

Role of cortical cannabinoid CB1 receptor in conditioned taste aversion memory

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Abstract

The brain endocannabinoid system has been shown to play a role in memory, though the extent to which this role generalizes over different types and processes of memory is not yet determined. Here we show that the cannabinoid receptor 1 (CB1) plays differential roles in acquisition, extinction and reconsolidation of conditioned taste aversion (CTA) memory in the rat insular cortex, which contains the taste cortex. Activation of the CB1 receptor in the insular cortex inhibits acquisition and reconsolidation but not extinction, whereas blockade of the CB1 receptor promotes memory and blocks extinction of CTA, while having no apparent effect on reconsolidation. The CB1 ligands used in this study were incapable of substituting the unconditioned stimulus in CTA training. All in all, the data raise the possibility that the state of activity of the CB1 receptor in the insular cortex contributes to the encoding of hedonic valence that enters into association with taste items.

Introduction

Ample evidence indicates that the endocannabinoid system, which is widespread in the mammalian brain (Wilson & Nicoll, 2002), plays a role in learning and memory. This evidence rests on manipulation of the function of the major brain endocannabinoid receptor, the cannabinoid receptor 1 (CB1; Howlett *et al.*, 2002). CB1 receptor agonists have been reported to impair memory formation or maintenance in some aversive and stressful conditioning protocols (Lichtman *et al.*, 1995; Pamlona & Takahashi, 2006). An antagonist blocked the effect of the agonist without having an effect by itself (Brodtkin & Moerschbaeher, 1997; Hampson & Deadwyler, 2000), except in social recognition, which a CB1 receptor antagonist improved (Terranova *et al.*, 1996). CB1-deficient mice have been shown to become resistant to experimental extinction in an aversive paradigm, without affecting memory encoding and consolidation (Marsicano *et al.*, 2002). Such mice were unimpaired, however, in extinction of appetitive memory (Holter *et al.*, 2005). In line with the effect of the CB1 deficiency, a CB1 receptor antagonist impaired extinction in fear conditioning (Suzuki *et al.*, 2004; Varvel *et al.*, 2005). Recently, a CB1 receptor agonist was reported to impair reconsolidation of activated long-term fear conditioning (Lin *et al.*, 2006), while the antagonist lacked an effect (Suzuki *et al.*, 2004).

Better understanding of the specific roles of CB1 receptor in the formation, maintenance and use of memory, could be obtained by investigating the effects of CB1 receptor agonists and antagonists in multiple phases of learning and memory in a single behavioural protocol. Toward that end, conditioned taste aversion (CTA) provides a suitable choice. In CTA, the subject learns to associate a taste with delayed malaise (Garcia *et al.*, 1966; Rosenblum *et al.*, 1993; Bures *et al.*, 1998). A single training trial can generate robust long-term CTA

memory, yet this memory is readily amenable to extinction (Berman & Dudai, 2001). If the training is intensified, the trace becomes resistant to extinction. This situation was found to permit unveiling the process of reconsolidation, in which the original trace regains transient susceptibility to amnesic agents, similarly to postencoding consolidation (Eisenberg *et al.*, 2003). In the rat, the formation of long-term CTA depends to a substantial degree on the gustatory cortex (Rosenblum *et al.*, 1993), which is part of the insular cortex (IC, Bahar *et al.*, 2004). Much attention has been recently assigned to the role of the cannabinoid system in learning in the hippocampus (Sullivan, 2000; Wilson & Nicoll, 2002) and in the amygdala (Barad *et al.*, 2006; Lin *et al.*, 2006); the use of CTA could hence provide an opportunity to unveil roles of cannabinoids in acquisition, extinction and reconsolidation of memory in cortex.

We describe here the effect of local administration of CB1 receptor agonist and antagonist into the IC of the behaving rat on CTA learning and memory. The data indicate that CB1 receptor plays differential roles in acquisition, extinction and reconsolidation of CTA memory in the rat cortex.

Materials and methods

Animals

Male Wistar rats (~60-days old, 250–300 g) were caged individually at 22 ± 2 °C under 12-h light : 12-h dark cycles. Water and food were available *ad libitum* unless otherwise indicated. All experiments were conducted with the approval of the Weizmann Institute Animal Experiments Committee.

Drugs

The CB1 receptor agonist, WIN55,212–2 (Tocris Bioscience, UK), was dissolved in 100% DMSO and was diluted to 0.4 µg/mL by

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addition of 45% (w/v) 2-hydroxypropyl-1- β -cyclodextrin (Cavasol[®], Sigma–Aldrich, Steinheim, Germany). The CB1 receptor antagonist, SR141716 (RTI International, Research Triangle Park, NC, USA) was dissolved in 100% ethanol to a concentration of 90 μ M, and then diluted with an equal volume of Cremophor EL (Fluka, Sigma–Aldrich, Steinheim, Germany). The emulsion was mixed, and diluted with saline to a final concentration of 0.9 μ M.

Behavioural procedures

Conditioned taste aversion (CTA, Garcia *et al.*, 1966; Bures *et al.*, 1998) was performed following the procedures of Rosenblum *et al.* (1993). In brief, saccharin (0.1% w/v, sodium salt) was used as the conditioned stimulus (CS), and i.p. injection of LiCl (0.15 M, 2% body weight) as the unconditioned stimulus (UCS). Rats were water deprived for 24 h and then pretrained for 3 days to get their daily water ration once a day for 10 min from two pipettes containing 10 mL of water each. On day 4, the rats were allowed to drink saccharin instead of water for 10 min. Forty minutes after the offset of the drinking period they were injected with LiCl (i.p.). This procedure was used as the standard, single trial CTA training (ST CTA). In the intensive, double trial CTA training (DT CTA, Eisenberg *et al.*, 2003), two ST CTA were performed 24 h apart. After completion of either ST CTA or DT CTA, the rats were presented for 10 min a day for three successive days with two pipettes containing 10 mL of water each. On day 8, a multiple-choice test was performed to quantify the acquired aversion. In this 10-min choice test, the rats were allowed free access to an array of six pipettes, three with 5 mL saccharin each and three with 5 mL water each. An aversion index (AI) was defined as in Rosenblum *et al.* (1993): $AI = 100 \times \text{ml water consumed} / (\text{ml water} + \text{ml saccharin consumed})$. Hence, $AI = 50$ is equal preference level, and $AI > 50$ implies higher preference to water over saccharin. Unless otherwise indicated the test was repeated once a day for two additional successive days ('extinction mode').

Surgery and drug microinfusion

Rats were anaesthetized with 4.8 mL/kg Equithesin (2.12% w/v MgSO₄, 10% v/v ethanol, 39.1% v/v propylene glycol, 0.98% w/v sodium pentobarbital, and 4.2% w/v chloral hydrate), restrained in a stereotactic apparatus (David Kopf Instruments, Tujunga, CA), and implanted bilaterally with stainless steel guide cannula (23 gauge, thin wall) aimed to 1.0 mm above the gustatory cortex in the insular cortex (IC) [AP +1.2 mm, L \pm 5.4 mm, V 5.4 mm relative to bregma, according to Paxinos & Watson (1986)]. The cannula were positioned in place with acrylic dental cement and secured by two skull screws. A stylus was placed in the guide cannula to prevent clogging. Animals were allowed 1 week to recuperate before being subjected to experimental manipulations. The stylus was removed from the guide cannula, and a 28-gauge injection cannula, extending 1.0 mm from the tip of the guide cannula, was inserted. The infusion cannula was connected via PE20 tubing to a Hamilton microsyringe driven by a microinfusion pump (CMA/100; Carnegie Medicin, Stockholm, Sweden). Microinfusion was performed bilaterally in a 1 μ L volume per hemisphere delivered over 1 min. The infusion cannula was left in position before withdrawal for an additional 1 min to minimize dragging of the injected liquid along the injection tract. Drugs were microinfused into the IC as indicated in the Results. Controls were microinfused with vehicles only.

Histology

Following completion of the experimental protocol, rats were deeply anaesthetized and 1 μ L of India ink was microinfused into the insular cortex. Rats were decapitated and the brain was quickly removed, frozen on dry ice and kept at -20°C . Examination of the location and the histological effects of cannulation and microinfusion on brain tissue was performed under a light microscope in Nissl-stained 30- μ m frozen sections of coronal cuts (Berman *et al.*, 2000).

Statistics

Unless otherwise indicated, repeated measure ANOVA was used.

Results

CB1 receptor agonist in cortex decreased CTA, whereas an antagonist intensified it

We have microinfused either the CB1 agonist WIN55,212–2 or the antagonist SR141716 into the IC of the behaving rat, immediately after the completion of the consumption of the conditioned stimulus, saccharin, in the standard, one-trial CTA training (ST CTA, see Materials and methods). When tested once, for the first time, 4 days after training, the agonist-treated rats displayed a significantly reduced CTA compared to control, vehicle-treated rats (Fig. 1A; $P < 0.001$, two-tailed *t*-test). In contrast, the antagonist-treated rats displayed a high and robust CTA, which unlike vehicle-treated rats, did not extinguish, as evident from repeated nonreinforced tests (Fig. 1B; main effect for group across test days, $F_{1,12} = 8.49$, $P < 0.05$).

CB1 receptor antagonist blocked extinction, but an agonist had no effect

When microinfused into the IC immediately after the first retrieval session, 4 days after completion of the one-trial, ST CTA training, the agonist had no significant effect on extinction (Fig. 1C; $F_{1,11} = 0.33$, $P = 0.577$). In contrast, the antagonist, similarly to its effect when administered in training, blocked extinction. (Fig. 1D; $F_{1,13} = 18.86$, $P < 0.001$).

CB1 receptor agonist blocked reconsolidation, whereas an antagonist had no effect

The one-trial, ST CTA fits well to investigate acquisition, consolidation and experimental extinction of CTA (Berman & Dudai, 2001; Eisenberg *et al.*, 2003). However, to detect reconsolidation in CTA, one must generate conditions under which the extinction trace (i.e. the inhibitory, CS-noUCS trace) does not dominate the control of the rat's behaviour after the retrieval test (Eisenberg *et al.*, 2003). To achieve this, one could intensify the training by multiplying the number of conditioning trials from one to two (DT CTA, see Materials and methods). We have hence trained rats in the DT CTA protocol and microinfused the CB1 receptor ligands into the IC immediately after the first retrieval test, 4 days after the completion of training. As can be seen in Fig. 1E, the CB1 receptor agonist led to blockade of the CTA trace ($F_{1,10} = 5.79$, $P < 0.05$). This could be interpreted either as accelerated extinction or blocked reconsolidation (Eisenberg *et al.*, 2003; Dudai, 2004). The interpretation of blocked reconsolidation is more plausible, given that other amnesic agents interfere with reconsolidation of CTA under the same conditions (Eisenberg *et al.*, 2003), and, particularly, that in the extinction experiment described above, the CB1 receptor agonist did not show any acceleration of

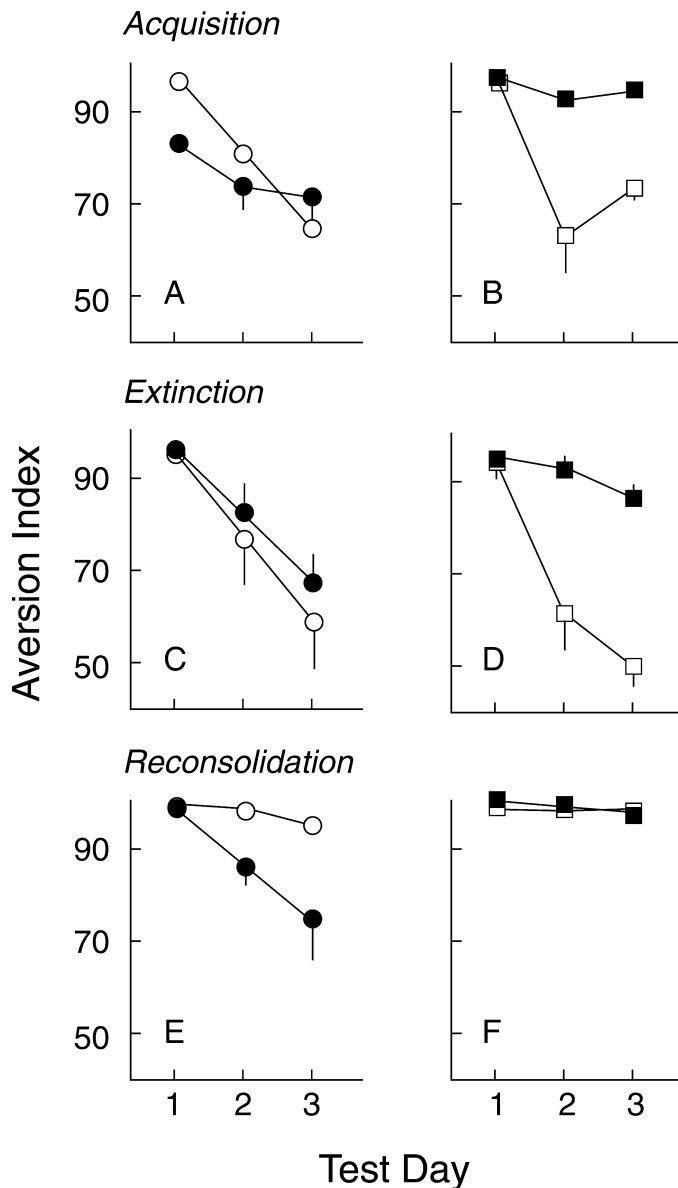


FIG. 1. Effect of cannabinoid agonist (A, C and E) and antagonist (B, D and F) on acquisition, extinction and reconsolidation of long-term CTA memory. Acquisition (A and B); compounds were microinfused into the IC immediately after saccharin consumption during standard CTA training. In these and the following panels, the test was performed for three successive days, once per day, starting 4 days after the completion of training (Test Day 1), as detailed in the Materials and methods; closed circles, the CB1 agonist WIN55,212-2; open circles, the vehicle; closed squares, the CB1 receptor antagonist, SR-141716; open squares, the vehicle. Extinction (C and D); the CB1 receptor ligands microinfused immediately after the first retrieval session, on day 4 (i.e. Test Day 1) after training on the single-trial, standard CTA training protocol. Reconsolidation (E and F); the CB1 receptor ligands microinfused as above but on day 4 (i.e. Test Day 1) after the intensified two-day CTA training.

experimental extinction (Fig. 1C). In contrast, the CB1 receptor antagonist had no apparent effect on the trace under these conditions (Fig. 1F; $F_{1,13} = 0.2$, n.s.).

The CB1 ligands per-se had no lingering effect on the ability to encode and express CTA

Postmortem histological examination of the IC in rats microinfused with either the CB1 agonist or the CB1 antagonist did not unveil

differences from vehicle-microinfused controls (data not shown). To further exclude the possibility that the microinfusion of the CB1 ligands had a nonspecific toxic effect on the IC that could escape the histological examination, we subjected rats to new CTA training one week after the microinfusion of either the CB1 agonist or the CB1 antagonist into the IC. Groups of rats microinfused into the IC with the vehicle a week before the CTA training were used as controls. CTA was tested as specified in the Materials and methods once a day, on three successive days. There was no difference between the experimental and their respective control groups throughout the tests, indicating no long-term effect on acquisition, memory and extinction of CTA (WIN55,212-2, $F_{1,8} = 0.57$, n.s.; SR141716, $F_{1,11} = 0.7$, n.s.).

Neither the CB1 receptor agonist nor the antagonist can substitute for the unconditioned stimulus in CTA training

As CTA tolerates a long interstimulus interval in training, one should consider the possibility that association of the microinfused ligand shortly after the retrieval test leads to long-term modification of taste preference, which might affect subsequent CTA performance. We have therefore presented naive rats with the CS, saccharin, as in the CTA training protocol. We have omitted, however, the application of the UCS (LiCl i.p.) and instead microinfused the CB1 receptor agonist or antagonist into the IC immediately after the taste consumption, to mimic the conditions of drug application in the retrieval session. A control group was microinfused with vehicle. The rats were then subjected to the taste-choice test as in the CTA testing protocol, 4 days after training. We found no evidence for any aversive or appetitive taste effects of microinfusions of CB1 receptor ligands into the IC under these conditions. Hence the aversion index of the agonist-treated, antagonist-treated, and control rats were 35 ± 7 , 31 ± 7 , and 29 ± 4 , respectively (one-way ANOVA, $F_{2,15} = 0.25$, n.s.).

Discussion

Our findings are relevant to three issues in memory research. One is the role of the CB1 receptor in learning and memory, the other is the potential role of this type of receptor in the encoding of hedonic valence of experience-dependent associations in the cortex, and the third is the potential mechanistic dissociations among consolidation, extinction and reconsolidation.

The present data confirm and extend earlier reports that the CB1 receptor plays a role in aversive and stressful conditioning (Lichtman *et al.*, 1995; Marsicano *et al.*, 2002; Suzuki *et al.*, 2004; Varvel *et al.*, 2005). However, whereas in fear conditioning the CB1 receptor is required for extinction only (Marsicano *et al.*, 2002; Suzuki *et al.*, 2004), in CTA it is obligatory for normal acquisition as well. Furthermore, in disparity with the report that a CB1 receptor antagonist blocks the agonist effect on memory but is ineffective by itself (Brodtkin & Moerschbaecher, 1997; Hampson & Deadwyler, 2000; Lin *et al.*, 2006), in CTA the antagonist renders the trace resistant to extinction. All in all, in CTA the CB1 receptor agonist inhibits acquisition and probably reconsolidation, whereas the antagonist promotes acquisition and/or storage and blocks extinction. These findings indicate roles for the endocannabinoid system in multiple phases of CTA memory.

The behavioural outcome of the antagonist effect in the context of CTA is high aversion. This is unlikely to be the outcome of a 'ceiling effect' of the ligand that could mask differential roles of CB1 in different memory phases. In fact, both the antagonist and the agonist at the doses used dissociated the role of the CB1 receptor in different CTA memory phases (and see below). The possibility can not be excluded

that other doses might alter the aforementioned differentiation, but such argument holds for any study that attempts to separate molecular mechanisms of learning and memory. Commonly, it is differentiation in performance under similar drug doses that paves the way to the disentangling of mechanisms. Further, the antagonist dose used in our studies is at the lowest range reported in intrabrain studies of the CB1 antagonist (Martin *et al.*, 1998; El-Banoua *et al.*, 2004; Meng & Johansen, 2004; Seagard *et al.*, 2004).

Under the conditions used in our study, we found no evidence that the state of activity of the CB1 receptor in IC affects taste experience in the absence of encoding or reactivation of a 'conventional' (i.e. taste-strong malaise) CTA trace; coincident activation of the CTA association is required. Possibly typical of the long CS–UCS interval tolerated by the CTA association, 'coincidental' in this case refers to the minutes range. This raises the possibility that the state of activity of CB1 receptor in cortex is a modulating or synergistic reinforcer. The fact that the effect of the CB1 receptor ligands is expressed in CTA alteration on repetitive tests, days after ligand application into the IC, indicates that the aforementioned reinforcer is required to act only transiently and further, that no state-dependency is involved. We hence wish to propose as a working hypothesis that in the context of the brain algorithm that culminates in the encoding or reactivation of CTA memory, endogenous activation of the CB1 receptor in cortex, which is mimicked in our experiments by microinfusion of the agonist, contributes to the representation of a positive hedonic valence. In contrast, lack of activation, or inhibition of the CB1 receptor, contributes to the representation of a negative hedonic valence. This could explain why the CB1 receptor agonist weakens the CTA acquisition as well as the postulated reconsolidation of the aversive trace, whereas the CB1 receptor antagonist augments the aversive association in or after acquisition and blocks the extinction of the trace. Indeed, the microinfusion of the antagonist in the acquisition phase has the same effect on CTA memory as enhancing the conditioned aversion training (Eisenberg *et al.*, 2003; see below); in both cases the trace becomes resistant to extinction. Further pharmacological and cellular analysis of the relevant circuits in the insular cortex is needed to test this hypothesis.

The CTA paradigm offers some advantages for the investigation of reconsolidation (Sara, 2000; Nader, 2003; Dudai, 2006), because a simple variation in training allows switching between extinction and reconsolidation modes after retrieval (Eisenberg *et al.*, 2003). Furthermore, encoding, consolidation, extinction and reconsolidation are all subserved by the IC. In the present study, we dissociate the contribution of the CB1 receptor to extinction and reconsolidation. The latter term is used here with an implicit caveat, because the interpretation of the postretrieval decline of performance after intensive training is construed as a blockade of reconsolidation on the basis of previous data that have demonstrated that this decline is highly unlikely to represent accelerated extinction (Eisenberg *et al.*, 2003; Dudai, 2004). These findings confirm and extend previous reports that identify differences in the engagement of receptors and intracellular signalling cascades in acquisition vs. extinction (Berman & Dudai, 2001), as well as in extinction vs. reconsolidation (Suzuki *et al.*, 2004; Lee *et al.*, 2006). Detailed elaboration of such differences could pave the way to the ability to differentially modulate one memory process but not another, e.g. extinction vs. postretrieval intensification of the trace in reconsolidation.

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Abbreviations

CB1, cannabinoid 1 (receptor); CS, conditioned stimulus; CTA, conditioned taste aversion; DT, double trial; IC, insular cortex; ST, single trial; UCS, unconditioned stimulus.

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