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Memory Encoding in Hippocampal Ensembles is Negatively Influenced by Cannabinoid CB1 Receptors

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Abstract

It has been previously demonstrated that the detrimental effect on performance of a delayed nonmatch to sample (DNMS) memory task by exogenously administered cannabinoid CB1 receptor agonist, WIN 55212-2 (WIN), is reversed by the receptor antagonist Rimonabant (Rmbt). In addition Rmbt administered alone elevates DNMS performance, presumably via suppression of negative modulation by released endocannabinoids during normal task performance. Other investigations have shown that Rmbt enhances encoding of DNMS task-relevant information on a trial-by-trial, delay-dependent basis. In the current study these reciprocal pharmacological actions were fully characterized by long-term, chronic intrahippocampal infusion of both agents (WIN and Rmbt) in successive 2 week intervals. Such long-term exposure allowed extraction and confirmation of task-related firing patterns where Rmbt reversed effects of CB1 agonists. This information was then utilized to artificially impose the facilitatory effects of Rmbt and reverse the effects of WIN on DNMS performance, by delivering multichannel electrical stimulation in the same firing patterns to the same hippocampal regions. Direct comparison of normal and WIN injected animals, in which Rmbt injections and ensemble firing facilitated performance, verified reversal of the modulation of hippocampal memory processes by CB1 receptor agonists, including released endocannabinoids.

Keywords

cannabinoids; intrahippocampal infusion; increased endocannabinoids; hippocampal ensembles; memory encoding; patterned stimulation; rat

Introduction

Endocannabinoids are released by several different neural systems and alter cell and circuit activity in different brain areas (Shen et al 1996; Tzavara et al. 2004; Foldy et al. 2006; Janero and Makriyannis 2009). Continual exposure to cannabinoids is an important factor in abuse and addiction so it is important to know whether the effects of exogenous cannabinoids or cannabinoid receptor antagonists are altered following long-term continuous

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exposure. It is also important to know that the effects of continued exposure are present in particular regions and not the result of changes in other brain areas. Finally, it is critical to understand how endocannabinoids that modulate behavioral performance alter neural activity related to encoding of task information (Deadwyler et al. 2007). Several recent investigations have implicated cannabinoid CB1 receptors in the control of glutamatergic transmission and have demonstrated modification of NMDA receptor function by altering the effects of MK801 on locomotor activity (Auclair et al. 2000; Bubeníková-Valesová et al. 2008; and Black et al. 2009). Other studies have demonstrated that cognitive changes produced by cannabinoids administered systemically, can be modified by drugs that affect other convergent neuronal systems (Ballmaier et al. 2007; Borowsky et al. 2005; D'Souza et al. 2005; Hajós et al. 2008; Lundqvist 2005). It is for these reasons that the current study was undertaken, to examine the effects of intrahippocampal infusion of CB1 receptor agonists and antagonists to determine whether acute actions subside or are enhanced by continued chronic exposure.

Methods

Subjects

Male Long-Evans rats (n=32) age 4–12 months were used as subjects. Animals were allowed free access to food, and fluid intake was adjusted daily for maintenance at 85% of free-feeding body weight. All animal care and experimental procedures, including water deprivation and surgery, conformed to IACUC and National Institutes of Health (NIH) and Society for Neuroscience (SFN) recommendations and Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) regulations for care and use of experimental animals.

Apparatus and Behavioral Training

Complete details of apparatus design and behavioral training have been reported previously (Deadwyler and Hampson 2004, 2008; Hampson et al. 2010). Briefly, the apparatus consisted of a Plexiglas behavioral testing chamber with two retractable levers mounted on one wall, positioned to either side of a water trough, and a nosepoke device mounted in the center of the wall on the opposite side of the chamber. The testing chamber was housed inside a commercially built sound-attenuated cubicle (Industrial Acoustics Co, Bronx, NY) with a video camera mounted above the testing chamber. Animals received a portion of their daily fluid intake during behavioral testing sessions (consisting of 100–150 trials) with the remainder supplied on return to their home cage. The DNMS task consisted of three main phases: Sample, Delay and Nonmatch. In the Sample phase either the left or right positioned lever was selected at random and extended into the testing chamber (Figure 1A). The animal responded by pressing the extended lever which constituted the Sample Response (SR). After the SR the lever was immediately retracted and the Delay phase of the task was initiated (Delay in Figure 1A). The Delay phase consisted of 1–30 s intervals with no levers extended and a cue light illuminated over the nosepoke device (NP) on the opposite wall. In the Delay phase the animal was required to nosepoke into a photo beam to terminate the Delay phase after the interval timed out: this turned off the cue light and produced extension of both levers signaling onset of the Nonmatch phase of the task (Figure 1A). A response on the lever *opposite* the position of the SR, i.e. a “nonmatch response” (NR), produced a reward consisting of a drop of water (0.04 ml) delivered to a trough located between the two levers (see Figure 1A). A 10 s intertrial interval (ITI) preceded onset of the next trial. An incorrect response in the Nonmatch phase, i.e. a response on the same lever as the SR, caused the chamber lights to be turned off for 5 s with both levers retracted, after which, the lights were re-illuminated, and the next trial initiated 5.0 sec later. Animals were trained to a

criterion performance of 90% correct responses on trials with delays of 1–5 s in sessions of 100–150 trials with delay durations of 1–30 s prior to initiation of experimental procedures.

Surgery

Animals were trained to criterion performance prior to surgery, and retrained to the same level after recovery. For implantation of electrode arrays animals were anesthetized with ketamine (100 mg/kg, I.P.) and xylazine (10 mg/kg, I.P.) and multi-neuron recording arrays, each consisting of sixteen 25 μ m wire electrodes (NBLabs, Denison, TX; Neuroline, New York, NY) aimed at the CA1 and CA3 subfields bilaterally in each hippocampus, centered at coordinates: 3.8 mm posterior to bregma (A/P), 3.0 mm left or right (M/L) of midline (Hampson *et al.* 1999). The longitudinal axis of the array was angled 30° from the midline, with posterior electrode sites more lateral than anterior sites, following the longitudinal axis of the hippocampus. Each 16-electrode array was lowered in 25–100 μ m steps to a depth (D/V) in which CA3 electrodes penetrated 3.0–4.2 mm from surface of the brain and CA1 electrodes 1.6–2.8 mm, as per pre-cut lengths. Single neuron firing from each electrode on the array was monitored during surgery to ensure placement in appropriate hippocampal cell layers.

All animals were additionally implanted with bilateral intracranial infusion cannulae (Micheau *et al.* 2004), although only nineteen received intrahippocampal drug infusion for this experiment (the others received only saline). Two intrahippocampal cannula (stainless steel, 26-gauge, L shaped) were lowered to place the respective tip coordinates adjacent to the left and right CA3 electrodes (A/P 3.8 mm, M/L 3.6 mm, D/V 4.0 – approximately 0.2 mm above and lateral to CA3 electrode placement; Figure 1B), and then connected via flexible polyethylene tubing to Alzet 2004 minipumps (Durect Inc.) containing artificial cerebrospinal fluid/saline, placed in a cavity below the skin of the neck. After surgery, the skin was replaced and sutured tight. To replace and exchange minipumps, animals were anesthetized, and the skin over the minipumps opened with a fresh incision. The polyethylene tubing was cut, the exhausted minipump removed and replaced with fresh filled minipumps, containing a suspension of Rmbt or WIN 55,212-2. After minipump replacement, the fresh incision was sutured and treated with antibiotic.

Following placement of the array and intrahippocampal infusion cannulae, the cranium was sealed with bone wax and dental cement, and animals allowed to recover for at least one week prior to resumption of behavioral testing. Scalp wounds and neck incisions were treated periodically with Neosporin antibiotic and a systemic injection of penicillin G (300,000 U, i.m.) was given to prevent infection. Animals received buprenorphine (0.01–0.05 mg/kg, IP) for pain relief for 4–6 hrs after all cranial surgeries.

Drug Preparation and Administration

Rimonabant (SR141716A, Sanofi-Aventis, provided by Research Triangle Institute, Cary, NC), WIN 55,212-2 (Tocris), URB597 (Tocris) or URB602 (Tocris) were prepared daily from a 20 mg/ml stock in ethanol. Rimonabant, WIN 55,212-2, URB597 or URB602 stock (0.5 ml) were added to 2.0 ml of a Pluronic F68 detergent (Sigma) in ethanol solution (20 mg/ml) in which 2.0 ml of saline (0.9%) was slowly added. The solution was stirred rapidly and placed under a steady stream of nitrogen gas to evaporate the ethanol (approx 10 minutes). This resulted in a detergent/drug suspension of 5.0 mg/ml which was then sonicated and diluted with saline to either final (IP) injection concentration of 2.0 mg/ml for Rmbt or WIN, or diluted to final concentration (Rmbt, Win, URB597 or URB602) for filling of minipumps (see below). Vehicle solutions were prepared in a similar manner, except the drug was omitted (i.e. final IP injection vehicle = 8 mg/ml pluronic in saline).

Five animals were treated for two weeks each with WIN (1.0 $\mu\text{g/day}$) then Rmbt (2.0 $\mu\text{g/day}$). Four other animals received the drugs in the reverse order (Rmbt 1.0 $\mu\text{g/day}$ then WIN 1.0 $\mu\text{g/day}$) for two weeks each. Five other animals received two weeks of URB597 (5.0 $\mu\text{g/day}$) followed by two weeks of URB597 plus daily i.p. injections of Rmbt (2.0 mg/kg) and a final set of five animals received the same schedule with two weeks of URB602 (5 $\mu\text{g/day}$) and then two weeks of URB602 plus daily i.p. injections of Rmbt (2.0 mg/kg). All chronic treatments above were via intrahippocampal minipump (Alzet 2004, 0.25 $\mu\text{l/hr}$, maximum 28 days). Each animal received three pump changes: the initial pump was filled with saline/ACSF at surgery and was maintained for 21–28 days, after which the first pump change installed the first drug treatment and delivered the drug for 15–18 days. With the exception of animals receiving URB602, the subsequent second pump change delivered a different drug for an additional 15–18 days. A final exchange of pumps washed out remaining drug with saline/ACSF (15–28 days) then the minipump was removed and cannula sealed to prevent infection.

An additional thirteen animals received WIN 55,212-2 (0.4–0.8 mg/kg) by daily i.p. injection. On drug administration days, animals were injected with 1.0 ml/kg of WIN/pluronic solution approximately 10 minutes prior to the start of the behavioral session. On vehicle injection days the same volume (1.0 ml/kg) of pluronic vehicle was administered prior to the session. At least one day of vehicle injection was imposed between each drug-testing session.

Analyses of Behavioral Data

The primary behavioral measure was mean % correct trials during the session and mean % correct trials at each delay interval (assessed in 5.0 sec increments). Multifactor analyses of variance (ANOVA) were employed, and main effects were examined further via adjusted pairwise linear contrasts for individual comparisons of drug effects at specific delays.

Multineuron Recording Technique

Extracellular action potentials and behavioral events within each DNMS trial were digitized on-line and time-stamped for computer processing (Deadwyler and Hampson 2004). Single neurons were isolated and selected for analysis from each of the 32 (16 per hemisphere) different hippocampal recording electrodes. Action potential waveforms were digitized at 40 kHz and isolated in real-time by derivation of individual waveform characteristics via a Plexon Multineuron Acquisition Processor (MAP, Plexon, Inc., Dallas, TX). A limit of two separately identified neurons recorded from a single electrode was included in ensemble analyses (Hampson et al. 1999).

Hippocampal Neuron Ensembles and Extraction of Encoding Features

Hippocampal ensembles consisted of 15–25 isolated neuronal action potentials that exhibited the same waveform and firing rate criteria over a minimum of 1000 consecutive trials (at least ten successive behavioral testing sessions) for each animal. CA1/CA3 pyramidal neurons with mean background firing rates of 0.25 – 2.0 Hz were utilized in analyses. Behavioral correlates were determined for each neuron via perievent histograms computed ± 1.5 s before and after the occurrence of the SR in the Sample phase of the DNMS task. Prior investigations have shown that ensemble firing during occurrence of the SR predicts the behavioral outcome of the task (Deadwyler et al. 1996; Deadwyler and Hampson 1997) due to the level of encoding of the position of the lever required for retrieval and decision in the Nonmatch phase of the DNMS task (Deadwyler and Hampson 2006, Hampson et al 2011). Neurons were only included in the analysis if they exhibited the same identified behavioral correlate across all testing sessions.

A canonical discriminant analysis (CDA) utilizing multivariate procedures (Stevens 2002; Deadwyler and Hampson 2008; Hampson et al. 2010) assessed ensemble neural firing using 3.0 s (± 1.5 s relative to time of event occurrence in 0.25 s bins) perievent histograms of the SR and NR trial events. The CDA extracted common sources of variance or Discriminant Functions (DFs) from a time X neuron matrix of ensemble firing rates for each (15–25 neuron) ensemble in each animal. DFs were computed by canonical correlation and eigenvector decomposition (Rao 2002) of the ensemble firing rate matrices compiled from data collected over five previous consecutive daily DNMS sessions (at least 500 total trials). Decomposition of the covariance matrix of ensemble firing by this means extracted five significant sources of variance (DFs), each of which represented a proportion of the total variance associated with a specified behavioral event. In a prior study (Deadwyler and Hampson 2008), it was determined that the fifth discriminant function (DF5) demarcated firing in Left vs. Right SRs on correct trials; therefore in the current application DF5 was extracted for each ensemble and analyzed for strength of SR encoding on single trials.

CA1 Multineuron Stimulation Parameters

A custom built 16-channel stimulator (Triangle BioSystems Inc. Durham, NC) was utilized to deliver patterns of electrical pulses bilaterally to the 8 CA1 electrodes in both hippocampal arrays. The stimulator delivered digital-to-analog (D/A) converted biphasic output pulses in specific patterns, simultaneously to CA1 electrodes in each array. Each output channel delivered one-half of a symmetric biphasic stimulation pulse of 1.0 ms duration to a pair of adjacent electrodes in CA1 allowing bipolar stimulation that was isolated from other electrodes on the same array. Stimulator pulses were electronically gated to produce square constant voltage outputs in the range of 0.1 to 15 V (20–100 μ A) and interpulse intervals of 0.5 ms on a given channel. The range of parameters employed on a single output channel was: biphasic, 1.0–4.0 V p-p, 1.0 ms, ≤ 10.0 Hz. Single pulse stimulation intensity was adjusted to produce reliable field potentials recorded from adjacent electrodes in the array. Stimulation patterns consisted of 8 channels of biphasic pulses simultaneously delivered to CA1 electrodes, in trains of 1.5–3.0 s duration that conformed to prior analyzed firing patterns from the same electrode locations during SR occurrence. Stimulation patterns were derived from common patterns of neural firing computed ± 1.5 s around SR events across 13 animals tested in this study, and 45 animals tested under similar conditions in a prior study (Berger et al. 2011).

Results

Previous studies from this laboratory showed that cannabinoids modulate task-related hippocampal encoding of information on a trial-by-trial basis in a delay nonmatch to sample (DNMS) short-term memory task (Deadwyler et al. 2007; Deadwyler and Hampson 2008). The same task was utilized here to examine the effects of chronic exposure to CB1 receptor agonists and antagonists, and to assess the corresponding changes in hippocampal ensemble encoding of task-specific information on a trial by trial basis (Hampson et al 2011).

Hippocampal Cannabinoid Receptor Modulation of DNMS Behavior

Thirty two Long Evans rats were trained to perform a DNMS task (Figure 1A) in order to assess hippocampal encoding of short-term, working memory. Animals were then treated with the potent CB1 agonist WIN and antagonist Rmbt to respectively suppress or enhance hippocampal encoding of DNMS events as shown in prior reports (Deadwyler and Hampson 2008; Goonawardena et al. 2010a, 2010b; Hampson and Deadwyler 2000; Hampson et al. 2003). To confirm the role of CB1 receptors confined to hippocampal CA1 and CA3 neurons, nine Long Evans rats were implanted with electrode arrays as well as bilateral infusion cannula targeting the CA3/CA1 regions of hippocampus. Figure 1B illustrates the

array/cannula arrangement and infusion protocols used for each group of animals. Figure 1C shows DNMS performance averaged over all nine animals for 10 daily DNMS sessions in each infusion condition irrespective of sequence of drug delivery. ANOVA confirmed a significant overall main effect of drug ($F_{(2,1890)} = 5.70$, $p < 0.001$), delay $F_{(6,1890)} = 4.17$, $p < 0.001$) and delay X drug interaction ($F_{(12,1890)} = 3.81$, $p < 0.001$). It can be seen that chronic intrahippocampal WIN infusion suppressed DNMS performance in a delay-dependent manner (overall mean% correct $\pm$ SEM: $70.8 \pm 2.2\%$) compared to ACSF/saline control infusion ($82.2 \pm 2.5\%$, $F_{(1,1890)} = 13.62$, $P < 0.001$) recorded 10 days prior to the first minipump change. Likewise, as reported previously for i.p. injections (Deadwyler et al. 2007) Rmbt improved overall DNMS performance ($87.5 \pm 1.3\%$, $F_{(1,1890)} = 9.44$, $P < 0.001$), most significantly at longer delays (asterisks, Figure 1C).

To confirm that there was no differential effect of the sequence of intrahippocampal drug infusion, analyses of the same performance data shown in Figure 1C as a function of the order of exposure (Rmbt-WIN vs. WIN-Rmbt) is depicted in Figure 1D. There was no significant difference due to sequence of exposure for Rmbt (left graph: $F_{(1,1890)} = 1.38$, NS) or WIN (right graph: $F_{(1,1890)} = 0.97$, NS) for either group.

Cannabinoid Modulation of Neural Encoding

Prior studies from this laboratory have demonstrated that canonical analysis yields discriminant functions which can be used to identify components of hippocampal ensemble activity that specifically encode spatial (lever position) and nonspatial (task phase and correct outcome) factors of the DNMS task (Deadwyler et al. 1996; Deadwyler and Hampson 1997, 2004, 2006; Hampson et al. 2003, 2010). It has also been verified that the discriminant function DF5 accounted for approximately 12–15% of the overall variance in ensemble firing and discriminated Left from Right SRs on correct trials (Deadwyler and Hampson 2008). Therefore a similar analysis using the DF5 discriminant function was performed here for sessions with Control, Rmbt and WIN intrahippocampal infusions. DF5 discriminant scores for position yielded a significant (ANOVA) main effect ($F_{(1,5859)} = 21.2$, $p < 0.001$) for DF5 discrimination of right (1.97 ± 0.22) vs. left (-2.02 ± 0.19) SRs, respectively.

The reciprocal effect of CB1 receptor activation and blockade on the strength of SR encoding is shown in Figure 2A which displays the 3D temporal distribution of DF5-weighted firing for a single ensemble of 12 CA1 neurons at the SR. The spatiotemporal color contour plots indicate different ensemble encoding strengths derived by multiplying the mean firing rate of each neuron by its respective DF5 coefficient with the resultant weighted firing ± 2.0 s relative to the occurrence of the SR (0.0s). The increased intensity of ensemble firing (yellow-to-red shading) in the center panel of Figure 2A is evidence for increased ensemble representation (i.e. encoding) of SR information and improved DNMS performance at long delays (Figure 1C) during Rmbt infusion. Decreased ensemble firing (Figure 2A lower) compared to both Rmbt and Control conditions likewise confirms reduced SR encoding associated with decreased DNMS performance following chronic WIN infusion (Figure 1C).

Figure 2B shows the distribution ensemble neuron DF5 scores (firing rate X DF5 coefficient summed over neurons and SR time frame shown in Figure 2A) for Left vs. Right SRs on correct trials. The top (blue) panel depicts the percentage and range of DF5 scores (-4.0 to $+4.0$) for left vs. right SRs during saline infusion sessions. The dotted lines indicate mean DF5 discriminant scores for right (1.97 ± 0.22) vs. left (-2.02 ± 0.19) SRs, respectively for correct trials. The center graph (Figure 2B, green) shows that the left and right DF5 score distributions during Rmbt infusion were skewed to the extremes and differed from control distributions as determined by chi square (χ^2) population analyses ($\chi^2_{(16)} = 35.7$, $p < 0.001$).

In contrast, infusion of WIN (Figure 2B, red) shifted DF5 scores in the opposite direction, toward zero, exhibiting a significant difference in from both Control ($\chi^2_{(16)} = 42.8$, $p < 0.001$) and Rmbt ($\chi^2_{(16)} = 65.2$, $p < 0.001$) distributions. Figure 2C depicts the same distributions in terms of magnitude of DF5 SR scores (absolute values) for each lever position shown in Figure 2A to illustrate on the same scale, the more “effective” SR encoding (i.e. higher DF5 scores) during Rmbt infusion relative to Control sessions and “ineffective” encoding associated with worse performance (Figure 1C) during WIN infusion (Figure 2C, lower scores). Figure 2D plots the mean (+ SEM) number of neurons per ensemble that showed significantly increased firing during the SR, after weighting by the coefficients of the respective discriminant functions (Hampson et al. 2008), to greater than 2 standard deviations above background firing rate (2.0–3.0 s prior to the SR) during Control, Rmbt and WIN infusion. Rimobant infusion significantly increased the number of neurons contributing to both left and right strong DF5 codes (Left SR: Control 9.2 ± 1.2 neurons, Rmbt 15.3 ± 1.8 neurons, $F_{(1,81)} = 15.4$, $p < 0.001$; Right SR: Control 11.0 ± 1.4 neurons, Rmbt 17.4 ± 1.5 neurons, $F_{(1,81)} = 17.0$, $p < 0.001$). WIN infusion slightly decreased the number of neurons contributing to strong codes (Left SR: WIN 7.2 ± 0.8 neurons, $F_{(1,81)} = 8.1$, $p < 0.01$; Right SR: WIN 9.7 ± 1.0 neurons, $F_{(1,81)} = 5.9$, $p = 0.02$).

Chronic Modification of Endocannabinoid Levels via Metabolic Inhibition

Figure 3A shows the effects of two week exposure to the MAG lipase inhibitor URB602 and FAAH inhibitor URB597, which produced behavioral and neural changes similar to the CB1 receptor agonist WIN. DNMS performance was reduced relative to Control levels (mean Control $82.9 \pm 2.2\%$ vs. mean URB597 $66.6 \pm 2.6\%$, $F_{(1,1890)} = 25.4$, $p < 0.001$; vs. mean URB602: $70.1 \pm 3.0\%$, $F_{(1,1890)} = 21.8$, $p < 0.001$) at all delays (Control: $82.9 \pm 2.2\%$, URB597: $66.6 \pm 2.6\%$, $F_{(1,1890)} = 25.4$, $p < 0.001$; URB602: $70.1 \pm 3.0\%$, $F_{(1,1890)} = 21.8$, $p < 0.001$) in animals with chronic minipumps, and could be reversed by injection of Rmbt (2.0 mg/kg) 10–15 min prior to the session (Rmbt+URB597: $83.0 \pm 2.6\%$, $F_{(1,1890)} = 22.7$, $p < 0.001$ vs. URB597, $F_{(1,1890)} = 1.7$, N.S. vs. Control; Rmbt+URB602: $80.0 \pm 2.4\%$, $F_{(1,1890)} = 20.4$, $p < 0.001$ vs. URB602, $F_{(1,1890)} = 1.5$, N.S. vs. Control). In addition, effects similar to chronic administration of WIN were observed for hippocampal ensemble encoding of the SR (Figure 3B URB597: $\chi^2_{(16)} = 29.4$, $p < 0.001$; URB602: $\chi^2_{(16)} = 37.3$, $p < 0.001$) and for the number of neurons incorporated in SR encoding (Figure 3C URB597: $F_{(1,81)} = 8.1$, $p < 0.01$; URB602: $F_{(1,81)} = 7.6$, $p < 0.01$). The fact that these latter agents, whose chemical action increases levels of endocannabinoids above normal levels (Karanian et al. 2005; Manwell et al. 2009), acted specifically and consistently when administered in a chronic manner, demonstrated by the reversal with Rmbt pre-injection, confirms that it was endocannabinoids that modulated DNMS performance and suppressed encoding of information in ensembles of hippocampal neurons.

Reinstatement of Cannabinoid-Suppressed DNMS Performance

While chronic infusion of WIN reduced DNMS performance in the above demonstrations, it has also been demonstrated that systemic i.p. WIN injections decrease performance in the same task (Deadwyler and Hampson 2008; Goonawardena et al. 2010a, 2010b; Hampson and Deadwyler 2000; Hampson et al. 2003). In order to understand the relationship of the process of disruption by both CB1 agonists and endocannabinoids a unique countermeasure was applied, on a session-by-session basis, to animals injected systemically (i.p.) with WIN. The procedure involved multichannel electrical stimulation delivered to CA1 via the same hippocampal recording arrays in a manner that mimicked the SR spatiotemporal firing pattern illustrated in Figure 2A. Recent results have shown that DNMS performance can be enhanced under normal conditions by applying pre-characterized SR patterns via electric pulses delivered bilaterally to the CA1 regions at the time of the SR via the same electrode array (Berger et al. 2011). It was also reported in the same investigation that performance

could be restored by application of similar electrical stimulation patterns when hippocampal neural activity was compromised via local infusion of disruptive pharmacological agents.

The same patterned stimulation procedure was tested in the current study to determine whether CA1 stimulation using “effective” SR firing patterns similar to those produced by Rmbt (Figures 2&3), were capable of restoring DNMS performance on an individual trial basis in animals with reduced performance via systemic (IP) injections of WIN. Effective CA1 firing patterns obtained from Rmbt sessions in the same animals (Figures 2&3) were used to program multichannel stimulation (Hampson et al. 2010) delivered bilaterally to the CA1 electrodes in hippocampal arrays (Figure 4A). Figure 4B shows mean ($\pm$ S.E.M.) DNMS performance summed across 13 animals exposed to both 0.4 mg/kg and 0.6 mg/kg doses of WIN (i.p.), which dose-dependently decreased DNMS performance (Control: $80.3 \pm 0.9\%$, WIN 0.4 mg/kg: $67.8 \pm 1.4\%$, WIN 0.6 mg/kg: $64.8 \pm 0.9\%$, $F_{(2,354)} = 7.8$, $p < 0.001$) at all delays longer than 5 sec (* $F_{(1,354)} = 6.9$, $p < 0.01$, ** $F_{(1,354)} = 11.5$, $p < 0.001$). Ensemble stimulation patterns were delivered to CA1 electrodes on 30% of long delay (>15 sec) trials during sessions in which animals received doses of WIN (i.p.) shown to impair performance at the same delays. Figure 4B shows that performance was improved in sessions employing stimulation (WIN + Stim) with both WIN dose levels, compared to WIN-only sessions (mean WIN + Stim: $72.8 \pm 2.5\%$, vs. WIN i.p. 0.4–0.6 mg/kg (see above), $F_{(1,354)} = 12.6$, $p < 0.001$), although performance remained slightly suppressed relative to nondrug Control levels ($F_{(1,354)} = 6.5$, $p < 0.01$). Stimulation pulses delivered to CA1 via effective SR ensemble firing patterns significantly improved performance at both WIN dose levels (WIN 0.4 + Stim: $74.2 \pm 2.2\%$, $F_{(1,354)} = 15.4$, $p < 0.001$; (WIN 0.6 + Stim: $71.4 \pm 2.3\%$, $F_{(1,354)} = 12.1$, $p < 0.001$).

Figure 4C shows the relative change in DNMS performance, indicated by difference scores at all delays for 1) WIN sessions (overall mean 0.4 mg/kg: $-9.7 \pm 2.0\%$; 0.6 mg/kg: $-12.5 \pm 1.5\%$) relative to Control sessions (Control vs. WIN) shown in Figure 4A and 2) WIN+Stim sessions calculated as the difference from the WIN session with the same dose level (WIN + Stim: mean % change $+8.7 \pm 1.4\%$). Figure 4D illustrates that the latter differences in performance were due to the marked improvement on > 15 s delay trials when effective stimulation was delivered (mean: No Stim $60.6 \pm 1.6\%$ vs. Stim $81.0 \pm 1.1\%$, $F_{(1,354)} = 38.2$, $p < 0.001$), whereas performance was suppressed on trials without stimulation (No Stim) at the same delays. Since DNMS performance was improved on only a select number of long delay (> 15 s) stimulation trials in WIN i.p. sessions, it reveals that CB1 receptor control of performance was via modulation of hippocampal encoding of SR lever position on a trial-to-trial basis during the session.

Discussion

The study reported here shows that cannabinoid CB1 receptor agonists or endocannabinoid reuptake inhibitors, continuously infused into the hippocampus over several days, chronically depress performance of a DNMS memory task. The results also show that such endocannabinoid suppression can be alleviated by subjecting the same ensembles of neurons to subsequent exposure to CB1 receptor antagonists for the same 2 week duration. Changes in the ability of hippocampal ensembles to encode task specific information were assessed during each chronic exposure period and shown to be directly related to performance efficacy. These findings provided the means to reverse the effects of systemic injections of CB1 agonists by electrically stimulating ensembles of hippocampal neurons in a manner defined by the firing patterns of the same ensemble on successful trials (Hampson et al. 2011). Results indicate that the detrimental effects of CB1 receptor modulation of information encoding by endocannabinoid release during performance of the DNMS task,

can be overcome by delivery of task-relevant ensemble-mimicked electrical stimulation patterns.

The above results using long-term chronic infusion of cannabinoids is similar to that observed with periodic systemic injections (Terranova et al. 1996; Lichtman et al. 1995; Hampson and Deadwyler 2000; Takahashi et al. 2005). The fact that chronic intrahippocampal infusion of WIN produced consistent suppression of hippocampal ensemble encoding of task-relevant information (SR), and correspondingly depressed DNMS performance, indicates that hippocampus is a specific target of CB1 receptor agonists and antagonists (Reibaud et al. 1999; Lichtman 2000; Abush and Akirav 2010). This lack of adaptation to the mnemonic detriments of CB1 receptor agonists makes it likely that prior demonstrations of altered ensemble encoding produced by transient i.p. injections (Deadwyler et al. 2007) were the result of direct actions on hippocampal cellular and synaptic processes (Fortin et al. 2004; Foldy et al. 2006). Figure 1 shows an important feature of the effects of cannabinoids, namely that the chronic effects of both WIN and Rmbt did not influence DNMS performance on short delay (1–5s) trials, but decreased or increased performance respectively in a delay-dependent manner irrespective of which drug or the order in which it was chronically infused over a two week time period.

Figure 2 verifies earlier reports of the modulation of hippocampal ensemble encoding during DNMS performance by CB1 receptor agonists and antagonists in the same manner as systemic IP administration (Heyser et al. 1993; Hampson and Deadwyler 1998, 1999, 2000; Hampson et al. 2003). However, in this case both direct CB1 receptor agonists and metabolic inhibitors (Figure 3) were chronically administered and restricted to hippocampus, and produced the same type of Rmbt-sensitive impairment in performance. The fact that the magnitude of discriminant SR scores in the same chronic drug conditions were either increased or decreased, in terms of respective changes in DNMS performance, clearly implicates CB1 activation or suppression as instrumental in modulating hippocampal function, as reported in several recent studies (Foldy et al. 2006; Deadwyler and Hampson 2007; Falenski et al. 2007; Hampson and Deadwyler 2008; Moreira et al. 2009; Abush and Akirav 2010). However, the current results also show that chronic infusion of Rmbt alone was capable of significantly enhancing hippocampal processing in the DNMS task (Figure 2), which shows that endocannabinoids modulate hippocampal activity during normal task performance, not just when agents are applied exogenously prior to individual sessions. Figure 2A confirms this distinction by showing that under Control (nondrug) conditions task-specific ensemble firing during the SR was midway between enhanced or suppressed levels produced by chronic intrahippocampal exposure to Rmbt or WIN, respectively. This chronic action of endogenous cannabinoids at CB1 receptors has been verified in other studies showing facilitation of different behavioral outcomes by blockade of endocannabinoids administered acutely to produce transient beneficial effects for the duration of action of the CB1 antagonists (Gaisler-Salomon and Weiner 2003; Gaisler-Salomon et al. 2008). It is not obvious at this time why endocannabinoid modulation of hippocampal activity occurs under normal testing conditions; however, suppression or alteration in the patterns of ensemble firing could reflect operations that are useful under conditions in which endocannabinoid release is provoked, such as regimented exploration during searching for food (Hampson et al 2008).

This specificity of endocannabinoid modulation of trial-by-trial DNMS performance was determined by the actions of two specific compounds, a FAAH Inhibitor (URB597) and a MAG lipase inhibitor (URB602), both of which have been shown to increase levels of endocannabinoids (Karanian et al. 2005; Manwell et al. 2009). The fact that the 'WIN-like' detrimental effects on performance were also reversed by Rmbt (Figure 3) provides further evidence for relegating the modulation of DNMS performance specifically to CB1 receptors

within the hippocampus. Further support for this specificity is shown by the fact that neither agent produced the same degree of disruption as WIN, a potent receptor agonist, since the only manner in which endocannabinoids could increase action was via secondary changes in levels due to inhibition of hydrolysis of anandamide (URB597), and also 2AG via MAG lipase inhibition (URB602).

The final demonstration that endocannabinoids act specifically to modulate task-dependent firing of hippocampal neurons is shown in Figure 4 in which substitution of ensemble firing with ensemble-derived patterned electrical stimulation (Berger et al. 2011) effectively reversed the WIN-produced deficit in DNMS performance. The convergence of two independent findings in the current study provides additional evidence for a common underlying cellular process modulated by endocannabinoids, capable of being reversed by different treatments (Fortin et al. 2004; Glickfeld and Scanziani 2006). First, chronically infused Rmbt produced enhanced performance under normal (nondrug) conditions by facilitating ensemble firing components identified by discriminant analyses to represent trial-specific SR encoding (Figures 1&2). Second, the same patterns delivered as electrical stimulation during the SR in WIN-Stim sessions not only enhanced performance, but also reversed the effects of i.p. WIN on selective long-delay trials in a manner similar to Rmbt (Figures 1&4). Figure 4C also shows that short-delay trials were facilitated in WIN-Stim sessions, even though stimulation was not delivered on those trials, which is consistent with earlier findings showing that enhanced ensemble SR encoding produced by injection of Rmbt eliminated endocannabinoid-induced sequential dependent performance errors following long-delay trials (Hampson and Deadwyler 2008).

As shown in Figure 4 the dominant control of performance by endocannabinoid levels can be substantially altered by administering electrical stimulation in the pattern of ensemble firing during the SR shown to be disrupted by CB1 receptor agonists (Figures 2&3). Figure 2 also shows that, for the duration of chronic intrahippocampal infusion of Rmbt, both the CB1 agonist-induced behavioral deficits and concomitant hippocampal ensemble SR encoding were reversed and elevated above normal (Control) levels, which provides further evidence that the SR ensemble firing pattern was the critical factor modulated by endocannabinoid levels during task performance (Watson and Stanton 2009). This is also consistent with the recent demonstration that NMDA-mediated calcium control of intracellular calcium release can be modulated directly by endocannabinoids and is facilitated by application of Rmbt in hippocampal slices (Hampson et al 2011a). Finally, showing ‘trial-specific enhancement’ of DNMS performance by delivery of electrical stimulation in effective ensemble SR encoding patterns *to the same neurons disrupted by CB1 receptor activation* (Figure 4C & D), clearly confirms that endocannabinoids play a major role in encoding of hippocampal memory in event-specific contexts.

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Figure 1.

Intrahippocampal infusion of cannabinoid receptor (CB1) agents chronically alters delayed-nonmatch-to-sample (DNMS) performance. **A:** Schematic of DNMS task. *Sample phase:* Rats are trained to press a single lever presented at random in left or right spatial position (Sample). A variable 1–30 s delay is interposed after the Sample response (SR) in which animals must nosepoke into a photocell (NP) on the opposite wall of chamber during the delay (Delay, dividing line). *Nonmatch phase:* Both levers are presented and rat must press lever opposite the SR to receive water reward (0.2 ml per trial). **B:** Hippocampal recording and infusion regimen. Rats implanted with bilateral recording arrays consisting of two rows of eight 25 μ m wire electrodes, with one row each aimed at CA1 and CA3 cell layers in hippocampus (Deadwyler et al. 2007). Bilateral intrahippocampal infusion cannula consisting of 26 ga. stainless steel were targeted 0.2 mm above and lateral to the midpoint of the CA3 electrodes. At surgery, intrahippocampal cannula were connected to Alzet 2004 (4-week) osmotic minipumps containing artificial cerebrospinal fluid (ACSF) then animals were allowed to recover and resume testing in the DNMS task. After 16–22 days original minipumps were removed and replaced with pumps containing either Rmbt (0.33 mg/ml, $n=5$ animals) or WIN 55,212-2 (0.16 mg/ml, $n=4$ animals). Animals resumed DNMS testing 2 days after pump exchange. All behavioral and electrophysiological data are reported for days 6–15 following minipump exchange to allow time for complete cannula flush and steady-state perfusion of infused drugs. After completion of testing for the first agent, minipumps were exchanged and Rmbt animals received WIN, and WIN animals received Rmbt infusions; behavioral and electrophysiological data collected again for days 6–15 following the 2nd minipump exchange. Upon completion of testing, minipumps were removed and cannulae sealed. **C:** DNMS performance assessed during intrahippocampal

infusion of Rmbt or WIN in the same animals. Daily sessions for all animals (n=9) shown by delay and drug condition were averaged over 1000 trials (10 sessions) in 5 s intervals. Mean ($\pm$ S.E.M.) percentage of correct responses on single DNMS trials is plotted for the Control (ACSF/saline), Rmbt and WIN intrahippocampal infusion periods summed across all animals. Durations of the delays were programmed for 1–30 s, however the nosepoke requirement to terminate the delay could increase the actual delay time on a given trial, hence delays >30 s are shown separately (“>30 s” interval). Asterisks (* p <0.01, ** p <0.001) indicate significant increase or decrease in DNMS performance compared to performance in the Control (ACSF/saline minipump) condition. D: Assessment of the sequence of intrahippocampal drug infusions. DNMS performance during infusion with each drug plotted as in C was assessed in different animals grouped according to sequence of drug infusions, Rmbt-WIN or WIN- Rmbt. There was no significant difference in the overall effect of Rmbt or WIN as a function of which drug was administered first in the series.

Figure 2.

Hippocampal ensemble encoding of Sample Response (SR) is differentially altered by chronic infusion of CB1 agonists and antagonists. **A.** Three dimensional displays of ensemble firing at SR (0.0s) adjusted by canonical discriminant DF5 (see Methods) derived from all trials shown in Figure 1. Surfaces are comparisons of peaks and troughs of DF5-adjusted firing rate during the three conditions depicted in Figure 1 with respect to chronic hippocampal infusion of WIN (lower), Rmbt (middle) or ACSF/saline control (upper). Peaks are color coded for high (red & yellow) and low (green & blue) rates of firing of indicated neurons ± 2.0 s relative to occurrence of the SR. Neural data were obtained from sessions showing corresponding differences in performance shown in Figure 1. **B:** Frequency distribution of single trial DF5 discriminant scores for SRs as a percentage of total DNMS trials (1000 per animal) under each condition. Dashed vertical lines indicate the mean DF5 score value for Left vs. Right SR events summed over all correct trials in Control (ACSF/saline infusion) sessions (upper graph). Asterisks (*p<0.01, **p<0.001) indicate significant shifts (by Chi-square analysis) in frequency distributions of WIN (DF5 absolute values, reduced) and Rmbt (DF5 values increased) compared to Control. **C:** Combined display of mean frequency of all DF5 scores in each of the 3 conditions (WIN, Rmbt, Control) on the same continuous scale irrespective of sign for comparison of the entire score distribution for all trials shown in Figure 2B. Scores in the range 0.5–1.5 are labeled “non-effective”, since those trials were typically errors at delays >15 s while scores >2.5 were considered “effective” since they were related to correct trials with delays of 16s to 30 s

when compared across 1000 trials per animal per condition. D: Mean (+SEM) number of neurons exhibiting significant changes in firing that contributed to the mean DF5 values for Left or Right SR events in each of the three conditions as shown in B&C. Neural ensemble firing averaged across ± 1.5 s at the SR was weighted by DF5 coefficients, then significant increases in firing identified by standard score (Z) based on peak firing rate relative to baseline rate. Bars indicate mean (+SEM) number of neurons that contributed significantly (i.e. positive coefficient-weighted firing to the SR) to increased (effective) SR values in each of the three conditions across animals. Asterisks (* $p < 0.01$, ** $p < 0.001$) indicate significant increase or decrease compared to Control.

Figure 3.

Chronic intrahippocampal infusion of MAG lipase inhibitor URB602 and FAAH inhibitor URB597. A: DNMS performance reduction at all delays by URB602 (left) and URB597 (right) was reversed by injections of Rimnabant (2.0 mg/kg) 10–15 min prior to the start of behavioral session. Mean ($\pm$ SEM) % correct performance shown for 5–10 sessions per animal over all 3 conditions. B: Distribution of SR DF5 values (see Figure 2C) for both URB602 (left) and URB597 (right) shows increased frequency of ineffective DF5 trials for both agents relative to Control and reversal to effective DF5 values with pre-injection of Rmbt. C: Chronic exposure to URB597 (upper) and URB602 (lower) reduced the number of hippocampal neurons encoding the SR, similar to WIN (see Figure 2D). This was also significantly reversed by prior injection of Rmbt (green bars).

Figure 4.

Reversal of cannabinoid suppression of DNMS performance using ensemble-SR-patterned stimulation of hippocampal CA1 cells. A. Effective SR CA1 firing patterns obtained from Rmbt sessions, as shown in Figure 2A, were used to model output of multichannel stimulator that delivered 1.0 ms biphasic pulses (0.25–1.0 V, 10–50uA) through pairs of CA1 electrodes in the same electrode array locations. Stimulation was delivered bilaterally in accordance with the effective firing patterns for 1.0–3.0 s commencing with the SR. Individual channel stimulation rates were limited to 20 Hz with a minimum of 50 ms between individual pulses as determined from prior investigations (see text). B. Dose-effect of IP WIN suppression of DNMS performance and partial recovery and improvement by delivery of effective electrical SR stimulation patterns shown in A. Animals (n=13) were first tested in sessions with i.p. WIN injections (0.4 mg/kg or 0.6 mg/kg) in which mean ($\pm$ SEM) percent correct DNMS performance was assessed for 2–4 sessions for each animal (open triangles, filled squares). The same animals were tested in 2–4 additional i.p. WIN sessions at the same doses in which patterned electrical stimulation was delivered to the CA1 cell layer on 30% of trials with delays >15 s (open diamonds). Asterisks (* p <0.01, ** p <0.001) indicate significant decrease in performance compared to Control. Daggers (‡ p <0.01, † p <0.001) indicate significant increase in performance compared to WIN-

injected sessions. C. DNMS performance in B re-plotted as mean difference ($\pm$ SEM) in % correct performance summed across animals for pairs of conditions. Control vs. WIN (0.4 & 0.6 mg/kg) = difference of % correct performance of Control minus values for WIN (0.4&0.6 mg/kg) sessions in B yielding negative mean percent changes from Control levels for each set of delay intervals. Stimulation vs. WIN = difference of % correct performance of WIN +Stim sessions in B minus performance in session with the corresponding WIN dose, yielding positive changes in performance at all delays compared to WIN sessions with no stimulation. Asterisks (* p <0.01, ** p <0.001) indicate significant increased or decreased % correct performance from no change (0%). D. Performance on stimulated vs. nonstimulated trials in WIN + Stim sessions in B and C were sorted according to delivery (Stim) or no delivery (No Stim) on individual DNMS trials >15 s. Asterisks (* p <0.01, ** p <0.001) indicate significant increase in performance on stimulation (Stim) trials compared to trials of the same delay in the same session that did not receive SR stimulation (No Stim).