

# Functional Interactions between Endocannabinoid and CCK Neurotransmitter Systems May Be Critical for Extinction Learning

Jasmeer P Chhatwal<sup>1</sup>, Alisa R Gutman<sup>1</sup>, Kimberly A Maguschak<sup>1</sup>, Michael E Bowser<sup>1</sup>, Yong Yang<sup>1</sup>, Michael Davis<sup>1</sup> and Kerry J Ressler\*<sup>1</sup>

<sup>1</sup>Departments of Psychiatry and Behavioral Sciences, Yerkes National Primate Research Center, Emory University, Atlanta, GA, USA

The endocannabinoid system and the cannabinoid type 1 receptor (CB1R) are required for the extinction of conditioned fear. CB1 antagonists have been shown to prevent extinction when delivered both systemically and within the amygdala. Anatomical studies suggest that CB1Rs in the basolateral amygdala (BLA) are expressed on GABAergic interneurons expressing the anxiogenic peptide cholecystokinin (CCK). Pre-synaptic CB1Rs inhibit neurotransmitter release, suggesting that CB1R activation during extinction may decrease CCK peptide release as well as GABA release. Thus, we examined whether extinction involves the CB1R modulation of CCK2 receptor activation. We found that intracerebroventricular administration of the CCK2 agonist pentagastrin dose-dependently impaired extinction of conditioned fear. Systemic administration of a CB1 antagonist, rimonabant (SR141716), also potently inhibited extinction learning. This effect was ameliorated with systemic administration of a CCK2 antagonist, CR2945. Furthermore, the extinction blockade by systemic SR141716 was reversed with intra-BLA, but not intrastriatal, infusion of CR2945. Lastly, as extinction usually leads to an increase in Akt phosphorylation, a biochemical effect antagonized by systemic CB1 antagonist treatment, we examined whether CR2945 co-administration would increase extinction-induced p-Akt levels. We observed that extinction-trained animals showed increased Akt phosphorylation following extinction, CB1 antagonist-treated animals showed p-Akt levels similar to those of non-extinction trained animals, and co-administration of CR2945 with SR141716 led to levels of p-Akt similar to those of vehicle-treated, extinction-trained controls. Together, these data suggest that interactions between the endocannabinoid and CCKergic transmitter systems may underlie the process of extinction of conditioned fear.

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## INTRODUCTION

Over the past 15 years, the endogenous cannabinoid system and the cannabinoid type 1 receptor (CB1R) have been linked to a staggering array of normal and pathologic functions of the CNS, ranging from excitotoxicity to nociception. Among the most striking behavioral findings regarding the cannabinoid system has been that genetic or pharmacologic antagonism of the CB1R leads to profound deficits in the extinction of conditioned fear (Marsicano *et al*, 2002). This systemic effect of CB1 antagonists has now been demonstrated to occur when an antagonist is delivered locally within the basolateral amygdala (BLA) (Roche *et al*,

2007). Furthermore, we have previously demonstrated that a cannabinoid reuptake inhibitor enhances extinction learning (Chhatwal *et al*, 2005). As disruptions in extinction learning are thought to be major obstacles in the treatment of a variety of psychiatric illnesses, including PTSD, specific phobias, and many anxiety disorders, the endogenous cannabinoid system has become a major therapeutic target in the treatment of fear and anxiety.

Anatomical studies of the CB1R in the CNS have demonstrated that they are often pre-synaptically located, where they are thought to be activated by retrograde diffusion of endocannabinoid (eCB) transmitters. Once activated, CB1Rs act to decrease the excitability of the pre-synaptic terminal, leading to decreases in neurotransmitter release.

High concentrations of CB1Rs have been observed on the pre-synaptic terminals of GABAergic interneurons expressing the anxiogenic neuropeptide cholecystokinin (CCK) in many brain regions (Katona *et al*, 1999; Marsicano and Lutz, 1999; McDonald and Mascagni, 2001). Several

\*Correspondence: Dr K Ressler, Department of Psychiatry and Behavioral Sciences, Yerkes Research Center, Emory University, 954 Gatewood Dr, Atlanta, GA 30329, USA, Tel: +1 404 727 7739, Fax: +1 404 727 8070, E-mail: kressle@emory.edu  
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electrophysiological studies have established that CB1R activation leads to decreases in GABA release and likely CCK release from interneurons in the hippocampus that contain both CCK peptide and CB1R (Katona *et al*, 1999; Beinfeld and Connolly, 2001; Burdyga *et al*, 2004; Fride, 2005).

With respect to extinction, Marsicano *et al* (2002) have demonstrated that re-exposure to conditioned cues in the absence of the original aversive unconditioned stimulus (ie extinction training) is a potent signal for the production of two major eCBs in the amygdala (Marsicano *et al*, 2002). This coupled with the known electrophysiological effects of CB1R activation suggests that activity-dependent reductions in neurotransmitter release from CCK + /CB1 + neurons in the amygdala may play a role in the neurobiology of extinction learning.

Administration of exogenous CCK peptide (usually given as a sulfated version of the terminal 4–8 peptides) to rodents and humans appears to be anxiogenic and, in some cases, panicogenic (Harro *et al*, 1993; Belcheva *et al*, 1994; Vasar *et al*, 1994; Bradwejn and Koszycki, 2001). The anxiogenic effects of CCK peptide agonists are believed to be mediated by the CCK2 (or CCK-B) receptor, a G-protein-coupled receptor expressed widely in the brain. There has also been considerable evidence that CCK2 receptors within the BLA are responsible for these anxiogenic effects (Josselyn *et al*, 1995; Frankland *et al*, 1996). Notably, Frankland *et al* (1996) have shown that intracerebroventricular (i.c.v.) or intra-amygdala administration of the CCK2 agonist pentagastrin leads to increased expression of baseline startle, suggesting that CCK2 receptors within the amygdala are important mediators of fear responses in addition to their role in unconditioned anxiety responses. Furthermore, the same authors have been able to show that administration of a CCK2 antagonist can lead to a decrease in the conditioned, fear-potentiated startle (FPS) response in the rat (Josselyn *et al*, 1995); this finding has been supported by more recent studies showing that two other CCK2 antagonists reduce post fear-conditioning freezing in mice, both with respect to contextual fear conditioning and conditioning to a discrete conditioned stimulus (CS; Izumi *et al*, 1996; Tsutsumi *et al*, 1999).

In this study, we examine the interaction between cannabinoid neurotransmission and CCKergic neurotransmission in the extinction of conditioned fear. Specifically, as the anatomy of the CB1 system suggests that it may be involved in producing activity-dependent, dynamic reductions in CCK and GABA release, we address the hypothesis that the CB1 antagonist-induced blockade of extinction learning may be mediated in part by an inability of eCBs to reduce CCK2 receptor activation during extinction.

## MATERIALS AND METHODS

### Animals

The procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Adult male Sprague–Dawley rats (Charles River, Raleigh, NC) weighing 350–500 g were used. Animals were housed in

pairs in a temperature-controlled (24°C) animal colony, with *ad libitum* access to food and water. They were maintained on a 12 h light/dark cycle with lights on at 0800, with all behavioral procedures performed during the rats' light cycle.

### Surgery

In studies utilizing i.c.v. drug administration, 22-gauge stainless-steel guide cannulae were implanted under ketamine/xylazine anesthesia, and secured using dental cement (coordinates: AP: 0, ML: –1.6, DV: –5.0; nosebar: +5.0). Habituation to the testing context and subsequent behavioral testing began 7–10 days following surgery. Similar procedures were used to implant bilateral cannulae aimed at the basolateral complex of the amygdala (22-gauge cannulae, AP: –3.1, ML: ±5.4, DV: –8.4; nosebar: –3.6). Additional control experiments were performed with cannulae aimed at the striatum (22-gauge cannulae, AP: –1.0, ML: ±4.0, DV: 5.0; nosebar: –3.6). Following behavioral testing, cannulated animals were killed and cannula placement was assessed on cryostat-sectioned tissue. Animals with amygdala-placed (or striatally placed) cannulae were included for analysis ( $n = 8$  each for vehicle and CR2945-treated groups).

### In Situ Hybridization

*In situ* hybridization was performed as described previously (Ressler *et al*, 2002). A cDNA clone containing the coding sequence of the rat cannabinoid receptor type 1 (IMAGE expressed sequence tag clone, GI accession no. 11375084) and pre-pro CCK (IMAGE expressed sequence tag clone, GI accession no. 4059800) were linearized after sequence verification. Antisense riboprobes were generated with T3 RNA polymerase. Slide-mounted sections of snap-frozen rodent brain tissue were post-fixed, proteinase K digested, and blocked followed by overnight hybridization of the tissue at 52°C with [<sup>35</sup>S]UTP-labeled riboprobes. After a stringent wash protocol, slides were apposed to autoradiography film.

Two-color fluorescent *in situ* hybridization was performed as described previously (Vosshall *et al*, 1999). In brief, digoxigenin (CCK) or fluorescein (CB1) was used to label CCK and CB1 riboprobes, respectively (Roche Diagnostics, Indianapolis, IN). Hybridization and wash protocols identical to those described above for radiolabeled probes were used. Following quenching of endogenous peroxidases and blocking (30 min in 1% bovine serum albumin diluted in 100 mM Tris-HCl, 150 mM NaCl), hybridized slides were incubated with peroxidase-tagged antibodies against fluorescein (1:500 dilution in 1% BSA, 100 mM Tris-HCl, 150 mM NaCl; Roche Diagnostics). Amplification was then performed using an FITC-tyramide conjugate (1:50; Perkin-Elmer, Wellesley, MA). Following quenching of the peroxidase activity from the first probe, similar procedures were employed to visualize the digoxigenin-labeled probe (CCK) via the use of a peroxidase-labeled antibody raised against digoxigenin (Roche Diagnostics) followed by amplification with a CY5-tyramide conjugate (Perkin-Elmer).

## Startle Apparatus

Animals were trained and tested in  $8 \times 15 \times 15$  cm Plexiglas and wire-mesh cages, with floors consisting of four 6.0-mm-diameter stainless-steel bars spaced 18 mm apart. Each cage was suspended between compression springs within a steel frame and located within a custom-designed  $90 \times 70 \times 70$  cm ventilated sound-attenuating chamber. Background noise (60-dB wide-band) was provided by a type 1390-B noise generator (ACO Pacific Inc., Belmont, CA) and delivered through high-frequency speakers (Radio Shack Supertweeter; Tandy, Fort Worth, TX) located 5 cm from the front of each cage. Sound level measurements (sound pressure level) were made with a Bruel & Kjaer (Marlborough, MA) model 2235 sound-level meter (A scale; random input) with the microphone (type 4176) located 7 cm from the center of the speaker (approximating the distance of the rat's ear from the speaker). Startle responses were evoked by 50-ms, 95-dB white-noise bursts generated by a Macintosh G3 computer soundfile (0–22 kHz), amplified by a Radio Shack amplifier (100 W; model MPA-200; Tandy), and delivered through the same speakers used to provide background noise. An accelerometer (model U321A02; PCB Piezotronics, Depew, NY) affixed to the bottom of each cage produced a voltage output proportional to the velocity of cage movement. This output was amplified (model 483B21; PCB Piezotronics) and digitized on a scale of 0–2500 U by an InstruNET device (model 100B; GW Instruments, Somerville, MA) interfaced to a Macintosh G3 computer. Startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 ms after the onset of the startle-eliciting stimulus. The CS was a 3.7-s light (82 lux) produced by an 8 W fluorescent bulb (100  $\mu$ s rise time) located 10 cm behind each cage. Luminosity was measured using a VWR light meter (Atlanta, GA). The US was a 0.5-s shock, delivered to the floorbars and produced by a shock generator (SGS-004; Lehigh Valley, Beltsville, MD). Shock intensities (measured as in Cassella and Davis, 1986) were 0.4 mA. The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads Inc., Newton, CT). Animals were pre-exposed to the chambers for 10 min on each of 2 days before training to habituate them to handling and the test chambers and to minimize the effects of contextual conditioning.

## Fear Conditioning

On 2 consecutive days following habituation, rats were returned to the same chambers and presented with 10 pairings of a light (3.7 s) co-terminating with a 0.4-mA, 0.5-s shock (3.6-min intertrial interval).

## Post-training Matching

Twenty-four hours following the last fear-conditioning session, animals were returned to the same chambers and presented with startle stimuli (50-ms, 95-dB white-noise bursts) in the presence or absence of the light-CS (15 light-startle compounds and 10 startle-alone trials). Increased startle in the presence of the light-CS was taken as a

measure of conditioned fear, and the magnitude of the fear response was calculated as the percentage by which startle increased when the light-CS was presented along with the startle stimulus vs when it was omitted (FPS). Using these measurements, animals were divided into groups displaying approximately equal levels of FPS before drug treatment and extinction training.

## Extinction Training (Retention Studies)

Five days following the last fear-conditioning trial, animals were injected intraperitoneally (i.p.) with a test compound or its vehicle in 1 ml/kg volumes and then immediately returned to the same chambers and presented with 90 presentations of the light-CS in the absence of footshock (3.7-s light, 30-s intertrial interval). Light presentations were preceded by 10 95-dB noise burst-alone trials with 30 s ITI to assess potential drug-induced alterations of baseline startle. At 48 and 96 h post-extinction training, animals were tested for the presence of FPS (15 light-startle compounds and 15 startle-alone trials).

## Extinction Training (Within-Session Extinction)

In the final experiment in this set of studies, the extinction training program was altered to allow for the assessment of reductions in conditioned fear during the extinction training sessions (within-session extinction). To do this, extinction training was altered to 30 light-startle and 30 startle-alone trials (3.7-s light, 95-dB startle, 30-s interstimulus interval) rather than 90 light-alone trials. FPS was calculated as in the other testing sessions, and results were grouped into 10 blocks of three trials each. To allow for direct comparison of within-session extinction across groups, FPS values were normalized such that each animal's fear response during the first block of trials was considered 100%, a normalization that compensated for variations in FPS before extinction.

## Assessment of Drug Effects on Baseline Startle and Unconditioned Fear

A group of behaviorally naïve animals were handled and habituated to the training and testing chambers for 2 consecutive days and then presented with a 15-trial test for the presence of unconditioned fear to the light (15 light-startle compounds and 15 startle-alone trials) and matched into four groups showing equivalent levels of unconditioned fear to the light and equivalent levels of baseline startle. Unconditioned fear to the light was observed to be quite low (<10% per matched group). Three days following this matching test, animals were injected with vehicle (100% DMSO), SR141716A (5 mg/kg), CR2945 (3 mg/kg), or a combination of SR141716A and CR2945 (5 and 3 mg/kg, respectively). In all cases, drugs were administered in volumes of 1 ml/kg. Twenty minutes following injection, animals were tested for alterations in baseline startle and unconditioned fear to light (5 light-startle compounds and 45 startle-alone trials), followed by two light-shock pairings to assess alterations in shock reactivity.

## Drugs

SR141716A (Rimonabant NIMH Drug Supply Program, Bethesda, MD) and CR2945 (Sigma-Aldrich, St Louis, MO) were dissolved in 100% DMSO. A 25 mg portion of pentagastrin (Sigma-Aldrich) was first dissolved in 2.5 ml of 100% DMSO and then serially diluted to generate 100 and 500 nM working solutions. I.c.v. infusions were performed using a flow rate of 1.0  $\mu$ l/min with a total infused volume of 5  $\mu$ l. For local infusion of CR2945, a 1 mg/ml solution of CR2945 in 100% DMSO was diluted to generate a working solution of 2  $\mu$ g/ $\mu$ l CR2945 in 5% DMSO/95% sterile PBS. Intra-BLA infusions were performed using a flow rate of 0.1  $\mu$ l/min with a total infused volume of 0.5  $\mu$ l per side (1  $\mu$ g drug/side).

## Western Blotting

Following extinction training, animals were killed using overdoses of isoflurane. Brains were blocked rapidly over ice and dissected into 2-mm-thick coronal sections. The BLA was removed bilaterally using a brain punch tool, and punches from each side were pooled and homogenized in buffer (5 mM HEPES, 0.32 M sucrose, protease inhibitors) and kept frozen at  $-80^{\circ}\text{C}$  until western blot assay. Whole-cell lysed samples were tested for protein concentration using a BCA assay (Pierce Biotechnology, Rockford, IL). Fifteen micrograms of protein per animal was loaded onto polyacrylamide-SDS mini-gels, separated electrophoretically, blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA), and blocked for 1 h in 2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, and 10 mM HEPES (pH 7.4) (NDM-HEPES). Membranes were incubated overnight at

$4^{\circ}\text{C}$  in a 1:1000 dilution of rabbit  $\times$  phospho-Akt (Ser473) antibody (Cell Signaling, Danver, MA, no. 9271) in NDM-HEPES buffer and then incubated in a 1:2000 dilution of an HRP-labeled secondary antibody for 60 min. The bound antibody was detected by SuperSignal West Chemiluminescence (Pierce Biotechnology) in an Alpha Innotech Fluorchem imaging system (Alpha Innotech, San Leandro, CA). Total blotted protein levels were assessed using levels of  $\alpha$ -tubulin (1:5000; Sigma), the detection of which was used to control for variations in protein loading.

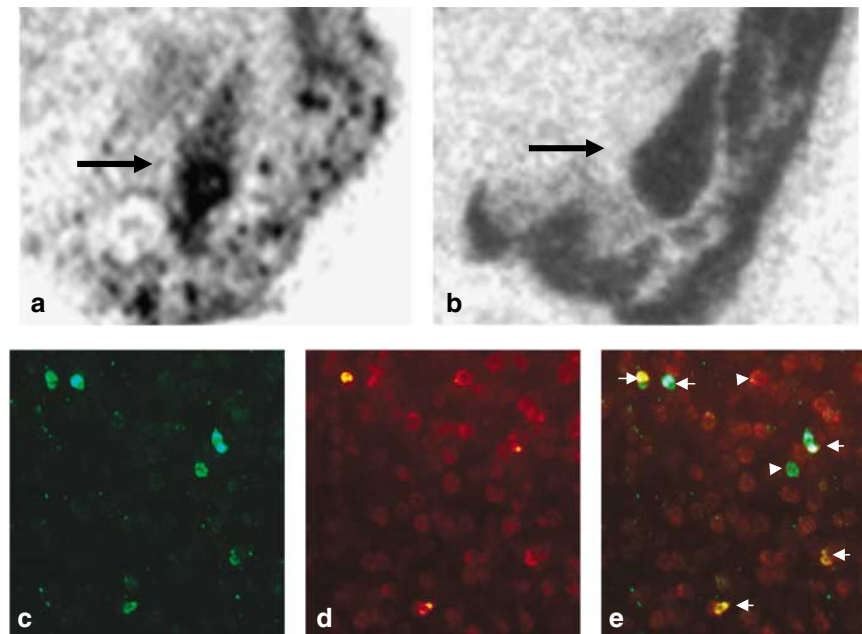
## Statistical Analysis

Comparisons were made across drug treatment groups at each test using an ANOVA or Student's *t*-test with drug or dose as the independent measure. In experiments involving multiple days of extinction training and subsequent testing, a repeated measures ANOVA was used to assess differences between drug groups and to assess drug treatment interactions with time and FPS. In both cases, Fisher's LSD was used for *post hoc* analysis.

## RESULTS

### CCK mRNA is Coexpressed with CB1 mRNA within the BLA

*In situ* hybridization was used to determine the patterns of CCK mRNA expression within the rat amygdala and to assess differential expression of CCK mRNA in the basolateral, medial, and central amygdaloid nuclei. In close agreement with previous studies, we observed that both CB1R (Figure 1a) and CCK (Figure 1b) mRNAs were highly

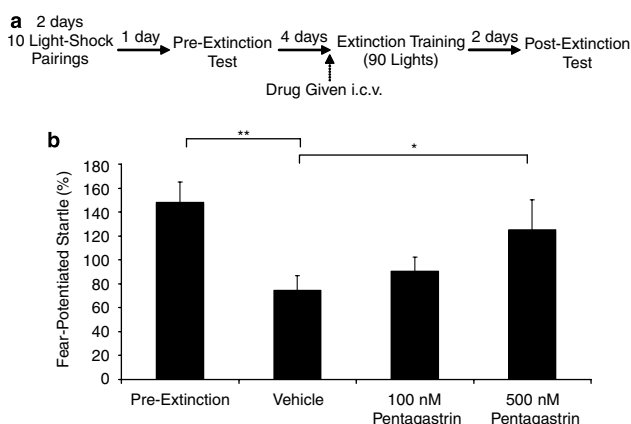


**Figure 1** CB1 and CCK mRNAs are highly expressed within the amygdala, and show a high degree of colocalization: the mRNAs for the CB1R (a) and pro-CCK peptide (b) are highly enriched in the basolateral complex of the amygdala (BLA), as compared to the central and medial nuclei of the amygdala, as shown by radiolabeled riboprobes. Using fluorescently labeled riboprobes, the mRNAs for CB1 and CCK were observed to be coexpressed in many of the same cells (arrows), as depicted in a double-labeled section of the BLA shown in panels c (CBI—green), d (CCK—red), and e (overlay). A smaller subset of neurons were observed to be expressing primarily only CBI or CCK (arrowheads).

expressed in the basal, lateral, and basolateral nuclei of the amygdala (collectively referred to as the basolateral complex of the amygdala (BLA)), whereas much lower levels of both mRNAs were observed in the central and medial amygdala. Using fluorescently labeled riboprobes, we examined whether CB1 and CCK mRNAs were expressed in the same neuronal cells within the BLA. In agreement with previous results (Marsicano and Lutz, 1999; McDonald and Mascagni, 2001), we found that many CB1-expressing cells within the BLA also expressed the mRNA for CCK (Figure 1c and d, arrows), although occasionally cells were observed to express only one of these mRNAs (Figure 1c and d, arrowheads). Notably, double-labeling for CCK and CB1 mRNA expression has not been reported in rats. Marsicano and Lutz (1999) found an overlap of CCK mRNA expression in CB1 mRNA-expressing neurons in the mouse ranging from 47 to 100% depending on the brain region and the level of CB1 expression. In this rat sample, we found that approximately 70% of neurons expressing high levels of CB1 mRNA also express CCK mRNA, consistent with reports in mice.

### Pentagastrin, a CCK2 Agonist, Impairs Extinction, Similar to a CB1 Antagonist

We hypothesized that CB1R activation, which is critical for extinction, acts through reduction in CCK release and results in subsequent reduction in CCK2 receptor activation. According to this hypothesis, increasing CCK2 activation by administering a CCK2 agonist should impair extinction. To test this hypothesis, rats were implanted with i.c.v. cannulae, allowed to recover for 7–10 days, fear-conditioned as outlined in Figure 2a, and matched into groups demonstrating similar levels of FPS before extinction. Thirty minutes before extinction training (90 lights without shocks), animals were infused with vehicle, 100 nM pentagastrin, or 500 nM pentagastrin (5  $\mu$ l/infusion).



**Figure 2** Pentagastrin, a CCK receptor agonist, impairs extinction. Animals were implanted with i.c.v. cannulae 7–10 days before behavioral training (a). Thirty minutes before extinction training, animals received 5 ml infusions of 0, 100, or 500 nM pentagastrin. Animals treated with pentagastrin showed higher levels of fear than vehicle-treated controls when tested off-drug, 48-h following extinction (b)  $n = 25$  for vehicle group,  $n = 8$  for 100 nM pentagastrin group,  $n = 17$  for 500 nM pentagastrin group; values shown are the averages of all test trials; error bars indicate  $\pm$  SEM; \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.01$ .

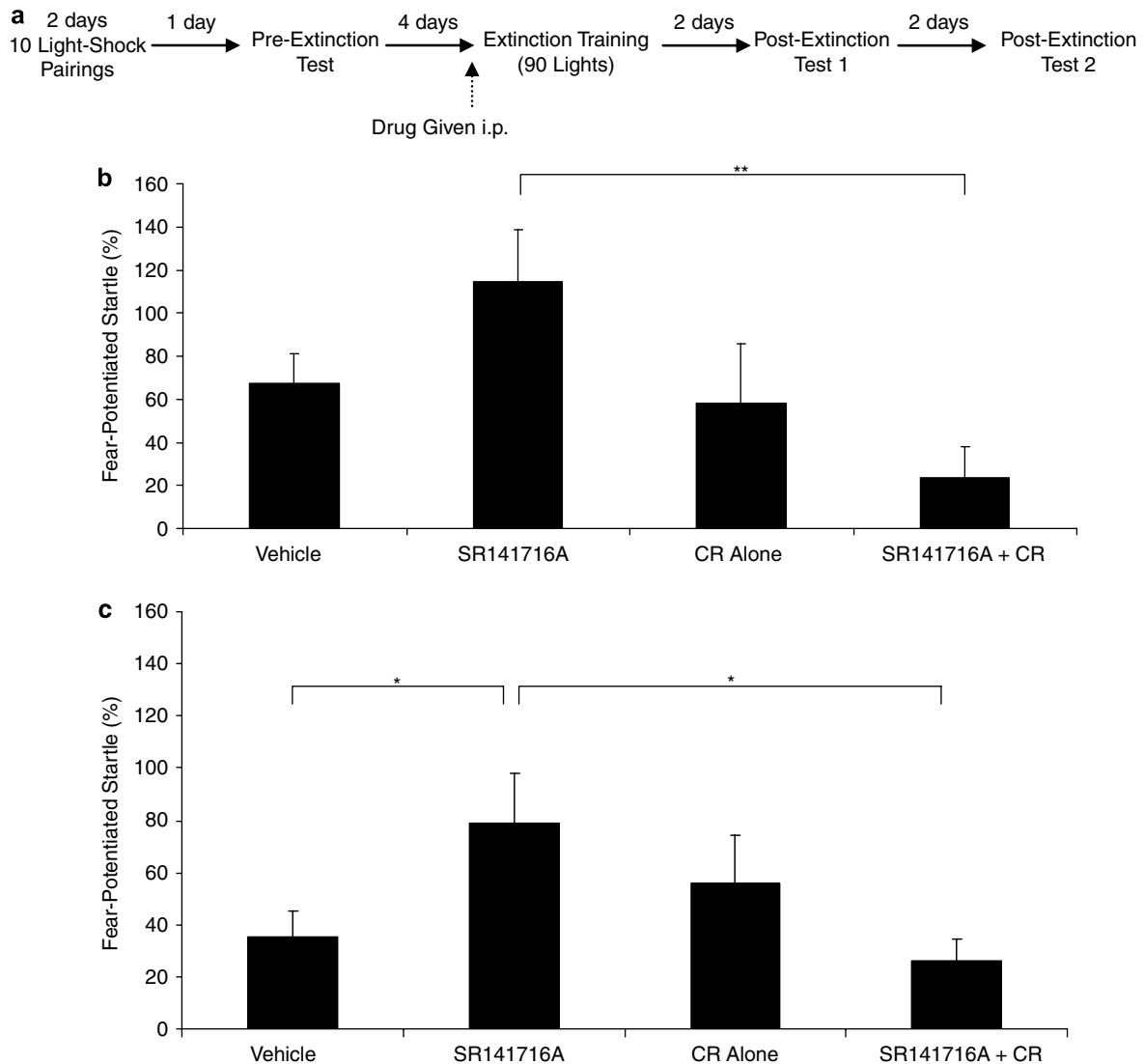
Two days following extinction training, animals were tested, in the absence of any drug, for the presence of FPS as a measure of conditioned fear. Vehicle-treated control animals showed the lowest levels of FPS following extinction, indicative of significant extinction. In contrast, animals receiving 500 nM pentagastrin at the time of extinction training showed significantly higher levels of conditioned fear than vehicle-treated controls. In animals receiving 100 nM pentagastrin, the levels of FPS were intermediate between vehicle- and 500 nM pentagastrin-treated animals, suggesting that the impairment of extinction retention with pentagastrin may be dose-dependent (Figure 2b; linear contrast ANOVA  $F_{(1,50)} = 5.074$ ; *post hoc* 500 nM vs vehicle,  $p < 0.05$ ). Notably, baseline startle in our training and testing paradigm was not significantly different in pentagastrin-treated animals as compared with controls, either immediately after pentagastrin administration (Supplementary Figure 1A) or 48 h post-extinction (Supplementary Figure 1B).

### Blockade of Extinction by a CB1 Antagonist is Reversed with a Systemic CCK2 Antagonist

It has previously been shown that a cannabinoid antagonist (SR141716A) prevents the normal extinction of conditioned fear when delivered systemically (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005) and through intra-BLA infusions (Roche *et al*, 2007). Activation of CB1Rs is thought to inhibit the release of GABA and CCK by decreasing the excitability of the pre-synaptic terminal. If preventing CCK release was a critical component of CB1-mediated effects on extinction, we predicted that antagonizing the CCK2 receptor would reverse the normal blockade of extinction seen with CB1R antagonists. Thus, we examined whether co-administration of a CCK2 antagonist (CR2945) might reverse the blockade of extinction seen with CB1 antagonist (SR141716A) treatment. In this series of experiments, animals were again fear-conditioned and matched into groups showing similar levels of FPS before extinction training (Figure 3a). Thirty minutes before extinction training (90 lights without shocks), animals were systemically administered vehicle (100% DMSO), SR141716A (5 mg/kg), CR2945 (3 mg/kg), or a combination of SR141716A and CR2945 (5 and 3 mg/kg, i.p., respectively).

In agreement with previous studies (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005), we observed that administration of SR141716A potently inhibited extinction learning, as animals receiving SR141716A showed higher levels of FPS than vehicle-treated controls when tested 48- and 96-h post-extinction (Figure 3b). Notably, rats treated with a combination of SR141716A and CR2945 showed significantly less fear than those receiving SR141716A alone and levels of FPS that were statistically similar to those of vehicle-treated controls (Figure 3b; first test  $F_{(3,24)} = 3.876$ ,  $p < 0.05$ ; second test  $F_{(3,24)} = 3.060$ ,  $p < 0.05$ ). Rats that received CR2945 alone before extinction training did not show enhanced extinction retention as compared to vehicle-treated animals.

Thus, CCK2 activation is an important downstream effect of CB1R blockade on extinction. Additionally, as we find that in the absence of CB1 blockade, CCK2 antagonism is



**Figure 3** Systemic administration of a CCKB antagonist reverses the blockade of extinction normally seen with CB1 antagonist treatment. Schematic representation of the behavioral paradigm used in these studies (a). Consistent with previous results, we observed that pre-extinction training administration of the CB1 antagonist SR141716a (5 mg/kg, i.p.) produced a profound blockade of extinction retention as measured 48 h (b) and 96 h (c) following extinction training. Vehicle-treated animals and animals co-administered the CCK2 antagonist CR2945 (CR, 3 mg/kg, i.p.) showed significantly less FPS 48 h (b, SR141716A + CR group) and 96 h (c, vehicle and SR141716A + CR group) following extinction training as compared to those receiving SR alone ( $n = 7$  per group; values shown are averages of all trials in each test; error bars indicate  $\pm$  SEM; \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.01$ ).

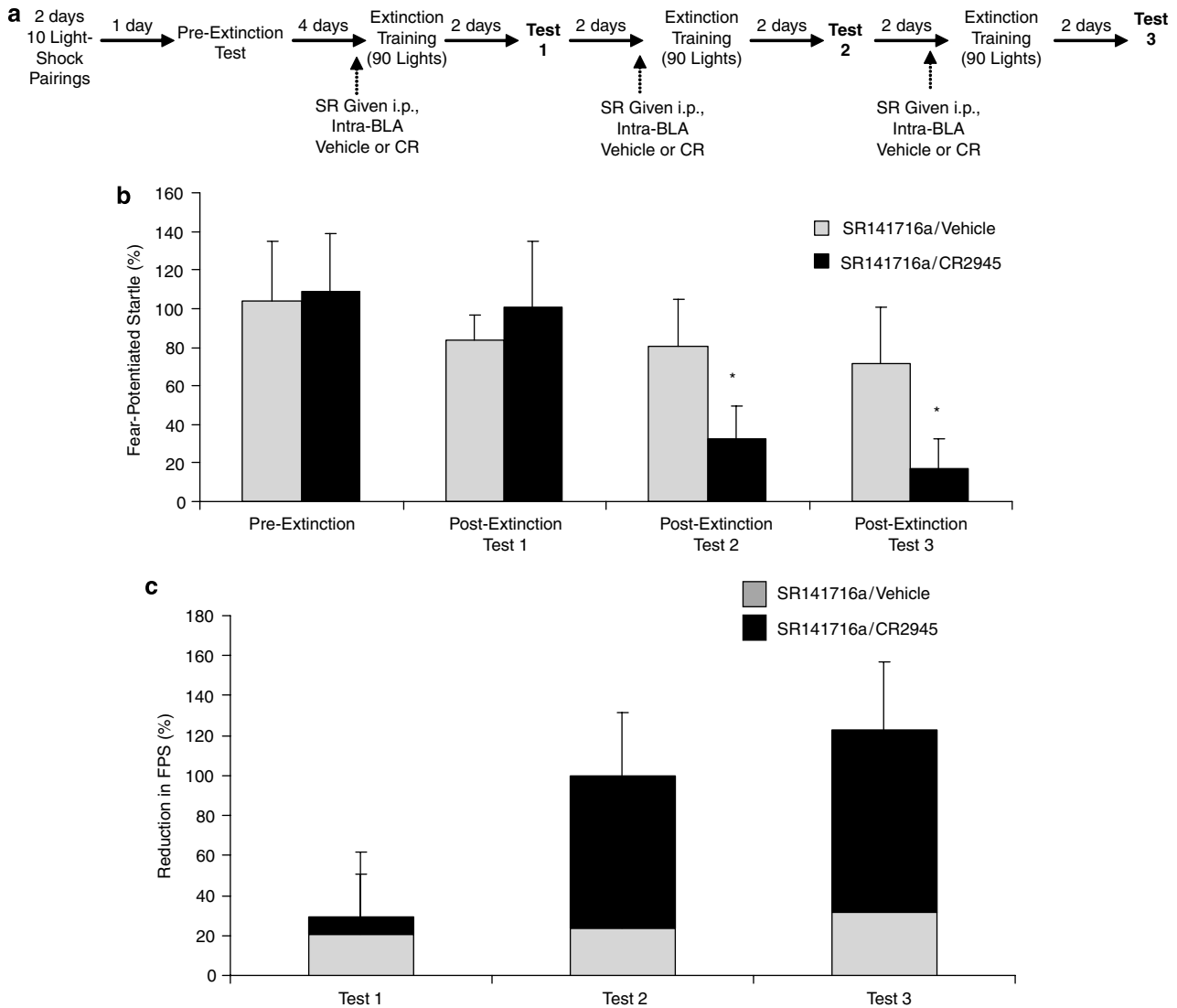
not sufficient to enhance extinction, other pathways (eg modulation of GABA release) are likely important as well in extinction modulation at baseline.

### Blockade of Extinction by a CB1 Antagonist is Reversed with an Intra-amygdalar CCK2 Antagonist

The BLA is known to be a critical site for extinction learning, and intra-BLA infusions of SR141716A have been shown to prevent extinction of fear (Roche *et al*, 2007). Here, we examined whether the local infusions of CR2945 into the amygdala would mitigate the blockade of extinction seen with systemic SR141716A administration. In these experiments, rats were implanted with bilateral cannulae aimed at the BLA and allowed to recover for 7–10 days. Subsequently, these animals were fear-conditioned and

matched into groups showing equivalent levels of startle, as in the aforementioned experiments. Thirty minutes before extinction training (90 lights without shocks), all animals were given i.p. injections of SR141716A (5 mg/kg) along with bilateral infusions of either vehicle (5% DMSO in PBS) or CR2945 (1  $\mu$ g/0.5  $\mu$ l/side).

Following extinction training, animals were tested for the presence of FPS 2 days following training. As the animals in these experiments showed relatively little extinction at the first post-extinction test, perhaps as a result of the stress involved in local amygdala infusions, two additional blocks of extinction training (with similar drug treatment) and testing were given (Figure 4a). Importantly, we focused on the amount of FPS demonstrated in the first five trials of each post-extinction testing session and used this to assess extinction retention, as a great deal of within-session



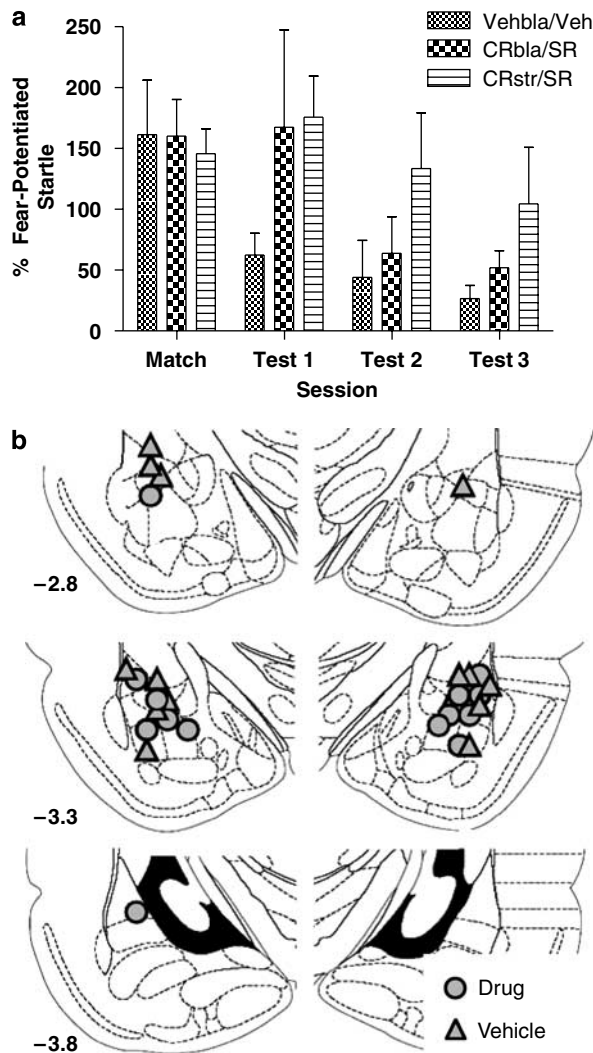
**Figure 4** Intra-amygdala infusion of a CCKB antagonist partially reverses the blockade of extinction seen with CB1 antagonist treatment. Animals were implanted with bilateral cannulae aimed at the BLA 7–10 days before behavioral training. Before each extinction session, all animals were injected with 5 mg/kg SR141716a; in addition to these i.p. injections, either vehicle or 1  $\mu$ g CR2945 (CR) was bilaterally infused into the BLA (outlined in (a), volume = 0.5  $\mu$ l over 5 min). Animals receiving intra-BLA CR showed significant extinction retention on post-extinction tests 2 and 3 (b). Animals receiving vehicle did not show significant extinction retention on any of the three post-extinction tests (b). Panel c depicts the change in FPS seen pre- vs post-extinction for each of the three tests. Animals receiving intra-BLA CR showed significantly greater reductions in FPS in tests 2 and 3 ( $n = 8$  per group; values shown are averages of the first five trials in each test; error bars indicate  $\pm$  SEM; \* denotes  $p < 0.05$  comparing pre-extinction FPS to post-extinction FPS within each group).

extinction was observed on days 2 and 3 of testing. Animals that received intra-BLA infusions of CR2945 showed significant extinction retention on the second and third post-extinction tests as compared to their first test and pre-extinction test values (significant main effect of testing day, repeated measures ANOVA  $F_{(3,18)} = 4.344$ ,  $p < 0.05$ ; *post hoc* tests comparing days 2 and 3 to pre-extinction test and first test,  $p < 0.05$ ; Figure 4b and c). In contrast, vehicle-infused controls failed to show significant extinction on any of the three testing days, suggesting that SR141716A was able to attenuate extinction (repeated measures ANOVA  $F_{(3,18)} = 0.383$ ,  $p = 0.766$ ). Notably, as the amelioration of the CB1 antagonist effect was slightly less pronounced with intra-amygdala CR2945 infusions compared to systemic administrations, sites other than the amygdala may also be important mediators of the CCK–CB1 interaction.

Nonetheless, these data suggest that intra-amygdala blockade of CCK2 receptors is sufficient to overcome the extinction deficit caused by systemic CB1 blockade.

### Striatal Infusions of CCK2 Antagonist do not Reverse the CB1 Antagonist-Induced Blockade of Extinction

To confirm that the effect of CR2945 on the reversal of the SR141716A-induced blockade of extinction was due to the amygdala and not another nearby subcortical site, a replication study was performed in exactly the same manner as the above experiment. In this study, cannulae were placed either in the BLA (as above; Figure 5b) or within the striatum as an intracerebral control injection site. Animals were trained and tested as in the preceding experiment, with three groups of animals: (1) those receiving vehicle



**Figure 5** Reversal of systemic cannabinoid antagonist blockade of extinction by local infusion of a CCK antagonist into the BLA but not the striatum. (a) Similar to Figure 4a, animals were implanted with bilateral cannulae aimed at the BLA or striatum (str) 7–10 days before behavioral training. Before each extinction session, all animals were injected with 5 mg/kg SR141716a systemically; in addition to these i.p. injections, either vehicle or 1  $\mu$ g CR2945 (CR) was bilaterally infused into the BLA or striatum (volume = 0.5  $\mu$ l over 5 min) before each extinction training session and then tested 48 h later off-drug. Animals given SR141716a systemically and CR2945 into BLA, but not striatum, demonstrate extinction levels similar to those of vehicle-treated animals by the second and third tests. (b) Cannula locations within the BLA (striatal cannula not shown) of rats included within this experiment.

systemically and vehicle intra-BLA (Vehbla/Veh group); (2) those receiving SR141716A systemically and CR2945 intra-BLA (CRbla/SR group); and (3) those receiving SR141716A systemically and CR2945 intrastriatum (CRstr/SR group). We found that the vehicle–vehicle group extinguished rapidly, as expected (Figure 5a; significant main effect of testing day, overall repeated measures ANOVA  $F_{(3,87)} = 5.902$ ,  $p < 0.01$ ; *post hoc* tests comparing day 1 to pre-extinction,  $p < 0.05$ , days 2 and 3 to pre-extinction,  $p < 0.001$ ). We also found that, as in the reversal experiment above, animals receiving CR2945 in the amygdala (CRbla/SR group) extinguished significantly faster than animals

receiving CR2945 infusions in the striatum (CRstr/SR group), which did not show significant extinction during the testing (Figure 5a, *post hoc* tests comparing the intra-amygdala group days 2 and 3 to pre-extinction,  $p < 0.05$ ). This experiment confirms and replicates the finding that CCK2 receptor blockade within the amygdala is sufficient to reverse the systemic effects of CB1 blockade on extinction learning. Furthermore, CCK2 blockade at an extra-amygdalar site, the striatum, does not reverse the effect on extinction of systemic CB1 blockade.

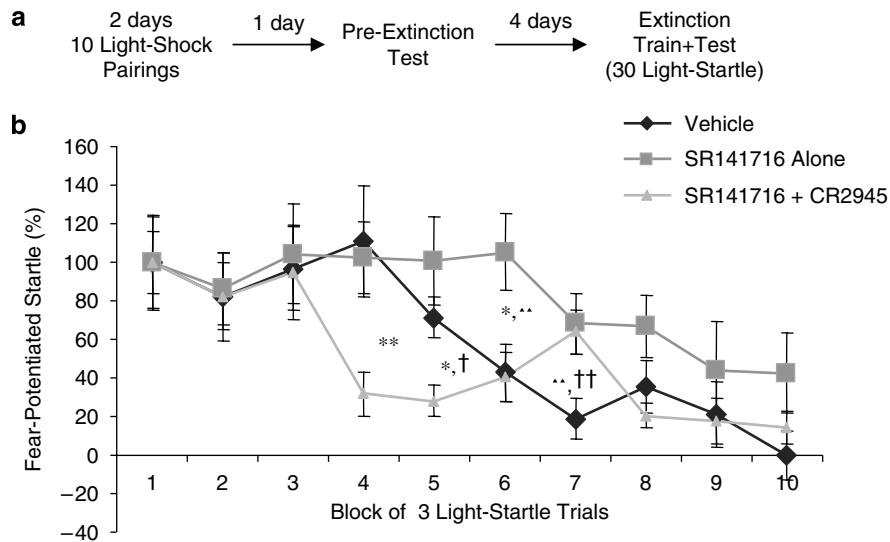
### Systemic CCK2 Antagonist Reverses CB1 Antagonist-Induced Deficits in Within-Session Extinction

Previous studies by Marsicano *et al* (2002) have demonstrated that extinction training induces the production of anandamide and 2-AG within the BLA and suggest that activation of CB1Rs during extinction training itself is required for the formation of stable extinction memories. Consistent with this, pre- but not post-training administrations of SR141716A inhibit long-term extinction (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005). Additionally, the behavior of CB1-knockout mice suggests that they may be impaired in achieving reductions in fear responses during extinction training (Marsicano *et al*, 2002), in turn suggesting that SR141716A may be blocking extinction retention by impairing the dynamic reduction in fear with increasing numbers of non-reinforced CS-alone extinction trials (ie within-session extinction).

As the aforementioned experiments suggested that CR2945 may reverse the effects of SR141716A on extinction retention, we next examined if SR141716A-treated animals would exhibit deficits in within-session extinction and if this impairment would be improved with CR2945 co-administration. In these experiments, rather than presenting 90 lights without shocks during extinction, we presented fear-conditioned animals with 30 light–startle trials intermixed with 30 startle-alone trials. This alteration in our extinction training paradigm allowed us to measure within-session extinction. Thirty minutes before this extinction training/testing session, animals were administered vehicle (100% DMSO), SR141716A (5 mg/kg), or a combination of SR141716A and CR2945 (5 and 3 mg/kg, respectively). To compare the rates of within-session extinction, each animal's FPS was normalized to 100% according to their behavior on the first block of light–startle trials, allowing for comparison across treatment groups and compensating for variations in fear levels at the outset of extinction.

All three groups showed within-session extinction (Figure 6; within-session extinction: overall ANOVA  $F_{(9,405)} = 9.891$ ,  $p < 0.001$ ; vehicle group  $F_{(9,153)} = 6.079$ ,  $p < 0.001$ ; SR141716A-alone group  $F_{(9,153)} = 2.594$ ,  $p < 0.01$ ; SR141716A + CR group  $F_{(9,99)} = 7.365$ ,  $p < 0.001$ ). However, significantly slower extinction was observed in SR141716A-treated animals than in vehicle-treated controls (Figure 6b), an effect that was especially prominent in the middle blocks of the extinction training/testing session. This observation agrees with previous studies (Marsicano *et al*, 2002; Suzuki *et al*, 2004) and provides further evidence that CB1 antagonism impedes within-session extinction.

In contrast, animals co-administered CR2945 and SR141716A showed rates of within-session extinction



**Figure 6** The CBI antagonist SR141716 impairs within-session extinction in a manner reversible by the CCKB antagonist CR2945. Animals were trained similarly to previous experiments (a), but in lieu of explicit extinction training (light-alone trials), a long (30 light–startle) test was used to determine the rates of within-session extinction in animals receiving vehicle, SR141716a alone (5 mg/kg), or SR141716a and CR2945 (SR141716a + CR; 5 and 3 mg/kg, respectively). FPS in all groups was normalized such that the FPS response during the first block of three trials represented 100% for that group, controlling for differences in starting levels of FPS. SR141716a-treated animals showed less within-session extinction than animals receiving vehicle or SR141716a + CR (b), an effect that was particularly evident in blocks 5–8. In some blocks, SR141716a + CR animals showed significantly different FPS compared to vehicle-treated controls ( $n = 18$  each for vehicle and SR141716a-alone groups,  $n = 10$  for SR141716a + CR group; error bars indicate  $\pm$  SEM; in comparisons of SR141716a + CR to SR141716a alone, \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.01$ ; in comparisons of vehicle to SR141716a alone,  $\blacktriangle$  denotes  $p < 0.01$ ; in comparison of vehicle to SR141716a + CR,  $\dagger$  denotes  $p < 0.05$ ,  $\dagger\dagger$  denotes  $p < 0.01$ ).

similar to (and, in some blocks, better than) those of vehicle-treated controls, suggesting that CR2945 may reverse SR141716A-induced deficits in within-session extinction (significant drug  $\times$  time interaction  $F_{(18,405)} = 1.633$ ,  $p < 0.05$ ; several *post hoc* differences noted in Figure 6b). Additionally, this result suggests that CCK2 antagonism may enhance the rates of within-session extinction.

As the assessment of CCK and CB1 modulation of within-session extinction in these experiments necessitated the testing of animals ‘on-drug,’ we performed a series of control experiments in which we examined whether the doses of SR141716A and CR2945 used in these studies could affect baseline startle (a measure of basal anxiety) as well as reactivity to footshock, a measure of nociception and general reactivity. Using animals that had been matched for equivalent levels of baseline startle and unconditioned fear to the light, we injected animals with vehicle, SR141716A alone (5 mg/kg), CR2945 (3 mg/kg), or a combination of SR141716A and CR2945 (5 and 3 mg/kg, respectively) 30 min before a test session in which baseline startle and shock reactivity were assessed. We observed that both baseline startle and shock reactivity were similar across all groups (Figure 7), suggesting that the effects of these drugs on within-session extinction proceed without gross alterations of nociception or baseline anxiety.

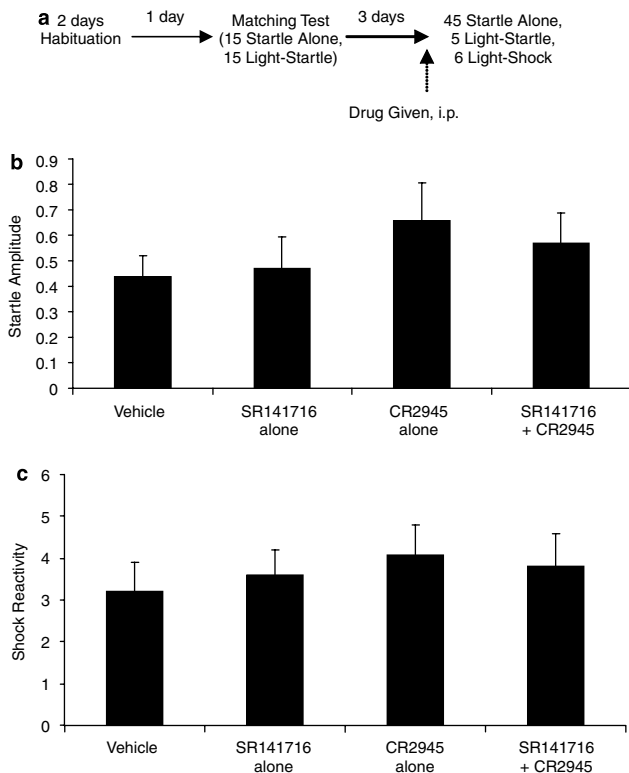
#### CCK2 Antagonist Co-administration Reverses CB1 Antagonist-Induced Blockade of Akt Activation Following Extinction

Extinction is an active learning process that is thought to involve many of the same intracellular signaling molecules

as fear acquisition. It has previously been shown that extinction training leads to increases in phosphorylated (active) Akt in the amygdala and that the extinction-induced activation of Akt was enhanced when extinction learning was pharmacologically facilitated (Yang and Lu, 2005).

We examined whether SR141716A would prevent the normal induction of phosphorylation of Akt following extinction and whether co-administration of CR2945 with SR141716A would reverse these effects. Rats were fear-conditioned and matched into groups demonstrating similar levels of FPS before extinction (Figure 8a). Thirty minutes before extinction (90 lights without shocks), animals were administered either SR141716A (5 mg/kg) or a combination of SR141716A and CR2945 (5 and 3 mg/kg, respectively). Two control groups were employed: one group received vehicle plus exposure to the training context for the same period of time as extinction-trained controls (context) and the other group received vehicle in addition to extinction training (vehicle group). These rats were then killed 2 h following extinction training and the BLA was rapidly dissected out over ice, homogenized, and stored at  $-80^{\circ}\text{C}$  until western blot assays were performed. The 2 h post-extinction time point used here was chosen based on preliminary studies showing that changes in the phosphorylation states of Akt were observable 2 h following extinction training. An overall significant effect of drug treatment and extinction training was seen (ANOVA  $F_{(4,51)} = 3.529$ ,  $p = 0.013$ ).

Levels of phosphorylated Akt appeared similar between SR141716A-treated animals and non-extinguished controls (SR compared to context,  $p = 0.44$ ), suggesting that SR141716A antagonized the extinction-induced activation



**Figure 7** Pre-testing administration of *SR141716a* and/or *CR2945* does not affect baseline startle or shock reactivity. Animals were handled and placed in the training/testing chambers for 2 days for habituation purposes. (a) Following habituation, animals were tested and matched into groups showing equivalent levels of baseline startle and unconditioned fear to the light-CS. Three days later, animals were injected with vehicle (100% DMSO), *SR141716a* (5 mg/kg), *CR2945* (CR, 3 mg/kg), or a combination of *SR141716a* and *CR2945* (Rim + CR, 5 and 3 mg/kg, respectively). Thirty minutes following injection, animals were presented with 30 startle-alone trials, followed by an intermixed session of 5 light–startle compounds and 15 startle-alone trials. At the end of this session, six light–shock compounds were administered to assess shock reactivity (ie accelerometer displacement in response to shock). All four groups showed similar levels of baseline startle (b) and similar levels of shock reactivity (c).

of Akt within the amygdala. However, animals co-administered *CR2945* with *SR141716a* showed significantly higher levels of p-Akt following extinction than animals receiving *SR141716a* alone ( $p=0.044$ ), whereas no significant differences were seen between the vehicle + extinction and the group co-administered SR + CR before extinction ( $p=0.52$ ). This suggests that co-administration of *CR2945* may allow extinction-induced increases in p-Akt, even in the presence of *SR141716a* treatment (Figure 8b and c), a biochemical measure that appears to parallel the behavioral effects of these drugs.

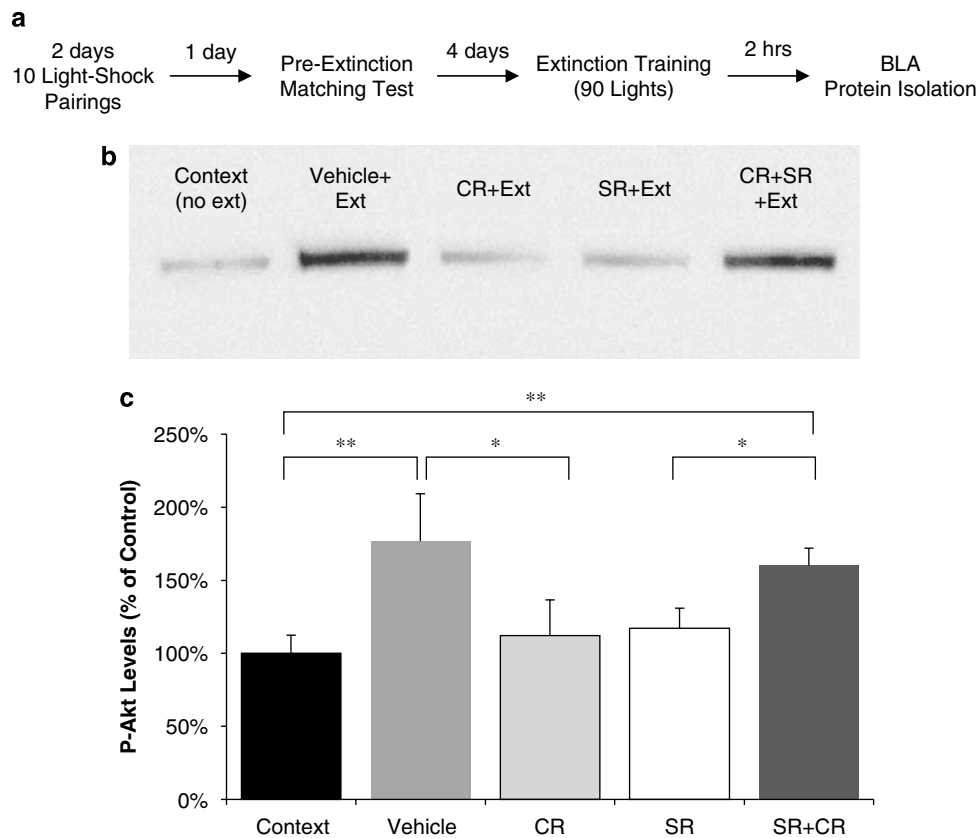
## DISCUSSION

We present several lines of evidence to suggest that interactions between the eCB and CCK neurotransmitter systems may play an important role in extinction and that CB1-mediated modulation of CCK2 receptor activation may be a critical component in the CB1 dependency of extinction learning. Specifically, these experiments demonstrate that

(1) CCK mRNA and CB1 mRNA are both expressed at a higher level within the BLA, whereas they are relatively absent in the CeA and MeA, (2) i.c.v. infusions of the CCK2 agonist pentagastrin dose-dependently impair extinction; (3) pre-extinction administration of the CB1 antagonist *SR141716a* potentially impairs extinction, and this effect is ameliorated with systemic co-administration of the CCK2 antagonist *CR2945*, (4) local infusion of *CR2945* into the amygdala partially reverses the blockade of extinction seen with systemic *SR141716a* treatment, (5) increased phosphorylation of Akt in the amygdala is observable 2 h post-extinction training, and this effect is reversed in animals pretreated with *SR141716a* before extinction; (6) co-administration of *CR2945* with *SR141716a* before extinction training leads to levels of Akt phosphorylation that are similar to control animals; and (7) *SR141716a*-treated animals showed slower rates of within-session extinction than vehicle-treated controls and animals co-administered *SR141716a* and *CR2945*.

In terms of understanding the neural circuitry underlying the process of extinction learning, the present study in combination with the aforementioned anatomical studies describing high concentrations of CB1Rs on the presynaptic terminals of CCK+ interneurons (Marsicano and Lutz, 1999; McDonald and Mascagni, 2001) suggests that the putative synapse of CCK/CB1+ neurons onto BLA pyramidal neurons may be an important locus of plasticity underlying extinction. It should be noted, however, that CB1 modulation of both GABAergic and glutamatergic neurotransmission in the BLA has been demonstrated, suggesting that the interaction between the CCKergic and eCB systems could involve a broad range of synapses (Azad *et al*, 2004). This raises the intriguing possibility that although many pyramidal BLA neurons express low levels of CB1 mRNA, CB1-mediated modulation of glutamate transmission likely plays an important role in extinction.

We have now found in the several experiments described in this paper that the CCK antagonist *CR2945* reverses the inhibition of extinction of the CB1 antagonist *SR141716a*, both when given systemically and via cannula directly into the amygdala. Interestingly, however, *CR2945* does not appear to significantly enhance extinction of fear when given alone. There are several possible explanations for this: first, it is possible that the level of extinction that we normally see in these studies is already ‘at ceiling,’ meaning that we cannot detect further facilitation of extinction or that *CR2945* would enhance extinction in animals with low levels of extinction-induced CB1 activation (such as may be the case in chronic stress or in animals with chronic exposure to CB1 agonists). As is the case in stress-related neural systems, there are some pathways that are not able to be modulated at baseline, but which respond differently in stress or anxiogenic situations. With this hypothesis, it is possible that blocking CB1 with systemic antagonist treatment mimics a naturally diminished CB1-mediated process. Similarly, in some studies, *D-cycloserine* (DCS), an NMDA partial agonist that enhances extinction, has been shown to be more efficacious in states of anxiety than at baseline (Bertotto *et al*, 2006). Additionally, as mentioned earlier, it is likely that CB1-mediated modulation of glutamatergic transmission is also likely to play an important role in extinction.



**Figure 8** Animals co-administered SRI41716A and CR2945 demonstrate higher levels of p-Akt within amygdala following extinction than animals receiving SRI41716A alone. (a) Temporal organization of the experiment. (b) Qualitative representation of western blot data from BLA following extinction. (c) A significant increase is found in phospho-Akt in extinction-trained animals, as compared to those receiving context exposure alone. Animals co-administered the CCKB antagonist CR2945 (3 mg/kg) with CB1 antagonist SRI41716 (5 mg/kg) before extinction training showed greater p-Akt in the BLA following extinction, as compared to animals who received SR + extinction (SR alone) and animals who were fear-conditioned but not extinction-trained (context) (error bars indicate  $\pm$  SEM; \* denotes  $p < 0.05$ , \*\* denotes  $p < 0.01$ ).

Furthermore, results from both human and animal literature suggest that although CCK antagonists reverse the anxiogenic effects of CCK activation, they do not consistently show such effects when administered alone (Harro, 2006). This is evident in clinical trials of CCK2 antagonists wherein no effect was found following the administration of several CCK2 antagonists in patients with generalized anxiety disorder or panic disorder (Adams *et al*, 1995; Kramer *et al*, 1995; van Megen *et al*, 1997; Pande *et al*, 1999). Several results from the animal literature also support the notion that CCK2 antagonists are not necessarily anxiolytic when administered alone (Dawson *et al*, 1995; Johnson and Rodgers, 1996). These results suggest that the CCK antagonist is not expected to facilitate extinction alone, and are consistent with our hypothesis that perturbation of the endogenous system with the CB1 antagonist is necessary to reveal the underlying behavioral effects of CCK on the extinction of conditioned fear. Further studies aimed at examining pre- and post-synaptic effects of CB1 and CCK2 manipulation on extinction learning hope to further dissect these interacting mechanisms.

The observation that pentagastrin impairs extinction seems to fit well with previous data indicating that CCK receptor agonist treatment is acutely anxiogenic, and with data showing that pentagastrin enhances conditioned fear responses, as measured by FPS (Frankland *et al*, 1996). In

the context of the present study, the enhancement of fear expression seen by Frankland *et al* (1996) is particularly interesting in that it suggests that pentagastrin-treated animals may have impairments in adequately reducing their fear responses during extinction training (ie within-session extinction)—a phenotype similar to that seen in CB1 knockouts and in animals administered CB1 antagonists (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005).

Such a connection may have important clinical implications, as pentagastrin has been shown to be anxiogenic and/or panicogenic in humans, and several modulators of the CB1 and CCK2 receptors are being considered for clinical use. As CCK and CB1 modulation of extinction seems to be a within-session effect (ie an acquisition of extinction effect), it is possible that CCK2 antagonists and/or eCB reuptake inhibitors could be given acutely before exposure-based psychotherapy. Such treatment may be particularly effective in patients manifesting deficits in within-session extinction (or who are especially avoidant in exposure-based sessions) as opposed to extinction retention. Indeed, our group and others are currently investigating the intriguing possibility that genetic differences in the eCB and/or CCKergic neurotransmitter systems may lead to altered susceptibility to trauma-induced psychopathology in humans. Additionally, there is potential for modulators of

eCB or CCKergic transmission to be given in combination with other drugs, such as DCS, which appear to enhance the consolidation of extinction (Walker *et al*, 2002; Ledgerwood *et al*, 2003; Ressler *et al*, 2004). Notably, recent evidence strongly suggests that the acquisition and consolidation of extinction are separable processes (Quirk *et al*, 2000; Santini *et al*, 2001; Chhatwal *et al*, 2006), suggesting that pharmacologic enhancement of either process or both processes may be possible. Although more work is needed to understand the sites at which centrally active CCK2 antagonists can decrease unconditioned and conditioned fear responses, the results of this study suggest that a more complete understanding of CCK2-mediated affective responses may be very useful in the development of anxiolytics that promote extinction learning.

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## DISCLOSURE/CONFLICTS OF INTEREST

KJR, MD, and JPC have filed a provisional patent application for the use of enhancers of eCB transmission to augment extinction in clinical settings. Notably, no drugs of this type were used in these studies. KJR has received research support from Lundbeck Pharmaceuticals, LLC. KJR has received consultation fees from Tikvah Pharmaceuticals, LLC.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)