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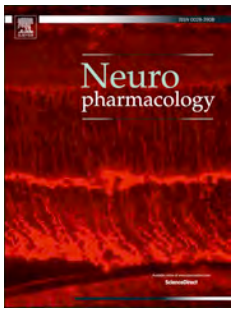

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TITLE PAGE

Sensitivity to cocaine in adult mice is due to interplay between genetic makeup, early environment and later experience

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Abstract

Although early aversive postnatal events are known to increase the risk to develop psychiatric disorders later in life, rarely they determine alone the nature and outcome of the psychopathology, indicating that interaction with genetic factors is crucial for expression of psychopathologies in adulthood. Moreover, it has been suggested that early life experiences could have negative consequences or confer adaptive value in different individuals. Here we suggest that resilience or vulnerability to adult cocaine sensitivity depends on a "triple interaction" between genetic makeup x early environment x later experience. We have recently showed that Repeated Cross Fostering (RCF; RCF pups were fostered by four adoptive mothers from postnatal day 1 to postnatal day 4. Pups were left with the last adoptive mother until weaning) experienced by pups affected the response to a negative experience in adulthood in opposite direction in two genotypes leading DBA2/J, but not C57BL/6J mice, toward an "anhedonia-like" phenotype.

Here we investigate whether exposure to a rewarding stimulus, instead of a negative one, in adulthood induces an opposite behavioral outcome. To test this hypothesis, we investigated the long-lasting effects of RCF on cocaine sensitivity in C57 and DBA female mice by evaluating conditioned place preference induced by different cocaine doses and catecholamine prefrontal-accumbal response to cocaine using a "dual probe" *in vivo* microdialysis procedure. Moreover, cocaine-induced c-Fos activity was assessed in different brain regions involved in processing of rewarding stimuli. Finally, cocaine-induced spine changes were evaluated in the prefrontal-accumbal system. RCF experience strongly affected the behavioral, neurochemical and morphological responses to cocaine in adulthood in opposite direction in the two genotypes increasing and reducing, respectively, the sensitivity to cocaine in C57 and DBA mice.

Keywords: unstable maternal environment, animal models, gene x environment interplay, resilience, vulnerability, cocaine.

Introduction

Recent evidence suggests an association between exposure to aversive events during the early postnatal life and increased vulnerability to a variety of neuropsychiatric and psychosocial disorders later in life (Heim and Nemeroff, 2001; Matthews and Robbins 2003; McEwen 2000; McLaughlin et al. 2015; Meaney, 2001; Nemeroff, 2004; Sánchez, et al., 2001; van der Veen, et al., 2008). Although the effects of early experiences may be strong and pervasive, large individual differences exist in their impact on health and behavior (Bakermans-Kranenburg and van Ijzendoorn, 2007; Belsky and Pluess, 2009; Belsky et al., 2015), suggesting that genetic background can modulate the capacity of an environmental risk factor to give rise to mental illness (Caspi and Moffitt, 2006). Finally, even if early aversive experiences are generally believed to confer a great risk for psychopathologies in adult life, different current points of view suggest that the same early events might determine either susceptibility or resilience to mental illness (Belsky and Pluess, 2009; Belsky et al., 2013; Der-Avakian and Markou, 2010; Maccari et al., 2014; Meaney, 2010; Parker and Maestripieri 2001; Pryce and Feldon, 2003; Rana et al., 2015; van der Veen et al., 2008), probably depending on the genetic makeup (Bakermans-Kranenburg and van Ijzendoorn 2007; Belsky et al., 2013; Di Segni et al., 2016).

Based on the "three-hit concept" of resilience and vulnerability (Daskalakis et al., 2013) as well as on the "plasticity genes" hypotheses (Belsky and Pluess, 2009), here we suggest that genetic background-early environment interplay increases vulnerability to development/expression of certain psychopathologies and resilience to others, also depending on the successive experiences in adult life.

We have recently reported that Repeated Cross Fostering (RCF) experienced by pups, affected adult behavioral and neurochemical responses to aversive events in opposite direction in two genotypes leading DBA2/J (DBA**)** mice toward an "anhedonia-like" phenotype and C57BL/6J **(**C57**)** mice toward an increased sensitivity for a natural reinforcing stimulus (Di Segni et al., 2016). In RCF condition, pups from the same litter spend the first postnatal day (PND0) with their biological

mother. On PND1, experimental litter are fostered by replacing the mother with a novel lactating female caring for pups of the same age; this procedure is repeated daily (4 times, from PND1 up to PND4) until the fourth adoptive mother was reached. Pups are left with the last adoptive mother until weaning (D'Amato et al., 2011; Ventura et al., 2013; Di Segni et al., 2016).

Instability of the early environment, modeled by the RCF procedure, is thus hypothesized also to affect the response to a rewarding stimulus (cocaine) in opposite way, depending on the genetic makeup. In particular we anticipate an increased and reduced, respectively, response to cocaine in RCF C57 and RCF DBA female mice compared to their Control groups.

Females were chosen as subjects for this study because of the significant incidence of gender differences in stress-related disorders and their greater stress sensitivity (Bale, 2006; Kendleret al., 1992; Nestler et al., 2002) as well as on the basis of previous data reported by our laboratory (Di Segni et al., 2016). Moreover, clinical research indicates that women show higher susceptibility to cocaine abuse (Kosten et al., 1996; Scott et al., 2006, Cotto et al., 2010) and relapse (Becker and Hu, 2008) in comparison to men. In fact, despite severity of childhood emotional abuse has been correlated with increased risk for substance abuse in both sexes (Hyman et al. 2006), in woman emotional abuse/neglect and maltreatment have been associated with a heightened severity of substance abuse and increased risk of relapse (Hyman et al. 2006). Consisting with these reports, preclinical studies suggest that female rodents are characterized by enhanced susceptibility to develop addiction-related pathological phenotypes (Hu et al., 2004; Lynch, 2006; Van Swearingen et al., 2013) and this outcome is increased by exposure to critical early life experiences (Kosten et al., 2004; 2006).

To test our hypothesis, three experiments were carried out in RCF and Control (Cont) female mice of the 2 strains. In the first experiment, we tested whether RCF experience affected the behavioral response to cocaine in adult mice in a genotype-dependent manner using cocaine-induced conditioned place preference (CPP), a drug seeking correlated behavioral test (Kiraly et al., 2010). Clinical and pre-clinical studies have demonstrated the impact of early postnatal experiences on

prefrontal-accumbal catecholamine circuit (Barros et al., 2004; Huppertz-Kessler et al., 2012; Jahng et al., 2010; Oswald et al., 2014; Peña et al., 2014; Rincón-Cortés and Sullivan, 2016; Ventura et al., 2013; Yang et al., 2014), a brain network known to be involved in processing of rewarding stimuli (Cabib and Puglisi-Allegra, 2012; Di Segni et al., 2016, Pascucci et al., 2007; Puglisi-Allegra and Ventura 2012; Siciliano et al., 2015; Ventura et al., 2007; 2008; 2013). Based on this evidence, in the second experiment, we evaluated the enduring effects of RCF on catecholamine prefrontal-accumbal response to cocaine injection in adult mice using a "dual probes" *in vivo* intracerebral microdialysis procedure (Di Segni et al., 2016). Moreover, c-Fos activity was assessed in different brain areas involved in processing of rewarding stimuli and, based on literature pointing to the role that dendritic spine alterations may play in drug-related behaviors (Kauer and Malenka, 2007; Kiraly et al., 2010; Pulipparacharuvil et al., 2008; Robinson and Kolb, 2004; Villalba and Smith, 2013), in the last experiment we investigated the effects of repeated cocaine injection on spine density on Medium-Sized Spiny Neurons (MSNs) in the NAc as well as on pyramidal neurons in mpFC, two brain areas strongly involved in the effects of drugs of abuse in both humans and rodents.

Materials and Methods

Animals

C57BL/6J and DBA2/J female mice (purchased when 6/7 weeks old from Charles River, Italy) were mated when 12 weeks old.

Mating protocol was as previously described (Di Segni et al., 2016). Each experimental group was composed by 6-8 animals. Adequate measures were taken to minimize pain or discomfort of mice. All experiments were carried out in accordance with Italian national law (DL 116/92 and DL 26/2014) on the use of animals for research based on the European Communities Council Directives (86/609/EEC and 2010/63/UE).

Repeated Cross-Fostering

As previously described (D'Amato et al., 2011; Di Segni et al., 2016; Ventura et al., 2013), pups from the same litter spent the first postnatal day (PND0) with their biological mother. On PND1, litters were randomly selected and assigned to experimental (RCF) or Control (Cont) treatment. RCF pups were fostered by introducing the entire litter into the home cage of a different dam whose pups had just been removed and moved to a different adoptive mother. This procedure was repeated daily (4 times, from PND1 up to PND4) until the fourth adoptive mother was reached. Pups were left with the last adoptive mother until weaning. Control litters were only picked up daily and reintroduced in their home cage, for the same period, and had their mothers returned within 30 sec. Animals were weaned at PND28, separated by sex and housed in groups of 4 littermates. All of the experiments were performed on 3-month-old adult female mice. To prevent potential estrous cycle group synchronization, experimental subjects for cross-fostered and control female groups were sorted by collecting not >2 individuals per cage/litter (Ventura et al., 2013).

Conditioned Place Preference

Conditioned place preference experiments were performed using a place conditioning apparatus as previously reported (Ventura et al., 2007; 2013). Briefly, on day 1 (pretest), mice were free to explore the entire apparatus for 20 min. During the following 8 days (conditioning phase), mice were confined daily for 40 min alternately in 1 of the 2 chambers. One of the patterns was consistently paired with drug (P; cocaine-Paired chamber (C57: 2.5, 5, 7.5, mg/kg i.p.; DBA: 5, 7.5, 10 mg/kg i.p.)) and the other one with vehicle (U; Unpaired chamber **(**saline 0.9%)). Different doses of cocaine were choose on the basis of a previous work indicating that C57 and DBA mice differ for the sensitivity to cocaine-induced CPP, and suggesting a different sensitivity to the positive incentive properties of drugs of abuse (Kutlu et al. 2015; Orsini et al., 2005). Testing for the

expression of CPP was conducted on day 10 using the pretest procedure. Behavioral data were collected and analyzed by the "EthoVision" (Noldus, The Netherlands) fully automated video tracking system (Spink et al., 2001). The digital data were analyzed by means of the EthoVision software to obtain "time spent" (seconds; sec.), which was used as raw data for preference scores in each sector of the apparatus of each subject. Each experimental group was composed by 6-8 animals.

In Vivo Microdialysis

"Dual probe" microdialysis experiments in mpFC and NAc were carried out as previously described (Di Segni et al., 2016). Animals were anesthetized with Zoletil and Rompun, mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) equipped with a mouse adapter. Zoletil 100 (Virbac, Milano, Italy (tiletamine HCl 50 mg/ml+zolazepam HCl 50 mg/ml)) and Rompun 20 (Bayer S.p.A Milano, Italy (xylazine 20 mg/ml)), purchased commercially, were dissolved in a volume of 4.1mg/ml and 1.6mg/ml respectively, in saline and injected in a volume of 7.3 ml/kg.

Bilateral (mpFC and NAc) probe implantation was counterbalanced in either the right or left hemisphere. Vertical concentric dialysis probes were prepared with AN69 fibers (Hospal Dasco, Bologna, Italy), according to the method of Di Chiara et al. (Di Chiara et al., 1996). Different coordinates for probe implantation were used for the 2 strains. The coordinates from bregma (measured according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) and Mouse Brain Atlases, The Mouse Brain Library, www.nervenet.org) were: mpFC: 2.5 Antero-Posterior (AP), ±0.6 Medio-Lateral (ML) (C57), and 2.0 AP, ±0.6 L (DBA); NAc: +1.6 AP, ±0.6 L (C57) and +1.1 AP; ±0.6 L (DBA). Different probe length was used for C57 and DBA NAc (mpFC: 3 mm shaft length, dialysis membrane length 2 mm o.d. 0.24 mm; NAc: 4.5 mm (C57) or 4.0 mm (DBA) shaft length, dialysis membrane length 1 mm o. d. 0.24 mm). The final depth of the probe was 3 mm for mpFC and 5.5 or 5.0 mm for C57 and DBA NAc, respectively, ventral to the skull surface.

The day before use, the membranes were tested to verify in vitro norepinephrine and/or dopamine recovery. The microdialysis system was as previous reported (Di Segni et al., 2016). Experiments were carried out 48 h after probe placement. The mean concentration of the 3 samples collected immediately before treatment (<10% variation) was taken as baseline concentration. Following, Cont and RCF C57 and DBA mice were injected (i.p.) with cocaine (C57: 2.5 mg/Kg; DBA: 5 mg/Kg). 2.5 and 5 mg/Kg cocaine doses were choose, respectively, in C57 and in DBA mice based on CPP experiments showing that these doses only induced different effects (discriminative dose) in RCF vs. Control groups of two strains. Dialysate was collected every 20 min for 120 min. Twenty microliters of the dialysate sample was analyzed by performance liquid chromatography, as previously reported (Di Segni et al., 2016; Latagliata et al., 2014; Ventura et al., 2013). Concentrations (pg/20 μL) were not corrected for probe recovery. Each experimental group was composed by 6-8 animals.

Immunohistochemistry

At the end of the microdialysis experiments, mice were sacrificed and c-Fos expression was evaluated in NAc core (NAcC) and shell (NAcS), caudate-putamen (CP), infralimbic cortex (ILC), prelimbic cortex (PRLC), Hippocampus (CA3, CA1, dentate gyrus (DG)), Amygdala (Lateral Amygdala (LA), Basolateral Amygdala (BLA), Central Amigdala (CEA)) in the contralateral areas of probes implantation. Brains were fixed overnight in paraformaldehyde (4%), cryoprotected in sucrose (30%), frozen with dry ice, and cut in transversal sections. For c-Fos immunohistochemistry, brain sections (40 μm) were collected approximately from 1.98 to -3.40 for C57 and from 2.40 to -2.80 for DBA from bregma, according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) and Mouse Brain Atlases (The Mouse Brain Library; www.nervenet.org/mbl/). Immunohistochemistry was performed as previously described (Conversi et al., 2004; Di Segni et al., 2016). Each experimental group was composed by 6-8 animals.

Morphologic analysis

Based on a previous work (Kiraly et al., 2010), mice from the different groups were given 4 daily injections of cocaine or saline (C57 Cont cocaine/saline, C57 RCF cocaine/saline, DBA Cont cocaine/saline, DBA RCF cocaine/saline). Different doses of cocaine (C57 2.5mg/kg; DBA 5mg/kg) were used based on the behavioral (CPP) and microdialysis experiments. Each experimental group was composed by 4-6 animals.

Thirty minutes after the last injection, mice were perfused and collected brains were impregnated with a standard Golgi-Cox solution as previously described (Andolina et al., 2011). Coronal sections of 120 µm were obtained using a vibratome, mounted on gelatinized slides, stained according to the Gibb and Kolb method (Gibb and Kolb, 1998) and covered with Eukitt (Kindler GmbH & Co., Germany). Measurements were performed on impregnated neurons identified under low magnification (20X/0.4 numerical aperture). MpFC and NAc analysis was restricted, respectively, to pyramidal-like (C57 from 1.98 to 1.58; DBA from 1.40 to 1.70) and medium-spiny neurons (MSNs) (C57 from 1.94 to 0.74; DBA from 1.70 to 0.74 from bregma, measured according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 1997) and Mouse Brain Atlases (The Mouse Brain Library; www.nervenet.org/mbl/)). Golgi-impregnated neurons were selected according to criteria proposed by Vyas (Vyas et al., 2002) as reported in Figure 1. An average of 3/4 neurons for each mouse were analyzed and randomly selected from both hemispheres. An experimenter blind to the experimental groups performed the morphological analyses. The analysis of total spine density, mature and immature spine density from apical and basal dendrite of pyramidal neurons from mpFC as well as total spine density, mature and immature spine density from accumbal MSNs was performed by 3D reconstruction of the selected neurons using the NeuroLucida image analysis system (mbf, Bioscience) connected to an Olympus BX53 microscope (100X/1.25 numerical aperture). By using the same NeuroLucida system (100X, 1.25 numerical aperture, Olympus BX53), all protrusions, respective of their morphological characteristics, were counted on each-order branches as spines if they were in direct continuity with the dendritic shaft.

On each dendrite, all protrusions with a clearly recognizable neck were considered as spines and were classified according to the categories proposed by Peters & Kaiserman-Abramof (1969) as stubby, mushroom and thin types. Stubby spines protrude from spiny dendrites with no neck visible, they have a length similar to the diameter of the neck and to the head width; mushroom spines have a neck diameter much smaller than the diameter of the head, head width >2 neck width; thin spines have a head width <2 neck width. Spine types were grouped as mature (stubby and mushroom) and immature (thin) (Figure 1).

Statistics

Statistical analysis for place conditioning experiments was performed on preference scores assessed by calculating the time spent in cocaine (Paired; P) and saline (Unpaired; U) compartments on the test day minus the time spent in the same compartments on the pretest session (Ventura et al., 2007; 2013). Data were analyzed, separately for each strain (C57, DBA) and dose (C57: 2.5, 5, 7.5 mg/kg; DBA: 5, 7.5, 10 mg/kg) by repeated-measures ANOVA with 1 between factor (early experience, 2 levels: Control, RCF) and 1 within factor (pairing, 2 levels: Paired, Unpaired). In order to evaluate the difference in the time spent in paired and unpaired chambers, repeated-measures ANOVA (pairing, 2 levels: Paired, Unpaired) was carried out separately for each experimental group.
C-Fos immunoreactivity in selected brain regions (NAcC, NAcS, CP, ILC, PRLC, Hip, Amy) was analyzed by Student's t-test within each strain.
In vivo microdialysis data were analyzed by repeated-measures ANOVAs with one between factor (early experience, 2 levels: Control, RCF) and one within factor (time, 7 levels: 0, 20, 40, 60, 80, 100, 120) within each strain for each brain area (mpFC, NAc). Statistical analyses were performed on raw data (concentration of pg/20 μL). Simple effects were assessed by one-way ANOVA for each time point. Baseline extracellular levels in mpFC (NE, DA) and NAc (DA) of Control and RCF animals were compared by Student's t-test within each strain.

Concerning morphological data, two-way ANOVA (early experience, 2 levels: Control, RCF; treatment, 2 levels: Saline, Cocaine) was conducted separately for each strain. Three parameters were analyzed for both mpFC and NAc: total spine density, immature spine density; mature spine density. For all experiments, individual between-group comparisons were performed when appropriate by post hoc test (Duncan's multiple range test).

Results

Conditioned Place Preference

The effects of RCF experience on CPP are shown in Figure 2 (A,B).

For C57 mice, repeated-measures ANOVA revealed that RCF animals showed a preference for the cocaine-paired compartment at all the doses: 2.5 mg/kg ($F(1,14) = 15.76$, $P < 0.001$), 5 mg/kg ($F(1,14)= 11.53$, $P < 0.005$) and 7.5 mg/kg ($F(1,14) = 9.37$, $P < 0.005$), while Control animals showed a significant preference for the cocaine-paired compartment only at the high doses, 5 mg/kg ($F(1,12) = 9.18$, $P < 0.005$) and 7.5 mg/kg ($F(1,14) = 25.26$, $P < 0.001$), but not at the lowest one (2.5 mg/kg ($F(1,12) = 3.02$, n.s.) (Figure 2A).

Concerning DBA mice, repeated-measures ANOVA revealed that RCF animals showed a significant preference for the cocaine-paired compartment only at the high doses, 7.5 mg/kg ($F(1,14) = 10.36$, $P < 0.001$) and 10 mg/kg ($F(1,14) = 16.44$, $P < 0.001$), but not at the lowest dose, 5 mg/kg ($F(1,12) =.752$, n.s.), while Control animals showed a significant preference for the cocaine-paired compartment at all the doses: 5 mg/kg ($F(1,12)= 11.34$, $P < 0.05$), 7.5 mg/kg ($F(1,14) = 17.35$, $P < 0.001$) and 10 mg/kg ($F(1,12) = 10.95$, $P < 0.05$) (Figure 2B).

These data strongly indicate that unstable maternal environment has opposite effects on adult C57 and DBA mice increasing and reducing, respectively, the sensitivity to cocaine.

Microdialysis

Medial Prefrontal Cortex

Data analysis for the effects of cocaine on prefrontal NE and DA outflow in C57 mice showed a significant early experience x time interaction for NE ($F(1,84) = 3.01$, $P < 0.05$) and a significant time effect for DA ($F(6,72) = 16.42$, $P < 0.005$).

Simple effect analyses revealed a significant time effect for DA and NE outflow in both (Cont, RCF) groups. Cont animals showed a time-dependent increase in NE and DA outflow reaching a maximal increase of □□□□□□and of □110□□at 20 min respectively. RCF animals showed a time-dependent increase in NE and DA outflow reaching a maximal increase of □□□□□□and of □150□□at 20 min respectively. Moreover, cocaine produced a higher NE increase in the RCF group compared to the Control group (Figure 3A); no significant difference in DA outflow between Cont and RCF groups was evident (Figure 3B).

Concerning DBA mice, data analysis for the cocaine effects showed a significant early experience x time interaction for both NE ($F(1,66)=2.36$, $P<0.05$) and DA ($F(1,78)=2.26$, $P<0.05$). Simple effect analyses revealed a significant time effect for NE and DA outflow in Cont animals and a significant time effect for NE outflow in RCF animals (Figure 3 D,E). Cont animals showed a time-dependent increase in NE and DA outflow reaching a maximal increase of □□□□□□and of □75□□at 20 and at 40 min respectively. RCF animals showed a time-dependent increase in NE outflow reaching a maximal increase of □70□ at 20 min. No significant increase in DA outflow was evident. A significant difference in NE and DA outflow was evident between Cont and RCF animals injected with cocaine (Figure 3 D,E).

Finally, analyses of baseline levels of catecholamine did not reveal a significant difference between Cont and RCF animals within each strain (NE: C57 Cont mean=.754; SE=.112; C57 RCF mean=.561; SE=.046) ($t_{(14)}$=-1.595, n.s.); DBA Cont mean=.637; SE=.182; DBA RCF mean=.512; SE=.118) ($t_{(11)}$=-.557, n.s.) and DA: C57 Cont mean=.538; SE=.073; C57 RCF mean=.480; SE=.124) ($t_{(12)}$=-.390, n.s.); DBA Cont mean=.479; SE=.071; DBA RCF mean=.314; SE=.055) ($t_{(13)}$=-1.797, n.s.).

Nucleus Accumbens

Data analysis for the effects of cocaine on accumbal DA outflow showed a significant time effect for both strains (C57: $F(1,6)=12.79$, $P<0.005$; DBA: $F(1,6)=2.86$, $P<0.05$).

Concerning C57 mice, simple effects analysis revealed a significant time effect for DA outflow in both (Cont, RCF) groups. Cont animals showed a time-dependent increase in DA outflow reaching a maximal increase of □100% at 20 min; RCF animals showed a time-dependent increase in DA outflow reaching a maximal increase of □180% at 20 min. Moreover, a significant difference in DA outflow between Cont and RCF mice injected with cocaine was observed (Figure 3C); cocaine produced a longer and sustained increase in the RCF group compared to the Cont group.

Concerning DBA mice, simple effects analysis revealed a significant time effect for DA outflow in both (Cont, RCF) groups. Both groups (Cont, RCF) showed a time-dependent increase in DA outflow reaching a maximal increase of □95% at 20 min (Figure 3F). However, no significant difference in DA outflow between Cont and RCF mice injected with cocaine was observed (Figure 3F).

Finally, analyses of baseline levels of dopamine did not reveal a significant difference between Cont and RCF animals within each strain (C57 Cont mean=.323; SE=.026; C57 RCF mean=.314; SE=.053) ($t_{(10)}$=-.179, n.s.); DBA Cont mean=.689; SE=.161; DBA RCF mean=,478; SE=.128) ($t_{(13)}$=-1.002, n.s.).

c-Fos Immunoreactivity

The effects of the early experience on c-Fos positive nuclei are schematically illustrated in Table 1.

Concerning C57 mice, t-test analysis revealed a significant increase of c-Fos in PRLC ($t_{(45)}$=-2.453, $P<0.05$), NAcC ($t_{(48)}$=-3.246, $P<0.01$), NAcS ($t_{(52)}$=-3.051, $P<0.05$), LA ($t_{(55)}$=-2.303, $P<0.05$) and BLA ($t_{(58)}$=-3.847, $P<0.005$), a significant reduced activity in CA1 ($t_{(60)}$=2.530, $P<0.05$) and DG ($t_{(56)}$=3.779, $P<0.001$) of RCF animals in comparison with Control animals. No significant

difference between RCF and Cont was evident in CP ($t_{(63)}$=-0.546, n.s.), CA3 ($t_{(51)}$=1.707, n.s.) and CEA ($t_{(58)}$= -0.537, n.s.) and ILC ($t_{(43)}$=0.270, n.s.).

Concerning DBA mice, t-test analysis revealed a significant reduced activity of c-Fos in ILC ($t_{(25)}$=3.432, P<0.01), PRLC ($t_{(23)}$=3.317, P<0.01), NAcC ($t_{(71)}$=2.179, P<0.05), NAcS ($t_{(21)}$=2.919, P<0.01), CP ($t_{(69)}$=2.152, P<0.05) DG ($t_{(62)}$=2.116, P<0.05), LA ($t_{(55)}$=3.522, P<0.01), BLA ($t_{(64)}$=3.145, P<0.01) and CEA ($t_{(62)}$=3.387, P<0.01) of RCF group in comparison with Control animals. No significant difference was evident in CA1 ($t_{(66)}$=-0.088, n.s.) and in CA3 ($t_{(67)}$=1.556, n.s.) between RCF and Cont groups.

Morphological analysis

Medial Prefrontal Cortex

The effects of cocaine on spine density and morphology of C57 and DBA mice are shown in Figure 4. Concerning C57 mice, two-way ANOVA for apical dendrites of prefrontal pyramidal neurons reveals a significant treatment x early experiences interaction for mature spine density (F(1,55) = 4.21, P<0.05) (Figure 4C) but not for total spine density (F(1,55) =.32, n.s.) (Figure 4A) and immature spine density (F(1,55) =.48, n.s.) (Figure 4B). Analysis conducted within each group (Cont, RCF) shows that cocaine increased mature spine density only in RCF mice (P< 0.05) (Figure 4C).

Two-way ANOVA for basal dendrites of prefrontal pyramidal neurons reveals a significant treatment x early experiences interaction for total spine density (F(1,57) = 6.12, P< 0.05), immature spine density (F(1,57) = 6.05, P< 0.05) and mature spine density (F(1,57) = 4.04, P< 0.05). Comparison within Cont and RCF C57 groups showed that cocaine produced a significant increase, compared to the respective saline-treated groups, in total spine density (P< 0.001) (Figure 4D), immature spine density (P< 0.001) (Figure 4E), and mature spine density (P< 0.05) (Figure 4F), in RCF C57 mice but not in Cont C57.

Concerning DBA mice, two-way ANOVA for apical dendrites of prefrontal pyramidal neurons of

mpFC reveals a significant treatment x early experiences interaction for total spine density ($F(1,67) = 7.72$, $P < 0.05$), immature spines ($F(1,67) = 7.53$, $P < 0.05$) and mature spines ($F(1,67) = 4.76$, $P < 0.05$).

Analysis conducted within each group (Cont, RCF) showed that cocaine induced an increase in total spine density ($P < 0.05$) (Figure 4G), immature spine density ($P < 0.05$) (Figure 4H) and mature spine density ($P < 0.05$) (Figure 4I) in Cont DBA but not in RCF DBA mice, in comparison with the respective saline-treated groups.

Two-way ANOVA for basal dendrites of prefrontal pyramidal neurons reveals a significant treatment x early experiences interaction for total spine density (Figure 4L) and immature spine (Figure 4M) (respectively: $F(1,67) = 4.09$, $P < 0.05$; $F(1,67) = 3.99$, $P < 0.05$) but not for mature spines ($F(1,67) = 2.10$, n.s.) (Figure 4N). Analysis conducted within each group (Cont, RCF) showed that cocaine produced in RCF DBA a reduction, in comparison with the saline-treated group, in total spine density ($P < 0.05$) (Figure 4L) and no significant effect in Control DBA, compared to the saline-treated group, in total spine density (Figure 4L) and immature spine density (Figure 4M).

Nucleus Accumbens

The effects of cocaine on morphological changes in the NAc of C57 and DBA mice are shown in Figure 5.

Concerning C57 mice, two-way ANOVA analysis conducted for MSNs in the NAc reveals a significant treatment x early experiences interaction for total spine density ($F(1,85) = 15.28$, $P < 0.001$) and immature spine density ($F(1,85) = 8.45$, $P<0.005$) but not for mature spine density ($F(1,85) = 1.65$, n.s.) (Figure 5A, B, C).

Comparison conducted within each group (Cont, RCF) showed that cocaine produced in RCF mice an increase, in comparison with the saline-treated group, in total spine density ($P < 0.001$) (Figure 5A) and immature spine density ($P < 0.05$) (Figure 5B), but not in mature spine density (Figure 5C).

No significant effect was evident for the same parameters in the Cont group (Figure 5 A,B,C). Concerning DBA mice, two-way ANOVA showed a significant treatment x early experiences interaction for total spine density ($F(1,65) = 9.53$, $P< 0.005$) and immature spine density ($F(1,65) = 9.28$, $P<0.005$), but not for mature spine density ($F(1,65) = 3.4$, n.s.) (Figure 5 D,E,F). Analysis conducted within each group (Cont, RCF) showed that cocaine produced a significant reduction in total spine density ($P< 0.001$) (Figure 5 D) and immature spine density ($P< 0.001$) (Figure 5 E) in Cont mice in comparison with the saline-treated group. No significant effect was evident for the same parameters in RCF group (Figure 5 D,E,F).

Discussion

This paper provides experimental data supporting the current point of view that early life aversive experiences affect response to drugs of abuse in adult life.

Contrasting data on long-term effects of postnatal events on responsiveness to psychostimulants in adulthood have been reported showing increased (Der-Avakian and Markou, 2010; Kosten et al., 2003; Meaney et al., 2002; Brake et al., 2004; Zhang et al., 2005) or reduced (Campbell et al., 1999; Ewing Corcoran and Howell, 2010; O'Connor et al., 2015) sensitivity, probably affecting catecholaminergic cortical-accumbal system (Brake et al., 2004; Moffett et al., 2007). Results presented in this paper showing that an early experience (RCF) may either increase or reduce response to cocaine in mice, permit to reconcile conflicting data supporting the point of view that complex gene-environment interplay may determine either susceptibility or resilience to drugs abuse (Der-Avakian and Markou, 2010; van der Veen et al., 2008).

Here we found that RCF C57 and DBA female mice show:

1) opposite behavioral response to cocaine as evaluated by cocaine-induced CPP;

2) different catecholamine outflow within the prefrontal-accumbal system induced by systemic cocaine injection, as well as an opposite c-Fos activity in cortical and subcortical structures;

3) opposite morphological changes induced by systemic repeated cocaine in the prefrontal-accumbens system.

CPP results indicate that unstable maternal environment induced by RCF produced opposite effects on behavioral response to cocaine in adult C57 and DBA mice. In agreement with previous reports, C57 strain shows a greater sensitivity to psychostimulants than DBA strain (Cabib et al., 2000; Orsini et al., 2005; Ventura e al., 2004) but, more importantly, our data indicate that early experience increased and reduced, respectively, the sensitivity to cocaine in adult C57 and DBA mice.

Neurochemical results confirmed behavioral data. Accordingly to previous reports on psychostimulants effects in C57 and DBA strains, Cont DBA mice showed lower sensitivity to cocaine effects on dopamine outflow in the NAc compared with Cont C57 mice (Ventura et al., 2004; Zocchi et al., 2001) as demonstrated by the similar mean percent increase of accumbal DA release shown by two strains using different doses (2.5 mg/kg for C57 and 5.0 mg/kg for DBA). Most importantly, here we found that RCF induced a significantly different prefrontal-accumbal response to cocaine in adult C57 and DBA mice paralleling behavioral results. Cocaine injection induced a lower NE and DA release in the mpFC of RCF DBA mice in comparison with their control and no significant difference in accumbal DA outflow between Cont and RCF groups. A different scenario was evident in RCF C57 mice showing higher prefrontal NE as well as accumbal DA release and no significant difference between Cont and RCF groups in prefrontal DA release. The different prefrontal-accumbal response observed in two strains is consistent with previous reports suggesting a facilitating and inhibitor role of prefrontal NE and DA release, respectively, on accumbal DA outflow (Darracq et al., 1998; Di Segni et al., 2016; Pascucci et al., 2007; Ventura et al., 2003; 2005; 2007). In fact, RCF C57 mice showed a parallel prefrontal NE and accumbal DA cocaine-induced increase. Concerning DBA groups, the reduced increase in both prefrontal NE and DA shown by RCF DBA in comparison with Cont DBA mice is associated with no significant difference in accumbal DA release between RCF and Cont groups. This apparently odd effect can

be easily explained by opposite role of prefrontal NE and DA on accumbal DA outflow (Puglisi-Allegra and Ventura 2012; Ventura et al., 2003; 2007; 2015). In fact, the facilitating effect of prefrontal NE release on accumbal DA outflow is probably counteracts by inhibitor effect of prefrontal DA increase, so producing the same "net" effect on accumbal DA release shown by RCF group compared to Cont group.

According to behavioral and neurochemical results, c-Fos results indicated an opposite cocaine-induced activity pattern in cortical and subcortical brain areas depending on the genotype. In fact, while RCF C57 mice showed an enhancement of c-Fos positive nuclei in most of the areas investigated in comparison with Cont C57, a reduction was evident in almost all brain areas (except for CA1 and CA3) of RCF DBA mice compared with their controls (Table 1).

Exposure of the developing nervous system to early adversity in life can shape synaptic plasticity and neurogenesis (Korosi et al., 2011; Lupien et al., 2009) permanently changing brain functioning and increasing the probability of dysfunctional behavior in adulthood (Cabib and Puglisi-Allegra, 2012; Coccurello et al., 2009; McClelland et al., 2001). Mesocorticolimbic system is critically involved in emotion and motivation related processes and dendritic features in mesocorticolimbic brain areas are very sensitive to many psychological conditions. Different levels of maternal care and stress exposure during early postnatal development in rodents, as well as early life trauma in humans, have been shown to induce long-lasting consequences on morphology, especially in corticolimbic areas (Maccari et al., 2014; Musazzi and Marrocco, 2016; Romano-López et al., 2015; Wang et al., 2012).

Moreover, neuroplastic changes induced by drug exposure have been suggested to mediate cocaine-related behaviors (Kauer and Malenka; 2007; Kiraly et al., 2010; Pulipparacharuvil et al., 2008; Robinson and Kolb, 2004).

Based on these evidences, in the last experiment we evaluated whether RCF experience affected, in a genotype-dependent manner, dendritic spine density induced by repeated cocaine injection. Also

for this experiment, we used the two discriminative cocaine doses able to induce conditioned place preference in C57 (2.5 mg/kg i.p.) and DBA (5 mg/kg i.p.) mice.

Repeated cocaine injection produced increased spine density in apical portion of prefrontal pyramidal neurons of animals belonging to the groups showing CPP (RCF C57: mature spine density; Cont DBA: total, immature and mature spine density) and increased spine density in basal portion of prefrontal pyramidal neurons in RCF C57 mice (total, immature and mature spine density). Increased spine density in mpFC induced by cocaine injection is in agreement with previous reports (Robinson and Kolb, 1999; 2004; Robinson et al., 2001). Concerning NAc neurons, cocaine injection induced increased spine density in RCF C57 (total and immature spine density) and reduced spine density (total and immature spine density) in Cont DBA mice. While results from C57 are in line with literature (Dumitriu et al., 2012; Martin et al., 2011), data from Cont DBA are more puzzling and unexpected. However, different interpretations could be proposed. First, cocaine-induced behavior has been suggested to be not necessarily related with increased spine density or also to be correlated with decreased spine density (Kiraly et al., 2010; Norrholm et al., 2003; Pulipparacharuvil et al., 2008; Taylor et al., 2007). Moreover, in agreement with our results, it has been recently reported that cocaine can induce opposite morphological modifications in rats depending on the genotype (Miguéns e al., 2015). Finally, note that this is the first study investigating the cocaine-induced morphological alterations in DBA mice and a comparison with other studies is to date not possible.

Adverse early experiences (i.e. maternal separation) have been shown to affect drug-related behaviors in adult animals inducing morphological changes in some areas of the corticolimbic system (Higley et al., 1991; Huot et al., 2001; Muhammad and Kolb, 2011).

According to these reports, our morphological results strongly confirm a critical role of early postnatal environment, in interaction with the genetic background, on cocaine response in adult animals.

Conclusions

It has been recently suggested that early life experiences could have negative consequences or confer adaptive value in different individuals (Rana et al., 2015), and we have previously suggested that RCF experience makes DBA a "model" of anhedonia-like state while increases sensitivity toward rewarding natural stimuli in C57 mice that appear instead resilient to develop an anhedonia-like state (Di Segni et al., 2016), thus supporting the hypothesis of differences in the direction rather than in the intensity of stress-induced effects (Alcaro et al., 2002; Cabib et al., 1997; 2000; Ventura et al., 2001) shown by these inbred strains. Present data confirm the opposite effects that the same early experience has in different individuals depending on the genetic makeup also shedding new light on the important role that later experiences in adulthood have on complex behavioral outcomes. Accordingly to the "three-hit concept" of resilience and vulnerability hypothesis (Daskalakis et al., 2013) as well as to the "plasticity genes" concept (Belsky and Pluess, 2009; Belsky et al., 2013), we suggest that the interaction between genetic factors (inbred strains) and early-life environmental condition (RCF experience) induces a sort of "programmed phenotype" that when exposed to following challenges may promote resilience to expression of a pathological phenotype but when exposed to a different challenge may push the same individual toward a higher vulnerability to develop a different kind of pathological phenotype. In particular, here we present data supporting the hypothesis that C57 mice subjected to RCF, that appear resilience to anhedonia-like phenotype when exposed to a stressful experience in adult life (Di Segni et al., 2016), show instead an increased sensitivity to cocaine whether they get in touch with its effects in adulthood.

The implicit concept that underlies our hypothesis is that when subjected to early aversive experiences, it is difficult to anticipate the subsequent development of resilience or vulnerability to a specific psychopathology *tout court* in adulthood, because it is challenging to forecast what type of subsequent experience with which an individual must cope, with the final outcome depending on the interaction between early and later events and his genetic makeup. Detailed molecular investigations will shed light on causal mechanisms responsible for the opposite effects observed in

the two strains.

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Conflict of Interest:

The authors declare no competing financial interests.

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Figure Legends

Figure 1. Schematic representation of medial pre-Frontal Cortex (mpFC; A) and Nucleus Accumbens (NAc; C) and relative photomicrographs of representative Golgi–Cox impregnated pyramidal (mpFC) and medium spiny neurons (NAc). Scale bar, 100 μm.
High-power photomicrographs (B) of the representativel dendritic segment and of categories of spines considered (mature: M=mushroom; S=stubby; and immature: T=thin). Scale bar, 10 μm.

Figure 2. Effect of RCF on cocaine-induced Conditioned Place Preference in C57 and DBA mice.
Results from experiments performed with different doses of cocaine (A; C57: 2.5; 5; 7.5 mg/kg i.p.; B; DBA: 5; 7.5; 10 mg/kg i.p.; lower line). All data are expressed as mean (X) of time spent (± SEM) in compartments Paired (P) / Unpaired (U**)** with cocaine on Test day minus the time spent in the same compartments during the Pre-Test session. * P<.05 in comparison with Unpaired compartment. [A; C57: *2.5 mg/kg*; Cont: P, X 45,634 ±30,853; U, X -41,794 ±39,715; RCF: P, X 89,95 ±33,979; U, X -98,65 ±33,183; *5 mg/kg*; Cont: P, X95,371 ±34,541; U, X -56,834 ±36,46; RCF: P, X 69,13 ±23,512; U, X -59,44 ±30,484; *7.5 mg/kg*; Cont: P, X 73,58 ±22,75; U, X -89,66 ±23,175; RCF: P, X 82,18 ±32,732; U, X -52,75 ±29,521; B; DBA: *5 mg/kg*; Cont: P, X 130,469 ±40,51; U, X -97,634 ±54,272; RCF: P, X 68,42 ±38,477; U, X -32,332 ±29,463; *7.5 mg/kg*; Cont: P, X 110,84 ±32,046; U, X -90,13 ±36,043; RCF: P, X 107,6 ±41,156; U, X -89,73 ±45,418; *10 mg/kg*; Cont: P, X 138,789 ±48,463; U, X -110,469 ±42,046; RCF: P, X 99,69 ±42,807; U, X-99,28 ±23,981].

Figure 3. Effect of RCF on cortico-accumbal cocaine-induced catecholamine release in C57 mice.
Results of cocaine administration in C57 (upper line; 2.5 mg/kg i.p.) and DBA (lower line; 5 mg/kg i.p.) mice on NE (A,D) and DA (B,E) release in mpFC and DA in NAc (C,F) in Control and RCF

mice and relative photomicrographs of representative crazy-violet impregnated brain coronal section; the arrow indicates the tip of the probe, scale bar = 500 μm. Cocaine was administrated at time 0. All data are expressed as percentage changes from baseline level of each group as mean ± SEM. * P<.05 in comparison with Control (Cont) group of same strain.

Figure 4. Cocaine effects on morphological changes in cortical Pyramidal neurons of RCF C57 and DBA mice

Results of cocaine (C) administration (A-F, C57; 2.5 mg/kg i.p.; G-N, DBA, lower portion; 5 mg/kg i.p.) on apical and basal Total Spine Density, Immature and Mature Spine Density. All data are expressed as mean (X) ± SEM. * P< .05 in comparison with saline (S)-treated mice of same group. [**A**; Cont: S, X 2,272 ±0,2; C, X 3,659±0,323; RCF: S, X 2,296±0,2; C, X 3,177 ±0,314; **B**; Cont: S, X 2,21±0,196; C, X 3,588±0,319; RCF: S, X 2,242±0,198; C, X 3,064±0,303; **C**; Cont: S, X 0,062±0,008; C, X 0,071±0,011; RCF: S, X 0,054±0,005; C, X 0,113±0,017; **D**; Cont: S, X 0,876±0,095; C, X 0,803±0,07; RCF: S, X 0,729±0,074; C, X 1,218±0,114; **E**; Cont: S, X 0,858±0,092; C, X 0,79±0,068; RCF: S, X 0,719±0,073; C, X 1,194±0,113; **F**; Cont: S, X 0,018±0,004; C, X 0,013±0,003; RCF: S, X 0,01±0,002; C, X 0,023±0,005_**G**; Cont: S, X 2,228±0,28 C, X 3,246±0,242; RCF: S, X 2,263±0,231; C, X 2,063±0,254; **H**; Cont: S, X 2,145±0,231; C, X 3,103±0,266; RCF: S, X 2,193±0,225; C, X 1,997±0,248; **I**; Cont: S, X 0,084±0,015; C, X 0,143±0,02; RCF: S, X 0,07±0,011; C, X 0,065±0,012; **L**; Cont: S, X 0,776±0,04; C, X 0,83±0,098; RCF: S, X 0,795±0,063; C, X 0,617±0,049; **M**; Cont: S, X 0,759±0,039; C, X 0,81±0,095; RCF: S, X 0,772±0,06; C, X 0,603±0,047; **N**; Cont: S, X 0,017±0,004; C, X 0,02±0,004; RCF: S, X 0,023±0,005; C, X 0,014±0,003]

Figure 5. Cocaine effects on morphological changes in accumbal Medium Spiny Neurons of RCF C57 and DBA mice

Results of cocaine (C) administration (A, B, C, C57; 2.5 mg/kg i.p.; D, E, F, DBA; 5 mg/kg i.p.) on

Total Spine Density, Immature and Mature Spine Density. All data are expressed as mean **(**X**)** ± SEM. * P< .05 in comparison with saline (S)-treated mice of same group. [**A**; Cont: S, X 0,896±0,141; C, X 0,828±0,087; RCF: S, X 0,738±0,077; C, X 1,82±0,143; **B**; Cont: S, X 0,876±0,141; C, X 0,804±0,084; RCF: S, X 0,845±0,148; C, X 1,416±0,15; **C**; Cont: S, X 0,019±0,004; C, X 0,04±0,007; RCF: S, X 0,024±0,005; C, X 0,054±0,01_**D**; Cont: S, X 1,156±0,114; C, X 0,645±0,046; RCF: S, X 0,835±0,113; C, X 1,041±0,13; **E;** Cont: S, X 1,101±0,109; C, X 0,634±0,038; RCF: S, X 0,783±0,104; C, X 0,979±0,12; **F;** Cont: S, X 0,061±0,016; C, X 0,036±0,01; RCF: S, X 0,041±0,009; C, X 0,062±0,014].

Tab 1. C-Fos expression in different brain structures

Cocaine-induced c-Fos expression in RCF C57 and DBA mice in comparison with the respective Control groups.

Results of cocaine administration (C57; 2.5 mg/kg i.p.; DBA; 5 mg/kg i.p.) on c-Fos immunoreactive cell nuclei in different brain areas (infralimbic Cortex (ILC), prelimbic Cortex (PRLC), Nucleus Accumbens Core (NAcC), Nucleus Accumbens Shell (NAcS), Caudate-Putamen (CP), Hippocampus (CA3, CA1, Dentate Gyrus (DG)), Amygdala (Lateral Amygdala (LA), Basolateral Amygdala (BLA), Central Amygdala (CEA)).

All data are expressed as trend arrows. *P<0.05 in comparison with Control (Cont) group of the same strain. [NAcC: DBA: Cont X 0.174±0.036, RCF X 0.505 ±0.082; C57: Cont X 0.695±0.0772, RCF X 0.121±0.013; NAcS: DBA Cont X 0.190±0.031, RCF X 0.489±0.0129; C57: Cont X 0.654±0.070, RCF X 0.114±0.141; LA: DBA Cont X 0.651±0.013 RCF X 0.579±0.010; C57: Cont X 0.107±0.020, RCF X 0.258±0.063; CEA: DBA Cont X 0.740±0.015, RCF X 0.506±0.011; C57: Cont X 0.132±0.314, RCF X 0.276±0.057; BLA: DBA Cont X 0.966±0.020, RCF X 0.110±0.033; C57: Cont X 0.131±0.02, RCF X 0.286±0.0401; CA1: DBA Cont X 0.652±0.018, RCF X 0.672±0.099; C57: Cont X 0.269±0.084, RCF X 0.4870±0.0238; CA3: DBA Cont X 0.131±0.0218,

945 RCF X 0.126±0.399; C57: Cont X 0.552±0.086, RCF X 0.329±0.098; DG: DBA Cont X
946 0.547±0.091, RCF X 0.358±0.045; C57: Cont X 0.926±0.022, RCF X 0.393±0.088; CP: DBA Cont
947 X 0.113±0.0172, RCF X 0.143±0.054; C57: Cont X 0.238±0.057, RCF X 0.457±0.010; IL: DBA
948 Cont X 0.762±0.092, RCF X 0.245±0.017; C57: Cont X 0.322±0.059, RCF X 0.270±0.044; PRLC:
949 DBA Cont X 0.964±0.012, RCF X 0.209±0.042; C57: Cont X 0.214±0.028, RCF X 0.409±0.059].

950 **Tab 1**. Effects of RCF on c-fos expression in different brain structures of C57 and DBA mice.

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Brain area	C57 RCF	DBA RCF
ILC	=	↓
PRLC	↑	↓
NAcC	↑	↓
NAcS	↑	↓
CP	=	↓
CA3	=	=
CA1	↓	=
DG	↓	↓
LA	↑	↓
BLA	↑	↓
CEA	=	↓

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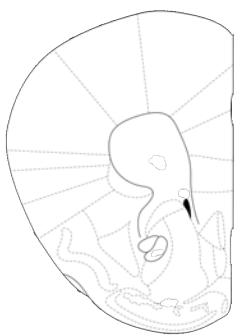
A



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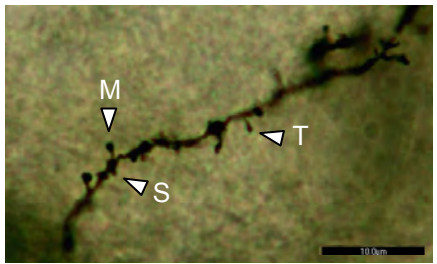

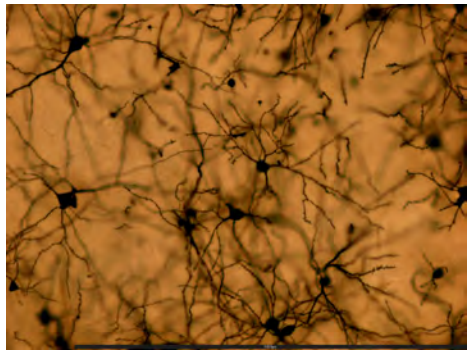

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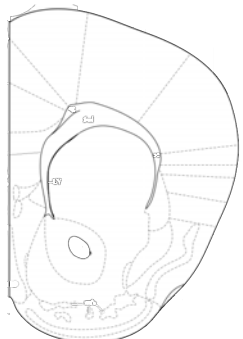

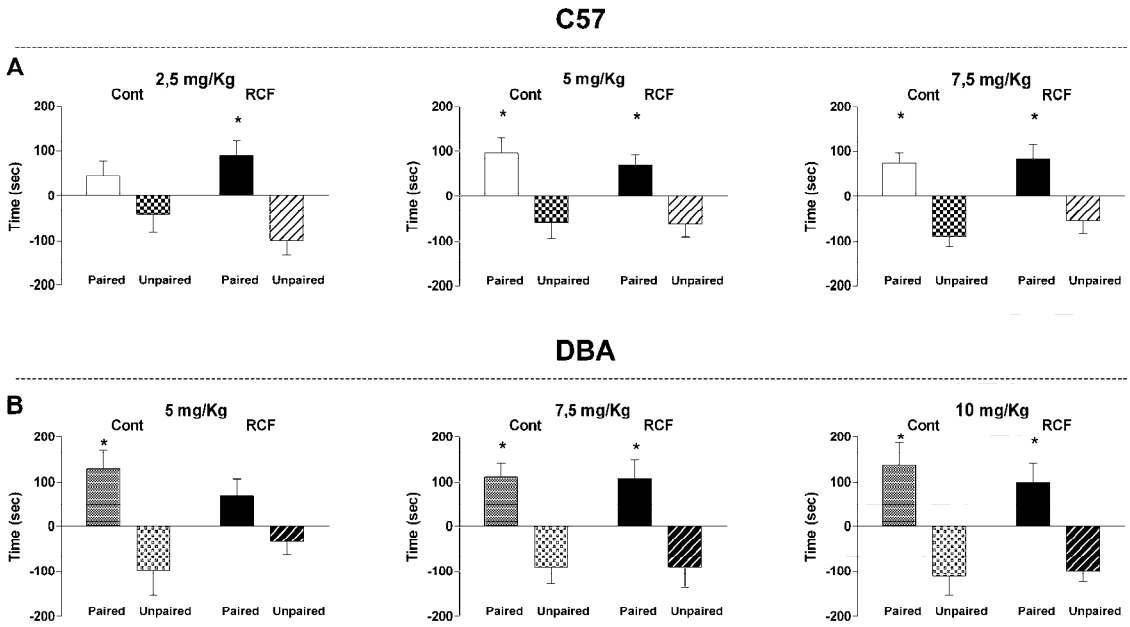

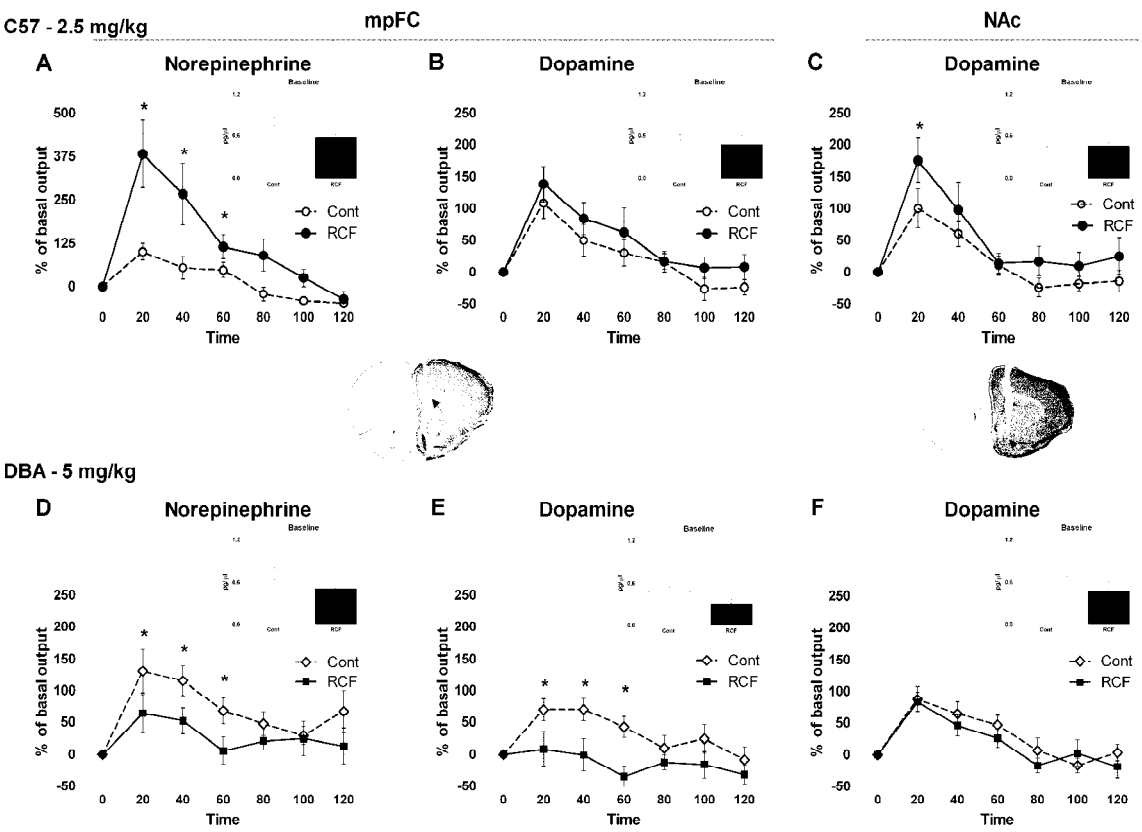

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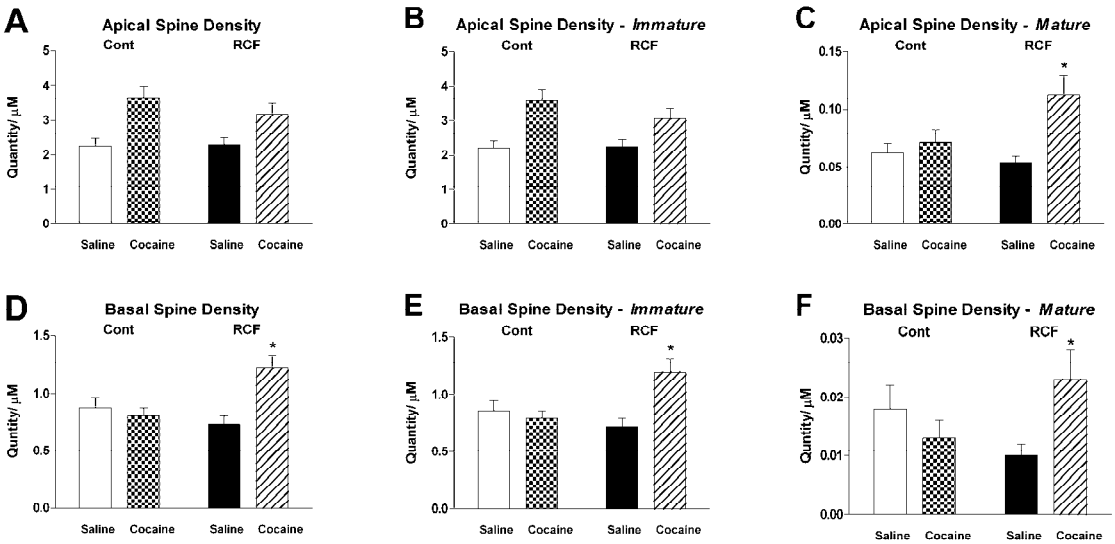

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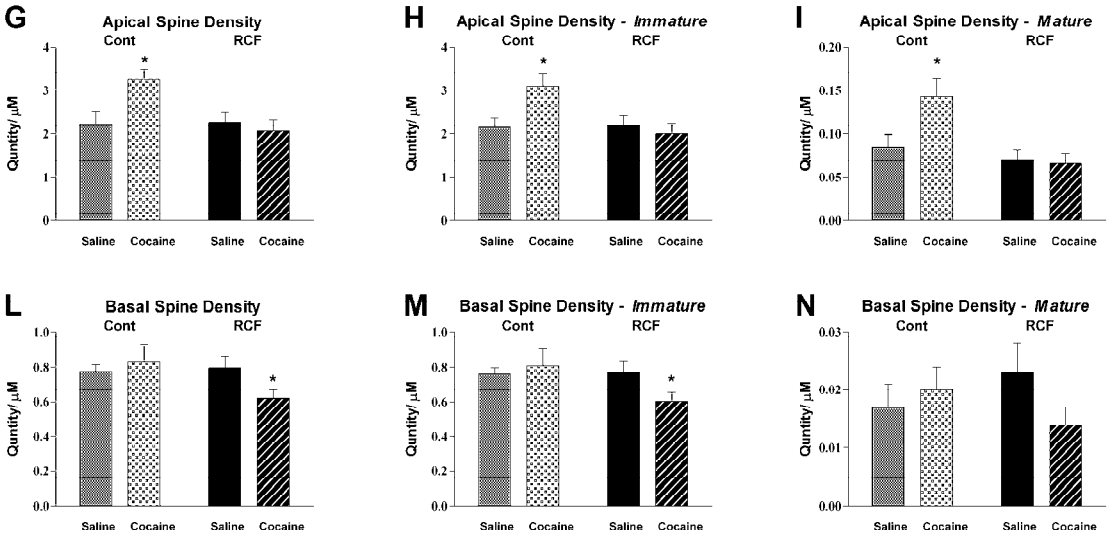

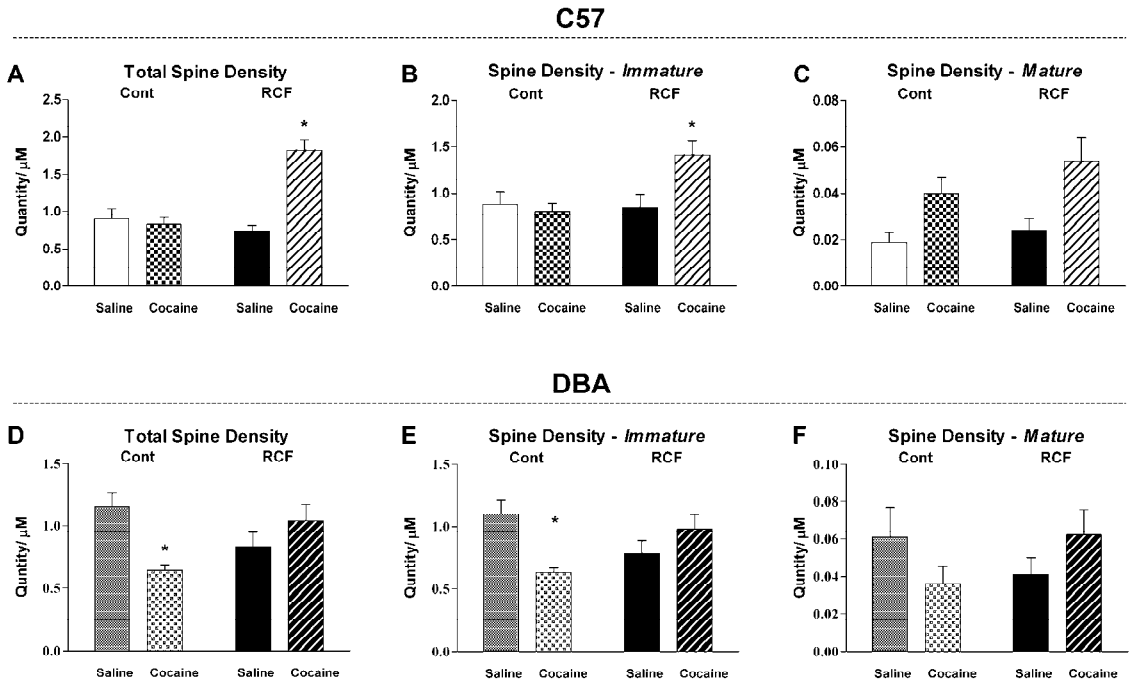

Highlights

Genetic makeup interacts with early experience and later experiences in adulthood

Early environment affects cocaine response in adulthood depending on the genotype

Early environment modifies behavioral, neurochemical and morphological response to cocaine

Early environment increases cocaine susceptibility in C57BL6/J mice