Addiction Biology

SSA SOCIETY FOR THE STUDY OF ADDICTION

ORIGINAL ARTICLE

doi:10.1111/adb.12373

Social threat exposure in juvenile mice promotes cocaine-seeking by altering blood clotting and brain vasculature

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ABSTRACT

Childhood maltreatment is associated with increased severity of substance use disorder and frequent relapse to drug use following abstinence. However, the molecular and neurobiological substrates that are engaged during early traumatic events and mediate the greater risk of relapse are poorly understood and knowledge of risk factors is to date extremely limited. In this study, we modeled childhood maltreatment by exposing juvenile mice to a threatening social experience (social stressed, S-S). We showed that S-S experience influenced the propensity to reinstate cocaine-seeking after periods of withdrawal in adulthood. By exploring global gene expression in blood leukocytes we found that this behavioral phenotype was associated with greater blood coagulation. In parallel, impairments in brain microvasculature were observed in S-S mice. Furthermore, treatment with an anticoagulant agent during withdrawal abolished the susceptibility to reinstate cocaine-seeking in S-S mice. These findings provide novel insights into a possible molecular mechanism by which childhood maltreatment heightens the risk for relapse in cocaine-dependent individuals.

Keywords acetylsalicylic acid, blood coagulation, brain vasculature, childhood maltreatment, cocaine, relapse risk factors, vWF.

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INTRODUCTION

Child maltreatment is defined as any act of commission or omission by a caregiver that results in harm, potential for harm or threat of harm to a child. Estimates suggest that 40 percent to 50 percent of those who experience childhood maltreatment (CM) will develop substance abuse problems (Dube *et al.* 2003). CM is associated with increased severity of substance use disorder (SUD) and frequency of relapse to drug-seeking after withdrawal (Khoury *et al.* 2010; Van Dam *et al.* 2014; Elton *et al.* 2014). Neuroimaging studies have begun to detect structural and functional changes in the reward circuitry that are induced by CM and related to the severity of SUD (Van Dam *et al.* 2014; Elton *et al.* 2014).

Nevertheless, the molecular mechanisms that are engaged during early traumatic events and mediate the risk of SUD and relapse remain poorly understood. The identification of new therapeutic targets for the prevention of SUD and relapse requires a greater understanding of these phenomena. To this end, the identification of noninvasive biomarkers that predict a subject's propensity toward severe SUD after a traumatic childhood is warranted and is essential for advancements in effective prophylactic and therapeutic strategies (Bough *et al.* 2014). Clinical research on this matter is difficult,

because genetic and environmental heterogeneity, comorbid states and polysubstance use do not allow the establishment of causal links between CM and subsequent SUD (Begemann *et al.* 2015), necessitating the use of preclinical models.

In rodents, acute and chronic stress influences the escalation of drug use (Sinha 2008). Exposure to stress during early postnatal life, such as during maternal separation and decreases in maternal care, modulates the rewarding effects of cocaine, amphetamines and morphine in adult mice and rats (Francis and Kuhar, 2008; Moffett *et al.* 2006; Lewis *et al.* 2013; Boasen *et al.* 2009). Interestingly, in humans, CM seems to be associated with SUD and increased frequency of relapse (Elton *et al.* 2014). Despite its relevance, the impact of this type of experience on drug seeking behavior has never been investigated in preclinical models. Mimicking CM in rodents could be a powerful tool to identifying the long-lasting molecular changes underlying the susceptibility to SUD and relapse to drug abuse.

Preclinical research trying to mimic early traumatic events in rodents has primarily focused on the first two weeks of life. However, recent studies have shown that during the juvenile period in mice (3–4 post-natal weeks), a large-scale reconfiguration in the neuronal epigenome (e.g. increased methylation in the non-CG context) and extensive synaptogenesis occurs, similar to what occurs in humans during childhood (2–3 years of age; Lister *et al.* 2013). Furthermore, this developmental time-window is characterized by the maturation of functions that are crucial for the interaction of a mouse with its environment, such as visual, motor and social abilities (Pellis & Pasztor 1999; Berardi *et al.* 2000; Rice & Barone 2000). Thus we hypothesize that exposure to social stress during the juvenile period may affect long-term epigenetic regulation of gene expression and brain functionality.

Here, we modeled CM by exposing juvenile mice to a threatening social environment (social stress, S-S) and studied the impact of this experience on cocaine drug-seeking behavior in adulthood. We also used this model to examine the molecular mechanisms underlying the susceptibility to cocaine abuse and identify risk factors of relapse that can be measured noninvasively and thus translated into clinical population.

Exposure to S-S promoted cocaine-seeking and relapse to cocaine-seeking after periods of withdrawal in mice. We compared differences in global gene expression in leukocytes in S-S and control mice during cocaine abstinence. Notably, in S-S mice, the expression of genes involved in blood clotting was altered, and these changes were associated with impairments in brain vasculature. Unexpectedly, treatment with an anticoagulant agent mitigated the susceptibility to reinstate cocaine-seeking in S-S mice. These findings implicate blood coagulation and brain vasculature in the risk of relapse to drug seeking.

MATERIALS AND METHODS

Animals and breedings

Seven-week-old CD-1 (CD1) male and DBA2/J @Ico (DBA) male and female mice were purchased from Charles River Laboratories (Calco, Italy). For the production of pups, DBA/2J male and female mice were mated at 12 weeks of age and fathers were removed before parturition. Manipulation protocols and behavioral testing were performed during the light cycle from 10:30 to 14:30. Mice were kept at constant temperature (21 ± 1°C) and humidity (55 ± 5 percent). Food and water were provided ad libitum, and mice were housed on a 12:12 light:dark cycle with lights on at 0700 h. All work with animals was conducted in accordance with European legislation (EEC no. 86/609), Italian national legislation (DL no. 116/92) governing the use of animals for research and the guidelines of the National Institutes of Health on the use and care of laboratory animals.

Juvenile stress procedure

Mouse pup litters (27 litters) were randomly assigned to unhandled control (UN; 5 litters), not Social Stress (NS-S; 10 litters) or Social Stress (S-S; 12 litters) group at postnatal day (PD) 14. In the UN group ($N = 26$) mothers and offspring were left undisturbed until weaning (PD22). In the NS-S group ($N = 62$) each pup was singly housed in a novel clean bedding cage for 30 min per day from PD14 to 21. In the S-S group ($N = 55$), each pup was housed in a cage with a resident adult CD1 male mouse (different every day) for 30 min per day from PDs 14 to 21 (Fig. 1a). To avoid killing of the pups, CD1 males were gonadectomized and singly housed one month before the manipulation protocol. Pup body weight was measured at PDs14 and 22 (see Supporting Information). The experimental sample size was determined with the aid of online available software (http://www.stat.ubc.ca/~rollin/stats/ssize). To perform this calculation, we used values (e.g. means and common standard deviation) obtained from similar experiments previously performed in our laboratory. Behavioral phenotype was evaluated at 10–12 weeks of age.

Drugs

Cocaine hydrochloride was purchased from Sigma (Milan, Italy). Cocaine was dissolved in saline (0.9 percent NaCl) and injected intraperitoneally (i.p.) in a volume of 10 ml/kg. To condition the mice a cocaine dose

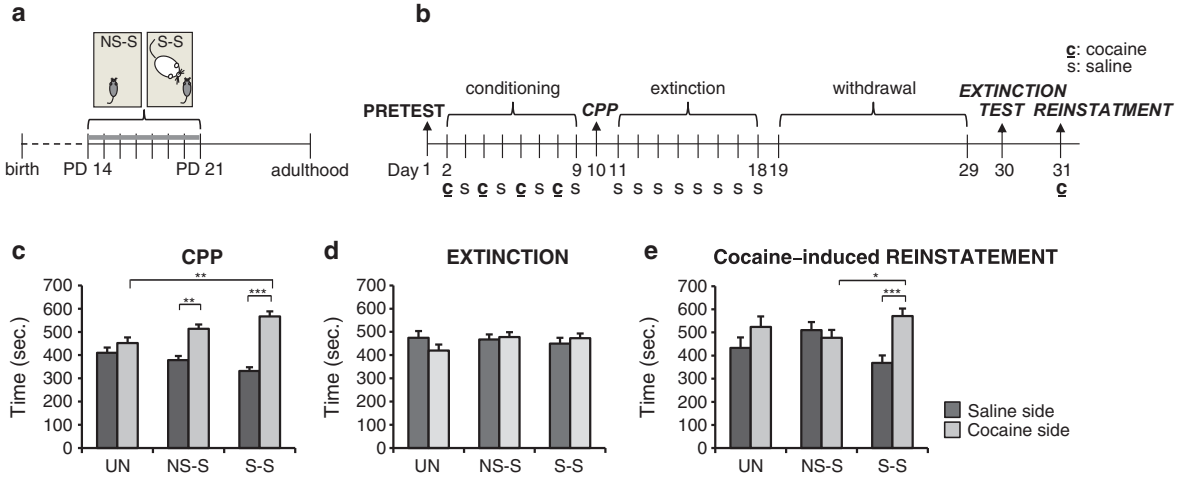

Figure 1 Exposure to a social threat in juvenile age promotes reinstatement to cocaine-seeking behavior after withdrawal in adulthood. (a) Stress protocols applied from postnatal days (PDs) 14 to 21. During social stress (S-S), pups were exposed daily to an adult CD1. No social stress (NS-S) mice experienced daily isolation in a novel environment in the same week. (b) The susceptibility to cocaine-seeking behavior and reinstatement after abstinence was measured by conditioned place preference (CPP) in under-threshold dose conditions. (c) In the CPP test, S-S and NS-S mice showed a significant preference for the cocaine-paired (cocaine side) versus saline (saline side) compartment. (d) This effect disappeared after extinction. (e) S-S but not NS-S mice showed cocaine-induced reinstatement (*N*: UN = 26, NS-S = 38, S-S = 25). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

of 5 mg/kg was used, whereas to test reinstatement a 1.25 mg/kg dose was used (Orsini *et al.* 2008). Previous studies have reported the absence of a dose-dependent effect on preference for the cocaine-paired compartment in DBA strain at both CPP and reinstatement test. We therefore reasoned that using a 'sub-threshold' dose, that fails to induce CPP in control DBA/2J mice, we would have been able to discriminate mice that, due their adverse early experience, would be more susceptible to the effects of cocaine.

Conditioned place preference protocol

Mice were tested for cocaine-seeking behavior as previously describe (Orsini *et al.* 2008) at 10 weeks of age (Fig. 1b). Briefly, on day 1 (pretest), mice were free to explore the entire unbiased apparatus for 20 min. On the following 8 days (conditioning phase), mice were injected and confined daily for 40 min in one of the two chambers. For each animal, during the conditioning phase, one of the patterns was consistently paired with a saline injection and the other one with a drug injection. On days 2, 4, 6 and 8, all animals received injections of cocaine immediately prior to the session and on the alternating days received saline injections the session. Testing was conducted on day 10 in a drug-free state and lasted 20 min. After conditioning and the initial CPP test, mice were given extinction training in which saline was paired four times with each of the two compartments, once per day, over 8 days. After extinction training, all mice were left undisturbed for 10 days (withdrawal) and as previous research has demonstrated the necessity of a withdrawal period for cocaine prime-induced reinstatement in DBA mice (Orsini et al., 2008). Animals were then retested to evaluate extinction learning. Twenty-four hours later, reinstatement was evaluated using a priming injection of cocaine (1.25 mg/kg i.p.) immediately before a CPP test. The behavioral data were obtained by means of three replications of the experiment (Fig. 1c–e; *N*: UN = 26; NS-S = 38; S-S = 25)

Leukocytes isolation and RNA preparation

For gene expression analysis, mouse blood was collected via sub-mandibular puncture in EDTA tubes after the extinction test during the CPP protocol. One day after blood collection mice were tested for cocaine-induced reinstatement to verify their behavioral phenotype (*N*: NS-S = 8; S-S = 8). Leukocytes were extracted from blood samples using Red Blood Cell (RBC) lysis buffer (Norgen Biotek, Thorold, Canada) according to the manufacturer's protocol and RNA was subsequently isolated using Total RNA purification Plus Kit (Norgen Biotek). RNA quantity was determined by absorbance at 260 nm using a NanoDrop UV–VIS spectrophotometer, and RNA quality was assessed by the use of Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA samples were stored at −80°C for further analysis.

Mouse gene expression microarray and data analysis

Leukocytes RNA samples (from 16 different mice) were hybridized on Agilent mouse gene expression microarrays (G4852A) according to the manufacturer's

procedure (Callegari *et al.* 2012) using the Low Input Quick-Amp Labeling Kit and one color design (Agilent Technologies) (cyanine 3-CTP). Images at 3-μm resolution were generated by Agilent scanner, and the Feature Extraction 10.7.3.1 software (Agilent Technologies) was used to obtain the microarray raw-data.

Data were analyzed with BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). Data were subjected to filtration based on signal intensity, spot quality and presence across the data set. A total of 27978 probes passed the filters and were further analyzed. Statistical analysis of genes differentially expressed S-S and NS-S was performed using Random Variance Model (Wright & Simon 2003) with parametric *p*-value threshold set to 0.005. Identified genes were further filtered according to fold change (LOG FC > 0.6 for up-regulated genes, LOG FC < -0.6 for down-regulated ones). False Discovery Rate of the whole gene list was calculated as the proportion of expected false positive observations on total significant observations. Gene Ontology (GO) analysis was performed on the above selected genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/; Huang *et al.* 2009). Significance of overrepresentation was adjusted for multiple comparisons to control the false discovery rate (FDR) by means of the Benjamini–Hochberg step-down procedure or the approximated FDR tools provided in DAVID. Fold enrichment was scalculated as the ratio between the number of genes belonging to the family observed in the gene list and the number of genes expected to be randomly present in the gene list. To avoid redundancy, selected statistically enriched GO Biological Processes (BPs) and Panther pathways were reported.

The microarray data obtained have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and are accessible through GEO Series accession number GSE69019 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69019).

Quantitative real time RT-PCR (qPCR)

qPCR was performed to validate microarray results (on the same RNA samples used in the microarray) and to measure vWF expression (*N*: NS-S = 6; S-S = 6). cDNA was obtained using the High Capacity Reverse Transcription kit (Applied Biosystems, Branchburg, NJ). cDNA templates (10 ng) were processed by qPCR using the 7900HT thermal cycler apparatus equipped with the SDS software version 2.3 (Applied Biosystems) for data collection. Taqman primer sets (Applied Biosystem, Supporting Information Table S1) were used for cDNA amplification, and Ct values were normalized to measures of TBP and PGK1 mRNA (Taqman primers code TBP: Mm00446974_m1; PGK1: Mm01225301_m1). All data were run in triplicate and analyzed using the $\Delta\Delta C(t)$ method. Correlation analyses between microarray and qPCR expression data were performed correlating (Pearson correlation) the expression data for each animal obtained with the two techniques.

Acetyl-salicylic acid treatment

NS-S and S-S mice were randomized to receive placebo (water) or acetyl-salicylic acid (ASA, 40 mg/l) in their drinking water, which was replaced with fresh solutions every day for 10 days during the abstinence phase in the CPP protocol (Days 19 to 31). Considering that each animal drinks an average 3 to 4 ml of water per day, this is approximately 120 to 160-μg ASA per day for a 30-g mouse (4 to 5.5 mg/kg/day). One day following the testing of cocaine-induced reinstatement, mice were sacrificed and blood and brain tissues isolated for coagulation and vasculature analysis, respectively (*N*: UN = 26; NS-S = 38; S-S = 25). The behavioral data were obtained by means of two replications of the experiment.

Prothrombin time analysis

On day 30 of CPP protocol (Fig. 1b), mice were anesthetized with ether, the thoracical cavity was opened and blood was drawn from the upper Vena cava into a syringe containing sodium citrate 4 percent solution (Citrate solution/blood 1/9 *v*/*v*). Blood was centrifuged within 10 min from collection at 2500 rpm for 15 min, RT. Plasma was removed, placed in new vials on ice and sent to a specialized veterinarian laboratory (Provet Lab, Latina, Italy) for Prothrombin time (PT) analysis. PT was automatically measured on a ACL 9000 coagulation analyzer (Beckman Coulter, Fullerton, CA) using the photometric method.

Immunohistochemistry

On day 30 of CPP protocol (Fig. 1b), NS-S and S-S mice were anesthetized (xylazine, 20 mg/ml, 0.5 ml/kg body weight, i.p.) and transcardially perfused with saline, followed by 4 percent paraformaldehyde (PFA). Brains were removed, post-fixed in PFA 4 percent, washed in PBS and transferred to a 30 percent sucrose solution at 4°C. A series of 30-μm-thick transverse sections from different regions including nucleus accumbens (NAc), striatum and hippocampus were obtained with a freezing microtome. For lectin staining (Thurston *et al.* 1996) brain sections containing NAc, striatum or hippocampus (dentate gyrus) were first incubated overnight at 4°C in PBS containing 0.3 percent Triton X-100 with biotin-conjugated *Lycopersicon esculentum* (tomato) lectin (1 μg/ml; BD Pharmingen, San Diego, CA) and rabbit

anti-NeuN (1:500; Millipore, Billerica, Massachusetts, USA). After three washes in PB, sections were incubated with streptavidin Alexa Fluor 488 conjugate (1:500; Life Technologies, Carlsbad, CA) and Alexa Fluor 555 donkey anti-rabbit IgG (1:200; Life Technologies) for 2 h at RT. After washes, the sections were counterstained with Neuro-Trace® 647 Fluorescent Nissl Stain (1:400; Life Technologies) and then were mounted using an anti-fade medium (Fluoromount; Sigma). For comparison of fluorescent intensities, sections from S-S and NS-S mice were stained in the same wells, placed onto the same glass slides and acquired under the same conditions. The brain areas were further determined in Nissl-stained sections according to Franklin and Paxinos' Atlas of Mouse Brain (Paxinos & Franklin, 1997).

Imaging and analysis

Specimens were examined under a confocal laser-scanning microscope (Zeiss CLSM700). The structures of interest were identified by using a 10× objective and captured by using a 20× objective. For acquisition of capillaries in the different brain structures, a series of z-sections were acquired at interval ranging from 0.87 to 1.2 µm. Specimens were captured using consistent settings for laser power and detector gain.

The densitometric analysis of lectin staining was performed with ImageJ software (http://rsb. info.nih.gov/ij/; National Institutes of Health) on confocal maximum intensity projection images. After background subtraction Lectin-associated signal was quantified in the areas of interest by manually outlining individual structures. Mean signal intensity of lectin staining (F) in the different structures was performed in a variable number of sections, in order to cover the whole extension of the areas of interest. Specifically, for each animal, in five sections for NAc, one every 150 µm, and in five sections for striatum and hippocampus, one every 300 µm. The F/A ratio defines mean fluorescence of individual samples (F) normalized to total cellular surface (A). Quantification was done on five sections per mouse per structure (four mice per group—total group samples $n = 20$).

Furthermore morphometric analysis of lectin-stained blood capillaries in the different structures was performed on confocal maximum intensity projection images and analyzed by using Neurolucida software (Neurolucida 7.5, MicroBright-Field, Germany). For capillary diameters analysis, a total of 240 capillaries per structure (from four mice per group) were considered, and the minor axes of capillaries were measured. The branch points of microvasculature (Czéh *et al.* 2010; Kiuchi *et al.* 2012) were numerated and counted with ImageJ software on confocal maximum intensity projection images. Data collecting for densitometric and morphometric analyses were performed by experimenters blind to the group analyzed.

Statistical analysis

All data obtained were checked for homogeneity of variance, with measures failing Levene's test analyzed by non-parametric Mann–Whitney procedures. All other parameters were subjected to parametric either Student's *t*-test or repeated-measure analysis of variance (ANOVA). ANOVA was followed, in cases of significance ($P < 0.05$), by post-hoc comparisons using Duncan's test. The comparisons between the capillary diameter population were performed using the Chi-square test on categorical measure (capillary diameter). All statistical analyses were carried out with the help of Statistica software Version 12.0 (StatSoft, Tulsa, OK, USA).

RESULTS

Exposure to a threatening social environment in juvenile mice increases cocaine-seeking behavior

We exposed DBA/2J mouse pups to a social threat (daily contact with an adult CD1 male mouse; Fig. 1 and Supporting Information Fig. S1a) during the third postnatal week (juvenile social stress, S-S). To analyze the specific effects of this exposure, S-S mice were compared with unhandled controls (UN) and mice that experienced only daily isolation in a novel environment in the same week [no social stress (NS-S), Fig. 1a]. The induction of a stress response by the S-S procedure in pups, versus the NS-S and UN control protocols, was measured and characterized by ultrasonic vocalization (USV), which is a behavioral index of distress (Jelen *et al.* 2003), and plasmatic corticosterone levels and body weight loss as physiological responses to the stress (see Supporting Information Fig. S1b-e).

Based on the robust relationship between CM and SUD in humans, we examined the long-term impact of S-S stress on adult cocaine-seeking behavior using CPP. We used an experimental CPP protocol (Orsini *et al.* 2005) that allowed us to model drug abuse and relapse to drug seeking after withdrawal (cocaine-induced CPP and cocaine-induced reinstatement, respectively; Fig. 1b; Supporting Information Fig. S2a).

During the CPP pretest session, there was no preference for any chamber in the apparatus in the 3 experimental groups (Pretest, *time effect*, $F[2,166] = 0.05$, $P > 0.05$). In the CPP test, the NS-S and S-S groups developed a significant preference for the cocaine-paired compartment, but of greater magnitude in the S-S group compared with NS-S and UN mice (CPP, *time × stress effect*, $F[4,166] = 4.03$, $P = 0.004$; Fig. 1c). After CPP testing, all groups underwent extinction, withdrawal

and retesting (Fig. 1d). Twenty-four hours later, mice were challenged with a subthreshold priming dose of cocaine (1.25 mg/kg) and evaluated for CPP reinstatement. Notably, only S-S mice showed cocaine-seeking behavior, suggesting that juvenile stress increased the propensity toward cocaine prime-induced reinstatement (Reinstatement, *time × stress effect*, $F[4,166] = 2.80$, $P = 0.030$; Fig. 1e).

Social stressed mice experience abnormal blood clotting and impairments in brain microvasculature during cocaine withdrawal

Preventing relapse to drug abuse is a significant goal in the treatment of SUD, for which effective preventive methods require knowledge of the risk factors for relapse that can be measured noninvasively (Bough *et al.* 2014). Taking advantage of the increased susceptibility to reinstatement in S-S mice, we performed a molecular examination of the cocaine withdrawal phase of CPP. We compared differences in global gene expression in the leukocytes in S-S ($n = 8$) and NS-S ($n = 8$) mice during cocaine abstinence. Blood was collected 24 h after the extinction test (Day 31 of the CPP protocol, Fig. 1b; Supporting Information Fig. S2a), and genome-wide mRNA levels in leukocytes were measured using an Agilent Mouse Gene Expression Microarray.

Microarray 60k database underwent quality control filtering and replicates spots reduction, thus resulting in 27 978 transcripts, corresponding to 14 625 annotated genes. By statistical analysis of this dataset, 1242 RNAs differed significantly between the S-S and NS-S groups (univariate *t*-test *p* value <0.005, Supporting Information Table S2), 1139 of which were upregulated (corresponding to 761 annotated genes, LOG FC >0.6) and 103 of which were downregulated (86 annotated genes, LOG FC < -0.6) in S-S versus NS-S mice (Supporting Information Fig. S3 and Table S1).

By Gene Ontology classification, using the DAVID web-based tool [http://david.abcc.ncifcrf.gov/] (irrespective of the direction of the change, modified Fisher exact *p*-value ≤ 0.005), this set of RNAs was enriched for genes that were involved in coagulation, wound healing, protein phosphorylation, intracellular signaling, platelet activation and small GTPase-mediated signal transduction. Our PANTHER pathway analysis revealed significant enrichment in blood coagulation, integrin signaling and VEGF signaling (Table 1).

These results prompted us to determine whether this expression pattern would alter blood coagulation. To test this hypothesis, we measured prothrombin time (PT), which reflects the tendency of blood to clot, and von Willebrand factor (vWF) expression. vWF (upregulated in S-S mice by microarray analysis; Supporting Information Tables S1 and S2) participates in blood coagulation and is frequently used as a diagnostic marker for hemostasis (Meyer & Girma, 1993). We collected blood from new NS-S and S-S groups during the withdrawal phase of the CPP protocol (Day 30; Supporting Information Fig. S2b). Consistent with increased blood coagulation, we observed lower PT values and confirmed the significant rise in vWF mRNA levels in S-S compared with NS-S mice (PT, $t[5] = 2.87$, $P = 0.035$; vWF, $t[9] = 2.50$, $P = 0.049$; Fig. 2a and b).

Based on earlier reports that have indicated a clinical association between circulating hemostatic measures (e.g. vWF), cerebral blood flow and cognitive impairments (Sabayan *et al.* 2014) we hypothesized that the alterations in the blood of S-S mice are linked to changes in the cerebral microvasculature. Thus, we examined the blood capillaries in three structures of the brain in our mice—the hippocampal dentate gyrus (DG), dorsal striatum (STR) and nucleus accumbens (NAC)—during withdrawal from cocaine (Day 30 of the CPP protocol; Supporting Information Fig. S2b). Capillaries were stained with *L. esculentum* (tomato) lectin (Fig. 2c), a glycoprotein that labels vascular endothelium in rodents (Thurston *et al.* 1996). In S-S mice, the capillary diameters (STR, $\chi 2 = 523.73$, $df = 241$, $P < 0.001$; NAC, $\chi 2 = 620.38$, $df = 241$), number of capillary branch points, and the overall intensity of the signals declined significantly (STR branches, $U = 83.00$, $P < 0.003$, $r = 0.479$; intensity, $t[37] = 4.08$, $P < 0.001$; DG branches, $t[37] = 2.69$, $P = 0.011$, intensity, $U = 103.00$, $P < 0.015$, $r = 0.389$; NAC branches, $t[37] = 2.14$, $P = 0.039$, intensity, $U = 49.00$, $P > 0.001$, $r = 0.632$; Fig. 2d–g), indicating a less extensive vasculature in all structures in S-S mice versus NS-S mice.

Anticoagulant treatment during withdrawal prevents the reinstatement of cocaine-seeking

Our results suggest that the increased susceptibility to cocaine-induced reinstatement in S-S mice is associated with reduced cerebral blood flow (CBF), as evidenced by a rise in blood coagulation and reduced brain vasculature (Fig. 2). Notably, regional CBF is lower in cocaine-abstinent individuals (Kosten *et al.* 2004; Strickland *et al.* 1993; Volkow *et al.* 1988) and is linked to cognitive impairments, possibly favoring a relapse to cocaine abuse (Kalivas *et al.* 2005).

Based on these findings, we hypothesized that enhancing blood flow in S-S mice would improve their behavioral performance and lower their susceptibility to cocaine-induced reinstatement. After being tested for cocaine-induced CPP, we treated new groups of S-S mice with low doses of the anticoagulant acetylsalicylic acid

Table 1 Gene Ontology (GO) analysis of gene expression changes in S-S versus NS-S mouse leukocytes. GO biological processes and Panther pathways showing significant categorical enrichment (*P*-value < 0.005 and Benjamini < 0.05) are shown. GO analysis was performed in DAVID for all differentially expressed genes (*t*-test, $P < 0.005$, FC > 1.5, irrespective of the direction of the change). For each family, significantly changed genes are also shown

GO term	%	*P* value	GenesBiological process	FoldEnr.	Benjamini
GO:0050817 coagulation	2.1	4.8×10^{-9}	F2RL2, PLEK, PIK3CB, F13A1, PF4, GP9, P2RY12, VWF, GP5, GP6, F5, P2RX1, GP1BB, GP1BA, TREML1, PROS1, F2R	6.4	1×10^{-5}
GO:0042060 wound healing	2.3	1.2×10^{-7}	F2RL2, PLEK, PIK3CB, F13A1, PF4, TIMP3, GP9, P2RY12, VWF, GP5, GP6, F5, P2RX1, GP1BB, PECAM1, GP1BA, TREML1, PROS1, F2R	4.6	6.6×10^{-5}
GO:0006468 protein amino acid phosphorylation	5.9	6.2×10^{-6}	HTATIP2, AURKC, ABI2, CHEK2, PRKG1, PRKAR2B, PTK2, WNK4, SPNB2, TLK1, FERT2, TIE1, PRKAA2, CDK16, AKT3, PRKCA, PDK1, BCR, TNIK, STK25, ROCK2, NLK, MAP2K3, PNCK, PICK1, STRADB, PDE6H, STK4, KDR, DAPK1, MAPK1, PRKCQ, CCND1, MAST2, MAPK6, FYN, GSK3B, CD81, CDC42BPA, CAMK1, GRK5, MYLK, KALRN, F2R	2	0.003
GO:0007242 intracellular signaling cascade	7.6	9.8×10^{-6}	CTNNAL1, RHOJ, ITPKB, ITSN1, RGL1, MCF2L, TRIAP1, ARL5A, WNK4, GUCY1A3, TLK1, RAB27B, AGPAT1, PRKCA, RAP2A, TNIK, BCR, PIK3CB, ROCK2, PIK3C2B, PICK1, PSD3, ARL3, DAPK1, PRKCQ, MAPK1, CCND1, GNAQ, CD81, RAB12, RAB13, NRGN, RAB10, RALGPS2, MRAS, CHEK2, TGM2, RHOBTB1, RAB11A, RASA3, CDC42EP5, PDK1, PLEK, NLK, MAP2K3, STRADB, PEA15A, TAX1BP3, PDE6H, P2RY12, NRAS, LAT, ADCY9, RAB37, GSK3B, PECAM1, CDC42BPA, F2R	7.6	0.003
GO:0030168 platelet activation	0.8	2.3×10^{-5}	P2RY12, VWF, PLEK, P2RX1, PIK3CB, PF4, TREML1	10.9	0.005
GO:0007264 small GTPase mediated signal transduction	3.1	5×10^{-5}	RHOJ, CTNNAL1, RALGPS2, MRAS, ITSN1, RGL1, MCF2L, ARL5A, RAB11A, RHOBTB1, RAB27B, CDC42EP5, RAP2A, ROCK2, PSD3, TAX1BP3, ARL3, LAT, NRAS, RAB37, PECAM1, RAB12, RAB13, RAB10	2.5	0.011
	%	***P* value**	**GenesPanther pathways**	**FoldEnr.**	**Benjamini**
P00011: Blood coagulation	1.6	5.7×10^{-5}	F2RL2, F13A1, ITGB3, GP9, VWF, GP5, F5, GP1BB, SERPINB2, GP1BA, PROS1, F2R, ITGA2B	3.9	0.006
P00034: Integrin signaling pathway	3.2	5.6×10^{-4}	LIMS1, ITGB3, ITGB1, VCL, PTK2, COL18A1, RAP2A, PIK3CB, MAP2K3, PIK3C2B, ACTN1, ITGA2, PTPN12, ARPC1A, MAPK1, NRAS, ITGA6, MAPK6, FYN, COL1A2, TGFB1I1, LAMC1, GRAP2, PARVB, ARHGAP10, ITGA2B	2	0.028
P00056: VEGF signaling pathway	1.6	1.2×10^{-3}	PRKCA, PIK3CB, PIK3C2B, KDR, SH2D4B, MAPK1, PRKCQ, NRAS, PTK2, PLA2G4A, MAPK6, TGFB1I1, AKT3	2.9	0.039

(ASA, 4 to 5.5 mg/kg/day in their drinking water) or water throughout the withdrawal phase of the CPP protocol (Days 19 to 31, Fig. 3a; Supporting Information Fig. S2c). We first noted that the pharmacological treatment improved blood flow in S-S mice, as confirmed by the increase in PT in the blood of ASA-treated S-S mice at the end of the treatment, compared with water-fed S-S mice ($t[7] = 12.49$, $P < 0.001$; Fig. 3b). Then, we examined cocaine-induced reinstatement in ASA-treated mice.

Whereas water-treated S-S mice relapsed to cocaine-seeking behavior (Pretest, *time effect*, $F[1,12] = 1.59$, $P > 0.05$; CPP, *time effect*, $F[1,12] = 22.20$, $P < 0.001$; Reinstatement, *time effect*, $F[1,12] = 9.43$, $P = 0.009$; Fig. 3c), as expected, the administration of ASA during withdrawal prevented the prime-induced preference for the cocaine-paired side (Pretest, *time effect*, $F[1,16] = 1$, $P > 0.05$; CPP, *time effect*, $F[1,16] = 17.28$, $P < 0.001$; Reinstatement, *time effect*, $F[1,16] = 1.78$, $P > 0.05$, Fig. 3d). Similar treatment with ASA in NS-S mice did not alter their phenotype in the reinstatement test (Supporting Information Fig. S4). These results implicated blood flow in the S-S-induced susceptibility to cocaine-induced reinstatement.

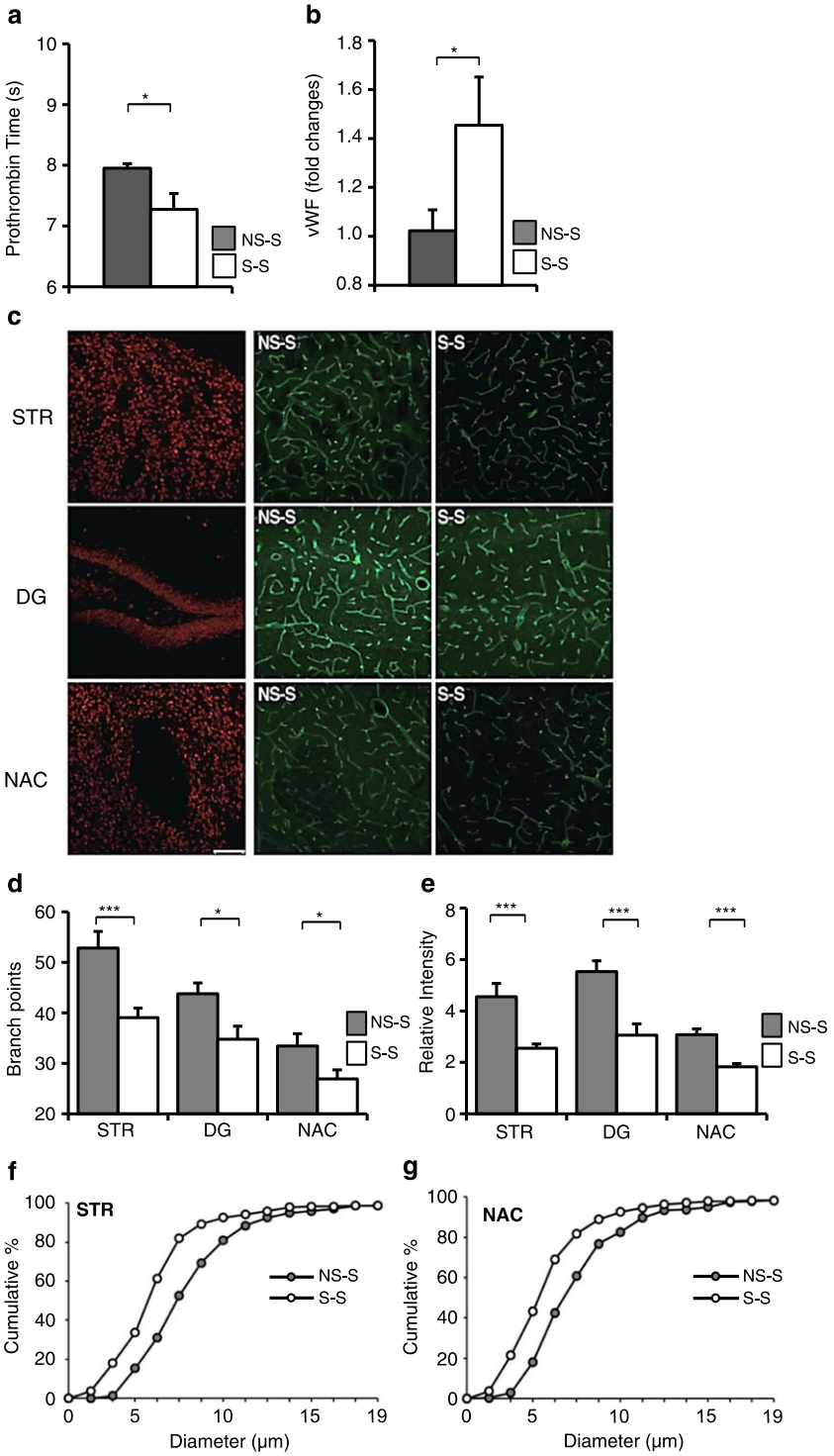

Figure 2 Increased blood coagulation and impairments in brain microvasculature in S-S cocaine-abstinent mice. (a) Decreased prothrombin time (PT) and (b) increased vWF mRNA expression were observed in cocaine-abstinent S-S mice (PT, *N*: NS-S = 3, S-S = 4; vWF, *N*: NS-S = 6, S-S = 5). (c) Representative images of NeuN immunostaining (left column) and blood vessels stained with L. *esculentum* (tomato) lectin of the dorsal striatum (STR; top), dentate gyrus (DG; middle) and nucleus accumbens (NAC; bottom) of S-S and NS-S mice (*N*: NS-S = 4, S-S = 4) during withdrawal. (d) Quantitative analysis of blood vessels showing that S-S mice had fewer capillary branch points and (e) lower signal intensity in the STR, DG and NAC. (f) Cumulative plots of distribution of capillary diameters in the STR and (g) NAC in NS-S (filled circle) and S-S (open circle) mice revealing smaller diameter vessels in S-S compared with NS-S mice. $P < 0.001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

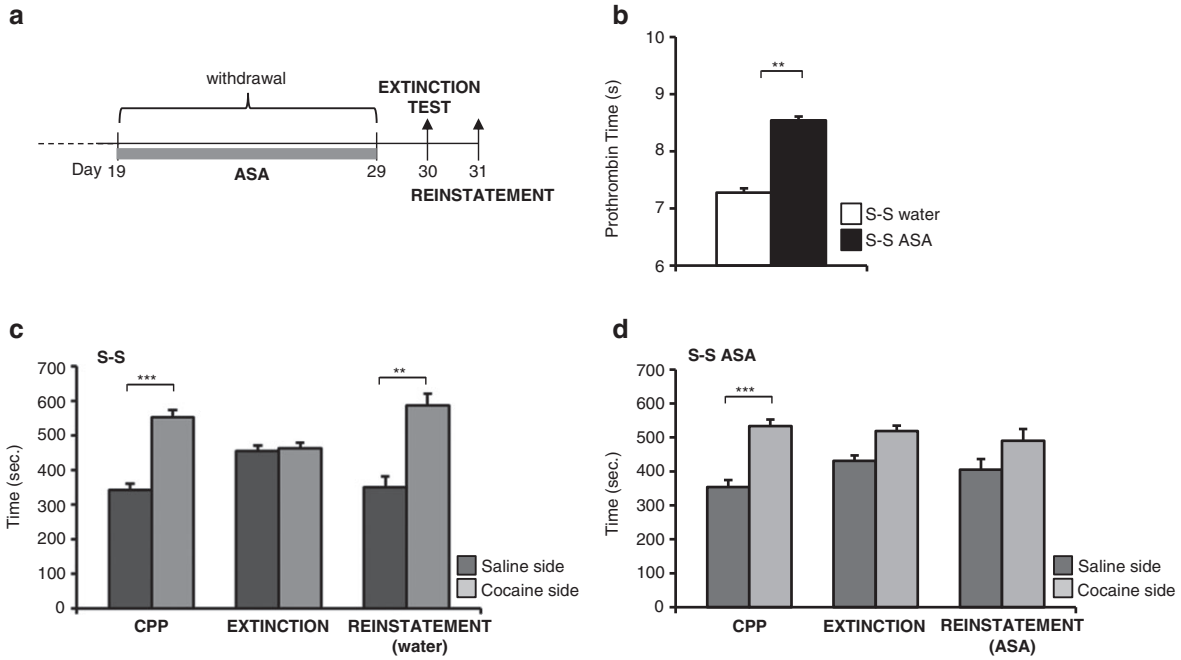

Figure 3 ASA treatment during withdrawal increases prothrombin time and abolishes the susceptibility to reinstate cocaine-seeking behavior in S-S mice. (a) ASA was administered during the withdrawal phase of the CPP protocol used in the study. (b) Prothrombin time was increased in S-S mice after ASA treatment (PT, *N*: S-S = 4, S-S ASA = 5). (c–d) Susceptibility to reinstate cocaine-seeking behavior in S-S mice (*N*: S-S = 14) was abolished on ASA treatment (*N*: S-S ASA = 10)

DISCUSSION

Our findings demonstrate that exposure to adverse social environments in juvenile mice stimulates cocaine-seeking behavior and promotes its reinstatement after abstinence from cocaine. In this respect, our results indicate a specific interaction between the type of stress that is experienced and susceptibility to prime-induced reinstatement. Further, only exposure to adverse social stimulation (S-S)—not to isolation in a novel environment (NS-S)—affects this susceptibility. This result supports recent clinical evidence that links CM to the severity of relapse to drug abuse (Van Dam *et al.* 2014; Elton *et al.* 2014) and underscores the significance of the type of trauma that is experienced.

The different impact of the two types of stress examined is consistent with the physiological and behavioral measurements performed in the pups during stress exposure. Both NS-S and S-S pups showed high number of USV at PD14, whereas only S-S mice vocalized on PD21. Both NS-S and S-S exposure produced an acute increase in corticosterone on PD14 and PD21. However, an increased level of baseline corticosterone was observed only in S-S pups.

Thus, the two types of stress differ profoundly. The S-S experience is likely to elicit intense fear because of physical contact with a 'real' threat. Yet, our procedure for limiting the aggressive behavior of the CD1 mouse (see Supporting Information) allowed us to avoid killing or physically injuring the pup, thus excluding the possible involvement of pain or inflammatory effects in the subsequent behavioral outcomes. In contrast, in the pup response to the novel, 'potentially' threatening environment during the NS-S experience, the anxiety component might predominate.

The two experiences likely involve disparate structures of the brain and differentially affect long-term biological mechanisms. Consistent with this possibility, the long-term behavioral effects in NS-S and S-S mice diverge in adulthood toward a depression-like phenotype (Lo Iacono *et al.* 2015) and increased cocaine-seeking behavior, respectively (Fig. 1c).

In parallel, we observed that in S-S mice, the susceptibility to cocaine-induced reinstatement correlates with alterations in blood coagulation and the brain vasculature. Cocaine induces vasoconstriction, endothelial damage (Sàez *et al.* 2011), platelet activation and aggregation (Kosten *et al.* 2003), and modulates circulating hemostatic parameters, such as vWF and fibrinogen (Siegel *et al.* 2002; Hobbs *et al.* 2013). In the human and rodent brain, chronic use of cocaine affects cerebral blood vessels (He *et al.* 1994; Barroso-Moguel *et al.* 1997; Ren *et al.* 2012) and disrupts regional CBF, likely contributing to the occurrence of ischemic stroke (Bough et al. 2014; Strickland *et al.* 1993; Gozzi *et al.* 2011).

Similar to cocaine, stress has effects on blood circulation parameters. Acute and chronic psychosocial stress

accelerates blood coagulation and elicits hemoconcentration, with changes in procoagulant parameters (Robicsek *et al.* 2011; Von Känel *et al.* 2009). Moreover, experiencing early adversity in life has been associated with increased fibrinogen concentration (Morley *et al.* 2000; Slopen *et al.* 2013) and vWF activity (Tietjen et al. 2012). In rodents, chronic stress induces platelet aggregation (Matsuhisa *et al.* 2014) and decreases the number of microvessels in the hippocampus (Czéh *et al.* 2010; Kiuchi *et al.* 2012).

Overall, these findings led us hypothesize that by influencing similar processes, the juvenile social adversity that was experienced by S-S mice—more extensive than the NS-S experience—amplifies the chronic effects of cocaine on blood circulation and the brain vasculature.

Recently, blood levels of molecules including brain-derived neurotrophic factor [(BDNF) Corominas-Roso *et al.* 2015; Viola *et al.* 2014], glial cell-line-derived neurotrophic factor [(GDNF), Viola *et al.* 2014], polyunsaturated fatty acids (Buydens-Branchey *et al.* 2008), tumor necrosis factor (TNF) alpha and TNF-related weak inducer of apoptosis (Levandowski *et al.* 2014) have been shown to be associated with relapse vulnerability in cocaine addicts. Notably, some of these molecules correlated with abstinence severity only in individuals with history of CM (Viola *et al.* 2014; Levandowski *et al.* 2014), suggesting that different molecular mechanisms modulate the increased risk of relapse in this clinical population.

In our study, the PANTHER pathway analysis revealed significant enrichment in integrin signaling and VEGF signaling (Table 1). Emerging evidence supports a relevant role for both pathways in the brain. Integrins are cell adhesion molecules involved in signal transduction between the extracellular and intracellular environment. Interestingly, a variation of the expression of integrins has been observed after both cocaine-induced reinstatement and psychosocial stress exposure (Wiggins et al. 2009). This alteration is thought to mediate the regulation of actin cycling as a compensatory neuroadaptation to stress or cocaine. VEGF signaling is critically involved in blood vessel growth in the developing and adult nervous system. Recent studies have demonstrated a role for VEGF in promoting neuronal patterning, neuroprotection, glial growth and stress-induced hippocampal neurogenesis (Rosenstein et al. 2010).

Ultimately, our study demonstrates that alterations in blood flow contribute to the susceptibility to relapse to cocaine abuse. Chronic treatment with low-dose ASA during withdrawal prevented cocaine-induced reinstatement in S-S mice. ASA improves CBF (Sabayan *et al.* 2014), and low doses of ASA have anticoagulant and mild anti-inflammatory effects, acting primarily at the platelet level, where it irreversibly blocks the formation of thromboxane A2 (TXA2), thus inhibiting platelet aggregation. We cannot exclude the possibility that a mild anti-inflammatory effect contributes to its effects on cocaine-seeking behavior. Future experiments will differentiate the molecular targets of ASA and identify the mechanisms involved in the mitigation of relapse to cocaine-seeking behavior in S-S mice. Further studies will also determine whether similar behavioral and molecular changes can be induced by administration of similar stressor (S-S) during adolescence or adulthood.

Our study increases knowledge of the mechanisms that underlie the increased risk of relapse in cocaine-dependent individuals who have experienced CM. Further, it suggests that regional hypoperfusion in brains of cocaine-abstinent individuals, with alterations in inhibitory control over drug-seeking, may be a risk factor for relapse to cocaine abuse. Notably, the non-invasive nature of the molecular and physiological alteration identified in this study (e.g. blood biomarkers and regional CBF) will provide for a smooth extrapolation of our findings to applied clinical research.

Acknowledgments

We thank Cornelius T. Gross, Simona Cabib and Diego Andolina for essential comments and criticism, and Perla Caputo, Maria Guendalina Alari, Ramona Furfaro and Laura Roscini for help with the experiments. The professional editorial work of Blue Pencil Science is also acknowledged. This work was supported by the Italian Ministry of Health, Young Researcher Grant (GR-2009-1576820) to V.C. and 'Ricerca corrente 2014' to S.P.A.

Author Contributions

The studies were conceived and designed by VC and LLI. AV, FVC, DO, FRDA and VC performed the behavioral experiments. OE helped to establish the protocol for the RNA extraction from PBMC. LLI performed qPCR, PT, corticosterone level analyses. EA and LC performed the bioinformatics analyses. MTV and AV performed immunohistochemistry. SPA. contributed reagents. The paper was written by VC and LLI and was edited by the other authors.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure 1 Exposure to a social threat in juvenile age promotes reinstatement to cocaine-seeking behavior after withdrawal in adulthood.

Figure 2 Increased blood coagulation and impairments in brain microvasculature in S-S cocaineabstinent mice.

Figure 3 ASA treatment during withdrawal increases prothrombin time and abolishes the susceptibility to reinstate cocaine-seeking behavior in S-S mice.

Table 1 Gene Ontology (GO) analysis of gene expression changes in S-S vs NS-S mouse PBMCs.