation) of conditioned fear memory in rodents (Chatwal et al. 2005; Pamplona et al., 2008) suggesting effectiveness of treatment in terms of mnemonic systems.

Here, we reasoned that if these compounds were applied to rats trained in a delayed nonmatch-to-sample (DNMS) task, the resulting effects on STM and hippocampal ensemble firing in DNMS relevant phases (Hampson and Deadwyler, 2000; Goonawardena et al., 20010b) could shed new light on the physiological role of the endocannabinoid system in behaviorally defined situations.

2. Material and methods

2.1. Animals

Adult male, Long-Evans rats (Harlan, USA) aged between 6 – 8 months (280–350g) were individually housed in a temperature-controlled (20–22°C) environment with a 12hr light/dark cycle (lights on 7 am – 7 pm). All animals were water-restricted throughout training and drug testing, but allowed free access to food (regular rat-chow). The volume of water consumed and weights were monitored daily to maintain ~83% of *ad libitum* body weight. Water consumption during behavioral testing was recorded, and a supplemental volume given immediately after the session to provide 20–22hrs of water deprivation. All animal care and experimental procedures including water regulation and surgery were approved by the Wake Forest University Institutional Animal Care and Use Committee and were in concordance with the National Institutes of Health (NIH) Guide for the Care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

2.2. Apparatus

The apparatus as described previously (Heyser et al., 1993; Deadwyler et al., 2007; Goonawardena et al., 2010a, 2010b) consisted of a 43 × 43 × 50cm Plexiglass behavioral testing chamber with two retractable levers (left and right) positioned on either side of a water trough in the front panel and a nose-poke device mounted at the center of the back panel. A cue light (28-V) was located immediately above the nose-poke device. The test chamber was illuminated by two 28-V incandescent house lights mounted into the ceiling next to a video camera which recorded the behavior of rats continuously. The entire apparatus was computer-controlled and housed inside a commercially built sound-attenuated cubicle (Industrial Acoustics Co., Bronx, New York, USA).

2.3. Behavioral training procedure

All animals were trained to perform the DNMS task (Deadwyler and Hampson, 2004; Deadwyler et al., 2007; Goonawardena et al., 2010a, 2010b). Each trial consisted of three main phases: *Sample*, *Delay* and *Nonmatch*. It was initiated at the *Sample* phase in which either the left or right lever was selected at random and extended (counterbalanced design). The animal responded by pressing the lever (Sample Response; SR), which was immediately retracted, thereby initiating the *Delay* phase of the task (1–30s). In the *Delay* phase a cue light over the nose-poke device on the opposite wall was illuminated and the animal was required to respond with at least one nose poke to terminate the delay phase. The last nose-poke at the end of the delay phase also turned out the cue light and extended both levers in the front panel, signaling the onset of the *Nonmatch* phase. Only a Nonmatch Response (NR) on the lever opposite to the position of the SR was rewarded. Reinforcement consisted of a drop of water (40µL) delivered to the trough immediately after the NR occurred. At the termination of the *Nonmatch* phase both levers were retracted for a 10s inter-trial interval (ITI) with house lights on, after which one lever was extended again and the next trial started with the onset of the *Sample* phase. An error consisting of a ‘Match Response’ resulted in no water reward and caused the house lights to be turned off for 5s with both levers retracted, after which house lights were re-illuminated for another 5s, and the next trial started. All animals were trained to criterion of 90% correct responding on trials with delays of 1–5s in sessions of 100 trials; delays varied from 1–30s prior to surgery (i.e. implantation of electrode arrays), and animals were re-trained to the same level after surgical recovery and before drug testing.

2.4. Surgery

Following training, animals were anesthetized under a constant flow of isoflurane (3% for induction, 1.5% for maintenance)/O₂ (100%) mixture and placed in a stereotaxic frame. A craniotomy (5mm diameter) was performed over the dorsal hippocampus so that the center pair of array electrodes consisting of sixteen stainless steel micro-wires (diameter: 40 µm each; Neuroline Corp., NJ, USA) was positioned 3.4 mm posterior and 3.0 mm lateral to Bregma (Paxinos and Watson, 1998). The longitudinal axis of the array was angled 30° to the midline, with posterior electrodes more lateral than anterior sites. The array was lowered in 25–100 µm steps to a depth of 3.0 – 4.0 mm from the cortical surface for CA3 leads (eight long wires), and CA1 leads (eight short wires) automatically positioned 1.2 mm higher along the longitudinal axis of the hippocampus. Electrodes were spaced 200 µm apart within each row and 400 µm between rows. Recordings from all micro-wire electrodes were monitored throughout surgery to ensure correct placement in appropriate hippocampal cell layers and the exposed cortex was kept moist with 0.9% sodium chloride. After array placement, the cranium was sealed with bone wax and dental cement (Hampson and Deadwyler, 2000; Deadwyler et al., 2007; Goonawardena et al., 2010a, 2010b) and given 0.3 mg/kg analgesic (buprenorphine). All animals were allowed to recover for 3–4 days with *ad libitum* food and water before retraining. All efforts were made to minimize animal suffering.

2.5. Drug preparation and experimental design

AM404 (*N*-(4-hydroxyphenyl)arachidonylethanolamide) and *R*-methanandamide (Cayman chemicals, Ann Arbor, MI) were obtained as solutions in ethanol. URB597 ([3-phenyl-3-(3-carbamoylphenyl)-N-cyclohexylcarbamate), Capsaicin (*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-6E-8-methyl-nonenamide) and Capsazepine (*N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide) (Cayman chemicals, Ann Arbor, MI) were purchased as crystalline solids and subsequently mixed in ethanol. Sufficient drug to make up the required concentration was mixed in Cremophor EL (Sigma, St Louis, MO) and saline (0.9%) at a ratio – ethanol (1 part) :

cremophor (1 part) : saline (18 parts). The solution was stirred rapidly and placed under a steady stream of nitrogen gas to evaporate the ethanol (approx. 10 minutes). The resulting drug/detergent/saline suspension was sonicated prior to intraperitoneal (i.p.) injection. Vehicle solutions were prepared in the same manner, with the drug omitted.

During *Experiment 1*, each subject ($n = 6$) received both a low (3.0 mg/kg) and high (10.0 mg/kg) dose of *R*-methanandamide on separate days alternated with vehicle-only control days (i.e. day 1: vehicle; day 2: low dose; day 3: vehicle; day 4: high dose). *Experiment 2* assessed the effects of *R*-methanandamide (10.0 mg/kg), Capsaicin (0.01 mg/kg) and *R*-methanandamide + Capsazepine (0.1 mg/kg) on separate days alternated with vehicle-only control days ($n = 14$). *Experiments 2* and *3* ($n = 6$ each), examined the effects of URB597 (low dose: 0.3mg/kg; high dose: 3.0mg/kg) and AM404 (low dose: 1.5mg/kg; high dose: 10.0mg/kg) similar to that described in *Experiment 1*. Note that all animals in each experiment were administered with vehicle in between drug(s) to allow behavioral performance to return to baseline prior to subsequent drug exposure. All injections were administered 20 – 30 min prior to the start of each behavioral and electrophysiological test session.

2.6. Analyses of behavioral data

Performance under the influence of each treatment was assessed using three primary measures: 1) mean percentage correct trials; 2) mean percentage correct trials at each 5s delay interval epoch, and 3) response latency. Overall statistical significance for the first measure was calculated by one-way repeated measures analysis of variance (ANOVA) across each treatment (i.e. vehicle, low and high dose) followed by Tukey's multiple comparison (post-hoc) to compare between treatments of interest. Two-way repeated measures ANOVA using treatment and delay interval as factors was employed for the second measure. Further planned paired *t*-tests were performed between treatments of interest to assess differences across each 5s delay interval epoch. Moreover, the mean latency to response levers (i.e. locomotor activity) was compared using one-way repeated measures ANOVA across treatment groups followed by Tukey's multiple comparison tests between treatment groups of interest. p 's < 0.05 were considered statistically significant.

2.7. Multi-neuronal recording

Extracellular action potentials (spikes) and behavioral events within each DNMS trial were recorded. Single neurons recorded from each of the 16 micro-wire electrodes were digitized at 40 KHz and time-stamped along with behavioral events for computer-processing within each DNMS session (Hampson and Deadwyler, 2000; Hampson et al., 2003; Goonawardena et al., 2010a, 2010b). Action potentials of individual neurons were isolated by time-amplitude window discrimination and computer-identified individual waveform characteristics using a multi-neuron acquisition processor (MAP; Plexon, Dallas, TX). Single-units from each electrode were recorded daily using waveform and firing characteristics for each trial following respective treatments. Only spike waveforms with associated discharge rates consistent with behavioral correlates across sessions were included in the analysis.

2.8. Analysis of neuronal data

Hippocampal principal cells (mean firing rate: 0.25 – 6 Hz) identified from selected wires in CA3 and CA1 sub-fields were recorded. Task specific activity during correct DNMS trials was characterized based on peak z-scores calculated from peri-event histograms (± 1.5 s) around sample and nonmatch responses as follows: (1) the peak bin (histogram maximum) was found (bin size: 100 ms); (2) the mean (M) and standard deviation (SD) of background bins (those bins that are equal or less than peak bin / 2) were calculated; (3) peak z-score =

(peak bin - M) / SD. All cells were selected only if the peak z-score around any task-specific event was equal or greater than 3.19 ($P < 0.001$). Thereafter, activity of these neurons was determined following vehicle and drug treatments. Peak z-scores of neurons that showed significant increases in firing during SR and NR were averaged across all sessions. Statistical significance was calculated using one-way repeated-measures ANOVA followed by Tukey's multiple comparison tests for differences between treatments of interest. Again, p 's < 0.05 were accepted as significant.

3. Results

3.1. Effects of R-methanandamide, URB597 and AM404 on delayed-nonmatch-to-sample performance

The mean overall DNMS performances (% correct responses) following administration of different doses of *R*-methanandamide in *Exp.1*, revealed a main effect of dose ($F_{(1,17)} = 3.95$, $p < 0.05$; Fig.1A) owing to a reliable impairment when animals were treated with 10 mg/kg of *R*-methanandamide relative to vehicle ($p < 0.05$). The 100 trials of each DNMS session were sorted by % correct responses and delay epochs averaged across 5s intervals for the respective treatments (Fig.1B). Intriguingly, only when administered with 10mg/kg at very long delays were animals impaired leading to a dose $\times$ delay interaction ($F_{(10,75)} = 2.45$, $p < 0.01$), apart from the normal delay effect ($F_{(5,75)} = 39.83$, $p < 0.001$). This delay-dependence was confirmed for 10 mg/kg of *R*-methanandamide at both 21–25s ($t = 2.95$, $p < 0.05$) and 25–30s ($t = 3.55$, $p < 0.01$) delay intervals in comparison to vehicle, and was not present at any other delay interval (t 's < 0.84 , p 's > 0.05), suggesting that the higher but not the lower dose of *R*-methanandamide was able to induce deficits in DNMS performance at delays greater than 20s. Moreover, the overall response latency was unaffected ($F < 1$), suggesting no effect of *R*-methanandamide on locomotor activity (Fig.1C).

Since *R*-methanandamide is not specific to CB1, but also stimulates TRPV1 (vanilloid) receptors, *Exp.2* explored whether *R*-methanandamide-induced deficits in STM can be reversed by blockade of TRPV1 with capsazepine. At the same time, a more specific activation of TRPV1 was probed using the agonist capsaicin to evaluate one selective contribution of the vanilloid system to DNMS performance. Data are depicted in Fig. 2 and confirm that i) the vanilloid system alone seems to play no part in DNMS performance since capsaicin did not affect STM (Fig.2A and B); ii) the impairment of *R*-methanandamide was confirmed; and iii) any TRPV1 receptor mediated action of *R*-methanandamide was excluded given that capsazepine did not reverse deficits. Consequently, we observed an overall main effect of treatment ($F_{(3,55)} = 14.90$, $p < 0.001$; Fig.2A) on DNMS performance (% correct responses) owing to reliable deficits produced by *R*-methanandamide ($p < 0.001$) and *R*-methanandamide + capsazepine ($p < 0.001$) treated groups relative to vehicle; capsaicin failed to cause significant impairments. A main effect of drug ($F_{(3,260)} = 11.01$, $p < 0.001$) and delay ($F_{(5,260)} = 58.83$, $p < 0.001$) was evident when each trial was sorted by 5s delay intervals (Fig.2B) and post-hoc analysis revealed that capsaicin failed to impair DNMS performance at all delay interval epochs (all p 's > 0.05). Moreover, the *R*-methanandamide + capsazepine treated group produced delay-dependent deficits at all delay intervals greater than 6s (i.e. 6–10s: $t = 4.02$, $p < 0.01$; 11–15s: $t = 4.89$, $p < 0.001$; 16–20s: $t = 4.50$, $p < 0.001$; 21–25s: $t = 2.47$, $p < 0.05$; 26–30s: $t = 3.29$, $p < 0.01$). These results suggest that co-administration of capsazepine was not able to reverse *R*-methanandamide induced deficits in DNMS performance. Furthermore, the overall response latency (Fig.2C) was unaffected ($F < 1$), suggesting that none of the treatments in *Exp.2* had any influence on locomotor activity.

These results were further supported in *Exp.3* following the treatment of different doses of the FAAH inhibitor, URB597, in which we also observed a dose-dependent impairment in

DNMS performance ($F_{(2,17)} = 10.31, p < 0.001$; Fig.3A) due to deficits after administration of the 3.0 mg/kg dose ($p < 0.01$). There was, however, no dose $\times$ delay interaction but we obtained main effects of dose ($F_{(2,75)} = 10.04, p < 0.001$) and delay ($F_{(5,75)} = 46.31, p < 0.001$; Fig 3B). Further planned comparisons confirmed that treatment with 3.0 mg/kg URB597 was detrimental for DNMS performance for all delays longer than 11s (all t 's > 2.1 ; p 's < 0.05), but not for shorter delay epochs (t 's < 1.33 ; p 's > 0.05). These URB597 induced deficits were not due to their effects on locomotion ($F_{(2,17)} < 1, p > 0.05$; Fig.3C).

In contrast and despite a small reduction in performance, AM404 failed to cause an overall main effect of treatment ($F_{(2,17)} = 2.09, p > 0.05$; Fig 4A) at doses that have been shown to affect behavior *in vivo* (Exp.4). This was also confirmed in terms of delay dependence; there was no effect with drug dose as a factor (F 's < 1.2), but a main effect of delay ($F_{(5,75)} = 34.78, p < 0.001$; Fig 4B) confirmed an overall decline in performance accuracy for longer delay intervals. A planned comparison, however, detected a small yet reliable reduction in correct responses at 25–30s delay intervals following both the 1.5 mg/kg ($t = 2.97, p < 0.05$) and 10 mg/kg dose of AM404 ($t = 2.56, p < 0.05$) treatments. Again, these effects were seen in the absence of any motor deficits ($F_{(2,17)} = 1.22, p > 0.05$; Fig 4C).

3.2. Effects of R-methanandamide, URB597 and AM404 on hippocampal ensemble activity during delayed-nonmatch-to-sample task specific events

Recordings and analyses of hippocampal ensemble activity in rodents performing the DNMS short-term memory task have been conducted over a number of years and have provided substantial insight into the functional nature of task-specific ensemble firing patterns (Deadwyler et al., 1996, 2007; Deadwyler and Hampson, 2004; Hampson et al., 2003; Goonawardena et al., 2010a,b). The DNMS task requires the rat to make a 'Sample' lever response (SR) at the start of the trial and memorize the lever position in order to make the correct 'Nonmatch' response (NR) choice after a temporal delay of 1–30s on different trials during the session. Here hippocampal neuronal activity (i.e. peak z-score) around SR and NR was assessed in conjunction with behavior (see 3.1 above) following drug treatment.

In line with behavioral performance, 10mg/kg *R*-methanandamide suppressed the z-score and its peak during the SR, but not the NR phase of the trial (Fig. 5). This was confirmed statistically in $n = 13$ hippocampal pyramidal neurons yielding a main effect of dose ($F_{(2,38)} = 3.93, p < 0.05$; Fig.5A) due to firing rate reductions in the high dose ($t = 2.79, p < 0.05$). This was different during the NR ($F < 1; n = 15$; Fig. 5B). These results confirm that *R*-methanandamide alters hippocampal ensemble activity at the high dose affecting specifically the encoding of the sample lever position. This is also exemplified by the firing rates of a principal cell which responded during the SR (Fig. 5C) and another cell which fired during the NR (Fig. 5D) that were tracked over days when different doses of *R*-methanandamide were administered.

A similar analysis was conducted for URB597 and again the higher dose of 3mg/kg significantly reduced hippocampal cell firing (overall: $F_{(2,38)} = 8.84, p < 0.01$; $t = 3.88, p < 0.01$ compared with vehicle; $n = 13$) around the SR (Fig. 6A). However, the lower dose of 0.3 mg/kg failed to produce any alterations in firing rate around SR ($p > 0.05$). No drug effect on NR firing was observed ($F < 1.6; n = 15$; Fig. 6B). Two representative example cells with selectively increased firing in response to the sample and nonmatch phases are presented in Fig. 6C and 6D respectively. They were tracked during control and drug conditions. Note the selective suppression of activity during SR, but not NR.

In contrast, AM404 failed to produce any changes to hippocampal ensemble activity (peak z-scores) either around SR ($F < 1; n = 12$, Fig.7A) or NR ($F < 1, n = 15$, Fig.7B) during DNMS performance. The firing frequencies of two individual cells that responded to the

sample and nonmatch phases following respective AM404 dosing are depicted in Figs. 7C and 7D respectively.

Overall these electrophysiological results corroborate the behavioral outcome and provide plausible mechanistic explanations for the failures in performance observed in some drug doses.

4. Discussion

We have previously concentrated our research efforts on synthetic and plant-derived cannabinoid function as drugs of abuse. From this work (Goonawardena et al., 2010a,b; Robinson et al., 2008; 2010) we concluded that exogenously applied agonists exert their effects through CB1 and non-CB1 receptor mediated actions. It is therefore important to disentangle these two actions and this is best achieved through selective modulation of the endocannabinoid system. The current work is a first attempt in this direction.

Three compounds with different pharmacological profiles were administered in a within-subject design and effects on STM assessed. While both the stable anandamide analogue, *R*-methanandamide, and more prominently the FAAH inhibitor, URB597, impaired task performance and neuronal encoding of the sample event, an inhibitor of the anandamide transporter AM404 was not effective. This difference is attributed to the different pharmacological targets of our compounds, but at the same time highlights cannabinoid release during the sample event as a limiting factor for STM in the DNMS task. We propose that high levels of endocannabinoid release in response to the sample lever presentation curtails the length of STM so that correct performance is at risk for long, but not short delay intervals.

4.1. Synaptic adaptation and long-delay performance are impaired by *R*-methanandamide

R-methanandamide caused delay-dependent deficits in DNMS performance following the administration of a high dose (10 mg/kg) as opposed to a low dose (3.0 mg/kg). This is in line with our previous work, which attests CB1 receptors in hippocampus (and adjacent brain regions) having a selective role in STM (Hampson & Deadwyler, 1999; 2000). It appears that they are functionally irrelevant in DNMS performance when the NR immediately follows SR, but gain relevance for longer delay intervals when the memory demand (i.e. cognitive workload) is high (Goonawardena et al., 2010b). These results are in agreement with other behavioral data that (i) *R*-methanandamide at similar doses impaired working memory in the water maze (Varvel and Lichtman, 2002; Varvel et al., 2006) or learning an operant conditioning task (Brodkin and Moerschbaecher, 1997), and (ii) anandamide itself can lead to memory deficits (Varvel et al. 2006; Castellano et al., 1997, 1999; Costanzi et al., 2003). Unexplored in all these studies were possible physiological responses to drug exposure. We here confirm that *R*-methanandamide indeed alters discharges during the sample event and thereby compromises encoding of the sample lever with the result that animals are more likely to fail in their NR and is thus in agreement with findings obtained following exposure to exogenous cannabinoids such as Δ^9 -THC and WIN-2 (Hampson & Deadwyler, 2000; Hampson et al., 2003; Goonawardena et al., 2010). Given that anandamide is able to inhibit hippocampal long-term potentiation (LTP) (Terranova et al., 1995; Lees & Dougalis, 2004) and block the formation of new synapses in hippocampal cultures via the activation of CB1 receptors (Kim and Thayer, 2001), it is tempting to speculate that an increase in firing rate during the sample (encoding) phase provides a strong high-frequency stimulus and induce LTP-like plastic changes that could survive the delay for accurate recall later, and that *R*-methanandamide disrupts this process. However, to date no concrete evidence suggests that this blockade of neuronal firing during encoding results from the ability of anandamide to limit LTP.

4.2 Heightened endocannabinoid levels affect DNMS performance and sample firing during encoding, but not during recall

The main focus of this study was on the function of endocannabinoids. Although *R*-methanandamide is quasi-identical with anandamide, its pharmacological actions are somewhat different and longer lasting. We therefore blocked the metabolism of anandamide by inhibition of its degradation by FAAH (URB597) or its putative re-uptake transporter (AM404). Although we expected both methods to yield similar outcome, results clearly indicate stronger efficacy of FAAH inhibition. URB597 produced both delay- and dose-dependent impairments in DNMS performance while AM404-induced deficits in DNMS performance were very subtle and not paralleled by physiological changes. This is despite doses used that have previously been confirmed to potentially elevate anandamide levels *in vivo* (Fegley et al., 2004; Pamplona et al., 2008; Abush and Akirav, 2010; Glaser et al., 2005; Fowler et al., 2004) and may be interpreted in two ways: 1) URB597 is more potent than AM404 or 2) their differential pharmacology has different effects on anandamide levels and so the behavioral/physiological outcome. While our experiments do not allow conclusive distinction between these theories, the dose selection was based on previous work and for URB597, there is compelling evidence for efficacy as low as 0.3mg/kg which impaired working memory (i.e. delayed-alternation task) in the T-maze in rats (Seillier et al., 2010). Similarly, systemic administration of 10 mg/kg or direct intra-cerebroventricular injection of AM404 facilitated the extinction of short-term contextual fear memory in rats (Pamplona et al., 2008; Bitencourt et al., 2008). In addition, Chhatwal and co-workers (2005) reported that AM404 (2–10 mg/kg) dose-dependently enhanced extinction of conditioned fear memory in rats. And although we take this as strong evidence for drug efficacy in the doses selected in our study, it remains unexplored whether doses required for impairment of operant conditioning with positive reward are similar to extinction of fear. Alternatively, the negative result in *exp.3* may be due to differential mechanisms activated in hippocampus and amygdala during the two tasks. Observed potent effects of URB597 relative to AM404 and *R*-methanandamide may lie with its (URB597) ability to inhibit FAAH and thereby prevent the breakdown of other endocannabinoids such as palmitylethanolamide (PEA) and oleoylethanolamide (OEA) that are also substrates of FAAH (Mazzola et al., 2009; Wise et al., 2008). PEA and OEA are co-synthesized with anandamide and can potentiate anandamide responses (so called ‘entourage effects’) via the activation of other cannabinoid-sensitive receptors such as GPR119 (G-protein coupled), TRPV-1 (Transient-Receptor-Potential-Vanilloid type-1) and PPAR α (Peroxisome-Proliferator-Activated-Receptor-alpha) receptors (Hansen, 2010; Ho et al., 2008; Ning et al., 2008). In addition, there is considerable debate as to whether the uptake of anandamide is mediated via an active transporter or passive diffusion (Glaser et al., 2003, 2005). If anandamide is taken up by facilitated (passive) diffusion, it should mediate bidirectional transport and be involved in both its release and re-uptake (Hillard et al., 1997). In fact previous work has shown that effects of transport inhibitors can result in a blockade of endocannabinoid-mediated effects and intracellular accumulation of anandamide and 2-AG, possibly due to the disruption of endocannabinoid release (Ronesi et al., 2004; Ligresti et al., 2004; Pillolla et al., 2007). Thus, the blockade of anandamide release may functionally counteract the blockade of FAAH by AM404 resulting in a lack of effect by this drug. This would explain the failure of AM404 to alter behavior and event-related firing during this particular STM task.

The finding that higher doses of URB597 (3.0 mg/kg) suppressed hippocampal neuronal firing during the sample but not the nonmatch phase suggests that elevation of anandamide disrupted the encoding of trial relevant information during DNMS performance. Similar results from *exp.1* with *R*-methanandamide therefore provide compelling evidence that anandamide modulation impinges on hippocampal neurophysiology and the alterations

observed in single unit firing indeed underlie the behavioral impairment. They add to similar and existing data from studies on exogenous cannabinoid exposure such as Δ^9 -THC and WIN55,212-2 and DNMS performance, in which similar changes in discharge patterns have been observed (Hampson and Deadwyler, 2000; Hampson et al., 2003; Goonawardena et al., 2010b). Additionally, this is the first *in vivo* study to demonstrate URB597 induced alterations in hippocampal ensemble activity in awake, behaving animals. Coomber and co-workers (2008) reported a dose-dependent attenuation of kainate-induced firing and bursting rates of hippocampal neurons *in vivo* following URB597 administration, but under anesthesia conditions.

4.3 R-methanandamide induced deficits in DNMS performance are not mediated by TRPV-1 receptors

Like anandamide, which is considered to be both a cannabinoid and an endovanilloid, *R*-methanandamide also acts as a ligand for TRPV-1 receptors (Roberts et al., 2008; Breyné and Vanheel, 2006) and these receptors are highly expressed on cell bodies and dendrites of hippocampal neurons (Toth et al., 2005). Owing to the recent finding that anandamide mediates a TRPV-1 triggered form of long-term depression (LTD) in the hippocampus, (Chavez et al., 2010) we explored whether TRPV-1 receptors may also have a modulatory role in hippocampal-dependent learning and memory, and possibly contribute to the *R*-methanandamide and URB597 induced deficits in DNMS performance. However, our results suggest TRPV-1 receptors do not seem to play an active role in this particular STM task since DNMS performance because neither capsaicin alone, nor capsaicin in conjunction with *R*-methanandamide exerted pharmacological efficiency.

Although the emphasis here was on anandamide, other endocannabinoids in the hippocampus, such as 2-AG (Stella et al., 1997) might also play important roles and thus explain the relatively weak potency of *R*-methanandamide and AM404. However, until recently no pharmacological tools were available to selectively modulate brain levels of 2-AG. The discovery of URB602, URB754 and JZL184 (King et al., 2007; Hillard et al., 2007; Long et al., 2009) as potent inhibitors of monoacylglycerol lipase (MAGL), the enzyme responsible in the metabolic breakdown of 2-AG, are of considerable significance in this regard, and require further examination.

In conclusion, this is the first study that reports STM deficits in an operant conditioning task with concurrent alterations in hippocampal neuronal activity following enhancement of brain anandamide levels via the blockade of its breakdown (URB597), but not re-uptake or release (AM404). We provide evidence for the congruent suppression of hippocampal single cell firing and task performance, which seems due to a lack of on-demand release of anandamide that normally enables encoding of task-relevant information. The differences in pharmacology between the applied compounds may explain the variations in results.

Abbreviations

2-AG	2-Arachidonylglycerol
DNMS	Delayed Non-Match to Sample
FAAH	Fatty Acid Amide Hydrolase
STM	Short-Term Memory
WIN-2	WIN55,212-2
WM	working memory

SR	sample response
NR	non-match response
TRPV-1	transient receptor potential vanilloid type-1

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Figure 1.

Overall percentage correct responses of DNMS performance across all delay intervals ranging from 1–30s (A), percentage correct DNMS responses sorted by length of delay in increments of 5sec (B), and latency to response lever (s) during DNMS performance (C) following the exposure of either vehicle, *R*-methanandamide (3.0 mg/kg) or *R*-methanandamide (10.0 mg/kg) ($n = 6$). Deficits in DNMS performance were observed only with the higher dose (10.0 mg/kg) and were most apparent at 21–25 and 26–30s delay intervals. Both doses had no effect on general locomotor activity. All values are represented as mean $\pm$ s.e.m. * $p < 0.05$; ** $p < 0.01$.

Figure 2.

Overall percentage correct responses of DNMS performance across all delay intervals ranging from 1–30s (A), percentage correct DNMS responses sorted by length of delay in increments of 5sec (B), and latency to response levers (s) during DNMS performance (C) following the exposure of either vehicle, or capsaicin (0.01 mg/kg), or *R*-methanandamide (10.0 mg/kg), or capsazepine (0.1 mg/kg), or *R*-methanandamide + capsazepine ($n = 14$). The TRPV-1 receptor agonist, capsaicin had no effect on DNMS performance. The *R*-methanandamide induced deficits in DNMS performance were not reversed by the TRPV-1 receptor antagonist, capsazepine. No effect on general locomotor activity was observed

following each of the treatments. All values are represented as mean $\pm$ s.e.m. *# $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3.

Overall percentage correct responses of DNMS performance across all delay intervals ranging from 1–30s (A), percentage correct DNMS responses sorted by length of delay in increments of 5sec (B), and latency to response levers (s) during DNMS performance (C) following the exposure of either vehicle, URB597 (0.3 mg/kg) or URB597 (3.0 mg/kg) ($n = 6$). DNMS deficits were observed only with the higher dose (3.0 mg/kg) and most evident at delays intervals greater than 10s. Both doses had no effect on general locomotor activity. All values are represented as mean $\pm$ s.e.m. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 4.

Overall percentage correct responses of DNMS performance across all delay intervals ranging from 1–30s (A), percentage correct DNMS responses sorted by length of delay in increments of 5sec (B), and latency to response levers (s) during DNMS performance (C) following the exposure of either vehicle, AM404 (1.5 mg/kg), or AM404 (10.0 mg/kg) ($n = 6$). A subtle deficit in DNMS performance was observed with both low (1.5 mg/kg) and high (10.0 mg/kg) doses at delay intervals greater than 25s. No effect on general locomotor activity was observed following the treatment of both doses. All values are represented as mean $\pm$ s.e.m. *# $p < 0.05$.

Figure 5.

Peak z-scores ((peak bin – mean firing rate of background bins) / standard deviation of background bins) of hippocampal principal cells that demonstrated increased firing during the sample (A) and nonmatch (B) response under vehicle conditions and following acute treatments of low (3.0 mg/kg) or high (10.0 mg/kg) doses of *R*-methanandamide. The firing rates and waveforms of two separate cells that responded to the sample and nonmatch events following each treatment are represented in (C) and (D) respectively. Note that the higher dose of *R*-methanandamide was able induce a suppression in firing frequency around the sample phase. All values in (A) and (B) are represented as mean $\pm$ s.e.m. * $p < 0.05$.

Figure 6.

Peak z-scores ((peak bin – mean firing rate of background bins) / standard deviation of background bins) of hippocampal principal cells that demonstrated increased firing during the sample (A) and nonmatch (B) response under vehicle conditions and following acute treatments of low (0.3 mg/kg) or high (3.0 mg/kg) doses of URB597. The firing rates and waveforms of two neurons that responded to sample and nonmatch events following each treatment are represented in (C) and (D) respectively. URB597 at the higher dose induced significant reductions in firing frequency around the sample phase. All values in (A) and (B) are represented as mean $\pm$ s.e.m. ** $p < 0.01$.

Figure 7.

Peak z-scores ((peak bin – mean firing rate of background bins) / standard deviation of background bins) of hippocampal principal cells that demonstrated increased firing during the sample (A) and nonmatch (B) response under vehicle conditions and following the acute treatments of low (1.5 mg/kg) or high (10.0 mg/kg) doses of AM404. The firing rate and waveforms of two separate neurons that responded to sample and nonmatch events following each treatment are represented in (C) and (D) respectively. Both doses failed to produce significant alterations in event-related firing frequencies during DNMS performance. All values in (A) and (B) are represented as mean ± s.e.m.