# Tolerance to the Memory Disruptive Effects of Cannabinoids Involves Adaptation by Hippocampal Neurons

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**ABSTRACT: The effects of chronic exposure to cannabinoids on short-term memory in rats were assessed during repeated daily injections of an initially debilitating dose (3.75 mg/kg) of the potent CB1 cannabinoid receptor ligand, WIN 55,212-2. Delayed nonmatch to sample (DNMS) performance was assessed over a 35-day exposure period in which performance was initially disrupted during the first 21 days of exposure but recovered by day 30 and was stable at pre-drug levels for 5 days thereafter. Withdrawal was precipitated by injections of the CB1 receptor antagonist SR141716A and transiently reduced performance for 2 days but was restabilized to pre-drug levels within 3–4 days. Concomitant recording from identified CA1 and CA3 hippocampal neurons demonstrated a marked correspondence in the time course of suppression of peak firing in the sample and delay phases of the task to the drug-induced performance deficits over the same days of exposure. Hippocampal encoding of task-relevant events and performance levels "tracked" each other on a daily basis throughout the chronic cannabinoid treatment and withdrawal regimen. However, hippocampal neuronal activity in the nonmatch phase of the task was unaffected by the chronic cannabinoid treatment or withdrawal, suggesting that only a select population of hippocampal neurons and synapses are involved in cannabinoid-sensitive short-term memory processes. *Hippocampus 2003;13:543–556.* © 2003 Wiley-Liss, Inc.**

**KEY WORDS: delayed-nonmatch-to-sample; behavior; withdrawal; electrophysiology; ensemble; aminoalkylindole; WIN 55,212-2; SR141716A**

## INTRODUCTION

Tolerance to repeated exposure to cannabinoids is of major significance, given the number of cellular and synaptic processes shown to be coupled to cannabinoid receptors in the brain (Deadwyler et al., 1993, 1995a; Twitchell et al., 1997; Katona et al., 1999; Mu et al., 1999, 2000; Hampson et al., 2000; Irving et al., 2000; Baker et al., 2000; DeSanty and Dar, 2001; Cichewicz et al., 2001; Pertwee, 2001; Vaughan, 2001). It has been known for some time that tolerance to cannabinoids can occur quite rapidly (Coutts et al., 2001; DeSanty and Dar, 2001; Rubino et al., 1988; Hsieh et al., 1999); however, the time course and degree of tolerance are often a function of the system being assessed. In some systems, cannabinoid tolerance cannot be demonstrated at all (Romero et al., 1999; Wu and French, 2000; Nava et al., 2001); in other systems, it may take weeks or months (Deadwyler et al., 1995b; Aceto et al., 2001; Costa and Colleoni, 2000). We previously reported that rats trained on a delayed-match-to-sample (DMS) short-term memory task, if treated once daily with a very debilitating dose (10 mg/kg) of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), exhibited recovery from the disruptive influences over a 35-day exposure period (Deadwyler et al., 1995b). We recently showed that cannabinoids affect DNMS performance by suppressing the activity of specific functional cell types (FCTs) in the hippocampus (Hampson and Deadwyler, 2000). It was therefore important to determine whether tolerance to the behavioral effects of cannabinoids involved the disruption and eventual recovery of this same class of hippocampal neurons.

In the present study, animals received chronic exposure to the potent CB1 cannabinoid receptor agonist WIN 55,212-2 (Ward et al., 1991; Pacheco et al., 1991; Compton et al., 1992), to assess the time course of recovery from disruption of DNMS performance. Once developed, tolerance to the drug was terminated by precipitation of withdrawal via administration of the CB1 receptor antagonist, SR141716A (Rinaldi-Carmona et al., 1994, 1995). Changes in hippocampal neuronal correlates of DNMS performance were recorded simultaneously in animals showing behavioral impairment and subsequent recovery from the disruptive effects of chronic cannabinoid exposure.

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## MATERIALS AND METHODS

### Subjects

Eight (n = 8) male Long-Evans rats ranging in age from 250–300 days were used as subjects. All animals were trained with the same DNMS performance criteria (90% at 1–5-s delays) before surgery and were retrained with the same criteria after surgery before receiving chronic drug exposure.

### Apparatus

The apparatus was the same as employed in other studies from this laboratory (Heyser et al., 1993; Deadwyler et al., 1996; Hampson and Deadwyler, 2000) and consisted of a 43 × 43 × 53-cm plexiglass behavioral testing chamber with two levers mounted on either side of a water trough on the same wall and a nosepoke (NP) device mounted in the center of the opposite wall. The entire apparatus was housed inside a commercially built sound-attenuated cubicle (Industrial Acoustics Co, Bronx, NY). The two retractable levers (Coulborn Instruments, Lehigh Valley, PA) were positioned 3.5 cm above the floor, separated by 14.0 cm, center to center. The NP device consisted of an infrared photodetector and light-emitting diode separated by a 2.5 × 1 × 1-cm opening in a delrin housing, mounted 4.0 cm above the chamber floor, in the center of the wall opposite the levers. A cue light (6 V, 10 W) was positioned immediately above the NP device, and a speaker was mounted overhead provided constant 85-db white noise. Two 12-V, 25-W incandescent lamps (house lights) were mounted on the top of the chamber. Video monitoring of the animal at all times was provided by a Sanyo CCD camera mounted above the chamber. PC computers controlled behavioral parameters and collected behavioral and neuronal data for offline analysis.

### Behavioral Training Procedure

Animals were water restricted, but allowed free access to food throughout DNMS training and testing. Volume of water consumed and daily weight gain were monitored to maintain 85–90% of ad libitum body weight. Water consumed during the behavioral session was recorded, and a supplemental volume was given immediately after the session, to provide 20–22 h of water deprivation. The DNMS task and pretraining were identical to those described by Hampson and Deadwyler (2000), consisting of three main phases: sample, delay, and nonmatch. At the initiation of a trial, either the left or right lever was extended (sample presentation) at 50% overall probability, and the animal responded (sample response) to complete the sample phase of the task. The lever was then immediately retracted, initiating the delay phase, signaled by an illuminated cue light over the NP device on the opposite wall. Duration of the delay interval varied randomly on each trial at 1–30 s, with equal likelihood for any duration at 1-s resolution. The animal was required to NP in the photocell device on the opposite wall at least once during the delay before the interval was terminated. The last nosepoke (LNP) after the delay timed out turned off the cue light and simultaneously extended both levers on the opposite wall, signaling the onset of the nonmatch phase of the task. In this phase, the animal was required to press the lever opposite to the response executed in the sample phase. If correct, the response operated a valve that delivered a drop of water to the trough between the two levers. The levers were immediately retracted for 10 s (intertrial interval [ITI]) before initiation of the next trial. On incorrect (error) trials in which the same lever press as in the sample phase occurred (i.e., a match response), a 5-s time-out (TO) period was initiated, and the house lights were turned off, leaving the chamber completely dark with both levers retracted. The house lights were then illuminated for an additional 5 s with levers still retracted (TO+ 5 s = ITI, 10 s, after error trials), after which the next trial was initiated. All animals were trained to a minimum criterion performance of 90–95% correct on trials with 1–5-s delays during pre-drug sessions (Deadwyler et al., 1996).

### Drug Preparation and Administration

The CB1 cannabinoid receptor agonist, WIN55,212-2, was obtained as mesylate powder (Sigma/RBI) and was dissolved in ethanol to a make a 20-mg/ml stock solution. The detergent vehicle was prepared from Pluronic F68 (Sigma) 20 mg/ml in ethanol. The WIN 55,212-2 stock (1.0 ml) was added to the detergent/ethanol solution; 2.0 ml of saline (0.9%) was then slowly added to this solution, stirred rapidly, and then placed under a steady stream of nitrogen gas for 10 min to evaporate the ethanol. This detergent/drug suspension (10.0 mg/ml) was sonicated and then diluted with saline to a final injection concentration of 3.75 mg/ml (pH 7.2). The CB1 receptor antagonist SR141716A (NIDA, Research Triangle Institute, NC) was prepared in the same vehicle solution, for a final concentration of 5 mg/ml (Hampson and Deadwyler, 2000). Animals were injected 1 h before behavioral testing with a volume of 1 ml/kg of the above solutions. Animals received 5 days of vehicle-only injections, then 35 successive days of a moderate to large dose (3.75 mg/kg) of WIN 55,212-2. On days 36–40, each animal received SR141716A (5.0 mg/kg) instead of WIN55,212-2 to precipitate withdrawal (Rubino et al., 1998). Finally, WIN 55,212-2 (3.75 mg/kg) was readministered on day 41 to test for the effects of SR precipitated withdrawal. All drug solutions were mixed fresh each day before injection.

### Analyses of Behavioral Data

Daily performance levels for each animal used two primary measures: mean percentage correct trials during the session, and mean percentage correct trials at each delay interval grouped into 5.0-s blocks. Analysis of variance (ANOVA) with adjusted pairwise contrasts for individual comparisons was employed to assess statistical significance (Stevens, 1992). Four of the eight animals tested showed signs of a respiratory virus around days 16–20, lasting about 5–7 days. For only the infected animals, there was a plateau in behavioral performance of the same duration; uninfected animals showed continuous performance improvement. The data for 5–7 days have been omitted from the time course of exposure graphs, to provide consistent behavior and electrophysiology comparison between the two groups.

## Surgery

When animals reached performance criteria on the DNMS task, they were surgically implanted with multi-neuron recording arrays consisting of 16 40-μm wire electrodes (NB Labs, Denison, TX) positioned in the CA1 and CA3 subfields of the hippocampus (Deadwyler et al., 1996). Animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) during implantation of the array, which was positioned such that the tips of the two rows of electrodes were located within the cell layers of the CA1 and CA3 subfields along the longitudinal axis of the hippocampus. The pair of electrodes at the center of the array was positioned at coordinates 3.8 mm posterior to bregma and 3.0 mm left of midline. The longitudinal axis of the array was angled 30 degrees to midline, driven in 25–100-μm steps to a depth of 3.0–4.0 mm for CA3 leads, with the CA1 leads automatically positioned 1.2–1.4 mm dorsal to that. Neural activity was monitored throughout surgery to ensure placement in appropriate structures. The cranium and array were sealed with dental cement. The animals were injected with penicillin G (300,000 U) to prevent infection and were allowed to recover for 1 week before retraining. All animal care and experimental procedures conformed to National Institutes of Health (NIH) and Society for Neuroscience guidelines for the care and use of experimental animals.

## Multineuron Recording

Extracellular action potentials, or "spikes" recorded from the microwire electrodes were digitized at 40 kHz and time-stamped along with behavioral events for computer processing within each DNMS session. Individual neuronal action potentials were isolated by time-amplitude window discrimination and computer-identified individual waveforms using a Plexon Multineuron Acquisition Processor (Plexon, Dallas, TX). Single neurons recorded at each array location were selected for analysis from the 16 different wire electrodes. Single neuron data from each electrode were tracked from session to session by waveform and firing characteristics (peri-event histograms [PEHs]) within the DNMS task across sessions. Only neurons with baseline firing rates of 0.5–2.0 Hz, corresponding to putative hippocampal pyramidal cells were analyzed. Neuronal recordings were obtained from six of the eight animals in the experiment.

## Analysis of Neuronal Data

Changes in neural firing rates were analyzed for statistically significant differences via two-way and three-way ANOVA. Measurements of single neuron activity included: mean (±SEM) firing rate within defined intervals (i.e., across the delay in 5–10-s blocks), mean firing rate before, during and after task-relevant events (i.e., ± 1.5 s for sample or nonmatch responses) and peak firing rate during either of the three phases: sample, delay or nonmatch. Background firing rate was computed from 3-s intervals during the ITI. Standard scores ($z = [\text{peak rate} - \text{background rate}] \div SD$) were computed to determine significant peak firing rates. Using this measure, 92% of cells recorded showed valid firing correlates in at least one phase of the DNMS task. Combined simultaneous multineuron ("ensemble") firing rates were also analyzed by multivariate statistical procedures to verify the relevance of functional cell types (FCTs) (Hampson and Deadwyler, 1998, 2000). FCTs were defined as individual neurons with significant encoding of individual behavioral events, and were sorted into three categories recorded throughout the chronic WIN55212-2 exposure regimen according to the following categories: phase of DNMS trial (sample or nonmatch), position of response (left or right) within a phase, or conjunctive firing consisting of specific combinations of phase and position firing (i.e., left-sample).

# RESULTS

## Chronic Cannabinoid Treatment and DNMS Performance

All rats were trained to criterion performance in the DNMS task, given daily injections of Pluronic F68 vehicle for 5 days, and then injected with WIN 55,212-2 (3.75 mg/kg) for 35 days. The 35-day exposure was followed by 5 days of withdrawal, precipitated by injections of the antagonist SR141716A (5 mg/kg) instead of WIN 55,212-2, and then one test day of the same dose (3.75 mg/kg) of WIN 55,212-2. The dose of WIN 55,212-2 was quite high (3.75 mg/kg) as judged by prior acute studies (Hampson and Deadwyler, 2000) and was sufficient to induce catalepsy in the early stages of the study (Chaperon and Thiebot, 1999; Sanudo-Pena et al., 2000; Meschler et al., 2000). Animals were placed in the DNMS task starting 1 h after injection, to ensure that the cataleptic effects of the drug had dissipated (Meschler et al., 2000). Mean performance (percentage correct responses) is shown in Figure 1, averaged across all eight animals (performance of the two animals without recording arrays is shown by the cross-hair symbols), and sorted by 5-day intervals during chronic cannabinoid exposure. Performance on pre-drug (vehicle) days 1–5 was 79.7 ± 2.7%, but immediately after onset of daily cannabinoid injections, decreased precipitously to 20.9 ± 4.2% ($F_{1,351} = 70.6$, $P < 0.001$). Performance gradually improved over the next 30 days and was not significantly different from pre-drug levels on days 30–35 (77.2 ± 2.7%, $F_{1,351} \geq 2.75$, $P = 0.09$). Also plotted is the curve (triangles) from a prior study (Deadwyler et al., 1995b), using $\Delta^9$-THC (10 mg/kg) over the same time course of exposure. The same initial disruption is depicted, only not as severe as with WIN 55212-2; and the basic recovery time is the same with respect to the development of tolerance.

Withdrawal from chronic cannabinoid exposure was precipitated on day 36, by injection of SR141716A (5.0 mg/kg) 1 h before the behavioral session. At 5–10 min after injection of the antagonist, all animals showed behavioral signs of withdrawal to varying degrees, as indicated in videotaped records by increased scratching movements and forelimb quivering, as well as increased grooming and other reported indices (Aceto et al., 1996; Rubino et al., 1998). DNMS performance was decreased significantly on the first 3 days of withdrawal compared with the last day of tolerance (day 35: 80.2 ± 3.0; day 36: 58.6 ± 3.1%, $F_{1,351} = 25.9$, $P < 0.001$; day

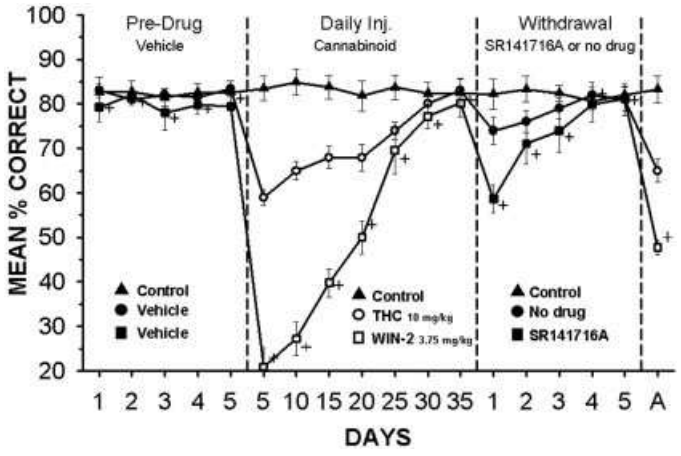


**FIGURE 1. Overall behavioral performance (mean percentage correct) at all delays during chronic exposure to cannabinoids. Control and WIN 55,212-2 results are from the current study; results with $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) are from a previous study (Deadwyler et al., 1995b). Animals received vehicle injections (filled symbols) during pre-drug baseline. Daily injections of $\Delta^9$-THC (10 mg/kg, n = 4 animals) or WIN 55,212-2 (3.75 mg/kg, n = 6) were administered on days 1–35 (unfilled symbols). Withdrawal was precipitated via injection of the CB1 receptor antagonist, SR141716A (5 mg/kg) or abstinence in the case of $\Delta^9$-THC, on days 36–40. Reversal of tolerance was demonstrated by the response to reexposure to the tolerant animals (A, 3.75 mg/kg) on day 41. Mean behavioral performance of two additional animals that were not implanted and recorded is shown by unconnected crosses. Control plot shows performance of the same animals over a similar time course of 40 days (before starting the cannabinoid exposure), in which only the vehicle was administered. Each point shows mean (±SEM) percentage correct performance during 100 trial sessions, averaged over eight animals for each drug. Below-chance behavioral performance is discussed in text.**

37: 71.1 ± 4.6, $F_{1,351} = 10.9$, $P < 0.001$; day 38: 74.0 ± 4.8%, $F_{1,351} = 7.5$, $P < 0.01$) but returned to baseline by the fourth day (day 39: 79.9 ± 3.9%, $F_{1,351} = 0.35$, $P = 0.55$). To test for reversal of tolerance, animals received a final injection of the same high dose of WIN55212-2 (3.75 mg/kg) on day 41 after withdrawal. DNMS performance was again markedly reduced (47.7 ± 1.7%, $F_{1,351} = 38.3$, $P < 0.001$) compared with pre-drug (day 0) levels.

These results were compared with a prior study (Deadwyler et al., 1995b) in which another cannabinoid ($\Delta^9$-THC, 0 mg/kg) was administered over the same time course. In comparing the two studies, Figure 1 shows that WIN 55,212-2 produced significantly greater deficits in DNMS performance under the same chronic exposure procedure (day 5, WIN 55,212-2: 20.9 ± 4.2%, $\Delta^9$-THC: 58.7 ± 1.7%, $F_{1,351} = 24.4$, $P < 0.001$). In the prior study, withdrawal consisted of discontinuing $\Delta^9$-THC for vehicle injections; however, SR141716A produced a greater decrement in DNMS performance during withdrawal than removal of $\Delta^9$-THC (WIN 55,212-2: 58.6 ± 3.1%; $\Delta^9$-THC: 74.2 ± 3.0%, $F_{1,351} = 18.5$, $P < 0.001$, Fig. 1, days 36–40).

The triangles in Figure 1 depict performance over a comparable 40-day period, while the same animals received vehicle-only injections. Mean performance across the entire control period was 81.3%, and there was no significant difference ($F_{1,351} = 1.7$, $P = 0.19$) from the pre-drug period (squares, Fig. 1). There was also no significant difference in performance throughout the 40-day control period ($F_{1,351} = 1.2$, $P = 0.27$). Figure 1 shows that during the first 20 days of chronic WIN 55,212-2 exposure, performance fell below chance (50%) levels. This resulted from a circumstance in which animals made more errors due to a drug-enhanced incompatible behavioral strategy, previously described in normal animals on error trials (Hampson et al., 1998), in animals under acute cannabinoid exposure (Hampson and Deadwyler, 1996, 2000), and documented behaviorally in hippocampal lesioned animals performing the DNMS task (Hampson et al., 1999). Basically, performance is below chance because after an error, animals tend to perseverate and "encode" the next subsequent sample lever on the next trial the same as the lever that was pressed erroneously in the nonmatch phase on the prior error trial (Hampson et al., 1998). They then respond accordingly but are likely to make a second consecutive error if the two lever positions are not the same, because the sample lever on the next trial will be "miscoded" 50% of the time (Deadwyler et al., 1996). However, even if the lever positions between the two trials are the same (i.e., correctly encoded), the animal is still at risk for making an error 50% of the time as a function of the strength of the code and the length of the delay on that trial (Hampson and Deadwyler, 1996, 2000). This means that the likelihood of an error, given an error on the prior trial, is 75%. The performance rate continues at 25% as long as the animal continues to perseverate after an error trial, which accounts for the combined probability of 25% correct or lower than chance performance in the early stages of chronic cannabinoid treatment shown in Figure 1 (see also discussion in Fig. 8).

## Delay Dependence of Chronic Cannabinoid Effects on DNMS Performance

DNMS trials were sorted by length of delay and were averaged in 5-s increments for all trials within a session across the time course of chronic WIN 55,212-2 exposure and withdrawal. Figure 2A compares DNMS delay performance for the pre-drug baseline on day 0 (PRE) with performance on days 5, 15, 20, 25, and 35, of chronic WIN 55,212-2 exposure. There was a significant overall decrease in DNMS performance at all delays across days ($F_{55,573} = 2.74$, $P < 0.001$), with a significant day-by-delay interaction ($F_{7,573} = 4.98$, $P < 0.001$). For the first 15 days of chronic drug exposure, DNMS performance was decreased significantly at even the shortest (1–5 s) delay intervals (Pre: 89.9 ± 0.7; day 5: 55.0 ± 9.7, $F_{1,573} = 17.54$, $P < 0.001$; day 15: 71.2 ± 3.3, $F_{1,573} = 9.35$, $P < 0.01$), but this did not persist beyond day 15 (all >80.8 ± 3.3%, $F_{1,573} < 4.52$, $P > 0.03$). However, performance remained significantly impaired on day 20 for delays of >5 s ($F_{1,573} > 13.8$, $P < 0.001$), and on day 25 for delays of >10 s ($F_{1,573} > 7.42$, $P < 0.01$). Figure 2A shows that by day 35 there was no significant difference from pre-drug performance (day 0) at any delay (all $F_{1,573} < 2.09$, $P > 0.14$) in the task, revealing complete tolerance to WIN 55,212,-2 injections.

Figure 1 shows that performance dropped by 20% from day 35 levels, to less than 60% correct responding on the first day of withdrawal precipitated by SR141716A (day 36, $F_{1,351} = 25.9$, $P < 0.001$). Figure 2B shows that this decline was due primarily to

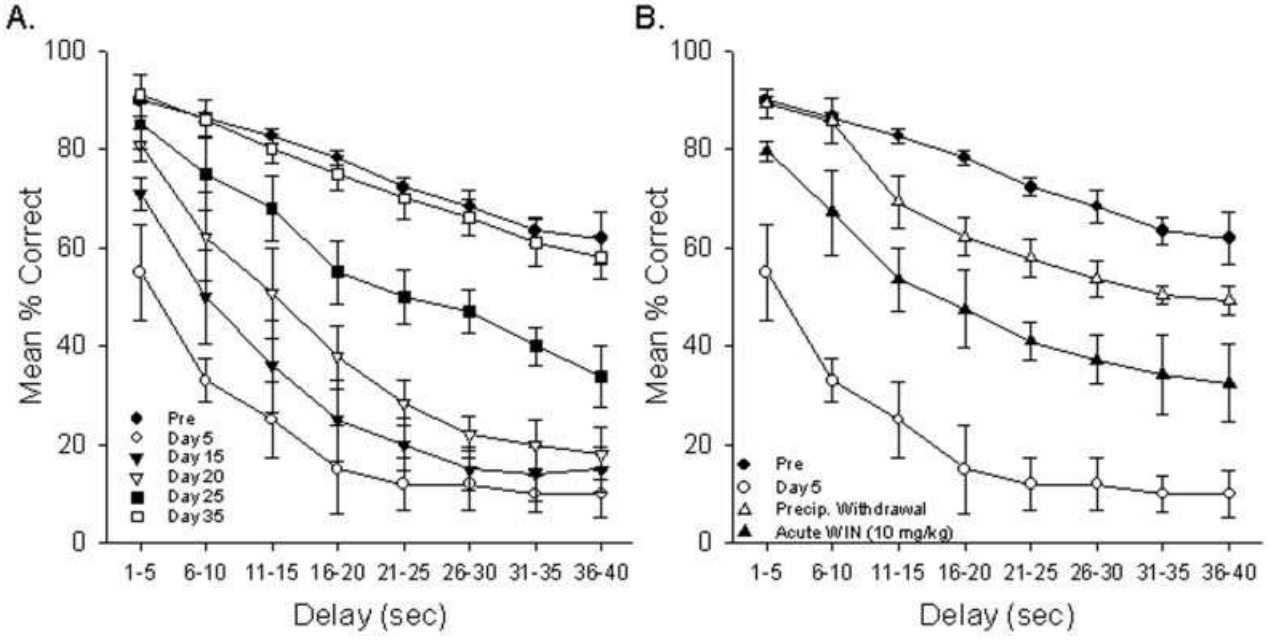


**FIGURE 2. Delay-dependent delayed nonmatch to sample (DNMS) performance during chronic WIN 55,212-2 exposure. DNMS trials were sorted by length of delay, and averaged within 5-s increments. Each curve represents mean (±SEM) performance across animals for a single session (100 trials). A: Comparison of delay-dependent DNMS performance before chronic WIN 55,212-2 (Pre) and at days 5, 15, 20, 25, and 35 (Fig. 1). B: Comparison of baseline (Pre) performance with the first day (day 36) of SR141716A precipitated withdrawal, and an acute day of cannabinoid exposure after withdrawal.**

a reduction in correct responding on trials with delays of >10 s (all $F_{1,573} > 6.3$, $P < 0.01$) that returned to pre-drug control levels by day 40 (5th day of withdrawal). Reexposure to WIN 55,212-2 (3.75 mg/kg) on day 41 again produced a significant decrease in performance at all delays (all $F_{1,573} > 5.2$, $P < 0.02$), indicating that withdrawal had been effected, and animals were no longer tolerant to the large dose of WIN 55,212-2.

## Effects of Chronic Cannabinoid Treatment on Hippocampal Neurons (FCTs)

A total of 62 identified hippocampal neurons were recorded from six animals to determine whether the initial decrease in DNMS performance and subsequent behavioral tolerance to the effects of WIN 55,212-2 were correlated with alterations in hippocampal cell firing. It was previously shown that DNMS performance is associated with activation of different CA1 and CA3 hippocampal FCTs during the task (Deadwyler et al., 1996; Hampson et al., 1999, 2002; Hampson and Deadwyler, 2000). Since the activity of specific FCTs reflects the differential encoding of DNMS information within the trial, it is important to assess how each class of FCT (see Materials and Methods) was affected by chronic exposure to WIN 55,212-2.

Figure 3 shows a sample phase FCT that fired at either lever position of the sample response. Single trial rastergrams and PEHs summed over the session show that increased firing for both right and left sample phase responses was eliminated for several days after initiation of chronic WIN 55,212-2 exposure (day 10), but partially returned by day 25 and eventually did not differ from pre-drug (day 0) levels by day 35 (Fig. 3). In marked contrast, Figure 4 shows a nonmatch phase FCT in which there was no effect on firing to either lever position response in this phase of the task on any of the corresponding days in which sample phase firing was reduced. Figure 5 shows an example of the firing of different conjunctive FCTs. Since these cells only fire during one phase of the task, Figure 5 shows the FCT-appropriate sample firing of a right-sample cell and the nonmatch firing of a right-nonmatch cell. Firing of the right-sample cell was markedly suppressed on day 10, partially returned by day 25 and recovered to pre-drug levels by day 35. Firing of the right-nonmatch cell was unaffected at similar periods and fired normally throughout the chronic drug regimen. Finally, Figure 6 depicts firing of the right position FCT, during either right-lever sample or right-lever nonmatch responses on different DNMS trials. Firing of this FCT was differentially affected by chronic cannabinoid exposure in that firing in the sample phase was suppressed in the same manner as other sample phase FCTs on day 10, partially recovered by day 25, and fully recovered by day 35 (Fig. 3). However, firing of this same (position) FCT was unaffected in the nonmatch phase on different trials within the same DNMS session (Fig. 6).

The dynamics of firing in simultaneously recorded ensembles of hippocampal FCTs is shown in Figure 7, using mean trial-based histograms (TBHs) with single trial rastergrams, at different time periods during chronic cannabinoid exposure. Rastergrams depict single-trial firing of a simultaneously recorded sample, delay, and nonmatch FCT from a single animal, TBHs reflect the ensemble (10 neurons) firing from which the FCTs were selected. Pre-drug

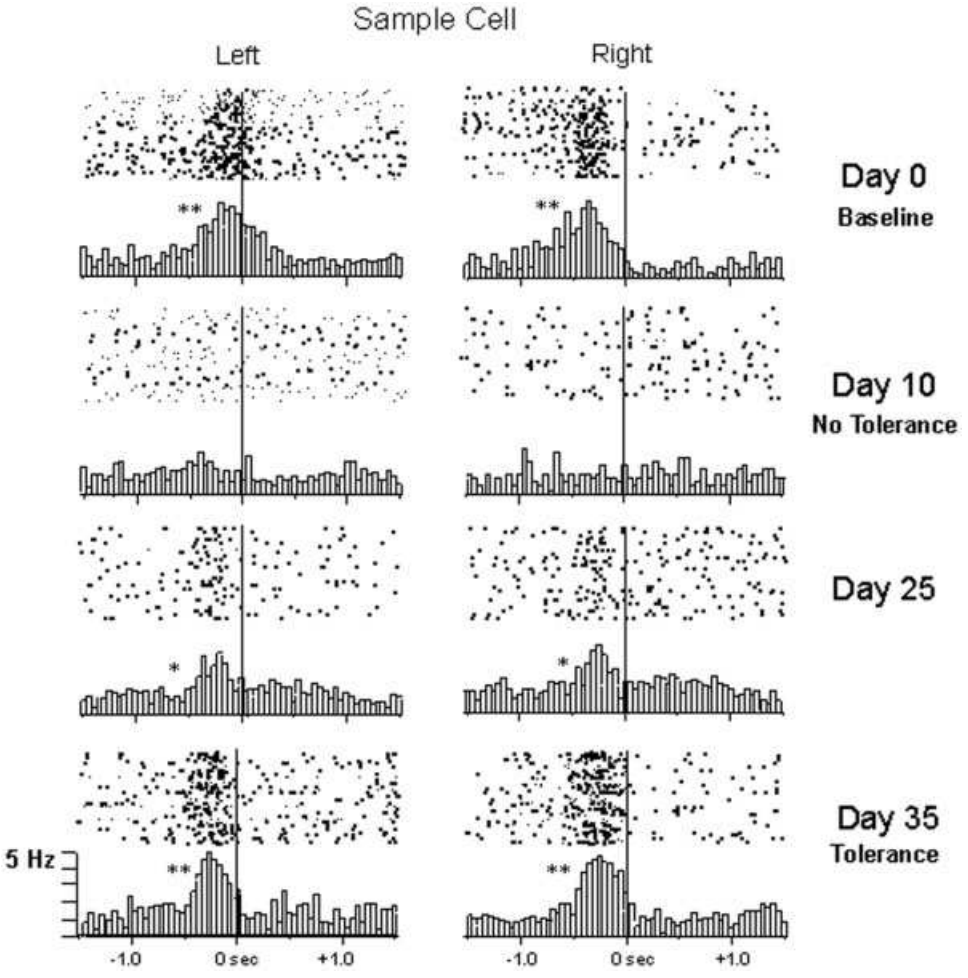


**FIGURE 3. Effects of chronic cannabinoid on sample phase functional cell type (FCT) in the delayed nonmatch to sample (DNMS) task. Peri-event histogram (PEH) reflects mean left and right sample phase firing which occurred on different DNMS trials (± 1.5 s around sample response (0 sec.); n = 50 trials). All PEHs are plotted to the same 5.0-Hz firing rate scale. Peri-event rastergrams above each PEH indicate cell firing on 20 individual trials in the same session. Each row represents a single trial, dots indicates each time the cell fired. PEHs and rastergrams are plotted individually for left and right responses during the sample phase. Sessions from days 0, 10, 25 and 35 (Fig. 1) are plotted to illustrate change in sample firing during chronic exposure to WIN 55,212-2. Significant peak firing indicated by asterisks (*$F_{4,25}$ >4.2, $P < 0.001$; **$F_{4,25}$ >6.5, $P < 0.001$).**

(day 0) firing shows an increase to 3.5 Hz in the sample phase FCT just before the response, followed by a return to baseline firing in all three cells, and then a gradual "ramping" up to a 3.0-Hz firing peak in the delay FCT across the 40-s delay interval until the occurrence of the LNP, which is accompanied by increased firing (≥5.0 Hz) of the nonmatch FCT at the time of the nonmatch response (Fig. 7). The consistency across trials in the rastergrams (Fig. 7) indicates the specificity of firing of different FCTs recorded in the same animals (Hampson et al., 1999; Hampson and Deadwyler, 2000). Figure 7 also shows the dramatic influence on this ensemble firing pattern of initial WIN 55,212-2 exposure at day10, in which sample and delay phase firing were virtually eliminated (1.8 ± 0.5 Hz, $F_{1,634} = 17.3$, $P < 0.001$ and 1.8 ± 0.3 Hz, $F_{1,634} = 13.4$, $P < 0.001$, respectively) but, as shown above, nonmatch phase firing was unchanged (4.9 ± 0.6 Hz, $F_{1,634} = 0.4$, $P = 0.53$). Although firing on match (error) trials is not shown, FCT firing during this phase did not differ from the nonmatch phase firing shown in Figure 7. By day 25, sample phase firing was somewhat recovered, but delay firing continued to be suppressed, and both remained significantly reduced relative to pre-drug (day 0) levels (sample phase: 2.4 ± 0.4 Hz, $F_{1,634} = 14.2$, $P < 0.001$; delay 1.8 ± 0.4 Hz, $F_{1,634} = 12.1$, $P < 0.001$) while nonmatch firing remained unaltered. However, after 35 days of chronic cannabinoid exposure, sample peak firing was not significantly different from pre-drug (day 0) levels (day 35; 3.4 ± 0.3 Hz, $F_{1,634} = 2.2$, $P = 0.14$) and delay firing was only slightly depressed (day 35; 2.5 ± 0.3 Hz, $F_{1,634} = 4.6$, $p < 0.05$), even though animals received the same high dose as on days 10 and 25 (Fig. 7). Thus, by day 35 of chronic exposure, hippocampal ensembles exhibited nearly complete tolerance to the initial suppressive effects WIN 55,212-2 (all $F_{1,634} < 3.8$, $P > 0.05$) on DNMS task-relevant firing.

This change in sample and delay firing across the time course of chronic exposure was also revealed in trial-by-trial measurements. We previously showed that sample firing rates<3.0 Hz were "at

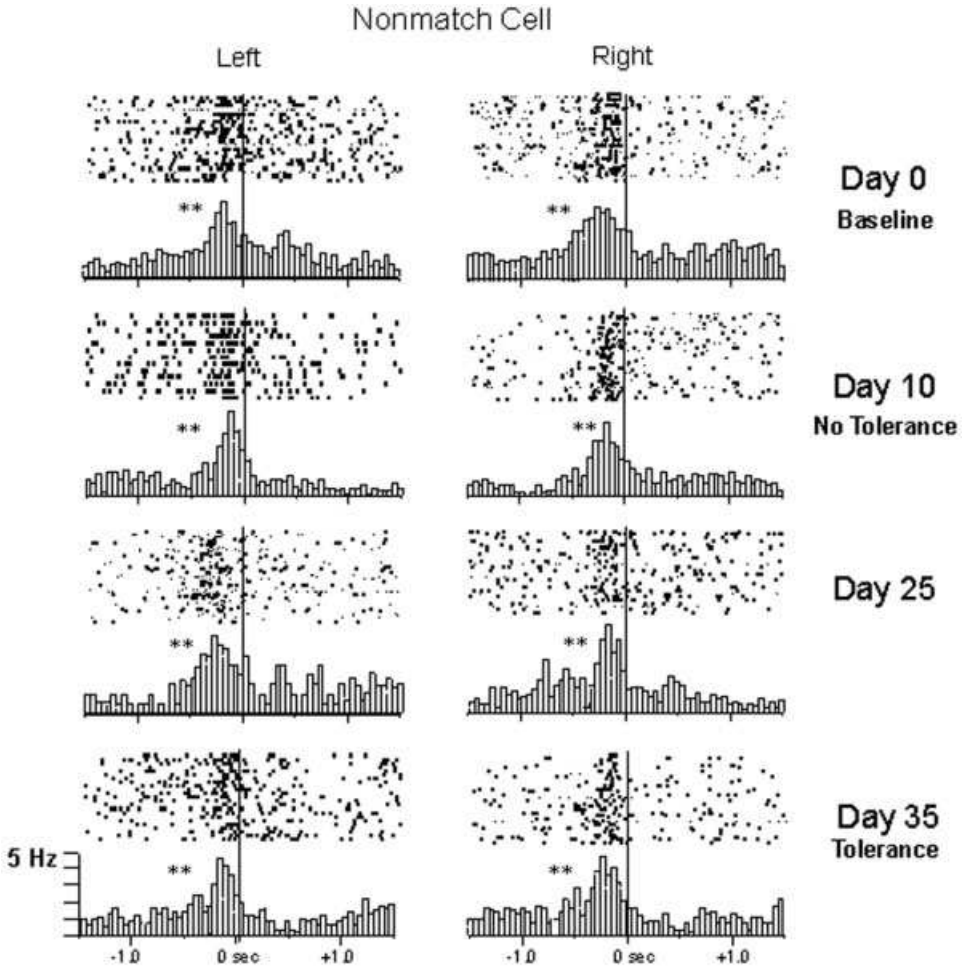


**FIGURE 4. Lack of chronic cannabinoid effect on nonmatch phase FCT. Firing of a single nonmatch cell is plotted for left and right lever responses in the nonmatch phase with the same parameters as in Figure 3. Scale and labeling is the same as in Figure 3. There were no effects of chronic WIN 55,212-2 exposure on the firing of this FCT. Significant peak firing is indicated by asterisks (*$F_{4,25}$ >4.2, $P < 0.001$; **$F_{4,25}$ >6.5, $P < 0.001$).**

risk" for errors on long delays (Hampson and Deadwyler, 1999), and that the distribution of sample phase firing rates on individual trials under control conditions was such that most trials had a sample firing of ≥3.0 Hz. Acute exposure to cannabinoids shifts the trial distribution such that more trials have low sample firing rates, and hence more errors occur as a consequence of this change in sample firing across trials (Hampson and Deadwyler, 2000). Figure 8 shows the distribution of trials with respect to sample firing rates across the chronic exposure period. Sample firing rates supporting correct performance at any delay are indicated by the white bars, while sample firing that results in errors irrespective of delay are indicated by black bars. Hence, trials "at risk" (bracket) for delay-dependent errors are indicated by the striped bars in Figure 8. On day 0, before cannabinoid exposure, the distribution of single trial sample firing is such that the majority of trials had firing rates of ≥3.0 Hz and resulted in correct performance, with only 25% in the error and "at risk" categories. However, on day 10, the distribution is shifted such that nearly 70% of trials had "at risk" sample phase firing. The predominance of trials "at risk" for errors due to low sample firing, combined with the tendency for animals to perseverate after an error trial, contributed to the below-chance performance seen on days 1–20 of chronic exposure (Fig. 1). The gradual improvement in performance from days 25 through 35 was accompanied by a shift in distribution of sample phase firing rates across the same interval (Fig. 8). The distributions were not significantly different from vehicle control (day 0) on day 35 ($t_{40} = 1.79$, $P < 0.08$). This trial-by-trial correlation between ensemble firing and behavior is also represented by the firing of specific FCTs across the time course of chronic cannabinoid exposure as discussed below.

## Correspondence Between Decreased DNMS Performance and FCT Impairment Over the Time Course of Chronic Exposure

Figure 9 shows the day-to-day time course of chronic WIN55,212-2 exposure on encoding by sample, delay, and nonmatch-specific FCTs across the entire 41-day period of the experiment for all six animals. What is immediately apparent is the correspondence between changes in sample and delay FCT firing

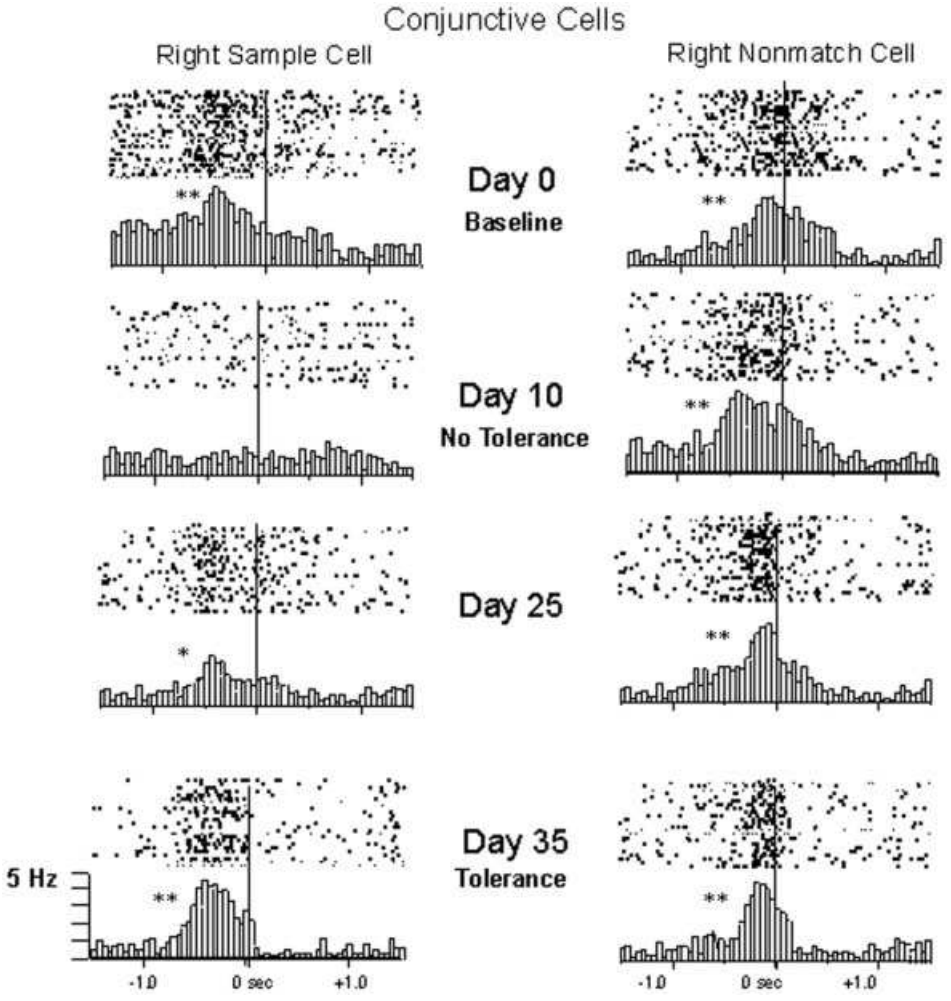


**FIGURE 5. Chronic WIN 55,212-2 exposure alters firing of sample conjunctive FCT, but not nonmatch conjunctive FCTs. Firing of two different right position conjunctive cells is shown. Peri-event histograms (PEHs) and rastergrams are constructed for the appropriate conjunctive events (i.e., right sample lever response for right sample cell; right nonmatch lever response for right nonmatch cell). Only the right sample conjunctive FCT was affected by chronic exposure to WIN 55,212-2. Note, however, that tolerance developed over 35 days of exposure. Significant peak firing is indicated by asterisks (*$F_{4,25}$ >4.2, $P < 0.001$; **$F_{4,25}$ >6.5, $P < 0.001$).**

and DNMS performance levels and the lack of associated changes in the nonmatch FCT firing over the same time course. This was supported by significant correlations between sample ($r^2 = 0.92$, $F_{1,16} = 11.37$, $P < 0.001$) and delay ($r^2 = 0.85$, $F_{1,16} = 10.44$, $P < 0.001$) FCT firing and DNMS performance and the lack of nonmatch FCT correlation ($r^2 = 0.17$, $F_{1,16} = 0.36$, $P > 0.50$) over the same time period. It is interesting that the alterations in sample and delay FCT firing did not coincide exactly during different periods of chronic exposure, delay firing changes appeared to be less severe in the early stages of treatment (days 1–15) but lagged behind recovery of sample phase FCT firing in the latter phases of chronic exposure (days 20–30, Fig. 9). In the current study, as well as in a previous report (Hampson and Deadwyler, 2000), we observed that cannabinoids selectively affected FCTs with sample firing correlates. Nearly all sample phase and sample conjunctive FCTs did not fire differentially after acute or chronic cannabinoid exposure, while FCTs that normally fired in both sample and nonmatch phases fired only during the nonmatch phase. The fact that there were no changes in the large population of nonmatch FCTs confirms the results illustrated by the single ensemble in Figure 7, showing that this cell type appears to be immune to cannabinoid drug influences on DNMS-relevant task firing.

## Effect of Precipitated Withdrawal on Hippocampal FCTs

The effects of 5 consecutive days of withdrawal (days 36–40) precipitated by substituted injections of SR141716A on DNMS performance (Figs. 1 and 2) and FCT firing are also shown in Figure 9. On the first day of precipitated withdrawal (day 36) there was a significant decrease in peak firing rate for sample and delay FCTs relative to pre-drug (day 0) levels (sample: 2.7 ± 0.4, $F_{1,634} = 8.3$, $P < 0.01$; delay: 2.1 ± 0.3 Hz, $F_{1,634} = 7.7$, $P < 0.01$) but not in nonmatch phase firing (nonmatch: 5.1 ± 0.5 Hz, $F_{1,634} = 3.1$, $P = 0.08$). By the 5th day of treatment (day 40) there were no significant differences from either day 0 or day 35 firing

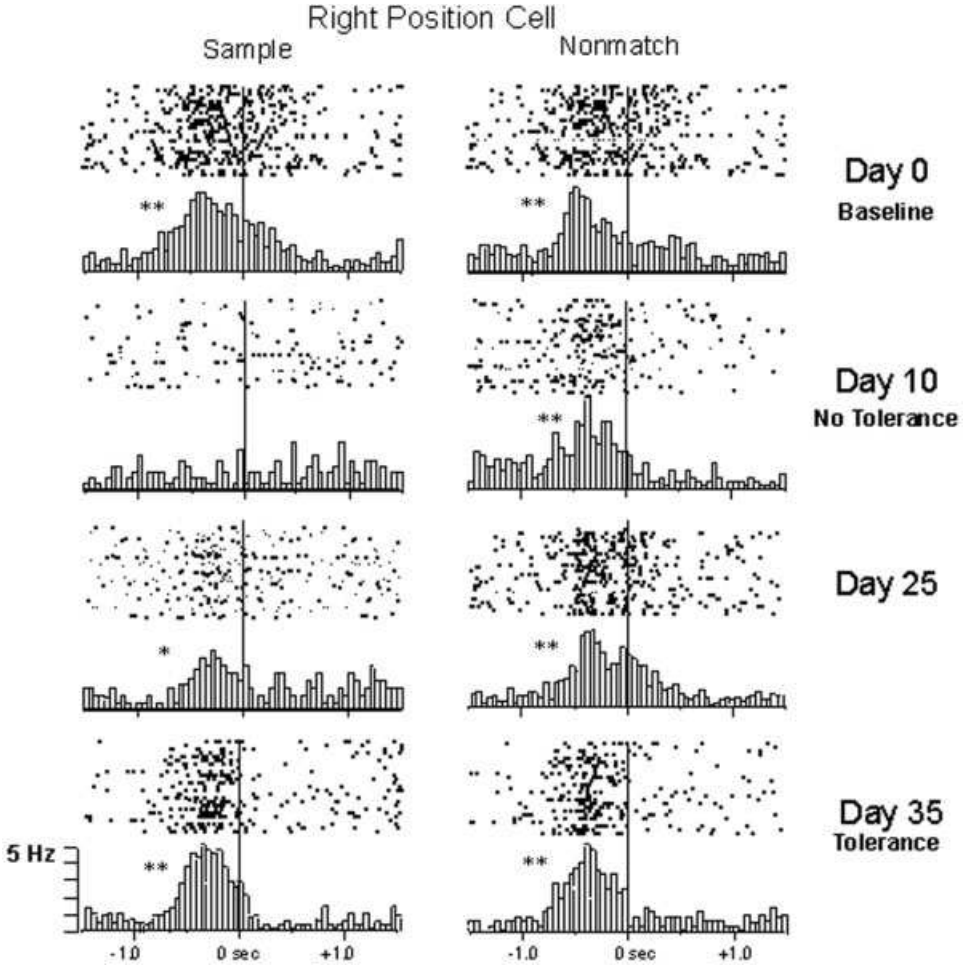


**FIGURE 6. Effects of chronic cannabinoid on right position FCT. Peri-event histograms (PEHs) and rastergrams are plotted for right sample and right nonmatch firing of the same cell (occurring on different trials) on days 0, 10, 25, and 35, as in Figures 3–5. Sample firing, but not nonmatch firing for the same neuron, was altered in response to chronic WIN 55,212-2 exposure. Significant peak firing is indicated by asterisks (*$F_{4,25}$ >4.2, $P < 0.001$; **$F_{4,25}$ >6.5, $P < 0.001$).**

levels for any of the FCT groups (all $F_{1,634} < 0.26$, $P > 0.10$). The test for effectiveness of withdrawal precipitated by SR141716A via a final injection of WIN 55,212-2 (3.75 mg/kg) on day 41 produced a recurrence of the marked reduction in sample and delay FCT firing (sample: 2.4 ± 0.4 Hz, $F_{1,634} = 14.3$, $P < 0.001$; delay: 2.1 ± 0.4 Hz, $F_{1,634} = 11.4$, $P < 0.001$). There was no effect on nonmatch FCT firing (5.0 ± 0.5 Hz, $F_{1,634} = 3.9$, $P = 0.06$), indicating that animals were no longer tolerant to this dose of WIN 55,212-2 (Fig. 9).

## DISCUSSION

The results presented here confirm and extend prior findings with $\Delta^9$-THC (Deadwyler et al., 1995b) with respect to the effects and time course of tolerance to cannabinoid effects on short-term memory (Fig. 1). The fact that similar behavioral results were obtained at lower doses with a more potent agonist WIN 55,212-2, and that those effects were reversed immediately by administering the antagonist SR141617A, indicates that the observed tolerance was mediated primarily by CB1 receptors. The potency of repeated WIN 55,212-2 injections in the initial suppression of DNMS performance was surprisingly three times greater than $\Delta^9$-THC at a dose that was calculated on the basis of the acute actions of these two drugs to be pharmacologically similar (Hampson and Deadwyler, 2000). However, both agents did have similar time courses of tolerance development (Fig. 1), which suggests that tolerance to the more severe effects of WIN 55,212-2, a full receptor agonist (Estep et al., 1991; Pacheco et al., 1991; Abood and Martin, 1996) may be more rapid than to $\Delta^9$-THC, a partial agonist (Mechoulam et al., 1992; Selley et al., 1996; Thomas et al., 1998). Similarly, withdrawal precipitated by SR141716A produced a more pronounced transient impairment in DNMS performance than abstinence withdrawal from $\Delta^9$-THC (Fig. 1), again suggesting that chronic exposure to the more potent agonist (WIN 55,212-2) altered DNMS processes more severely than $\Delta^9$-THC in the prior study (Deadwyler et al., 1995b).

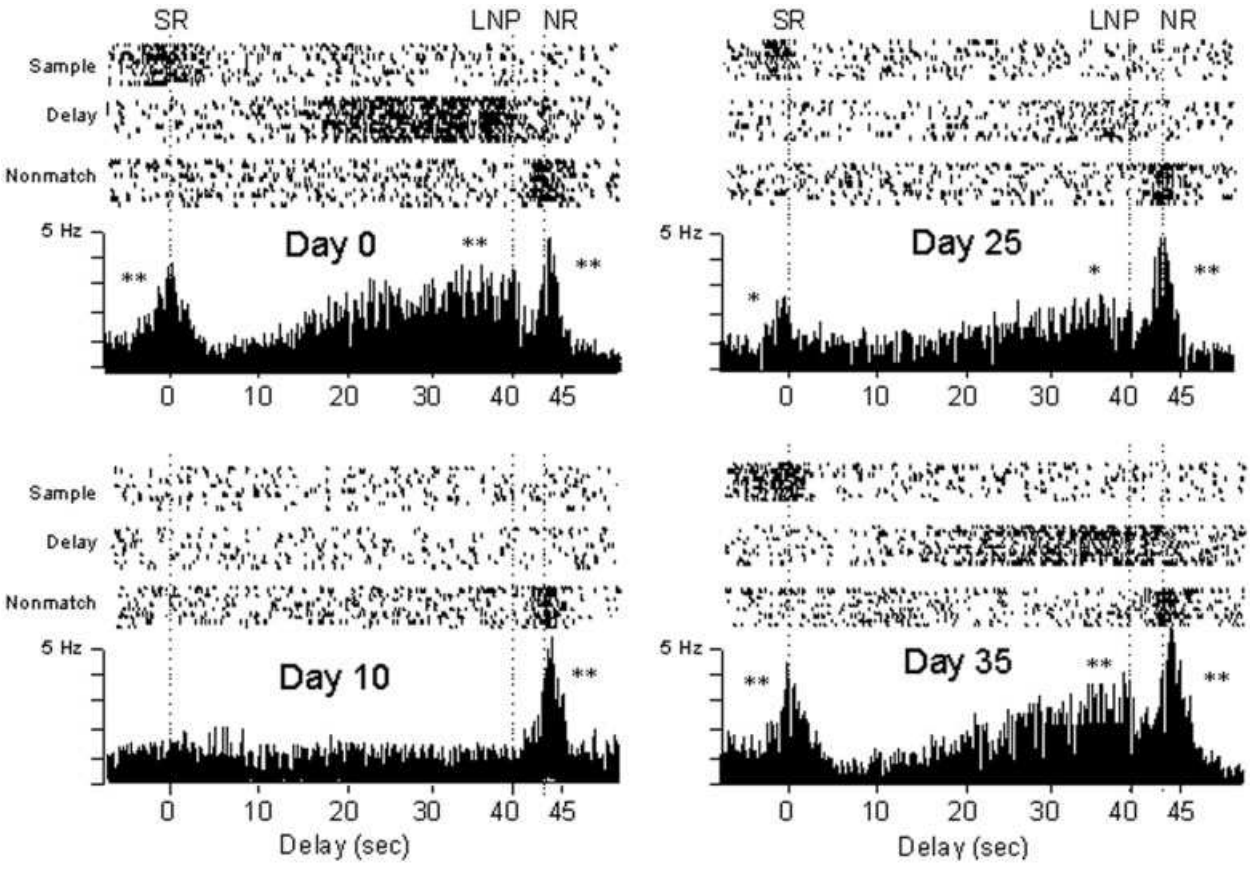


**FIGURE 7. Single trial activity (raster plots, above) and averaged trial-based histograms (TBH, below) depicting encoding of different phases of the delayed nonmatch to sample (DNMS) task, by simultaneously recorded neurons from the same animal at different stages of chronic WIN 55,212-2 exposure. Trial-based rastergrams indicate firing of three functional cell types (FCTs) that fired during sample, delay, and nonmatch phases, respectively (10 trials are shown for each neuron). TBHs below each rastergam depict mean ensemble firing for 10 neurons (inclusive of the above three FCTs) recorded simultaneously from this same animal on 25 or more DNMS trials with 30–40-s delays during the same sessions. SR, sample response; LNP, last nosepoke during delay; NR, nonmatch response. Mean ensemble TBHs were recorded on days 0 (pre-drug), 10, 25, and 35. Significant sample or nonmatch peak firing as well as significantly elevated delay firing indicated by asterisks (*$F_{4,25} > 4.2$, $P < 0.001$; **$F_{4,25} > 6.5$, $P < 0.001$).**

In the current study, several factors suggest that the suppression and recovery of DNMS performance was linked to altered firing of a select population of hippocampal neurons whose firing was specific to the sample and delay phases of the DNMS task (Figs. 3–4 and 7–9). Sample and delay FCT firing was tightly correlated with DNMS performance across the chronic exposure period, while nonmatch FCTs were almost never affected during performance impairment or developed tolerance. Figure 8 confirmed that the behavioral and electrophysiological effects were consistent on a trial-by-trial basis. The specificity of the deficit was profoundly demonstrated at the single neuron level by position FCTs that fired in both the sample and nonmatch conditions, but following cannabinoid exposure suppressed firing only in the sample phase and not in the nonmatch phase in the same daily sessions (Fig. 6).

The high correlation between the drug induced deficit in sample and delay FCT firing over the time course of impairment and recovery of DNMS performance strongly suggests a functional linkage that is selectively disrupted by cannabinoid receptor processes (Hampson and Deadwyler, 2000). One likely target for cannabinoid actions in this context would be decreased synaptic input to sample and delay FCTs from peri- and postrhinal cortical regions via relays from entorhinal projections to CA1 and CA3 (Burwell, 2000). The entorhinal cortex (layers 1 and 2) has been reported to contain large numbers of cannabinoid receptors (Marsicano and Lutz, 1999; Moldrich and Wenger, 2000) that could regulate these inputs by several of the recently proposed cellular and synaptic mechanisms (Hoffman and Lupica, 2000; Wilson et al., 2001).

Tolerance to high levels of exogenously administered cannabinoids has long been recognized (Ferraro and Grilly, 1974; Hollister, 1978; Branch et al., 1980; Dewey, 1986). Both the degree and time course of such tolerance are dependent on the type of ligand, the measures employed and the system in which it is assessed (Abood and Martin, 1996; Romero et al., 1999; Pertwee, 2001). Measures of tolerance to physiological and pharmacological effects are typically within the range of 3–7 days (Lichtman et al., 1998, 2001). Tolerance to the disruptive effects associated with learning paradigms has not been as thoroughly characterized (Adams and Martin, 1996); however, a number of studies have been published using chronic exposure regimens similar to that employed in this

study to examine various neurobiological, molecular, and pharmacological factors that could underlie the behavioral and neurophysiological tolerance reported here. The following processes have been shown to be altered over a 20–40-day exposure to cannabinoids: decreased CB1 receptor number (Breivogel et al., 1999), decreased activation of GTP-γ-S (Sim et al., 1996), a transient change in CB1 receptor message (Zhuang et al., 1998), and altered gene expression of several proteins (Kittler et al., 2000). Many of the above processes reflect major alterations in cannabinoid receptor linked signaling pathways, suggesting that the state of several of these systems after chronic exposure in the manner employed here would be quite different in tolerant vs naive animals (Pertwee, 1997, 1999). This was confirmed by the significant change in performance and electrophysiological parameters during withdrawal precipitated by SR141617A (days 35–40) and the return to susceptibility to acute injections of the "tolerated" dose of WIN 55,212-2 on day 41 (Figs. 1 and 9).

The effects of precipitated withdrawal with SR141716A were similar to those produced by abstinence from chronic exposure to $\Delta^9$-THC in the prior study (Fig. 1). It is not intuitively obvious why in both circumstances, animals that were tolerant to cannabinoids should be affected negatively in terms of DNMS performance and FCT firing upon withdrawal (Fig. 9). However, the fact that the animals were definitely in withdrawal was supported by video taped observations of the behavioral signs immediately after injection (Rubino et al., 1998) at 1 h before the start of the DNMS session. It is not insignificant that both sample and delay FCTs continued to be correlated with the transient change in DNMS performance throughout the 5-day withdrawal period in the same manner as during the 35-day development of tolerance to the disruptive effects of WIN 55,212-2.

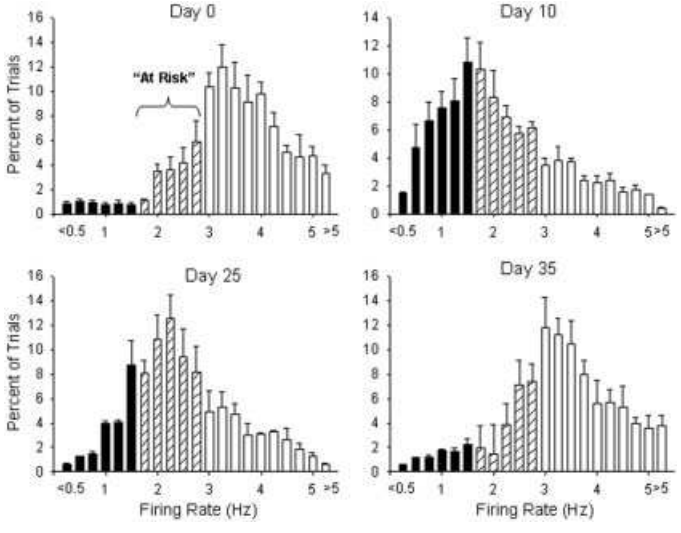


**FIGURE 8. Frequency distribution of sample phase firing within individual trials at different stages of chronic cannabinoid exposure. Trials were sorted according to strength of sample phase firing as evidenced by sample phase and conjunctive FCT. The frequency distribution was calculated over 100 trials each from six animals on days 0, 10, 25, and 35. The bar graph indicates mean and SEM for all animals. Trials with sample firing rates of <1.5 Hz (black bars) resulted in errors irrespective of delay, while trials with firing >3.0 Hz (white bars) were correct at any delay. Striped bars indicate trials "at risk" (bracket) for errors on trials with delays of >10 s (Hampson and Deadwyler, 1996, 1999, 2000). All trials with sample firing of <0.5 Hz or >5 Hz were combined in the indicated bars at the right and left of the plots.**

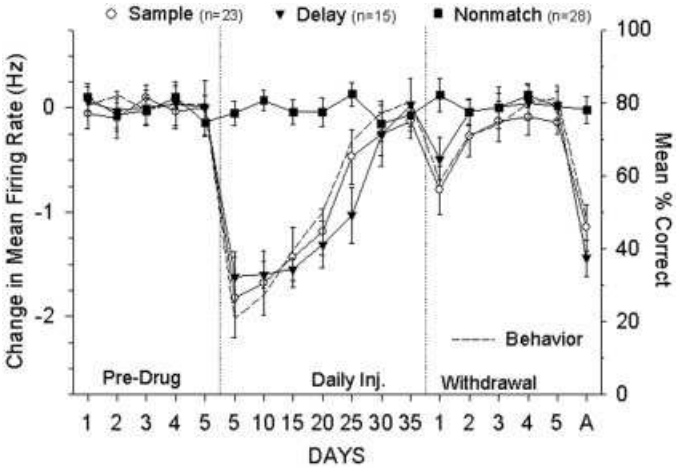


**FIGURE 9. Mean (±SEM) for all FCTs that encoded different features of DNMS task during chronic cannabinoid exposure. FCTs were grouped according to firing during sample (n = 23, includes sample phase, position, sample conjunctive, and trial-type cells, range 2–5 neurons per animal ensemble), delay (n = 15, including trial-type, and nonmatch conjunctive cells with delay firing, range 1–4 neurons per ensemble), or nonmatch phases (n = 28, including nonmatch phase, position and trial-type and nonmatch conjunctive FCTs without delay firing, range 3–7 neurons per ensemble). Time course of behavioral change (dashed line: n = 6 animals) is plotted for comparison with mean firing rate across animals. Sample and nonmatch FCT firing were calculated from peak rates at ±1 s surrounding the sample or nonmatch responses (see Figs. 3–6), delay FCT firing was calculated from mean peak rate in the terminal 3 s of 30–40-s delay trials (Fig. 7).**

Any proposed neural basis of cannabinoid tolerance must address recent findings regarding the demonstrated actions of endogenous cannabinoids at hippocampal pyramidal cell synapses (Wilson et al., 2001). Given the fact that cannabinoid receptors in hippocampus are located on a select population of γ-aminobutyric acid (GABA)ergic interneurons (Katona et al., 1999; Wilson et al., 2001) and that the action of these receptors is to decrease release of GABA via retrograde activation of presynaptic CB1 receptors by release of endogenous cannabinoids after postsynaptic cellular activation (Wilson and Nicoll, 2001; Morishita and Alger, 1999), it is likely that such a mechanism would be sensitive to chronic cannabinoid exposure.

However, several important issues need to be resolved before this explanation can be assumed to be the basis for either the acute actions of cannabinoids (Hampson and Deadwyler, 2000) or the development of tolerance to those effects as demonstrated here. First, it is unclear how exogenous cannabinoids produce a selective decrease in peak firing in sample and delay FCTs, since a decrease in GABAergic activity by the above proposed mechanism, would likely result in an enhanced rather than decreased peak firing in pyramidal cells (Alger et al., 1996). Therefore, the demonstrated

cannabinoid action of suppressing sample and delay FCT firing is not consistent with its demonstrated role of decreasing release of GABA from interneurons via depolarization-induced suppression of inhibition (i.e., DSI; Martin et al., 2001; Morishita and Alger, 1999, 2001; Wilson and Nicoll, 2001). Second, there is no evidence that select populations of GABAergic interneurons that have CB1 receptors in the hippocampus (Wilson et al., 2001) are involved in shaping sample and delay FCT firing patterns, although this remains a cogent possibility (see below). Third, if a reduction in the retrograde synaptic actions of cannabinoids were responsible for sample and delay FCT firing, it would have to be selective for some synapses and not others on the same neuron, as indicated by the fact the same position FCTs exhibit suppressed firing in the sample phase, but fire normally in the nonmatch phase of the task (Fig. 6). Finally, there is no evidence to indicate whether this retrograde action of cannabinoids exhibits tolerance after repeated cannabinoid exposure.

One aspect of the current findings, however, that is directly supportive of the notion of involvement of the retrograde actions of cannabinoids is that only certain classes of GABAergic interneurons were found by Wilson et al. (2001) to be sensitive to CB1 receptor-mediated DSI, primarily those that produced fast and large IPSCs on hippocampal pyramidal cells. In the present study, only two types of the three identified classes of FCTs, sample and delay, were sensitive to and exhibited tolerance for cannabinoid induced suppression of firing. Nonmatch FCT firing was almost completely immune to cannabinoid actions in the same animals (Figs. 6–7 and 9). This finding supports the notion that a selective class of hippocampal pyramidal cells are influenced by activation of cannabinoid receptors and that this selectivity may even extend to specific synapses on particular neurons (Fig. 6). In the case of nonmatch firing FCTs, neither acute nor chronic administration of WIN 55,212-2 nor injection of the antagonist SR141716A affected firing to a meaningful extent during the task, even though performance of the task was severely disrupted. This suggests that cannabinoid receptors are located on specific interneurons or other cell types that differentially influence certain FCT (pyramidal cell) firing patterns during the DNMS trial.

Irrespective of how the retrograde synaptic influence is manifested in FCT firing, or in what signaling pathway tolerance to the behaviorally disruptive effects of cannabinoids develops, the fact that the return of sample and delay firing was critical to recovery from the memory impairing effects of a moderate dose of WIN 55,212-2 was more than suggestive from the data (Figs. 8 and 9). Because behavioral tolerance developed over a considerable period of time (30–35 days), and because the initial disruption was significantly prolonged (15–20 days), it is quite likely that WIN 55,212-2 affected processes responsible for encoding (sample FCTs) and retrieving (delay FCTs) information critical to DNMS performance (Hampson and Deadwyler, 2000). The selective influence on long vs short delay trials with respect to recovery from the initial effects of the large dose of the drug (Fig. 2) is consistent with the well documented delay-dependent disruption of DNMS and DMS performance previously reported for $\Delta^9$-THC (Heyser, 1993; Deadwyler et al., 1995b) and supports the hypothesis that cannabinoids induce a reversible hippocampal lesion in this type of task (Hampson et al., 1999). The basis of this effect appears to be elimination of the ability of sample and delay FCTs to encode and maintain trial-specific information (Hampson and Deadwyler, 2000), a process that, in strong correlation with performance, becomes tolerant over time to repeated cannabinoid exposure.

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