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ORIGINAL INVESTIGATION

Adolescent cannabinoid exposure effects on natural reward seeking and learning in rats

H. Schoch[1] · M. Y. Huerta[1] · C. M. Ruiz[1] · M. R. Farrell[1] · K. M. Jung[2] · J. J. Huang[1] · R. R. Campbell[1] · D. Piomelli[2] · S. V. Mahler[1]

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Abstract
Rationale Adolescence is characterized by endocannabinoid (ECB)-dependent refinement of neural circuits underlying emotion, learning, and motivation. As a result, adolescent cannabinoid receptor stimulation (ACRS) with phytocannabinoids or synthetic agonists like "Spice" cause robust and persistent changes in both behavior and circuit architecture in rodents, including in reward-related regions like medial prefrontal cortex and nucleus accumbens (NAc).
Objectives and methods Here, we examine persistent effects of ACRS with the cannabinoid receptor 1/2 specific agonist WIN55-212,2 (WIN; 1.2 mg/kg/day, postnatal day (PD) 30–43), on natural reward-seeking behaviors and ECB system function in adult male Long Evans rats (PD 60+).
Results WIN ACRS increased palatable food intake, and altered attribution of incentive salience to food cues in a sign-/goal-tracking paradigm. ACRS also blunted hunger-induced sucrose intake, and resulted in increased anandamide and oleoylethanolamide levels in NAc after acute food restriction not seen in controls. ACRS did not affect food neophobia or locomotor response to a novel environment, but did increase preference for exploring a novel environment.
Conclusions These results demonstrate that ACRS causes long-term increases in natural reward-seeking behaviors and ECB system function that persist into adulthood, potentially increasing liability to excessive natural reward seeking later in life.

✉ S. V. Mahler
mahlers@uci.edu

[1] Department of Neurobiology & Behavior, University of California, 1203 McGaugh Hall, Irvine, CA 92697, USA

[2] Department of Anatomy & Neurobiology, University of California, Irvine, CA, USA

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Introduction

Adolescence is a dynamic period of neural circuit development, when subcortical emotion and motivation circuits mature, in part via activity-dependent endocannabinoid (ECB) signaling (Bossong and Niesink 2010; Brenhouse and Andersen 2011; Dow-Edwards and Silva 2017; Renard et al. 2014). Unfortunately, adolescence is also when many people first experiment with cannabis and synthetic cannabinoid agonist drugs like "Spice" or "K2" (Chen and Kandel 1995; Cuttler and Spradlin 2017; Mokrysz et al. 2016; Taylor et al. 2017). In humans, early use of these drugs is associated with cognitive and emotional deficits lasting into adulthood (Meier et al. 2012; Patton et al. 2002; Rubino et al. 2012; Scott et al. 2017), though causality is difficult to prove in these associational studies. In rodent models, exogenous adolescent cannabinoid receptor stimulation (ACRS) causes marked changes in neural circuit connectivity, gene expression, and other anatomical and biochemical features within cognition-, emotion-, and motivation-related brain circuits (Caballero and Tseng 2016; Hurd et al. 2014; Jager and Ramsey 2008; Lee and Gorzalka 2012; Renard et al. 2016a).

ACRS increases susceptibility to the rewarding effects of other drugs of abuse in adulthood (Doremus-Fitzwater and Spear 2016; Spear 2016), mediated by changes in brain reward systems including mesocorticolimbic dopamine pathways, endogenous opioids in nucleus accumbens (NAc), and the ECB system. ACRS with intermittent doses of Δ^9-THC increase adulthood heroin (male rats) and cocaine (female rats) self-administration, stress-induced relapse of heroin seeking (Stopponi et al. 2014), as well as morphine

conditioned place preference (Ellgren et al. 2007; Higuera-Matas et al. 2008; Tomasiewicz et al. 2012). In part, this may involve ACRS-induced persistent potentiation of mesocorticolimbic dopamine circuits, including increased basal (Behan et al. 2012; Gomes et al. 2015) and drug-induced activity in ventral tegmental area (VTA) dopamine neurons (Gomes et al. 2015; Pistis et al. 2004; Wegener and Koch 2009), and their forebrain projections (Renard et al. 2017). Numerous changes in ECB signaling molecules and receptors have also been reported after ACRS (Caballero and Tseng 2012; Ellgren et al. 2008; Renard et al. 2016b), with likely consequences for conditioned and unconditioned drug and natural reward seeking that is mediated in part by corticolimbic cannabinoids (Achterberg et al. 2016; Laviolette and Grace 2006; Maldonado et al. 2006; Vlachou and Panagis 2014). Such ACRS-induced changes could put individuals at risk of developing psychiatric disorders including schizophrenia, depression, or addiction (Lubman et al. 2015; Renard et al. 2014; Renard et al. 2016a; Rubino and Parolaro 2016; Spear 2016), and would also be expected to facilitate responsiveness to natural rewards like palatable foods or novelty.

Instead, several reports show decreases in seeking of natural rewards like palatable foods and social interaction after escalating dose adolescent cannabinoid exposure—an effect interpreted as depression-related anhedonia (Rubino et al. 2012). For example, social interaction is decreased after ACRS, as is preference for a weak (1–2%) sucrose solution over water, intake of a salty, fatty snack, and stress-induced suppression of chow intake in food-restricted rats (Bambico et al. 2010; Realini et al. 2011; Rubino et al. 2008). However, it is not clear to what extent these effects are mediated by alterations in learning, hedonic, appetitive, feeding-specific, or energy homeostasis processes, or instead by interactions between reward seeking and anxiety, or other emotional dysregulation caused by ACRS. Since dopamine and opioid circuits differentially mediate reward seeking and hedonics in mesolimbic regions like NAc (Baldo et al. 2013; Smith et al. 2010), and since ACRS affects both of these systems (Behan et al. 2012; Tomasiewicz et al. 2012), this begs the question of how ACRS affects specific behavioral assays of natural reward pursuit and consumption, and interactions between these processes and anxiety.

Here, we examined how ACRS with the CB1/2 agonist WIN55, 212-2 (WIN) affects specific assays of reward cue learning, palatable food intake, hunger and satiety effects on feeding, responses to novelty, and anxiety, tested in adulthood after WIN washout. We also characterize ECB levels in medial prefrontal cortex (mPFC), NAc, and cerebellum following acute food restriction. Results suggest that ACRS with WIN causes major changes in natural reward-seeking behavior that could indicate an addiction-prone phenotype later in life.

Methods

Subjects Male Long Evans rats (n = 50) were obtained from Harlan/Envigo (Indianapolis, IN, USA) post-weaning, arriving in our vivarium at postnatal day (PD) 21–22. Since sex differences in the effects of ACRS and in the reward-related behaviors tested here are well-known (Becker 2009; King et al. 2016; Rubino et al. 2008; Silva et al. 2016; Wiley and Burston 2014), in the initial experiments reported here, we restricted analyses to one sex only. Rats were subsequently housed in groups of four in wire-top polycarbonate tub-style cages (48 × 20 × 27 cm) with bedding, paper nesting material, and ad libitum food and water in a climate controlled vivarium on a 12-h reverse light-dark cycle. At PD 58, rats were separated into pairs ahead of behavioral testing. All procedures were approved by the University of California Irvine Institutional Animal Care and Use Committee, and are in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

ACRS protocol Starting at PD 30, rats were treated with 14 daily i.p. injections of WIN (0 or 1.2 mg/kg), a moderate-dose chronic adolescent treatment protocol known to impact neural circuit development and behavior, followed by a homecage washout period of 14+ days, adapted from (Abboussi et al. 2014; Bambico et al. 2010; Schneider 2008) (Fig. 1a). Rats receiving WIN or vehicle were housed with cagemates receiving the same treatment. During ACRS treatment and subsequent washout, rats were weighed daily. After the 14th injection (PD 43), > 50 g of standard rat chow was provided, and intake (grams consumed in 2.5 h) was measured for each cage containing four rats receiving WIN or vehicle.

Drugs WIN55-212,2 (mesylate) (#10009023, Cayman Chemical. Ann Arbor, MI, USA) was dissolved in 5% polyethylene glycol, 5% Tween 80, and physiological saline daily prior to injection.

Behavioral testing Staring at PD 60 (17+ days after the final WIN injection, at the onset of young adulthood (Schneider and Koch, 2003)), a battery of behavioral tests occurred (order of testing shown in Fig. 1b). Three cohorts of animals were tested, with all undergoing sign-/goal-tracking training, and subsets undergoing other behavioral tests (detailed below). All behavioral testing was conducted during the dark phase of the light cycle.

Sign/goal tracking We used a sign-/goal-tracking task in which a discrete cue was repeatedly paired with delivery of a palatable food pellet into an adjacent food cup. Over repeated training, rats develop conditioned approach responses to the lever (sign tracking) and/or food cup magazine (goal tracking)—an individual difference that predicts a variety of other

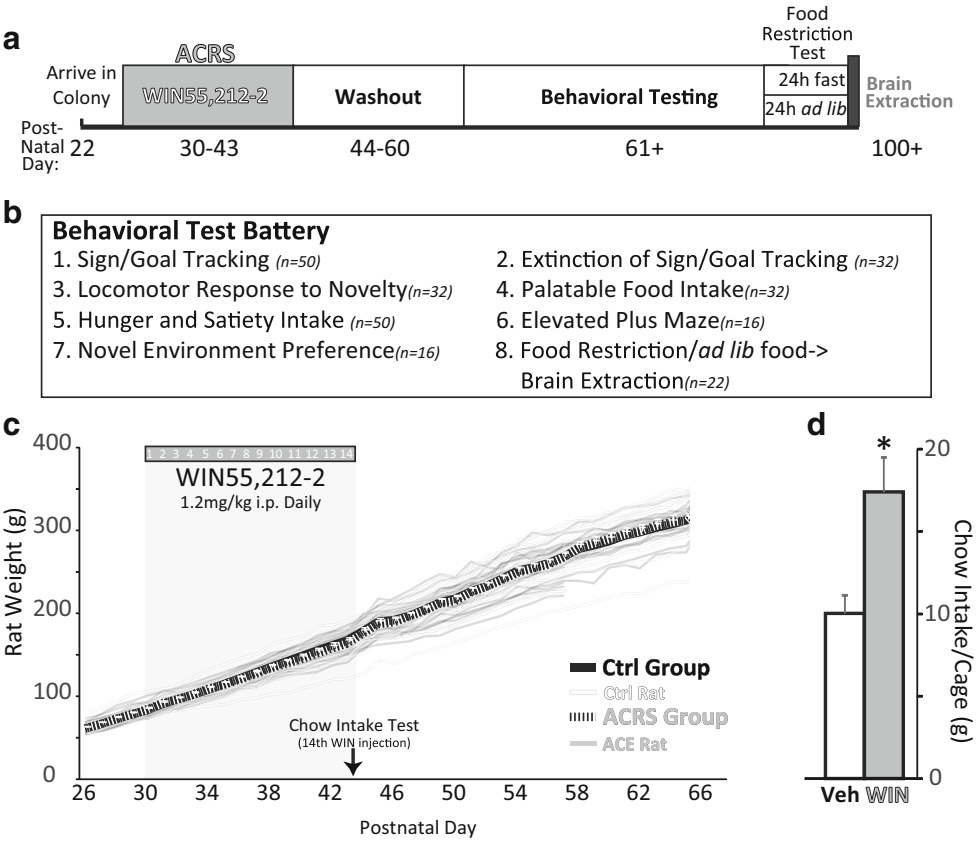

Fig. 1 Adolescent cannabinoid receptor stimulation procedure. **a** Timeline of experimental procedures is shown, with age (postnatal day) shown at bottom. **b** List of behavioral tests, number of animals tested in each, and the order in which they were conducted. **c** Body weights during WIN ACRS period (shaded box), and for 3 weeks thereafter. Group m+SEM displayed with solid black (ACRS) and gray (control) lines, and individual rats shown with semi-transparent lines of the same colors. **d** Chow intake in the 2.5-h after the 14th WIN injection (PD 43) is shown for vehicle (white bar) or WIN (gray bar) groups (m+SEM). *$p < 0.05$ vehicle vs. WIN cages

reward-seeking behaviors for natural and drug rewards (Robinson et al. 2014; Saunders and Robinson 2013). Individual rat bias toward sign or goal tracking is operationalized with a Pavlovian conditioned approach (PCA) score, which ranges from + 1 (exclusive sign tracking) to − 1 (exclusive goal tracking) (Meyer et al. 2012). This score is computed from the average of three behavioral variables indicating cue preference: (1) response bias (number of lever deflections minus number of cup entries/total interactions); (2) probability bias (per session probability of at least one deflection of the CS+ lever during a cue presentation, minus probability of cup entry); and (3) latency bias (average latency to deflect the lever minus average latency to enter the food cup). We defined PCA scores between − 1 and − 0.3 as reflecting predominant goal tracking, − 0.3 to + 0.3 as "intermediate" behavior, and + 0.3 to + 1 as predominant sign tracking. PCA scores were computed for each rat on each training day.

Following procedures described in Mahler and Berridge (2009)), we trained ad lib fed rats on the sign-/goal-tracking task for eight consecutive days. Rats were first habituated to 45 mg banana-flavored palatable food pellets [containing carbohydrate (52%), fat (6.3%), and protein (20.2%; Dustless Precision Pellets, #F0059; Bio-Serv, Flemington NJ, USA)] in their home cages, then magazine trained in 1–2 sessions, where 25 pellets were individually delivered into a food cup on a 30-s variable interval schedule, until > 90% of pellets were retrieved by all rats. On the next 8 days, rats received training sessions on which extension of an illuminated lever, accompanied by a tone emitted from a speaker at the top of the box, was presented for 8 s, followed immediately by delivery of a palatable banana-flavored pellet into a food cup positioned adjacent to the lever cue. Twenty-five such cue/reward pairings were presented on each 35–45-min training session. Importantly, pressing the CS+ lever had no effect on the timing or probability of reward delivery. An inactive control lever was continually available throughout each session, and while interactions with this lever were recorded, they also did not affect reward delivery. All training sessions were video recorded for subsequent coding of rears and CS+ lever, food cup, and inactive lever interactions during CS+ periods on acquisition days 1–3 (early), and 7–8 (late).

Extinction of sign-/goal-tracking On the day after the 8th sign-/goal-tracking training session, two cohorts of rats (control $n = 17$, ACRS $n = 17$) underwent a final ~ 2-h session on which they received 75 presentations of the CS+ cue on the same 90-s variable interval schedule without delivery of palatable food in order to examine within-session extinction of sign-/goal-tracking behavior.

Locomotor response to novelty 48 h after the sign-/goal-tracking extinction test, the same rats (control $n = 17$, ACRS

n = 17) were placed into a novel environment; a 43 × 43 × 30.5 cm Med Associates locomotor testing chamber without bedding or food/water. Distance traveled was automatically scored by infrared beam breaks and analyzed in 15-min bins throughout the 120-min session.

Familiar and novel palatable food intake The same rats (control n = 17, ACRS n = 17) were habituated to the same locomotor testing box on the following day for 90 min and were then given a 30-min access to a plastic weigh boat filled with pre-weighed 45 mg banana-flavored palatable food pellets used previously in sign-/goal-tracking training. Food intake was recorded by weight. On the following day, a 30-min intake of a novel chocolate food (M&Ms. brand candy) was similarly tested after a 90-min baseline habituation period to the same chamber, followed by an identical final test on the following day, with the now-familiar chocolate reward.

Hunger and satiety modulation of sucrose intake Next, all three cohorts of rats (control n = 25, ACRS n = 25) were habituated for 1 h/day for 6 days to a clear 48 × 20 × 27 cm polycarbonate tub cage with bedding and two bottles containing water or 15% sucrose solution, with intake of water and sucrose recorded daily by weight. On the following day, they were acutely food restricted (chow removed) 6 h prior to another 1 h test, with water intake recorded over this homecage restriction period, and water and sucrose intake measured during the 1 h two-bottle "acute hunger" test. Two additional habituation sessions without restriction were conducted on the next 2 days to re-stabilize intake, then a similar procedure was employed to acutely satiate rats with sucrose, by allowing them 6 h access to sucrose (without food or water) in their homecage prior to the 1 h "acute satiety" test.

Elevated plus maze In one cohort of rats (control n = 8, ACRS n = 8), an elevated plus maze (arms: 50.8 × 12.7 cm, closed arm wall height: 30.5 cm, elevated 73.7 cm above the floor) was used to examine anxiety-related behavior in ACRS and controls. Rats were placed in the closed center compartment, and entries into, and time spent (all four paws) on open and closed arms was measured in the 5-min test via offline hand scoring.

Novel place preference 48 h after elevated plus maze testing, the same rats (control n = 8, ACRS n = 8) were trained on two daily 30-min sessions to familiarize them with one side of a three chamber Med Associates rat conditioned place preference box. On the next day, they were allowed to explore all three chambers of the box in a 15-min test. Animal position was scored automatically by infrared beam breaks, and time spent in the familiar or novel compartments was quantified.

Quantification of video recorded behavior Behavior in sign-/goal-tracking and hunger/satiety regulation of food intake was coded by observers blind to ACRS history. For the sign-/goal-tracking experiment, bouts and duration of contact with the lever CS+ cue, and the always-present control lever and food cup, as well as rears, were quantified in the 8-s prior to and during cue presentations on training days 1–3. For the sucrose acute hunger/satiety intake experiment, latency to first drink from the sucrose or water bottles on each session was also recorded.

Acute food restriction and tissue collection Following all behavioral tests, 22 rats from two cohorts were habituated to wire bottom cages to prevent coprophagia for 48 h. Control and ACRS rats were randomly assigned to 24 h food restriction or no restriction (not restricted: control n = 4, ACRS n = 8; restricted: control n = 6, ACRS n = 4) (Kirkham et al. 2002). Rats were isoflurane anesthetized, and brains extracted and rapidly flash frozen in liquid nitrogen. Frozen brains were sectioned into 1 mm coronal sections, and discrete 2–10 mg samples of brain regions of interest were scalpel dissected for analysis of ECB content. Samples with ECB levels more than 2SD from group means were excluded from analyses (4 total, group sizes shown in Fig. 6).

Endocannabinoid analysis Procedures were previously described (Astarita et al. 2009; Wei et al. 2015). Tissue samples were homogenized in methanol containing internal standards for $^{2}H_4$-anandamide ($^{2}H_4$-AEA), $^{2}H_4$-oleoylethanolamide ($^{2}H_4$-OEA), and $^{2}H_8$-2-arachidonoyl-sn-glycerol ($^{2}H_8$-2-AG). Lipids were separated by a modified Folch-Pi method using chloroform/methanol/water (2:1:1) and open-bed silica column chromatography. For liquid chromatography/mass spectrometry (LC/MS) analyses, we used an Agilent 1200 LC system coupled to a 6410 triple quadrupole MS system (Agilent Technologies, Palo Alto, CA). The column was a ZORBAX Eclipse XDB-C18 (4.6 × 50 mm, 1.8 μm, Agilent Technologies). We used a gradient elution method as follows: solvent A consisted of methanol with 0.25% acetic acid and 5 mM ammonium acetate, and solvent B consisted of water with 0.25% acetic acid and 5 mM ammonium acetate. Lipids were eluted with a gradient of methanol in water (from 90 to 100% in 5 min, to 100% in 7 min, and to 90% in 8 min) at a flow rate of 1 mL/min. Column temperature was held at 40 °C. MS detection was in electrospray ionization (ESI) and positive ionization mode, with capillary voltage at 3.5 kV and fragmentor voltage at 135 V. N_2 was used as drying gas at a flow rate of 12 L/min and temperature of 350 °C. Nebulizer pressure was set at 50 psi. Quantifications were conducted by an isotope dilution method, monitoring $[M + H]^+$ in the selected ion monitoring (SIM) mode. The multiple reaction transitions monitored were as follows: anandamide, m/z 348 → 62; $^{2}H_4$-anandamide, m/z 352 → 66; OEA, m/z 326 → 62;

$^{2}H_4$-OEA, *m/z* 330 → 66; 2-AG, *m/z* 379 → 287; $^{2}H_8$-2-AG, *m/z* 387 → 295 (*m/z*, mass-to-charge ratio). Detection and analysis were performed using Mass Hunter Workstation software (Agilent).

Statistics For analysis of ACRS effects on sign-/goal-tracking behavior and locomotor response to novelty, mixed model ANOVAs with day as within subjects, and ACRS group as between subjects variables were used, along with Tukey posthoc analyses, and Greenhouse-Geisser correction for sphericity as needed. Initial sign-/goal-tracking acquisition was separately analyzed with χ^2 tests comparing frequency of behaviors of each scored type emitted during initial training trials, and mixed model ANOVAs with ACRS group X trial block (average of cues 1–5 versus 21–25 on days 1, 2, or 3) factors. Late-training (average of days 7–8) PCA score, palatable familiar, novel, or habituated food intake, two-bottle sucrose or water intake and latency to drink (after ad lib food, acute hunger, or acute satiety), elevated plus maze percentage open arm time, and novelty preference percent session time on novel side were tested with independent samples *t* tests comparing ACRS to control rats. For ECB analyses, 2-AG and AEA levels were tested with two-way ANOVAs with food restriction and ACRS group as factors.

Results

Acute WIN effects Daily WIN treatment did not affect body weight during either the 14-day exposure period (no main effect of adolescent treatment) or in the 14-day washout period (Fig. 1c). On the 14th daily WIN treatment (PD 43), WIN treated rats ate more chow in the 2.5-h following injection than vehicle injected rats ($t_6 = 3.13$, $p = 0.02$, Fig. 1d).

Sign-/goal-tracking acquisition On an initial magazine training day, where food pellets were dispensed on a 30-s variable interval schedule, ACRS and control rats entered the food cup to a similar extent and left a similar number of pellets uneaten during this 20–30-min habituation session. ACRS and control rats did, however, differ in their response to the initial presentation of the novel lever/tone cue on sign-/goal-tracking training day 1, with most control rats (56%) investigating the novel (and as yet unpaired with food) lighted lever and none entering the food cup during this initial 8-s cue period. In contrast, only 44% of ACRS rats investigated the novel lever, and 32% entered the food cup during the first ever cue presentation (20% interacted with both lever and food cup; difference in likelihood of food cup entry on first ever cue $\chi^2 = 38.72$, $p < 0.001$; Fig. 2a). Accordingly, ACRS rats had more cup entries during the initial 5 cue presentations on day 1 ($t_{32} = 3.48$, $p = 0.001$), but by the last 5 cues of the day (cues 21–25), groups approached the cup similarly (interaction of group X cue block; $F_{1,32} = 4.96$, $p = 0.033$; Fig. 2b). No other differences between ACRS and controls in cue period CS+ lever interactions, control lever interactions, food cup entries, or rearing was observed over the first 3 training days, when behavior was being acquired (no group effect, or group X day interaction; Fig. 2b–d).

Established sign/goal tracking As expected, all control rats show a clear bias toward preferential conditioned approach and interaction with either the CS+ lever (i.e., sign tracking; PCA score > 0.3) or the food-delivering cup (i.e., goal tracking; PCA score ≤ 0.3; Fig. 3a–c) (Peterson et al. 1972; Robinson et al. 2014). In contrast, over a third of ACRS rats developed cue approach that was intermediate between sign and goal tracking over 8 days of training (group X day interaction; $F_{7,336} = 2.44$, $p = 0.019$) (Fig. 3a). ACRS similarly affected each of the cue bias metrics composing the PCA score, including probability bias (interaction of group X day; $F_{7,336} = 2.71$, $p = 0.01$), response bias ($F_{7,336} = 2.14$, $p = 0.039$), and trended for latency bias ($F_{7,336} = 1.91$, $p = 0.067$). In the last 2 days of training (days 7 and 8), three quarters (76%) of control rats came to predominantly sign track during CS+ periods (PCA score m(SEM) = 0.61(0.03)), and the remainder goal tracked (PCA: m = − 0.55(0.08); overall control group PCA score m(SEM) = 0.332(0.11)). In contrast, 36% of ACRS rats (compared with 0% of control rats) approached both cues indiscriminately by days 7 and 8 (PCA score ≥ 0.3 and < 0.3), and were therefore classified as "intermediate" in phenotype (intermediate group m(SEM) = − 0.07(0.06)). The remaining ACRS rats either sign tracked (44%; PCA m = 0.56(0.04)), or goal tracked (20%; PCA m = − 0.63(0.1); Fig. 3c).

ACRS did not affect food cup entry rate during non-cue periods (Fig. 2d), rearing during cues on the first 3 days of training, or on the last day (Fig. 2b), or pressing of the control inactive lever on any training day during cue periods or non-cue periods (Fig. 2b). In addition, when effects of ACRS was examined only in rats that came to preferentially sign track (control $n = 19$; ACRS $n = 11$) or goal track (control $n = 6$; ACRS $n = 5$) by late training, no effects of ACRS were present (no main effect of ACRS in sign trackers or goal trackers, or group X day interactions). Therefore, the primary effect of ACRS was to create a relatively unusual behavioral phenotype, in which some rats approached both the CS+ lever and the food cup during most cue presentations.

Sign-/goal-tracking extinction Following the 8th day of cue training, we subjected two cohorts of rats ($n = 17$/group) to a single extinction session, in which 75 unrewarded cue presentations occurred on a VI-90-s schedule. ACRS and control rats extinguished their CS+ lever interactions similarly across this session [Main effect of cue block (three 25 cue blocks): $F_{2,64} = 54.26$, $p < 0.001$, no interaction of group X cue block, Fig. 3d]. However, ACRS rats entered the cup more than

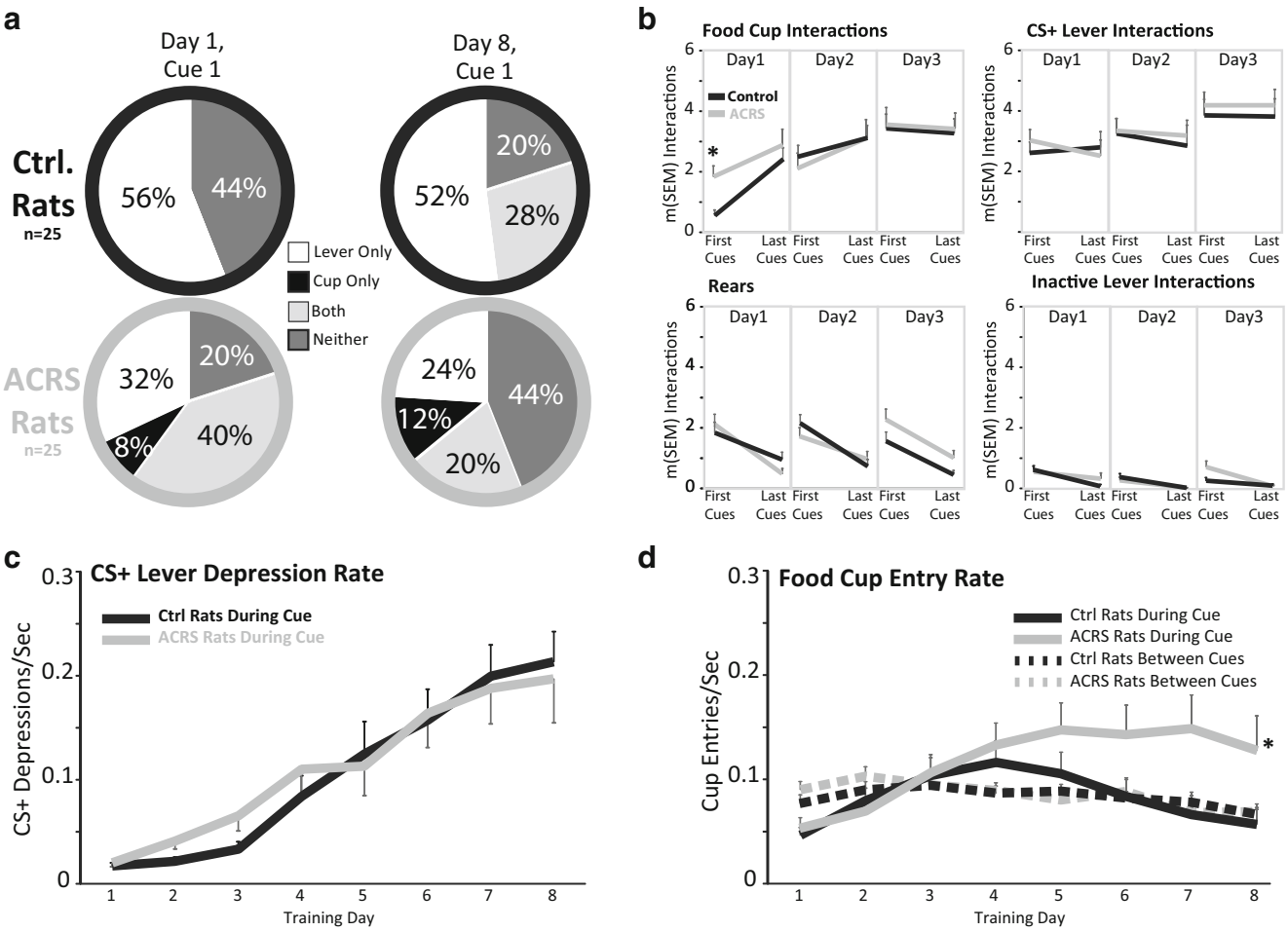

Fig. 2 Acquisition of sign and goal tracking to food-predictive cues. **a** The percentage of rats in control (top row) and ACRS (bottom row) that interacted with the lever cue only (white), cup only (black), both lever and cup (light gray), or neither stimulus (dark gray) on the first ever lever extension on sign-/goal-tracking training day 1 (left column), or the first cue presentation on the 8th training day (right column). **b** In each panel, m+SEM hand-scored interactions with the food cup (top left), cue lever (top right), or inactive lever (bottom right), or rears (bottom left) are shown during the first 5, or last 5 cue presentations on sign-/goal-tracking training days 1, 2, and 3. *$p < 0.05$, interaction of ACRS X cue block. **c** m+SEM rate of CS+ lever depressions (depressions/s) on each training day in control (black line) and ACRS rats (gray line). **d** m+SEM rate of food cup entries during cues (solid black, gray lines) or in non-cue periods (dashed black, gray lines). *$p < 0.05$, ACRS group X day interaction

controls during extinction (main effect of ACRS; $F_{1,32} = 5.77$, $p = 0.022$; group X block interaction: $F_{2,64} = 2.93$, $p = 0.061$; Fig. 3d), likely related to the more frequent cup entries made by ACRS rats on prior (rewarded) training days.

Familiar and novel palatable food intake Next, a subset of ACRS and control rats were presented with the opportunity to freely consume palatable foods for 30 min, following a 90-min habituation period on the same day, and the 120-min novel environment locomotion test in the same box on the prior day. ACRS rats ate more familiar banana-flavored palatable pellets than controls on the first test day ($t_{32} = 2.06$, $p = 0.048$, and more of a novel candy-coated chocolate reward (M&Ms) on the second day ($t_{32} = 2.49$, $p = 0.018$), but did not eat more than controls on test day 3, when again offered the now familiar chocolate reward (Fig. 4a). All rats ate significantly less of the novel chocolate reward than the familiar banana pellet reward, and this neophobia was equivalent in ACRS and control rats (main effect of group: $F_{1,32} = 6.83$, $p = 0.014$; no interaction of group X food type).

Hunger/satiety modulation of sucrose intake Next, we examined effects of prior ACRS on hunger and satiety regulation of food intake, by examining modulation of voluntary sucrose solution intake. Intake of 15% sucrose, water, or the ratio of sucrose/water was not significantly different in ACRS and control rats during the initial 6-day habituation and intake stabilization period (average daily ratio of sucrose/water: control m = 23.4(1.77), ACRS m = 26.7(2.26)). During this habituation period, latency to sample the sucrose bottle

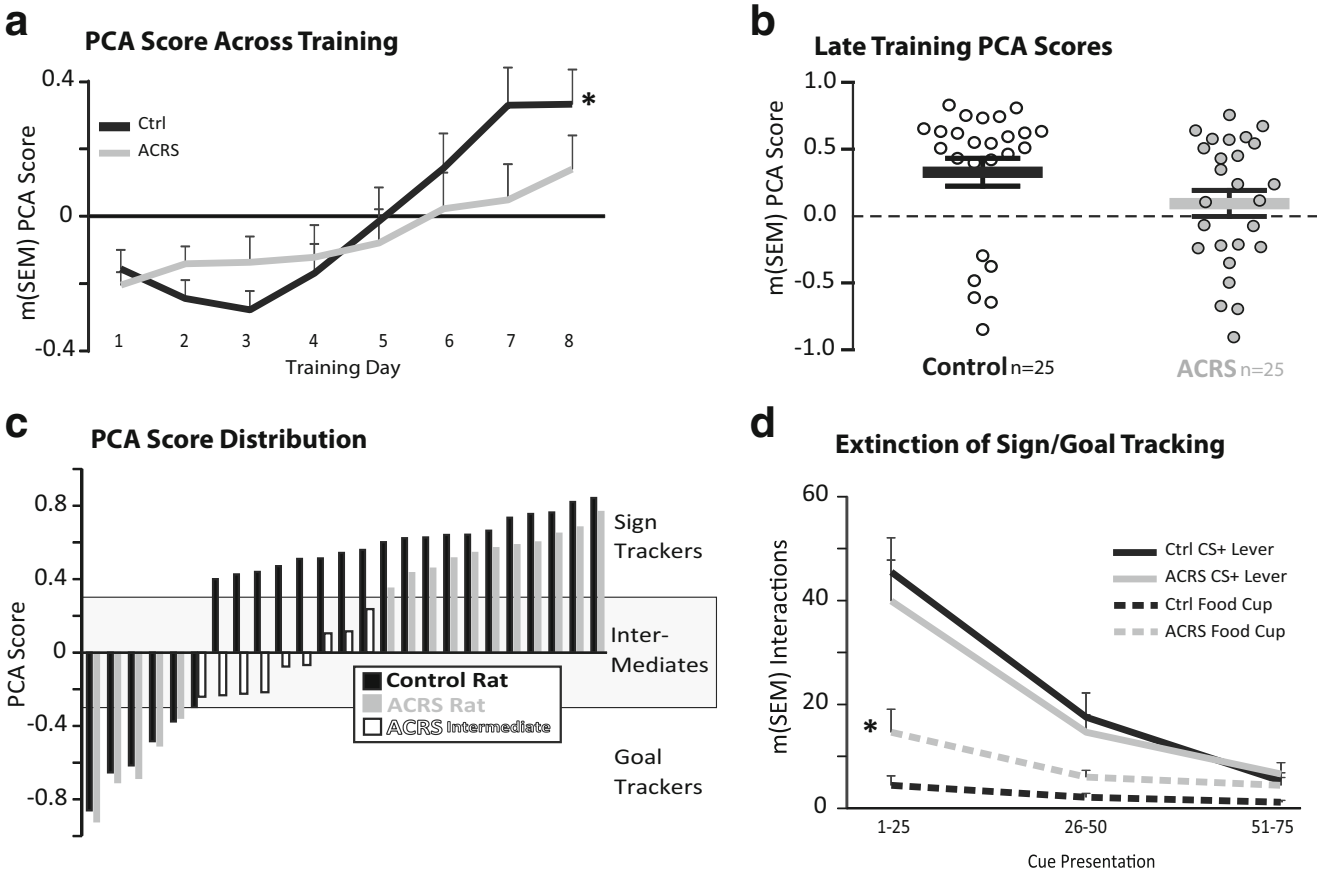

Fig. 3 Cue preference in ACRS and control rats. **a** Pavlovian conditioned approach (PCA) score across training days. Black line = control, gray line = ACRS. Positive values indicate bias toward the CS+ lever (sign tracking), negative values indicated bias toward food cup (goal tracking) *$p < 0.05$, group X day interaction. **b** Mean + SEM PCA score on training days 7 and 8 show with black (control) or gray (ACRS) horizontal lines and error bars. Individual rats' data are represented with white or gray circles. **c** Rat-by-rat PCA scores, ranked from most negative to most positive. Control rats = black bars, ACRS rats = gray bars, ACRS rats defined as intermediates (PCA < 0.3 and ≥ 0.3) = white bars). **d** CS+ lever (solid black, gray lines) and during cue food cup entries (dashed black, gray lines) emitted during the first, second, or third 25-cue blocks on sign-/goal-tracking extinction training test. *$p < 0.05$, main effect of ACRS on food cup entries

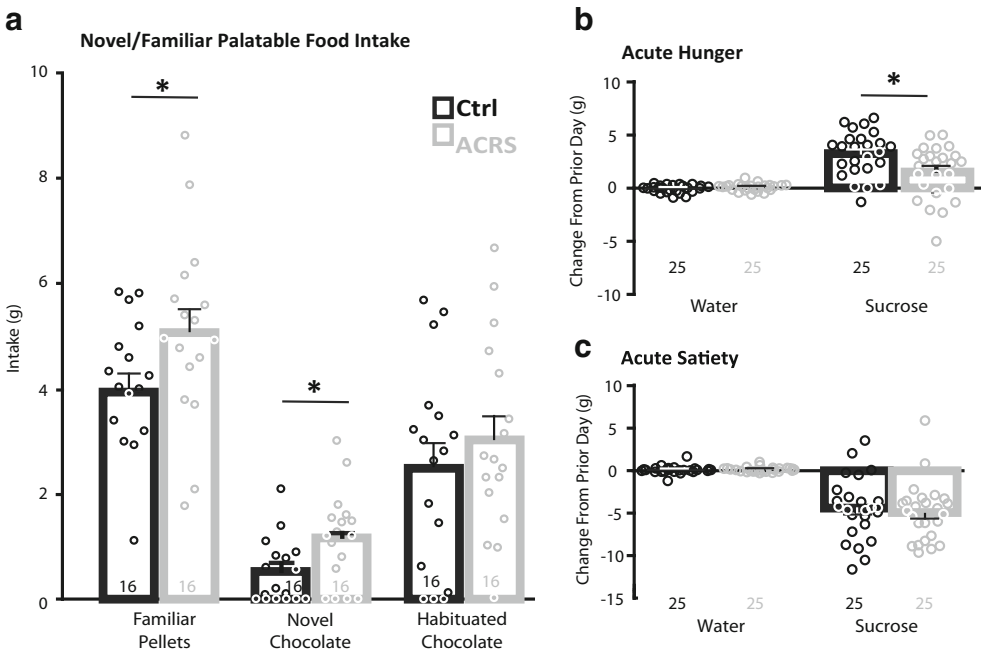

Fig. 4 ACRS effects on palatable food intake. **a** In 30-min intake tests held separate days, consumption of familiar banana-flavored pellets, a novel chocolate reward, or the same chocolate reward, now habituated, is shown for each group (black bars = control m + SEM), gray bars = ACRS m + SEM, circles represent individual control (black) or ACRS rats (gray). *$p < 0.05$, control versus ACRS. **b** Intake of water (left) or 15% sucrose solution (right) after acute food restriction in a two-bottle choice test. *$p < 0.05$, control versus ACRS. **c** Intake of water or sucrose after acute sucrose satiety. Group ns shown below bars

decreased from day 1 to day 6 of habituation ($F_{1,32} = 27.97$, $p < 0.001$), to a similar extent in ACRS and controls (no group X day interaction). Next, 1 h sucrose and water intake was measured after acute 6 h food restriction (access to water only in home cage), or acute sucrose satiety (access to 15% sucrose in homecage). Intake of water or sucrose in this 6-h pre-testing period was similar in ACRS and controls [water: control m = 6.8(1.0), ACRS m = 7.3(0.7); sucrose: control m = 45.1(3.2), ACRS m = 48.3(2.7)]. In control rats, acute food restriction resulted in increased intake of sucrose, but not water, compared to stable performance over the prior 2 days (increased sucrose intake during hunger compared to baseline: $F_{1,48} = 53.73$, $p < 0.001$; no similar effect on water; Fig. 4b). No such hunger-induced increase in sucrose intake was observed in ACRS rats (group X liquid interaction: $F_{1,48} = 8.53$, $p = 0.005$; sucrose intake: $t_{48} = 2.66$, $p = 0.011$; Fig. 4b). Latency to first drink sucrose was very short but was statistically unaffected by either acute hunger or ACRS history (no change in latency to drink sucrose from baseline to hunger day, no interaction of day X group, or main effect of ACRS). Both ACRS and control rats showed normal sucrose satiety-induced suppression of feeding (decreased sucrose intake during satiety compared to baseline: $F_{1,48} = 119.38$, $p < 0.001$, increased water intake compared to baseline: $F_{1,48} = 4.76$, $p = 0.034$; no group X liquid interaction; Fig. 4c). Acute satiety resulted in increased latency to drink sucrose, but did so similarly in both groups (no group X liquid interaction).

Locomotor response to novelty When rats were tested for locomotor activity in a novel testing environment (Bardo et al. 2013; Belin et al. 2011; Deminiere et al. 1989), ACRS and controls were nearly identical (Fig. 5a).

Novel place preference ACRS and control rats showed similar locomotor responses during habituation to the familiar, black-walled chamber. On the novelty preference test, rats were allowed to spend time in this familiar chamber, or explore the novel gray-walled center area, and white-walled chamber environments. ACRS rats spent more time in the novel, white-walled chamber (53.1(0.02)% of the test session) than controls (45.9(0.02)%; group X side interaction: $F_{1,21} = 5.07$, $p = 0.035$; percent session time on novel side: $t_{21} = 2.26$, $p = 0.034$; Fig. 5b).

Elevated plus maze No difference in anxiety, as measured in a plus maze task, tested in well-handled rats, was observed after ACRS, relative to controls (Fig. 5c).

Endocannabinoid response to food restriction ECB levels in gross forebrain dissections are dynamically altered by food restriction (Kirkham et al. 2002), and we found that ACRS decreases hunger-induced intake of sucrose solution. Therefore, we examined whether ACRS and/or acute food restriction alters levels of the ECBs AEA, 2-AG, or OEA in specific dissections of mPFC, NAc, or cerebellum. ACRS increased NAc AEA ($F_{1,15} = 7.4$, $p = 0.016$) and NAc OEA ($F_{1,15} = 5.2$, $p = 0.037$), and decreased cerebellum AEA ($F_{1,15} = 6.6$, $p = 0.021$). Food restriction increased NAc OEA ($F_{1,15} = 10.5$, $p = 0.006$), and decreased cerebellum AEA ($F_{1,15} = 14.6$, $p = 0.002$). An interaction between ACRS and restriction was seen on NAc OEA

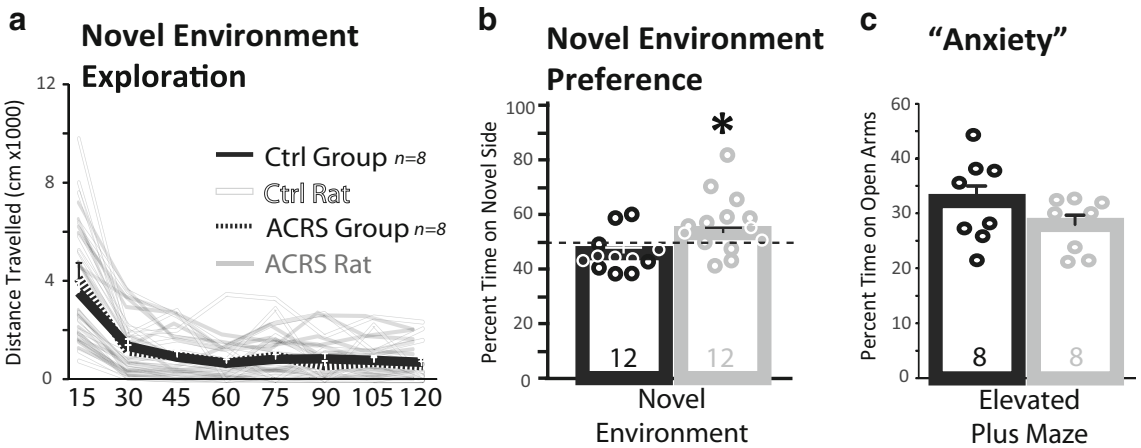

Fig. 5 ACRS effects on novelty-induced locomotion and novel environment preference. **a** m + SEM distance traveled on a 1-h novel environment locomotion test is shown in 15-min bins for control (black line; individual rats shown with faded black lines) and ACRS rats (m + SEM = dashed line, individual rats shown with faded white lines). **b** In a novel environment preference test, ACRS rats (gray bar; circles represent individual rats) spent a greater percentage of the session time on the novel side than controls (black bar; circles represent individual rats). **c** In an elevated plus maze task, percent of session time spent on the open arms of the apparatus, an index of anxiety, is shown. Group size shown in bars. *$p < 0.05$, vehicle versus ACRS. Group ns shown in bars

($F_{1,15}$ = 6.4, p = 0.024), and a trend toward interaction was seen in NAc AEA ($F_{1,15}$ = 4.3, p = 0.056). No other ACRS or restriction effects on ECB levels in the measured regions were observed (Fig. 6).

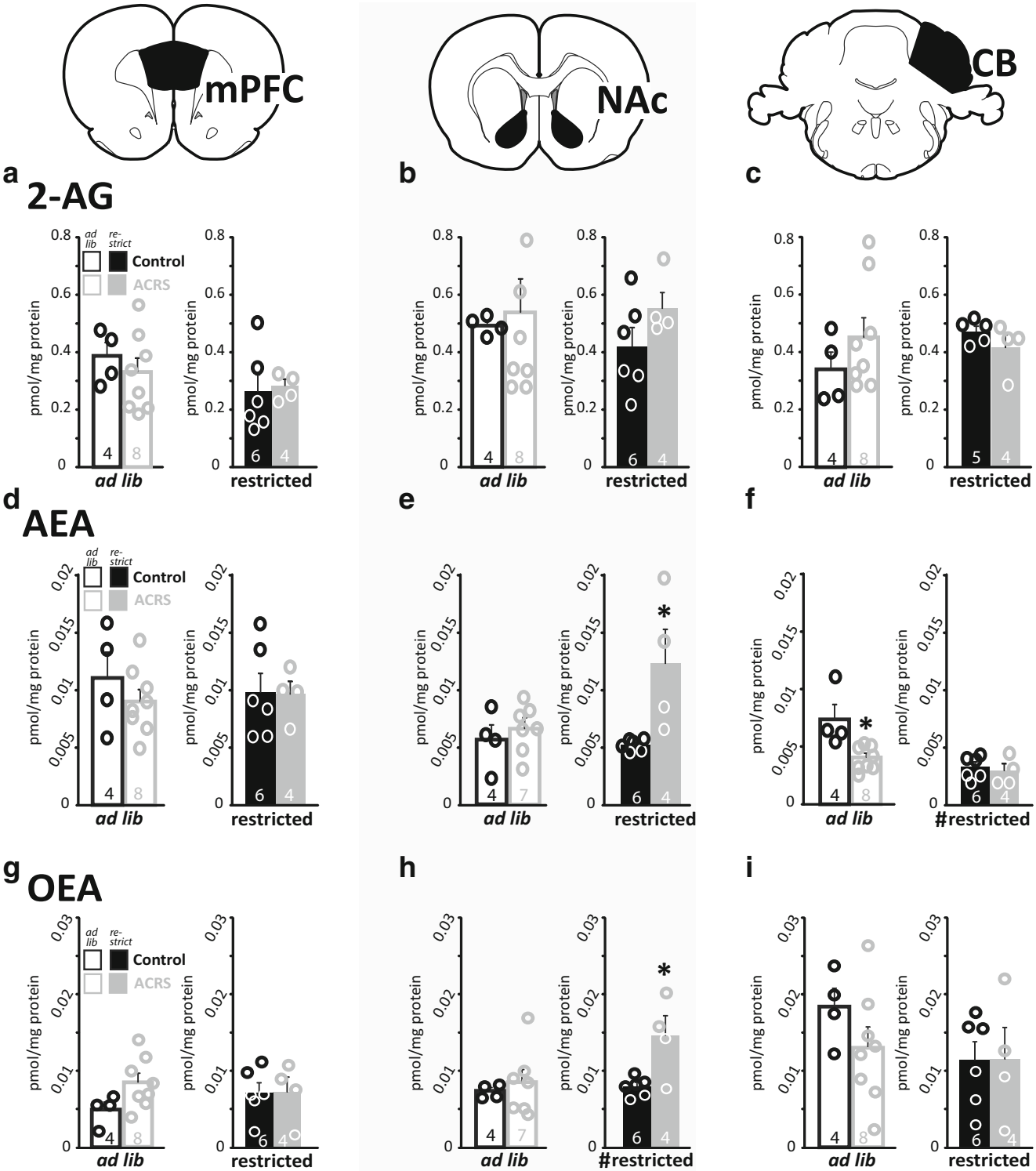

Fig. 6 ACRS and food restriction effects on brain endocannabinoid levels: m + SEM levels of **a**–**c** 2-AG, **d**–**f** AEA, or **g**–**i** OEA observed in **a**, **d**, **g** medial prefrontal cortex, **b**, **e**, **h** nucleus accumbens, or **c**, **f**, **i** cerebellum are shown in ad libitum fed rats (left panels; white bars; circles represent individual rats) or food-restricted rats (right panels; solid bars; circles represent individual rats). Control rats are represented with black borders or filled bars, ACRS rats represented with gray bordered or filled bars. ECB levels in dissected samples normalized by protein content in sample. Sample size shown in bars. *p < 0.05, main effect of ACRS/control. #p < 0.05, main effect of restriction

Discussion

We found that chronic adolescent CB1/2 receptor stimulation with WIN in male rats causes persistent changes in conditioned and unconditioned natural reward-seeking behavior. Alterations in food cue learning, increased binge-like intake of palatable food, suppression of hunger-induced sucrose intake, and increased preference for a novel environment are suggestive of a behavioral phenotype that could put individuals at risk of developing compulsive appetitive disorders like obesity or addiction later in life. These results show that ACRS with WIN causes nuanced changes in natural reward processing and contribute to the expanding list of behavioral phenotypes observed after ACRS in rodents.

ACRS with WIN robustly altered sign- and goal-tracking behavior, an assay of Pavlovian conditioned approach to reward cues that is thought to represent attribution and targeting of incentive salience (Mahler and Berridge 2009; Robinson et al. 2014). Rats spontaneously vary in their propensity to assign incentive salience to reward-predictive cues (i.e., sign track) in this task (Jenkins and Moore 1973; Saunders and Robinson 2013), and sign tracking behavior is associated with greater susceptibility to the relapse-promoting properties of response-contingent drug cues (Saunders and Robinson 2011; Saunders et al. 2013), and to phasic responses of VTA dopamine neurons to food-predictive cues (Flagel et al. 2011). In contrast, goal-tracking behavior instead predicts reactivity to multimodal contextual drug cues, and motivational gating effects of drug discriminative stimuli (Pitchers et al. 2017; Robinson et al. 2014). The primary effect of WIN ACRS here was to increase goal-tracking behavior in rats that also exhibited significant sign tracking, creating in over one third of ACRS rats (but no control rats) a relatively unusual "intermediate" phenotype, where rats approach and interact with both the reward-predicting lever cue, and the reward-delivering (but always present) food cup. This could indicate ACRS-induced changes in attention, reward expectation, or incentive salience targeting, all processes linked with individual differences in sign/goal tracking (Lovic et al. 2011; Robinson et al. 2014). Further study using more specific behavioral tasks is needed to disentangle these (non-mutually-exclusive) psychological changes due to adolescent exposure to WIN.

We also observed consistent changes in unconditioned palatable food intake in ACRS rats, relative to controls. WIN itself increased chow intake acutely in adolescents, but body weight was not affected during or after 14-day WIN treatment, demonstrating overt integrity of energy homeostasis mechanisms. In adulthood, ACRS rats consumed 130% of control levels of familiar palatable food (banana-flavored pellets used in sign-/goal-tracking tests containing sugar, fat, and protein), when tested in a habituated environment in a 30-min test. When a novel chocolate reward was introduced on the following day, both ACRS and control rats showed the expected neophobia of the new reward, decreasing their intake far below that of the familiar pellet reward offered on the prior day. However, even in the face of this neophobia, ACRS rats ate more chocolate than controls, consistent with enhancement of hedonic reward-based feeding mechanisms. Interestingly, increased intake was not observed under all conditions, since ACRS and controls ate similar amounts of chocolate upon a second exposure to this food, and drank similar amounts of 15% sucrose in daily 1 h water/sucrose preference tests, and a 6-h homecage access test. In contrast, THC and WIN in adolescence decreased homecage 1–2% sucrose preference (Bambico et al. 2010; Rubino et al. 2008), and escalating dose THC increased neophobia for a salty, fatty snack food (Realini et al. 2011), and reduced intake of chow presented to hungry rats in a stressful environment (Bambico et al. 2010), potentially indicating a difference between WIN ACRS and adolescent THC exposure, and/or methodological differences like testing environment, nutrient composition (sugar, fat, and protein), or restricted/continuous access conditions. Regardless, our data confirm that WIN ACRS effects on adulthood food intake depend on an interaction between increased palatability and other factors like novelty, expectancy, and food type (e.g., solid versus liquid, presence or absence of fat). Notably, anxiety on the elevated plus maze task was not altered in ACRS rats here, as previously reported (Bambico et al. 2010; Biscaia et al. 2003; Rubino et al. 2008).

In contrast to increased palatable food intake, hunger-induced drinking of a 15% sucrose solution was attenuated in ACRS rats. Control rats appropriately increased their intake after 6 h food restriction (6 h water pre-exposure), while ACRS rats did not. This could indicate altered hunger-induced feeding in ACRS rats, which may be mediated by changes in central and/or peripheral sensors of physiological calorie need, which are regulated importantly by ECBs (Di Marzo et al. 2009; Lau et al. 2017; Solinas et al. 2008), and are altered by ACRS (Llorente-Berzal et al. 2011). In contrast, ACRS and control rats showed similar sucrose satiety-induced suppression of intake. Therefore, ACRS-induced dysregulation of food intake and seeking is therefore far more nuanced than previously realized—with increases in palatability- and incentive motivation-related behaviors, and decreases in hunger-induced sucrose drinking.

To ask whether ECB system dysregulation within brain reward circuits might underlie altered hunger-induced food intake behavior in ACRS rats, we examined levels of AEA, OEA, and 2-AG in mPFC, NAc, and cerebellum of ACRS and control rats. Following acute 24 h food restriction or ad lib food, precise scalpel dissections of ACRS and control rat mPFC, NAc, and cerebellum were collected and analyzed via LC/MS for ECB content. We modeled our protocol based on (Kirkham et al. 2002), which showed increased AEA and 2-AG after food restriction in much less specific dissections of the limbic forebrain, a sample containing mPFC, NAc, and

several other reward circuit structures (but not cerebellum). Here, we did not find changes in 2-AG, AEA, or OEA in the mPFC, NAc, or cerebellum of acutely food-restricted control rats, relative to ad lib fed controls. In contrast, in ACRS rats, food restriction did increase NAc AEA and OEA levels in ACRS rats, relative to control-restricted rats, and to unrestricted ACRS rats. Neither AEA in mPFC and cerebellum, nor 2-AG in any measured region was affected by food restriction or ACRS. It is not clear whether hunger-associated NAc AEA/OEA recruitment in ACRS rats is related to the failure of these rats to appropriately increase sucrose intake when hungry. Since AEA in NAc has been linked specifically to assigning palatability (or "liking") to food rewards (Mahler et al. 2007; Shinohara et al. 2009), it is also possible that altered NAc AEA signaling during hunger in ACRS rats relates to the increased palatable food intake-/goal-tracking phenotype we observed in these animals.

ACRS also selectively increased preference for a novel, slightly stressful environment—a behavior linked both to novelty seeking in humans, and to propensity to develop addiction-like compulsive seeking of cocaine in rodents (Belin et al. 2011). When rats were allowed to choose to spend time in either a familiar habituated chamber or a novel one, ACRS rats spent more time exploring the novel environment. Such propensity to explore a novel environment predicts the subsequent transition of cocaine self-administration from goal-directed (recreational drug taking) to compulsive and punishment-resistant (addiction-like seeking) (Belin and Deroche-Gamonet 2012). Another commonly tested measure of rat novelty response is locomotion in a novel environment. Rats showing the most locomotor activity in this test (high responders) acquire cocaine self-administration faster than low responders (Cain et al. 2005; Wingo et al. 2016), but ACRS and control rats here did not differ on this measure. Since novelty-induced locomotion is uncorrelated with novel environment preference when tested in the same animals (Belin et al. 2011; Cain et al. 2005), we propose that the specific increase in preference for novelty after ACRS reveals a specific enhancement of novelty-seeking-like behavior (Belin and Deroche-Gamonet 2012), a trait related to addiction vulnerability in humans.

This report has several limitations that require additional study to address. First, only one dosage of WIN was tested here, chosen based on prior reports of persistent cognitive and emotional effects after adolescent administration (Abboussi et al. 2014; Bambico et al. 2010; Schneider 2008). Persistent effects of adolescent exposure to cannabinoid drugs are known to be dependent upon dose, sex, and cannabinoid agonist used [importantly, agonists vary in their affinity for cannabinoid CB1 and CB2 receptors, and non-cannabinoid receptors (De Petrocellis et al. 2012; Lowin et al. 2016)]. Therefore, interactions between these factors and persistent effects of ACRS on reward seeking are likely (Rubino and Parolaro 2015; Schneider et al. 2008). In addition, it is possible that WIN ACRS effects may in part involve acute behavioral effects of adolescent cannabinoid agonists on sociality, memory, food intake, or other behavioral factors (Blanco-Gandia et al. 2015; Renard et al. 2016a; Rubino and Parolaro 2008; Trezza et al. 2014)—calling attention to the need for further characterization of such effects in ACRS studies.

In sum, these results indicate that chronic ACRS with the cannabinoid receptor direct agonist WIN yields robust alterations in natural reward-seeking behaviors in rats, including increased palatable food intake, altered targeting of incentive salience to food cues, and decreased hunger-induced sucrose intake accompanied by upregulation of AEA and OEA in NAc. These findings suggest that adolescent exposure to moderate-dose WIN causes a behavioral phenotype that could put ACRS individuals at risk of later-life appetitive disorders including obesity, eating disorders, or addiction, and may have implications for understanding roles of cannabinoid receptor signaling in adolescent development of mesocorticolimbic reward circuits.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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